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# ポリグルタミン病の分子病態機序に基づく
# 分子標的治療法の開発

平成22年度　総括研究報告書

研究代表者　辻　省次

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# 厚生労働科学研究補助金(障害者対策総合研究事業)
# 総括研究報告書

## ポリグルタミン病の分子病態機序に基づく分子標的治療法の開発

研究代表者　辻　省次　東京大学医学部附属病院　神経内科教授

研究要旨:本研究では，歯状核赤核淡蒼球ルイ体萎縮症(DRPLA)の治療法開発を目指して，病因遺伝子の産物であるDRPLA protein の生理的機能を解明し，その知見に基づき分子病態機序明らかにし，その上で，有効な治療法の対象となる分子標的を定め，分子標的治療法を実現することを目的としている．DRPLA protein の機能部位が細胞核であり，DRPLA タンパクの核移行阻害が本疾患の治療法の一つになるという考えから，GFP-DRPLA protein の定常発現株を用いたスクリーニング系を構築し，低分子化合物ライブラリーを用いて，局在変化を指標にスクリーニングを実施した．このスクリーニングにより得られた化合物について，DRPLA トランスジェニックマウスを用いて，治療効果の検討を行い，最適な投与量を決定するための予備実験を実施し，体重の増加，寿命の増加が観察される投与量を決定した．

分担研究者

後藤順:東京大学医学部附属病院神経内科講師

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### A　研究目的

歯状核赤核・淡蒼球ルイ体萎縮症(以下DRPLA)は常染色体優性遺伝形式をとる進行性の神経変性疾患で、翻訳領域内にあるCAG繰り返し配列の伸長によって引き起こされるポリグルタミン病の一病型である。DRPLA タンパクは，近年その機能が転写調節に関わっているデータが蓄積しつつあるが、その機能はいまだ十分には解明されていない。われわれは，これまでの研究で，DRPLA タンパクの機能部位が細胞核であること，さらにこれまでの研究で伸長ポリグルタミン鎖を有する変異タンパクが時間依存性に，部位特異的に核内集積することが，病態機序として重要であることを見出している．従って，DRPLA タンパクの核移行阻害が本疾患の治療法の一つになるという考えから、本疾患の治療候補薬剤を探索する目的で、アメリカ食品医薬品局(FDA)により USA で臨床試験にまで到達した化合物のライブラリー1040 ( US-drug Collection. MicroSource Discovery Systems, Inc)を用いて GFP-DRPLA protein の定常発現株に添加し、DRPLA タンパクの局在変化と GFP 蛍光強度を指標として screening を実施した。このスクリーニングにより得られた化合物について，DRPLA モデルマウスを用いて，その治療効果を検討する．また、ETO/MTG8との共発現からDRPLAタンパクが核マトリクス分画により移行することから、その

移行にどのようなタンパクが関与するかについて，検討をし，DRPLA protein の核内凝集を抑制する方法を見出すことも目的とした．

B　研究方法

低分子化合物ライブラリーのスクリーニング：米国で臨床試験まで到達した化合物 1040 種について， GFP-DRPLA protein の定常発現株を用い，1 化合物につき 500uM, 167uM, 56uM, 19uM の 4 段階濃度(1 プレートにつき 23 化合物+DMSO) 計 46 種/week. 1 化合物の反応を 24 時間後、48 時間後に観察した．5 points/well について Hoechst, GFP 画像を取得し，すべての画像について，DRPLAp の局在変化等を確認した．変化が見られた化合物については、共焦点顕微鏡で確認した．

DRPLA モデルマウス（Q129）に対する経口投与実験：DRPLA トランスジェニックマウス Q129 および non-Tg 6 週齢を用いた．候補化合物の中から，中枢神経系への移行が確認されている薬剤を選択して，化合物濃度 0, 3, 10, 30, 60, 120ppm で，餌を調製，各群（n = 6-7）の体重、餌の摂取量および生存期間を記録した。

DRPLA protein の核内局在機構に関する実験：これまでに，DRPLA protein と結合するタンパクを多数見出している．これらのタンパクについて，GFP-DRPLA protein の定常発現株にトランスフェクションにより発現させ，DRPLA protein の核内局在を変化させるかどうかについて検討した．

