なお，抗うつ薬で神経新生が増加することから[13]，神経新生もうつ病に関係するという説もある．しかし，死後脳では神経新生に差がないこと[14]，ラットの実験では，電気ショック後，学習性無力になってもならなくても神経新生が低下している一方[15]，ショック後の神経新生低下は，むしろ外傷後ストレス障害（PTSD）様の行動変化と関係していること[16]などから，今のところ神経新生とうつ病の関係については，やや否定的と考えられる[7]．

こうした神経細胞の形態学的変化については，当初は海馬を中心に研究されていたが，その後の研究では，部位によって異なる結果が報告されている．すなわち，ストレスは，前頭葉や海馬では，神経細胞の萎縮や樹状突起スパイン（神経細胞の樹状突起上にある棘状の構造で，軸索からの入力を受ける部分）の減少を引き起すが，扁桃体では，スパインをむしろ増加させるのである[17]．

うつ病患者では，扁桃体の体積増加や，情動関連刺激による扁桃体の血流増加が報告されている．また，扁桃体の機能が亢進している者ほど，認知療法が有効であるという[18]．

認知療法が治療対象としている，“すべてか無か思考”“過剰な一般化”“ラベリング”といったうつ病に特徴的な認知パターンは，限られた情報をもとに両極端の結論を短時間で導き出すという情動の特徴によく合致している．これらを考え合わせ，ストレスにより神経回路の再構築が生じ，扁桃体を介する情動の回路が，皮質，海馬を介する認知の回路よりも優性となってしまうのがうつ病なのではないか，という仮説が提案されている[7]．

2．非定型うつ病

従来診断では，神経症的機制によるうつ状態は，内因性うつ病と区別されていたが，実際には，こうした発症メカニズムにより鑑別しようとしても，診断信頼性が高くないことから，DSM では，発症メカニズムにより分類することは避け，ある程度重症なうつ状態はすべて大うつ病性障害と分類されることとなった．

とは言え，非定型うつ病，すなわち，DSM-Ⅳの“大うつ病性障害，非定型の特徴を伴うもの”は，モノアミン酸化酵素阻害薬に反応する患者の特徴として見いだされた“類ヒステリー不機嫌症”に端を発しており，パーソナリティー障害や不安障害を伴う，従来診断では神経

症的と考えられていたものを多く含むと考えられる．

非定型うつ病では，虐待などの早期の発達環境が危険因子と考えられ，この危険因子はパーソナリティー障害，不安障害とも共通である．メランコリー型では，ストレスを誘因とするうつ状態がその本質と考えられるのに対し，非定型うつ病は，ストレス脆弱性そのものが中心的な病態であるというべきかも知れない．

デキサメサゾン抑制試験で非抑制パターンを示すメランコリー型うつ病に対し，非定型うつ病では逆に過抑制パターンを示す[19]．

動物モデル研究では，母仔分離飼育によるうつ病モデルが検討されており，こうしたモデルは，非定型うつ病的な側面を研究していると言えるかも知れない．早期養育環境によるグルココルチコイド受容体の DNA メチル化変化が，こうしたストレス脆弱性に関与しているという，エピジェネティックプログラミング仮説も提示されている[20]．

セロトニン仮説との関連

これまで，うつ病の病態仮説としては，セロトニン仮説が最も有力であった．すなわち，うつ病ではセロトニン神経系の機能が低下しており，これを抗うつ薬が改善するという考え方である．長い間こうした考えが広く啓発されてきた背景には，これが抗うつ薬の作用の説明に都合が良かった，という事情があったのではないかという指摘もある[21]．

抗うつ薬の神経新生への作用はセロトニン受容体を介していることから，抗うつ薬が BDNF を増やすのは，セロトニントランスポーター阻害作用を介していると考えられる[13]．すなわち，抗うつ薬がセロトニントランスポーターを阻害することによって作用するという考えは，神経可塑性説と矛盾するものではない．

しかしながら，抗うつ薬がセロトニンを介して効いているからと言って，うつ病の原因がセロトニン欠乏とは言えない．抗コリン薬がパーキンソン病に有効だからと言って，パーキンソン病がアセチルコリン過剰症とは言えないのと同様である．

ただし，うつ病患者では，セロトニンの前駆体であるトリプトファンの欠乏によりうつ状態が誘発されるというデータや，分子イメージングによるセロトニントランスポーターの増加の報告などもあり，う

つ病の病態には，やはり何らかの形でセロトニンも関与している可能性がある[7]．

その他のうつ病

その他，血管性うつ病では脳虚血とうつ病の因果関係が問題になっている[7]．季節性うつ病では，サーカディアンリズムの問題が推定されている．また，認知症の前駆症状として現れる，神経変性過程に伴ううつ病もあるであろう．

いずれにせよ，うつ病は1つの病気ではなく，症候群であり，その病因も多様であることは間違いない．

加藤忠史

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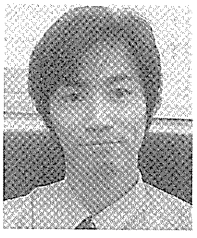
ミトコンドリアDNAと気分障害

Mitochondrial DNA and mood disorder



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◎ミトコンドリア病では精神症状，とくに気分障害(うつ病および双極性障害)を伴うことが多い．とくに，常染色体優性遺伝慢性進行性外眼筋麻痺(CPEO)およびその関連疾患において気分障害を伴うことが多く報告されている．双極性障害患者において死後脳でmtDNA変異蓄積が報告されていること，および脳にmtDNA変異が蓄積するPolg1トランスジェニックマウスが双極性障害様の行動異常を呈することから，ミトコンドリアDNA変異が脳に蓄積することにより気分障害を発症する可能性が示唆される．これらの知見は気分障害の発症メカニズムの解明にも寄与すると期待されている．

Key word：うつ病，双極性障害，ミトコンドリア病，ポリメラーゼγ

双極性障害と母系遺伝

双極性障害に遺伝要因が関与することは，双生児研究，養子研究，家族研究から明らかである．一方，うつ病では遺伝の関与はより低いことから，気分障害のなかでも遺伝学的研究は双極性障害を対象とした研究が先行してきた．

古典的な遺伝研究で，双極性障害では男性から男性への遺伝がまれであることが指摘され，X連鎖の可能性が検討されたが，WinokurはX連鎖のみではその遺伝様式は説明できないとして“細胞質遺伝”が関与する可能性を考えたという[1]．奇しくもミトコンドリアDNA(mtDNA)の発見と同じころのことであった[2]．その30年後，McMahonは連鎖解析用に集められた家系をもとに，双極性障害における遺伝の男女差について再検討し，母系遺伝にみえる家系が(仮想的な)“父系遺伝”にみえる家系よりも有意に多いこと，母方親族では父方親族よりも罹患者が多いことなどを報告し，mtDNAによる母系遺伝またはゲノムインプリンティングが関与している可能性を指摘した[3]．

