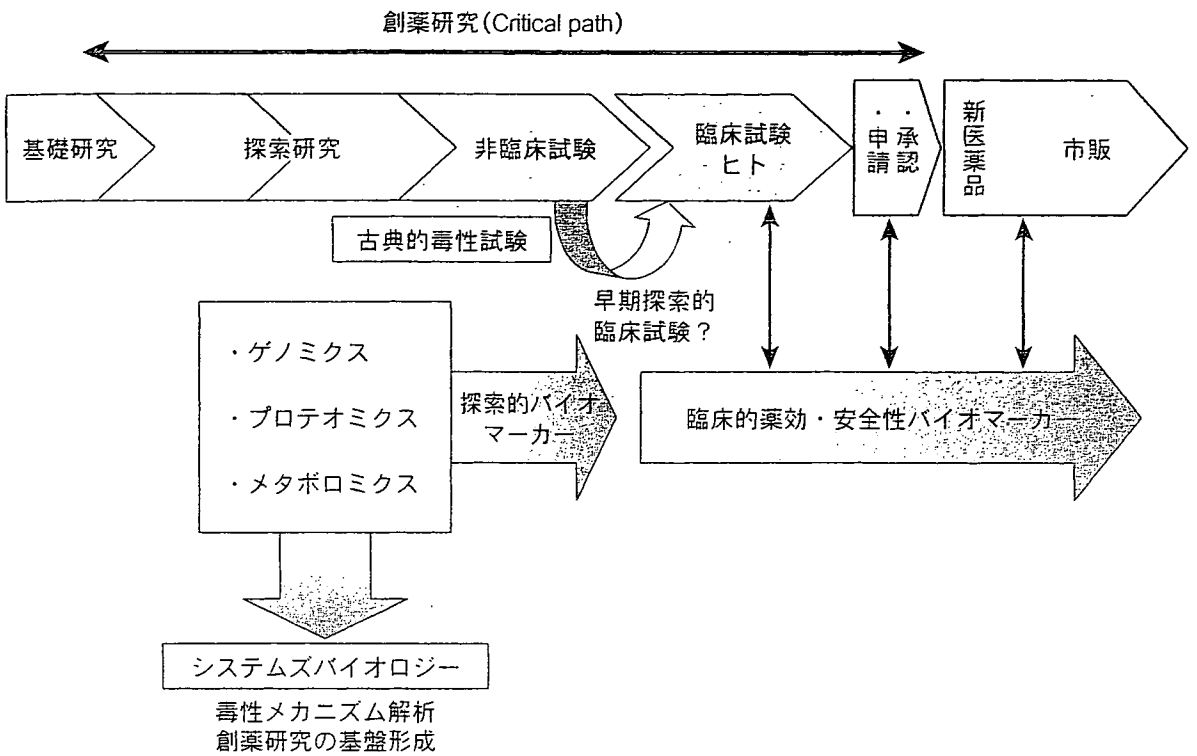

図2　創薬研究における安全性研究の関わり

古典的毒性学的研究が必要であることは変わりないが，ここにオミクステクノロジーを導入することによって毒性メカニズムの解析が進み，より成功確率の高い候補品が選択できる。望むらくはこの段階から，臨床の各段階での使用に耐えるバイオマーカーの創出につなげたい。動物実験と治験の間の大きなギャップを埋めるべく提案された早期探索的臨床試験は，現状の安全性研究に限っていえばまだ未知数である。

マイクロドーズ臨床試験よりは高いが，薬効用量以下の用量を用い，有害作用・薬効のいずれも現れないと想定される「準薬効用量早期探索的臨床試験」，及び（3）有害作用は現れないが，薬効は現れると想定される用量での「薬効用量早期探索的臨床試験」に大別される。現在わが国でガイダンス案が検討されているのは，このうち「マイクロドーズ臨床試験」であり，得られる情報もほぼ薬物動態に限られる。従って，安全性研究の立場からいえば，マイクロドーズ臨床試験で要求される安全性試験の妥当性，および将来，より高用量の試験に拡大されたときの安全性予測精度向上の可能性の２点が問題となり，現在はまだ臨床での安全性予測に直結するものとはいえない。

「マイクロドーズ臨床試験」とは，健常人に開発候補物質を「毒性試験におけるNOAEL[脚注]，及び薬効を発現するための予測投与量の1/100を超えない用量と，100μgのいずれか小さい方」を用いた極低用量（約2μg/kg）で単回投与する臨床試験である。本稿執筆の時点でまだガイドラインは確定していないが，欧米の例からみて，必要な前臨床試験としては拡大型単回投与毒性試験（一種の雌雄げっ歯類を用いた単回投与後２週間観察，各種毒性学的検査）を行って最小毒性量を求めればよく，かなり軽減されるようにみえる。問題は，臨床における薬効用量の推定と，放射性標識体による被験者内部被曝の安全性保証にあろう。特に後者は主目的が薬物動態であるため，極微量の試験薬を検出する必要から避けることが難しい。これはマイクロドーズ臨床試験を行わなければ生じない問題であり，放射性物質の規制が厳しく，一般人の心理的抵抗が高いわが国では，かなり高い

NOAEL：no observed adverse effect level；無毒性量

ハードルであると思われる。マイクロドージングを行えば通常の毒性試験や第Ⅰ相試験を省略できるのであればともかく，世界同時開発が通常である現在，最も規制のゆるい国で施行されることは確実で，わが国でわざわざマイクロドーズ臨床試験を行うメリットがあるとすればPET（ポジトロン断層撮影診断）用の診断薬ぐらいのものであろうという意見も聞かれる。

筆者の主観であるが，少なくともわが国の安全性研究においてマイクロドーズ臨床試験が大きなインパクトをもつことはまだないと思われる。将来的な問題は，用量を増加した早期探索的臨床試験が行われる場合の安全性研究である。2006年3月にロンドン近郊の病院において実施された完全ヒト化抗CD28モノクローナル抗体（TGN1412）の事件[5]はまだ記憶に新しい。第Ⅰ相試験において，投薬を受けた6名全員がICU（集中治療室）に搬送されるという，絶対に起きてはならない事態であり，「所詮動物実験による安全性予測精度が低いのならば，早期に人体実験に移行した方がよい」という一部の意見に，冷水を浴びせることになった。治験のプロトコールに見出された問題や，前臨床試験の充実でこれが予測できたか否かなど論点が多く，この場でこの問題を論じるのは避けるが，最大のポイントは，臨床における有害作用を時間的・用量的に予測可能なバイオマーカーが利用可能かどうかというところに帰着する。その意味から，これまで述べてきた種々の技術の応用がますます重要になってくることは明らかである。

以上，安全性医薬品開発における安全性研究の最近の動向を概観してみた。かつての創薬研究において安全性がネックになっていたことは否めないが，近年のオミクステクノロジーの進歩を取り入れることによってブレークスルーが可能になってきたとの感がある。現在の最大の課題は，これを臨床開発で活用できるバイオマーカーとして結実させることであるにちがいない。（図2）。

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レクチャーノート：
トキシコゲノミクスプロジェクト(2)
研究戦略

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前回述べたような経緯でトキシコゲノミクスプロジェクトの基本コンセプトが決定されたわけであるが，プロジェクト発足の2002年当時，マイクロアレイ技術は発展途上にあったものの，すでに米国では，その2年前にNational Center for Toxicogenomicsのプロジェクトが開始されており，ベンチャー企業の動きも盛んであった．また我が国でも，前年度から，新エネルギー・産業技術総合開発機構によって，マイクロアレイ技術による化学物質の発がん性予測を企図したプロジェクトが開始されていた．それらの先行プロジェクト，特にマイクロアレイデータを用いた安全性予測を請け負うとする欧米のバイオベンチャーに対抗して，本プロジェクト特有のメリットを付加する必要があった．そこで立てられた戦略は以下の4点であった．

1) 定量性に優れたAffymetrix社GeneChipを採用し，DNA量に基づいたSpike RNAを添加して細胞1個あたりのmRNA量を評価する手法(percellome)[1]を採用する．

2) 全被検化合物150は標準的医薬品を中心とし，副作用によって開発・市販中止となった薬物や，参加企業提供の独自化合物を含める．

3) 実験動物としてラットを採用し，単回・反復それぞれ4時点・4用量と，十分な用量・時間設定のもとに得られた各種毒性学的データのフルセットを，遺伝子発現データとリンクさせ，かつ関連情報と有機的に結びつけ，統合データベースとして構築する．

4) ラット一次培養肝細胞とヒト凍結肝細胞の系を用いて，種差のブリッジングを考慮する．

5年が経過した現在，データのデータベースへの格納が完了し，解析・予測システムとして，TG-GATEs: Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project Japanがほぼ完成した．そこで，これらについて振り返って検証してみたい．

1)については，厳密なQCプロトコールを確立し，信頼性のある定量的遺伝子発現データが得られ，検証済のデータの格納が完了した．マイクロアレイデータは所詮信頼性に乏しく，最終的には定量PCRによって確認すべきであるとの意見が旧来の研究者から聞かれることがあるが，少なくとも適切に管理された環境で取得されたGeneChipデータに関しては，PCRと同等，あるいはそれを凌駕する定量性と再現性が得られるというのが我々の感触であり，これは国際的にも認められつつある[2]．また，実験開始当初，毒性発現時のような極端な条件下では遺伝子発現が極端に変動し，グローバル補正では発現値を標準化できないのではという危惧があり，percellome補正を併用して解析を行ってきた．しかしながら，データが蓄積されるにつれ，明瞭なフェノタイプが認められる条件下であっても，少なくとも*in vivo*の肝臓においての薬物影響を対照群との比較において解析する場合は，グローバル補正で問題なく行えることが確認できた．従って本プロジェクトの基本的な解析はグローバル補正法に基づいて行なわれている．ただし，組織や器官が異なる場合の解析にはpercellome補正法が有用である場合もあり[3]，必要に応じて細胞(DNA)当たりの発現量を計算できる環境を整えてある．

