表3　Phase Ⅱにあるうつ病対象化合物

分類	化合物名	開発会社	作用機序[a]	開発状況		
				米国	EU	日本
臨床試験参加率（%）[b]				70.0 (14/20)	30.0 (6/20)	0.0 (0/20)
モノアミン類関連	SEP-225289	Sepracor	TRI	phase Ⅱ	−	−
	GSK-372475	GlaxoSmithKline	TRI	−	phase Ⅱ	−
	LU-AA24530	Lundbeck	mixed serotonin modulator	−	phase Ⅱ	−
	Tyrima™	CeNeRx BioPharma	MAO A inhibitor	phase Ⅱ	−	−
タキキニン類関連	GW597599B	GlaxoSmithKline	NK1 antagonist	phase Ⅱ	−	−
	GW-679769	GlaxoSmithKline	NK1 antagonist	phase Ⅱ	phase Ⅱ	−
CRF 関連[c]	GSK-561679	GlaxoSmithKline	CRF1 antagonist	phase Ⅱ	phase Ⅰ	−
	BMS-562086	Bristol-Myers Squibb	CRF1 antagonist	phase Ⅱ	−	−
Vasopressin 関連	SSR149415	Sanofi-Aventis	vasopressin V1b antagonist	phase Ⅱ	phase Ⅱ	
グルタミン酸関連	AZD-6765	AstraZeneca	NMDA antagonist	phase Ⅱ	−	−
	ORG-26576	Schering-Plough	AMPA agonist	phase Ⅱ	−	−
コリン類関連	TC-5214	Targacept	nonselective nicotinic acetylcholine receptor antagonist	phase Ⅱ	−	−
	BCI-540	BrainCells	choline uptake enhancer	−	−	−
その他	GW-856553X	GlaxoSmithKline	p38 kinase inhibitor	−	−	−
	SA-4503	M's Science	opioid σ1 agonist	−	phase Ⅱ	−
	ORG-34517	Schering-Plough	glucocorticoid receptor antagonist	phase Ⅱ	−	−
	SSR-411298	sanofi-aventis	FAAH inhibitor	−	−	−
不明	RO-4917523	Roche	−	phase Ⅱ	−	−
	R-228060	Johnson & Johnson	−	phase Ⅱ	−	−
	AZD-2327	AstraZeneca	−	phase Ⅱ	−	−

serotonin や noradrenaline だけでなく dopamine の再取り込み阻害作用を有する TRI，神経ペプチド類（NK1，CRF1，vasopressin）関連の化合物，グルタミン酸（NMDA，AMPA）関連の化合物が phase Ⅱの開発段階にある。phase Ⅱにあるうつ病対象化合物の臨床試験には本邦は参加していない。

a）「明日の新薬」（http://www.technomics.co.jp/asusin/index.html）を使用した調査結果。
TRI：triple reuptake inhibitor（serotonin-noradrenaline-dopamine reuptake inhibitor），NK1：neurokinin 1，FAAH：fatty acid amide hydrolase，NMDA：N-methyl-D-aspartic acid，AMPA：α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

b）臨床試験参加率（%）＝各国で臨床試験が実施されている品目数 / 最高フェーズが phase Ⅱの全うつ病対象化合物数（20 品目）

c）CRF：corticotrophin-releasing factor

5HT1 antagonist である GSK-163090 のみであり，神経ペプチド受容体に関連する NK1 antagonist である orvepitant，NK2 antagonist である SAR-10279，NK2/NK3 antagonist である SSR-241586，CRF1 antagonist である GSK-586529 及び GW-876008 等が存在した。

3．うつ病対象化合物の臨床試験実施状況

2009 年 1 月 15 日時点で本邦，米国，EU において実施中のうつ病対象化合物の臨床試験の実施数を**図2**に示した。ここでは製薬会社が主導する臨床試験で phase Ⅱ及び phase Ⅲとして実施されているものを

表4　海外で phase Ⅰ実施中のうつ病対象化合物

化合物名	開発会社	作用機序[a)]
GSK-586529	GlaxoSmithKline	CRF1 antagonist
GW-876008	GlaxoSmithKline	CRF1 antagonist
orvepitant	GlaxoSmithKline	NK1 antagonist
SAR-10279	sanofi-aventis	NK2 antagonist
SSR-241586	sanofi-aventis	NK2/NK3 antagonist
GSK-163090	GlaxoSmithKline	5HT1 antagonist
SSR-101010	sanofi-aventis	FAAH inhibitor
TC-2216	Targacept	nicotinic receptor antagonist

神経ペプチド（CRF1，NK1，NK2，NK2/NK3）関連の化合物等が phase Ⅰの開発段階にある。
a）「明日の新薬」（http://www.technomics.co.jp/asusin/index.html）を使用した調査結果。
CRF：corticotrophin-releasing factor，NK：neurokinin，FAAH：fatty acid amide hydrolase

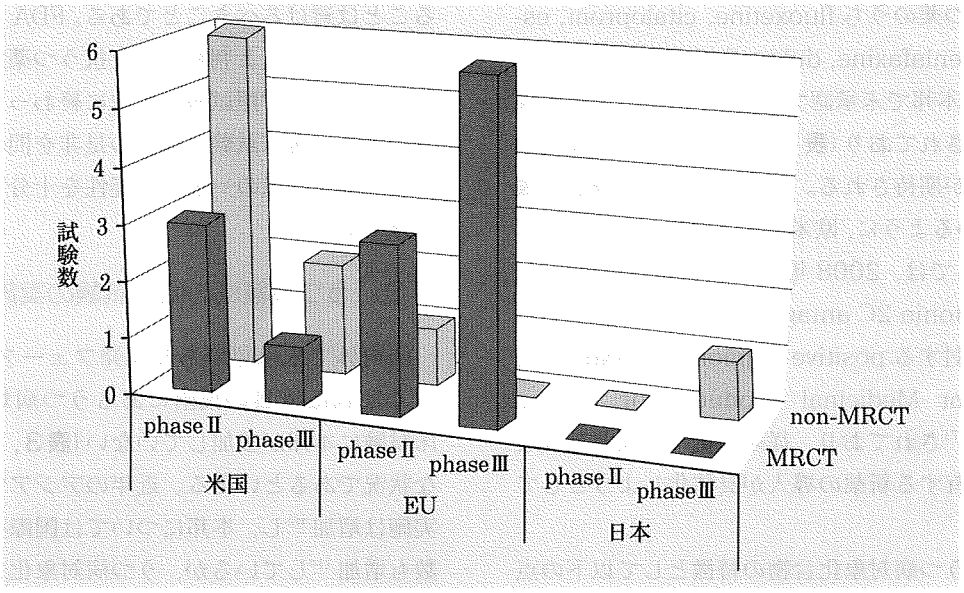

図2　うつ病対象化合物の臨床試験実施数（2009年1月15日時点）
米国（12試験）とEU（10試験）の実施数は同程度であったが，本邦での実施数（1試験）は少なかった。米国は国内試験が多かったのに対し，EUは国際共同による試験が多かった。

集計しており，海外で既承認でありかつ国内で未承認の抗うつ薬も含まれる。国際共同で実施している試験（**MRCT**：multi-regional clinical trial）と各国内のみで実施している試験（non-MRCT）別に図示した。EUについても，単独の国のみで行われている臨床試験をnon-MRCTとして扱った。うつ病を対象として実施中の臨床試験は，米国で12試験（phase Ⅱ：9試験，phase Ⅲ：3試験），EUで10試験（phase Ⅱ：4試験，phase Ⅲ：6試験）と試験数は同程度であったが，本邦では1試験（phase Ⅱ：0試験，phase Ⅲ：1試験）と少なかった。米国での試験は国際共同試験より国内単独によるものが多く（MRCT：4試験，non-MRCT：8試験），逆にEUでは国際共同による試験が多かった（MRCT：9試験，non-MRCT：1試験）

MRCT（multi-regional clinical trial）

のが特徴であった。本邦が参加しているのは，desvenlafaxine の第Ⅲ相 1 試験のみであったが，“Clinical Trials.gov”（http://www.clinicaltrials.gov/）に登録されていない本邦単独で開発が進められている臨床試験も存在する可能性はある。

Ⅳ．考察

1．国内外の抗うつ薬の承認状況と開発状況

本邦での SSRI 導入から 10 年が経過しようとしており，SSRI（paroxetine，fluvoxamine，sertraline），そして SNRI（milnacipran）の使用経験は十分に蓄積できている。欧米で標準治療薬[3~6]として位置づけられている抗うつ薬のうち fluoxetine，citalopram，escitalopram，venlafaxine，duloxetine，mirtazapi-ne，bupropion は本邦で未承認であるが，その一部も新薬承認申請がなされており（**表1**），うつ病の治療環境のさらなる向上が期待される。本調査の結果（**表2，表3**）からもわかるように，欧米での SSRI の開発は終了している。EU では，2008 年 11 月に melatonin 1/2 agonist，serotonin 2C antagonist である agom-elatine の承認に対する positive opinion が **CHMP**（The Committee for Medicinal Products for Human Use）から提出[7]されており，従来の抗うつ薬とは異なる作用機序を有する新薬の導入が具体化しようとしている。