C　研究結果

低分子化合物ライブラリーのスクリーニング：GFP-DRPLA protein の定常発現株を用いて細胞内局在変化が起こる Drug screening を実施し，FDA の承認した薬剤 1040 種類の screening を完了した．GFP-DRPLA protein が細胞質に存在する薬剤として 22 種見出した．一部同じ骨格を持つ薬剤が 2 群あった。さらに，GFP-DRPLA が核内の局在を変化させる薬剤が 12 種見出した

DRPLA モデルマウス（Q129）に対する経口投与実験：低分子化合物ライブラリーのスクリーニングで同定された，合計 34 種類の化合物について，基本骨格を共有する化合物群に着目した．これらの化合物については，中枢神経系への移行が確認されており，この中の1つについて，DRPLAモデルマウス（Q129）を用いて，治療研究の予備実験を進めた．化合物濃度 0, 3, 10, 30, 60, 120ppm について検討したところ，体重低下，食事摂取量低下に対して，30ppm 投与群で抑制効果がみられた。また，軽度ではあるが生存期間の中間値も 30ppm 投与群が長くなった。

DRPLA protein の核内局在機構に関する実験：これまでに，DRPLA protein と結合するタンパクを多数見出しているタンパクについて，GFP-DRPLA protein の定常発現株にトランスフェクションにより発現させ，DRPLA protein の核内局在を変化させるかどうかについて検討した．その結果，DRPLA protein に結合するタンパクの一つが，DRPLA の核マトリックスへの移行を促進することが見出された．

D　考察

細胞質に局在変化する化合物のうち同じ骨格をもった化合物群が 2 種存在した。このことから，共通する骨格が各移行阻害に重要な役割を果たしていると考えられる． これらの化合

物については，中枢神経系への移行が確認されており，この中の1つについて，DRPLA モデルマウス（Q129）を用いて，治療研究の予備実験を進めた．その結果，体重低下，食事摂取量低下に対して，30ppm 投与群で抑制効果がみられた。また，軽度ではあるが生存期間の中間値も 30ppm 投与群が長くなった。

本年度の研究で，DRPLA モデルマウスを用いた治療実験のシステムを確立することができた．最初に投与した化合物については，体重定価，食事摂取量低下に対して効果が認められ，軽度であるが生存期間の中間値も延長が観察された．この結果は，本化合物の治療効果を示唆するものである．今後，これまでに見出している他の化合物についても体系的に，治療研究を進めていく．

DRPLA の核マトリックスへの移行に関わるタンパクを1つ同定することができた．ノックダウンによってこのタンパクの発現量を低下させた場合に，DRPLA protein の核マトリックスへの移行がどのように変化するかなどを含めて，さらに検討を行う必要がある．

## E　結論

モデルマウスを用いて治療効果を判定する実験系が構築できた．これまでに見出されている候補化合物についてスクリーニングを進める．核内局在機構についてはさらに解析を進める．

## F　健康危険情報

## G　研究発表

1 論文発表

該当なし

2 学会発表

伊達　英俊，辻　省次．DRPLA 治療薬探索を目指した Cell-based high-throughput screening. Neuro2010（第 33 回日本神経科学大会，第 53 回日本神経化学会大会，第 20 回日本神経回路学会大会　合同大会）　2010 年 9 月 2 日（木）～4 日（土）に神戸コンベンションセンター

## H　知的財産権の出願・登録状況

1.特許取得

2.実用新案登録

3.その他

## P2-o12 Distribution of pathogenic androgen receptor aggregations is influenced by heat shock factor-1 in model mouse of spinal and bulbar muscle atrophy (SBMA)

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Objective:Spinal and bulbar muscle atrophy (SBMA) is a hereditary motor neuron disease caused by CAG triplet repeat expansion in the androgen receptor (AR) gene. In animal models of SBMA, heat shock proteins (Hsps) have been shown to play a protective role in the neurodegenerative process. Heat shock factor 1 (Hsf-1) induces the expression of Hsps, such as Hsp70, in response to various kinds of cellular stress. To elucidate the role of Hsf-1 in SBMA, here we investigate the mouse and cell models of this disease.Methods:We performed immunohistochemistry of various neuronal and non-neuronal tissues from wild-type and SBMA mice using anti-Hsf-1 and anti-polyglutamine antibodies. The distribution of Hsf-1 and that of mutant AR were compared. We knocked down the expression of Hsf-1 using small interference RNA (siRNA) in SH-SY5Y cells stably expressing human AR-97Q and analyzed cell viability and frequency of AR aggregation.Results:There was no aggregation of mutant AR in liver, kidney and testis of SBMA mice, where Hsf-1 was expressed at a high level. Conversely, in the tissues with mutant AR accumulation, the expression level of Hsf-1 expression was faint. In the cerebellum Hsf-1 staining was detected in Purkinje cells but not in granule cells. In contrast, the accumulation of mutant AR was detected in cerebellar granule cells but not in Purkinje cells. The WST assay revealed that viability of the cells treated with Hsf-1 siRNA was lower than that of control cells and that the aggregation of pathogenic AR increased in Hsf-1 knocked-down cells compared with control cells.Conclusions:Our observations suggest that Hsf-1 might influence the distribution of pathogenic AR accumulation and cellular viability. The finding that mutant AR accumulation is rarely detected in the tissues which have a high physiological expression of Hsf-1 suggests the role of this transcriptional factor for the determination of the pathological tissue selectivity in SBMA.