2)に関しては，前回述べたとおり，選択した化合物のターゲットは広範な治療領域をカバーし，典型的な薬物はほぼ網羅されている．世界的にも，実際に使用された，また現在も使用されている医薬品のトランスクリプトームデータをこの規模で揃えたデータベースは他に類を見ない．ただしここで問題となるのが「毒性とは何か」という問題である．環境毒性物質であればNo Observed Effect Level (NOEL)とNo Observed Adverse Effect Level (NOAEL)を区別して定義できる．しかしながら医薬品を対象にした場合，原理的にNOELはあり得ず，観測された効果がadverse effectか否か，あるいは，期待される薬効と比較して許容範囲か否か，を決定する必要がある．トランスクリプトームデータに基づいて新規開発候補品の毒性発現を避けるのは，ある意味で簡単なことである．「疑わしい」遺伝子の動きを示した薬物の開発をすべて中止すればよいのである．しかしこのようなことをすれば，有用な医薬品が世に出ることがなくなり，人類の福祉にとって逆効果となるのは明らかであろう．この問題に対しては現在明確な答えをもっていないが，プロジェクトでは，毒性発現機序に基づいた解析を可能とするために，典型的な肝・腎毒性物質(四塩化炭素，ヘキサクロロベンゼンなど)や，毒性パスウェイが分子レベルで解明されつつある物質類(サイトカイン，タンパク合成阻害薬，グルタチオン枯渇薬など)のデータも取得してきた．TG-GATEsを活用して，毒性メカニズムが分子レベルで明らかになっていくことにより，将来，この問題に解答が得られることが期待される．

3)の用量・時間水準の充実であるが，この点が本プロジェクトの最大のメリットであると考えられる．マイクロアレイによって実質的に全遺伝子(5桁のオーダーの数)の発現変動を一気に測定できるとしても，コストを考える

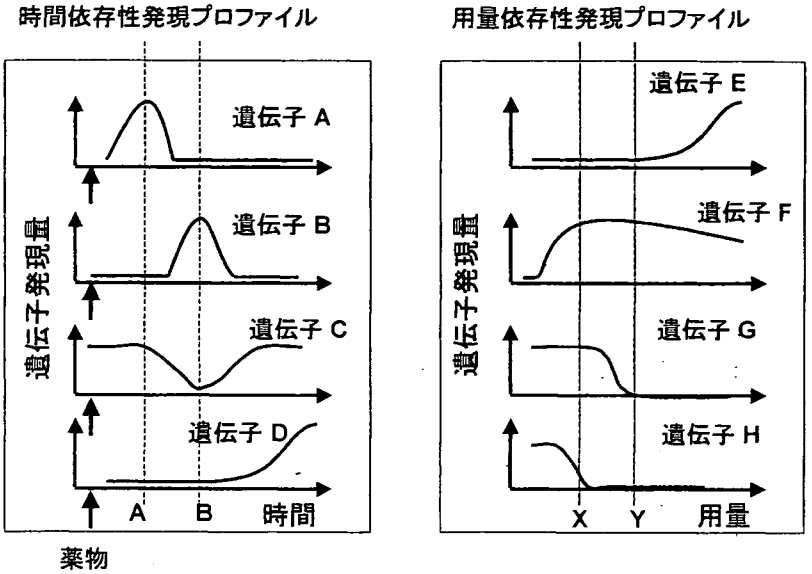

図　仮想的な遺伝子発現パターン

と，時点や用量水準は制限される．各測定点においての例数は通常一桁であり，数万の遺伝子の変動解析に対して統計学は無力である．遺伝子発現解析において，個々の遺伝子の生物学的重要性は大きく異なるため，一律の統計学的処理では重要な変化を見逃す確率が高い．また，仮に各測定点における変動を正確に把握できたとしても，大きな問題が残される．図に，仮想的な遺伝子発現パターンを示した．遺伝子発現は，ある事象に次々に応答する連続的なパターンを示すことが予想され，図左側の時点AとBの測定では，連続した事象のある断面しか得られない．また，用量反応性に関しても同様のことが成り立ち，用量XとYでは異なった効果のように見えてしまう．仮に時点A，用量Xのデータから予測アルゴリズムを構築できたとしても，これは時点B，用量Yでのものとは全く異なるはずである．毒性メカニズムが同一の薬物ならば同様の時間・用量依存性を示す，ということが成り立つのであれば補正が可能であろうが，容易に推測できるように，メカニズムが同一でも，例えば薬物動態が異なっていれば，全く違う像が見えてしまうであろう．

本プロジェクトの目標が「医薬品の開発初期段階における肝・腎毒性の予測」である以上，いつ，何が起こるかわからない毒性を予測する場合，前もって用量や時点を制限してしまったようなデータベースの利用価値は限られたものになってしまう．実際，毒性学的に重要な多くの遺伝子発現が，同じ薬物であってさえも，種々の条件によって応答時間・用量に大きな差が出ることが見出されてきている[4]．この問題を計算科学的にどう克服していくかはこれからの課題であるが，少なくとも，リファレンスとなる薬物群について，充実した用量・時点でのデータを集積した今回のデータベースの価値は非常に高いといえるであろう．

4）種差のブリッジングの問題は，計画当初から困難が予想されていた．人体実験が不可能である以上，人型組織を持つ動物か *in vitro* のモデル系の2つしか可能性がない．前者はモデル系としてまだ未成熟であると考え，*in vitro* のモデル系を選択することとした．cell line は，系の均一性・再現性という点では優れているが，正常組織と cell line の遺伝子発現パターンはあまりにも異なるため，結果的に一次培養細胞を選択せざるを得なかった．すなわち，肝毒性に関して，ラット *in vivo*→ラット一次培養細胞系→ヒト凍結肝細胞培養系→ヒト臨床，というブリッジングを企図したのである．データが集積され，解析を行ってみると，種差も大きいが，*in vitro* と *in vivo* の差も非常に大きく，単純なブリッジングの戦略は通用しないことが次第に明らかとなってきた．これはトキシコゲノミクス手法の限界というよりも，生物学的な限界である．しかしながら，フェノタイプや毒性パスウェイそれぞれについて，「種差を越えた適用可能」「種差が大きい」「*in vivo* と *vitro* のブリッジング不可能」などの分類・評価は可能であり，それだけでも有用な情報といえる．

生体は複雑系であり，それに対する薬物影響は実質上計算不能な事象である．最初からある生物学的応答に注目した場合は，絞られたプロトコールから得られた絞られた遺伝子の発現値を用いた定型的な予測アルゴリズムが設定可能であるかもしれないが，「新規化合物の未知の毒性」という，全く手がかりのない状態からの予測の場合，現在の生物学のレベルからいって，定型的アルゴリズムは設定不可能であるし，むしろ，採用してはならない戦略であろう．本プロジェクトで構築した TG-GATEs は，それぞれ

の化合物の特徴を多方面から捉え，それぞれの特徴がデータベース中でどのような位置づけにあるかを概観した後，研究者がそれぞれの切り口・視点で効率的に解析・予測して行くことを可能としたシステムである．予測困難な「毒性」という曖昧な実体を，研究者が多方面からの gates をくぐって探索することにより，理想的な薬物創製に役立てる — TG-GATEs にはそのような思いがこめられている．次回は，この TG-GATEs の内容を紹介する．

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Research Report

β-Estradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells

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ABSTRACT

We investigated the effect of β-estradiol (E2) on synaptogenesis in the hippocampus using organotypic hippocampal slice cultures and subregional hippocampal neuron cultures. E2 increased the expression of PSD95, a postsynaptic marker, specifically in stratum lucidum of Cornu Ammonis 3 (CA3SL) in cultured hippocampal slices. E2 also increased the spine density at the proximal site of CA3 apical dendrites in CA3SL and PSD95 was clustered on these spine heads. The effects of E2 on the expression of PSD95 and the spine density disappeared when the dentate gyrus (DG) had been excised at 1 day in vitro (DIV). FM1-43 analysis of subregional hippocampal neuron cultures which were comprised of Ammon's horn neurons, DG neurons, or a mixture of these neurons, revealed that E2 increased the number of presynaptic sites in the cultures that contained DG neurons. K252a, a potent inhibitor of the high affinity receptor of brain-derived neurotrophic factor (BDNF), and function-blocking antibody to BDNF (BDNFAB) completely inhibited the effects of E2 in hippocampal slice cultures and subregional neuron cultures, whereas ICI182,780 (ICI), a strong antagonist of nuclear estrogen receptors (nERs), did not. Expression of BDNF in DG neurons was markedly higher than that in Ammon's horn

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Abbreviations: ACM, astrocyte-conditioned medium; ANOVA, analysis of variance; AraC, cytosine β-D-arabino-furanoside; BDNF, brain-derived neurotrophic factor; BDNFAB, function blocking antibody to BDNF; BSA, bovine serum albumin; CA1, Cornu Ammonis 1; CA3, Cornu Ammonis 3; cAMP, 3′–5′-cyclic adenosine monophosphate; CNS, central nervous system; CREB, PKA/cAMP-responsive element binding protein; DG, dentate gyrus; DIC, differential interference contrast; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DIV, day(s) in vitro; DMSO, dimethylsufoxide; E2, β-estradiol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; ER, estrogen receptor; FM1-43, (*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide; GABA, γ(gamma)-aminobutyric acid; HBSS, Hank's balanced salt solution; HS, horse serum; ICI, ICI182,780; IgG, immunoglobulin G; LDCVs, large dense-core vesicles; L-Glu, L-glutamate; LTP, long-term-potentiation; MEK, MAP kinase kinase; MEM, minimal essential medium; mER, membrane estrogen receptor; NB, neurobasal medium; nER, nuclear estrogen receptor; NeuN, neuronal nuclear antigen; OD, optical density; P3, postnatal day 3; P8, postnatal day 8; PB, phosphate buffer; PBS, phosphate buffered saline; PDZ, PSD-95-Disks large-zona occludens 1/2; PFA, paraformaldehyde; PKA, cAMP-dependent protein kinase A; PSD95, postsynaptic density protein of 95 kDa; Rp-cAMP, Rp-adenosine 3′, 5′-cyclic monophosphorothioate triethylammonium salt; SDS, sodium dodecyl sulphate; S.E.M., standard error of the mean; SL, stratum lucidum; SO, stratum oliens; SP, stratum pyramidale; SR, stratum radiatum; TBS, Tris-buffered saline; TrkB, the high affinity receptor for several neurotrophins; TTX, tetrodotoxin

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neurons and E2 did not affect these expression levels. E2 significantly increased the BDNF release from DG neurons. KT5720, a specific inhibitor of 3′–5′-cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), and Rp-adenosine 3′, 5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMP), a non-hydrolyzable diastereoisomer and a potent inhibitor of PKA, completely suppressed the E2-induced increase in BDNF release, whereas ICI and U0126, a potent inhibitor of MAP kinase kinase (MEK), did not. These results suggest that E2 induces synaptogenesis between mossy fibers and CA3 neurons by enhancing BDNF release from DG granule cells in a nER-independent and PKA-dependent manner.