開発途上のうつ病対象化合物の特徴として以下の点が挙げられる。

- モノアミン類関連の化合物は，serotonin や noradrenaline だけでなく dopamine の再取り込み阻害作用を有する TRI が登場していること。
- モノアミン類関連の化合物として，受容体に直接作用する化合物（serotonin antagonist や serotonin agonist 等）が登場していること。
- 神経ペプチド類関連の化合物（NK1 antagonist，NK2 antagonist，NK2/NK3 antagonist，CRF1 antagonist，vasopressin V1b antagonist），グルタミン酸関連（NMDA antagonist，AMPA agonist）そして **FAAH**（fatty acid amide hydrolase）inhibitor 等のモノアミン類関連以外の化合物が登場していること。

当然のことながら，今回の調査で提示した化合物（**表2，表3，表4**）全てが新薬として承認されるわけではない。中枢領域の化合物での臨床試験の成功確立は，phase Ⅱ及び phase Ⅲとも 40 ～ 50%であり，初回の臨床試験からの成功確率は 10%未満[8]であることを考慮すると，臨床現場に導入されるのはこれらの化合物のうち一部になると思われる。

今回の調査対象となった化合物についても試験成績が論文等でも公表され始めているが，臨床現場の医師にとって注意すべき点は，統計量のみで有効性を判断したり，1 試験の失敗の結果をもって有効性を否定することは避けるべきことである。FDA での審査で最終的に有効であると判断された抗うつ薬でさえも，約半数のプラセボ対照試験が失敗に終わっている[9]のも事実である。臨床試験の成功の是非を問わず，試験計画の妥当性と結果の一般化可能性を十分に吟味し判断すべきである。

2．うつ病領域の臨床試験の実施状況

国内外で未承認であり開発フェーズが phase Ⅱもしくは phase Ⅲの段階にあるうつ病対象化合物の臨床試験に本邦が参加していない（**表3，表4**）のは深刻な状況であるといえる。近年のアジアでの臨床試験の実施は増加[10]し，本邦については国際共同治験の実施数も増加[11]しているが，うつ病対象化合物に関しては phase Ⅱ等の用量反応性を探索的に検討する段階の試験に本邦はまだ十分に参加できていない。新薬開発においては，海外の臨床試験成績のみでなく日本人での用量反応性を検討することは，有効性と安全性を評価する上で重要な過程[12~14]であり，これらの医薬品をより適切な形で速やかに本邦に導入するためには，欧米で進められている開発により早期の段階から参加することが重要と考えられる。

3．本調査の方法論的限界

本調査は，臨床試験の現在の実施状況を調査するこ

CHMP（The Committee for Medicinal Products for Human Use）
FAAH（fatty acid amide hydrolase）

とができ，一般にアクセス可能であるデータベースである“ClinicalTrials.gov”（http://www.clinicaltrials.gov/）を主体に調査したが，方法論的限界として，全ての臨床試験が登録されていない可能性と最新の情報に更新されていない可能性が残る。

V．おわりに

今回の報告では，国内外のうつ病を対象とした化合物の開発状況を説明した。日本におけるドラッグ・ラグを改善するためには，治験の早期開始，治験実施期間の短縮及び承認審査の迅速化が必要と考えられており，産官学にわたる種々の努力がなされている[15]。臨床開発着手の遅れは，本邦のうつ病領域の開発にも依然として認められ，治験の早期開始に向けた取組みを行政，製薬企業，治験実施機関等の関係者で引き続き進めていくことが必要と考えられる。最終的な目標は当然のことながら治療環境の向上であり，このためには新薬として承認された市販後においても継続的に検討していくことも望まれるが，最近公表された標準治療薬と位置付けられる抗うつ薬の臨床試験成績のメタアナリシス[16]には日本人患者も含まれており，今後も国際的な医薬品評価の中に積極的に関与していくことが重要ではないかと考える。

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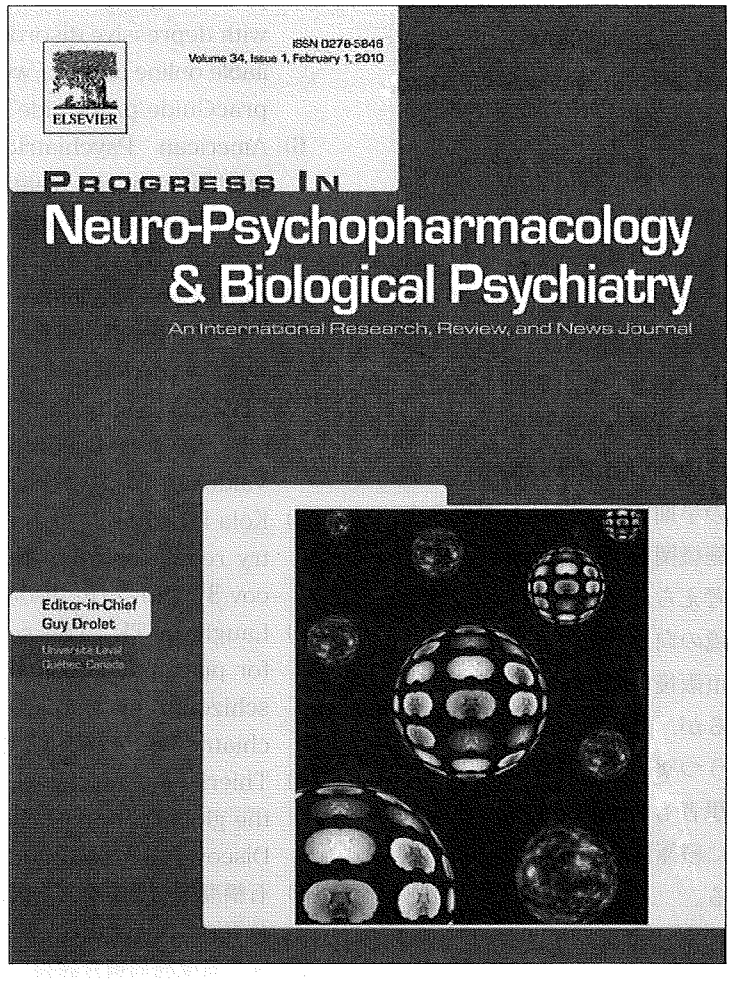

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Prednisolone causes anxiety- and depression-like behaviors and altered expression of apoptotic genes in mice hippocampus

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ABSTRACT

Glucocorticoids are known to cause psychiatric disorders including depression. Prednisolone (PSL) is one of the most widely used synthetic glucocorticoids to treat various medical diseases; however, little is known about PSL-induced behavioral changes and its molecular basis in the brain. Growing evidence has implicated that hippocampal remodeling or damage play a role in the pathogenic effect of glucocorticoids. In this study, mice were administered PSL (50 or 100 mg/kg) or vehicle for 6 or 7 days and subjected to a series of behavioral tests, i.e., open field, elevated plus maze, prepulse inhibition, forced swim, and tail suspension tests. Hippocampal tissues were subject to microarray analysis using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix) containing 45,101 probes of transcripts. Increased anxiety- and depression-like behaviors assessed with open field, elevated plus maze, and tail suspension tests were observed. Microarray analysis detected 108 transcripts with a fold change of >2.0 or <0.5 in which many cell-death-related genes were found. The microarray data was validated by quantitative reverse transcriptase-polymerase chain reaction analysis. Our results demonstrated that PSL causes anxiety- and depression-like behaviors, and suggest that altered gene expressions related to hippocampal remodeling or damage are involved in the effect of PSL on such behaviors.

1. Introduction

The hypothalamus–pituitary–adrenal (HPA) axis plays a major role in the pathophysiology of depressive disorder (Ströhle and Holsboer, 2003; de Kloet et al., 2005). Many clinical studies (for review: Ising et al., 2005; Sher, 2006), including ours (Kunugi et al., 2004; Kunugi et al., 2006), have shown that hyperactivity of the HPA can be found in as many as 80% of patients with major depression. Such hyperactivity is, at least in part, state-dependent (Kunugi et al., 2006), and high levels of glucocorticoid might be involved in the pathogenesis of depression. In accordance, patients with Cushing's syndrome often develop depression; approximately 50% of such patients develop major depression (Sonino et al., 1998). In rodents, repeated administration of corticosterone induces depression-like behaviors (Hill et al., 2003; Kalynchuk et al., 2004; Gregus et al., 2005; Zhao et al., 2008). Furthermore, therapeutic administration of synthetic glucocorticoids has been known to have adverse psychiatric effects (i.e., steroid-induced psychosis), including depressed mood, euphoria, and other symptoms (Bolanos et al., 2004; Brown et al., 1999a, 2004, 2007; Lewis and Smith, 1983; Ling et al., 1981; Patten, 2000). Prednisolone (PSL) is one of the most widely used synthetic glucocorticoids to treat various allergic, inflammatory, and autoimmune disorders. To our knowledge, however, there have been no animal experiments focusing on PSL-induced behavioral changes and its molecular basis in the brain, although there is one study reporting that a similar drug, prednisone, induces anxiety and glial changes in rats (Gonzalez-Perez et al., 2001).

A possible mechanism of the pathogenic effect of hyperactivity in the HPA-axis is hippocampal remodeling, damage, or atrophy (Brown et al., 1999b; McEwen, 2005). Long-term exposure to high levels of glucocorticoid due to chronic stress may cause hippocampal damage, which in turn results in an impaired negative feedback system of the HPA-axis. Dysfunction of the negative feedback may then maintain the heightened HPA-axis. Indeed, decreased volume of the hippocampus has frequently been observed in major depression, Cushing syndrome,

Abbreviations: PSL, prednisolone; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; HPA, hypothalamus–pituitary–adrenal; OFT, open field test; EPT, elevated plus maze test; FST, forced swimming test; TST, tail suspension test; PPI, prepulse inhibition; GR, glucocorticoid receptor.

