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化学物質リスク研究事業

化学物質の標的としての膜機能タンパク質発現系を利用したリスク評価法に関する研究に関する研究

平成１７年度～平成１９年度　総合研究報告書

主任研究者　大和田　智彦

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（注意事項）
本報告書は
○平成１７年度～平成１９年度　総合研究報告書
○平成１９年度　総括・分担研究報告書
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平成１９年度　総括・分担研究報告書の「研究成果の刊行物･別刷」は
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「研究成果の刊行物･別刷」と同一ですので，総合研究報告書に
添付したものを参照してください。

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Ⅰ．総合研究報告

厚生労働科学研究費(化学物質リスク研究事業)
総合研究報告書

化学物質の標的としての膜機能タンパク質発現系を利用したリスク評価法に関する研究

主任研究者　大和田　智彦　東京大学大学院薬学系研究科薬化学教室・教授

研究要旨

膜タンパク質は外来化学物質の最初の作用点である。内分泌かく乱物質として研究された物質，エストロゲン類縁化合物は汎用される基本的な化学構造単位をもつ物質が多い。一方、これらの物質の非ステロイド作用，すなわち受容体，イオンチャネル，トランスポーターなどのユビキタスな膜タンパク質への影響について系統的な研究がなされていない。潜在的なリスク化合物の膜タンパク質への作用のメカニズム・特異性や化学物質の構造特性は不明である。本研究課題では(目的1)潜在的なリスク化合物ライブラリーの合成と構築(目的2)化学物質のユビキタス膜タンパク質の機能に対するリスク評価系の開発(目的3)化学物質の膜タンパク質への作用点・作用メカニズムの解明，化学物質の構造特性の解明を行った。

分担研究者

中澤　憲一　国立医薬品食品衛生研究所・薬理部・部長

赤羽　悟美　東邦大学医学部医学科薬理学講座・准教授

A. 研究目的

化学物質のリスクの作用点の解明および評価系開発を目的として，ヒトおよびラット等の膜タンパク質をcDNAより細胞系に発現させ，膜タンパク質の機能に対する各種化合物の影響を検討した。膜タンパク質は化学物質の最初の作用点である。汎用される基本的な化学構造単位をもつ物質がユビキタスな膜タンパク質への影響について系統的な研究がなされていない。潜在的なリスク化合物の膜タンパク質への作用のメカニズム・特異性や化学物質の構造特性は不明である。本研究課題では(目的1)潜在的なリスク化合物ライブラリーの合成と構築(目的2)化学物質のユビキタス膜タンパク質の機能に対するリスク評価系の開発(目的3)化学物質の膜タンパク質への作用点・作用メカニズムの解明，化学物質の構造特性の解明を行った。

B. 研究方法

エストロゲンアンタゴニストであるタモキシフェンに含まれるジアリールエチレン構造を有する化合物を合成しリスク化合物ライブラリーとして非ステロイド作用としてユビキタスに存在する膜タンパク質に対する作用を調査した。また製紙表面加工等に汎用され日常生活に豊富に存在する松脂の成分であるデヒドロアビエチン酸の誘導体の化学合成を行い，天然資源誘導体が持つイオンチャネルに対する作用を調査した。膜タンパク質としてユビキタスに存在するイオン・チャネル型ATP受容体および複数のイオンチャネルを対象として，アフリカツメガエル卵母細胞発現系とヒト由来培養細胞(HEK293 細胞)を用いた発現系をそれぞれ構築し化学物質の効果を評価した。また，イオン・チャネル以外の膜タンパク質としてグルタミン酸トランスポーター(GLAST)への効果も検討した.

