特集
抗うつ薬治療における増強療法と併用療法
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抗うつ薬と電気けいれん療法の併用療法

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抄録：電気けいれん療法 electroconvulsive therapy（ECT）は，パルス波治療器導入に伴い，より安全性の高い使用法が広がり，うつ病の治療として再びその有効性が期待されるようになった。薬物療法に抵抗性を示したうつ病にも効果を認め，治療抵抗性うつ病の治療として有効であり，その効果発現は薬物療法より早いため急性期の治療としても有効である。一方，その効果が持続しないという問題点があり，ECT治療後の維持療法として薬物療法を行うことや，薬物療法だけでは寛解状態を維持できない時は，薬物療法に維持継続ECTを併用することが望まれる。しかしながら，麻酔のリスクだけでなく認知障害などの副作用の軽減，作用機序の解明，より効果的な使用法の統一などの課題も残されており，今後さらなる研究が必要であろう。 臨床精神薬理 12：221–227, 2009

Key words：*depression, combination therapy, electroconvulsive therapy (ECT), antidepressants*

Ⅰ．はじめに

電気けいれん療法 electroconvulsive therapy（ECT）は，電気的刺激を与えて脳にてんかん様けいれん発作を誘発することで治療効果を発揮するものとして，うつ病などで用いられている。歴史的にはECTが初めて精神科の治療として欧米に登場したのは1938年で，1940年代よりけいれん発作時の骨折事故をへらすために筋弛緩薬が，さらに発作時の恐怖感を回避する目的で静脈麻酔薬が用いられるようになった。1950年代から静脈麻酔薬，筋弛緩薬，酸素投与を用いた修正型ECT（modified ECT：mECT）が普及した。

わが国では，早くも1939年にECTが導入され，1958年筋弛緩薬を使用したECTの報告がなされたが，その後安全面を含め評価，改良，一般化が行われず，第一線の治療ではなくなっていった。ようやく1980年代にリエゾン精神医学の進展に伴い，麻酔科医と連携してmECTを行うことが総合病院や大学病院で広がった。以前はサイン波刺激のみであったが，2002年に新たにパルス波治療器が認可された。パルス波治療器の使用に当たっては，ECT実務者委員会の講習が義務付けられ，全身麻酔と筋弛緩薬使用下に限定するなど使用法についても統一されたことで[18,19]，ECT治療がより安全に行われるようになり，普及してきている。

うつ病にECTを用いる意義は何であろうか。うつ病は「治る」病気と考えられていたが，その考え方を変えないといけないことが分かってきた。Keitnerらによると，薬物療法での反応率は50～65%，寛解率は28～47%，精神療法での反応率は50～58%，寛解率は30～48%であった[11]。実に初回の治療で寛解に至るのは半分以下である。定義が未だ一貫していない（わが国では異なる2

Combination therapy of electroconvulsive therapy and antidepressants.

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種類の抗うつ薬を十分量十分期間使用して無効である場合を言うことが多い）点に注意する必要があるが，治療抵抗性うつ病の問題も大きくなっている[5]。これまで治療抵抗性うつ病はうつ病の10～15％と見積もられていたが，最近のメタ解析では約40％を占めるという[11]。Sequenced Treatment Alternatives to Relieve Depression（STAR*D）研究の結果，抗うつ薬投与で寛解に至らず，さらに別の抗うつ薬への置換，増強療法，精神療法などの治療段階を経ても寛解に至らない患者が約3分の1いることが分かった[24]。現在，このような「治りにくい」うつ病に対する治療戦略が大きな課題となっており，ECTはこれまでSTAR*Dに組み込まれていなかったが，「治りにくい」うつ病に対する治療法として期待されるようになった[16]。本稿では，うつ病に対するECTの有効性に関する文献的報告とともに，当院におけるECTについて紹介し，最後に抗うつ薬とECTの併用療法について言及する。

Ⅱ．ECTの効果

1．ECTは治療抵抗性うつ病にも有効である

Keitnerらのメタ解析によると，ECTの反応率は53～80％，寛解率は27～56％であった[11]。しかし，ECTの施行方法が報告によって異なるため，結果に幅があると考察されている[14]。では，治療抵抗性うつ病に対する効果はどうであろうか。Folkertsらによる治療抵抗性うつ病患者に対するECT（右片側性週3回）の反応率は71％であった[6]。当院においては，2006年に治療抵抗性うつ病の患者63人にECT（両側性週2回）を施行したところ，反応率が93％，寛解率は74％という高い効果を認めた。また，一般に抗うつ薬に対して治療反応性の乏しい精神病像を伴う重症うつ病にもECTは有効である[3,26,31]。

うつ病患者に対して，プラセボ，シミュレーションECT，抗うつ薬と比較してECTの方が治療効果が優れていると，多くのメタ解析で報告されてきた[10,27,34]。各抗うつ薬との比較では，ECTと三環系抗うつ薬（tricyclic antidepressants：TCA）やmonoamine oxidase inhibitors（MAOI）を比較した研究がいくつかあり，TCAやMAOIよりECTの方が有効であることが示されてきた[10,27,34]。新しい抗うつ薬とECTを比較した研究は未だ少ないが，従来薬と同様，ECTの方が有効である可能性が高いと思われる。先述のFolkertsらによる研究[6]では，治療抵抗性うつ病の患者39人を，無作為にECT群（21人）とparoxetine群（18人）に分け，ECT群で59％，paroxetine群で29％のうつ状態の改善を認めた。ECT群でより高い反応率（71％でハミルトンうつ病評価尺度：HAM-D総得点の50％減少）を認め，paroxetineと比較してもECTがより有効であった。

2．ECTは効果発現が早い

先述したFolkertsらは，治療抵抗性うつ病患者でECTとparoxetineの効果発現の早さについても比較検討している[6]。ECT群ではparoxetine群と比較し，治療1週間後よりうつ状態の有意な改善を認めた。Husainらは，うつ病の患者に対し週3回のペースでECTを施行し反応や寛解の速さを検討したところ，ECTは平均4回の施行（1.3週間）で効果発現を認め，平均8回（約2.5週間）の施行で寛解に至ったと報告している[9]。一方，抗うつ薬の効果発現には2～4週間かかり[20]，一般的に寛解に至るには約4～8週間を必要とする。早急な抗うつ効果が必要とされるカタトニアで全身状態が悪化している患者や，深刻な自殺念慮があり自殺企図リスクが高い患者などには，薬物療法より効果発現や寛解に至るまでが早いECTがより有効な治療であると考えられる。

3．ECTの効果は持続しない

ECTの治療持続性はどうであろうか。継続治療を行わない場合の再発率は50％以上で，ほとんどの再発が治療後の6ヵ月以内に起こり[2]，その効果が持続しないという問題点がある。ECT後に再発しやすくなるリスクファクターとして，抗うつ薬への抵抗性や，精神病症状の合併，Double Depressionが報告されている[2]。

4．ECTの効果はその施行方法に影響を受ける

ECTの効果に影響を与えうる因子として，刺

激用量と電極の位置（両側性か片側性か）がある。刺激用量が高いほど効果があるが，副作用である認知障害を起こす確率は高くなる[34]。電極の位置は，両側性の方が片側性よりも効果があるとする報告が多い。しかし，Sackeimらは刺激用量の十分高い右片側性ECTは両側性と比較しても効果に差がなく，認知機能への影響が少ないのでより適切であると報告している[29]。波形については，パルス波刺激とサイン波刺激の両者の間に効果の面で有意な差を認めなかったとするメタ解析がある[34]。

5．ECTに禁忌はないが，いくつかのリスクがある

ECTに絶対的な医学的禁忌は存在しない。しかし麻酔下で行うため，潜在的な麻酔のリスクがあるので，麻酔科医と連携し，術前に全身状態や合併症について評価する必要がある。ECTを第一選択の治療法としない理由の一つは，全身麻酔による致死的副作用のリスクがゼロではないからである。

ECTの通電直後の副作用としては，けいれん重積，遷延性けいれん，発作後せん妄，遷延性無呼吸，交感・副交感神経刺激による心血管性合併症（不整脈など）がある。また，覚醒後に出現し数時間持続する副作用として，頭痛，筋肉痛，嘔気，見当識障害，せん妄がある。

ECTの副作用として問題となる認知障害には，前向性健忘と逆行性健忘がある。前向性健忘は速やかに回復するのに対し，逆行性健忘は回復に時間がかかることがあり，まれに残存することもある。片側性より両側性が，薬物は低用量より高用量の方が[34]，波形はパルス波よりサイン波の方が[36]，認知障害の頻度がやや高いという報告がある。しかし，ECTを反復して施行することによる器質的障害の発生については否定的と考えられている[4]。

Ⅲ．ECTの作用機序

ECTの効果発現にかかわる物質として，コルチゾールや，副腎皮質刺激ホルモン，コルチコトロピン放出因子，甲状腺刺激ホルモン，プロラクチン，オキシトシン，バソプレッシン，dehydroepiandrosterone sulfate（DHEA），そして最近ではtumor necrosis factor αが報告されている[15]。しかしながら，これらがどのように作用して治療に有効なのかは未だ明らかになっていない。

最近，ECTの神経保護作用が注目されている。神経細胞の可塑性，再生，維持に重要とされる神経栄養因子brain-derived neurotrophic factor（BDNF）への関心が高まっている[33]。Maranoらは，ECTによるBDNFの増加を確認し，BDNF増加とHAM-D総得点減少が相関すると報告した[15]。BDNFはセロトニンの発現を増加させる可能性があるので[17]，セロトニンを介する機序が示唆される。またPereraらは，霊長類を用いた研究で，ECTにより海馬での神経新生が促進されたことを確認した[28]。

Gamma-aminobutyric acid（GABA）はうつ状態で減少していると報告されている神経伝達物質であるが，magnetic resonance spectroscopy（MRS）を用いた研究で，ECTにてGABAが増加することが示されている。ECTの施行を繰り返すとけいれん時間の減少やけいれん閾値の上昇がみられ，これには脳内におけるGABAの増加が関係していると考えられている[1]。

脳血流の変化に関する報告はいくつかあるが未だ一定した見解はない。TakanoらはECT直前と比較してECT施行中に基底核，脳幹，間脳，扁桃体，前頭葉，側頭葉で血流が増加し，ECT施行直後には視床で血流が増加し前帯状回で低下したと報告している[32]。

以上のようにECTの作用機序を研究することは，うつ病の病態の解明につながる可能性もあり重要である。

Ⅳ．mECTの実際

1．適応の判断

ここでは当院で行われているECTの実際について述べる。当院のうつストレスケア病棟には，センター病院としての役割もあり，他院にて抗うつ薬を何剤か試されて十分な改善を示さなかった

表1　電気けいれん療法（ECT）が適応となる状態

一次的使用	二次的使用
精神症状の型（緊張病状態など） 症状が重篤（深刻な焦燥感など） 自傷他害の危険（自殺企図など） ECTが効果的であった治療歴 全身状態（全身衰弱など） 他の治療より高い安全性（高齢者，妊娠中など） 患者希望	薬物療法への乏しい反応性 副作用，忍容性においてECTが優れる場合