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and glucocorticoid therapy (Brown et al., 2004; Campbell et al., 2004; Hickie et al., 2005; Sapolsky, 2000; Sheline, 2003; Videbech and Ravnkilde, 2004).

The aim of the present study is to examine the PSL-induced behavioral changes and its molecular basis in the hippocampus in mice. Since growing evidence has suggested that hippocampal damage is involved in such psychiatric adverse effects, we hypothesized that altered expression of genes related to cell death (apoptosis) in the hippocampus might be involved in the behavioral changes.

2. Materials and methods

2.1. Animals

Male C57BL/6n mice (9–10 weeks of age) weighing 24 to 27 g were purchased from CLEA Japan, Inc. and housed in standard polycarbonate mouse cages (16×22×14 cm; 3 or 4 mice per cage) for at least 2 weeks prior to the experimental procedures. All mice were kept under standard laboratory conditions: 12 h light/dark cycle (lights on at 16:30 h), 21 to 24 °C of room temperature, and free access to tap water and standard mouse diet. The study protocol was in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the ethics review committee for animal experiments in the National Institute of Neuroscience, Japan. All efforts were made to minimize the number of animals used and their suffering.

2.2. Experimental design

Mice were injected subcutaneously (s.c.) with 2 doses of PSL (50 or 100 mg/kg) for 6 to 7 days. Six to 8 animals for each group were subject to the behavioral tests. Open field, elevated plus maze, prepulse inhibition, forced swimming, and tail suspension tests (OFT, EPT, PPI, FST, TST, respectively), were performed on days 6 and 7 in the administration period (Fig. 1). For the microarray analysis and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), the hippocampus of the animals was sampled on day 6. The duration of the PSL treatment (6 to 7 days) was chosen because mood was changed by 5-day treatment in healthy volunteer subject (Wolkowitz et al., 1990) and anxiety was induced by 5-day-predonisone treatment in rats (Gonzalez-Perez et al., 2001).

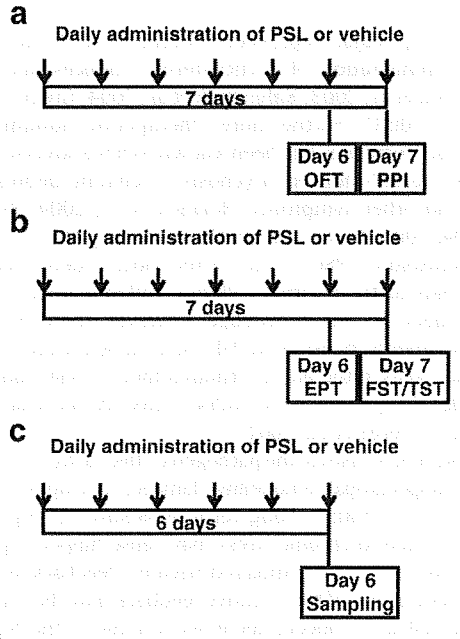


Fig. 1. Schematic representation of experimental schedules. a. Schedule for animals receiving open field test (OFT) and prepulse inhibition (PPI). b. Schedule for animals receiving elevated plus maze (EPT), tail suspension test (TST), and forced swim test (FST). c. Schedule for sampling brain.

2.3. Behavioral testing

All behavioral tests, except TST, were performed by apparatuses and computer programs (O'hara & Co., Tokyo, Japan) which yielded behavioral measures automatically.

2.3.1. Open field test

An open field (50×50×35 cm) with a 16-square grid floor was used. The illumination was maintained at 30 lx. Total distance traveled and time spent in the central 4 squares were automatically measured by a program, Image OF, during the 15 min test session. Total distance traveled indicates locomotor activity. Decreased time spent in the central area indicates anxiety-like behavior.

2.3.2. Elevated plus maze test

The elevated plus maze consisted of two open arms (25×5 cm) with 3-mm-high ledges and two enclosed arms of the same size, in 20-cm-high transparent walls. The plus arms were made of white plastic plates and were elevated to a height of 33 cm above the floor. At the beginning of the test, mice were placed in the edge of the closed arm and their behaviors were recorded. Total distance traveled and time spent in open and closed arms were automatically measured by a program, Image EP, during the 10 min test session. An increase in time spent on the closed arms indicates anxiety-like behavior.

2.3.3. Prepulse inhibition test

PPI of the startle response was measured using the startle reflex measurement system. A test session began by placing a mouse in a Plexiglas cylinder and leaving the mouse undisturbed for 5 min. The startle stimulus was white noise for 20 ms at 110 or 120 dB, and the background noise level was 65 dB. The prepulse sound was presented 100 ms before the startle stimulus, for 20 ms, and its intensity was 78, 82 or 88 dB. Six combinations of prepulse and startle stimuli were used (78/110, 82/110, 88/110, 78/120, 82/120 and 88/120). Six blocks of eight different trial types (six trial types with the combinations of prepulse and startle stimulus and two startle stimulus only trials) were presented in a pseudorandom order such that each trial type was presented once within one block. The intertrial interval was chosen from 10 to 20 s in a pseudorandom order to prevent the animals from habituating to the startle response. The magnitude of startle response and %PPI were automatically calculated by a program, SR-9020.

2.3.4. Forced swim test

Mice were forced to swim in a chamber filled with 50 cm of 27 °C water. Time spent by the mouse in active movement (struggling to escape chamber or swimming) and that of immobility was measured automatically by a program, Image FZ, during a 5 min test session. Increased time spent immobile indicates depression-like behavior.

2.3.5. Tail suspension test

Mice were suspended by the tail to a horizontal bar (distance from the floor: 80 cm), using adhesive tape affixed 2 cm from the tip of the tail. Time spent by the mouse in immobility was measured from a videotaped image by an observer who was blind to the treatment status.

2.4. Microarray analysis

2.4.1. Sampling

The animals were euthanized in a glass container using carbon dioxide gas. The whole brains were removed and immersed in the cold PBS buffer for a few minutes. The hippocampi were sampled from

the whole brain on the filter paper immersed in the cold PBS. The hippocampi were then frozen and stored at −80 °C until use.

2.4.2. Microarray procedures

Total RNA from hippocampi of the animals was extracted using an RNeasy Plus Mini Kit (Qiagen, Hilden, Land Nordrhein-Westfalen, Germany). The GeneChip Mouse Genome 430 2.0 Array containing probes of 45,101 transcripts (Affymetrix, Santa Clara, CA, USA) was used for microarray analysis. Four arrays were prepared for 2 PSL-treated (100 mg/kg) and 2 control, samples. Double-stranded cDNA derived from 5 μg of total RNA was used to produce biotinylated cRNA, and labeled cRNA targets were hybridized to GeneChips. Hybridization was allowed to proceed 16 h at 45 °C, followed by washing and staining with streptavidin–phycoerythrin. Hybridization assay procedures including preparation of solutions were carried out as described in the Technical Manual of the manufacturer. The distribution of fluorescent material on the array was obtained using GeneArray Scanner 3000 (Affymetrix, Santa Clara, CA, USA). GeneChip Operating Software supplied by Affymetrix was used to perform gene expression analysis.

2.4.3. Data analysis

We used Genespring GX (Agilent Technologies, CA, USA) for data analysis of the microarray data. "Per chip normalization" was performed, i.e., expression levels of all probes were divided by the median value. Then "per gene normalization for specific sample" was performed according to the manual of the manufacturer. Briefly, the ratio of normalized expression level of PSL-treated sample to that of vehicle-treated sample was calculated for each gene in two arbitrary selected pairs of PSL- and vehicle-treated samples, yielding two ratios for each gene. The mean of these ratios was considered to be the fold change. When flag of probes showed A (absent) or standard deviation of normalized values of vehicle-treated samples was 0.5 or more, expression data was excluded from the analysis. This procedure excluded 17,817 of the initial 45,101 probes. In the remaining 27,914 probes, 108 probes with a fold change of >2.0 or <0.5 were identified as "focus genes".

2.4.4. Network/functional analysis

One hundred eight identified transcripts were subjected to the network and functional analysis, using the Ingenuity Pathways Knowledge Base (Ingenuity systems, CA, USA). Detailed procedures of the analysis are described in the Supplementary text under the heading of "Network/functional analysis". To summarize, "Network Generation", "Functional Analysis of an Entire Data Set", "Functional Analysis of a Network", and "Network/My Pathways Graphical Representation" procedures were carried out.

2.4.5. qRT-PCR

Among the 108 focus genes, 12 were identified as cell-death-related genes by functional analysis of the entire microarray data (see supplemental Table S4). Among them 8 genes (*CDKN1A*, *SGK1*, *SGK3*, *EMILIN2*, *FKBP5*, *PLEKHF1*, *UCP2* and *TSC22D3*) were arbitrarily selected and subject to qRT-PCR analysis in order to confirm altered expression in the hippocampus of PSL-treated animals ($n=8$). Gene-specific primers for real time qRT-PCR were designed (for sequences see supplemental Table S1). Detailed descriptions of PCR conditions are shown in Supplemental methods. All gene amplifications were normalized to mouse *GAPDH*.

