厚生労働科学研究費補助金

こころの健康科学研究事業

気分障害の神経病理学に基づく分類を目指した脳病態の解明に関する研究

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平成２１年度　総括研究報告書

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平成21年度　総括研究報告書

研究代表者　加藤忠史
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研究要旨：

うつ病は、大きな社会負担、急増する患者数等から社会問題となっているが、双極スペクトラム、非定型うつ病、血管性うつ病など、多様な病型が存在し、その診療には混乱も生じている。本研究の目的は、遺伝要因、早期養育環境、ストレス、加齢、動脈硬化などのうつ病の危険因子によって引き起こされる脳病態を、死後脳研究、脳画像解析、疫学研究、および動物モデルにおける神経科学的解析などによって明らかにし、脳病態に応じた気分障害の分類を目指すことである。

本研究の特色は、うつ病の危険因子による脳病態を多角的に明らかにすると共に、これを患者死後脳で確認することである。気分障害の疾患概念を病理学に基づいて再構築し、病型に応じた診断法、治療法を開発することができれば、精神医学が病理学に基づく医学へと進化する第一歩となる。現在混沌としている「うつ」が種々の脳疾患と心の悩みに明確に分類され、適切に対応されれば、自殺者数の減少、円滑な人事管理など、多くの波及効果が期待でき、国民の生活の質を向上させると期待される。

平成21年度は、動物実験では、ミトコンドリアDNA異常を持つモデルマウスにおいて、脳病変部位を探索し、候補部位として、視床室傍核および下辺縁皮質を同定した。また、ストレスに反応して活動する脳部位を探索し、前辺縁／下辺縁皮質など、前頭辺縁系の諸部位がストレスに反応することを見出した。また、免疫学的なうつ病モデルを用いて、脳病態を検討し、活性化ミクログリアの関与について検討した。地域疫学研究では、地域住民におけるメタボリック症候群とうつ病の関連について検討を行い、男性ではメタボリック症候群がうつ病の危険因子となる可能性が考えられた。また、血管性うつ病の長期予後研究では、血管性うつ病群では非血管性うつ病群に比して寛解を継続した割合が低く、認知症に進展した割合が高いことが判明した。気分障害患者の死後脳研究では、モデルマウスにおいて見出された候補脳部位である視床室傍核の検討が可能かどうか、予備的な検討を行った。高齢者ブレインバンクでは、新たに2例のうつ病患者の剖検を行い、1例では神経原線維変化のみ、もう1例では神経原線維変化、老人班およびLewy小体を認めた。更に、これまでに高齢者ブレインバンクに蓄積された症例のうち、うつ病の病歴がある者31例を同定し、気分障害に伴う神経病理学的所見に関する調査の準備を開始した。

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A 研究目的

社会生活障害を引き起こす疾患として最大の要因であるうつ病の患者数は近年急速に増加し、社会問題となっている。しかし、抗うつ薬によく反応するメランコリー型に加え、抗うつ薬により悪化する双極スペクトラム、抗うつ薬が奏効しにくく心理療法も重要となる非定型うつ病、難治化しやすい血管性うつ病など、治療抵抗性のうつ病が増加し、うつ病診療は混沌とした状況にある。面接に頼った現在の診断法には限界があり、現状の打開には、神経病理学所見に基づいて疾患概念を再構築すると共に亜型分類を確立し、脳病態診断に基づいて治療を最適化する他ない。

うつ病の発症には遺伝、養育、ストレス、加齢など、種々の危険因子が関与する。加藤らはミトコンドリアDNA異常が脳に蓄積する遺伝子改変マウスが双極性障害様の行動異常を示すことを見出した。山下らは基底核に脳血管障害が存在するとうつ病の中でも意欲低下が優勢となることを見出し、血管性うつ病の神経学的基盤を示した。神庭らはインターロイキン1(IL-1)が脳内モノアミン系に影響することや、インターフェロンがIL-1 β 上昇を介して海馬神経新生を低下させることを見出し、うつ病の神経免疫仮説を確立した。また久山町の65歳以上の全住民を対象としてうつ病の調査を行い、有病率を明らかにした。村山らは1万名近い脳を有する高齢者ブレインバンクを構築し、千例以上の検討で、加齢に伴い徐々にLewy小体が蓄積し、脳機能障害が引き起こされることを示した。

上記の他多くの研究から、遺伝要因によるミトコンドリア機能障害、ストレスによる神経内分泌免疫学的変化、加齢に伴う脳血管障害および異常蛋白蓄積など、各危険因子による脳機能障害がうつ病を引き起こすと考えられる。本研究では、こうした脳病態を臨床研究および動物モデルを用いて明らかにすると共に、これを患者死後脳で検討する。

本計画の特徴は、うつ病の異種性の高さを前提とし、まず各危険因子と脳病態の関連を明らかにし、次に脳病態を臨床診断（亜型分類）と対応づけることにより、気分障害の神経病理学的な分類の基盤を固めることである。本研究によりうつ病の神経病理学的所見に基づく分類が可能となれば、診療やメンタルヘルス管理の最適化が可能となり、自殺者減少、生活の質の向上につながる。

B. 研究方法

動物実験では、ミトコンドリアDNA変異が脳内に蓄積するモデルマウスを用いて、ミトコンドリアDNA蓄積を指標として、気分障害類似の行動異常と関連する脳の異常を同定する。また、ストレスによる神経病理学的変化を探索するため、c-fos免疫染色を用いて、ストレス反応に関与する脳部位を探索する。また、免疫学的うつ病モデル動物を用いて、脳病態を検討する。

地域疫学研究では、うつ病のスクリーニングを行い、耐糖能異常や認知機能障害との関係を調べる。本研究で得られた精神医学的診断のデータは、将来剖検所見が得られた際に、病理所見との関係を調べるためにも用いることができる。また、脳血管障害とうつ病発症の関連を検討する。

脳バンクより供与を受けた気分障害患者の凍結死後脳を用い、候補脳部位において、遺伝子発現解析を行い、気分障害の脳病態を検討する。また、高齢者ブレインバンクの登録症例で、うつ病の調査を行うと共に、新たな剖検例について、神経病理学的検索を行う。

C.研究結果

ミトコンドリアDNA(mtDNA)合成酵素の変異を脳特異的に発現させた双極性障害モデルマウスにおいて、mtDNA変異の蓄積部位を探索するため、マウスの脳切片をレーザーキャプチャーマイクロダイセクション法により小片に細断し、各小片のDNAを用いて、定量的PCR法により、mtDNA欠失量を定量する、定量的mtDNA欠失マッピング法(quantitative Mapping of Deleted Mitochondrial DNA [qMDMD])を開発した。この方法により、モデルマウスにおいてmtDNA欠失をマッピングし、半網羅的に検討した結果、視床室傍核、下辺縁皮質などに、特に多くの欠失mtDNAを認めた。

