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学 位 論 文 題 名

Glucocorticoids and lithium reciprocally regulate the proliferation of adult dentate gyrus-derived neural precursor cells through GSK-3β and β-catenin/TCF pathway

(グルココルチコイドとリチウムはGSK-3βとβ-catenin/TCF pathwayを介して成体海馬歯状回由来神経前駆細胞の増殖を相反的に調節する)

学位論文内容の要旨

【Background and Objective】

It has been well established that neurogenesis occurs in adult brain of various animal species, including human. Neurogenesis mainly takes place in two discrete regions of adult brain: subventricular zone of lateral ventricles and subgranular zone of the dentate gyrus (DG) in hippocampas. 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. 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. In human, elevated levels of glucocorticoids is one of causal events in stress-related disorders. In contrast, the administrations of lithium (Li), which is used for treatment of stress-related disorders, increase adult hippocampal neurogenesis. These studies suggest that neurogenesis might be involved in the therapeutic action of Li. Therefore, to elucidate how glucocorticoids and lithium regulate neurogenesis might lead to further understanding of the pathophysiology of stress-related disorders and the development of new therapeutic targets. Li is an inhibitor of GSK-3β, and GSK-3β is widely known as a key regulator of β-catenin/TCF pathway. We here established the culture system of adult rat DG-derived neural precursor cell (ADP) and showed that dexamethasone (DEX), an agonist of glucocorticoid receptor, decreased ADP's proliferation and Li recovered it. In addition, we demonstrated this reciprocal effect between DEX and Li on the proliferation of ADP were regulated by GSK-3β and β-catenin/TCF pathway.

【Materials and Methods】

(Isolation and culture of ADP) Dentate gyrus was dissected from brains of adult male Sprague-Dawley Rats (8 weeks old, 250g) under a microscope. Then the tissues were digested by proteases and DNase I . The fraction containing ADP was isolated by Percoll-gradinent centrifugation and used for monolayer-culture in non-serum medium with bFGF. It takes about 4-5 weeks to get enough amounts of cells for assays.

(Characterization of ADP) Expression of neural precursor cell-specific markers, nestin, GFAP, SOX2 and doublecortin (DCX) was investigated with immunocytocemistry and RT-PCR. Self-renewal was estimated by immunocytochemistry for BrdU. Multipotency was estimated by immunocytochemistry for Tuj1, GFAP and O4 after induction by BDNF, retinoic acid, and IGF, respectively.

(Proliferation Assay) ADPs were seeded on 96-well plates and incubated for 3days after each drug was added. Then, the effects of DEX and Li on the proliferation of ADP were estimated by Alamar

Blue Assay (Invitrogen). Statistical analysis was performed with one-way ANOVA and Dunnet's post hoc test.

(Quantitative RT-PCR) ADPs were seeded on 6-well plates and incubation for 3days after each drug was added. Then, total RNA was purified by RNeasy mini kit (Qiagen) and cell lysate was prepared by Cell Lysis kit (Sigma). Quantitative RT-PCR was performed with Quantitect Reverse Transcription kit (Qiagen) and SYBR GreenER qPCR Suoer Mix for ABI PRISM (Invitrogen). GAPDH was used as a control. Statistical analysis was performed with one-way ANOVA and Dunnet's post hoc test.

(Western Blotting) ADPs were seeded on 6-well plates and incubation for 3days after each drug was added. Then, preparation of total proteins was performed with the Mammalian Cell Lysis Kit (Sigma), and preparation of nuclear proteins was performed with the Nuclear Extract Kit (Active Motif). GAPDH was used as a control. Statistical analysis was performed with one-way ANOVA and Dunnet's post hoc test.

【Results】

(Characterization of ADP) Most cells were flat and round, but slightly elongated shape, and were phase-dark. mRNA expressions of Nestin, GFAP, SOX2 were detected, but that of DCX was not detected in ADP. Most cells co-expressed Nestin, GFAP and SOX2. BrdU was positive in ADP and the ratio of BrdU-positive cells in DAPI-positive cells was 60-70%. All BrdU-positive cells expressed Nestin. ADPs differentiated into Tuj1 (a marker of neuron)-positive cells, GFAP (a marker of astrocyte)-positive cells, 04 (a marker of oligodendrocyte)-positive cells under each condition.

(Effects of DEX and Li on the proliferation of ADP) DEX decreased ADP's proliferation. Li had no effect on ADP's proliferation in the absence of DEX, but recovered ADP's proliferation decreased by DEX.

(Effects of SB415286 and quercetin (Que) on the proliferation of ADP) SB415286, a specific inhibitor of GSK-3β, had no effect on the proliferation of ADP in the absence of DEX, but recovered the proliferation of ADP decreased by DEX. Que, a specific inhibitor of β-catenin/TCF pathway, had no effect on the proliferation of ADP decreased by DEX in the absence of Li, but abolished the recovery effect of Li on it.

(Effects of DEX, Li and Que on nuclear β-catenin) DEX decreased nuclear β-catenin. Li recovered nuclear β-catenin decreased by DEX, and Que abolished the recovery effect of Li on it.

(Effects of DEX, Li and Que on cyclin D1 expression) DEX decreased cyclin D1 expression in both mRNA and protein levels. Li recovered cyclin D1 expression decreased by DEX, and Que abolished the recovery effect of Li on it.

(Effects of DEX, Li on the phosphorylation state of GSK-3β) DEX had no effect on Ser^{9} phosphorylation on GSK-3β, which renders it inactive. DEX significantly increased Tyr^{216} phosphorylation on GSK-3β, which renders it active. Li had no effect on both Ser^{9} and Tyr^{216} phosphorylations on GSK-3β.