しかし，こうした解析においては，たとえば父親よりも母親のほうが面接を受ける機会が多いといったさまざまなバイアスが関与することや，うつ病の罹患率が女性で2倍であることなど，多くの攪乱因子が介在することから，こうした家系の分離分析によって双極性障害のような複雑疾患における母系遺伝の関与を検討することは無理があると考えられた[4]．

ミトコンドリア病患児の母親15名を，同程度の重症度の劣性遺伝病の母親である対照群17名と比較したところ，うつ病などの精神疾患が前者では15名中10名と，対照群(17名中2名)より有意に多く，mtDNAがうつ病の脆弱性に関連していると考えられた[5]．

双極性障害とmtDNA多型

McMahonらは母系遺伝に矛盾しない双極性障害家系の発端者でmtDNAの塩基配列解析を行い，見出された多型のうち4つが患者で多くみられたが，多重検定の補正を行うと有意ではない，と報告した[6]．このうち，アミノ酸置換を伴うものは10398 A>Gの1つだけであった．10398 G多型は，アジア人に多くみられるマクロハプログループMを特徴づけるものであり[7]，著者らは10398 G/8701 Gのハプロタイプ，すなわちマクロハプログループMをもつサイブリッド(mtDNA

をもたないρ^0細胞を被験者の血小板と融合させて作った融合細胞．核遺伝子の影響を除外して，特定の mtDNA 排列による機能変化を調べるために用いられる）では 10398 A/8701 A に比べてミトコンドリア内のカルシウムレベルが有意に低いことを見出した[8]．なお，その後のより細かくハプログループを分けた検討では，5460 G>A などに特徴づけられるハプログループ G3/G をもつサイブリッドでカルシウム濃度が低いことや，13651 A>G で特徴づけられる D4a ではカルシウム濃度が高いことがわかった[9]．

当初，著者らの変異検索の結果でも 10398 A>G との関連がみられたが[10]，他のサンプルではこの関連は支持されていない[11,12]．また，双極性障害と関連するハプログループを検討したところ，ハプログループ N9a が多くみられた[13]．N9a を特徴づける 12358 A>G をもつサイブリッドでは細胞質のカルシウム反応が高いことから双極性障害との関連を調べたところ，12358 G が有意に関連していたが，これも独立サンプルでは確認されなかった[13]．

2007 年以来，数千人単位でのゲノムワイド関連解析（GWAS）が報告されるようになり，これまで報告された双極性障害における数百人単位での遺伝子多型の研究は擬陽性所見が多かったと考えられるようになってきた．いまのところ，GWAS では核ゲノムのみに焦点が当てられているが，GWAS に用いられているアレイには 100 個以上の mtDNA のプローブが含まれていることから，近い将来，数千人単位での mtDNA の関連解析の結果が報告されると期待される．

ミトコンドリア病との併発

1992 年に，Suomalainen らは制止の強い，重症の反復性うつ病を主症状とする家族性慢性進行性外眼筋麻痺（CPEO）患者を報告した[14]．家系内の他の罹患者も，うつ病を有していた．この症例では心筋，骨格筋よりも多くの mtDNA 欠失が脳で見出されたことから，mtDNA の蓄積が気分障害を引き起こすことが示唆された．

その後，気分障害を併発するミトコンドリア病の症例は多く報告されているが[15,16]，症状記載にとどまっている報告が多く，信頼性のある精神科診断が行われた報告はほとんどなかった．Fattal らは構造化面接法である MINI（Mini International Neuropsychiatric Interview）を用い，ミトコンドリア病患者 36 名において DSM-IV（アメリカ精神医学会が作成し，事実上の世界標準となっている診断基準）に基づいて診断を行った[17]．その結果，54％が大うつ病，17％が双極性障害と，高率で気分障害を併発していた．

気分障害を併発するミトコンドリア病

気分障害を併発するミトコンドリア病としては MELAS の報告も多いが[18]，常染色体優性遺伝 CPEO（adCPEO）において気分障害を伴うことは多くの教科書にも記されており[19,20]，ほぼコンセンサスになっているといえよう．adCPEO の原因遺伝子のうち，*ANT1*[21]，*Twinkle*[22]，*POLG1*[23]，*RRM2B*[24]，および *OPA1*[25]において，うつ病や双極性障害の併発が報告されている．もう 1 つの CPEO の原因遺伝子，*POLG2*[26]については，まだ報告が少なく，気分障害併発例の報告はない．*POLG1*（ポリメラーゼγ）の変異はミトコンドリア劣性失調症候群（mitochondrial recessive ataxia syndrome：MIRAS）を引き起こすことも報告されているが，MIRAS 患者の半数以上がうつ病を含む精神症状を呈することが報告されている[27]．

これらの研究では DSM 診断がなされていることがほとんどないため，“うつ”の記載があっても精神医学的に“うつ病（大うつ病）”なのか，“双極性障害”なのかは不明である．DSM-Ⅳ診断が行われた研究では，家系中の存命中の CPEO 患者 4 名全員が CPEO 発症前から双極性障害を発症していた[21]．

双極性障害とミトコンドリアDNA変異

うつ病を併発する CPEO 患者における脳内 mtDNA 欠失蓄積の報告[14]に着目した Stine らは，双極性障害患者死後脳大脳皮質で mtDNA 欠失を Southern blot 法により探索したが，欠失は認めなかった[28]．しかし，同じサンプルで定量的 PCR 法により再検討したところ，双極性障害患者中 2 名，うつ病患者 1 名で，0.5％程度の共通欠失が認めら

れた[29]．その後，スタンレー脳バンクの前頭葉サンプルを用いた検討では，さまざまな測定法で欠失に差がないと報告されている[30-32]が，別の前頭葉サンプルを用いた検討でmtDNAの共通欠失が増加していたという報告もある[33]．測定方法や脳部位の差も考えられるが，対象者の差によるものかもしれない．

Munakataらは，双極性障害発症後にミトコンドリア病様の身体症状を呈したが，ミトコンドリア病は否定された患者6名で，mtDNA全周解析を行った．その結果，1名が比較的まれなホモプラスミー多型3644Cを有しており，この多型を有するサイブリッドではミトコンドリア膜電位が低下していた．また3644Cは，関連研究で双極性障害患者に多くみられた(患者：9/630，対照群：1/734)．これらのことから3644Cは，双極性障害の危険因子であると考えられた[12]．しかし，この所見も独立サンプルで確認する必要があろう．

ミトコンドリア関連遺伝子発現

双極性障害患者の死後脳ではミトコンドリア関連の核遺伝子の発現が低下しており，これがミトコンドリア機能障害を示していると報告された[34]．死後脳のpHは生前の状態や投薬の影響を強く受けるため[35]アーチファクトと考えられたが[36-38]，mtDNAハプログループの影響を受けるとも報告され[39]，死後脳のpH低下自体が病態を反映するとの説もあり[37]，結論は得られていない．