表2　電気けいれん療法の相対的禁忌

■最近起きた心筋梗塞，不安定狭心症，非代償性うっ血性心不全，重度の心臓弁膜症のような不安定で重度の心血管系疾患
■血圧上昇により破裂する可能性のある動脈瘤または血管奇形・脳腫瘍やその他の脳占拠性病変により生じる頭蓋内圧亢進
■最近起きた脳梗塞
■重症の骨折
■重度の慢性閉塞性肺疾患，喘息，肺炎のような呼吸器系疾患
■米国麻酔学会，水準4または水準5と評価される状態
　水準4：日常生活を大きく制限する全身疾患があり常に生命を脅かされている状態
　水準5：手術をしなくとも24時間以上生存しないと思われる瀕死の状態

治療抵抗性うつ病患者が多く入院してくる。当院ではアルゴリズムを用いてうつ病の治療を行っている。まず「見かけ上の」治療抵抗性を否定するために，診断（双極性障害など）や治療（内服はできていたかなど）の見直しを行う。異なる種類の抗うつ薬を2剤以上，十分量十分期間使用しても寛解に至らない「本当の」治療抵抗性うつ病と診断された場合は，lithiumや甲状腺ホルモンなどの増強療法の使用を検討する。次に，非定型抗精神病薬や，ドーパミンアゴニスト，気分安定薬（carbamazepineやvalproate）などの使用を検討する。認知行動療法は必要に応じて併用する。以上で寛解に至らない場合，ECTの適応の有無を検討する（表1の二次的使用の場合）[23]。ただし，緊張病状態など表1の一次的使用に当てはまる状態の場合は，積極的にECTの適応を考慮してい。これらの判断は精神科医師2名により行うが，相対的禁忌の疾患（表2）を合併している場合は，麻酔科医へECTの適応について相談している。

2．mECTの施行方法

当院では，麻酔科医による全身麻酔下で筋弛緩薬を用い，パルス波治療器によりmECTを施行している。mECT治療を効率的かつ安全に施行するために，mECTマニュアル[22]とクリニカルパスを作成した。まず，患者本人や保護者（扶養義務者）へ書面を用いて十分な情報提供を行う。mECTの治療内容だけでなく，維持療法の重要性や期間など治療方針の十分なインフォームドコンセントを行う。原則として患者本人から同意を得る必要があるが，医療保護入院や措置入院の場合は少なくとも保護者か扶養義務者の同意を必要としている。同意を得たら，クリニカルパスに従い術前検査や患者情報のチェックを行う。

mECTの施行はECTユニットと呼ばれる専用の準手術室で，精神科医，麻酔科医，看護師のチームで行う。mECTの施行は，ECT実務者委員会の講習に参加し承認を得た精神科医により行われる。パルス波治療器を用い，初回の刺激強度は半年齢法（患者の年齢の半分の刺激強度）により決定する。電極はせん妄や認知障害が発生するリ

スクが高い場合は原則として片側で行うが，適切な刺激強度で4～6回施行しても十分な効果が得られない場合は両側へ変更する。静脈麻酔や呼吸管理は麻酔科医が行う。静脈麻酔薬としては，thiopental や propofol が一般に使われるが，当院で ketamine を使用したところ，うつ状態がより早く改善する傾向がみられた[25]。今後は ketamine の使用をより積極的に考慮してもよいと思われる。

V．抗うつ薬と ECT の併用療法

最後に抗うつ薬と ECT の併用療法について述べる。ECT は寛解を維持する効果は乏しいので，一般に抗うつ薬による維持療法が用いられる。抗うつ薬の抗うつ効果発現には週単位の時間がかかるため，実際には ECT 施行前から抗うつ薬を開始する。抗うつ薬の種類によって ECT の維持効果が異なると報告されている。Lauritzen らの報告では，ECT 施行後の維持療法としてプラセボと imipramine，paroxetine とを比較し，6ヵ月以内の再燃はプラセボ群65%に対し，imipramine 群30%，paroxetine 群10%であり薬剤による差を認めた[13]。ECT 施行前に効果を認めなかった薬剤は再発予防の維持療法としての効果も乏しい[2]という報告がある一方，それを否定するような次のような報告もある。van den Broek らは，TCA(imipramine を含む）や lithium，MAOI などの薬剤に治療抵抗性の患者に対し ECT 施行後の維持療法として imipramine を使用した randomized controlled trial（RCT）を行ったところ，24週間後にプラセボ群は80%が再発したのに対して，imipramine 群は18%で有意に再発率が低かったと報告しており[35]，ECT により治療抵抗性が改善した可能性が示唆された。また，lithium の併用療法が有効との報告もある。Sackeim らは，ECT 施行後24週間後にプラセボ群では84%が再発したのに対して，nortriptyline 群は60%，nortriptyline と lithium 併用群が39%と有意に低く，抗うつ薬の単剤投与より lithium の併用が維持療法として有効であったと報告している[30]。

最近 ECT を維持療法として使用して効果を認めたという報告がいくつか出てきている。維持継続 ECT の施行方法としては，初めの1ヵ月は週に1回，次の1～2ヵ月は2週に1回，それ以後は月に1回で継続する方法の報告がある[12,21]。当院では1週間に2回を1クールとして，3ヵ月毎に1クールの維持継続 ECT を行う方法を用い，寛解を維持しているケースが多い。Kellner らはうつ病の維持療法として，維持継続 ECT 群と，nortriptyline に lithium を加えた薬物療法群とを比較した研究を行った[12]。6ヵ月後，維持継続 ECT 群の46.1%，薬物療法群の46.3%が寛解を維持した。この結果はプラセボコントロール群に比べ有意に再燃率が低く，維持継続 ECT の有効性が示された。また，Gagné らは，急性期に ECT を使用し寛解に至った治療抵抗性うつ病患者に対して，併用群（維持 ECT と薬物療法）と薬物療法単独群とを比較する後ろ向きケースコントロール研究を行った[8]。経過2年の時点で，併用群では寛解率が93%，薬物療法単独群では52%，経過5年の時点では，併用群の寛解率73%，薬物療法単独群18%と，併用群において優れた寛解維持効果を示した。さらに Navarro らは，急性期に ECT が有効であった高齢者の精神病像を伴う治療抵抗性うつ病患者に対して，併用群（維持 ECT に nortriptyline）と nortriptyline 単独群を比較した[21]。2年目の時点で，併用群では17人中11人（65%）が，nortriptyline 群では17人中5人（29%）が寛解を維持し，併用群が薬物療法単独群より有効であり，しかも有害な副作用は認めなかった。また維持 ECT は，60歳以上の高齢者に対し忍容性があることも示唆された。以上より，長期予後の点からも維持 ECT は，特に薬物との併用で優れた治療効果が期待される。

Frederikse らは，ECT の維持療法としての有効性を示す報告をまとめて，抗うつ薬の効果が不十分な場合などに ECT 維持継続を行うことを推奨している[7]。その際，ECT 単独ではなく薬物療法を併用する方が寛解を維持する可能性が高い[21]。大規模スタディの実施や，維持 ECT の施行方法（頻度や併用する薬物など）について，今後検討する必要があると思われる。

Ⅵ. おわりに

ECTはうつ病患者，特に治療抵抗性の場合でも有効性が期待される治療であり，今後さらなる貢献が期待されている。しかしながら，問題点もいくつかある。ECTは麻酔科医や手術室に準じた施設が必要となるため限られた医療機関でしか行えない治療であることや，入院が必要でありアクセスビリティがよくないこと，さらには施行方法や施設により効果に差があることなどが問題である。ECTは急性期のみならず，維持療法としても効果が期待できるが，その機序が明らかとはなっていない。また薬物療法との併用の方法や，機序についても不明な点が多く，今後さらなる研究が必要であろう。

文　献

1) Bajbouj, M., Lang, U. E., Niehaus, L. et al. : Effects of right unilateral electroconvulsive therapy on motor cortical excitability in depressive patients. J. Psychiatr. Res., 40 : 322–327, 2006.
2) Bourgon, L. N., Kellner, C. H. : Relapse of depression after ECT : a review. J. ECT, 16 : 19–31, 2000.
3) Buchan, H., Johnstone, E., McPherson, K. et al. : Who benefits from electroconvulsive therapy? Combined results of the Leicester and Northwick Park trials. Br. J. Psychiatry, 160 : 355–359, 1992.
4) Devanand, D. P., Dwork, A. J., Hutchinson, E. R. et al. : Does ECT alter brain structure? Am. J. Psychiatry, 151 : 957–970, 1994.
5) Fava, M. : Diagnosis and definition of treatment-resistant depression. Biol. Psychiatry, 53 : 649–659, 2003.
6) Folkerts, H. W., Michael, N., Tölle, R. et al. : Electroconvulsive therapy vs. paroxetine in treatment-resistant depression – a randomized study. Acta Psychiatr. Scand., 96 : 334–342, 1997.
7) Frederikse, M., Petrides, G., Kellner, C. : Continuation and maintenance electroconvulsive therapy for the treatment of depressive illness : a response to the National Institute for Clinical Excellence report. J. ECT, 22 : 13–17, 2006.
8) Gagné, G. G. Jr., Furman, M. J., Carpenter, L. L. et al. : Efficacy of continuation ECT and antidepressant drugs compared to long-term antidepressants alone in depressed patients. Am. J. Psychiatry, 157 : 1960–1965, 2000.
9) Husain, M. M., Rush, A. J., Fink, M. et al. : Speed of response and remission in major depressive disorder with acute electroconvulsive therapy (ECT) : a Consortium for Research in ECT (CORE) report. J. Clin. Psychiatry, 65 : 485–491, 2004.
10) Janicak, P. G., Davis, J. M., Gibbons, R. D. et al. : Efficacy of ECT : a meta-analysis. Am. J. Psychiatry, 142 : 297–302, 1985.
11) Keitner, G. I., Ryan, C. E., Solomon, D. A. : Realistic expectations and a disease management model for depressed patients with persistent symptoms. J. Clin. Psychiatry, 67 : 1412–1421, 2006.
12) Kellner, C. H., Knapp, R. G., Petrides, G. et al. : Continuation electroconvulsive therapy vs pharmacotherapy for relapse prevention in major depression : a multisite study from the Consortium for Research in Electroconvulsive Therapy (CORE). Arch. Gen. Psychiatry, 63 : 1337–1344, 2006.
13) Lauritzen, L., Odgaard, K., Clemmesen, L. et al. : Relapse prevention by means of paroxetine in ECT-treated patients with major depression : a comparison with imipramine and placebo in medium-term continuation therapy. Acta Psychiatr. Scand., 94 : 241–251, 1996.
14) Lisanby, S. H. : Electroconvulsive therapy for depression. N. Engl. J. Med., 357 : 1939–1945, 2007.
15) Marano, C. M., Phatak, P., Vemulapalli, U. R. et al. : Increased plasma concentration of brain-derived neurotrophic factor with electroconvulsive therapy : a pilot study in patients with major depression. J. Clin. Psychiatry, 68 : 512–517, 2007.
16) McCall, W. V. : What does Star*D tell us about ECT? J. ECT, 23 : 1–2, 2007.
17) Mössner, R., Daniel, S., Albert, D. et al. : Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). Neurochem. Int., 36 : 197–202, 2000.
18) 本橋伸高：電気けいれん療法の過去・現在・未来. 精神経誌，106 : 537–545, 2004.