視床室傍核におけるmtDNA欠失蓄積および遺伝子発現変化について、気分障害患者の死後脳で検討すべく、スタンレー脳バンクの凍結視床切片を用いて、トルイジンブルー染色による検討を行った。予備的な検討では、視床室傍核が失われている場合があることがわかった。視床室傍核は、第三脳室に面しているため、脳を左右に切り分ける際に、損傷する場合が少なくないと考えられた。しかし、トルイジンブルー染色のみでは視床室傍核の同定は容易でないため、免疫組織化学的手法により、視床室傍核の同定を行うべく検討を進めている。

ストレスにより賦活される神経系を同定するた

め、マウスに強制水泳試験を行った後、脳を摘出し、灌流固定を行って、c-Fos 免疫組織化学による検討を行った。その結果、前辺縁／下辺縁皮質、外側中隔、扁桃体基底外側核、内嗅領皮質、視床室傍核など、前頭辺縁系の諸部位に賦活が見られた。中でも、辺縁／下辺縁皮質の賦活は、尾懸垂試験では見られず、強制水泳に特異的な所見と考えられた。

また、免疫学的なうつ病モデルとして、エンドトキシンである LPS(lipopolysaccharide)単回投与による行動変化および組織化学的変化の検討を開始した。Iba-1 によるミクログリアの分布を調べ、視床下部弓状核に活性化ミクログリアが多いなどの予備的結果を得た。また、抗うつ薬の活性化ミクログリアに対する影響を in vitro で調べた結果、いくつかの抗うつ薬が活性化ミクログリア由来の NO や炎症性サイトカイン産生を抑制することがわかった。これらの結果を元に、in vivo での検討を行う予定である。

久山町における疫学研究では、地域住民 3025 名のうち、CES-D によりうつ病と評価された者は、男性 3.3%、女性 4.7%であった。男性では、メタボリック症候群を持つ者におけるうつ病の頻度は 7.3%と、非メタボリック症候群の者（2.8%）より有意に高かった。女性では差は見られなかった。これらのことから、男性では、メタボリック症候群がうつ病の危険因子となる可能性が考えられた。

血管性うつ病の長期予後研究では、広島大学病院で入院加療をおこなった 50 歳以上で初発のうつ病患者 172 例でカルテおよび本人・家族への調査票によって、気分障害に関する長期予後および生命予後、認知症、その他の神経学的障害の有無を調査した。5 年以上の経過観察が可能であった 88 例について、頭部 MRI により潜在性脳梗塞の有無を評価し、血管性うつ病群および非血管性うつ病群の 2 群に分類し、長期的な予後について比較した。

その結果、血管性うつ病群では、寛解を継続した割合（5%）が、非血管性うつ病群（36%）に比して有意に低かった。10 年転帰では、血管性うつ病では、認知症に進展した割合が有意に多かった。

高齢者ブレインバンクでは、この 1 年で、2 名のうつ病の剖検例があり、死後脳の病理学的な検索を行った。

症例 1 は、死亡時 81 歳女性である。死亡の 1 年 7 ヶ月前、他人に強く叱責され、精神的不安・不眠・記憶力減退が出現した。認知症初期の疑いとして精神科に紹介されたが、薬物治療で軽快した。うつ状態、不安神経症と診断された。死亡半年前より食事がとれなくなったが、近医で精神的なものと診断された。その後、悪性リンパ種のため、死亡した。

神経病理学的検索の結果、神経原線維変化はステージ 4、老人斑はステージ 1、嗜銀顆粒ステージ 1、レヴィー小体は認めず、との結果であり、神経原線維変化優位型の老年性変化と考えられた。

症例 2 は、 死亡時 70 歳女性である。長女が統合失調症である。50 歳ころより動悸、気分の落ち込みがあり、更年期のうつ症状として、デパスを常用していた。66 歳時、気分の落ち込みが強くなったため、ドグマチールを処方され、パーキンソン症状が出現し、動けなくなった。内服を中止したが改善せず、左優位の安静時振戦、筋強剛より、Yahr II 度の本態性パーキンソン病と診断された。抗パーキンソン病薬に反応し、改善した。67 歳、注意集中力障害、幻視のため、アリセプトを開始した。69 歳時、ADL は車いすレベルで、尿失禁、便秘も出現。70 歳時、Yahr V 度の状態で入院し、肺炎で死亡した。

神経原線維変化ステージ 4、老人斑はステージ 3、嗜銀顆粒ステージ 1、レヴィー小体ステージ 5、との結果であり、病理学的にはアルツハイマー型のびまん性 Lewy 小体病に矛盾しない結果であった。

一方、これまでの剖検例のうち、双極性障害の既往を調べたところ、3 名が該当し、1 例（88 歳男性）は Binswanger 病、1 例（67 歳男性）は軽度低酸素虚血性変化、1 例（79 歳男性）は大脳皮質基底核変性症の病理診断であった。

これらの結果より、老年期のうつ病および双極性障害では、前頭側頭型認知症、アルツハイマー病、脳虚血性変化、大脳皮質基底核変性症など、さまざまな神経病理学的変化を伴う場合があることがわかった。

更に、高齢者ブレインバンクにおけるこれまでの剖検例において、予備的にうつ病の病歴を探索したところ、31 例（うち 8 例は凍結サンプルも保存）でうつ病の病歴が確認された。これらの症例についても、今後、神経病理学的所見を検討していく予定である。

D.考察

動物モデル研究では、気分障害の原因脳部位として、視床室傍核、前辺縁／下辺縁皮質などの脳構造の関与が疑われた。

一方、疫学研究および動物モデル研究から、免疫系、メタボリック症候群、脳血管障害など、多彩な因子が気分障害の危険因子となっていることがわかってきた。

これらの結果生じる脳病理学的変化として、老

年期の気分障害患者における検討からは、神経原線維変化、老人斑、Lewy 小体、脳虚血性変化など、さまざまな神経病理学的過程が関与する可能性が示唆された。

これらの病理学的変化は、高齢者の気分障害で見られたものであり、気分障害患者全般にも見られるものではないかも知れない。また、これらの病理学的な所見は、うつ病に特異的なものではない。しかしながら、本研究で明らかになった気分障害関連脳部位に注目し、更に検索を進めることで、うつ病の病態に迫ることができる可能性も考えられる。