Munakataらは双極性障害患者の死後脳で変化しているミトコンドリア関連核遺伝子を調べ，*LARS2*(mitochondrial leucyl tRNA synthase)の発現増加に着目した[40]．*LARS2*は，mtDNA3243変異をもつサイブリッドで発現が増加していた．3243変異は$tRNA^{LeuUUR}$のアミノアシル化を障害することが報告されており，$tRNA^{LeuUUR}$のアミノアシル化を行う酵素の遺伝子である*LARS2*の増加は代償的なものと考えられた．そこで，双極性障害患者の死後脳で3243変異を高感度法で定量したところ，双極性障害患者2名および統合失調症患者1名で増加が検出された[40]．これらの症例では，肝でも3243変異の増加がみられた．

モデル動物

上記のmtDNAの解析結果に加え，双極性障害では磁気共鳴スペクトロスコピーでCPEO患者に類似したエネルギー代謝の障害が報告されていること[41,42]，気分安定薬であるリチウムとバルプロ酸が，ともにミトコンドリア外膜の抗アポトーシス蛋白であるBcl-2を増加させ[43]ミトコンドリア膜電位を高めることから[44]，気分安定薬の作用点の1つがミトコンドリアであると考えられていること[45]と考えあわせ，脳におけるmtDNAの変異蓄積は双極性障害の原因になりうると考えられた．

そこでKasaharaらは，CPEOの原因遺伝子である*Polg1*の変異を脳特異的に発現させたトランスジェニックマウスを作成した．このマウスでは脳にmtDNA欠失が蓄積していた[46]．感覚，運動，記憶，学習，情動には明確な異常はなかった．輪回し行動量の解析では明るくなってもしばらく輪回しを続け，暗くなる前から動きはじめるといった日内リズムの異常がみられた．こうした日内リズムの異常は電気けいれん刺激(週3回，2週間)で顕著に改善する一方[47]，三環系抗うつ薬で悪化し，一部のマウスは躁転様の行動変化を示した．また，メスマウスは4〜5日周期での輪回し行動量の変化を認め，これはリチウム投与により改善した．これらのことから，このマウスは表面的妥当性(症状の類似性)，予測妥当性(患者に有効な薬の有効性)，構成的妥当性(メカニズムの類似性)の3つの基準を満たし，双極性障害のモデルマウスといってよいと考えられた．

ミトコンドリアはエネルギー産生のほか，カルシウム制御にも関与するが，このマウスの脳由来単離ミトコンドリアではカルシウム取込み速度が増加していた．また，海馬錐体細胞では代謝型グルタミン酸受容体アゴニスト刺激による細胞質のCa^{2+}反応が減弱していた[48]．遺伝子発現解析で，ミトコンドリア遷移性透過の構成成分であるシクロフィリンD(CypD)が低下していたことから，この所見はCypD低下によるものと考えられた[48]．

おわりに

このように，ミトコンドリア DNA と双極性障害の関連がさまざまな方向から支持されてきたが，すべての報告が一致した方向を示しているわけではなく，さらなる検討が必要であろう．

また，ミトコンドリア機能障害は当然ながら双極性障害に特異的なものではなく，多くの神経変性疾患[49]や身体疾患にも関係する現象である．とはいえ，双極性障害と統合失調症の遺伝的背景の類似性が指摘されているなか，双極性障害が神経変性疾患と共通した病態をもつことは，あらたな方向性を指し示しているともいえる．パーキンソン病では黒質のドパミンニューロンに mtDNA 欠失が蓄積していると報告されており[50]，脳内のどこに mtDNA 変異が蓄積することが気分障害と関連するのかが今後の課題であろう．

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ARTICLE

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Therapeutic implications of down-regulation of cyclophilin D in bipolar disorder

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Abstract

We previously reported that neuron-specific mutant *Polg1* (mitochondrial DNA polymerase) transgenic (Tg) mice exhibited bipolar disorder (BD)-like phenotypes such as periodic activity change and altered circadian rhythm. In this study, we re-evaluated two datasets resulting from DNA microarray analysis to estimate a biological pathway associated with the disorder. The gene lists were derived from the comparison between post-mortem brains of BD patients and control subjects, and from the comparison between the brains of Tg and wild-type mice. Gene ontology analysis showed that 16 categories overlapped in the altered gene expression profiles of BD patients and the mouse model. In the brains of Tg mice, 33 genes showed similar changes in the frontal cortex and hippocampus compared to wild-type mice. Among the 33 genes, *SFPQ* and *PPIF* were differentially expressed in post-mortem brains of BD patients compared to control subjects. The only gene consistently down-regulated in both patients and the mouse model was *PPIF*, which encodes cyclophilin D (CypD), a component of the mitochondrial permeability transition pore. A blood–brain barrier-permeable CypD inhibitor significantly improved the abnormal behaviour of Tg mice at 40 mg/kg.d. These findings collectively suggest that CypD is a promising target for a new drug for BD.

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Key words: Bipolar disorder, cyclophilin D, DNA microarray, mtDNA, NIM811.

Introduction

Bipolar disorder (BD) is a serious mental disorder accompanied by extreme mood swings from mania to depression. Recent genome-wide association studies identified candidate genes related to calcium signalling, such as *ANK3* and *CACNA1C* (Ferreira *et al.* 2008) as being involved in the development of BD. Lithium can prevent relapse, but many patients do not respond to it or cannot tolerate the side-effects. Other drugs currently used to treat BD such as carbamazepine, valproate, or atypical antipsychotics were initially developed for treatment of epilepsy or schizophrenia. So far there has been no instance of successful development of a new mood stabilizer based on the pathophysiological mechanism of the disease (Kato, 2007). This is mainly ascribed to a paucity of animal models.

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Several studies have suggested that mitochondria play a role in the pathophysiology of BD (Kato, 2008; Kato & Kato, 2000; Stork & Renshaw, 2005) based on abnormalities found by using magnetic resonance spectroscopy (Dager *et al.* 2004; Kato *et al.* 1993; Kato *et al.* 1994; Stork & Renshaw, 2005). These abnormalities resemble those of mitochondrial diseases. Recently, an elevated lactate level in cerebrospinal fluid was also reported in BD patients (Regenold *et al.* 2009). Moreover, a hereditary mitochondrial disease, chronic progressive opthalmoplegia (CPEO), sometimes exists as a comorbidity with BD or depression (Kasahara *et al.* 2006; Suomalainen *et al.* 1992). Mitochondrial DNA (mtDNA) polymerase (polymerase γ; *Polg1*) is one of the causative genes for CPEO. We generated transgenic (Tg) mice with forebrain-specific expression of mutant *Polg1* (*mutPolg1*) as a putative animal model for BD (Kasahara *et al.* 2006). The mutant mice showed distorted diurnal rhythm and periodic fluctuation of activity level in long-term recording of wheel running. Mitochondria isolated from the brains of these mice showed an

enhanced Ca^{2+} uptake rate (Kubota *et al.* 2006). These findings suggest that the *mutPolg1* Tg mouse could serve as an animal model for BD.

However, most BD patients do not have *POLG1* mutations. To establish a new drug target by using the animal model, we believe it is crucial to identify the downstream event of the *Polg1* mutation that relates to BD-like phenotypes. The aim of this study was to identify pathways that may be involved in the pathophysiology of BD and potential drug targets for BD.