3. Results

3.1. Behavioral effect of repeated PSL

In OFT, there was a significant main effect of time course ($F=4.2$, $df=2, p=0.023$) and dose of PSL ($F=7.5$, $df=2, p=0.005$) on distance traveled (repeated measures ANOVA), showing habituation of locomotor activity during the 15 min test session and PSL-induced decreased locomotion (Fig. 2a). Post-hoc analysis revealed that there was a significant difference in distance traveled between PSL- and vehicle-

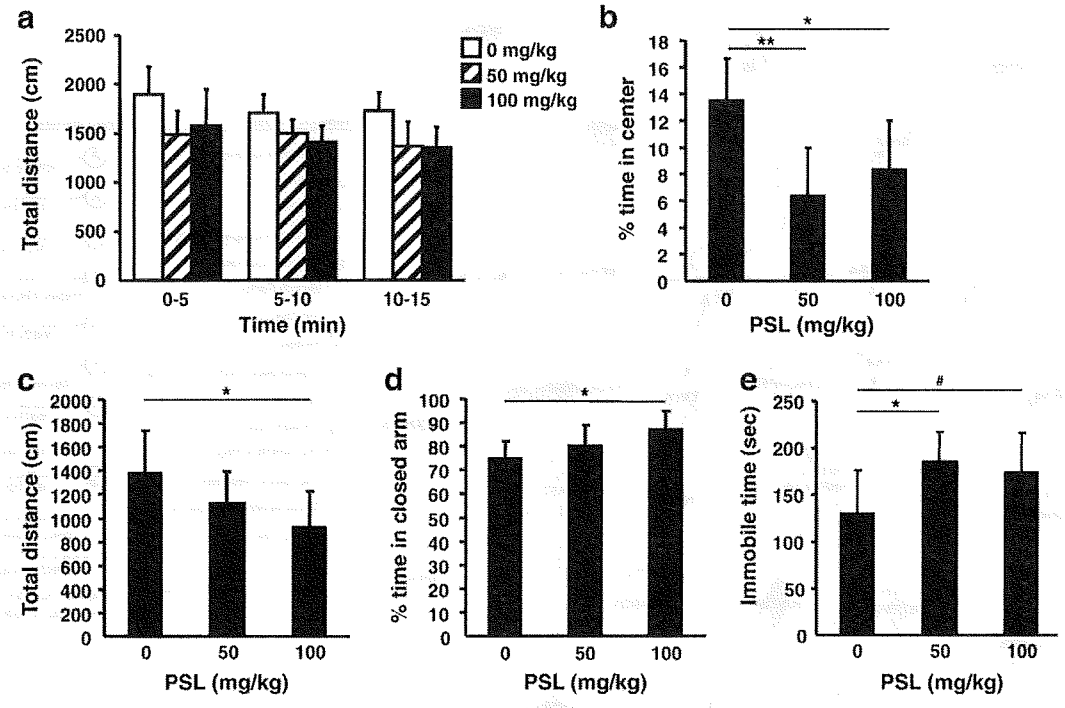
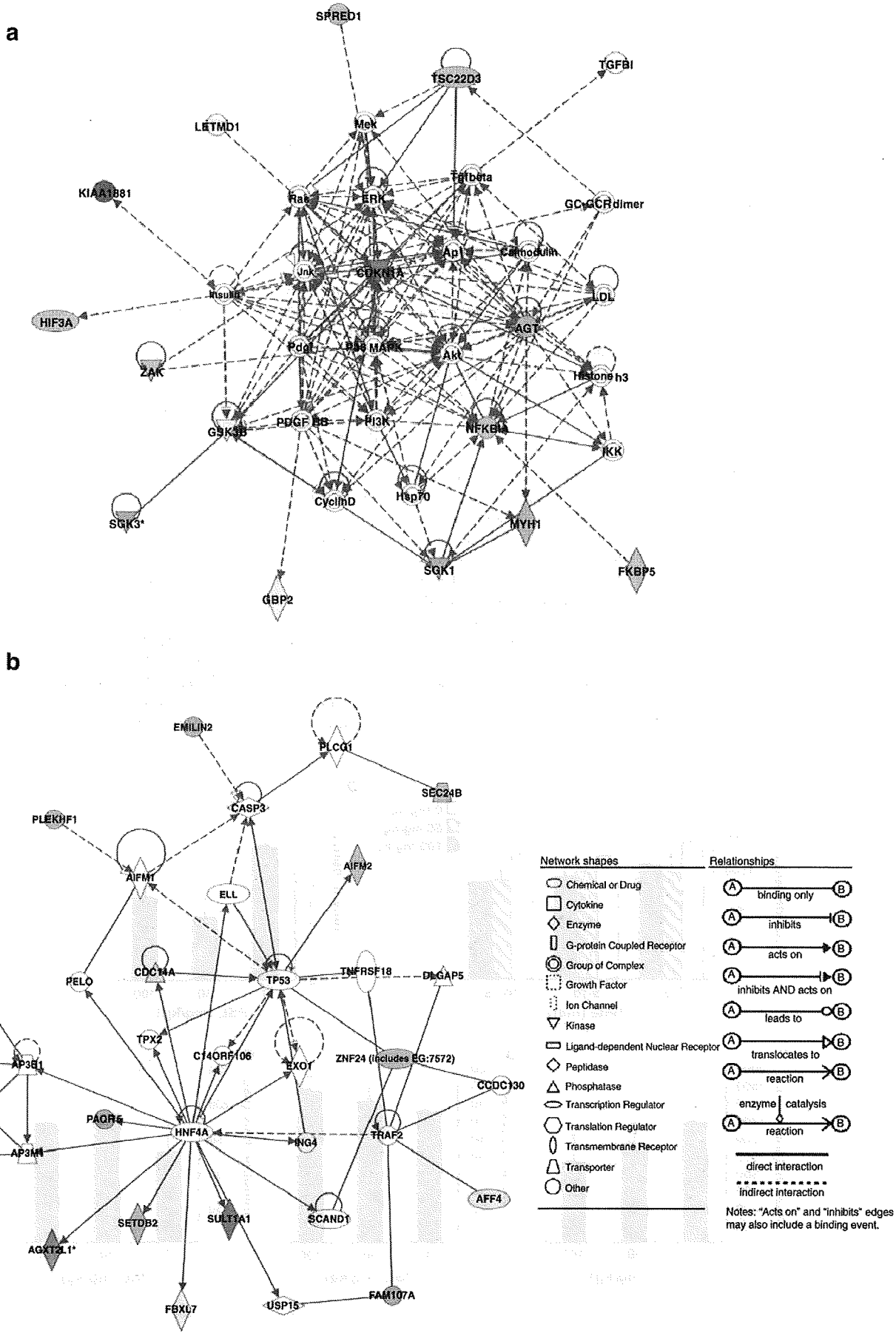


Fig. 2. Results of behavioral tests in vehicle- or PSL-treated (50 or 100 mg/kg) mice. PSL administration of both doses (50 and 100 mg/kg) for 6 days significantly decreased distance traveled (a) and time in the center area (b) in OFT. In EPT, the group receiving 100 mg/kg of PSL showed a significant decrease in total distance traveled (c) and the proportion of time spent in open arms (d), compared with the vehicle-treated animals. PSL increased the immobility time in TST (e). Bars and error-bars represent means and standard deviations. #: $p<0.10$, *: $p<0.05$, **: $p<0.01$.



administered mice for both doses of PSL ($p=0.009$ for 50 mg/kg and $p=0.006$ for 100 mg/kg by Dunnett multiple comparison). In addition, there was a significant effect of dose of PSL ($F=7.5$, df = 2, $p=0.005$) on time spent in the center area (one-way ANOVA, Fig. 2b). Post-hoc analysis revealed that there was a significant decrease in time spent in the center area between PSL- and vehicle-administered mice for both doses ($p=0.004$ for 50 mg/kg and $p=0.025$ for 100 mg/kg by Dunnett multiple comparison).

In EPT, there was a significant effect of dose of PSL ($F=4.1$, df = 2, $p=0.032$) on distance traveled (one-way ANOVA, Fig. 2c). Post-hoc analysis revealed that there was a significant decrease in distance traveled between PSL- and vehicle-administered mice for the 100 mg/kg group ($p=0.190$ for 50 mg/kg and $p=0.019$ for 100 mg/kg by Dunnett multiple comparison). There was a significant effect of dose of PSL ($F=3.9$, df = 2, $p=0.036$) on time spent in the closed arm (one-way ANOVA, Fig. 2d). Post-hoc analysis revealed that there was a significant decrease in time spent in closed arm between PSL- and vehicle-administered mice for the 100 mg/kg group ($p=0.384$ for 50 mg/kg and $p=0.021$ for 100 mg/kg by Dunnett multiple comparison).

In PPI test, no significant difference was observed in any measure of startle response or %PPI in the three groups (data not shown). In FST, there was no significant effect of administration of PSL for 7 days on the immobility time (data not shown). In TST, however, there was a significant effect of dose of PSL ($F=4.23$, df = 2, $p=0.030$) on immobility time (one-way ANOVA, Fig. 2e). Post-hoc analysis revealed that there was a significant increase in immobility time between PSL- and vehicle-administered mice for the 50 mg/kg group and a similar trend for the 100 mg/kg group ($p=0.022$ for PSL of 50 mg/kg and $p=0.097$ for 100 mg/kg by Dunnett of multiple comparison).