一方、気分障害の責任部位と考えられる微小脳部位は、脳保存時に損傷されやすいという問題点も判明した。

E.結論

これまでのうつ病研究では、抗うつ薬の作用機序などに注目される場合が多く、脳のどの部位にどのような病理学的異常が生じるのかは、わかっていなかった。

本研究の結果、気分障害に視床室傍核や下辺縁皮質などの関与が疑われること、炎症、メタボリック症候群、血管性変化、神経変性など、さまざまな要因による病理学的変化がうつ病の背景となっている可能性が示唆された。

今後、更に症例を増やし、これらの要因の関与について、より詳細に検討していく予定である。

F.研究発表

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G.知的財産権の出願・登録状況

1. 特許取得
   なし
2. 実用新案登録
   なし

3. その他

研究成果の刊行一覧表（平成21年度）

加藤　忠史

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資　　料

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Regular Article

Mutation screening and assessment of the effect of genetic variations on expression and RNA editing of serotonin receptor 2C in the human brain

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Aim: Serotonin receptor 2C (*HTR2C*) has been postulated as being involved in the etiology or pathophysiology of mental disorders such as bipolar disorder, major depression and schizophrenia. We previously revealed the altered mRNA expression and RNA editing of *HTR2C* in the postmortem brains of patients with mental disorders. Here we examined the relationship between genetic variations and expression level or RNA editing level of *HTR2C* in the human brain.

Methods: We performed mutation screening of the *HTR2C* gene by sequencing all exons, exon–intron boundaries, and promoter region in the same cohort used for expression and RNA editing studies ($n = 58$). Using the detected genetic variations, we examined the relationship between genetic variations and expression or RNA editing level.

Results and conclusion: We did not find novel mutations or single nucleotide polymorphisms that were specific to patients. Genotype and haplotype-based analyses revealed that genetic variations of *HTR2C* did not account for observed altered expression or RNA editing level of *HTR2C* in the brain.

Key words: bipolar disorder, depression, HTR2C, postmortem brain, schizophrenia.

SEROTONIN IS A monoamine neurotransmitter that plays important roles in regulation of mood, appetite, sexual behavior and anxiety. Serotonin receptor 2C (*HTR2C*) encodes a G protein-coupled receptor that activates phospholipase C. Knock-out mice for this gene showed various phenotypic and behavioral alterations including seizure, enhanced exploration of a novel environment, and dysregulation of anxiety-related behaviors,[1–3] implicating the involvement of this gene in the pathophysiology of mental disorders. Several studies also reported the significant associations between genetic variations of *HTR2C* and major depression (MD), bipolar disorder (BD),[4,5] increased risk for lifetime hospitalization in schizophrenia (SZ),[6] and psychotic symptoms of Alzheimer' s disease.[7,8] Among the known 14 serotonin receptors, *HTR2C* has a unique feature in that its pre-mRNA undergoes adenosine-to-inosine (A-to-I) RNA editing by adenosine deaminases.[9] In A-to-I RNA editing, the translation machinery recognizes inosine as guanosine, resulting in changes in amino acid sequences. In cases of *HTR2C*, there are five editable positions (termed sites A to E) in exon 5 (Fig. 1), which theoretically result in a generation of up to 24 different HTR2C, in terms of amino acid sequences.[9,10]

In the studies using postmortem brains, decreased expression of *HTR2C* was reported in patients with SZ,[11,12] and altered RNA editing in patients with SZ,[13] MD,[14] and BD[15] as well as an animal model of depression.[16] Consistent with these results, we have also

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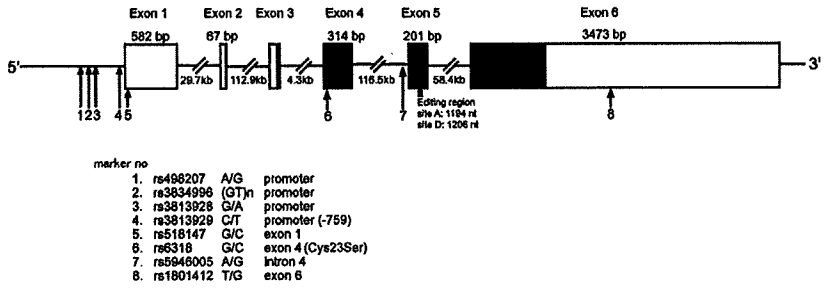

Figure 1. Genomic structure of the serotonin receptor 2C (*HTR2C*) gene. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. SNP detected in this study are indicated by arrows. SNP are denoted as major allele/minor allele. RNA editing sites are indicated by a black bar.

revealed decreased expression of *HTR2C* in brains of patients with SZ and BD,[17] and altered RNA editing status in major depression and suicide victims.[18] To examine the possible cause of the altered status of expression and RNA editing, we here performed mutation screening of the *HTR2C* gene in the same cohort used in previous studies. We then examined the effect of genetic variations on expression and RNA editing level of *HTR2C* in the brain.

METHODS

Postmortem samples

Postmortem liver samples corresponding to prefrontal cortex samples, using previous expression[17] and RNA editing[18] studies, were donated by the Stanley Medical Research Institute (SMRI). This sample set, called Consortium Collection, consisted of patients with BD, MD and SZ, as well as control subjects. Each diagnostic group consisted of 15 subjects. Diagnosis had been made according to the DSM-IV criteria. Detailed information about subjects are found elsewhere.[19] Of the 60 liver samples, we successfully extracted genomic DNA from 58 samples (15 BD, 15 MD, 14 SZ, and 14 controls) by standard phenol-chloroform extraction method.

Polymerase chain reaction and DNA sequencing

To screen the mutations in the *HTR2C* genomic region, we sequenced all exons, exon–intron boundaries, and the promoter region (Fig. 1). The polymerase chain reaction (PCR)-amplified regions, primer pairs and PCR conditions are available upon request. PCR products were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3730xl DNA analyzer (Applied-Biosystems, Foster City, CA). The number of dinucleotide repeats at a microsatellite (rs3834996) was determined by 3130xl Genetic Analyzer (Applied Biosystems) using primers FAM-labeled 5′-TGCTGTTTGTTGAAATGAAATG-3′ and 5′-GTTTCTTGAGAGGGAAGGCTTTCTCAA-3′.