1. Introduction

Estrogens have diverse effects on structure and function of the central nervous system (CNS) (for review, McEwen et al., 2001; Scharfman and MacLusky, 2005; Segal and Murphy, 2001). These effects include enhancement of glutamate-mediated transmission (Woolley, 1998), decreased afterhyperpolarization (Kramar et al., 2004), facilitation of memory (Tyler et al., 2002), increased dendritic spine and spine synapse numbers (Segal and Murphy, 2001), promotion of DG neurogenesis (Tanapat et al., 1999), and increased seizure susceptibility (Woolley and Schwartzkroin, 1998). Such diversity arises because estrogens have multiple mechanisms of action. They modulate gene transcription by interacting with 2 types of nERs, ERα and ERβ. In addition, recent reports clarified nongenomic mechanisms that act via receptors associated

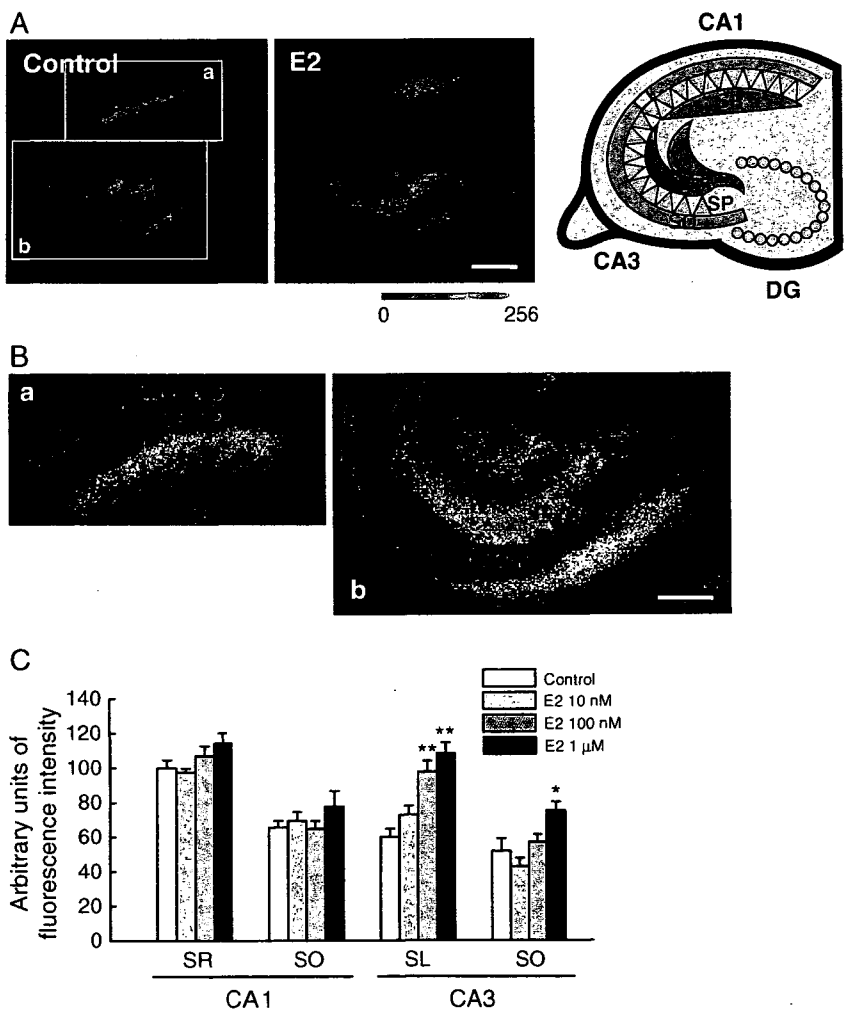

Fig. 1 – Effects of E2 on the expression of PSD95 in cultured hippocampal slices. (A) PSD95 immunoreactive signals in the control slice (left) and the slice treated with E2 (1 μM, 24 h) (middle). Bar=500 μm. (B) Magnified gray-scale images of a and b in A. CA1SR, CA1SO, CA3SL, and CA3SO appeared as fluorescent compartments. Bar=250 μm. (C) Effects of E2 (10 nM–1 μM, 24 h) on the expression of PSD95. E2 increased the expression level of PSD95 dose-dependently in CA3SL. *: $p<0.05$, **: $p<0.01$ vs. the control group in each region. $N=8$, Tukey's test following ANOVA.

with or integral to plasma membrane (mERs), thereby activating signaling cascades distinct from those of nERs (Beyer et al., 2003; Kelly and Levin, 2001; Segars and Driggers, 2002). We previously reported that pretreatment with estrogens increased neuronal sensitivity to L-glutamate (L-glu) specifically in CA3 in organotypic hippocampal slice cultures. In the same study we found that these effects were mediated by the mechanisms that did not involve nERs (Sato et al., 2002). These results raised the possibility that estrogens affect synaptic contacts in CA3. In the present study, we therefore investigated the effects of E2 on synaptogenesis in the hippocampus and explored the underlying mechanisms using 2 experimental systems. Firstly, we investigated the effects of E2 on the expression of PSD95, a postsynaptic marker, and the spine density in cultured hippocampal slices. Secondly, we investigated the effects of E2 on the number of presynaptic release sites in subregional hippocampal neuron cultures, which were comprised of Ammon's horn neurons, DG neurons, or a mixture of these neurons. It has been reported that in the hippocampus the highest concentration of BDNF occurs in DG granule cells, especially in their axons, mossy fibers (Dieni and Rees, 2002; Scharfman et al., 2003), from the prenatal period through to adulthood (Dieni and Rees, 2002). Although BDNF is known to promote synaptogenesis (Aguado et al., 2003; Alsina et al., 2001; Seil and Drake-Baumann, 2000), it has not been elucidated whether the BDNF in DG granule cells has a role in hippocampal synapse formation. For this reason, we also investigated the relationship between endogenous BDNF in DG granule cells and the effects of E2 in CA3. We here provide evidence showing that E2 induces synaptogenesis between mossy fibers and CA3 neurons by enhancing BDNF release from DG granule cells in a nER-independent and PKA-dependent manner.

2. Results

2.1. *Effects of E2 on postsynaptic sites in cultured hippocampal slices*

We first examined the effect of E2 on the expression of PSD95 in cultured hippocampal slices immunohistochemically. PSD95 is one of the PDZ (PSD-95-Disks large-zona occludens 1/2) domain-containing proteins (Craven and Bredt, 1998; Garner et al., 2000) and is an integral protein of the postsynaptic density. In the control group, the fluorescent signals for PSD95 were apparent in the major hippocampal synaptic sites, i.e., stratum radiatum (SR), stratum oriens (SO), SL and the dentate hilar region (Fig. 1A, left). Because in this study slices were cultured after removing entorhinal cortex, we quantified the expression of PSD95 in CA1SR, CA1SO, CA3SL, and CA3SO, the synaptic sites which maintain the intact presynaptic and postsynaptic cells. Because CA1SR, CA1SO, CA3SL, and CA3SO appeared as fluorescent compartments (Figs. 1B, a and b) in magnified gray-scale mode images, we regarded the averaged fluorescence intensity of each compartment (an outlined area) as the expression level of PSD95 of each synaptic site (see Experimental procedures). When we compared the effects of E2 on the PSD95 expression in CA1 and CA3, E2 (24 h) increased the expression of PSD95 dose-dependently in CA3SL and the effects were significant at 100 nM and the higher concentration (Figs. 1A middle and B). Although E2 also increased the PSD 95 expression in CA3SO at 1 μM (145±9.75% of control), the effect was weaker than that in CA3SL (180±10.2% of control at 1 μM). The distribution pattern of PSD95 signals (including area) in each region was not affected by E2. We then investigated the effect of E2 on the spine density in CA3SL using 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) staining. E2 (1 μM, 24 h) markedly increased the spine density at the proximal site of CA3 apical dendrites in CA3SL (296±24.3% of control; Figs. 2A and B). E2 also increased the spine density at the proximal site of CA1 apical dendrites in CA1SR (132±4.49% of control), although to a much lesser extent than that in CA3SL (Fig. 2A). Fig. 2B shows typical images of the proximal sites of CA3 apical dendrites in the control slice (left) and in the E2-treated slice (right). When we immunostained the E2-treated slices with anti-PSD95 antibody after DiI staining, most PSD95 signals in CA3SL clustered on the spine heads (Fig. 2B, right). These results indicate that E2 increased the number of postsynaptic sites in CA3SL. CA3SL is the region in which mossy fibers (DG granule cell axons) make synapses with CA3 pyramidal neurons. We then investigated the effect of E2 on the expression of PSD95 and the spine density in CA3 in DG (−) slices, i.e., the slices of which DG had been excised at 1 DIV. As shown by