3.2. Microarray analysis

We identified 108 "focus genes" with a fold change of >2.0 or <0.5. These genes are listed in supplemental Tables S2 and S3. The data was analyzed using the Ingenuity Pathway Analysis (IPA) based on the Ingenuity Pathway Knowledge Base (IPKB) that uses a genome-scale biological knowledge base and generates multiple biological networks with associated functional analysis. The pathway analysis can be a useful tool to help uncover relationships between focus genes, such that multiple members of the list may be regulated by the same biological function. When seeded with the focus genes, the pathway analysis produced networks of genes with functional relationship.

Fig. 3 shows the two highest ranked networks. Each includes focus genes with very strong evidence of connectivity between the genes. The top ranked network identified by IPA (Fig. 3a) is associated with cell death/apoptosis and proliferation. Network 1 includes 16 focus genes (i.e., *CDKN1A*, *NFKBIA*, *AGT*, *SGK1*, *SGK3*, *MYH1*, *GSK3B*, *ZAK*, *TGFBI*, *TSC22D3*, *SPRED1*, *LETMD1*, *KIAA1881*, *HIF3A*, *GBP2* and *FKBP5*) and also contains cell death/apoptosis related molecules in the central, *Jnk*, *P38/MAPK*, *PI3K* and *Akt*. Network 2 includes 14 focus genes (i.e., *EMILIN2*, *PLEKHF1*, *SEC24B*, *AIFM2*, *CDC14A*, *ZNF24*, *PAQR5*, *AP3S2*, *AGXT2L1*, *SETDB2*, *FBXL7*, *SULT1A1*, *FAM107A* and *AFF4*) and also contains genes related to cell death/apoptosis, *TP53* and *CASP3*.

Functional analysis revealed that there were 12 genes that were categorized as involved in "cell death": *GSK3B*, *SLC22A6*, *AGT*, *CDKN1A*, *NFKBIA*, *SGK1*, *SGK3*, *EMILIN2*, *FKBP5*, *PLEKHF1*, *UCP2* and *TSC22D3*. *GSK3B* and *SLC22A6* were reduced, while the remaining 10 genes were increased in PSL-treated mice. These genes were also shown to be involved in other functions such as "Cell cycle", "Neurological disease", "Cellular growth and proliferation", and "Behavior".

Table 1
Comparisons of fold changes between qRT-PCR and microarray in 8 genes.

Gene symbol	Fold change (qRT-PCR)	Fold change (microarray)
CDKN1A	5.37 (4.65–6.19)	4.26
SGK3	4.02 (3.24–5.00)	2.96
UCP2	2.51 (2.16–2.92)	2.09
SGK1	2.25 (1.88–2.69)	2.39
FKBP5	1.77 (1.39–2.26)	2.04
EMILIN2	1.62 (1.26–2.10)	2.22
TSC22D3	1.70 (1.30–2.22)	2.22
PLEKHF1	1.41 (1.18–1.69)	2.35

Table 1 shows results of qRT-PCR of the 8 genes involved in "cell death". As shown, results of qRT-PCR are fairly consistent with those obtained by microarray analysis.

4. Discussion

We found that administration of PSL (50 or 100 mg/kg) for 6 to 7 days gave rise to behavioral changes such as decreased distance traveled and time in the center area in OFT, decreased total distance traveled and decreased proportion of time spent in open arms in EPT, and increased immobility time in TST. In the microarray analysis, we found that a number of genes showed substantial alterations (a fold change of >2.0 or <0.5) in expression. Functional and network analyses demonstrated that several genes functioning in "cell death" and gene networks related to "cell death" were altered in the hippocampus of PSL-treated mice.

Our behavioral findings indicate that administration of PSL for 6 to 7 days results in anxiety- and depression-like behaviors. A drawback might be that immobility time in FST representing "behavioral despair" was not significantly elevated in PSL-treated mice, which was inconsistent with the result of TST. However, biological substrates mediating performance in these two behavioral tests may not be identical (Bai et al., 2001), which explains the differential results. Our finding is consistent with the previous study of Gonzalez-Perez et al. (2001) who showed that administration of prednisone (5 or 60 mg/kg), a synthetic glucocorticoid similar to PSL, for 5 days caused anxiety-like behaviors assessed with OFT (decreased distance traveled) and EPT (decreased time in open arms). Furthermore, several studies showed that repeated administration of corticosterone induces depression-like behaviors (Hill et al., 2003; Kalynchuk et al., 2004; Gregus et al., 2005; Zhao et al., 2008). Since a portion of individuals receiving corticosteroids develop psychotic symptoms (Lewis and Smith, 1983; Patten, 2000), we examined PPI as a marker for psychotic (schizophrenia-like) symptoms in our mice. However, we found no significant effect of PSL on PPI. Taken together, chronic administration of glucocorticoids in rodents could cause predominantly anxiety- and depression-like condition.

The microarray analysis supports our study hypothesis that altered expression of genes related to cell death (apoptosis) in the hippocampus might be involved in the behavioral changes induced by PSL. The network analysis, the central gene in Network 1 identified by IPA, *CDKN1A* is one of the representative apoptotic genes. *CDKN1A* is a regulator of cell-cycle progression and related to stress response (Rodriguez and Meuth, 2006). Network 2 also includes genes related to cell death/apoptosis, *TP53* and *CASP3*. The functional analysis of focus genes detected 12 cell-death-related genes (*GSK3B*, *SLC22A6*, *AGT*, *CDKN1A*, *NFKBIA*, *SGK1*, *SGK3*, *EMILIN2*, *FKBP5*, *PLEKHF1*, *UCP2*

Fig. 3. Network generation of focus genes. Pathway analysis based on the Ingenuity Knowledge Base (IPKB). The two highest scoring networks from the focus genes are shown. A gene network generated by the focus genes converging to *NFKB*, *P38MAPK*, and *AKT* all of which play a crucial role in cell death/apoptosis (a). Another network converging to *TP53* and *CASP3* both of which also play an important role in cell death/apoptosis, and proliferation (b). Genes (or gene products) are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, a textbook, or canonical information stored in the IPKB. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

and *TSC22D3*). Among these genes, *GSK3B* and *SLC22A6* were reduced while the remaining 10 genes were increased in PSL-treated mice. We examined expression of 8 arbitrary selected genes by qRT-PCR and obtained results being fairly consistent with those obtained by microarray analysis, which validates the microarray data.

Characteristics of the 12 cell-death-related genes are summarized in Table 2. Among them 7 genes seem to facilitate cell death/apoptosis, while 5 genes (including *SLC22A6*) may have a protective effect against cell death/apoptosis. Up-regulation of cell-death-facilitating genes and down-regulation of the protective gene may have damaged hippocampus in the PSL-treated mice, while up-regulation of protective genes and down-regulation of the cell-death-facilitating gene may have occurred to minimize such cell death. Of note, a recent study found that genetic variations in *FKBP5* were associated with response to antidepressants and the recurrence of depressive episodes in patients with depression (Binder et al., 2004). They were also associated with incomplete normalization of the stress-elicited cortisol secretion as well as increased self-reported anxiety after psychosocial stress in healthy subjects (Ising et al., 2008). Our result of an increased expression of *FKBP5*, a possible apoptosis-facilitating molecule, in PSL-treated mice further points to the key role of *FKBP5* in the pathogenesis of glucocorticoid or stress-induced depressive behavior.

The molecular changes, particularly those of cell-death-related genes, are likely to be involved in hippocampal remodeling or damage, which may result in the PSL-induced anxiety- and depression-like behaviors demonstrated in the present study. This is in accordance with previous findings that decreased volume of the hippocampus has frequently been observed in major depression, Cushing syndrome, and glucocorticoid therapy (Brown et al., 2004; Campbell et al., 2004; Hickie et al., 2005; Sapolsky, 2000; Sheline, 2003; Videbech and Ravnkilde, 2004).

A limitation of this study might be that the observed behavioral and molecular changes were induced by relatively high dose (50 or 100 mg/kg) of PSL compared to therapeutic dose in humans; approximately 1 mg/kg of PSL is recommended to treat refractory systemic lupus erythematosus in humans (American College of Rheumatology Ad Hoc Committee on Systemic Lupus Erythematosus Guidelines, 1999), for example. However, higher doses may be required to obtain adequate effects in mice; 30 mg/kg rather than 3 mg/kg of PSL was required to adequately suppress the inflammation in collagen-induced arthritis in mice (Geiger et al., 1994), which may be attributable to the species difference in the hepatic metabolism of glucocorticoid (Tomlinson et al., 1997). Regarding behavioral changes, anxiety-like behaviors were observed after subchronic administration of 5 or 60 mg/kg of prednisone, a similar drug to PSL in rats (Gonzalez-Perez et al., 2001). Thus relatively high doses of glucocorticoids, comapred to those for clinical use in humans, might be required to obtain behavioral changes and neuronal damage caused by PSL in rodents, which was a rationale for the use of the doses (50 or 100 mg/kg) in the present study. Nonetheless, it is possible that the observed changes may have been exaggerated due to the use of relatively high dose of PSL, which requires caution in interpretation of the data.

5. Conclusions

The present study suggests that subchronic administration (6 to 7 days) of PSL (50 or 100 mg/kg) causes anxiety-like behaviors observed in OFT and EPT, and depression-like behaviors in TST. However, no significant behavioral changes were observed in FST or PPI. When changes in gene expression in hippocampus after subchronic administration of PSL (100 mg/kg) were examined using cDNA microarray, 108 "focus genes", of which 12 genes were related to cell death, were identified. The pathway analysis of focus genes also pointed to the

Table 2
Characteristics of 12 cell-death-related genes.