In this study, we searched for genes whose expression was commonly altered in the brains of *mutPolg1* Tg mice and in the post-mortem brains of BD patients, without limiting the analysis to mitochondria-related genes. Although we searched for such genes using a comprehensive, unbiased approach, we finally determined that *Ppif*, encoding cyclophilin D (CypD) or mitochondrial peptidyl-prolyl *cis-trans* isomerase, is the only gene altered in both *mutPolg1* Tg mice and BD patients. Thus, we further investigated whether a CypD inhibitor improves the BD-like phenotypes in Tg mice. We found that the CypD inhibitor ameliorated the behaviour of Tg mice, which suggests that CypD inhibition may be a possible new treatment strategy for BD.

Materials and methods

Animals

Mutant *Polg1* lacking proofreading activity due to a D198A mutation was attached with the promoter of calmodulin kinase IIα. The method for generating *mutPolg1* Tg mice was as previously described (Kasahara *et al.* 2006). Male mutant mice were used for mating to avoid possible transmission of mtDNA mutations from the maternal side. All experimental procedures involving animals were approved by the RIKEN Brain Science Institute (BSI) Animal Committee. Male and female *mutPolg1* Tg mice used in the present study were aged 18–50 wk at the beginning of the experiments. For DNA microarray analysis, five pairs of littermates were used.

DNA microarray analysis in mutPolg1 *Tg mice*

All of the procedures were as previously described (Kubota *et al.* 2006). Briefly, the bilateral frontal cortices and hippocampi were dissected from five pairs of male *mutPolg1* Tg mice and their wild-type littermates. RNA samples were extracted with TRIzol reagent (Invitrogen, USA). Five micrograms of total RNA from each sample was reverse-transcribed into cDNA, and biotinylated cRNA was synthesized from the cDNA by *in-vitro* transcription.

DNA microarray experiments were performed with the MG_430 2.0 array (Affymetrix, USA). The hybridization signal on the chips was scanned by a GeneArray scanner and processed by GeneSuite software (Affymetrix). The raw data were initially analysed by MAS5 (Affymetrix) and then imported into GeneSpring 7.3.1 software (Silicon Genetics, USA). The fluorescence intensity of each probe on the chips was divided by its median value and normalized by GeneSpring. For statistical analysis, a two-tailed paired *t* test was performed between the *mutPolg1* Tg mice and their littermates; $p<0.05$ was considered statistically significant.

Quantitative real-time polymerase chain reaction (qRT–PCR)

qRT–PCR analysis was performed with commercially available probe-primer sets (TaqMan technology; Applied Biosystems, USA) as previously described (Kakiuchi *et al.* 2003). The relative expression levels of each mRNA were normalized to the corresponding expression levels of β-actin mRNA level. Each reaction was performed in quadruplicate. Results were presented as mean$\pm$S.E.M., and $p<0.05$ was considered statistically significant.

DNA microarray analysis in post-mortem brain samples

Samples of post-mortem prefrontal cortex (Brodmann's area 46) were donated by the Stanley Medical Research Institute (SMRI) from the institute's Array Collection. Detailed information of the original set of subjects may be found on the SMRI website (http://www.stanleyresearch.org/dnn/BrainResearchLaboratorybrBrainCollection/ArrayCollection/tabid/89/Default.aspx). The gene expression profile of these samples obtained with an Affymetrix HGU133A array was as previously reported (Iwamoto *et al.* 2005) and is available through the SMRI website. Among the expression data of 33 BD patients and 34 control subjects that we profiled, we chose only high-pH samples (pH$\geqslant$6.5) for data analysis in this study to avoid any effect of agonal factors on gene expression (Iwamoto *et al.* 2005; Li *et al.* 2004; Tomita *et al.* 2004). These samples accounted for 18 BD patients and 25 control subjects. Detailed information on these high-pH samples has been described previously (Iwamoto *et al.* 2005).

The mouse MG430 2.0 probe IDs were converted to human HGU133A probe IDs by the NetAffyx analysis centre website (http://www.affymetrix.com/analysis/index.affx). To examine the expression change in patients, we used the *t* test ($p<0.05$). In order to consider the possible effect of the confounding factors on gene expression, we used Pearson's correlation ($p<0.05$) for continuous variables including age, age at onset, duration of illness, and post-mortem interval. For categorical variables including sex, medication, and suicide status, we used the Mann–Whitney *U* test ($p<0.05$). For each of these variables, statistical analysis was performed by using all available high-pH samples regardless of the individuals' diagnoses.

Gene ontology (GO) analysis of DNA microarray data in humans and mice

GO terms were investigated among the differentially expressed genes by using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) annotation tool, version 2.0 (Dennis *et al.* 2003; Huang da *et al.* 2009). The Affymetrix probe IDs that differentially expressed in the frontal cortex of the BD patients compared to the control subjects or those differentially expressed in frontal cortex of Tg mice compared to wild-type mice were converted to the list of DAVID gene IDs. Only GO categories with enrichment scores $\geqslant 1.4$ were considered for further analysis. Fisher's exact test was adopted to measure the gene enrichment in annotation terms in DAVID. A Bonferroni correction for multiple testing was applied by multiplying the *p* value by the number of GO terms tested. The analysis determined overrepresentation of GO terms by computing the probability ($p<0.05$). A statistical analysis was individually applied to the number of categories of biological processes, cellular components, and molecular functions.

Recording of wheel-running activity

The methods for analysing wheel-running activity are described in detail elsewhere (Kasahara *et al.* 2006). In brief, male and female *mutPolg1* Tg mice were individually housed in cages (width 24 cm, depth 11 cm, height 14 cm) equipped with a steel wheel (width 5 cm, diameter 14 cm) (O'Hara & Co., Japan). Wheel-running activity was monitored by measuring the rotation of the wheel (3 counts/1 rotation). The animals were maintained under a 12-h light/dark cycle (lights on 08:00 hours JST) with food and water available *ad libitum*. Data from the initial 7–10 d were not included in the analysis. The basal wheel-running activity was calculated for 14 d before vehicle or drug treatment. The delayed activity index (DAI), referring to the wheel-running activity during the initial 3 h of a light phase, was calculated as previously described (Kasahara *et al.* 2006). Six pairs of Tg and wild-type littermates were placed in either the vehicle- or drug-treatment groups. For statistical comparison of the vehicle- and drug-treatment groups, the averages of the activity levels during day 7 and day 16 were used as the values after the treatment. In order to assess the effect of drug treatment by excluding the inter-individual difference of basal activity, we standardized DAIs for each day by the averages of the DAIs before the treatment for each mouse.

Treatment with CypD inhibitor

NIM811 (*N*-methyl-4-isoleucine-cyclosporin) was kindly provided by Novartis Pharma (Switzerland). NIM811 (5–50 mg/kg) or vehicle was injected intraperitoneally once a day under light ether anaesthesia. A stock solution of 50 mg/ml NIM811 dissolved in vehicle containing 76% cremophore EL (Nakarai Chemicals, Japan) and 24% ethanol was prepared in advance, and then diluted to the final concentration of 5 mg/ml with saline immediately prior to administration. The injection was given at $13{:}00 \pm 2$ hours for 16 d. Two hours after the final injection, the animals were perfused with phosphate buffer including 0.1% EDTA (pH 7.4). Then the brain was removed, weighed, and stored at -80 °C until preparation.