Data analysis

In the genotype- and haplotype-based analyses, one-way ANOVA was conducted using the genotype or haplotype as the factor variable, and expression level or RNA editing efficiency as the dependent variable using SPSS 11.0J software (SPSS Japan). In cases where the number of informative genotypes or haplotypes was too small (≤ 2), we omitted that group from the ANOVA. We employed the Student's *t*-test for two-group comparison. $P < 0.05$ was considered to be significant. Previously determined *HTR2C* expression level[17] and RNA editing efficiency[18] in postmortem prefrontal cortex (Brodmann's Area 10) were used for data analysis. Of the five edited sites, editing efficiency of two sites (sites A and D) were available.[20] Power analysis was performed using G*power software.[21]

RESULTS

The genomic structure of the *HTR2C* gene (Xq24) is shown in Fig. 1. We sequenced all six exons, exon–intron boundaries and the promoter region (about 500 bp upstream region from exon 1) in a total of 58 subjects (15 BD, 15 MD, 14 SZ, and 14 controls). We detected seven SNP and a dinucleotide (GT) repeat polymorphism in these subjects (Fig. 1). All polymorphisms are known genetic variations and could be found in the SNP database. Among them, the rs3813929 (–795C/T) was reported to alter transcriptional activities by *in vitro* reporter assays, and the rs6318 (Cys23Ser) was shown to alter receptor activ-

ity.[22] First, we examined whether each of the eight polymorphisms affected the mRNA expression or RNA editing level of *HTR2C* in the brain. We did not find significant effects of polymorphisms on the expression level of *HTR2C*, including −795C/T (Fig. 2) and Cys23Ser functional polymorphisms (data not shown). Similarly, we did not find significant effects of genetic variations on the RNA editing efficiency at site A or D (data not shown). We also confirmed that there were no significant effects of genetic variations on expression or RNA editing level using only control subjects (data not shown).

We then performed haplotype-based analysis using five polymorphisms in the promoter region (SNP #1–5 in Fig. 1). We conducted ANOVA using the three representative haplotype combinations (diplotypes; $A(GT)_{16}GCG/A(GT)_{16}GCG$ or $A(GT)_{16}GCG/-$, $A(GT)_{16}GCG/G(GT)_{13}ATC$, and $G(GT)_{13}ATC/G(GT)_{13}ATC$ or $G(GT)_{13}ATC/-$) as factor variables. However, we could not detect significant differences of expression or RNA editing efficiency across three diplotypes (Fig. 3). We then performed haplotype-based analysis using all detected polymorphisms (SNP #1–8 in Fig. 1) in the *HTR2C*. Although there were 12 diplotypes within 58 subjects, six diplotypes were omitted from analysis because of the small number of subjects ($n \leq 2$). Using the remaining six diplotypes as factor variables, we performed ANOVA again. However, we could not detect significant differences of expression or RNA editing efficiency across haplotypes (data

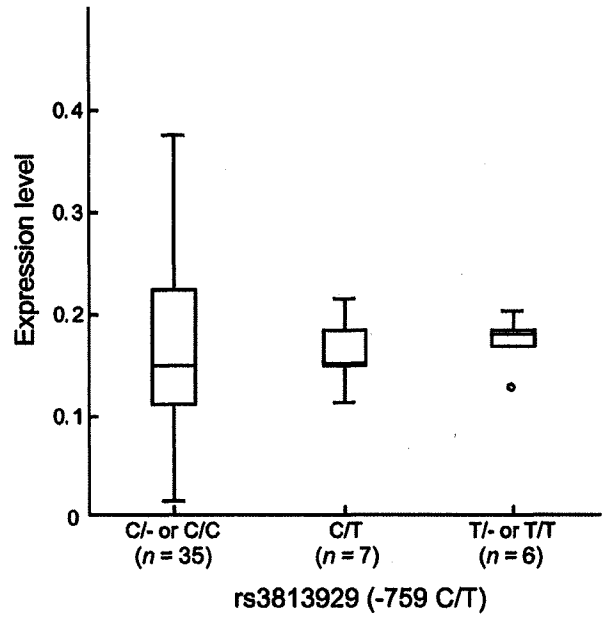

Figure 2. The rs3813929 (−795C/T) genotype and serotonin receptor 2C (*HTR2C*) mRNA expression level. In the box plot, top, bottom, and middle bars represent maximum, minimum, and median values, respectively. The top and bottom box represent third and first quartiles, respectively. Note that *HTR2C* is located on the X chromosome.

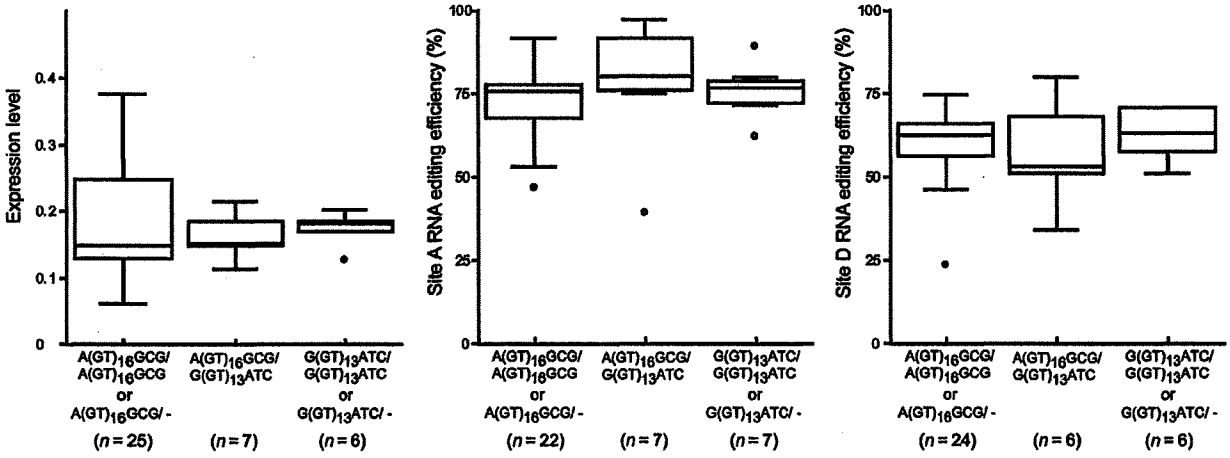

Figure 3. Promoter diplotype, mRNA expression level and RNA editing efficiency of the serotonin receptor 2C (*HTR2C*). Diplotypes were constructed using five promoter SNP (SNP #1–5 in Fig. 1).

not shown). We also examined the relationship between expression level and RNA editing efficiency of *HTR2C* in the brain, which was not addressed in the previous studies, but we found no significant correlations between them ($R = 0.118$, $P = 0.450$ for site A, and $R = 0.163$, $P = 0.297$ for site D). Because *HTR2C* is located on chromosome X, we then performed the same analyses in male and female groups, respectively. However, we did not find significant relationship between these variables.