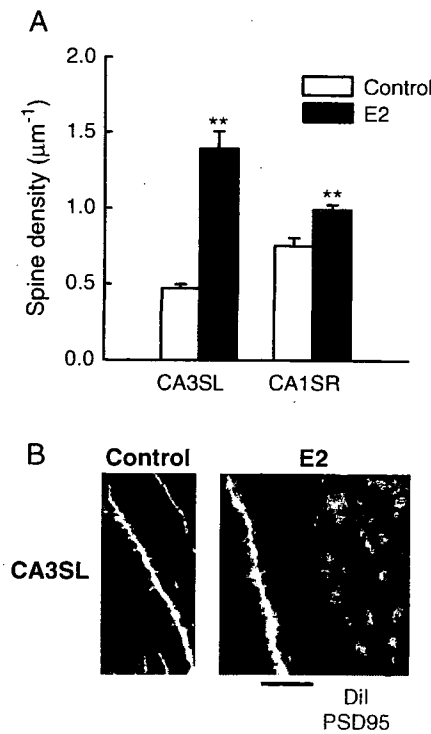

Fig. 2 – Effects of E2 on the spine density in cultured hippocampal slices. (A) E2 (1 μM, 24 h) markedly increased the spine density in CA3SL. **: $p<0.01$ vs. the vehicle control group in each region. $N=8$, Student's t test. (B) Typical images of the DiI-labeled CA3 apical dendrites in the control slice (left) and the E2-treated slice (right). Double staining with DiI and anti-PSD95 antibody revealed that in the E2-treated slice most PSD95 signals (green) were clustered on the spine-heads of the CA3 apical dendrites (red). Bar=5 μm.

Nissl staining, the viability of CA3 pyramidal neurons was not altered by the dissection of the DG (Fig. 3A). The distribution pattern of the PSD95 signals was not affected, either (Fig. 3B). E2 (1 μM, 24 h) affected neither the expression level (Fig. 3C) nor the distribution pattern of PSD95 in DG (–) slices (data not shown). The effect of E2 (1 μM, 24 h) on the spine density in CA3SL was also abolished in DG (–) slices (Fig. 3D). Taken together, these results suggest that E2 induces synaptogenesis between mossy fibers and CA3 pyramidal neurons.

2.2. *Effects of E2 on presynaptic sites in subregional hippocampal neuron cultures*

We next investigated the effect of E2 on the number of presynaptic sites using subregional hippocampal neuron cultures, which were comprised of Ammon's horn neurons, DG neurons, or a mixture of these neurons, respectively. We quantified the number of presynaptic sites by counting the number of sites in which depolarization-induced uptake and release of (*N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide (FM1-43) (Cochilla et al., 1999) had occurred (see Experimental procedures). Fig. 4A shows the typical morphologies of neurons in the Ammon's horn neuron culture (left) and in the DG neuron culture (middle). Most cells in the Ammon's horn neuron culture were large and spindle-shaped, whereas most cells in the DG neuron culture were small and granular. As shown in Fig. 4B, E2 (1 μM, 24 h) significantly increased the number of presynaptic sites in the mixed neuron culture (199±9.18% of control). E2 also increased the number of presynaptic sites in the DG neuron culture (170±12.1% of control), but not in the Ammon's horn neuron culture. Fig. 4C shows the typical fluorescent images of presynaptic sites (red puncta) in the control group (top left) and in the E2-treated group (top right) in the mixed neuron culture. We confirmed that E2 had no effect on the number of surviving neurons in each culture by immunostaining with anti-NeuN antibody (data not shown). These results indicate that E2 increased the number of presynaptic sites in the hippocampal neuron cultures and that DG neurons are indispensable for this effect.

2.3. *The effects of E2 in hippocampal slice cultures and subregional hippocampal neuron cultures were mediated by the mechanism which is independent of nERs and dependent on endogenous BDNF*

Pharmacological experiments were performed to investigate and compare the mechanisms underlying the effects of E2 in hippocampal slice cultures and subregional hippocampal neuron cultures (the mixed neuron culture) (Fig. 5). First, we examined the contribution of nERs using ICI, a strong antagonist to both of ERα (Ki: 1.5 nM) and ERβ (Ki: 6.4 nM) (Kuiper et al., 1997). ICI at a concentration of 1 μM did not alter the effect of E2 on the expression of PSD95 expression, the spine density, and the number of presynaptic sites (Figs. 5A–C). It has been reported that DG granule cells have the highest concentration of BDNF in the hippocampus, especially in the mossy fibers (Dieni and Rees, 2002; Scharfman et al., 2003). Because BDNF is known to enhance synapse formation (Aguado et al., 2003; Alsina et al., 2001; Seil and Drake-

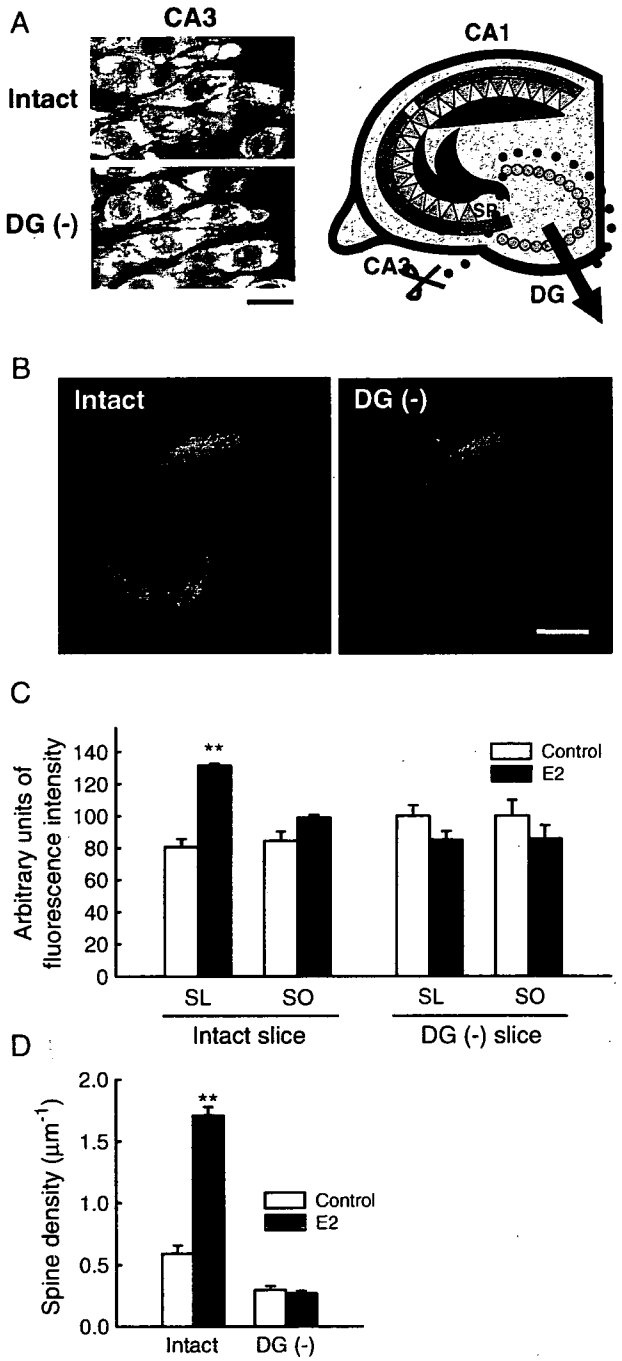

Fig. 3 – Effects of E2 on the expression of PSD95 and the spine density in cultured hippocampal slices of which DG had been excised at 1 DIV. (A) The viability of CA3 pyramidal neurons in DG (–) slices. Nissl staining revealed that their viability was not affected by the dissection of DG. Bar=20 μm. (B) Immunoreactive signals of PSD95 in a DG (–) slice. The distribution pattern of the PSD95 signals was not affected by the dissection of DG. Bar=500 μm. (C) The effect of E2 on the expression of PSD95 in DG (–) slices. E2 (1 μM, 24 h) did not affect the expression of PSD95 in CA3 in DG (–) slices. (D) The effect of E2 on the spine density in CA3SL in DG (–) slices. E2 (1 μM, 24 h) did not affect the spine density in CA3SL in DG (–) slices.

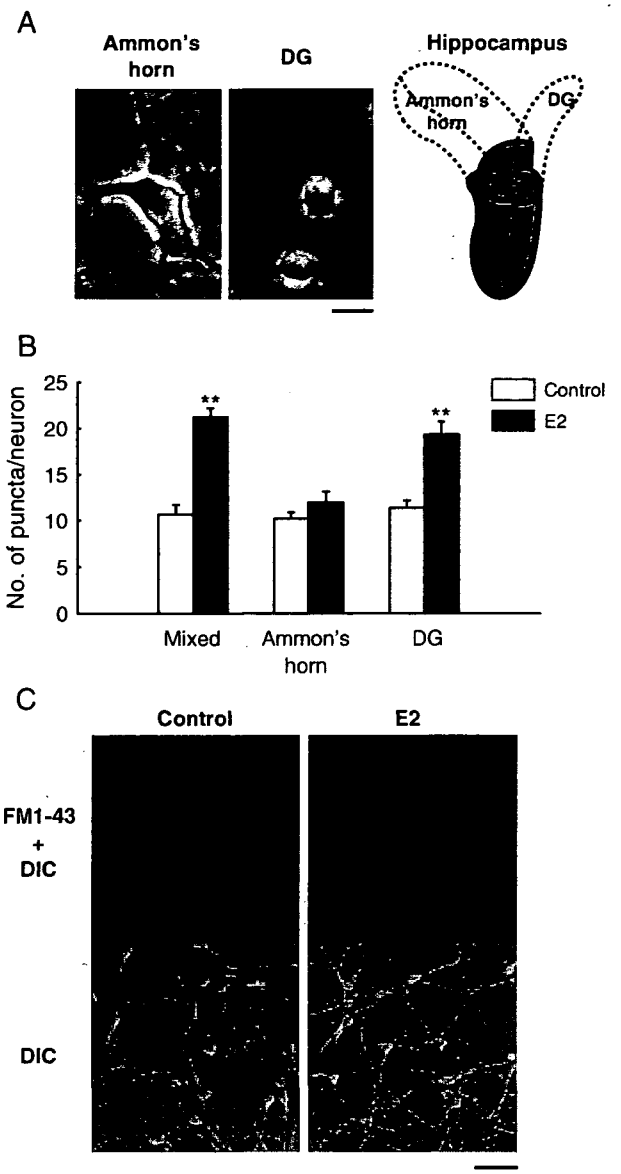

Fig. 4 – Effects of E2 on the number of presynaptic sites in subregional hippocampal neuron cultures. (A) Typical cell morphologies in the Ammon's horn neuron culture (left) and in the DG neuron culture (middle). Bar=20 μm. (B) E2 (1 μM, 24 h) significantly increased the number of presynaptic sites in the mixed neuron culture and in the DG neuron culture. **: $p<0.01$ vs. the control group in each culture. $N=8$, Student's t test. (C) Typical images of presynaptic sites visualized by FM1-43 (red puncta) in the control group (top left) and in the E2-treated group (top right) in the mixed neuron culture. DIC images of the same microscopic views were also shown (bottom left and bottom right). Bar=50 μm.