倫理面への配慮　動物実験は各研究者の所属する研究機関および実験実施施設における動物実験の指針に基づき，各機関における承認のもと実施し，実

験動物に対する動物愛護上の配慮を行った。

C. 研究結果

タモキシフェン(1)誘導体に含まれ，かつ一般に汎

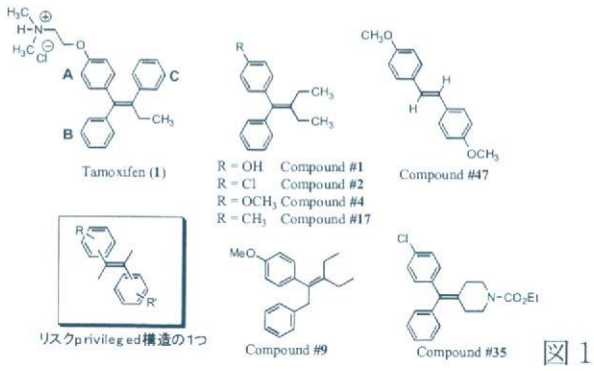

図1

用されている化学物質の部分構造となりうるジアリールエチレン構造を抽出してその誘導体(図1)の合成を行った。また製紙表面加工等に汎用され自然界に豊富に存在する松脂の成分であるデヒドロアビエチン酸の誘導体の化学合成を行った(図2)。アンチエストロゲンであるタモキシフェンおよびその関連化合物のイオン・チャネル型 ATP 受容体に対する作用をアフリカツメガエル卵母細胞発現系で行ない，この

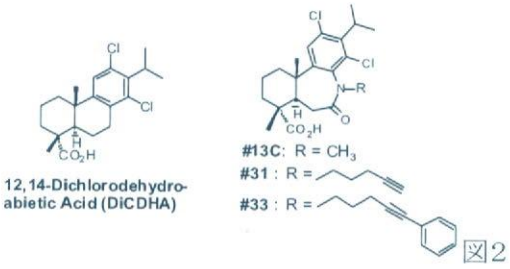

図2

系が各種化学物質のリスク評価に有用となりうることを示した。また，nM という低濃度で作用を示す化合物も確認された。この低濃度での作用，および複雑な濃度-作用が認められる例があることから，有害作用を検討する上で注意が必要であると考えられた．また，このような作用はアセチルコリン受容体チャネルでは認められなかった。

リスクライブラリ化合物の内，タモキシフェン及びその類縁物質に L-glu トランスポーターの取り込み活性を阻害する化合物を見いだした。この作用にはエストロゲン受容体との相互作用，およびその下流の PI3K が関与していた。

また，Ca^{2+}依存性 K^+チャネル(hSlo α，rSlo α，hSlo β_1，rSlo β_1，hSlo β_4)、遅延整流性 K^+チャネル(HERG, KvLQT1, minK)，電位依存性 Ca^{2+}チャネル($Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, $Ca_V2.2$)，電位依存性 Na^+チャネル(SCN5A)，Na^+-Ca^{2+}交換体(NCX1.1)をpcDNA3にサブクローニングし，哺乳動物細胞を用いた一過性発現系の構築を行った。さらにこの系を用いてタモキシフェン類縁化合物およびスチルベン誘導体ならびに松脂の成分ついて化合物(図2)の作用を評価した。

タモキシフェンおよびこれに構造が類似した化合物7 種のイオンチャネル型受容体に対する作用を検討し細胞外 ATP の受容体である P2X2 受容体を介するイオン電流に対し，検討した化合物のほとんどが影響を与えた。検討した化合物のうち，compound #2 以外はタモキシフェンより低濃度で作用を示した。compound #1, 2, 4, 17 の 4 つの化合物は置換基がひとつ異なるだけで構造的に近いが，共通して増強作用をしめすものの，作用を示す濃度にはばらつきが見られた。タモキシフェン類似化合物はニコチン様アセチルコリン受容体に対して抑制的に作用したが，P2X2 受容体に対するような低濃度での作用は認められなかった.