DISCUSSION

In the sample set used in this study, *HTR2C* expression showed a 1.55-fold decrease in BD and a 1.46-fold decrease in SZ.[17] We also reported a tendency toward an increase of RNA editing efficiency at site D in MD subjects and at site A in suicide victims.[18] Sequence analysis of *HTR2C* in the same cohort revealed that genetic variations of *HTR2C* did not affect the expression or RNA editing status in the brain. A similar result with regard to *HTR2C* expression in the brain was also reported by Castensson *et al.*[11] They compared the effect of haplotypes, which were similar to this study on the expression level of *HTR2C* in the brain, and they did not find significant differences either.[11]

In contrast, *in vitro* reporter assays revealed that promoter genotype or haplotype of *HTR2C* affected the transcriptional activity.[23–26] Among the studies, McCarthy *et al.* and Hill *et al.* used the haplotype constructs that were comparable to our study, for reporter assays.[25,26] They reported that haplotype $(GT)_{16}GCG$ showed higher promoter activity than $(GT)_{13}ATC$. The cause of discrepancy might be due to the relatively small sample size ($n = 58$) in this study, which lacks the statistical power to detect the small effects. Indeed, power analysis revealed that, in our sample size, we could detect a 'large' effect size ($f = 0.4$)[27] at the power of 0.55 in ANOVA. However, it is also clear that the observed decreased expression of *HTR2C* in brains of patients cannot be fully explained by the genetic variations of *HTR2C*.

Therefore, other factors may be involved in the observed altered expression and RNA editing statuses of *HTR2C* in patients with mental disorders. Because we did not detect correlations between expression and the RNA editing level of *HTR2C*, independent factors may contribute to their altered statuses in the brain.

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Effect of mood stabilizers on gene expression in lymphoblastoid cells

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Abstract Lithium and valproate are widely used as effective mood stabilizers for the treatment of bipolar disorder. To elucidate the common molecular effect of these drugs on non-neuronal cells, we studied the gene expression changes induced by these drugs. Lymphoblastoid cell cultures derived from lymphocytes harvested from three healthy subjects were incubated in medium containing therapeutic concentrations of lithium (0.75 mM) or valproate (100 μg ml^{-1}) for 7 days. Gene expression profiling was performed using an Affymetrix HGU95Av2 array containing approximately 12,000 probe sets. We identified 44 and 416 genes that were regulated by lithium and valproate, respectively. Most of the genes were not commonly affected by the two drugs. Among the 18 genes commonly altered by both drugs, vascular endothelial growth factor A (*VEGFA*), which is one of the *VEGF* gene isoforms, showed the largest downregulation. Our findings indicate that these two structurally dissimilar mood stabilizers, lithium, and valproate, alter *VEGFA* expression. *VEGFA* might be a useful biomarker of their effects on peripheral tissue.

Keywords Lithium · Valproate · Lymphoblastoid cells · DNA microarray · Vascular endothelial growth factor A

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Introduction

Bipolar disorder is a severe mental disorder characterized by recurrent episodes of mania and depression. Although the pathogenesis of bipolar disorder is unclear, mood stabilizers such as lithium and valproate can prevent its recurrence (Kato 2007). Lithium is the mainstay of treatment for bipolar disorder in many countries, both for acute mania and as prophylaxis for recurrent manic and depressive states (Geddes et al. 2004).

The molecular mechanism of their clinical actions as mood stabilizers has been extensively studied. Nonaka et al. (1998) and Chen et al. (1999c) demonstrated neuroprotective effects of lithium and valproate. These two mood stabilizers exert the same effects on neurons, such as growth cone enlargement (Williams et al. 2002) and enhanced adult neurogenesis (Chen et al. 2000; Hao et al. 2004). The effects may be mediated by depletion of inositol (Williams et al. 2002), upregulation of BCL-2 (Chen et al. 1999c), inhibition of glycogen synthase kinase 3b (Chen et al. 1999b; Klein and Melton 1996), altered glutamate receptor trafficking (Du et al. 2003), upregulation of BAG-1 (Zhou et al. 2005), inhibition of glucocorticoid receptor function (Basta-Kaim et al. 2004), upregulation of glutathione S-transferase (Cui et al. 2007), upregulation of brain-derived nerve growth factor (Yasuda et al. 2009), and activation of Notch signaling (Higashi et al. 2008). Inhibition of histone deacetylase (Leng et al. 2008) might also have a role in the neuroprotective effect of valproate.

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In most of these studies, neuronal cell cultures or neuronal cell lines were used, and very few studies examined the effect of these drugs on peripheral tissues or non-neuronal cell lines. The effects of these mood stabilizers on non-neuronal cells might also play a role in their clinical effects. Furthermore, a common molecular reaction between neurons and peripheral blood cells relevant to their clinical effects may be useful as a peripheral marker of the therapeutic response.

Although the usefulness of blood-derived samples such as lymphoblastoid cells (LCs) for studying mental disorders and pharmacogenetics is controversial (Choy et al. 2008; Matigian et al. 2008), several gene expression profiling studies of mental disorders have been performed using lymphocytes or LCs (Kakiuchi et al. 2003, 2008; Matigian et al. 2008; Sun et al. 2004; Vawter et al. 2004). Various phenotypes, such as abnormal Ca^{2+} levels (Emamghoreishi et al. 1997; Kato et al. 2003), altered endoplasmic reticulum stress response (Hayashi et al. 2009; Kakiuchi et al. 2003; So et al. 2007), and altered inositol levels (Belmaker et al. 2002), have been reported in LCs derived from patients with bipolar disorder.

Here we performed a comprehensive DNA microarray analysis of genes regulated by lithium and valproate in the LCs to identify their effects on peripheral tissue. Our findings indicated that lithium and valproate have distinctive as well as common effects on gene expression in LCs. Among the genes whose altered expression was common to lithium and valproate, vascular endothelial growth factor A (*VEGFA*) was the most downregulated. *VEGFA* might be a useful biomarker of their effects on peripheral tissue.

Materials and methods

Subjects

For DNA microarray and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), we used LCs derived from three unrelated healthy Japanese males (33, 42, and 49 years old). This study was approved by the Research Ethics Committee of RIKEN.

Lymphoblastoid cell culture

Lymphocytes from peripheral blood cells were transformed by Epstein–Barr virus using standard techniques as described previously (Kato et al. 2003). Briefly, lymphocytes were separated from peripheral blood and cultured with RPMI 1640 medium containing 20% fetal bovine serum (GIBCO, Carlsbad, CA, USA), penicillin and streptomycin (50 μg ml^{-1} each), and supernatant of the B95-8 cell culture infected by Epstein–Barr virus. The cells were passaged every week until the cells showed stable growth. Thereafter, the cells were passaged two or three times a week using similar medium, except for the addition of 10% fetal bovine serum.