19) 本橋伸高, 栗田主一 : ECT 推奨事項. 厚生労働科学研究費補助金　心の健康科学研究事業「精神疾患治療ガイドラインの策定等に関する研究」, 平成13年度～15年度.
20) 中村 純 : 抗うつ薬の早期効果発現へのアプローチ. 臨床精神薬理, 11 : 1741–1747, 2008.
21) Navarro, V., Gastó, C., Torres, X. et al. : Continuation/maintenance treatment with nortriptyline versus combined nortriptyline and ECT in late-life psychotic depression : a two-year randomized stud. Am. J. Geriatr. Psychiatry, 16 : 498–505, 2008.
22) 野田隆政, 澤田由紀子, 幸地洋子 他 : mECT(修正型電気けいれん療法)マニュアル. 国立精神・神経センター病院, 2004.
23) 野田隆政, 岡本長久 : 電気けいれん療法(electroconvulsive therapy ; ECT). エビデンスに基づく難治性うつ病の治療(野村総一郎, 樋口輝彦 編), pp. 82–104, 新興医学出版社, 東京, 2006.
24) 岡本長久 : 難治性うつ病への対応.　医学のあゆみ, 219 : 955–962, 2006.
25) 岡本長久, 佐藤真由美, 中井哲慈 : ケタミンと電気けいれん療法の併用による難治性うつ病の治療. Depression Frontier, 2008印刷中.
26) O'Leary, D., Gill, D., Gregory, S. et al. : Which depressed patients respond to ECT? The Nottingham results. J. Affect. Disord., 33 : 245–250, 1995.
27) Pagnin, D., de Queiroz, V., Pini, S. et al. : Efficacy of ECT in depression : a meta-analytic review. J. ECT, 20 : 13–20, 2004.
28) Perera, T. D., Coplan, J. D., Lisanby, S. H. et al. : Antidepressant-induced neurogenesis in the hippocampus of adult nonhuman primates. J. Neurosci., 27 : 4894–4901, 2007.
29) Sackeim, H. A., Prudic, J., Devanand, D. P. et al. : A prospective, randomized, double-blind comparison of bilateral and right unilateral electroconvulsive therapy at different stimulus intensities. Arch. Gen. Psychiatry, 57 : 425–434, 2000.
30) Sackeim, H. A., Haskett, R. F., Mulsant, B. H. et al. : Continuation pharmacotherapy in the prevention of relapse following electroconvulsive therapy : a randomized controlled trial. JAMA, 285 : 1299–1307, 2001.
31) Sobin, C., Prudic, J., Devanand, D. P. et al. : Who responds to electroconvulsive therapy? A comparison of effective and ineffective forms of treatment. Br. J. Psychiatry, 169 : 322–328, 1996.
32) Takano, H., Motohashi, N., Uema, T. et al. : Changes in regional cerebral blood flow during acute electroconvulsive therapy in patients with depression. Br. J. Psychiatry, 190 : 63–63, 2007.
33) Taylor, S. M. : Electroconvulsive therapy, brain-derived neurotrophic factor, and possible neurorestorative benefit of the clinical application of electroconvulsive therapy. J. ECT, 24 : 160–165, 2008.
34) UK ECT Review Group : Efficacy and safety of electroconvulsive therapy in depressive disorders : a systematic review and meta-analysis. Lancet, 361 : 799–808, 2003.
35) van den Broek, W. W., Birkenhäger, T. K., Mulder, P. G. et al. : Imipramine is effective in preventing relapse in electroconvulsive therapy-responsive depressed inpatients with prior pharmacotherapy treatment failure : a randomized, placebo-controlled trial. J. Clin. Psychiatry, 67 : 263–268, 2006.
36) Weiner, R. D., Rogers, H. J., Davidson, J. R. et al. : Effects of stimulus parameters on cognitive side effects. Ann. N Y Acad. Sci., 462 : 315–325, 1986.

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Glucocorticoids and Lithium Reciprocally Regulate the Proliferation of Adult Dentate Gyrus-Derived Neural Precursor Cells Through GSK-3β and β-Catenin/TCF Pathway

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Adult hippocampal neurogenesis is decreased in rodent models for stress-related disorders at least partly through an elevated level of glucocorticoids. On the other hand, the mood stabilizer lithium (Li) commonly used for their treatment increases it. This effect is thought to be one of the therapeutic actions of Li, but the molecular mechanism has been poorly understood. Here we established the culture system of adult rat dentate gyrus-derived neural precursor cells (ADPs) and examined the effects of dexamethasone (DEX), an agonist of glucocorticoids receptor, and Li on ADP proliferation. It is possible for ADP to be a type 2a cell, which corresponds to the second stage in a model of four differentiation stages in adult hippocampal neural precursor cells. DEX decreased ADP proliferation, but Li did not have any effect on it. However, Li recovered ADP proliferation decreased by DEX. The recovery effect of Li was abolished by quercetin, an inhibitor of β-catenin/TCF pathway. The intranuclear translocation of β-catenin and expression of cyclin D1 are reciprocally regulated by DEX and Li in a way similar to proliferation. In addition, DEX increased the phosphorylation of Tyr^{216}, which renders glycogen synthase kinase-3β (GSK-3β) active on it. These results suggest that GSK-3β and β-catenin/TCF pathway might be important in the reciprocal effects between DEX and Li on ADP proliferation and are new targets of therapeutic agents for stress-related disorders.
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Keywords: neurogenesis; stress; hippocampus; dexamethasone; mood stabilizer; Wnt

INTRODUCTION

It has been well established that neurogenesis occurs in the adult brain of various animal species, including humans (Eriksson *et al*, 1998; Gould *et al*, 1997; Gage, 2000). Neurogenesis takes place mainly in two discrete regions of the adult brain: the subventricular zone of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002) and the subgranular zone of the dentate gyrus (DG) in the hippocampus (Kempermann *et al*, 2006). It has been shown that neurogenesis in DG is affected by many factors, including environment, stress, hormones, and drugs. For example, adult neurogenesis in DG is decreased in rodent models for stress-related disorders (Gould *et al*, 1997; Malberg and Duman, 2003; Pham *et al*, 2003; Jayatissa *et al*, 2006). Although it remains unclear how neurogenesis in DG is decreased in these models, some studies have suggested that glucocorticoids might be involved in the decrease of adult hippocampal neurogenesis (Gould *et al*, 1997; Cameron and McKay, 1999; Kim JB *et al*, 2004). In humans, elevated levels of glucocorticoids constitute one of the causal events in stress-related disorders (de Kloet *et al*, 2005; Swaab *et al*, 2005). In contrast, the administration of lithium (Li), which is used for treatment of stress-related disorders, increases adult hippocampal neurogenesis (Chen *et al*, 2000; Son *et al*, 2003; Kim JS *et al*, 2004). These studies suggest that neurogenesis might be involved in the therapeutic action of Li. Therefore, elucidating how glucocorticoids and Li regulate neurogenesis might lead to a further understanding of the pathophysiology of stress-related disorders and the development of new therapeutic targets. Li is an inhibitor of glycogen synthase kinase-3β (GSK-3β; Klein and Melton, 1996; Stambolic *et al*, 1996), and GSK-3β is widely known as a key regulator of β-catenin/TCF pathway (Aberle *et al*, 1997; Orford *et al*, 1997).

In this study, we established the culture system of adult rat dentate gyrus-derived neural precursor cells (ADPs) and showed that dexamethasone (DEX), an agonist of glucocorticoid receptor (GR), decreased ADP proliferation and Li recovered it. In addition, we showed that this reciprocal

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effect between DEX and Li on ADP proliferation was regulated by GSK-3β and the β-catenin/TCF pathway.

MATERIALS AND METHODS

Isolation and Culture of ADP

Adult male Sprague–Dawley rats (8-week old) were deeply anesthetized with sodium pentobarbital and decapitated. The brains were removed and washed with ice-cold phosphate-buffered saline (PBS; pH 7.4). The coronal sections with a thickness of 1 mm were cut using a Brain Slicer (Muromachi, Tokyo, Japan) and immersed into dishes containing ice-cold PBS, and the regions containing DG were dissected carefully under a dissecting microscope to exclude all regions containing subependymal tissues (Figure 1a). These tissues were digested with a mixture of papain, dispase II and Dnase I as described earlier (Ray and Gage, 2005). The fraction containing ADP was isolated by Percoll (GE Healthcare, Buckinghamshire, UK) gradient centrifugation and seeded in DMEM: F12 (Sigma, St Louis, MO), including 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin-streptomycin (Invitrogen) on a noncoating dish, and incubated at 37°C in 5% CO_2. After overnight incubation, the medium was changed with a proliferation medium consisting of Neurobasal (Invitrogen), B27 supplement minus vitamin A (Invitrogen), 1 mM L-glutamine (Invitrogen), penicillin-streptomycin, and 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). The cultures were maintained with a proliferation medium at 37°C on laminin- (Invitrogen) and ornithin (Sigma)-coated dishes and fed with a new medium every 2 or 3 days by replacing 50% of the medium. When cell confluency reached 80–90%, the cells were passaged by trypsinization

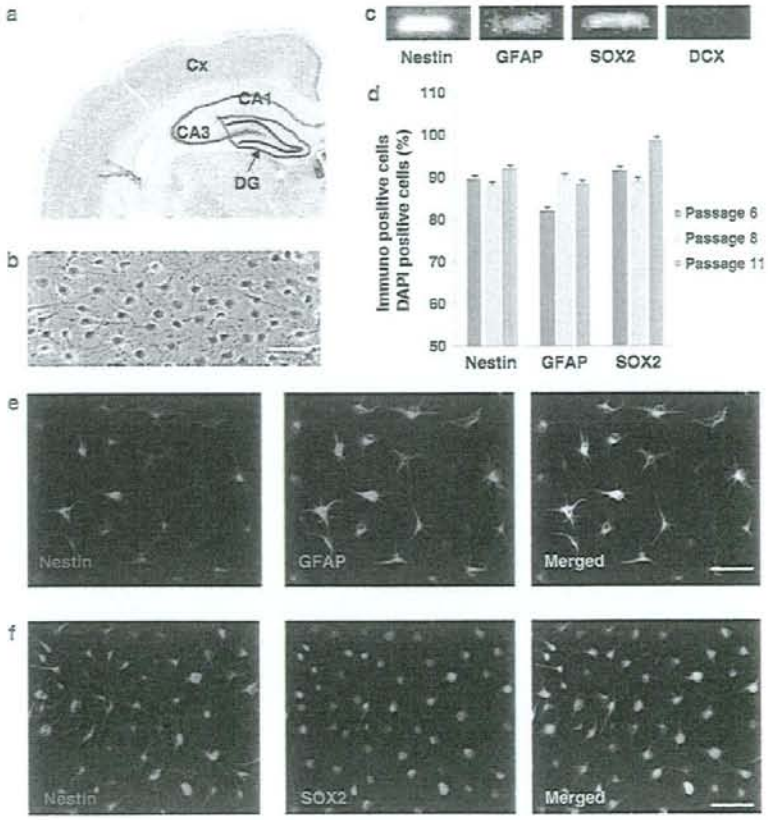

Figure 1 Isolation and characterization of adult rat dentate gyrus-derived neural precursor cells (ADP). (a) Coronal section through dissected regions. The dentate gyrus (DG) of adult rat was dissected as a red line, and cells were cultured as described in 'Materials and Methods'. Dissection was made carefully to exclude all other regions containing subependymal tissues. (b) The shape of cells. Phase-contrast image was shown at 3 days after passage. Cells grew as a monolayer. Scale bar = 90 μm. (c) Detection of the genes involved in neural precursor cell and immature neuron with RT–PCR. mRNAs of nestin, GFAP, and SOX2 were detected, but DCX was not. (d) The ratio of immunopositive cells with markers for neural precursor cells in passages 6, 8, and 11. Values are shown as the ratio of each marker-positive cell vs 4′,6-diamino-2-phenylindole (DAPI)-positive cell. Data are shown as the means ± SEM. (e, f) Immunocytochemistry for nestin, glial fibrillary acidic protein (GFAP), and SOX2. (e) Nestin (red), GFAP (green). Scale bar = 120 μm. (f) Nestin (red), SOX2 (green). Most cells coexpressed nestin, GFAP, and SOX2. Scale bar = 120 μm.

and the cell density for seeding was approximately 1×10^4 cells per cm^2. The cells were monitored with an IX-70 optical microscope (Olympus, Tokyo, Japan).