一方、タモキシフェン誘導体(Fig.1)およびデヒドロアビエチン酸誘導体(Fig.2)の一部が Ca^{2+}依存性 K^+チャネルを開口させる作用を示すことを見出した。タモキシフェン(10^{-5}M)ならびに#47(10^{-6}～10^{-5}M)は増強作用を示したが、化合物 #48 は作用を示さなかった。一方、12,14 ジクロロデヒドロアビエチン酸(10^{-5}M)および#31 は弱い増強作用を示した。化合物#13C はほとんど作用を示さなかったが、#33C は著明な Ca^{2+}依存性 K^+チャネル開口作用を示した。#33C は遅延整流性 K^+チャネルに対しては弱い遮断作用を示した。また、これらの化合物は、電位依存性 Ca^{2+}チャネル、電位依存性 Na^+チャネル、Na^+-Ca^{2+}交換体に対しては顕著な作用を示さなかった。よって、イオンチャネルのサブタイプに特異的に遮断作用あるいは開口作用を示すことが明らかとなった。

さらにプリン受容体(P2Y 受容体)を介したカルシウムシグナル応答に対するこれらの化合物の作用も合わせて検討したところ、化合物 #1 および#47 が HEK293 細胞のプリン受容体を介した細胞内 Ca^{2+}濃度上昇を 10^{-11}～10^{-7}M の濃度において抑制した。こ

れに対し、化合物 #2 および #9 は顕著な作用を示さなかった。

また，チャネル以外の膜タンパク質である GLAST に対しては，タモキシフェンおよび 16 種の関連化合物のうち，タモキシフェンと 1 つの関連化合物が 1 pM より，また他の 1 つの関連化合物が 1 μM より阻害作用を示した．

D. 考察

イオンチャネル型 ATP 受容体に対して 10 nM で増強を示した compound #1, 47 は基本部品構造は共通しているが結合構造は異なるため，1) これらの化合物の作用点はひとつではない，さらに，2) 化合物の作用態度がひとつではない，などの可能性が考えられる。compound #1 のように高濃度側で増強作用が減弱する，あるいは，compound #35 のように濃度を次第に上げていくと作用がジグザク状に増減する，という複雑な濃度依存性が見られたことから考えて，これらの化合物の作用態度が増強だけではなく，この増強を自ら抑制すると推察される。このような作用態度は古典的な薬理学の濃度-作用関係から逸脱しているが，化学物質のリスクを考える上で正しく評価する必要があると考えられる。compound #1, 47 などが 1 nM あるいは 10 nM という極めて低い濃度で効果を示したことは注目すべきである。非ホルモン性の急性の作用がこのような低濃度で認められることはこれまで看過されていた可能性もあり，種々の化学物質の有害性を検討する上で考慮に入れる可能性も考えられる。

ニコチン様アセチルコリン受容体に対する検討では，7 つのタモキシフェン関連化合物のうち，5 つが抑制作用を示した．抑制を示さなかった compound #2 および compound #17 は母核が共通で，1 か所の置換基のみが互いに異なる化合物である．抑制を示した化合物のうち compound #1 および compound #4 も同様の構造を有するが，この 2 つの化合物では compound #2 あるいは compound #17 とは異なり置換基に酸素原子が含まれている．このこと抑制作用の有無に関係するのかも知れない．抑制を示した残りの 2 つの化合物は上記 4 つとは母核を異にするが，母核を構成する 2 つのベンゼン環の配置に類似しており，この構造も抑制に関与する可能性が考えられる．compound #47 ではその構造に含まれるベンゼン環の配置が上記の 6 つとは異なっており，このことが抑制作用における複雑な濃度依存性に関わる一因であるかも知れない．compound #47 に見られた複雑な濃度依存性は，昨年度検討した ATP 受容体チャネル (P2X 受容体) では比較的多く見られたこれらの化合物の複雑な作用態度と類似している．しかし，ATP 受容体チャネルへの作用には nM レベルという低濃度領域で観察されるものが含まれており，これと比較すると，ニコチン様アセチルコリン受容体への作用は弱いと言える．