To investigate the effects of the mood stabilizers, we used LCs derived from three unrelated healthy Japanese men. The cells were cultured in medium containing therapeutic concentrations of lithium (0.75 mM) and valproate (100 μg ml^{-1}) or drug-free control medium for 7 days.

DNA microarray procedure

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and cleaned using an RNeasy column (Qiagen, Valencia, CA, USA). DNA microarray analysis was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA) as described previously (Iwamoto et al. 2004). Briefly, 8–10 μg total RNA was used to synthesize cDNA by SuperScript II reverse transcriptase (Invitrogen), which was then used to generate biotinylated cRNA. cRNA was fragmented and first applied to the TestChip (Affymetrix) to assess the sample quality, and then applied to the HU95Av2 chip (Affymetrix), which contained approximately 12,000 probe sets. The hybridization signal on the chip was scanned using a scanner (HP GeneArray scanner, Hewlett-Packard, Palo Alto, CA, USA), and processed with microarray suite 5 (MAS5, Affymetrix).

DNA microarray data analysis

DNA microarray raw data were imported into GeneSpring GX10 software (Agilent Technologies, Santa Clara, CA, USA). Data from each array were then normalized by dividing each value by the median whole gene expression value. We then conducted a principal component analysis (PCA) to visualize the relationship among experiments using the expression values of all probe sets on the array. To examine quantitative expression changes in response to drug treatment, we used probe sets whose flag statuses were present or marginal in at least five of nine samples for statistical analysis. Of approximately 12,000 probe sets, 5,656 probe sets passed this filtering process. Genes that were differentially expressed compared with control samples were identified using one-way analysis of variance [ANOVA ($df = 2$, lithium-treated, valproate-treated, and control samples)] followed by post hoc Tukey's test. A P value of less than 0.05 was considered significant. Genes with significantly different levels of expression between drug-treated and control samples were selected. Gene ontology analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis et al. 2003; Huang da et al. 2009). The significantly enriched terms were identified using Bonferroni's correction for multiple comparisons with a cutoff of $P < 0.05$.

Real-time quantitative RT-PCR

After DNase I treatment, 5 μg total RNA was used for cDNA synthesis by oligo(dT) 12–18 primer and Super-Script II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR using SYBER/GREEN I dye (Applied Biosystems, Foster City, CA, USA) was performed with ABI PRISM 7900HT (Applied Biosystems). After denaturation at 95°C for 10 min, the PCR conditions were 95°C for 45 s and 60°C for 45 s for 40 cycles. The comparative C_t method was used to quantify transcripts according to the manufacturer's protocol (User Bulletin #2, Applied Biosystems). Each sample was quantified in triplicate. Amplification of single products was confirmed by monitoring the dissociation curve and by agarose gel electrophoresis. We used the expression level of cofilin 1 as an internal control, as validated previously (Iwamoto et al. 2004). Primer pairs for cofilin 1 were reported previously (Iwamoto et al. 2004). Primer pairs used for qRT-PCR were as follows: *VEGFA*, 5′-TACTGCCATCCAATCGAGACC-3′ and 5′-GGTTTG ATCCGCATAATCTGC-3′. Two-tailed paired *t*-tests were used for the statistical analysis of the results of the control and lithium or valproate-treated samples.

Results

LCs established from three unrelated male subjects were cultured with therapeutic concentrations of lithium or valproate. After 7 days culture, total RNA was extracted from the cells and used for DNA microarray analysis. After data normalization, we first performed a PCA to visualize the relationship among the lithium- or valproate-treated samples and control samples (Fig. 1). The lithium-treated samples differed little from their respective control samples. On the other hand, the valproate-treated samples were quite different from the control samples. These results suggest the slight and drastic effects of lithium and valproate, respectively, on global gene expression in LCs in our experimental condition.

To identify differentially expressed probe sets, we performed an ANOVA followed by Tukey's test. Lithium induced altered expression in 44 probe sets, of which 17 were downregulated and 27 were upregulated (see Supplementary Table 1). On the other hand, valproate induced altered expression in 416 probe sets, of which 222 were downregulated and 194 were upregulated (see Supplementary Table 2). The ten most downregulated and upregulated probe sets affected by lithium and valproate treatment are listed in Tables 1 and 2, respectively, as representatives. Among the identified probe sets, the expression of 18 probe sets was altered by both lithium and valproate. Alterations

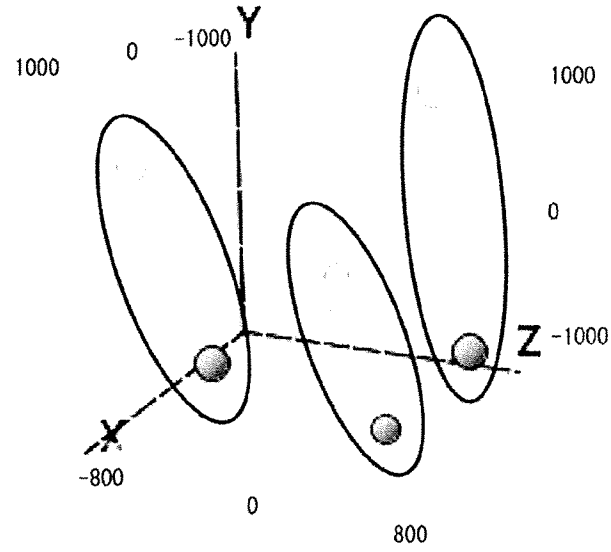

Fig. 1 Results of the PCA analysis. *X*, *Y*, and *Z* axes show the first, second, and third components, respectively. *White*, *gray*, and *black spheres* show control, lithium, and valproate-treated samples, respectively. Each ellipse surrounds the data of the same individual

of all of these 18 probe sets were in the same direction (Table 3).

A gene ontology analysis was then performed using the DAVID software (Table 4). The differential gene expression induced by valproate was significantly enriched in metabolic process-related and cellular process-related terms in the biologic process category, and nucleic acid-binding and protein-binding terms in the molecular function category. In the cellular component category, genes seemed to be enriched in every part of the cell, including the nucleus, cytoplasm, and organelles. We also performed a gene ontology analysis of the differential gene expression induced by lithium and that induced by both drugs. No significant enrichment was observed, however, after multiple testing corrections, possibly due to the small number of genes used for analysis (data not shown).