Brain tissue was homogenized in distilled water in a Teflon-glass homogenizer (1000 rpm, 5 strokes); sonicated for 30 s by a probe-type sonicator (VCX-130-PB; Sonics & Materials, USA) on ice; and then centrifuged at 15000 rpm for 10 min. The concentration of NIM811 in the supernatant was measured (SRL Inc., Japan) by radioimmunoassay (Diasorin Inc., USA) with the use of a gamma scintillation counter (ARC-950; Aloka, Japan). The NIM811 content was normalized by wet tissue weight. A standard curve was constructed by using brain homogenates from untreated wild-type mice. Mixtures of serial dilutions of NIM811 in the range of 0–2 μg/ml with the brain homogenates were analysed in duplicate to determine known amounts of the drug.

Student's *t* test and two-way repeated-measures analysis of variance (rm-ANOVA) with a between-group factor of drug (NIM811 or vehicle) and a within-group factor of time (before and after drug treatment) were used. When a significant interaction was found by rm-ANOVA, a paired *t* test was applied for *post-hoc*

analysis. Statistical calculation was performed with KyPlot version 4.0 (KyensLab Inc., Japan) and SPSS software version 16.0 (SPSS Inc., USA).

Results

Comparison of gene expression profiles between post-mortem brains of BD patients and mutPolg1 *Tg mice*

To search for similarity of a pathophysiological process in the brain between BD patients and BD model mice, we compared the gene expression profiles between patients and the mouse model, according to two strategies, GO analysis of differentially expressed genes, and gene level analysis.

We previously conducted a DNA microarray analysis in the post-mortem prefrontal cortex of BD patients and control subjects. The expression levels of 764/11 920 transcripts were significantly different in the frontal cortex. GO analysis was applied to this dataset. We also previously performed gene expression analysis of the frontal cortex and hippocampus in *mutPolg1* Tg and wild-type mice. For comparison, GO analysis was applied to the genes differentially expressed in the frontal cortex in the Tg mice (1471 out of 22 643 transcripts).

We found that 30 categories in the human dataset and 30 categories in the mouse dataset were significantly overrepresented. Among them, 16 categories of the GO terms overlapped in the human and mouse datasets (Table 1). The overrepresented categories included functional modules related to RNA processing and organelles, as well as other general biological processes (Table 1).

Differentially expressed genes in the brains of mutPolg1 *Tg compared to wild-type mice*

Previously we had reported a preliminary analysis of the difference in the expression levels of mitochondria-related genes in *mutPolg1* Tg mice compared to wild-type mice (Kubota *et al.* 2006). In the present study, we re-evaluated the gene expression changes without limiting the analysis to mitochondria-related genes in order to identify a similarity to gene expression in BD. Among the transcripts differentially expressed in the hippocampus (922 transcripts) and the frontal cortex (1471 transcripts) between *mutPolg1* Tg mice and wild-type mice, 60 showed a common alteration in the frontal cortex and the hippocampus. In these two regions, 33 transcripts were altered in the same direction; 15 were commonly up-regulated and 18 were commonly down-regulated (Table 2). Notably, the glucocorticoid receptor (GR) gene [nuclear receptor subfamily 3, group C, member 1 (*Nr3c1*)] was down-regulated in the two regions. The change was slightly larger in the frontal cortex but statistically more significant in the hippocampus ($p<0.001$) as confirmed by qRT–PCR ($p=0.02$).

Shared gene expression changes in mutPolg1 *Tg mice and post-mortem brains of BD patients*

We then searched for the genes with altered expression in both Tg mice and BD patients. The 33 mouse probes showing consistent alteration in the cortex and the hippocampus corresponded to 39 human probes, and three of the latter showed statistically significant changes ($p<0.05$) in post-mortem brains of BD patients compared to control subjects. These probes corresponded to two genes; one probe for *SFPQ* and two probes for *PPIF* showed expression changes in the same direction as that of Tg mice (Tables 2 and 3). *SFPQ* is included in several GO categories related to RNA processing in humans and mice, and *PPIF* is included in several GO categories related to organelles, in humans.

Expression levels of the two *PPIF* probes in the post-mortem brains were not significantly affected by confounding factors such as age, age at onset, duration of illness, post-mortem interval, and sex (data not shown). Although the number of samples ($n=4$) was too small to apply statistical analysis, these probes also showed a tendency for decreased expression in the medication-free BD patients. Taken together, these results suggest that decreased expression of *PPIF* was a change shared by the patients and the animal model.

In the present study, we further focused on the pharmacological analysis of *PPIF in vivo* using Tg mice.

Treatment of mutPolg1 *Tg mice with CypD inhibitor*

CypD encoded by *Ppif* is a component of the mitochondrial permeability transition pore (PTP) and regulates the PTP opening. Cyclosporin A (CsA) is a well-known CypD inhibitor, but it does not penetrate the blood–brain barrier (BBB) because of its high affinity to the P-glycoprotein transporter (Sakata *et al.* 1994). CsA also inhibits calcineurin. In contrast, NIM811 is a BBB-permeable CsA analog that potently inhibits CypD but has negligible effect on calcineurin. We confirmed that NIM811 potently inhibited PTP in mitochondria isolated from brains of wild-type mice (data not shown). Treatment with NIM811 is protective against mitochondrial dysfunction *in vitro* (Hansson *et al.* 2004; Waldmeier *et al.* 2002).

Table 1. Gene ontology (GO) analysis of DNA microarray datasets in human and mouse