さらに、compound #1 および compound #47 は上記のチャネルに加えてカルシウム依存性カリウムチャネルやプリン受容体応答に対しても低濃度において作用を示したことから、さまざまな膜蛋白の機能に影響を与えるリスクの高い基本骨格であると考えられる。

グルタミン酸トランスポーターに対して明瞭な阻害作用を示した化合物のうち，タモキシフェンおよび関連化合物の 1 つは母核のベンゼン環の数は異なる (それぞれ 3 個と 2 個) が，置換基は種類は違うものの同等な位置に存在する 1 個のみである．これらと同様の構造を有する他の関連化合物では明瞭な作用が認められなかったものの，この構造は阻害作用の基本の 1 つであるかも知れない．阻害作用を示した残りの 1 つは，母核の 4 つのベンゼン環を含むが，唯一の置換基の位置は上記の 3 つの化合物と同等である．

一方、デヒドロアビエチン酸誘導体の中にもイオンチャネルに対して特異的でかつ強力な作用を示す基本構造が見出されたことから、膜蛋白の特定の構造に選択的に結合して作用を及ぼすリスク化合物であると考えられる。

以上，このような検討を通じ，この発現系は各種化学物質の細胞機能への影響を評価するのに有用であることが示された。またリスク作用を起こす共通部分構造 (リスク privileged 構造) が存在することが示唆された。

E. 結論

アフリカツメガエル卵母細胞を用いたイオンチャネル型ATP受容体およびアセチルコリン受容体の発現系，および，哺乳類細胞発現系を用いた各種イオンチャネル（Ca^{2+}チャネル，Na^{+}チャネル，K^{+}チャネル，Na^{+}-Ca^{2+}交換体）の一過性発現系を構築し，これらの系が各種化学物質のリスク評価に有用となりうることを示した。また，イオンチャネル以外の膜タンパク質といてグルタミン酸トランスポーターへの影響を検討し，評価系としての有用性を示した．アンチエストロゲンであるタモキシフェンから派生した基本構造化合物群中にnMという低濃度で作用を示す化合物も確認された。この低濃度での作用，および複雑な濃度−作用が認められる例があることから，有害作用を検討する上で注意が必要であると考えられた。

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G. 知的財産権の出願・登録状況

1. 特許取得

大和田智彦, 赤羽悟美 ら

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佐藤薫, 中澤憲一, 大和田智彦

発明名称：グルタミン酸トランスポーター阻害剤

出願日：2008/02/14
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2. 実用新案登録
なし

3. その他
なし

II. 研究成果の刊行に関する一覧表

雑誌

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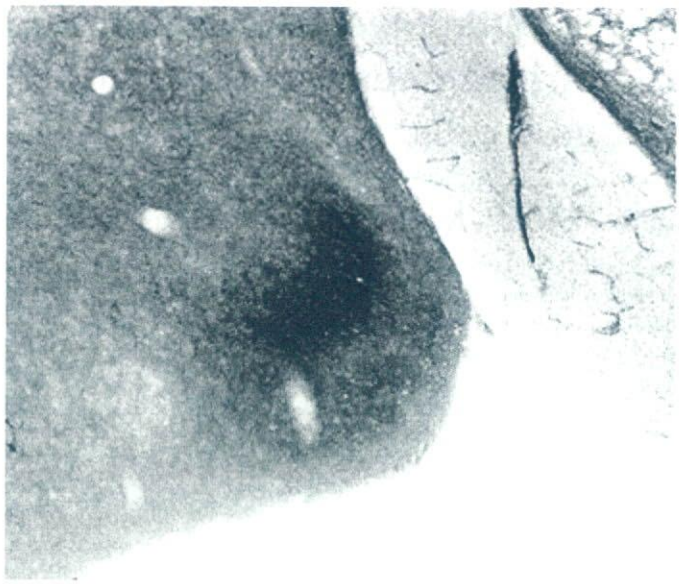

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Research Report

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

Kaoru Sato[a,*], *Tatsuhiro Akaishi*[b], *Norio Matsuki*[c], *Yasuo Ohno*[a], *Ken Nakazawa*[a]

[a]*Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b]*Laboratory of Pharmacology, Faculty of Pharmacy and Research Institute of Pharmaceutical Sciences, Musashino University, 1-1-20 Shinmachi, Nishitokyo-shi, Tokyo 202-8585, Japan*
[c]*Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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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

* *Corresponding author*. Fax: +81 3 3707 6950.
E-mail address: kasato@nihs.go.jp (K. Sato).