Gene symbol	Formal name	Fold change	Characteristics	Effect on cell death
CDKN1A	Cyclin-dependent kinase inhibitor 1A/P21	4.26	*CDKN1A* is activated by p53, and mediates arrest of cell cycle (Rokudai et al., 2009). Such arrest of cell cycle is involved in neuronal damage in the hippocampus as an apoptotic pathway (Alva-Sánchez et al., 2009).	↑
SGK3	Serum/glucocorticoid-induced protein kinase-3	2.96	Proliferation is reduced, and apoptosis is increased in hair follicles of mice lacking SGK3 (Alonso et al., 2005).	↓
SGK1	Serum/glucocorticoid-induced protein kinase-1	2.39	Glucocorticoid receptor-mediated protection from apoptosis (Mikosz et al., 2001).	↓
PLEKHF1	Lysosome-associated apoptosis-inducing protein containing the pleckstrin homology (PH) and FYVE domains (*LAPF*)	2.35	Overexpression of PLEKHF1 in L929 cells induces apoptosis and also increases cell sensitivity to tumor necrosis factor α-induced apoptosis (Chen et al., 2005).	↑
AGT	Angiotensinogen	2.28	AGT could play an important role in preventing neuronal cells from apoptosis; for example, AGT knock-out mice revealed the decreased density in granular layer cells of hippocampus and cerebellum (Kakinuma et al., 1997).	↓
EMILIN2	Elastin microfibril interface-located protein 2	2.22	EMILIN2 is an extracellular matrix glycoprotein (Doliana et al., 2000) and binds to the TRAIL receptors DR4 and DR5, which induces activation of caspase and subsequent apoptosis (Mongiat et al., 2007).	↑
TSC22D3	TSC22 domain family, member 3/glucocorticoid-induced leucine zipper	2.22	TSC22D3 is a negative regulator of Ras- and Raf-induced proliferation (Ayroldi et al., 2007). Transgenic mice overexpressing TSC22D3 reduced expression of antiapoptotic B-cell leukemia XL (Bcl-xL) and increased activation of caspase-8 and caspase-3 (Delfino et al., 2004).	↑
UCP2	Uncoupling protein 2	2.09	UCP2 was identified as an inducible neuroprotective protein in rat hippocampi after ischemia (Mattiasson et al., 2003). In mice overexpressing human UCP2, brain damage was diminished after experimental stroke and traumatic brain injury, and neurologic recovery was enhanced (Mattiasson et al., 2003).	↓
FKBP5	FK506 binding protein 5	2.04	FKBP5 interacts with GR and inhibits activation of an apoptosis-inhibiting molecule, NF-κB (Bouwmeester et al., 2004).	↑
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	2.01	NFKBIA inactivates NF-κB (Baldwin, 2001; Kucharczak et al., 2003).	↑
GSK3B	Glycogen synthase kinase 3β	0.49	GSK3B is abundant in the central nervous system, particularly in the hippocampus, plays a pivotal role in facilitating apoptosis, and is an important target molecule of mood stabilizers (Jope and Bijur, 2002).	↑
SLC22A6	Solute carrier family 22A6/organic anion transporter 1 (OAT1)	0.20	SLC22A6 is involved in the sodium-dependent transport and excretion of drugs, endogenous substances, and toxins. The role of SLC22A6 has predominantly been studied in renal excretion (Nigam et al., 2007); the potential role in the brain and whether glucocorticoids are substrates for it are unclear, which warrant future studies.	↓?

importance of gene networks related to cell death/apoptosis and proliferation. Such molecular changes are likely to be involved in hippocampal remodeling or damage, which may result in the PSL-induced anxiety- and depression-like behaviors.

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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.pnpbp.2009.10.018.

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RESEARCH ARTICLE

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Sexually Dimorphic Effect of the Val66Met Polymorphism of *BDNF* on Susceptibility to Alzheimer's Disease: New Data and Meta-Analysis

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Conflicting results have been reported as to whether genetic variations (Val66Met and C270T) of the brain-derived neurotrophic factor gene (BDNF) confer susceptibility to Alzheimer's disease (AD). We genotyped these polymorphisms in a Japanese sample of 657 patients with AD and 525 controls, and obtained weak evidence of association for Val66Met ($P = 0.063$), but not for C270T. After stratification by sex, we found a significant allelic association between Val66Met and AD in women ($P = 0.017$), but not in men. To confirm these observations, we collected genotyping data for each sex from 16 research centers worldwide (4,711 patients and 4,537 controls in total). The meta-analysis revealed that there was a clear sex difference in the allelic association; the Met66 allele confers susceptibility to AD in women (odds ratio = 1.14, 95% CI 1.05–1.24, $P = 0.002$), but not in men. Our results provide evidence that the Met66 allele of BDNF has a sexually dimorphic effect on susceptibility to AD.

Key words: Alzheimer's disease (AD); brain-derived neurotrophic factor (BDNF); meta-analysis; polymorphism; sex difference

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease and is neuropathologically characterized by loss and atrophy of basal forebrain cholinergic neurons and the limbic structures [Mattson, 2004]. Mutations in several genes are known to cause familial AD, namely those encoding amyloid precursor protein [Goate et al., 1991], presenilin-1 [Sherrington et al., 1995], and presenilin-2 [Levy-Lahad et al., 1995]. The ε4 allele of the apolipoprotein E (*APOE*) gene confers susceptibility to familial and sporadic AD [Saunders et al., 1993]. However, AD is a genetically complex disorder and these genetic markers for AD cannot explain the overall genetic susceptibility. Thus, additional genes may be involved in the development of AD.

Since neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) promote the development, regeneration, survival, and maintenance of function of neurons [Reichardt, 2006], polymorphisms of the genes encoding these proteins may confer susceptibility to neurodegenerative diseases. Several lines of evidence have suggested that *BDNF*, in particular, is an important candidate gene for susceptibility to AD. Reduced *BDNF* mRNA levels were observed in postmortem hippocampi and temporal cortices of patients with AD [Connor et al., 1997], and lower protein levels of BDNF in the entorhinal cortex were reported in AD [Hock et al., 2000]. Immunohistochemical and Western blotting studies revealed a selective decline of the BDNF/TrkB neurotrophic signaling pathway in the frontal cortex and hippocampus in AD [Ferrer et al., 1999].

Based on these observations, a number of genetic association studies have been performed for two polymorphisms of *BDNF*, Val66Met, and C270T. The non-synonymous polymorphism, Val66Met, is a functional single-nucleotide polymorphism (SNP), G to A substitution at nucleotide 196, which results in the Val66-to-Met amino acid change in the 5′ pro-region of the human BDNF protein [Ventriglia et al., 2002]. Two studies reported that the Met66 allele was significantly associated with an increased risk of AD [Saarela et al., 2006; Tsai et al., 2006], while one study reported that the Val66 allele was the risk-increasing allele [Matsushita et al., 2005]. The majority of studies, however, have found no significant association (Supplementary Fig. 1) [Ventriglia et al., 2002; Bagnoli et al., 2004; Combarros et al., 2004; Nacmias et al., 2004; Bian et al., 2005; Bodner et al., 2005; Desai et al., 2005; Lee et al., 2005; Li et al., 2005; Nishimura et al., 2005; Vepsalainen et al., 2005; Akatsu et al., 2006; Forero et al., 2006; Zhang et al., 2006; He et al., 2007; Huang et al., 2007]. The C270T polymorphism in the non-coding region of *BDNF* was detected by our group and found to be associated with late-onset AD [Kunugi et al., 2001]. Subsequently, two other groups reported that the T270 allele was significantly associated with an increased risk of AD [Nishimura et al., 2004; Olin et al., 2005], while one group reported the opposite [Saarela et al., 2006]. Other studies reported no significant association (Supplementary Fig. 2) [Riemenschneider et al., 2002; Bagnoli et al., 2004; Nishimura et al., 2004; Bodner et al., 2005; Desai et al., 2005; Lee et al., 2005; Matsushita et al., 2005; Akatsu et al., 2006; Tsai et al., 2006; Zhang et al., 2006; Huang et al., 2007]. These conflicting results require further investigation.

METHODS

Case–Control Study Sample

We genotyped 657 patients with AD (427 females; 73.5 years [SD] 8.7) and 525 healthy controls (305 females; 67.1 years [SD] 10.3) who were recruited around the Tokyo Metropolitan area, Japan. Diagnoses were made by neurologists according to the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [McKhann et al., 1984] for "probable AD." The numbers of individuals with and without a family history of dementia were 211 and 425, respectively, while the remaining 21 individuals had undetermined family histories. Controls were interviewed and those who had a family history of dementia within their first-degree relatives were not enrolled in the study. All subjects were biologically unrelated Japanese individuals. After description of the study, written informed consent was obtained from every subject. The study protocol was approved from the ethics committee of the National Center of Neurology and Psychiatry, Japan.

Genotyping

The two SNPs of *BDNF* were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay. TaqMan probes of the "Assay-On-Demand" (C__11592758_10) for Val66Met (rs6265) and TaqMan primers (forward: GGAGCCAGAATCGGAACCA; reverse: CCAGCGCTTGCCTACCT) and probes (VIC: CTCACGGGTCCCCG; FAM: CTCACGAGTCCCCG) of the "Assay-by-Design" for C270T and Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA). Thermal cycling conditions for the polymerase chain reaction (PCR) were one cycle at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 58°C for 1 min. After amplification, the allele-specific fluorescence was measured on ABI PRISM 7900 Sequence Detection

System (Applied Biosystems). Genotype data were read blind to the case–control status. We also genotyped the subjects for the *APOE* gene, according to the methods of Wenham et al. [1991].