Baumann, 2000), we examined the involvement of BDNF in the effects of E2. K252a (200 nM), a potent inhibitor of the high affinity receptor of BDNF (TrkB) (Squinto et al., 1991; Bothwell, 1995), significantly inhibited the effects of E2 on the expression of PSD95 expression, the spine density, and the number of presynaptic sites (Figs. 5A–C). Furthermore BDNFAB (10 μg/ml) significantly inhibited the effects of E2 in these experiments (Figs. 5A–C). These inhibitors alone had no effects in each case. These results indicate that the effects of E2 in hippocampal

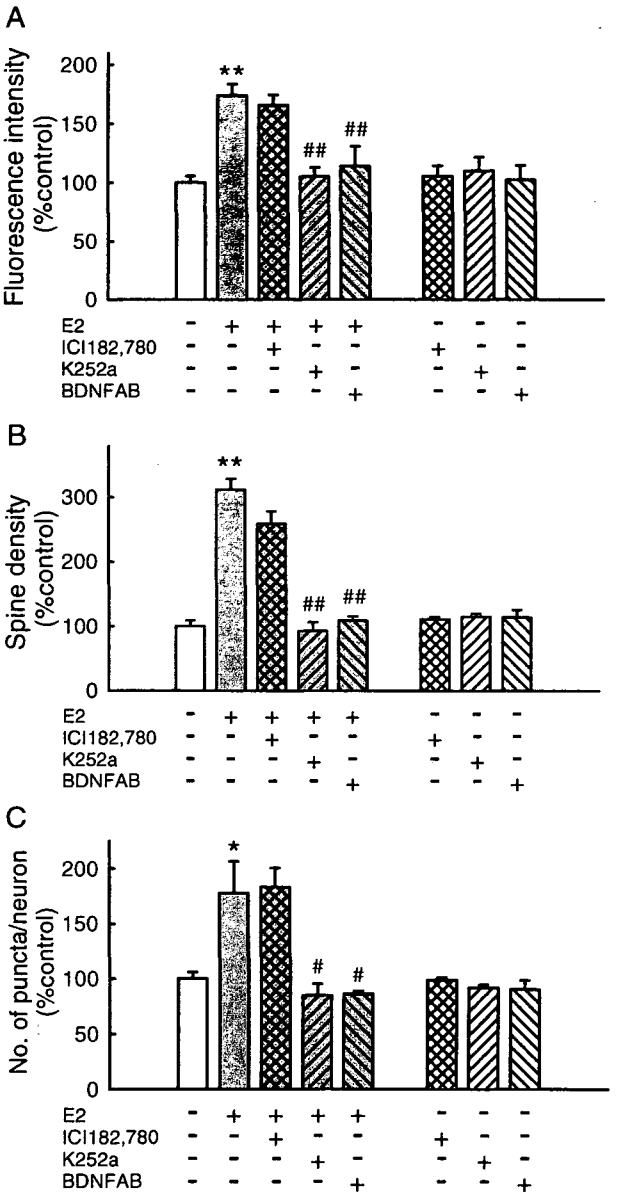

Fig. 5 – Effects of ICI, K252a, and BDNFAB on the effects of E2 in hippocampal slice cultures and subregional hippocampal neuron cultures. (A) K252a (200 nM) and BDNFAB (10 μg/ml) significantly inhibited the effect of E2 on the expression of PSD95 in cultured hippocampal slices, whereas ICI (1 μM) did not. **: $p<0.01$ vs. the control group, ##: $p<0.01$ vs. the E2-treated group. $N=8$, Tukey's test following ANOVA. (B) K252a (200 nM) and BDNFAB (10 μg/ml) significantly inhibited the effect of E2 on the spine density in cultured hippocampal slices, whereas ICI (1 μM) did not. **: $p<0.01$ vs. the control group, ##: $p<0.01$ vs. the E2-treated group. $N=8$, Tukey's test following ANOVA. (C) K252a (200 nM) and BDNFAB (10 μg/ml) significantly inhibited the effect of E2 on the number of presynaptic sites in the mixed neuron culture, whereas ICI (1 μM) did not. *: $p<0.05$ vs. the control group, #: $p<0.05$ vs. the E2-treated group. $N=8$, Tukey's test following ANOVA.

slice cultures and subregional neuron cultures were mediated by the common mechanism which is independent of nERs and dependent on endogenous BDNF, suggesting the involvement of BDNF in DG granule cells in the synaptogenic effect of E2 in CA3SL.

2.4. *E2 enhanced BDNF release from DG granule cells via nER-independent and PKA-dependent mechanisms*

We further examined the association between the effects of E2 and BDNF using subregional hippocampal neuron cultures. The expression levels of BDNF were confirmed for both the Ammon's horn neuron culture and the DG neuron culture by Western blot analysis and enzyme linked immunosorbent assay (ELISA) (Fig. 6). In Western blot analysis, BDNF immunoreactive bands were detected in the control lanes for both cultures, but the OD for the DG neurons was markedly higher than that for the Ammon's horn neurons (Fig. 6A). E2 (1 μM, 24 h) did not affect the expression levels of BNDF in Ammon's horn neurons or DG neurons. ELISA also showed that the

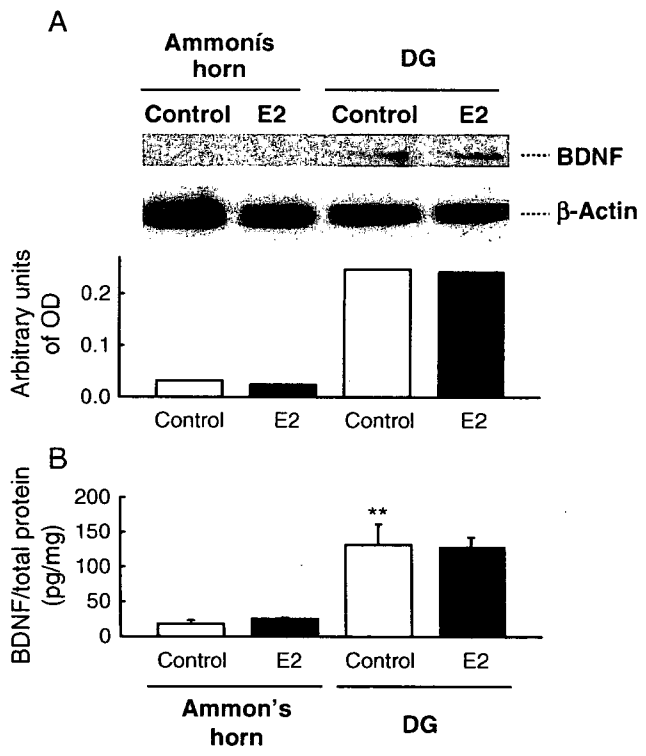

Fig. 6 – The expression of BDNF in subregional hippocampal neuron cultures. (A) Western blot analysis of BDNF in subregional hippocampal neuron cultures. The expression level of BDNF of DG neurons was much higher than that of Ammon's horn neurons. E2 (1 μM, 24 h) had no effect on the BDNF expression level. The same results were obtained in 3 independent experiments. (B) ELISA detection of BDNF in subregional hippocampal neuron cultures. The expression level of BDNF in DG neurons was significantly higher than that in Ammon's horn neurons. E2 (1 μM, 24 h) had no effect on the BDNF expression level. **: $p<0.01$ vs. the control group of Ammon's horn neurons. $N=4$, Tukey's test following ANOVA.