			Human				Mouse			
Category		Term	Count[a]	%	Fold enrichment	*p* value[b]	Count[a]	%	Fold enrichment	*p* value[b]
RNA processing										
GOTERM_MF_ALL	GO:0003723	RNA binding	65	8.9	2.49	<0.00001	80	5.6	2.07	<0.00001
GOTERM_BP_ALL	GO:0006396	RNA processing	44	6.1	2.64	0.00006	57	4.0	2.34	0.00002
GOTERM_BP_ALL	GO:0006397	mRNA processing	31	4.3	3.27	0.00012	38	2.6	2.56	0.00117
GOTERM_BP_ALL	GO:0008380	RNA splicing	30	4.1	3.62	0.00002	32	2.2	2.74	0.00268
GOTERM_BP_ALL	GO:0016071	mRNA metabolic process	36	5.0	3.19	0.00001	42	2.9	2.49	0.00057
Organelle										
GOTERM_CC_ALL	GO:0031090	Organelle membrane	95	13.1	1.86	<0.00001	81	5.6	1.91	0.00002
GOTERM_CC_ALL	GO:0031967	Organelle envelope	48	6.6	2.33	0.00010	55	3.8	1.95	0.00247
GOTERM_CC_ALL	GO:0044422	Organelle part	211	29.0	1.63	<0.00001	250	17.4	1.50	<0.00001
GOTERM_CC_ALL	GO:0044446	Intracellular organelle part	209	28.8	1.62	<0.00001	248	17.3	1.49	<0.00001
Others										
GOTERM_CC_ALL	GO:0005737	Cytoplasm	329	45.3	1.43	<0.00001	512	35.6	1.42	<0.00001
GOTERM_CC_ALL	GO:0044444	Cytoplasmic part	204	28.1	1.49	<0.00001	309	21.5	1.43	<0.00001
GOTERM_CC_ALL	GO:0012505	Endomembrane system	63	8.7	1.78	0.00940	55	3.8	2.14	0.00013
GOTERM_CC_ALL	GO:0031975	Envelope	48	6.6	2.32	0.00011	56	3.9	1.97	0.00140
GOTERM_CC_ALL	GO:0044428	Nuclear part	81	11.1	2.07	<0.00001	117	8.1	1.64	0.00007
GOTERM_BP_ALL	GO:0033036	Macromolecule localization	58	8.0	1.89	0.02083	86	6.0	1.64	0.03115
GOTERM_BP_ALL	GO:0015031	Protein transport	51	7.0	2.01	0.01894	75	5.2	1.70	0.03654

Among 764 probe sets (*t* test $p<0.05$), 727 probe sets were used for GO analysis in human study.

Among 1471 probe sets (paired *t* test $p<0.05$), 1,437 probe sets were used for GO analysis in mouse study.

[a] Count: number of genes in the gene list mapping to a specific term.

[b] EASE score (a modified Fisher's exact *p* value) with Bonferroni correction was used to determine statistical significant GO terms ($p<0.05$) in DAVID annotation tool.

Table 2. Differentially expressed genes in the brains of *mutPolg1* Tg mice compared to wild-type mice

		Hippocampus		Frontal cortex			
Symbol	Gene title	Fold change	*p* value[a]	Fold change	*p* value[a]	Public ID	Probe set ID
Sfpq	Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	1.26	0.003	1.27	0.039	BG061796	1439058_at
Erdr1 protein	Clone IMAGE: 3983821	2.12	0.005	1.98	0.022	BC021831	1427820_at
Top1mt	DNA topoisomerase 1, mitochondrial	1.13	0.005	1.19	0.002	AF362952	1460370_at
Zc3h13	Zinc finger CCCH type containing 13	1.18	0.006	1.18	0.031	AW536655	1434894_at
Pspc1	Paraspeckle protein 1	1.37	0.013	1.27	0.009	BB590675	1423192_at
Hist2h2aa1	Histone cluster 2, H2aa1	1.33	0.032	1.48	0.024	BC010564	1418367_x_at
ImmP2l	Mitochondrial inner membrane protease subunit 2 (IMP2- like protein).	1.38	0.039	1.27	0.029	BB291417	1458099_at
EG633640	Predicted gene, EG633640	1.28	0.039	1.12	0.040	BG068672	1426607_at
Slc35e1	Solute carrier family 35, member E1	1.20	0.041	1.21	0.038	BB041864	1434103_at
Ube3c	Ubiquitin protein ligase E3C	1.22	0.041	1.29	0.019	BE690666	1444562_at
Rbm25	RNA binding motif protein 25	1.18	0.042	1.31	0.024	AI159652	1437862_at
Ptprn2	Protein tyrosine phosphatase, receptor type, N polypeptide 2	1.16	0.045	1.45	0.009	U57345	1425724_at
Polg	Polymerase (DNA directed), gamma	1.42	0.045	1.23	0.007	BG064799	1423272_at
4930447A16Rik	RIKEN cDNA 4930447A16 gene	1.28	0.046	1.35	0.027	BB012182	1431671_at
Tradd	TNFRSF1A-associated via death domain	1.20	0.047	1.39	0.050	AA201054	1452622_a_at
Nr3c1	Nuclear receptor subfamily 3, group C, member 1	−1.12	<0.001	−1.33	0.035	NM_008173	1421866_at
Ero1l	ERO (endoplasmic reticulum oxidoreductin) 1-like (S. cerevisiae)	−1.08	0.001	−1.21	0.043	BM234652	1419029_at
Med26	Mediator complex subunit 26	−1.46	0.005	−1.62	0.007	AK017726	1452282_at
Npdc1	Neural proliferation, differentiation and control gene 1	−1.12	0.008	−1.12	0.028	NM_009849	1418259_a_at
2900052N01Rik	RIKEN cDNA 2900052N01 gene	−1.36	0.009	−1.32	0.026	AU067665	1436231_at
Kif5c	Kinesin family member 5C	−1.32	0.009	−1.50	0.047	AI844677	1450804_at
Flnb	Filamin, beta	−1.15	0.016	−1.29	0.002	AW538200	1426750_at
Rapgef6	rap guanine nucleotide exchange factor (GEF) 6	−1.10	0.017	−1.11	0.038	BQ177183	1427412_s_at
Usp31	Ubiquitin specific peptidase 31	−1.09	0.017	−1.19	0.038	BM227490	1442099_at
Prmt3	Protein arginine N-methyltransferase 3	−1.21	0.020	−1.24	0.005	AK008118	1431768_a_at
Aif1l	Allograft inflammatory factor 1-like	−1.22	0.035	−1.07	0.016	BC024599	1424263_at
Cntn3	Contactin 3	−1.09	0.036	−1.13	0.024	NM_008779	1420739_at
Ppif	Peptidylprolyl isomerase F (cyclophilin F)	−1.12	0.038	−1.18	0.008	NM_134084	1416940_at
Copg	Coatomer protein complex, subunit gamma	−1.16	0.044	−1.13	0.009	BC024686	1415670_at
Enoph1	Enolase-phosphatase 1	−1.10	0.044	−1.28	0.002	BC021429	1423705_at
Smad3	MAD homolog 3 (Drosophila)	−1.25	0.045	−1.28	0.019	BI150236	1450472_s_at
BC003266	cDNA sequence BC003266	−1.04	0.049	−1.08	0.039	NM_030252	1449189_at
Glud1	Glutamate dehydrogenase 1	−1.10	0.049	−1.19	0.040	BI329832	1416209_at

[a] Paired *t* test (<0.05).

Furthermore, its neuroprotective effect has been proven *in vivo* (Korde *et al.* 2007; Ravikumar *et al.* 2007).

Mood stabilizers effective for BD are known to have neuroprotective effects (Chen *et al.* 1999). Thus, we hypothesized that the down-regulation of *PPIF* is an

Table 3. Shared gene expression changes in the brains of *mutPolg1* Tg mice and post-mortem brains of the bipolar disorder patients

		pH ⩾ 6.5 samples (bipolar $n=18$, control $n=25$)		All samples (bipolar $n=33$, control $n=34$)				
Symbol	Gene title	Fold change	p value[a]	Fold change	p value[a]	Public ID	Probe set ID	Locus
PPIF	Peptidylprolyl isomerase F (cyclophilin F)	−1.20	0.016	−1.19	0.005	NM_005729	201490_s_at	10q22-q23
PPIF	Peptidylprolyl isomerase F (cyclophilin F)	−1.17	0.042	−1.11	0.095	BC005020	201489_at	10q22-q23
SFPQ	Splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)	1.33	0.002	1.10	0.215	AV705803	221768_at	1p34.2

[a] t test (<0.05).