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

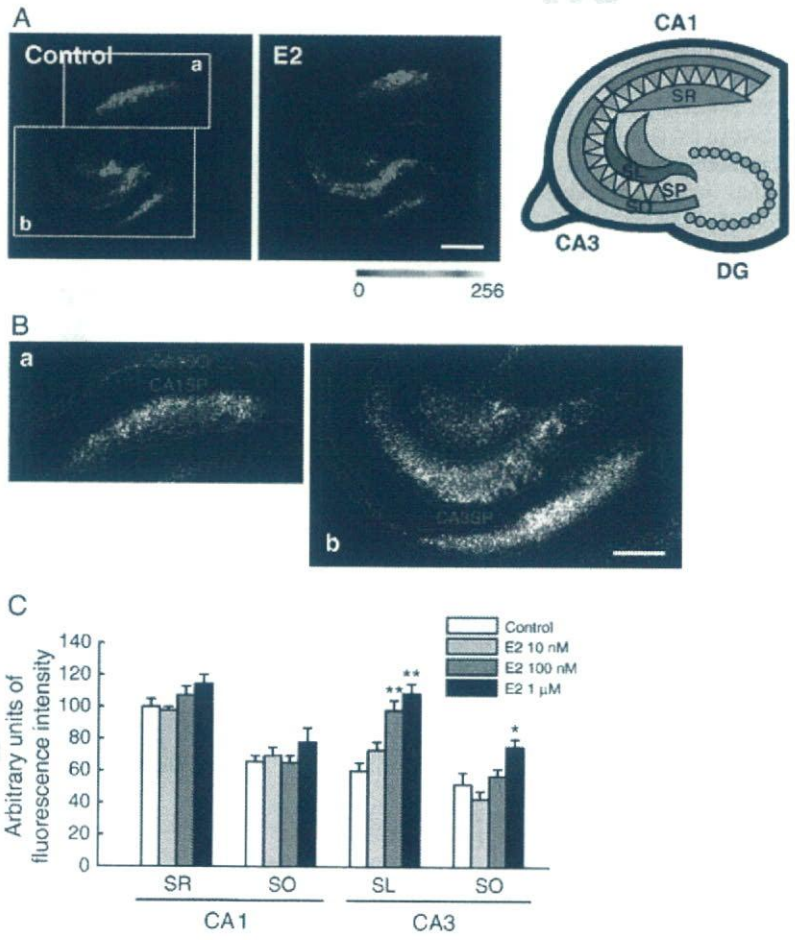

Fig. 1 – Effects of E2 on the expression of PSD95 in cultured hippocampal slices. (A) PSD95 immunoreactive signals in the control slice (left) and the slice treated with E2 (1 μM, 24 h) (middle). Bar=500 μm. (B) Magnified gray-scale images of a and b in A. CA1SR, CA1SO, CA3SL, and CA3SO appeared as fluorescent 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