We also analyzed the expression level of *VEGFA*, which was the gene most downregulated by both lithium and valproate, using real-time quantitative RT-PCR (Fig. 2). We confirmed significant downregulation of *VEGFA* in valproate-treated LCs ($P = 0.02$). We also observed downregulation of *VEGFA* in lithium-treated LCs, but this result was not significant ($P = 0.21$).

Discussion

Both lithium and valproate are widely used in the treatment of bipolar disorder as mood stabilizers. These two structurally unrelated drugs have similar clinical efficacy for

Table 1 The ten genes most downregulated and upregulated by lithium

Probe ID	Accession	Fold change	Gene symbol	Gene title
2067_f_at	D44466	−1.86	BAX	BCL2-associated X protein
2065_s_at	U67092	−1.70	BAX	BCL2-associated X protein
37632_s_at	U19599	−1.62	ZRF1	Zuotin related factor 1
38837_at	L22473	−1.59	TXNDC13	Thioredoxin domain containing 13
450_g_at	L22475	−1.57	CGRRF1	Cell growth regulator with ring finger domain 1
1997_s_at	X78283	−1.57	BAX	BCL2-associated X protein
36639_at	AC002985	−1.51	ADSL	Adenylosuccinate lyase
36101_s_at	AF060798	−1.45	VEGFA	Vascular endothelial growth factor A
41003_at	X79201	−1.37	PFDN4	Prefoldin subunit 4
34417_at	D63390	−1.35	DPY19L2P2	dpy-19-like 2 pseudogene 2 (*C. elegans*)
32445_at	AF057160	2.03	PAFAH1B2	Platelet-activating factor acetylhydrolase, isoform Ib, beta subunit 30 kDa
31872_at	X98260	1.87	SS18	Synovial sarcoma translocation, chromosome 18
38365_at	X80754	1.72	PEX1	Peroxisome biogenesis factor 1
41601_at	D25547	1.68	–	Transcribed locus
37837_at	AB020670	1.67	ADNP2	ADNP homeobox 2
940_g_at	D87449	1.64	NF1	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)
37888_at	AB007858	1.57	SLC35D1	Solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1
31804_f_at	Y10055	1.50	SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1
31868_at	AF026086	1.47	STK16	Serine/threonine kinase 16
33731_at	AA552140	1.47	SLC7A7	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7

Among the 27 probe sets identified, the top ten downregulated and upregulated probe sets are listed as representative

bipolar disorder, and thus we searched for a common molecular effect of these drugs. Although these drugs share clinical efficacy for classical mania, valproate has specific efficacy for irritable or dysphoric mania (Swann et al. 2002). Therefore, lithium and valproate may have common and drug-specific molecular effects. At the gene expression level in the LCs, the effect of lithium was modest compared with that of valproate. These two drugs showed distinctive gene expression changes with altered expression of only a very small number of common genes.

Gene expression changes induced by lithium

In our experiments, the effect of lithium on gene expression was relatively small. In recent studies, using mouse brain (Du et al. 2003) or human neuronal cell lines treated with lithium (Seelan et al. 2008), altered expression of more genes was reported. These differences may reflect the differences in the tissues or treatment methods used. The expression of three different probe sets for BCL-2 associated X protein (*BAX*) was consistently downregulated by lithium (Table 1). BAX is a BCL-2 family protein that promotes apoptosis by enhancing cytochrome c release (Graham et al. 2000). Several studies have reported that lithium upregulates anti-apoptotic BCL-2 and downregulates pro-apoptotic BAX (Chen and Chuang 1999; Ghribi et al. 2002; Somervaille et al. 2001). Our findings are consistent with these findings in the brain and neuronal cells.

On the other hand, the gene most upregulated by lithium was platelet-activating factor acetylhydrolase, isoform 1b, beta subunit (*PAFAH1B2*), which encodes one of the catalytic subunits of the PAFAH1B complex, alpha2. The PAFAH1B alpha complex is an enzyme composed of alpha2, alpha1 encoded by *PAFAH1B3*, and Lis1 encoded by *PAFAH1B1* (Hattori et al. 1993). Among them, *PAFAH1B3* has been identified as one of eight genes that show specific alterations in the postmortem brains of bipolar individuals (Nakatani et al. 2006). These enzymes interact biochemically and genetically with the Reelin (*RELN*) pathway, and RELN and Lis activities are important for neuronal migration and cellular layer formation in the brain (Assadi et al. 2003; Hirotsune et al. 1998). The Pafah1b alpha2 subunit encoded by the *Pafah1b2* gene reportedly does not affect cortical layer formation, but is critically involved in the development of hydrocephalus in double mutant mice defective in Reelin and Lis signaling (Assadi et al. 2008). Considering the possible role of the

Table 2 The ten genes most downregulated and upregulated by valproate

Probe ID	Accession	Fold change	Gene symbol	Gene title
39827_at	AA522530	−2.72	DDIT4	DNA-damage-inducible transcript 4
36398_at	W28729	−2.71	–	Human retina cDNA
36101_s_at	M63978	−2.52	VEGFA	Vascular endothelial growth factor A
37326_at	U93305	−2.51	PLP2	Proteolipid protein 2 (colonic epithelium-enriched)
35530_f_at	X92997	−2.45	–	Immunoglobulin lambda locus
282_at	L16782	−2.45	MPHOSPH1	M-phase phosphoprotein 1
40790_at	AB004066	−2.28	BHLHB2	Basic helix-loop-helix domain containing, class B, 2
300_f_at[a]	–	−2.23	–	–
40329_at	AL031228	−2.23	SLC39A7	Solute carrier family 39 (zinc transporter), member 7
38010_at	AF002697	−2.22	BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
35778_at	AB011103	6.20	KIF5C	Kinesin family member 5C
668_s_at	L22524	5.20	MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)
36874_at	M26004	4.34	CR2	Complement component (3d/Epstein–Barr virus) receptor 2
1575_at	M14758	3.85	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
40862_i_at	X15334	3.63	CKB	Creatine kinase, brain
1358_s_at	U22970	3.40	IFI6	Interferon, alpha-inducible protein 6
915_at	M24594	3.09	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
32814_at	M24594	3.05	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1
38584_at	AF026939	2.89	IFIT3	Interferon-induced protein with tetratricopeptide repeats 3
33232_at	AI017574	2.85	CRIP1	Cysteine-rich protein 1 (intestinal)/hypothetical protein LOC100127933

Among the 416 probe sets identified, the top 10 downregulated and upregulated probe sets are listed as representative

[a] 300_f_at was not annotated

PAFAH1B2 gene in brain development, an increase in *PAFAH1B2* may contribute to the mechanism of action of mood stabilizers, especially lithium.