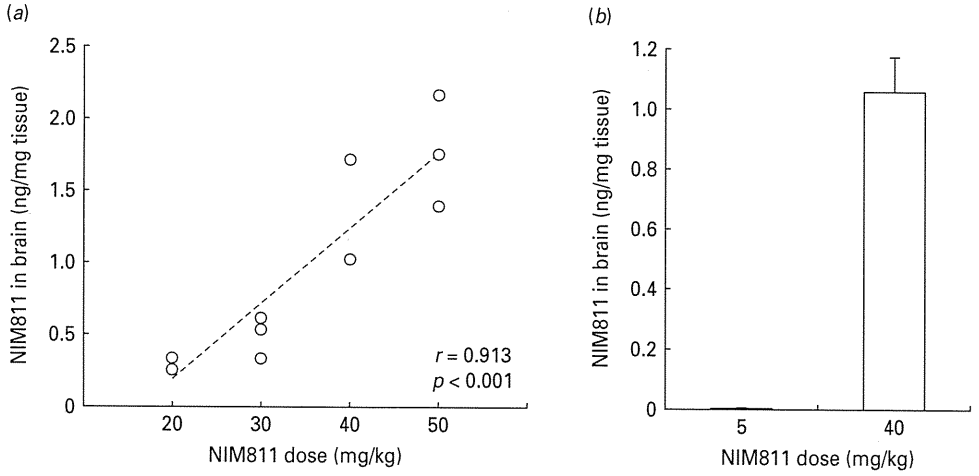

Fig. 1. Detection of NIM811 in the brain after chronic treatment. (*a*) Each dose of NIM811 (20, 30, 40, 50 mg/kg) was injected intraperitoneally into wild-type mice for 12 d. The animals were housed in home cages. Values indicate mean ± S.E.M. (20, 30, 50 mg/kg, $n=3$; 40 mg/kg, $n=2$). The NIM811 concentration in the brain was well correlated with the dose ($r=0.913$, $p<0.001$, by Pearson's correlation coefficient). (*b*) The *mutPolg1* Tg mice were injected with 5 or 40 mg/kg of NIM811 for 2 wk. After their wheel-running activity was recorded, the drug concentration was measured as described in the Materials and methods section. For detection of NIM811 in the brain after 5 mg/kg treatment, three brains were homogenized together and analysed in duplicate, because the drug content was undetectable in our analysis when individual tissue was used (data not shown). The NIM811 concentration was corrected for tissue weight. Values indicate mean ± S.E.M. (5 mg/kg, $n=2$; 40 mg/kg, $n=6$).

adaptive change associated with mitochondrial dysfunction, and that CypD inhibitor may be effective against the behaviour of *mutPolg1* Tg mice.

First, we determined the tissue concentration of the drug in mice treated with 20–50 mg/kg NIM811 for 2 wk (Fig. 1*a*). The concentration of NIM811 increased dose dependently ($r=0.913$, $p<0.001$) in the brain after the injection, implying that NIM811 can penetrate into the brain at higher doses. Similar to the levels in the brain, there was a dose-dependent increase of the drug concentration in the liver (data not shown).

With regard to the effect of CsA, the lower dose is reportedly therapeutic as an immunosuppressant for patients after liver transplantation (Rasmussen *et al.* 1996). NIM811 at a lower dose was only reported to be effective in a case accompanied by a breach of the BBB

(Sullivan *et al.* 2000). In the present study, we examined the effect of NIM811 on the behaviour of the Tg mice at two doses, 5 and 40 mg/kg. However, the lower dose (5 mg/kg), did not achieve a detectable concentration in the brain, possibly due to the intact BBB.

NIM811 treatment at a lower dose (5 mg/kg) did not significantly affect the DAI, the measure of excessive wheel-running activity at the beginning of the light phase, compared to the vehicle group. Rm-ANOVA showed no significant interaction between drug (NIM811 and vehicle) and time (before and after the treatment) (d.f. $=1$, $F=0.811$, $p=0.383$) (Fig. 2*e*). On the other hand, after the 40 mg/kg NIM811 treatment, there was a significant interaction between drug and time (d.f. $=1$, $F=18.15$, $p=0.002$) (Fig. 3*e*). The DAI was decreased in the NIM811 group (Post/Pre $=0.658\pm0.102$) whereas it was increased in the vehicle group (Post/Pre $=1.720\pm0.289$). There was a significant difference in the Post/Pre ratio between the two groups ($p<0.05$) (Fig. 3*f*).

Discussion

GO analysis of the genes differentially expressed in BD patients and model mice showed some overlap of the enriched GO categories. This might suggest the similarity of gene expression patterns between the patients and the animal model.

Notably, we found that *PPIF* encoding CypD, mitochondrial peptidyl-prolyl *cis-trans* isomerase, was consistently down-regulated both in *mPolg1* Tg mice (Table 2) and in BD patients (Table 3). Furthermore, pharmacological inhibition of CypD by NIM811 ameliorated the behavioural phenotype of the *mutPolg1* Tg mice (Fig. 3). Because low-dose NIM811 did not have an effect against the behavioural phenotype of the *mutPolg1* Tg mice, this effect was regarded as reflecting a direct effect on the brain (Fig. 2).

CypD is a component of mitochondrial PTP and is localized in a mitochondrial matrix. A Ca^{2+} overload induces CypD binding to the adenine nucleotide translocator resulting in the opening of the PTP, which has a key role in apoptotic or necrotic cell death. CsA, a potent CypD inhibitor, inhibits PTP opening. We confirmed that CsA enhanced mitochondrial Ca^{2+} uptake in isolated mitochondria (Kubota *et al.* 2006). NIM811 also inhibits PTP opening (Hansson *et al.* 2004; Waldmeier *et al.* 2002). In addition, NIM811 reduces the infarct volume and the release of cytochrome *c* from mitochondria after ischaemia (Korde *et al.* 2007; Ravikumar *et al.* 2007) and is effective for experimental traumatic brain injury (Mbye *et al.* 2008). Furthermore, brains of CypD knockout mice are resistant to ischaemia/reperfusion injury (Baines *et al.* 2005; Basso *et al.* 2005; Nakagawa *et al.* 2005; Schinzel *et al.* 2005). Cell death in animal models of neuromuscular diseases was also attenuated by crossbreeding with CypD knockout mice (Forte *et al.* 2007; Millay *et al.* 2008; Palma *et al.* 2009). In a mouse model of Alzheimer's disease, lack of CypD restores synaptic and cognitive function (Du *et al.* 2008). These findings suggest that CypD inhibition is a key therapeutic approach against central nervous system diseases.