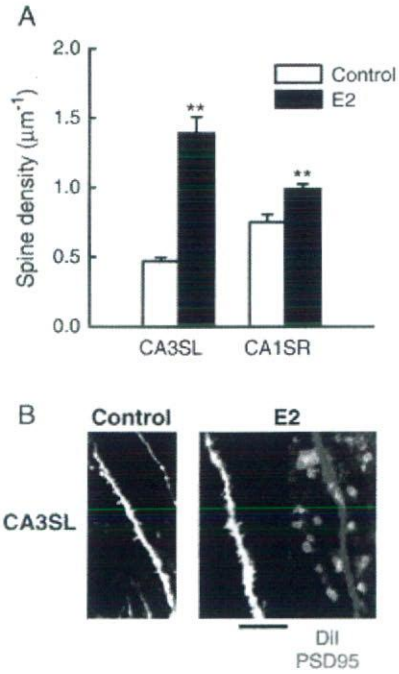

Fig. 2 – Effects of E2 on the spine density in cultured hippocampal slices. (A) E2 (1 μM, 24 h) markedly increased the spine density in CA3SL. **: $p<0.01$ vs. the vehicle control group in each region. $N=8$, Student's t test. (B) Typical images of the DiI-labeled CA3 apical dendrites in the control 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-

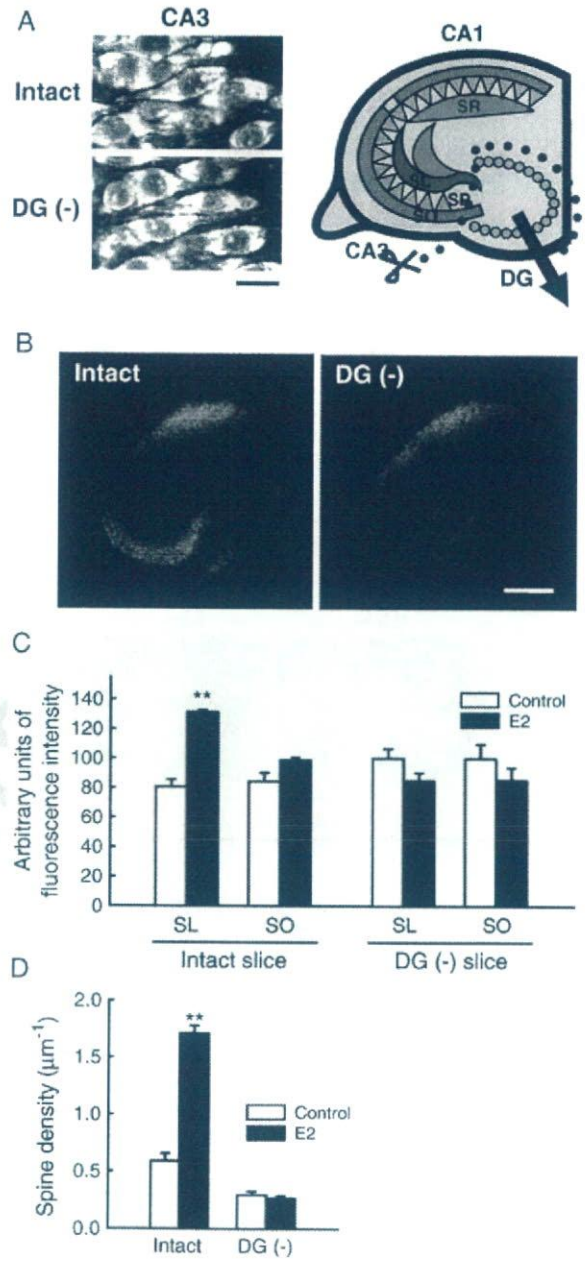

Fig. 3 – Effects of E2 on the expression of PSD95 and the spine density in cultured hippocampal slices of which DG had been excised at 1 DIV. (A) The viability of CA3 pyramidal neurons in DG (–) slices. Nissl staining revealed that their viability was not affected by the dissection of DG. Bar=20 μm. (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.