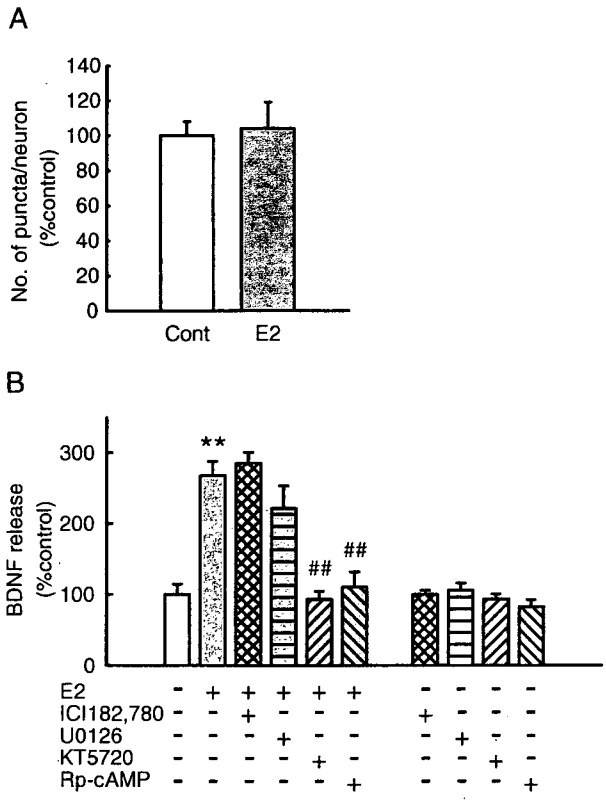

Fig. 7 – Effects of E2 on the BDNF release in the DG neuron culture. (A) Treatment for 10 h with E2 (1 μM) had no effect on the number of presynaptic sites in the DG neuron culture. (B) E2 (1 μM, 10 h) significantly enhanced BDNF release in the DG neuron culture. KT5720 (200 nM) and Rp-cAMP (10 μM) inhibited the effect of E2, whereas ICI (1 μM) and U0126 (10 μM) did not. **: $p<0.01$ vs. the control group, ##: $p<0.01$ vs. the E2-treated group. $N=4$, Tukey's test following ANOVA.

expression level of BDNF in DG neurons was remarkably higher than that of Ammon's horn neurons and E2 had no effect on the expression levels in both cultures (Fig. 6B). These results indicate that subregional neuron cultures reflect in vivo pattern of BDNF expression in the hippocampus, in which the highest concentration of BDNF occurs in DG granule cells (Dieni and Rees, 2002; Scharfman et al., 2003). We next examined the possibility that E2 enhances BDNF release from DG granule cells without affecting BDNF expression. The amount of BDNF released into the culture medium of the DG neuron culture was measured by ELISA. We performed ELISA after 10 h of treatment with E2, at the time point when the effect of E2 on the number of presynaptic sites was not yet apparent (Fig. 7A). E2 (1 μM, 10 h) remarkably increased the BDNF release (267 ± 20.5% of control; Fig. 7B). Neither ICI (1 μM) nor U0126 (10 μM) (Ki: 72 nM for MEK1, 58 nM for MEK2) (Duncia et al., 1998), influenced the effect of E2. In contrast, KT5720 (200 nM) (Ki: 56 nM for PKA) (Kase et al., 1987) and Rp-cAMP (10 μM) (Ki: 11 μM for PKA) (Rothermel and Parker Botelho, 1988), suppressed the effect of E2 to the control level. These inhibitors alone had no effects on the basal BDNF release. These results indicate that E2 enhanced BDNF release from DG

granule cells via nER-independent and PKA-dependent mechanisms, which may underlie the effects of E2 described above.

3. Discussion

In this study, we provided evidence showing that E2 induces synaptogenesis between mossy fibers and CA3 neurons by enhancing BDNF release from DG granule cells in a nER-independent and PKA-dependent manner.

We used subregional hippocampal neuron cultures to investigate the effects of E2 in detail. That these cultures sufficiently maintain their region-specific characters is supported by the following evidence: 1) the morphology of neurons in the Ammon's horn neuron culture was clearly different from that in the DG neuron culture (Fig. 4A). Most cells in the Ammon's horn neuron culture were large and spindle-shaped, which is typical for pyramidal neurons. Most cells in the DG neuron culture were small and granular, which is typical for DG granule cells. 2) DG neurons isolated and cultured using a similar procedure maintain their in vivo physiological properties (Ikegaya et al., 2000). 3) The expression level of BDNF of the cultured DG neurons is much higher than that of the cultured Ammon's horn neurons, reflecting in vivo pattern of BDNF expression in the hippocampus, in which the highest concentration of BDNF occurs in DG granule cells (Dieni and Rees, 2002; Scharfman et al., 2003).

In our study, we prepared hippocampal slices from both genders of P8 rat pups and cultured for 10 days with medium supplemented with horse serum (HS) collected from gelding horses, in which steroid concentrations were under the limits for detection. Because the increases in the expression level of PSD95 and the spine density in CA3 were observed in all slices treated with E2, we consider that the effects of E2 in our study are gender-independent. Currently we are investigating whether or not there is gender difference in the extents of the effects of E2. Organotypic hippocampal slice cultures of P5–9 rat brains are well-established, stable model for investigating hippocampal function including developmental synaptogenesis because neurons maintain synaptogenic ability in each region (CA1, CA3, and DG) (De Simoni et al., 2003; Mizuhashi et al., 2001; Qin et al., 2001). It has been reported that during postnatal development, the capacity of estrogen binding protein is high enough to lower the concentrations of serum estrogens to nonphysiological levels (Germain et al., 1978). This suggests that the conditions for the hippocampal slice culture in the present study more closely represent the postnatal developmental stage. Recently it was clarified that E2 is synthesized from endogenous cholesterol by P45017α and P450 aromatase in hippocampal neurons (Hojo et al., 2004) and that it plays an essential role in the maintenance of synapses (Kretz et al., 2004). The effects of E2 shown here might be achieved by locally synthesized E2 at the postnatal developmental stage. Two previous studies reported the effects of E2 on spinogenesis in cultured hippocampal slices (Kretz et al., 2004; Pozzo-Miller et al., 1999), but their results are conflicting, perhaps because of the effects of various steroids included in the HS in the culture medium.

Our findings suggest that BDNF in DG granule cells mediates the effects of E2. It has been reported that in the hippocampus the highest concentration of BDNF occurs in DG granule cells, especially in their axons, mossy fibers (Dieni and Rees, 2002; Scharfman et al., 2003), from the prenatal period through to adulthood (Dieni and Rees, 2002). The significance of BDNF in DG granule cells, however, had been unknown until Scharfman et al. showed that endogenous BDNF in mossy fibers affected the excitability of CA3 neurons in adult female rats (Scharfman et al., 2003). On the other hand, BDNF has long been known to promote synaptogenesis by maturation of presynaptic sites (Aguado et al., 2003; Seil and Drake-Baumann, 2000). Real-time monitoring revealed that BDNF increases the number of presynaptic sites (Alsina et al., 2001). Presynaptic maturation can induce postsynaptic maturation, as shown by mossy fiber induction of postsynaptic maturation including assembly and clustering of PSD95 on CA3 apical dendrites (Qin et al., 2001). In the present study, BDNF released from DG granule cells may have first increased the number of presynaptic sites by autocrine/paracrine mechanisms, thereby inducing the maturation of postsynaptic sites. In addition to the communication with CA3 pyramidal neurons through giant boutons, mossy fibers also communicate with local circuit interneurons in CA3 through filopodial extensions and en passant boutons (Acsady et al., 1998; Lawrence and McBain, 2003). Although the number of these small terminals is greater than that of giant boutons, we consider that E2 predominantly promoted the synaptogenesis between mossy fibers and CA3 pyramidal neurons in this study because of the following reasons: 1) E2 increased the number of giant boutons, which were identified as mossy fiber terminals containing Zn^{2+} in our previous report (Sato et al., 2002), and 2) the major population of BDNF-positive mossy fiber terminals is those with giant boutons (Danzer and McNamara, 2004). Further experiments using interneuron-specific markers will be necessary to identify the effect of E2 on synaptogenesis between mossy fibers and CA3 interneurons.

E2 enhanced BDNF release from DG granule cells in a nER-independent and PKA-dependent manner. Besides the genomic effects via nERs (ERα and ERβ), recent reports have described the nongenomic effects of estrogens mediated by mERs (Beyer et al., 2003; Kelly and Levin, 2001; Segars and Driggers, 2002). Although the membrane localization of the E2 binding sites is widely accepted, mERs still await isolation and gene cloning. One of the candidate mERs is membrane-localized ERα/β that can activate signal transduction pathways distinct from nERα/β (Razandi et al., 2004; Thomas et al., 2004). Although the mode of action has not been elucidated precisely, ERα has been localized to the neuronal plasma membrane in the hippocampus (Clarke et al., 2000). On the other hand, several reports suggest that the proteins, which are completely different from ERα/β, function as mERs in hypothalamus (Cambiasso and Carrer, 2001), midbrain (Beyer and Karolczak, 2000; Beyer et al., 2002), and neocortex (Toran-Allerand et al., 2002). The effects of E2 observed in our study may have been mediated by one or more mechanisms other than nERs.

It has been reported that E2 modulates the expression of BDNF by genomic (Sohrabji et al., 1995) or nongenomic mechanisms (Ivanova et al., 2001). Unexpectedly, in this

study BDNF expression levels were not affected by E2 (Fig. 6). Instead, E2 enhanced BDNF release from DG granule cells via the activation of the PKA pathway. The PKA/cAMP-responsive element binding protein (CREB) pathway has been shown to lie downstream of mERs in midbrain dopamine neurons (Beyer and Karolczak, 2000; Beyer et al., 2002). The effects of E2 in this study might be mediated by the same type of mERs as those in midbrain dopamine neurons. There are 2 major BDNF secretory pathways (for review, Lessmann et al., 2003): one is the Ca^{2+}-independent constitutive pathway and the other is the Ca^{2+}-dependent regulated pathway. In the regulated pathway, BDNF is sorted to large dense-core vesicles (LDCVs) (Wu et al., 2004) and released in an activity-dependent manner (Haubensak et al., 1998) following slow kinetics typical for protein secretion (Hartmann et al., 2001). BDNF plays an important role in long-term synaptic plasticity (for review, McAllister et al., 1999). BDNF is released selectively by electrical stimulation patterns that induce long-term-potentiation (LTP), thereby modulating the activity-dependent neuronal plasticity (Balkowiec and Katz, 2002; Gartner and Staiger, 2002). cAMP triggers BDNF release in such LTP-inducing condition (Patterson et al., 2001), so E2 might affect synaptic plasticity by way of cAMP-dependent BDNF release.