Meta-Analysis

To examine whether there was a possible sex difference in the effect of these polymorphisms on AD in a larger sample, we organized a multi-center collaborative study and performed a meta -analysis. We searched for published case–control association studies of the Val66Met or C270T polymorphism with AD in the PubMed database (National Center for Biotechnology Information; NCBI; www.ncbi.nlm.nih.gov/), using combinations of terms "BDNF," "brain-derived neurotrophic factor," "polymorphism," "Val66Met," "C270T," "C-270T," and "Alzheimer." Additionally, reference lists of these and relevant articles, and the AlzGene Database (www.alzforum.org/) [Bertram et al., 2007] were referred to. As a result, 23 association studies of AD with Val66Met (Supplementary Table III) and 18 with C270T (Supplementary Table IV) were identified. Then an e-mail calling for participation in the collaborative study was sent to corresponding and first authors. Sixteen research groups for the Val66Met and 12 for the C270T responded and participated in this study. Genotype data with information on sex were combined.

Genotypes in the control groups from all research groups were in Hardy–Weinberg equilibrium. In the meta-analysis, heterogeneity, publication bias, sensitivity analysis, and Rosenthal's failsafe N were determined. Meta-analytic procedures were carried out using Comprehensive Meta-Analysis v.2.0 (Biostat, Inc., Englewood, NJ). To confirm that there was no significant difference in the allele distributions of patients and controls between the collected and the uncollected data (i.e., studies whose authors did not respond to us), Breslow–Day tests were performed using R software (R Development Core Team, 2007). With respect to Val66Met, the summary data for the Breslow–Day tests are shown in Supplementary Table V. There was no significant difference between the collected and uncollected data ($\chi^2 = 2.0$, df = 1, $P = 0.15$).

RESULTS

Case–Control Study

Genotype distributions for Val66Met, C270T, and *APOE* were in Hardy–Weinberg equilibrium for both patients and controls (data not shown). Genotype distributions for *APOE* were significantly different between the patients and controls as expected ($P = 2 \times 10^{-18}$) (Supplementary Table I). Genotype and allele distributions for Val66Met are shown in Table I. There was a trend towards an increased frequency of the Met66 allele in patients compared to controls ($P = 0.063$). When men and women were examined separately, the allele distribution differed between the two groups in females (odds ratio [OR] = 1.30, 95% CI = 1.05--1.60, $P = 0.017$), but not in males (OR = 1.02, 95% CI = 0.78--1.32, $P = 0.91$) (Table I).

The genotype and allele distributions for C270T are shown in Supplementary Table II. There was no significant difference in the genotype or allele distribution between the patients and controls.

TABLE I. Genotype and Allele Distributions for the Val66Met Polymorphism of BDNF in Patients With Alzheimer's Disease and Controls

	Genotype distribution								Allele distribution					
	Patients				Controls				Patients			Controls		
	n	Val/Val	Val/Met	Met/Met	n	Val/Val	Val/Met	Met/Met	*P*-value, df = 2	Val	Met	Val	Met	*P*-value, df=1
Total	657	218 (0.33)	319 (0.49)	120 (0.18)	525	197 (0.38)	249 (0.47)	79 (0.15)	0.18 ($\chi^2 = 3.4$)	755 (0.57)	559 (0.53)	643 (0.61)	407 (0.39)	0.063 ($\chi^2 = 3.5$)
Female	427	142 (0.33)	205 (0.48)	80 (0.19)	305	122 (0.40)	143 (0.47)	40 (0.13)	0.057 ($\chi^2 = 5.7$)	489 (0.57)	365 (0.60)	387 (0.63)	223 (0.37)	0.017 ($\chi^2 = 5.7$)
Male	230	76 (0.33)	114 (0.50)	40 (0.17)	220	75 (0.34)	106 (0.48)	39 (0.18)	0.96 ($\chi^2 = 0.09$)	266 (0.58)	194 (0.44)	256 (0.58)	184 (0.42)	0.91 ($\chi^2 = 0.01$)

TABLE II. Genotype and Allele Distributions for the Val66Met Polymorphism of *BDNF* in Female Subjects

		Genotype distribution								Allele distribution			
		Patients				Controls				Patients		Controls	
Study	Ethnicity	n	Val/Val	Val/Met	Met/Met	n	Val/Val	Val/Met	Met/Met	Val	Met	Val	Met
Akatsu et al. [2006]	Asian	58	16	36	6	86	30	42	14	68	48	102	70
Bian et al. [2005]	Asian	108	20	67	21	105	36	47	22	107	109	119	91
He et al. [2007]	Asian	318	92	152	74	332	97	170	65	336	300	364	300
Matsushita et al. [2005]	Asian	340	117	170	53	321	104	154	63	404	276	362	280
Tsai et al. [2006]	Asian	84	19	50	15	101	33	50	18	88	80	116	86
Current study	Asian	427	142	205	80	305	122	143	40	489	365	387	223
Subtotal		1,335	406	680	249	1,250	422	606	222	1,492	1,178	1,450	1,050
Combarros et al. [2004]	Caucasian	161	107	47	7	155	105	44	6	261	61	254	56
Desai et al. [2005]	Caucasian	669	449	201	19	411	287	115	9	1,099	239	689	133
Li et al. [2005] (UCSD)[a]	Caucasian	87	51	32	4	226	150	67	9	134	40	367	85
Li et al. [2005] (WashU)[b]	Caucasian	248	163	81	4	215	150	60	5	407	89	360	70
Li et al. [2005] (UK)[c]	Caucasian	265	178	73	14	270	192	73	5	429	101	457	83
Nacmias et al. [2004]	Caucasian	58	36	19	3	61	39	22	0	91	25	100	22
Saarela et al. [2006]	Caucasian	68	45	21	2	56	46	10	0	111	25	102	10
Subtotal		1,556	1,029	474	53	1,394	969	391	34	2,532	580	2,329	459
Desai et al. [2005]	African-American	46	42	4	0	33	31	2	0	88	4	64	2
Forero et al. [2006]	Mixed	73	51	20	2	115	90	23	2	122	24	203	27
Lee et al. [2005]	Unknown	61	31	28	2	38	20	14	4	90	32	54	22
Total		3,071	1,559	1,206	306	2,830	1,532	1,036	262	4,324	1,818	4,100	1,560

[a]UCSD sample from the University of California, San Diego.
[b]WashU sample from the Washington University.
[c]UK sample from Cardiff University, Wales College of Medicine and King's College London.

TABLE III. Genotype and Allele Distributions for the Val66Met Polymorphism of *BDNF* in Male Subjects

		Genotype distribution								Allele distribution			
		Patients				Controls				Patients		Contorls	
Study	Ethnicity	n	Val/Val	Val/Met	Met/Met	n	Val/Val	Val/Met	Met/Met	Val	Met	Val	Met
Akatsu et al. [2006]	Asian	37	9	22	6	22	5	11	6	40	34	21	23
Bian et al. [2005]	Asian	95	29	46	20	134	37	68	29	104	86	142	126
He et al. [2007]	Asian	195	63	93	39	243	68	115	60	219	171	251	235
Matsushita et al. [2005]	Asian	147	54	77	16	150	46	69	35	185	109	161	139
Tsai et al. [2006]	Asian	91	24	42	25	88	31	45	12	90	92	107	69
Current study	Asian	230	76	114	40	220	75	106	39	266	194	256	184
Subtotal		795	255	394	146	857	262	414	181	904	686	938	776
Combarros et al. [2004]	Caucasian	76	42	31	3	63	38	23	2	115	37	99	27
Desai et al. [2005]	Caucasian	329	216	98	15	260	169	82	9	530	128	420	100
Li et al. [2005] (UCSD)	Caucasian	94	54	38	2	126	81	39	6	146	42	201	51
Li et al. [2005] (WashU)	Caucasian	140	88	45	7	134	87	45	2	221	59	219	49
Li et al. [2005] (UK)	Caucasian	72	46	26	0	89	56	28	5	118	26	140	38
Nacmias et al. [2004]	Caucasian	25	12	10	3	36	16	16	4	34	16	48	24
Saarela et al. [2006]	Caucasian	29	16	13	0	45	35	7	3	45	13	77	13
Subtotal		765	474	261	30	753	482	240	31	1,209	321	1,204	302
Desai et al. [2005]	African-American	18	17	1	0	12	11	1	0	35	1	23	1
Forero et al. [2006]	Mixed	28	21	7	0	53	41	11	1	49	7	93	13
Lee et al. [2005]	Unknown	34	14	19	1	32	12	16	4	47	21	40	24
Total		1,640	781	682	177	1,707	808	682	217	2,244	1,036	2,298	1,116

Also when sexes were examined separately, no significant association was found for either sex.