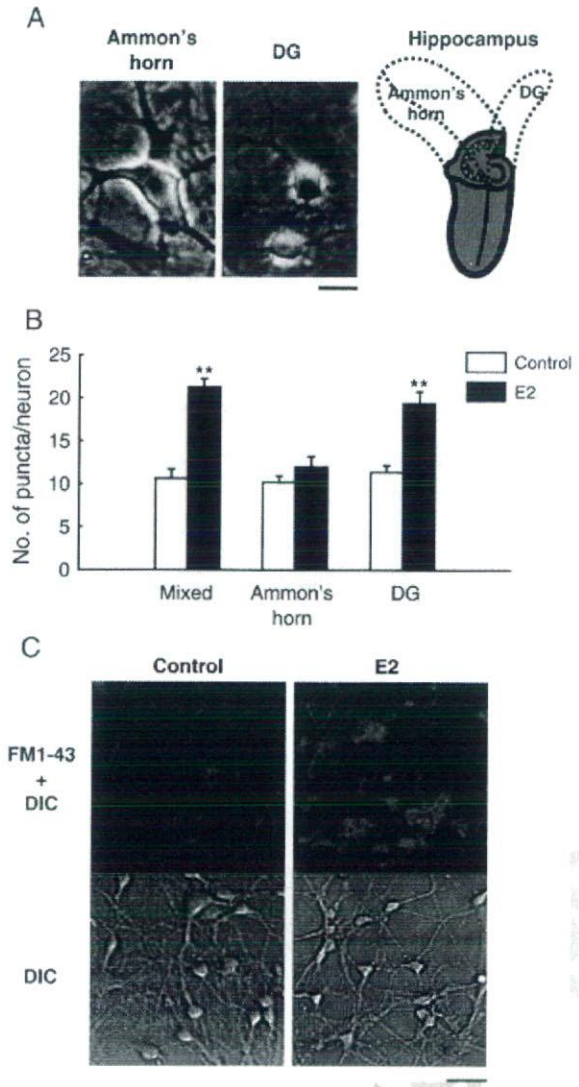

Fig. 4 – Effects of E2 on the number of presynaptic sites in subregional hippocampal neuron cultures. (A) Typical cell morphologies in the Ammon's horn neuron culture (left) and in the DG neuron culture (middle). Bar = 20 μm. (B) E2 (1 μM, 24 h) significantly increased the number of presynaptic sites 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

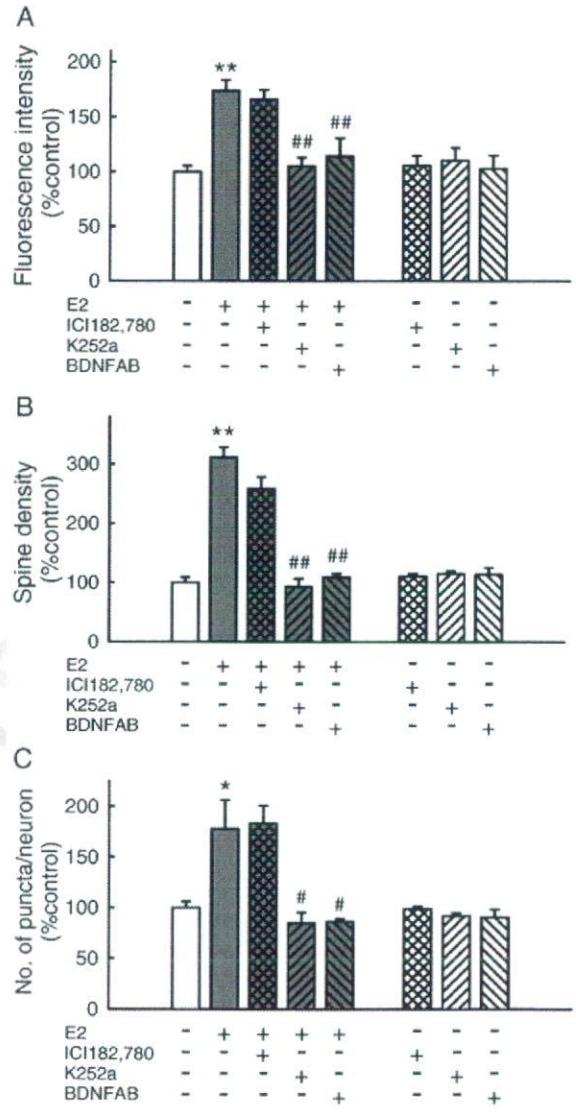

Fig. 5 – Effects of ICI, K252a, and BDNFAB on the effects of E2 in hippocampal slice cultures and subregional hippocampal neuron cultures. (A) K252a (200 nM) and BDNFAB (10 μg/ml) significantly inhibited the effect of E2 on the expression of PSD95 in cultured hippocampal slices, whereas ICI (1 μM) did 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 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