In ovariectomized adult female rats, E2 enhances the spinogenesis of apical dendrites in CA1 but not in CA3 (Gould et al., 1990). Recent studies have revealed that Akt (protein kinase B) activation via mERs mediates the spinogenesis in CA1 in adult rats (McEwen et al., 2001; Znamensky et al., 2003). On the other hand, there is evidence for another mechanism of E2-induced spinogenesis in embryonic hippocampal neuron cultures. In this system E2 acts via nERs to suppress BDNF expression in γ-aminobutyric acid (GABA) ergic interneurons and to decrease GABAergic inhibition, thereby inducing spinogenesis (Murphy et al., 1998a; Murphy et al., 1998b). It is possible that these mechanisms were also active in our study because E2 increased the spine density in CA1SR in cultured hippocampal slices. But clear differences were observed between the effect in CA1SR and that in CA3SL. The spinogenic effect in CA1SR was much weaker than that in CA3SL (Fig. 2) and the expression of PSD95 in CA1SR was not changed by E2 (Fig. 1). The local assembly of PSD95 is spatially and temporally correlated with the maturation of spine morphogenesis (Okabe et al., 2001: Jontes and Smith, 2000). PSD95 clusters are found in one-half of dendritic filopodia, but in most mature spines (Takahashi et al., 2003). Thus, the spines induced by E2 in CA1SR may be more immature compared with those in CA3SL. The effects of E2 in CA3 through BDNF derived from DG granule cells may be stronger than that in CA1 through the mechanisms described above. The absence of the effect of E2 in CA3 in previous reports (Gould et al., 1990; Znamensky et al., 2003) can be explained if the mechanism that we indicated here is not active in adulthood or the mechanisms demonstrated in the previous reports are active predominantly in CA1.

Our results strongly suggest that E2 induces synaptogenesis between mossy fibers and CA3 neurons by the enhancement of BDNF release from DG granule cells in a nER-independent and PKA-dependent manner. These data provide evidence that BDNF in DG granule cells has a role in synaptogenesis, and that E2 can modulate this synaptogenic function of BDNF.

4. Experimental procedure

4.1. Materials

Millicell-CM was from Millipore (Bedford, MA). Minimal essential medium (MEM), Neurobasal medium (NB) and B-27 supplement were from Gibco Invitrogen Co. (Carlsbad, CA). Donor HS (gelding) was from C-C Biotech Corporation (Valley Center, CA). Paraformaldehyde (PFA), polyoxyethylene (10) octylphenyl ether (Triton X-100), ammonium chloride, dimethylsufoxide (DMSO), L-glutamine, glycine, Tween 20 and sodium azide were from Wako Pure Chemical (Osaka, Japan). K252a was from Calbiochem (Darmstadt, Germany). Anti-BDNF antibodies (AB1534SP and AB1513P) and Chemikine BDNF Sandwich ELISA kit were from Chemicon (Temecula, CA). ICI was from Tocris (Ballwin, MO). Mouse monoclonal immunoglobulin G (IgG) to PSD95 (K28/43) was from Upstate Biotechnology (Lake Placid, NY). Alexa Fluor 488 rabbit anti-mouse IgG, NeuroTrace fluorescent Nissl, DiI and FM1-43 were from Molecular Probes (Eugene, OR). E2, poly-L-lysine, cytosine β-D-arabino-furanoside (AraC), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulphonyl fluoride, leupeptin, antipain hydrochloride, aprotinin, Trizma hydrochloride, bovine serum albumin (BSA), rabbit polyclonal IgG to β-actin, peroxidase-conjugated anti-rabbit IgG, tetrodotoxin (TTX), KT5720, and Rp-cAMP were from Sigma (St. Louis, MO). U0126 was from Promega (Madison, WI). Sodium dodecyl sulphate (SDS) was from Nacalai tesque (Kyoto, Japan). ADVASEP-7 was from Biotium (Hayward, CA). Enhanced chemiluminescence (ECL) plus Western blotting detection kit was from Amersham Biosciences (Arlington Heights, IL). Fluorescent images were obtained using a BioRad μ-Radiance laser scanning confocal system (Hercules, CA) attached to Nikon inverted microscope (Tokyo, Japan). Image analysis was performed using Adobe Photoshop 7.0 (Mountain View, CA).

4.2. Organotypic hippocampal slice culture

All animal procedures were in accordance with the guidelines of the National Institute of Health Sciences, Japan, to minimize pain or discomfort. Organotypic slice cultures of both genders of P8 Wistar rat hippocampi were prepared according to the method of Sato et al. (2002). Briefly, horizontal medial hippocampal slices (300-μm thick) were placed on Millicell-CM transmembranes and cultured with 0.7 ml of the culture media (50% [vol/vol] MEM, 25% [vol/vol] Hank's balanced salt solution [HBSS], and 25% [vol/vol] HS [gelding] supplemented with 6.5 mg/ml glucose, 50 U/ml penicillin G potassium and 100 μg/ml streptomycin sulphate). All experiments were performed at 10 days in vitro (DIV) because cultured hippocampal slices recover from damage by sectioning and complete the trisynaptic neuronal circuitry (DG→CA3→CA1) at 10–14 DIV (Nakagami et al., 1997).

4.3. *Immunohistochemistry*

Immunostaining of cultured hippocampal slices was performed according to the method of Qin et al. (2001) with modifications. Slices were fixed with ice-cold 4% (wt/vol) PFA in 0.1 M phosphate buffer (PB) for 10 min at 4 °C, washed with phosphate buffered saline (PBS) (5 min×3), and treated with 1% (vol/vol) Triton X-100 in PBS overnight at 4 °C. Slices were then blocked with 50 mM ammonium chloride for 30 min at 4 °C and 20% HS in PBS for 30 min at 4 °C. Subsequent steps were carried out using PBS containing 1% HS. Slices were treated with mouse monoclonal IgG to PSD95 (1:1000) overnight at 4 °C, washed (15 min×3), and treated with Alexa Fluor 488 rabbit anti-mouse IgG (1:1000) overnight at 4 °C. After washing (15 min×3), fluorescent images were obtained by confocal microscopy (BioRad μ-Radiance laser scanning confocal system) using a 4×objective. Black level was set so that the averaged fluorescence intensity of 5 independent squares (20 μm×20 μm) placed at the medial position of CA1 stratum pyramidale (SP) of the control slice had the same value as that of the outside of the slice. Gain level was set so that the averaged fluorescence intensity of 5 squares (20 μm×20 μm) placed at the medial position of CA3SL of the control slice was at the half-maximum level. In gray-scale mode under these settings, the major synaptic sites appeared as fluorescent compartments as shown in Fig. 1B. When we outlined these compartments as indicated in Fig. 1B and calculated the areas, the values were constant regardless of the treatment (data not shown), so, we measured the averaged fluorescence intensity of each compartment (an outlined area) and subtracted the back ground intensity to quantify the expression level of PSD95 of each synaptic site. Because slices were cultured after removing entorhinal cortex, we quantified the expression of PSD95 in CA1SR, CA1SO, CA3SL, and CA3SO, the synaptic sites which maintain the intact presynaptic and postsynaptic cells (Fig. 1B).

4.4. *DiI staining*

Cultured hippocampal slices were fixed with 4% PFA for 30 min at 4 °C. The fixative above the transmembrane was removed and DiI crystals were embedded into CA1SO and CA3SO under the light microscope. After 3 days of incubation at 4 °C, fluorescent images were obtained by confocal microscopy using a 60×objective. Horizontal optical sections were taken at 0.5 μm steps and the resultant z-series images were summed into a flat image. Spines (both dendritic filopodia and mature spines) were counted at the proximal sites of apical dendrites projecting from pyramidal cell bodies. For double labeling with DiI and PSD95 immunostaining, slices were immunostained after 3 days of incubation with DiI crystals.

4.5. *Fluorescent Nissl staining*

Cultured hippocampal slices were fixed with 4% PFA for 60 min at 4 °C. Subsequent steps were carried out at room temperature. After washing with PBS (15 min×3), the slices were treated with 0.1% Triton X-100 in PBS for 60 min, washed with PBS for 10 min, and incubated with NeuroTrace fluorescent Nissl (1:30 in PBS) for 40 min in a dark room. The incubation was terminated by a 10 min wash with 0.1% Triton X-100 in PBS, followed by 2 h wash with PBS. Fluorescent images were obtained by confocal microscopy using a 60×objective.

4.6. *Subregional hippocampal neuron culture*

Subregional neuron cultures of both genders of P3 Wistar rat hippocampi were prepared according to the method of Ikegaya et al. (2000). Ammon's horn and DG were isolated from hippocampi with extreme care so as not to mix these 2 regions (Fig. 4A, right). Dissociated cells from Ammon's horn, DG, or a combination of these regions were suspended in a 1:1 mixture of astrocyte-conditioned medium (ACM) and NB/B27 medium (2% [v/v] B-27 supplement and 73 μg/ml L-glutamine in NB) and plated onto 48-well plates coated with poly-L-lysine. After 24 h, the medium was changed to ACM-free NB/B-27 medium containing 2 μM AraC. Cells derived from each region were cultured for 7 days at the same cell density (2×10^4 cells/cm^2 for FM1-43 analysis, 5×10^5 cells/cm^2 for Western blot analysis and ELISA detection of BDNF). All surviving cells were immunohistochemically confirmed to be neurons using anti-NeuN antibody (data not shown).

4.7. *FM1-43 analysis*

After 1 h of incubation with HBSS at 37 °C, cultured neurons were treated with 10 μM FM1-43, a styryl pyridinium dye (Cochilla et al., 1999) in high K^+-HBSS (20 mM KCl; osmolarity maintained by concomitant decrease in sodium concentration) for 2 min and washed gently with HBSS for 1 min. To reduce background staining, neurons were washed with 20 μM ADVASEP-7, a sulphobutylated derivative of β-cyclodextrin (Tait et al., 1992) for 1 min. ADVASEP-7 has a higher affinity for FM1-43 than plasma membranes and has been shown to greatly reduce background staining in brain slices (Kay et al., 1999). After the incubation with 10 μM TTX for 30 min, three images ([1] stained image; [2] destained image obtained after the treatment with high K^+-HBSS; and [3] differential interference contrast [DIC] image) were obtained for each microscopic field of view using confocal microscopy with a 10× objective. The second image was subtracted from the first, which revealed the presynaptic sites where depolarization-specific release had occurred (Fig. 4C, top panels). The fluorescent puncta in each microscopic field of view were counted. The number of synapses per neuron was estimated by dividing the total number of puncta by the number of neurons observed in the third (DIC) image (Fig. 4C, bottom panels).