【Discussion】

ADPs express nestin, GFAP and SOX2, but not DCX. ADP also have multipotency and limited proliferation potency. Therefore, ADPs may correspond to type-2a cells in four developmental stages of neural precursor cells in adult DG. It's 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 antidepressant and can increase adult neurogenesis in DG. Therefore, it might be beneficial to examine the reactivity of type-2a cells to various drugs and ADPs could be a good model for type-2a cells.

We found that both cyclin D1 expression and nuclear β-catenin are reciprocally regulated by DEX and Li as well as the proliferation of ADP. In addition, DEX activated GSK-3β, a negative regulator of β-catenin/TCF pathway, through the phosphorylation of Tyr^{216}. These results suggest the involvement of GSK-3β and β-catenin/TCF pathway in the reciprocal effects between DEX and Li on the proliferation of ADP. β-catenin/TCF pathway is also well known as canonical Wnt pathway. It has been already shown that canonical Wnt pathway regulates the proliferation of embryo-derived neural precursor cells in vitro and adult hipppocampal neurogenesis in vivo. However, it has been shown that canonical Wnt pathway regulates the proliferation of DCX positive and elongated cells, which may correspond to type-3 cells, and they are in the late differentiation stages of neural

precursor cells. Therefore, our present study is the first report to indicate the involvement of GSK-3β and β-catenin/TCF pathway in the proliferation of hippocampal neural precursor cells in the early differentiation stages.

【Conclusion】

We have succeeded in establishing the culture system of adult rat dentate gyrus-derived neural precursor cells and have shown that DEX and Li reciprocally regulates ADP's 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 further understanding of stress mechanism and the development of new therapeutic targets for psychiatric disorders.

学位論文審査の要旨

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（グルココルチコイドとリチウムはGSK-3βとβ-catenin/TCF pathwayを介して成体海馬歯状回由来神経前駆細胞の増殖を相反的に調節する）

成体海馬歯状回における神経細胞新生の気分障害の病態やその治療薬の作用機序への関与が多くの研究によって示唆されているが、その詳細は不明である。この論文では、向精神薬が成体海馬歯状回の神経前駆細胞に及ぼす直接効果およびそのメカニズムを明らかにすることを目的に、成体ラット海馬歯状回由来神経前駆細胞（ADP）の培養系を確立し、ストレス関連物質であるグルココルチコイドと代表的な気分安定薬であるリチウムがADPの増殖に及ぼす影響について検討を行った。その結果、グルココルチコイドはGSK-3βのTyr216のリン酸化を増加させることでβ-catenin/TCF pathwayを抑制してADPの増殖を抑制すること、リチウムは活性化されたGSK-3βを阻害することでグルココルチコイドによるADPの増殖抑制を回復させることを見い出した。

学位申請者が上記の内容について発表を行った後、吉岡充弘教授から、リチウムが単独でcyclin D1の発現量に影響を及ぼさないのか否か、cyclin D1の発現量をリチウム投与によって増加させることはADPのガン化を促進する心配はないのか、グルココルチコイドによるGSK-3βの活性化のメカニズムについて現在申請者はどう考えているのか、GSK-3βは薬剤標的として適切と考えるか、という質問があった。これらの質問に対し、学位申請者は、リチウムは単独ではcyclin D1の発現量に影響を及ぼさないこと、ADPの培養条件では既に増殖能が最大に達していることや実験で用いた濃度以上の高濃度ではリチウムは毒性を有することからガン化の心配はないこと、GSK-3βのTyr216のリン酸化に関与しているチロシンキナーゼPYK2が海馬に多く局在しておりグルココルチコイドによるGSK-3βの活性化に関与しているのかもしれない、GSK-3βはユニバーサルに発現しておりこれを薬物標的とすることは副作用の危険性が高く適切ではないと考えており、GSK-3βの上流の因子で海馬歯状回に発現が限局しているものがあれば薬物の標的として望ましいと考えている、と回答した。次いで小山司教授から、リチウム以外の他の気分安定薬がADPの増殖に及ぼす影響はどうなのか、申請者の研究はHPA-axisの亢進が関与するストレス関連精神疾患の病態においてどういう位置づけであるか、について質問があった。これらの質問に対し、学位申請者は、リチウム以外の代表的な気分安定薬であるバルプロ酸、カルバマゼピン、ラモト

リジンがADPの増殖に及ぼす影響について既に検討を行っており、これらの中でバルプロさんのみがリチウムと同様にグルココルチコイドによるADPの増殖抑制を回復させることを見いだしたこと、今回の研究はあくまでもin vitroの研究であり、今回の研究で得られた知見が実際にストレスを負荷した動物モデルにおける神経細胞新生や行動変化に関与しているのかどうかを、in vivoの研究によって明らかにしていくことによってストレス関連精神疾患の病態を明らかにできるのではないかと考えている、と回答した。次いで渡辺雅彦教授から、気分障害の病態における神経細胞新生の関与はどの程度であるか、ステロイド治療によって精神疾患が悪化しうるのか、という質問があった。これらの質問に対し、神経細胞新生の気分障害の病態への関与の程度はまだ不明であるが、症状よりは疾患への脆弱性や再発し易さといった長期的な経過に関与しているのではないかと学位申請者は考えており、ステロイド治療を受けている患者にステロイド精神病という精神疾患が発症し、その症状は実際に気分障害に類似している、と回答した。

この論文は、新たな神経前駆細胞の培養系を確立したこと、グルココルチコイドとリチウムが神経前駆細胞の増殖に及ぼす影響およびそのメカニズムについて非常に興味深い知見を見いだしたことで高く評価され、今後、気分障害の病態解明や新たな治療標的の発見を目指して更に研究が発展することが期待される。

審査員一同は、これらの成果を高く評価し、申請者が博士（医学）の学位を受けるのに十分な資格があるものと判定した。