Analysis of ADP

For detecting the expression pattern of markers, 2×10^4 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated Lab-Tek II 8-chamber slides (Nalge Nunc International, Naperville, IL). After overnight incubation, immunocytochemistry was performed as described below. For testing proliferation potency, bromodeoxyuridine (BrdU; Sigma) was added into the medium at 10 nM. After 24 h, immunocytochemistry was performed. For testing multipotency, cells were treated in differentiation medium (proliferation medium without bFGF) with 10 ng/ml brain-derived neurotrophic factor (BDNF; Sigma), 1 μM retinoic acid (Invitrogen)/0.5% fetal bovine serum, or 500 ng/ml insulin-like growth factor (IGF; Invitrogen). After 7 days, immunocytochemistry was performed.

Cells were fixed with 4% paraformaldehyde for 15 min. In cases involving detecting the expression pattern of markers and testing for multipotency, permeabilization was performed with PBS containing 0.2% Triton X-100 for 30 min. In the cases of testing for proliferation potency, permeabilization was performed as follows: Cells were immersed with 50% formamide in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 h, incubated in 2 N HCl for 30 min at room temperature, and rinsed in 0.1 M borate buffer for 10 min. Subsequently, the cells were incubated in PBS containing 3% goat serum (Vector Laboratories, Burlingame, CA) for 20 min and then with primary antibodies containing 3% goat serum at 4°C overnight. The primary antibodies used were: mouse anti-nestin (1:2000; BD Biosciences, Franklin Lakes, NJ), rabbit anti-nestin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:2000; Dako, Glostrup, Denmark), rabbit anti-SRY (sex-determining region Y)-related high mobility group-box gene 2 (SOX2, 1:2000; Chemicon, Temecula, CA), rabbit anti-brain lipid-binding protein (BLBP, 1:300; kindly gifted by Masahiko Watanabe, Hokkaido University Graduate School of Medicine), rabbit anti-Prox1 (1:2000; Chemicon), mouse anti-Tuj1 (1:5000; Covance, Princeton, NJ), and mouse anti-O4 (1:2000; Chemicon). Cells were incubated in PBS containing secondary antibodies for 1 h at room temperature. The secondary antibodies used were: anti mouse Cy3 and anti-rabbit FITC (1:100; Jackson Immuno Research, West Grove, PA). Samples were coverslipped with Vectashield containing 4′,6-diamino-2-phenylindole (DAPI; Vector Laboratories). Fluorescent signals were detected using the IX-71 fluorescent microscope system (Olympus).

Cell Counting with Alamar Blue Assay

Alamar Blue assay is a rapid and simple nonradioactive assay to measure the number of cells. Alamar Blue dye is a fluorogenic redox indicator that is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent, and the fluorescence in Alamar Blue assay reflects the number of cells (Ahmed *et al*, 1994; Nakayama *et al*, 1997). In addition, we confirmed that fluorescence in Alamar Blue assay is surely proportional to the simply counted number of ADPs (data not shown). BrdU-based assays were often used for cell counting, but BrdU-positiveness reflects the duplication of DNA, but not the number of cells. Therefore, we used Alamar Blue assay to estimate the effects of drugs on the number of cells. A total of 1×10^4 cells per well were seeded in 100 μl per well of proliferation medium on laminin- and ornithin-coated 96-well plates. After overnight incubation, cells were treated with DEX (Sigma), Li chloride (Wako, Osaka, Japan), SB415286 (Tocris, Ellisville, MO), and/or quercetin (Que; Calbiochem, San Diego, CA) at each concentration. After 3 days, 10 μl per well of Alamar Blue solution (Invitrogen) was added into the medium and cells were incubated at 37°C for 3 h. The medium (50 μl) was dispensed into plates, and the fluorescence intensity of samples was measured by Fluoroscan (Dainippon Sumitomo Pharma, Osaka, Japan) and calculated as described in the manufacturer's manual. Statistical analysis was performed by one-way ANOVA and Dunnet's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means ± SEM.

RNA Extraction and Quantitative RT-PCR Analysis

A total of 1×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated six-well plates. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, total RNA was extracted from cells with the RNeasy extraction kit (Qiagen, Hilden, Germany). Total RNA was converted to cDNA with the Quantitect Reverse Transcription kit (Qiagen). PCR was performed with the SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA). The conditions of PCR were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The sequences of forward and reverse primers used were: AGCTGGTCATCAATGGGAAA and ATTTGATGTTAGCGGGATCG for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), CTGAGGCCTCTCTT CTTCCA and ATCAGATTCCCCACCCTGA for nestin, TTTCTCCAACCTCCAGATCC and GGTGGCCTTCTGACAC AGAT for GFAP, GAGGAGAGAAAAAGAAAGGAGAGAA and AAGTGCAATTGGGATGAAAAA for SOX2, TAAAAGC CCAGGCCCAAT and TGACTGCTAGAAGTTCCATTTGC for doublecortin (DCX), CTGCTTTGCTCCTGATCTGA and TTC ATAGGATACCTGCAATCTTTG for GR, GATAGAGGCCAA ATTAATCTTTCAA and CCTTCTGCGTCAGCTTAGGT for mineralcorticoid receptor (MR), CAACGCACTTTCT TTCCAGAG and AGGGCTTCAATCTGTTCCTG for cyclin D1, respectively. GAPDH was used as a control. The results were analyzed by using SDS 2.0 software (Applied Biosystems). Statistical analyses were performed by one-way ANOVA and Dunnet's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means ± SEM.

Western Blotting

For preparation of total proteins, 1×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated six-well plates. After overnight incubation, cells were

treated with each drug at each concentration. After 3 days, lysis of cells and preparation of total proteins were performed with the Mammalian Cell Lysis Kit (Sigma). For preparation of nuclear proteins, 4×10^5 cells per well were seeded in a proliferation medium on laminin- and ornithin-coated 100 mm dishes. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, lysis of cells and preparation of nuclear proteins were performed with the Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein concentration was measured by the Protein Assay Kit (Pierce, Rockford, IL), and an equal amount of proteins (20 μl per well) was loaded onto a 10% SDS gel. The gel was transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI) and incubated with primary antibodies that were used at the following concentrations: mouse monoclonal anti-cyclin D1 antibody (1 : 200; Santa Cruz Biotechnology), mouse monoclonal anti-β-catenin antibody (1 : 200; Santa Cruz Biotechnology), mouse monoclonal anti-GAPDH antibody (1 : 200; Santa Cruz Biotechnology), rabbit polyclonal anti-GSK-3β antibody (1 : 1000; Cell Signaling, Danvers, MA), rabbit polyclonal anti-pGSK-3β (Ser^9) antibody (1 : 1000; Cell Signaling), rabbit polyclonal anti-pGSK-3β (Tyr^{216}) antibody (1 : 1000; Biosource, Camarillo, CA). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary anti-mouse IgG antibody (1 : 4000; GE Healthcare) or horseradish peroxidase-conjugated secondary anti-rabbit IgG antibody (1 : 4000; GE Healthcare). Protein expression was detected with the Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). The pictures were converted to digital files and the intensity of each band was analyzed with ImageJ (National Institutes of Health, Bethesda, MD). Statistical analyses were performed by one-way ANOVA and Dunnet's *post hoc* test. Significance was defined as $p < 0.05$. Data are expressed as the means ± SEM.

RESULTS

Isolation and Culture of ADP

Tissues from DG of adult Sprague–Dawley rats were dissected carefully to exclude all other regions containing subependymal tissues in which neural stem cells exist (Figure 1a). Cells were dissociated from these tissues as described in Materials and methods, and cultured in a proliferation medium on laminin- and ornithin-coated dishes. In this condition, cells grew at monolayer and did not form any neurospheres. Most cells had a flat and round but slightly elongated shape with short branches, and were phase dark (Figure 1b). It took approximately 4–5 weeks (equal to 5–6 times passages) to get an adequate number of cells for assays. After around 12 times passages, the proliferation potency of cells was decreased, and the shape of cells began to change into smaller and more round ones or more elongated and branched ones. Therefore, we used cells for all assays from P5 to P11.

Characterization of ADP

First, we saw the mRNA expression of three common neural precursor cell markers, such as nestin, GFAP, and SOX2, and an immature neuron marker, DCX, in the cells with RT–PCR. The mRNA expressions of nestin, GFAP, and SOX2 were detected, but that of DCX was not (Figure 1c). Next, we performed immunocytochemistry for nestin, GFAP, and SOX2. The protein expression of each marker was detected in around 90% of the cells through different passages (Figure 1d). Moreover, most of the cells were immunolabeled by both nestin and GFAP antibodies at cytoplasm (Figure 1e), and nestin-positive cells were also immunolabeled by SOX2 antibody at the nucleus (Figure 1f). These results indicated that the cells dissociated from adult rat DG expressed nestin, GFAP, and SOX2 in both mRNA and protein, and were uniform in the shape and expression pattern through the passages we examined. In addition, we performed immunocytochemistry for BLBP, another neural precursor cell marker, and Prox1, another immature neuron marker. Protein expression of BLBP was detected as in the cases of nestin, GFAP, and SOX2, but that of Prox1 was not detected (data not shown). Next, we examined whether the nestin-positive cells are dividing or not. Most of the nestin-positive cells were immunolabeled by BrdU antibody (Figure 2A). The ratio of BrdU-positive cells/DAPI-positive cells was about 60–70% (Figure 2B).