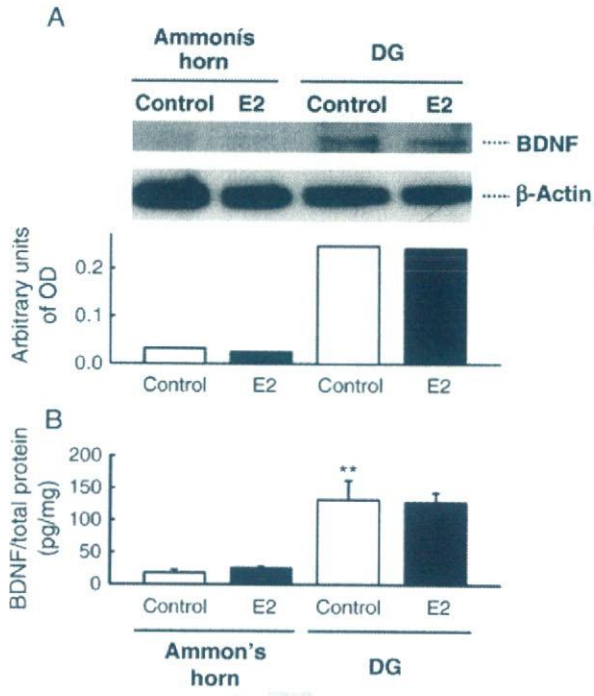

Fig. 6 – The expression of BDNF in subregional hippocampal neuron cultures. (A) Western blot analysis of BDNF in subregional hippocampal neuron cultures. The expression level of BDNF of DG neurons was much higher than that of Ammon's horn neurons. E2 (1 μM, 24 h) had no effect on the BDNF expression 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.

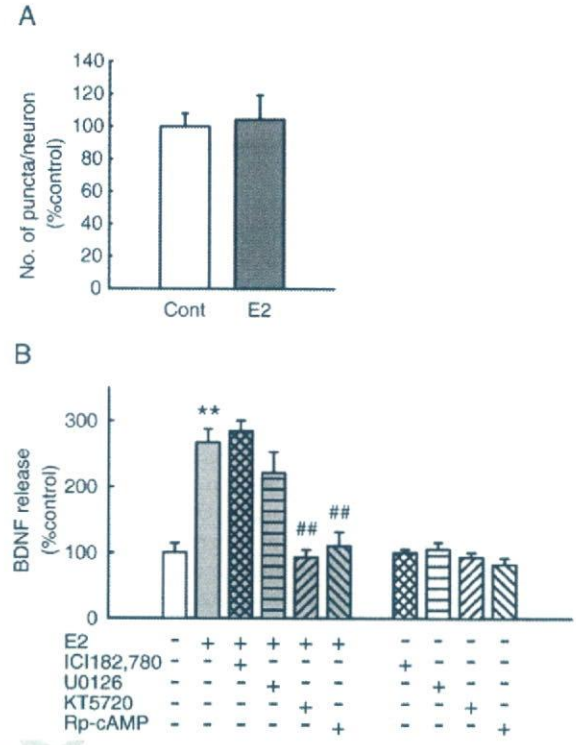

Fig. 7 – Effects of E2 on the BDNF release in the DG neuron culture. (A) Treatment for 10 h with E2 (1 μM) had no effect on the number of presynaptic sites in the DG neuron culture. (B) E2 (1 μM, 10 h) significantly enhanced BDNF release in the DG neuron culture. KT5720 (200 nM) and Rp-cAMP (10 μM) inhibited 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.

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).