4.8. *Western blot analysis*

Cultured neurons were washed twice with ice-cold PBS and then harvested on ice with 50 mM Tris buffer (pH 7.2) containing 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 mM leupeptin, 1 μg/ml antipain and 1 μg/ml aprotinin. After intense sonication (23 kHz, 1 min×3), the cell suspension was centrifuged at 800×*g* for 5 min at 4 °C. An

aliquot of this supernatant was removed for the protein assay. Another aliquot was diluted in SDS sample buffer. Protein samples containing an equal amount of protein were separated by electrophoresis on 10% polyacrylamide-SDS gels and transferred onto polyvinyliden difluoride membranes in 49.6 mM Tris, 384 mM glycine and 0.01% (wt/vol) SDS at 30 V overnight followed by 80 V for 1 h. The membranes were incubated with Tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween 20, 5% (wt/vol) skim milk, 2% (wt/vol) BSA, and 0.1% (wt/vol) sodium azide for 1 h, followed by overnight incubation with protein A purified rabbit anti-BDNF polyclonal antibody (AB1534SP, Chemicon) (1:1000) or rabbit polyclonal IgG to β-actin (1:1000) at 4 °C. After washing (30 min), the membranes were then incubated with peroxidase-conjugated anti-rabbit IgG (1:1000) for 1 h at room temperature. Immunoreactive bands were visualized using the ECL kit. Optical densities (ODs) of immunoreactive bands were measured based on a gray scale of 0–256 arbitrary units. Background was subtracted from the OD and this corrected value was normalized to the corrected value of the β-actin band obtained from the same sample.

4.9. *ELISA detection of BDNF*

In comparison of BDNF contents in cultured DG neurons and cultured Ammon's horn neurons, cells were washed twice with ice-cold PBS and then harvested on ice with homogenization buffer consisting of 100 mM Trin/HCl (pH7), containing 2% (wt/vol) BSA, 1 M NaCl, 4 mM EDTA.Na2, 2% (vol/vol) Triton X-100, 0.1% (wt/vol) sodium azide, 5 μg/ml aprotinin, 0.5 μg/ml antipain, 157 μg/ml benzamidine, 0.1 μg/ml pepstatin A and 17 μg/ml phenylmethyl-sulphonyl fluoride. After intense sonication (23 kHz, 1 min×3), the homogenates are centrifuged at 14,000×*g* for 30 min. An aliquot of this supernatant was removed for the protein assay. Another aliquot was subjected to the calculation of BDNF concentration by the Chemikine BDNF sandwich ELISA kit. The plates, which were pre-coated with monoclonal antibodies against BDNF, were incubated with 100 μl of supernatant in each well overnight, followed by incubation with the secondary antibody for 3 h and color developing procedures for 1 h. Immediately after the stop solution included in the kit was added, the ODs of 450 nm were measured. A standard curve was run for each plate and linearity was confirmed for all detections. Because the lower detection limit of the kit is 7.8 pg/ml, we used data from the experiments in which the control value was higher than this limit. The concentration of BDNF was normalized to the total amount of protein. In the calculation of BDNF contents in the culture media, the culture media were collected after 10 h of incubation with E2, centrifuged at 1500×*g*, and the concentration of BDNF in the supernatants was determined by ELISA. Because in this case the values of the control group varied from experiment to experiment by several folds, we set 'basal value' in each experiment. 24 h after medium change, BDNF concentrations in the culture media were calculated and averaged for 4 wells in one experiment. This value was taken as the 'basal value' and the data were normalized to this 'basal value' in each experiment.

4.10. *Drug treatment*

E2 was dissolved at 100 mM in ethanol and diluted to the final concentrations with the culture medium. For PSD95 immunohistochemistry and FM1-43 analysis, cultured slices and cells were treated with various concentrations of E2 for 24 h. ICI (Ki: 1.5 nM for ERα, 6.4 nM for ERβ; Kuiper et al., 1997) was dissolved at 1 mM in ethanol and co-applied at 1 μM with E2. K252a (Squinto et al., 1991; Bothwell, 1995) was dissolved at 1 mM in DMSO and co-applied at 200 nM with E2. This concentration completely blocks the effect of BDNF in cultured hippocampal slices (Koyama et al., 2004). BDNFAB (protein A purified sheep anti-BDNF polyclonal antibody, AB1513SP, Chemicon) was dissolved in the culture medium at 10 μg/ml. This concentration blocks the effect of endogenous BDNF (Rasika et al., 1999; Matsunaga et al., 2004). For ELISA detection of the released BDNF, cultured cells were treated with E2 for 10 h. KT5720 (Ki: 56 nM) (Kase et al., 1987) was dissolved in the ethanol at 1 mM and co-applied at 200 nM with E2. Rp-cAMP (Ki: 11 μM) (Rothermel and Parker Botelho, 1988) was dissolved in PBS at 10 mM and co-applied at 10 μM with E2. U0126 (Ki: 72 nM for MEK1, 58 nM for MEK2) (Duncia et al., 1998) was dissolved in DMSO at 10 mM and co-applied at 10 μM with E2. We also confirmed beforehand that 0.1% ethanol or 0.1% DMSO (the maximal concentration used for vehicle in our experiments) alone had no effects in cultured hippocampal slices and subregional hippocampal neuron cultures (Fig. S1).

4.11. *Data analysis*

All data regarding the expression level of PSD95, the spine density, and the number of FM1-43 positive puncta, were quantified in a blinded manner. For quantification of PSD-95 signals, the fluorescence intensities in the synaptic sites were averaged for 4 slices in one experiment. These values were then averaged for 8 independent experiments (separate platings) and statistical analysis was performed using one-way repeated-measure analysis of variance (ANOVA) and the post hoc Tukey's test for multiple pairwise comparisons. Data are shown as the values normalized to that of CA1SR in the control group. The spine densities (the number of spines per μm of dendrite) averaged for 8 to 10 neurons per slice were averaged for 4 slices in 1 experiment. These values were then averaged for 8 independent experiments (separate platings) and statistical analysis was performed using the Student's t test. For FM1-43 analysis, the numbers of presynaptic sites (per neuron) were averaged for 4 wells in 1 experiment. These values were then averaged for 8 independent experiments (separate platings) and statistical analysis was performed using the Student's t test. In multiple pharmacological treatments, data were collected according to the methods described above, and statistical analysis was performed by one-way repeated-measure ANOVA and the post hoc Tukey's test for multiple pairwise comparisons. Data were shown as the values normalized to that of the control group. For ELISA detection of BDNF expression, the normalized values (BDNF/total protein) were averaged for 4 wells in one experiment. These values were then averaged for 4 independent experiments (separate platings) and statistical analysis was

performed by one-way repeated-measure ANOVA and the post hoc Tukey's test for multiple pairwise comparisons. For ELISA detection of the released BDNF, the values normalized to the basal value were averaged for 4 wells in one experiment. These values were then averaged for 4 independent experiments (separate platings) and statistical analysis was performed using one-way repeated-measure ANOVA and the post-hoc Tukey's test for multiple pairwise comparisons. Values of $p<0.05$ were considered significant.

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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.brainres.2007.02.093.

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ORIGINAL ARTICLE

Assessment of the human Cell Line Activation Test (h-CLAT) for Skin Sensitization; Results of the First Japanese Inter-laboratory Study

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Abstract

The human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on the enhancement by sensitizers of CD86 and/or CD54 expression on THP-1 cells. The aim of this study is to confirm the transferability and reproducibility of the h-CLAT protocol. Seven Japanese laboratories participated in this h-CLAT ring study. First, two well-known sensitizers (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) and one non-sensitizer (sodium lauryl sulfate (SLS)) were evaluated at each laboratory with the same protocol at the same application dose. All laboratories correctly evaluated the skin sensitization potential of these three chemicals. Next, four sensitizers and one non-sensitizer were tested as a second trial. There were two false-negatives (ethylene diamine and eugenol) in some laboratories. Finally, chemicals tested in the second trial were re-evaluated with doses individually determined by each laboratory as a third trial. The results were almost the same as the results obtained when all the laboratories tested the same application doses. These results suggest that for more precise evaluation of difficult samples (e. g., unstable or water-insoluble chemicals), modifications of the protocol and prediction model are needed. However, the protocol was easily transferred to all laboratories and there were only a few false-negatives among 56 tests (8 chemicals at 7 laboratories).

Key words: *Skin sensitization, alternatives, THP-1, reproducibility, h-CLAT*

Introduction

Because of increasing social concern about animal welfare and the use of animals in testing, many alternative, non-animal tests have been proposed. There is particular interest in developing alternative methods for skin sensitization testing (De Silva et al., 1996). Measuring phenotypic changes, such as CD86 or CD54 expression on dendritic cells, induced by sensitizers is an important approach for developing alternative methods of evaluating skin sensitization potential (Aiba et al., 1997; Hopper et al., 1995). However, the effects of chemicals on the surface phenotype of dendritic cells are dependent on the source of peripheral blood used to obtain the cells; in other words, the effect varied from donor to donor (Aiba et al., 1997; Rougier et al., 2000). Furthermore, it is not easy to obtain sufficient fresh peripheral blood. In order to overcome these problems, we tested human leukemia cell lines, such as THP-1, as surrogates for dendritic cells. We have reported that THP-1 cells, which show enhanced CD86 and/or CD54 expression when treated with sensitizers, can be used in an in vitro skin sensitization test