In addition, we examined whether the cells differentiated to neuron or glia with the appropriate medium. The cells were treated with 10 ng/ml BDNF, 1 μM retinoic acid/0.5% fetal bovine serum, or 500 ng/ml IGF for 7 days. Cells were immunostained by anti-Tuj1 antibody for neuron, anti-GFAP antibody for astrocyte, or anti-O4 antibody for oligodendrocyte. Most cells treated with BDNF elongated axon-like branches and expressed Tuj1 (Figure 2C, a). Some cells treated with retinoic acid/fetal bovine serum changed like spread, less-rounded, and expressed GFAP (Figure 2C, b). These cells did not express nestin (data not shown), and their shape was larger and less round than that of untreated cells (Figures 1e and 2C, b), although the untreated cells also expressed GFAP (Figure 1e). A small number of cells treated with IGF formed many of the short dendrites and expressed O4 (Figure 2C, c). These results indicate that the cells dissociated from DG of adult rat brains express three common markers of neural precursor cells and have both proliferation potency and multipotency. Therefore, the cells were identified as neural precursor cells, and we call them ADPs.

DEX and Li have Reciprocal Effects on ADP Proliferation

Glucocorticoids can bind to both GR and MR (Sousa and Almeida, 2002). To elucidate the expression pattern of these GRs, we examined the mRNA expression of GR and MR in ADP. RT–PCR showed that the mRNA expression of GR was detected, but not that of MR in ADP (Figure 3a). Thus, it is likely to be adequate to use DEX, a specific agonist of GR, for evaluating the effects of glucocorticoids on ADP proliferation. We investigated the effect of DEX on ADP proliferation with Alamar Blue assay. The number of cells was not significantly different between control and 0.5, 1, 2, 5, 10 μM DEX treatment for 1 day or 2 days (data not shown). However, it was significantly decreased with 2, 5, 10 μM DEX treatment for 3 days (Figure 3b). The decreased

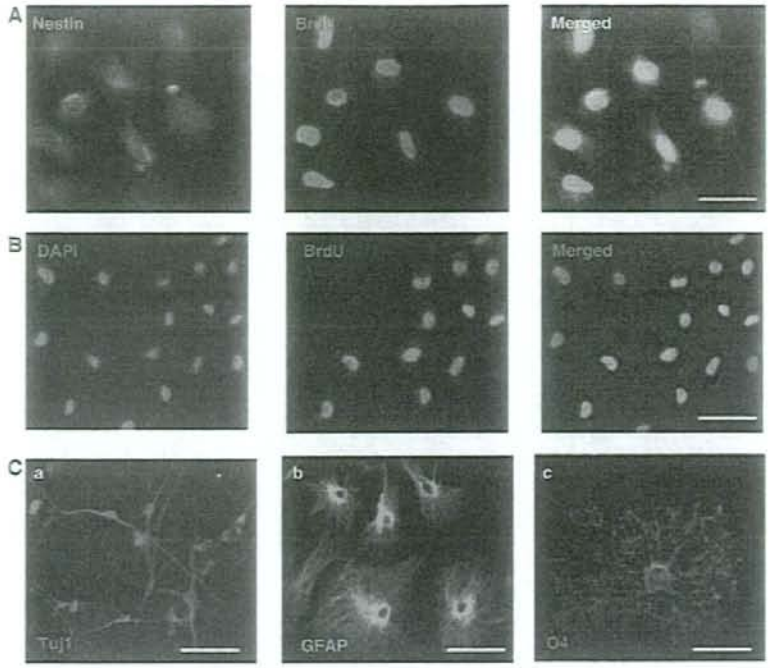

Figure 2 Tests for proliferation potency and multipotency. (A, B) Cells showed proliferation potency. Cells were incubated in 10 nM bromodeoxyuridine (BrdU) for 24 h and immunostained with anti-BrdU antibody and anti-nestin antibody. All of the BrdU-positive cells were nestin positive. The ratio of BrdU-positive cells was approximately 60–70%. Scale bar = 30 μm (A), 90 μm (B), respectively. (C) Cells showed multipotency. Cells were treated with (a) 10 ng/ml BDNF, (b) 1 μM retinoic acid/0.5% fetal bovine serum (FBS), and (c) 500 ng/ml insulin-like growth factor (IGF) for 7 days, respectively. Subsequently, cells were immunostained with anti-Tuj1 antibody (a) for neuron, anti-glial fibrillary acidic protein (anti-GFAP) antibody (b) for astrocyte, anti-O4 antibody (c) for oligodendrocyte. Most of the brain-derived neurotrophic factor (BDNF)-treated cells were Tuj1-positive (a). Some of the cells treated with retinoic acid/FBS were GFAP-positive (b). The small number of IGF-treated cells was O4-positive (c). Each of them showed characteristic shape. Scale bar = 90 μm (a), 60 μm (b), 20 μm (c), respectively.

number of cells has three possibilities: decreased proliferation, accelerated differentiation, and apoptosis. To test the possibility of accelerated differentiation, we performed immunostaining with Tuj1, nestin, GFAP, and O4 antibodies, and there were no Tuj 1-positive cells, nestin-negative and GFAP-positive cells, and O4-positive cells (data not shown). To test the possibility of accelerated apoptosis, we performed dUTP nick-end labeling (TUNEL) assay, and any TUNEL-positive cells were not seen (data not shown). In addition, we confirmed that DEX decreased ADP proliferation with BrdU immunocytochemistry (data not shown). Therefore, DEX had no effect on both differentiation and apoptosis in ADP, and we concluded that DEX decreases ADP proliferation. Next, we evaluated the effect of Li on ADP proliferation. We treated cells with Li in 0, 0.1, 0.3, 1, and 3 mM for 3 days, both in the absence and presence of 5 μM DEX, and performed Alamar Blue assay. Li had no effect on ADP proliferation at any concentrations in the absence of DEX (Figure 3c). On the other hand, Li recovered ADP proliferation decreased by DEX to the control level at 1 and 3 mM in the presence of 5 μM DEX (Figure 3d). Taken together, these results suggested that DEX and Li had reciprocal effects on ADP proliferation.

Involvement of GSK-3β and β-Catenin/TCF Pathway in the Reciprocal Effects Between DEX and Li on ADP Proliferation

Li works as an inhibitor of GSK-3β through the direct inhibition of its catalyzed reaction (Klein and Melton, 1996; Stambolic *et al*, 1996; Davies *et al*, 2000). However, Li also works as an inhibitor of inositol monophosphatase (Atack *et al*, 1995). To confirm that the effect of Li on ADP proliferation is mediated by the direct inhibition of GSK-3β, we examined the effects of SB415286, a specific inhibitor of GSK-3β (Coghlan *et al*, 2000). We found that SB415286 mimicked the effects of Li on ADP proliferation both in the absence of DEX (Figure 4a) and in the presence of 5 μM DEX (Figure 4b). Therefore, the direct inhibition of GSK-3β might be involved in the recovery effect of Li on ADP proliferation.

GSK-3β is widely known as a key regulator of β-catenin/TCF pathway (Aberle *et al*, 1997; Orford *et al*, 1997). Therefore, we investigated the involvement of β-catenin/TCF pathway in the reciprocal effects between DEX and Li on ADP proliferation with Que, which inhibits β-catenin/TCF pathway through mainly the inhibition of

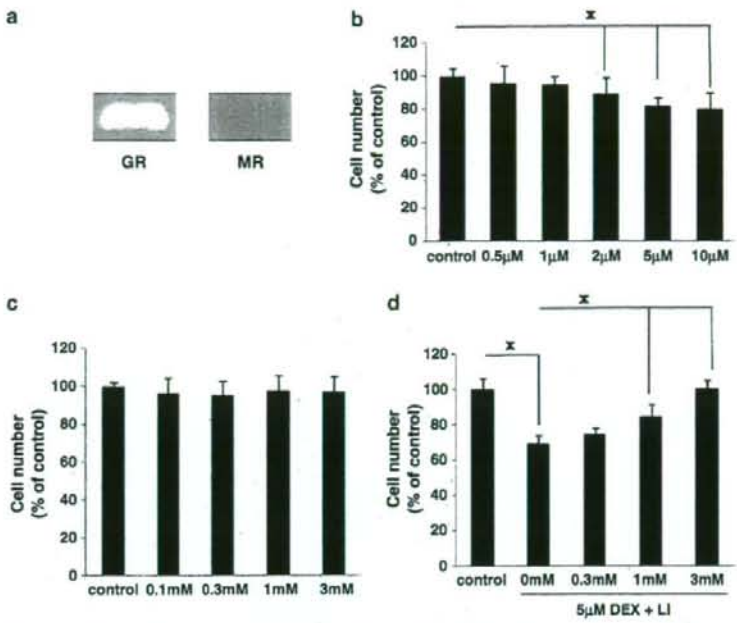

Figure 3 Dexamethasone (DEX) and lithium (Li) have reciprocal effects on adult rat dentate gyrus-derived neural precursor cell (ADP) proliferation. (a) Detection of mRNA of glucocorticoid receptor (GR) and mineralcorticoid receptor (MR) with RT–PCR. mRNA of GR was detected, but mRNA of MR was not detected. (b) DEX decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. *$p < 0.05$, compared with control. (c) Li had no effect on ADP proliferation in the absence of DEX. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. (d) Li recovered DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. *$p < 0.05$, compared with control or 0 mM.

nuclear translocation of β-catenin (Park *et al*, 2005). Cells were treated with DEX, Li, and Que for 3 days, and Alamar Blue assay was performed. Que had no effect on ADP proliferation decreased by DEX in the absence of Li (Figure 4c). On the other hand, Que significantly abolished the recovery effect of Li from ADP proliferation decreased by DEX at 10 and 30 μM (Figure 4d). These results indicated that β-catenin/TCF pathway was involved in the reciprocal effects between DEX and Li on ADP proliferation.

Cyclin D1 Expression is Reciprocally Regulated by DEX and Li Through β-Catenin/TCF Pathway

Cyclin D1 is a common cell-cycle regulator that controls G_1–S transition through controlling cyclin-dependent kinases, and its expression is regulated by β-catenin/TCF pathway (Tetsu and McCormick, 1999; Shtutman *et al*, 1999). We investigated the effect of DEX, Li, and Que for cyclin D1 expression in ADP. Cells were treated with 5 μM DEX, 3 mM Li, and/or 30 μM Que for 3 days, and quantitative RT-PCR and western blotting were performed. Quantitative analysis showed that DEX significantly decreased cyclin D1 expression in both mRNA (Figure 5a) and protein (Figure 5b) and that Li recovered it to the control level. However, Que abolished the recovery effect of Li in both mRNA (Figure 5a) and protein (Figure 5b). Li had no effect on cyclin D1 expression in ADPs in the absence of DEX (data not shown). Taken together, these results indicated that the expression level of cyclin D1 might be reciprocally regulated by DEX and Li through β-catenin/TCF pathway as in the case with ADP proliferation.

Nuclear Translocation of β-Catenin is Reciprocally Regulated by DEX and Li

β-Catenin is translocated from the cytosol to the nucleus and works as a transcription factor after binding to TCF (Salic *et al*, 2000). We measured the effects of DEX, Li, and Que on nuclear β-catenin in ADP. Cells were treated with 5 μM DEX, 3 mM Li, and/or 30 μM Que for 3 days. After that, the nuclear proteins were extracted and western blotting was performed. The protein level of nuclear β-catenin was decreased with DEX and Li recovered it to the control level (Figure 6a and b). Moreover, Que abolished the recovery effect of Li (Figure 6a and b). The treatment with Li alone had no effect on the protein level of nuclear β-catenin (data not shown). These results suggested that the nuclear translocation of β-catenin might be reciprocally regulated by DEX and Li, as in the cases of ADP proliferation and cyclin D1 expression.