## P2-o13 Gene mutations causing autosomal dominant cerebellar ataxia in Japan.

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Autosomal dominant cerebellar ataxia, also called as spinocerebellar ataxia (SCA), is caused by many different mutations. The largest fraction of patients is due to expansion of trinucleotide (CAG) repeat encoding polyglutamine, called as polyglutamine disease. The second type of mutation is non-coding repeat expansion disorders. The final type is static mutation which is conventional mutations, such as missense, frameshift, or deletion mutation, in important genes. To gain insight into molecular pathophysiology of autosomal dominant cerebellar ataxias, it is important to know the clinical phenotypes caused by different mutations. In this study, we analyzed all polyglutamine diseases, non-coding repeat disorders, and SCA14, and 15 in our cohort of index patients with SCA. Sixty-eight families collected between January, 2008 and December, 2009 were analyzed. Polyglutamine diseases and non-coding repeat disorders were tested as described elsewhere. SCA14 was tested for hotspots in the causative gene protein kinase C γ. SCA15 was tested by quantitative PCR to see the gene dosage of causative gene, inositol tri-phosphate receptor 1. Machado-Joseph disease, SCA6 and SCA31 were the three most common disorders in our cohort followed by dentatorubral-pallidoluysian atrophy (DRPLA), SCA1, and SCA2. As far as we studied, we did not see any SCA7, 10, 12, 14, or 15 in our cohort, suggesting that these are very rare in Japanese SCAs. The remaining 30% of patients were not found to have mutations. SCA28, recently identified to cause pure cerebellar syndrome, needs to be tested. We conclude that polyglutamine diseases and SCA31 are the frequent causes of SCAs in Japanese while static mutations are very rare. It should be noted that the causes of a significant portions of SCAs are still unidentified.

## P2-o14 A high-throughput screening assay for drug discovery in SOD1-mediated ALS targeting the transcription of SOD1

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Familial Amyotrophic lateral sclerosis (ALS) accounts for 10% of ALS cases and about 25% of familial ALS are due to mutations in the Cu/Zn superoxide dismutase (SOD1). Mutant SOD1-mediated ALS is caused by a gain of toxic function of the mutant protein. The amount of SOD1 level in non-neuronal neighbors including astrocytes determines the progression of ALS (non-cell-autonomous toxicity). Therefore, we hypothesize that a small compound to decrease SOD1 proteins in astrocytes may slow the progression of mutant SOD1-mediated ALS. We have developed and optimized a cell-based high-throughput screening assay using a library of 9,600 compounds to identify small compounds that reduce the SOD1 expression transcriptionally in astrocytoma cells. We have identified some hit compounds that down-regulate the SOD1 expression specifically with no non-specific cellular toxicity. This assay will provide a powerful strategy to discover novel therapy for familial SOD1-mediated ALS.

## P2-o15 A cell-based high throughput-screen for Dentatorubral-pallidoluysian atrophy (DRPLA)

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Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder caused by unstable expansion of a CAG trinucleotide repeat in exon 5 of the DRPLA gene, which codes for a polyglutamine stretch. We have recently shown that time-dependent and CAG repeat length-dependent neuronal intranuclear accummulation of mutant proteins with expanded polyglutamine stretches plays a pivotal role in the neuronal dysfunctions in DRPLA. DRPLA protein is located in the nucleus and functions as a transcriptional co-regulator. Thus, the nuclear transport and subcellular localization of DRPLA protein are considered to be potential molecular targets for development of efficacious treatment for DRPLA. To accomplish this aim, we have developed a cellular model which allows efficient high throughput screening of small compound libraries. The assay measures the ability of drugs to inhibit nuclear transport of DRPLA protein leading to altered subcellular localization of DRPLA protein. Using this assay, we started screening using a library of 1040 compounds compiled by the NIDS the NIH Custom Collection. Each compound was tested at four concentrations. We have completed the screening of 1040 compounds, and found 4 compounds preventing nuclear translocation of GFP-DRPLA protein and 10 causing altered localization in the nucleus. These compounds are candidates for further investigation.