Meta-Analysis

With respect to Val66Met, individual studies contained 64–998 patients with AD and 45–671 controls, and the combined sample consisted of 4,711 patients and 4,537 controls (Supplementary Table III). There was no heterogeneity across studies (total: $Q = 26.7$, $df = 21$, $P = 0.18$; men: $Q = 16.0$, $df = 15$, $P = 0.38$; women: $Q = 13.5$, $df = 15$, $P = 0.56$). Thus, we performed the fixed effects meta-analyses (Fig. 1, Tables II and III). The meta-analysis showed no significant association between AD and the Met66 allele (OR = 1.05, 95% CI = 0.98–1.11; $Z = 1.43$, $P = 0.15$; Supplementary Fig. 1). Meta-analysis of data in men and women separately revealed a significant association in women (OR = 1.14, 95% CI = 1.05–1.24; $Z = 3.05$, $P = 0.002$; Fig. 1A), but not in men (OR = 0.97, 95% CI = 0.87–1.08; $Z = -0.54$, $P = 0.59$; Fig. 1B). In the sensitivity analysis, the association of the Met66 allele with AD remained significant after removal of any one study (Supplementary Table VI): even if our data were removed, there remained a significant association for women (residual OR = 1.11,

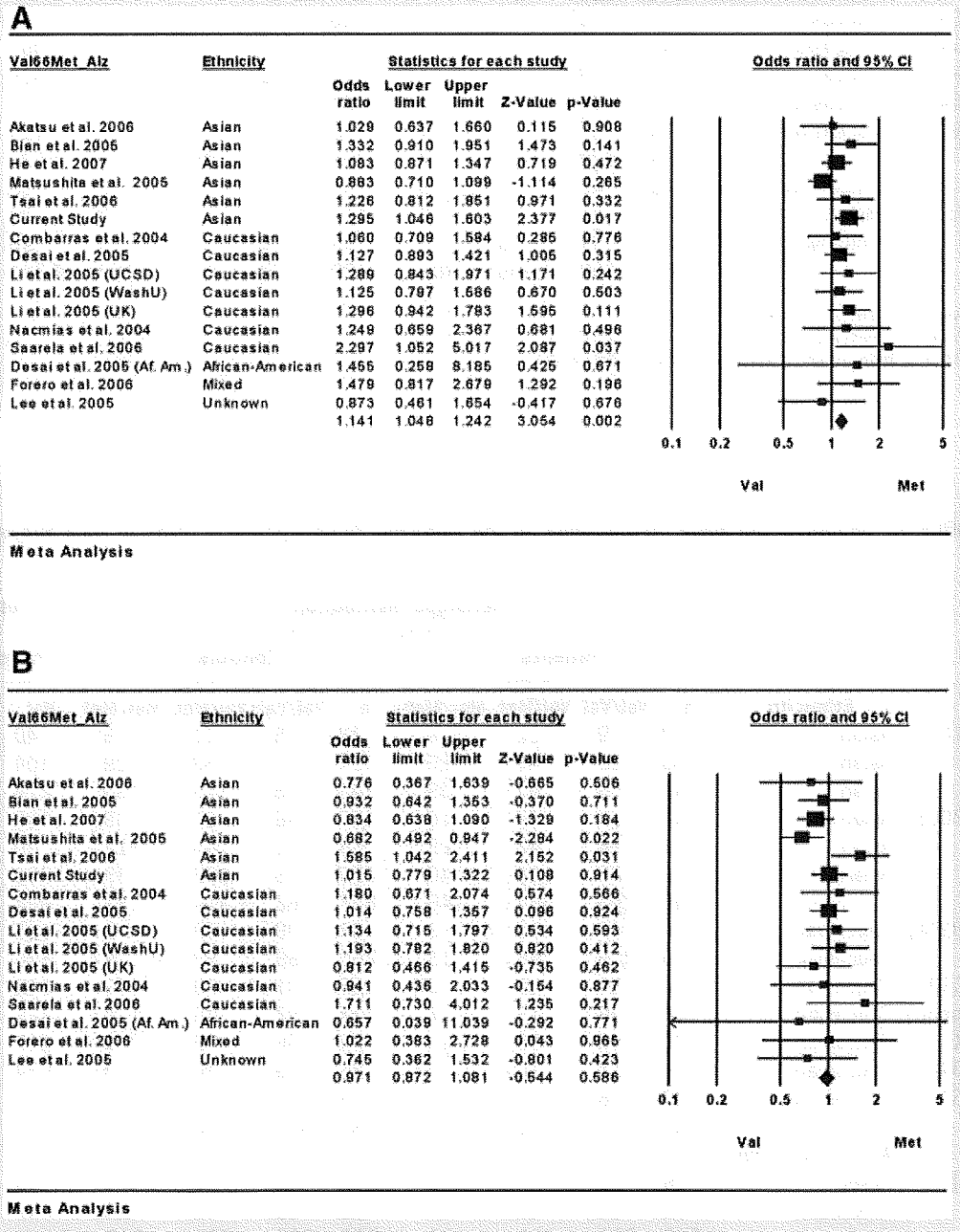

FIG. 1. Forest plots of meta-analysis on the possible association between the Val66Met polymorphism of *BDNF* and Alzheimer's disease in female (A) and male (B) subjects.

95% CI = 1.02–1.22; Z = 2.30, $P = 0.022$). The Rosenthal failsafe N for women was 31 studies. No evidence of publication bias was indicated by Egger's test (intercept = 0.80, 95% CI = −0.37 to 0.54, $t = 1.46$, $P = 0.17$).

Meta-analysis for the C270T polymorphism was performed in the same way. Eighteen studies were identified, of which 12, including ours, participated in the meta-analysis. The individual studies contained 58–722 AD cases and 42–525 controls, and the combined sample consisted of 2,963 subjects with AD and 2,756 controls (Supplementary Tables IV, VII, and VIII). There was a significant heterogeneity between studies (total: Q = 44.7, df = 17, $P < 0.01$; men: Q = 18.8, df = 11, $P = 0.065$; women: Q = 30.2, df = 11, $P < 0.01$). Thus, we performed the random effects meta-analyses (Supplementary Fig. 2). Our meta-analysis did not show significant association of AD with the T270 allele (random-effect pooled OR = 1.07, 95% CI = 0.83–1.39; Z = 0.54, $P = 0.59$; Supplementary Fig. 2A). Also when men and women were examined separately, our meta-analysis revealed no significant association with AD in women (OR = 1.08, 95% CI = 0.70–1.67; Z = 0.37, $P = 0.72$; Supplementary Fig. 2B) or in men (OR = 1.19, 95% CI = 0.77–1.84; Z = 0.78, $P = 0.43$; Supplementary Fig. 2C).

DISCUSSION

We showed, for the first time, a significant allelic association between the Val66Met of *BDNF* and AD in women in our Japanese sample ($P = 0.017$). In contrast, we did not observe such an association in men. When the multi-center study was organized, the sexually dimorphic effect of the Val66Met on the development of AD was similarly observed in the much larger sample (4,711 patients and 4,537 controls) from 16 research centers worldwide. These results provide evidence suggesting that the Met66 allele has a risk-increasing effect on AD in women, but not in men.

The Met66-BDNF protein has been shown to be associated with reduced transport of BDNF from the Golgi region to appropriate secretory granules in neurons, compared with the Val66-BDNF protein [Egan et al., 2003; del Toro et al., 2006]. It is reasonable to assume that the Met66 is associated with lower secretion of BDNF, which could result in attenuation of the survival signal of BDNF, compared with the Val66. In accordance with this, individuals carrying the Met66 allele have been reported to have decreased brain structures (e.g., hippocampus) than those individuals who did not carry the allele [Pezawas et al., 2004; Szeszko et al., 2005; Agartz et al., 2006; Bueller et al., 2006; Ho et al., 2006; Nemoto et al., 2006; Frodl et al., 2007; Liguori et al., 2007]. Of note, we found that female individuals carrying the Met66 allele showed more widespread age-associated volume reduction in the dorsolateral prefrontal cortices than male Met66 carriers [Nemoto et al., 2006].

Several lines of evidence suggest the sexual dimorphic effects of *BDNF*. The study of *BDNF* conditional knockout mice demonstrated sexually dimorphic effects in depression- and anxiety-related behavior [Monteggia et al., 2007]. A recent sexually stratified meta-analysis reported that the Val66Met was more important in the development of major depressive disorder in men than in women [Verhagen et al., 2008]. In Parkinson's disease as well, a sex difference in the effect of *BDNF* was reported [Foltynie et al., 2005]. Many epidemiological studies reported higher prevalence and incidence of AD in women than in men [Fratiglioni et al., 1997]. In an animal model of neurodegenerative diseases, aged female mice were more sensitive to kainic acid-induced excitotoxicity to neurons, compared with aged males [Zhang et al., 2008]. These findings are in line with our observations of the sexually dimorphic effect of *BDNF* on AD. Indeed, estrogen plays an important role in the expression of BDNF. Estrogen receptors co-localize with BDNF-synthesizing neurons in the forebrain [Miranda et al., 1993] and estrogen induces BDNF expression through the estrogen response element [Sohrabji et al., 1995].

With respect to the C270T, we obtained no evidence for an association with AD in our sample alone or in the combined sample. We observed a significant heterogeneity across studies in the meta-analysis. In addition, the allele frequency of the risk allele (T270) reported in the original study [Kunugi et al., 2001] was quite low (0.03 in total), indicating the possibility of type II error due to lack of statistical power. Thus, further studies are required to draw any conclusion.

In conclusion, we provided the first meta-analytic evidence that the Met66 allele of *BDNF* has a sexually dimorphic effect on susceptibility to AD. Studies elucidating the molecular mechanisms underlying this association are warranted.

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