Effects of DEX on the Phosphorylation States of GSK-3β

Activated GSK-3β phosphorylates β-catenin and inhibits its nuclear translocation (Aberle *et al*, 1997; Orford *et al*, 1997). The activity of GSK-3β is regulated by two

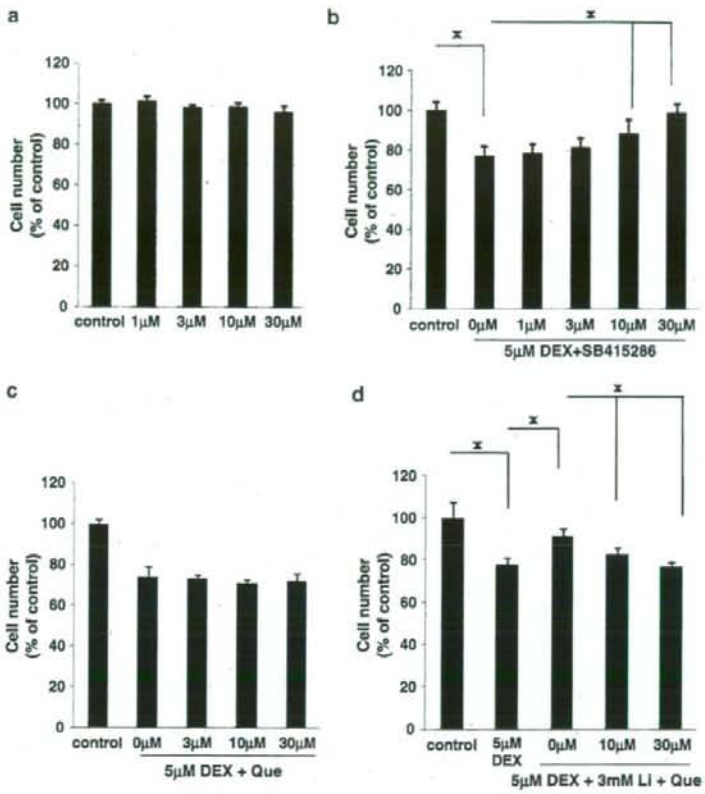

Figure 4 The involvement of glycogen synthase kinase-3β (GSK-3β) and β-catenin/TCF pathway in the reciprocal actions between dexamethasone (DEX) and lithium (Li) on adult rat dentate gyrus-derived neural precursor cell (ADP) proliferation. (a) SB415286 had no effect on ADP proliferation in the absence of DEX. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. (b) SB415286 recovered DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. $p<0.05$, compared with control or 0 μM. (c) Quercetin (Que) had no effect on ADP proliferation in the absence of Li. Alamar Blue assay was performed 3 days after the treatment. Data are the means ± SEM of four independent cultures. (d) Que abolished the recovery effect of Li on DEX-decreased ADP proliferation. Alamar Blue assay was performed 3 days after the treatment. Data are shown as the means ± SEM of four independent cultures. *$p<0.05$, compared with control, 5 μM DEX or 0 μM.

phosphorylated residues; Ser^9 to render it inactive (Cross *et al*, 1995) and Tyr^{216} to render it active (Hughes *et al*, 1993). Moreover, Li works as an inhibitor of GSK-3β through the direct inhibition of its catalyzed reaction (Klein and Melton, 1996; Stambolic *et al*, 1996). Therefore, we investigated whether DEX and Li affect the expression of GSK-3β and the phosphorylation of these residues. Cells were treated with DEX and/or Li for 3 days, and western blotting was performed. Ser^9 is well phosphorylated, but Tyr^{216} is little in the control condition (Figure 7a). DEX increased the phosphorylation of Tyr^{216} remarkably, but it had no effect on the expression of GSK-3β and the phosphorylation of Ser^9 (Figure 7a and b). Conversely, the treatment of Li alone or those of DEX and Li had no effect on all of them (data not shown). Taken together, these results suggested that DEX might decrease ADP proliferation by increasing the phosphorylation of Tyr^{216} on GSK-3β and that Li might recover ADP proliferation, decreased by DEX through the direct inhibition of its catalyzed reaction, not changing the phosphorylation of Tyr^{216} on it.

DISCUSSION

Here we have established the culture system of ADP. ADP is carefully isolated from the dissected DG of adult rats to exclude any subependymal tissues, grows at monolayers in the described condition, and can be passaged 10–11 times with the same character. ADP expresses nestin, GFAP, SOX2, and BLBP, but not DCX and Prox1. In addition, ADP has proliferation potency and multipotency. Some culture systems of neural precursor cells derived from the adult rodent hippocampus have already been reported (Palmer *et al*, 1997, 1999; Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005; Babu *et al*, 2007). Our culture system is based on them, but is different in some ways as shown below. Both Seaberg's and Bull's culture systems are derived from mouse

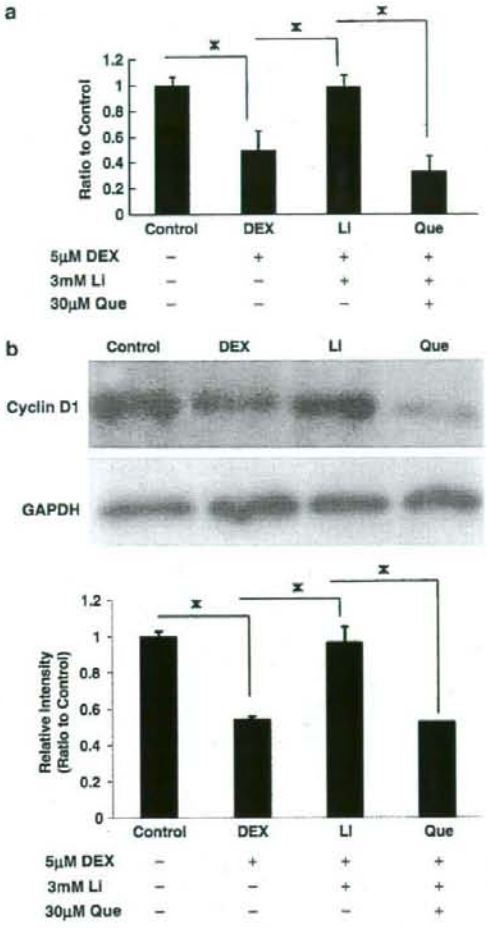

Figure 5 Cyclin D1 expression is reciprocally regulated by dexamethasone (DEX) and lithium (Li) through the β-catenin/TCF pathway. (a) DEX decreased mRNA expression of cyclin D1, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μM DEX, 3 mM Li, and/or 30 μM Que. After 3 days, RNA isolation and quantitative RT-PCR were performed. Values are shown as the ratio of cyclin D1 mRNA *vs* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Data are shown as the means ± SEM of three independent samples. *$p<0.05$, compared with control, DEX, or Li. (b) DEX decreased protein expression of cyclin D1, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μM DEX, 3 mM Li, and/or 30 μM Que. After 3 days, nuclear proteins were prepared, 20 μg of them was loaded into each lane, and western blotting was performed. Pictures are shown from a typical experiment that was repeated three times. Data are shown as the means ± SEM of three independent cultures. *$p<0.05$, compared with control, DEX, or Li.

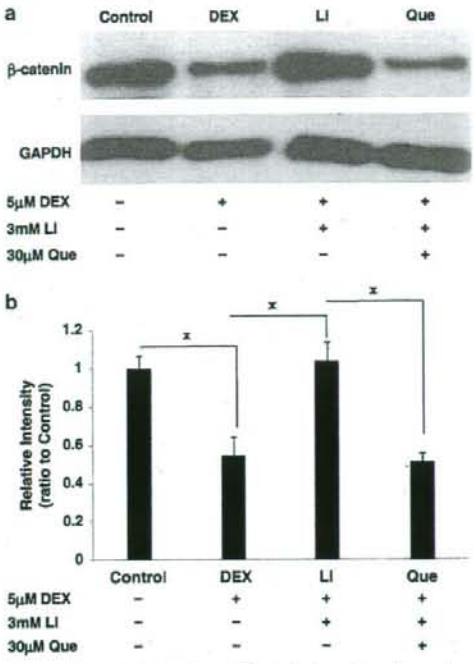

Figure 6 Nuclear translocation of β-catenin is reciprocally regulated by dexamethasone (DEX) and lithium (Li). (a, b) DEX decreased nuclear β-catenin, Li recovered it, and Que abolished the recovery effect of Li. Cells were treated with 5 μM DEX, 3 mM Li, and/or 30 μM Que. After 3 days, nuclear proteins were prepared, 20 μg of them was loaded into each lane, and western blotting was performed. In (a), pictures are shown from a typical experiment that was repeated three times. In (b), data are shown as the means ± SEM of three independent cultures. *$p<0.05$, compared with control, DEX, or Li.

DG, but their culture conditions differ from ours. Babu's culture system is relatively close to ours, but it is derived from mouse DG and can be passaged many more times than ours. In addition, the properties of adult mouse-derived neural precursor cells may be different from those of adult rat-derived ones (Ray and Gage, 2005). Meanwhile, Palmer's culture system is derived from rat entire hippocampus, but not dissected DG.

Four differentiation stages of precursor cells, including stem and progenitor cells, have been proposed in adult rodent hippocampus *in vivo* (Seri *et al*, 2001; Fukuda *et al*, 2003; Kempermann *et al*, 2004; Steiner *et al*, 2006): Cells in the first stage (type-1 cells) express nestin, GFAP, SOX2, and BLBP. Cells in the second stage (type-2a cells) express nestin, SOX2, and BLBP. The expression of GFAP in this stage is vague. Cells in the third stage (type-2b cells) express nestin, DCX, and Prox1, but not GFAP and SOX2 (Kempermann, 2006; Gage *et al*, 2008). Therefore, the expression pattern of markers on ADPs corresponds to type 1 cell or type 2a cell *in vivo*. The shape of ADP is different from that of type 1 cell, with an elongated and blanched shape, and is similar to that of type 2a cell with a flat and round shape (Kempermann *et al*, 2004). The proliferation potency of ADP is limited; that of type 1 cell is probably unlimited, and that of type 2a cell is limited (Kempermann *et al*, 2004). Taken together, ADP could possible be type2a cells. It is poorly understood which stage of neural precursor cells contributes to the reactivity of drugs to neurogenesis. However, a recent study indicated that type 2a-like cells might be a target of fluoxetine, which is an

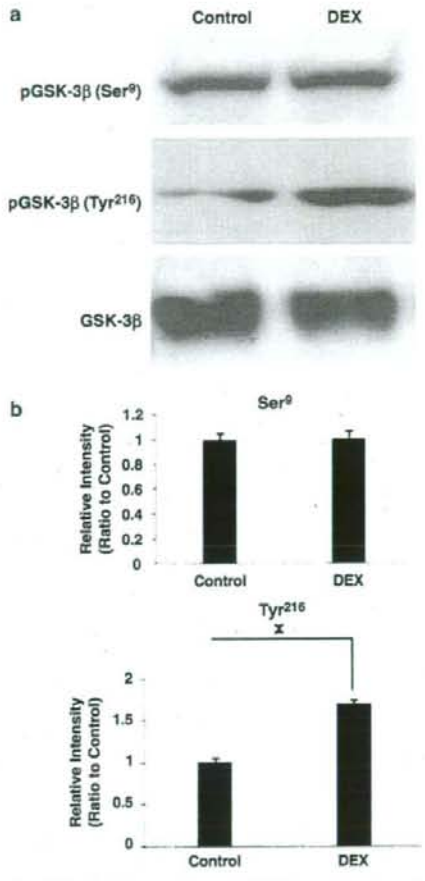

Figure 7 Effects of dexamethasone (DEX) on two phosphorylated residues: Ser^9 and Tyr^{216} of glycogen synthase kinase-3β (GSK-3β). (a, b) DEX increased the phosphorylation of Tyr^{216}, not but Ser^9 of GSK-3β. Cells were treated with 5 μM DEX. After 3 days, 20 μg of cell lysates were loaded into each lane and western blotting was performed. In (a), pictures are shown from a typical experiment that was repeated three times. In (b), data are shown as the means ± SEM of three independent cultures. *$p < 0.05$, compared with control.

antidepressant and can increase adult neurogenesis in DG (Encinas *et al*, 2006). Therefore, it might be beneficial to examine the reactivity of type 2a cells to various drugs and ADPs could be a good model for type2a cells.