## P2-o16 Molecular analysis and prenatal prediction of spinal muscular atrophy in Chinese by the combination of RFLP, DHPLC, HRMA ,MLPA and linkage analysis

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Background: Spinal muscular atrophy (SMA) is one of the most common inherited and incurable severe lower motor neuron diseases. The system applied in molecular analysis and prenatal diagnosis of SMA is not perfect yet. Methods: 155 patients and 212 parents from 148 Chinese SMA families were screened for SMN1 deletion using RFLP, DHPLC and high-resolution DNA melting analysis (HRMA). The patients without SMN1 deletion were quantified the SMN1 copy number by real-time fluorescence quantitative PCR and MLPA, then further screened for subtle mutations by direct sequencing. Prenatal prediction was performed by RFLP, DHPLC, HRMA,MLPA and linkage analysis on request for 21 fetuses from 20 families, in which the proband had been detected to have SMN1 deletion. Furthermore, the fetuses without SMN1 deletion were quantified the SMN1 copy number and discriminated SMA carriers by DHPLC, real-time fluorescence quantitative PCR and MLPA. The aborted fetuses and the born babies were reconfirmed, the born babies were follow-up and physically examined twice a year.Results: 141 patients from 134 families were detected to have SMN1 deletion, thus the frequency of SMN1 deletion detected is 90.5% (134/148). 2 subtle mutations were detected by direct sequencing in the other 14 patients. 6 out of 21 fetuses were detected to have SMN1 deletion and aborted. The other 15 fetuses, 10 carriers and 5 normal individuals, were born under suggestion. The aborted fetuses and the born babies were reconfirmed, and the results were completely consistent with those of prenatal prediction. The 15 born babies were follow-up until now and all of them showed normality.Conclusions: The combination of RFLP, DHPLC, HRMA and MLPA makes the molecular and prenatal diagnosis of SMA more perfect and the result more accurate. Also, both DHPLC and MLPA are reliable and rapid methods to discriminate SMA carriers. The diagnosis system established here can be used as a routine diagnosis method in clinics.

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## P2-o17 New strategy to treatment of myasthenia gravis caused by MuSK antibodies using a synchronized experimental animal model

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Myasthenia gravis caused by antibodies (Abs) against muscle-specific kinase (MuSK-MG) is frequently a severe disease requiring emergent and aggressive therapies. Although acetylcholinesterase inhibitors (AChE-I) are often used as symptomatic treatment and are effective for myasthenia gravis caused by antibodies against AChR (AChR-MG), MuSK-MG patients are frequently unresponsive or develop cholinergic crises characterized by increasing muscle weakness, however the pathophysiology of the outcome is remained to understand.

Previously, we generated a new mice model, which synchronizedly develop experimental autoimmune MG (EAMG) at 100% efficiency after immunization of MuSK protein using complement-deficient mice. This model showed that MuSK is required for interacting signaling between pre- and post-synaptic membrane at mature NMJs and interference of them by MuSK-Abs caused MG.

In this study, we demonstrated that this model gave us to assess and develop the appropriate medication for MuSK-MG. AChE-I (neostigmine) treatment to MG-affected mice induced occurrence of abnormal electomyographic (EMG) pattern, as observed in MuSK-MG patients receiving AChE-I and congenital myasthenia with AChE deficiency, demonstrating that abnormal sensitivity to ACh could be reproduced in mice with MuSK-EAMG. In immunofluorescence staining of NMJs, reductions in expression levels of AChE and ColQ, which is associated with AChE and anchored to MuSK, were observed, showing that MuSK is required for AChE clustering at NMJs in vivo, and interference by MuSK-Abs caused abnormal sensitivity to AChE-I treatment. Furthermore, treatment of 3,4-diaminopyridine, which potentiates ACh release from motor nerve terminals, induced more effective reversal of decremental pattern in EMG than that of neostigmine treatment, demonstrating a possibility of new medication to MuSK-MG patients.