Although simple logic suggests that down-regulation of CypD, being a downstream event of *Polg1* mutation, should contribute to the abnormal phenotypes observed in Tg mice, then, an activator, instead of an inhibitor of CypD, should be used to increase CypD level to improve the phenotypes. However, most of the drugs that are effective for maintenance treatment of BD, such as lithium (Nonaka *et al.* 1998), valproate (Jeong *et al.* 2003), olanzapine and quetiapine (Qing *et al.* 2003), reportedly have neuroprotective effects. Thus, it would be plausible to assume that a CypD inhibitor is effective for BD, and its down-regulation observed in the BD model mice is compensatory in nature.

Our result that NIM811, a CypD inhibitor, is effective for the BD-like phenotype in the animal model is consistent with the previous findings that pharmacological inhibition or genetic ablation of CypD has neuroprotective effects.

In heart-specific *mutPolg1* Tg mice, heart mitochondria are reportedly resistant to PTP opening, and CsA, a PTP inhibitor, prevented heart failure (Mott *et al.* 2006). This suggests that the resistance to PTP opening in Tg mice might not be a cause of heart failure but is instead a compensatory phenomenon caused by accumulation of mtDNA deletions.

Thus, the down-regulation of CypD in the brain in BD patients or in neuron-specific *mutPolg1* Tg mice might be an adaptive response to mitochondrial dysfunction, rather than the cause of the disorder, and thus blockade of CypD might have counteracted the BD-like behaviour of the *mutPolg1* Tg mice. Indeed, the behavioural phenotypes of CypD knockout mice, such as enhanced anxiety (Luvisetto *et al.* 2008) and cognitive impairment (Mouri *et al.* 2009), are different from those of *mutPolg1* Tg mice.

Two mood stabilizers, lithium and valproate, up-regulate B-cell lymphoma protein-2 (*Bcl-2*), an anti-apoptotic factor (Chen *et al.* 1999; Corson *et al.* 2004; Hiroi *et al.* 2005). Similar to CypD down-regulation, up-regulation of *BCL-2* also inhibits PTP opening.

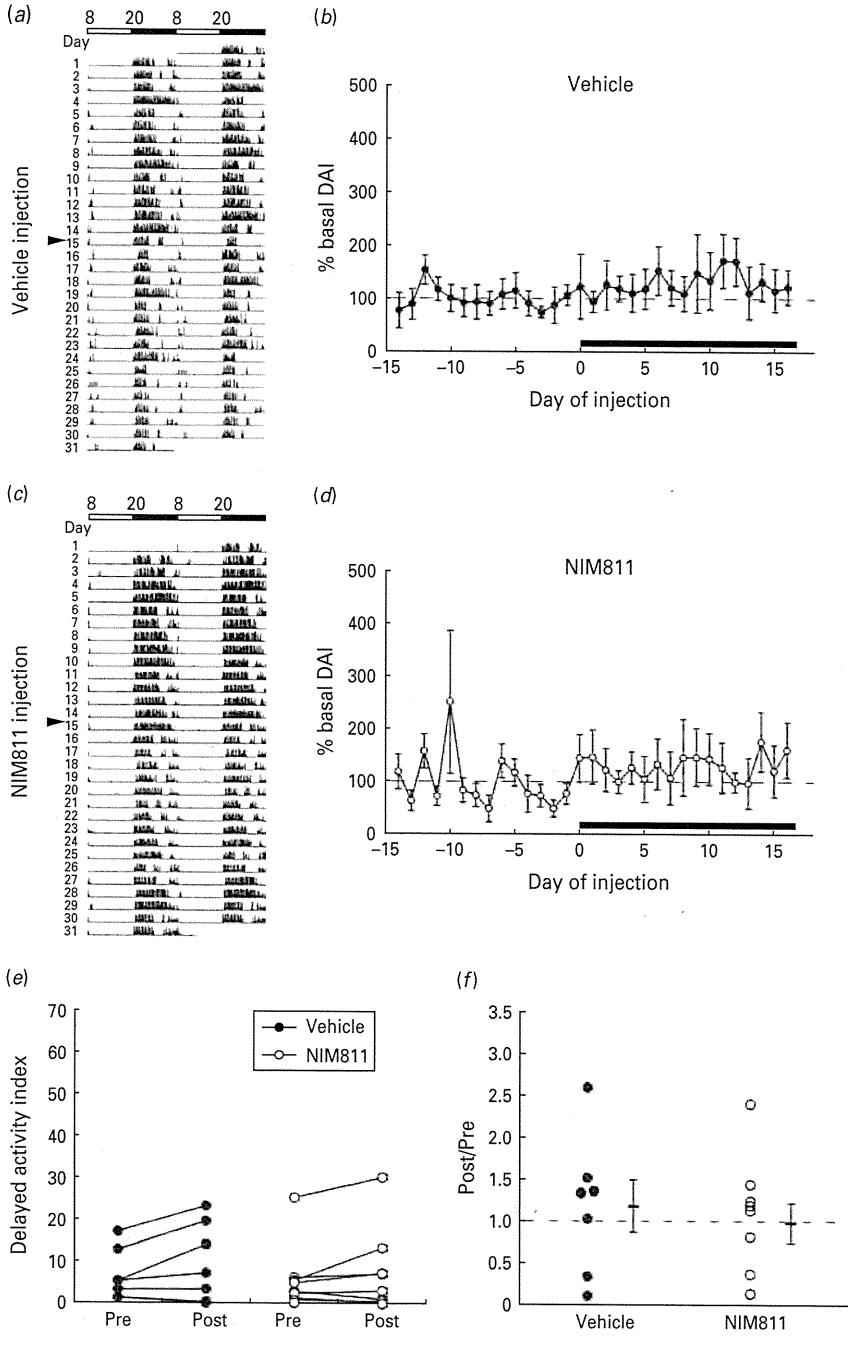

Fig. 2. Effect of 5 mg/kg NIM811 on wheel-running activity in *mutPolg1* Tg mice. Individual activity record of (*a*) vehicle-treated or (*c*) NIM811-treated *mutPolg1* Tg mice. The light and dark periods (12:12 hours) are indicated by white and black bars. Each bar represents the total count of wheel running in a 10-min interval. An arrowhead indicates the first day of injection (day 15). Standardized delayed activity index (DAI) of (*b*) vehicle-treated or (*d*) NIM811-treated *mutPolg1* Tg mice. DAI during the treatment (Post) was standardized by the mean value before the treatment (Pre). A broken line shows the basal activity level. Day 0 indicates the first day of injection. A horizontal bar represents the period of the drug injection. (*e*) Change of DAI of individual animals. The index before the treatment was averaged over 14 d (Pre: days −14 to −1) and after the treatment for 10 d (Post: days 7–16). (*f*) Effect of the vehicle or NIM811 treatment on DAI. The effect of drug treatment was estimated by the ratio of the index values before (Pre) and after (Post) the treatment. Values indicate mean ± S.E.M. (vehicle treatment group, $n=7$; NIM811 treatment group, $n=9$). A broken line shows the averaged level of DAI before the treatment.