Some studies have shown that Li increases adult neurogenesis in DG of rodents *in vivo* (Chen *et al*, 2000; Son *et al*, 2003; Kim JS *et al*, 2004). In addition, it has been shown that Li directly increases neural precursor cells derived from the embryo (Kim JS *et al*, 2004) and adult entire hippocampus (Wexler *et al*, 2008). However, it remains unclear whether Li directly affects the proliferation of neural precursor cells in DG of adult rodents. To elucidate it, we investigated the effect of Li on ADP proliferation. Although Li had no effect on ADP proliferation in the absence of DEX, Li recovered the ADP proliferation decreased by DEX to the control level. Interestingly, these findings are different from Wexler's results in spite of the closeness of the source of cells. The discrepancy might be due to the difference of the source and character of cells as well as culture condition. We have no answer regarding which culture condition and reactivity to Li is closer to those of *in vivo* neural precursor cells in adult DG, and further investigation to answer this question might lead to a further understanding of their character, including the reactivity to various drugs.

It has been shown that the activation of β-catenin/TCF pathway leads to increasing cyclin D1 expression in tumor-derived cell lines (Tetsu and McCormick, 1999; Shtutman *et al*, 1999). In this study, we found that both cyclin D1 expression and the protein level of nuclear β-catenin are reciprocally regulated by DEX and Li as well as ADP proliferation. These results suggest the involvement of β-catenin/TCF pathway in the reciprocal effects between DEX and Li on ADP proliferation. β-Catenin/TCF pathway is also well known as the canonical Wnt pathway. It has already been shown that the canonical Wnt pathway regulates the proliferation of embryo-derived neural precursor cells *in vitro* (Hirsch *et al*, 2007) and adult hippocampal neurogenesis *in vivo* (Lie *et al*, 2005). However, Lie *et al* showed that the canonical Wnt pathway regulates the proliferation of DCX-positive and elongated cells, which may correspond to type 3 cells and are in the late differentiation stages of neural precursor cells (Kempermann, 2006; Gage *et al*, 2008). Therefore, our present study is the first report to indicate the involvement of β-catenin/TCF pathway in the proliferation of hippocampal neural precursor cells in the early differentiation stages.

Moreover, we first clarified the involvement of glucocorticoids in β-catenin/TCF pathway in adult DG-derived neural precursor cells. There is the possibility that GR directly represses the transcription of cyclin D1 because GR is a transcription factor that can promote or repress the transcription of various genes through direct binding to their promoters (Schoneveld *et al*, 2004). Our results do not exclude this possibility, and this direct mechanism could regulate ADP proliferation in cooperation with β-catenin/TCF pathway.

We found that DEX significantly increases the phosphorylation of Tyr^{216} and has no effect on the phosphorylation of Ser^9 on GSK-3β. These results suggest that DEX might negatively regulate β-catenin/TCF pathway through the phosphorylation of Tyr^{216} on GSK-3β. Although it has been shown that glucocorticoids negatively regulate cell proliferation through decreasing the phosphorylation of Ser^9 in osteoblasts (Smith *et al*, 2002), nothing else is known about the effects of glucocorticoids on the phosphorylation states of GSK-3β. Therefore, our present study is the first report to indicate the effects of glucocorticoids on the phosphorylation of Tyr^{216} on GSK-3β.

GSK-3 is well known to be encoded by two different genes, *GSK-3*β and *GSK-3*α (Frame and Cohen, 2001; Grimes and Jope, 2001). As most studies of the function of GSK-3 activity in neurogenesis and cell proliferation have focused on the more abundant GSK-3β, our present study also focused on GSK-3β. However, some studies have highlighted a role for GSK-3α in neuronal disease (Phiel *et al*, 2003). In addition, both Li and SB415286 can inhibit not only GSK-3β but also GSK-3α. Therefore, the limitation of

our present study is that it does not exclude the involvement of GSK-3α.

We have shown that DEX and Li reciprocally regulate ADP proliferation through GSK-3β and β-catenin/TCF pathway; DEX activates GSK-3β through the phosphorylation of Tyr^{216}; GSK-3β activated by DEX inhibits β-catenin/TCF pathway, and Li recovers it through inhibiting GSK-3β activated by DEX. However, it remains unclear how DEX increases the phosphorylation of Tyr^{216} on GSK-3β. To elucidate it might lead to a further understanding of stress mechanism and the development of new therapeutic targets for psychiatric disorders.

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DISCLOSURE/CONFLICTS OF INTEREST

All the authors declare that they have no conflict of interest.

REFERENCES

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997). Betacatenin is a target for the ubiquitin–proteasome pathway. *EMBO J* **16**: 3797–3804.

Ahmed SA, Gogal Jr RM, Walsh JE (1994). A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H]thymidine incorporation assay. *J Immunol Methods* **170**: 211–224.

Alvarez-Buylla A, Garcia-Verdugo JM (2002). Neurogenesis in the adult subventricular zone. *J Neurosci* **22**: 629–634.

Atack JR, Broughton HB, Pollack SJ (1995). Inositol monophosphatase—a putative target for Li^+ in the treatment of bipolar disorder. *Trends Neurosci* **18**: 343–349.

Babu H, Cheung G, Kettenmann H, Palmer TD, Kempermann G (2007). Enriched monolayer precursor cell cultures from microdissected adult mouse dentate gyrus yield functional granule cell-like neurons. *PLoS ONE* **2**: e388.

Bull ND, Bartlett PF (2005). The adult mouse hippocampal progenitor is neurogenic but not a stem cell. *J Neurosci* **25**: 10815–10821.

Cameron H, McKay RDG (1999). Restoring production of hippocampal neurons in old age. *Nat Neurosci* **2**: 894–897.

Chen G, Rajkowska G, Du F, Seraji-Bozorgzad N, Manji HK (2000). Enhancement of hippocampal neurogenesis by lithium. *J Neurochem* **75**: 1729–1734.

Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ *et al* (2000). Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* **7**: 793–803.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**: 785–789.

Davies SP, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351**: 95–105.

de Kloet ER, Joels M, Holsboer F (2005). Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* **6**: 463–475.

Encinas JM, Vaahtokari A, Enikolopov G (2006). Fluoxetine targets early progenitor cells in the adult brain. *Proc Natl Acad Sci USA* **103**: 8233–8238.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA *et al* (1998). Neurogenesis in the adult human hippocampus. *Nat Med* **4**: 1313–1317.

Frame S, Cohen P (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* **359**: 1–16.

Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003). Two distinct subpopulation of nestin positive cells in adult mouse dentate gyrus. *J Neurosci* **23**: 9357–9366.

Gage FH (2000). Mammalian neural stem cells. *Science* **287**: 1433–1438.

Gage FH, Kempermann G, Song H (2008). *Adult Neurogenesis*. Cold Spring Harbor Laboratory Press: New York.

Gould E, McEwen BS, Tanapat P, Galea LAM, Fuchs E (1997). Neurogenesis in dentate gyrus of adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* **17**: 2492–2498.

Grimes CA, Jope RS (2001). The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol* **65**: 391–426.

Hirsch C, Campano LM, Wöhrle S, Hecht A (2007). Canonical Wnt signaling transiently stimulates proliferation and enhances neurogenesis in neonatal neural progenitor cultures. *Exp Cell Res* **313**: 572–587.

Hughes K, Nikolakaki E, Plyte SE, Totty NF, Woodgett JR (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *EMBO J* **12**: 803–808.

Jayatissa MN, Bisgaard C, Tingström A, Papp M, Wiborg O (2006). Hippocampal cytogenesis correlates to escitalopram-mediated recovery in chronic mild stress rat model of depression. *Neuropsychopharmacology* **31**: 2395–2404.

Kempermann G (2006). *Adult Neurogenesis*. Oxford University Press: Oxford.

Kempermann G, Chesler EJ, Lu L, Williams RW, Gage FH (2006). Natural variation and genetic covariance in the adult hippocampal neurogenesis. *Proc Natl Acad Sci USA* **103**: 780–785.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004). Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* **27**: 447–452.

Kim JB, Ju JY, Kim JH, Kim TY, Yang BH, Lee YS *et al* (2004). Dexamethasone inhibits proliferation of adult hippocampal neurogenesis *in vivo* and *in vitro*. *Brain Res* **1027**: 1–10.

Kim JS, Chang MY, Yu IT, Kim JH, Lee SH, Lee YS *et al* (2004). Lithium selectively increases neuronal differentiation of hippocampal neural progenitor cells both *in vitro* and *in vivo*. *J Neurochem* **89**: 324–336.

Klein PS, Melton DA (1996). A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* **93**: 8455–8459.

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A *et al* (2005). Wnt signaling regulates adult hippocampal neurogenesis. *Nature* **437**: 1370–1375.

Malberg JE, Duman RS (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology* **28**: 1562–1571.

Nakayama GR, Caton MC, Nova MP, Parandoosh Z (1997). Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *J Immunol Methods* **204**: 205–208.

Orford K, Crockett C, Jensen JP, Weissman AM, Byers SW (1997). Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. *J Biol Chem* **272**: 24735–24738.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999). Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of adult CNS. *J Neurosci* **19**: 8487–8497.

Palmer TD, Takahashi J, Gage FH (1997). The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci* **8**: 389–404.

Park CH, Chang JY, Hahm ER, Park S, Kim HK, Yang CH (2005). Quecertin, a potent inhibitor against β-catenin/TCF signaling in SW480 colon cancer cells. *Biochem Biophys Res Commun* **328**: 227–234.

Pham K, Nacher J, Hof PR, McEwen BS (2003). Repeated restraint stress suppresses neurogenesis and induces biphasic PSA-NCAM expression in the adult rat dentate gyrus. *Eur J Neurosci* **17**: 879–886.