Gene expression changes induced by valproate

Valproate is characterized as a histone deacetylase inhibitor (Phiel et al. 2001). Histone deacetylases control the transcription of a large number of genes by altering chromosome dynamics. In the present study, the PCA revealed more extensive gene expression alterations induced by valproate than by lithium. Statistical analysis also revealed that valproate affected a larger number of differentially expressed probe sets than lithium. Gene ontology analysis revealed that valproate affected the expression of genes with wide-ranging biologic functions. Consistent with previous studies in the brain (Bosetti et al. 2005; Chetcuti et al. 2006), we determined that valproate also affects multiple signaling pathways in LCs. BCL-2/adenovirus E1B 19 kDa interacting protein (*BNIP3*) was one of the ten most downregulated genes by valproate (Table 2). *BNIP3*, which is a BCL-2 family member, is upregulated during stress such as hypoxia and induces cell death by inducing mitochondrial dysfunction (Kim et al. 2002; Vande Velde et al. 2000). Downregulation of *BNIP3* may also contribute to the cytoprotective effect of valproate.

Altered gene expression commonly induced by lithium and valproate

Eighteen probe sets were identified as differentially expressed genes common to lithium and valproate. *VEGFA*, strongly associated with angiogenesis in the *VEGF* gene family (Ferrara 2002), was the gene most downregulated by both lithium and valproate. The expression level of *VEGFA* was significantly decreased by valproate, and tended to be decreased by lithium in real time RT-PCR.

VEGF was initially identified as a critical regulator of angiogenesis and a potent inducer of vascular permeability in many types of tissues, including the blood brain barrier (Ferrara et al. 2003; Gora-Kupilas and Josko 2005; Rigau et al. 2007). *VEGF* also has neurogenic and neuroprotective effects (Jin et al. 2002; Schanzer et al. 2004; Storkebaum et al. 2004). Recent studies indicate that *VEGF* may be involved in the molecular mechanisms of the pathogenesis and response to treatment for depression. In animal studies, *VEGF* is induced by antidepressant drugs (Warner-Schmidt and Duman 2007) and electroconvulsive shock

Table 3 The list of common differentially expressed genes by lithium and valproate

Probe ID	Accession	Fold change		Gene symbol	Gene title
		Lithium	Valproate		
36101_s_at	D44466	−1.45	−2.52	VEGFA	Vascular endothelial growth factor A
41003_at	X79201	−1.37	−1.39	PFDN4	Prefoldin subunit 4
34417_at	AL049437	−1.35	−1.63	DPY19L2P2	dpy-19-like 2 pseudogene 2 (*C. elegans*)
37737_at	H93123	−1.33	−1.46	PCMT1	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase
41595_at	AF074382	−1.22	−1.25	KIAA0947	KIAA0947 protein
38073_at	M63978	−1.15	−1.16	RNMT	RNA (guanine-7-) methyltransferase
31872_at	D25547	1.87	1.83	SS18	Synovial sarcoma translocation, chromosome 18
940_g_at	D87449	1.64	1.62	NF1	Neurofibromin 1 (neurofibromatosis, von Recklinghausen disease, Watson disease)
37888_at	AB007858	1.57	1.64	SLC35D1	Solute carrier family 35 (UDP-glucuronic acid/UDP-*N*-acetylgalactosamine dual transporter), member D1
38706_at	Y10055	1.47	1.49	E2F4	E2F transcription factor 4, p107/p130-binding
381_s_at	AA552140	1.45	1.90	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide
38945_at	X78710	1.34	1.66	MTF1	Metal-regulatory transcription factor 1
39434_at	AB011164	1.33	1.45	FAM21A/B/C/D	Family with sequence similarity 21, member A/B/C/D
35783_at	AF071504	1.30	1.57	VAMP3	Vesicle-associated membrane protein 3 (cellubrevin)
41047_at	U41816	1.26	1.24	C9orf16	Chromosome 9 open reading frame 16
36004_at	AI885170	1.22	1.29	IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
39858_s_at	AB023164	1.21	1.37	STX11	Syntaxin 11
1314_at	D12625	1.20	1.32	PSMD1	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1

(Altar et al. 2004; Newton et al. 2003; Segi-Nishida et al. 2008). Clinical studies demonstrated a higher expression level of *VEGF* mRNA and a decrease in these levels after treatment with antidepressants was observed in the peripheral leukocytes of depressed patients (Iga et al. 2007), but other studies reported conflicting results (Dome et al. 2009; Ventriglia et al. 2009).

Lithium increases myocardial levels of *VEGF* following ischemic insult (Kaga et al. 2006), promotes *VEGF* expression in brain endothelium and astrocytes (Guo et al. 2009), and prevents *VEGF* reduction in immature neurons of the hippocampus after chronic mild stress in rats (Silva et al. 2007). *VEGF* is decreased in a human neuronal cell line continuously maintained on therapeutic levels of lithium (Seelan et al. 2008). In addition, valproate promotes tumor growth arrest by downregulating *VEGF* (Gao et al. 2007; Zgouras et al. 2004).

In our experiments, both lithium and valproate decreased the gene expression of *VEGFA*. As described above, the direction of *VEGF* expression changes differed across studies. The discrepancy might be due to differences in the experimental methodology, such as in vivo or in vitro, the type of tissues, and treatment method. We used LCs derived from peripheral blood lymphocytes, and therefore, our findings suggest that the mechanisms of action of these mood stabilizers may be mediated in part by their effects on *VEGF* in non-neuronal cells. Valproate inhibits angiogenesis in endothelial cells, thereby protecting endothelial cells from stress-induced apoptosis (Michaelis et al. 2006). It is possible that the common antiangiogenic effect of lithium and valproate mediated by VEGF contributes to the neuroprotective effects in bipolar disorder.

Limitations

Although we used LCs in our experiment, the effect of mood stabilizers on non-neuronal cells might differ among various tissues. In addition, because the difference in drug sensitivity between non-neuronal cell and neuronal cell is suggested (Chen et al. 1999a; Gilad and Gilad 2007), we should examine lower and higher (toxic) dose of these drugs to explain whether the results are based on the treatment effects or toxic effects. The effects of mood stabilizers on other non-neuronal cells should also be studied. In our experiment, we used LCs rather than fresh lymphocytes because LCs are easily handled and cellular heterogeneity can be minimized. Whether this effect is also detected in non-transformed peripheral lymphocytes and can be used as a peripheral biomarker of the effects of mood stabilizers requires further study. The microarray data results did not cover the entire genome because the number of probe sets contained in the HU95Av2 chip is smaller than that of newer chip versions. Although we