Phiel CJ, Wilson CA, Lee VM, Klein PS (2003). GSK-3alpha regulates production of Alzheimer's disease amyroid-beta peptides. *Nature* **423**: 435–439.

Ray J, Gage FH (2005). Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. *Mol Cell Neurosci* **31**: 560–573.

Salic A, Lee E, Mayer L, Kirschner MW (2000). Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. *Mol Cell* **5**: 523–532.

Schoneveld OJLM, Gaemers I, Lamers WH (2004). Mechanisms of glucocorticoid signaling. *Biochim Biophys Acta* **1680**: 114–128.

Seaberg RM, van der Kooy D (2002). Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. *J Neurosci* **22**: 1784–1793.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001). Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* **21**: 7153–7160.

Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R *et al* (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc Natl Acad Sci USA* **96**: 5522–5527.

Smith E, Coetzee GA, Frenkel B (2002). Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase-3β. *J Biol Chem* **277**: 18191–18197.

Son H, Yu IT, Hwang SJ, Kim JS, Lee SH, Lee YS *et al* (2003). Lithium enhances long-term potentiation independently of hippocampal neurogenesis in the rat dentate gyrus. *J Neurochem* **85**: 872–881.

Sousa N, Almeida OF (2002). Corticosteroids: sculptors of hippocampal formation. *Rev Neurosci* **13**: 59–84.

Stambolic V, Ruel L, Woodgett JR (1996). Lithium inhibits glycogen kinase synthase-3 activity and mimics wingless signaling in intact cells. *Curr Biol* **6**: 1644–1668.

Steiner B, Klempin F, Wang L, Kott M, Kettenmann H, Kempermann G (2006). Type-2 cells as link between glial and neuronal linage in adult hippocampal neurogenesis. *Glia* **54**: 805–814.

Swaab DF, Bao AM, Lucassen PJ (2005). The stress system in the human brain in depression and neurodegeneration. *Aging Res Rev* **4**: 141–194.

Tetsu O, McCormick F (1999). β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**: 422–426.

Wexler EM, Geschwind DH, Palmer TD (2008). Lithium regulates adult hippocampal progenitor development through canonical Wnt pathway activation. *Mol Psychiatry* **13**: 285–292.

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Changes in amygdala neural activity that occur with the extinction of context-dependent conditioned fear stress

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ABSTRACT

The purpose of the present study was to characterize functional changes in the amygdala that accompany the extinction of context-dependent conditioned fear stress in a rat, an animal model of anxiety. Specifically, the effect of extinction of conditioned fear-induced cyclic AMP responsive element-binding protein (CREB) phosphorylation in the amygdala was investigated using immunohistochemistry. Experiments demonstrated that CREB phosphorylation in the basal nucleus of the amygdala decreased with the extinction of context-dependent conditioned fear-induced freezing behavior. These data suggest that the basal nucleus of the amygdala plays an essential role in the expression of context-dependent conditioned fear. Further, this is the first study to demonstrate that CREB phosphorylation in the basal nucleus of the amygdala changes in parallel with the extinction of context-dependent conditioned fear.

1. Introduction

Past studies have demonstrated that the amygdala plays a crucial role in anxiety and fear (Ono and Nishijo, 1992; LeDoux, 2000) and that the amygdala may be a target for the action of various kinds of anxiolytic drugs (Beck and Fibiger, 1995; Menard and Treit, 1999; Inoue et al., 2004). We recently reported that conditioned fear stress (CFS), an animal model of anxiety in rats, specifically induced c-Fos expression in the basal nucleus of the amygdala and that the administration of citalopram, a selective reuptake inhibitor, attenuated this increase in c-Fos expression (Izumi et al., 2006).

Conditioned fear stress is a type of classical conditioning (Fanselow, 1980) distinguished by acquisition, expression, and extinction (Myers and Davis, 2002). Acquisition occurs when a sensory stimulus (CS, conditioned stimulus), such as light, tone, or exposure to the test box (context), is paired with an aversive stimulus (US, unconditioned stimulus), such as footshock. Expression occurs when the animal is re-exposed to the CS without the US, and it elicits a variety of autonomic, hormonal, and behavioral conditioned responses. Extinction occurs when the CS is repeatedly presented in the absence of the US, and it decreases the amplitude of conditioned responses.

Extinction is thought to be an active learning process (Myers and Davis, 2002). Several studies have attempted to characterize the effect of a prefrontal cortex lesion on extinction, but the results have varied (Gewirtz et al., 1997; Morgan and LeDoux, 1999; Quirk et al., 2000). Further, administration of a *N*-methyl-D-aspartate (NMDA) receptor glycine site agonist facilitated extinction, while administration of a NMDA receptor antagonist, benzodiazepine receptor agonist, benzodiazepine receptor inverse agonist, muscarinic receptor antagonist, or dopamine-1 receptor agonist inhibited extinction (reviewed by Myers and Davis, 2002; Davis and Myers 2002).

The goal of the present study was to characterize changes in the amygdala neural activity that occur with the extinction of context-dependent CFS, using cAMP responsive element-binding protein (CREB) phosphorylation as an index of cellular activity.

2. Methods

This study was approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and all protocols complied with the Guide for the Care and Use of Laboratory Animals of the Hokkaido University School of Medicine.

2.1. Animals

Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 250–300 g, were used. Four rats were housed per cage (38×33×17 cm), in a 12-h light:12-h dark cycle and a temperature-controlled environment (22±1 °C) with free access to food and water. Experiments were initiated after a 14-day adaptation period.

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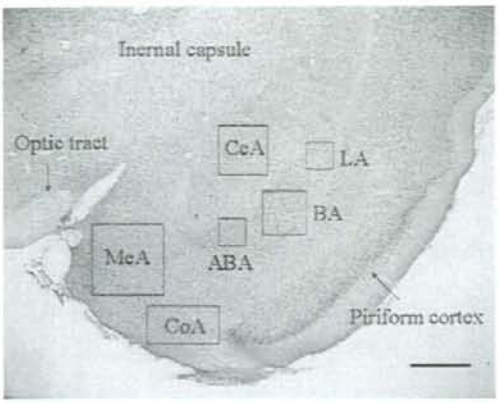

Fig. 1. Nissl staining of the amygdala (−3.14 mm to Bregma). BA, basal nucleus of the amygdala; ABA, accessory basal nucleus of the amygdala; CeA, central nucleus of the amygdala; CoA, cortical nucleus of the amygdala; LA, lateral nucleus of the amygdala; MeA, medial nucleus of the amygdala. Bar=500 μm.

2.2. CFS-induced freezing

Each rat was placed in a shock chamber (19×22×20 cm) and underwent 5 min of inescapable electric shocks (scrambled shocks of 0.2-mA intensity and 30-s duration, five times at variable intervals). Twenty-four hours after the footshock, the rats were again placed in the shock chamber and observed for 5 min without any shock application. During the 5-min observation period, freezing behavior was recorded using a time-sampling procedure (Fanselow, 1980), in which the animal behavior was classified as either "freezing" or "activity" at every 10-s interval. Freezing was defined as the lack of any observable movement of the body and the vibrissae, with the exception of movements related to respiration. Percentage scores for freezing were calculated for a 5-min observation period. Analysis of the freezing behavior was performed by an investigator who was blinded to the treatment.

2.3. Immunohistochemistry

Rats were anesthetized by pentobarbital injection (40 mg/kg, intraperitoneally) and perfused with saline and then by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were sectioned at 30-μm thickness. Immunohistochemistry was performed on free-floating coronal sections (Umino et al., 1995). After 24-h incubation in 0.01 M phosphate-buffered saline and normal goat serum, the sections were incubated for 48 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and rabbit anti-phospho-CREB antibody (Upstate Biotechnology, NY, 1:1000 dilution). The sections were incubated for 1 h in 0.01 M phosphate-buffered saline containing 0.2% Triton X-100 and biotinylated goat anti-rabbit IgG (Vector Labs) and then were incubated for 1 h in 0.01 M phosphate-buffered saline and avidin–biotinylated horseradish peroxidase complex (Vector Labs, Vectastain Elite ABC Kit). The reaction product was visualized by transferring the sections to a 50 mM Tris–HCl buffer (pH 7.6) containing 0.05% diaminobenzidine, 0.6% nickel ammonium sulfate and 0.01% H_2O_2.

2.4. Semiquantitative cell counting

According to the atlas of Paxinos and Watson (1997), the section that was located −3.14 mm posterior from the bregma was selected for semiquantitative evaluation of phospho-CREB (pCREB) immunoreactivity with a densitometric video image analysis system (MCID system, Imaging Research, CA, USA), according to the method of Bilang-Bleuel et al. (2002). The unit areas (200×200 μm) of the lateral nucleus, basal nucleus, accessory basal nucleus, central nucleus, medial nucleus, and cortical nucleus of the amygdala (Fig. 1) were digitally recorded by a CCD camera (CCD-IRIS, Sony, Japan) connected to a photomicroscope (B× 50, Olympus, Japan). The number of pCREB positive cells was assessed by automated selection of those cells within the unit areas that satisfied the following criteria: (1) the gray value of the cell nucleus was higher than the threshold value (threshold gray

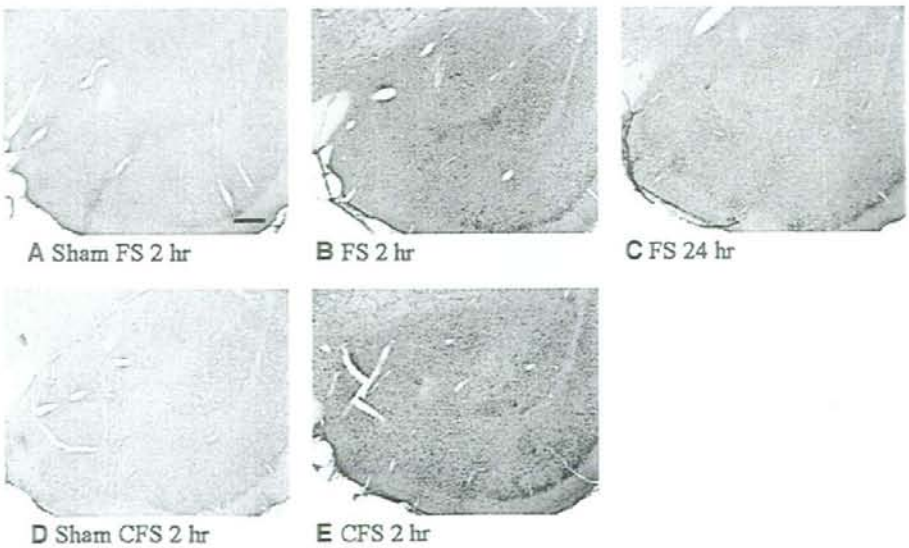

Fig. 2. Photomicrographs of the amygdala showing the expression of footshock and conditioned fear stress-induced phosphorylated CREB-like immunoreactivity. (A) 2 h after sham FS; (B) 2 h after FS; (C) 24 h after FS; (D) 2 h after sham CFS; (E) 2 h after CFS. FS, footshock; CFS, conditioned fear stress. Bar=200 μm.