様　式　Ｃ－１９、Ｆ－１９、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　２８　年　　５　月　３１　日現在

機関番号：　８２４０１
研究種目：　若手研究(B)
研究期間：　2014～2015
課題番号：　２６８３００５２

研究課題名（和文）自閉症関連遺伝子欠損による神経活動依存的な翻訳異常のプロテオミクス解析

研究課題名（英文）Proteomic analysis of activity-dependent protein translation and defects caused by autistic spectrum disorder (ASD)-related mutations

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交付決定額（研究期間全体）：（直接経費）　　3,000,000円

研究成果の概要（和文）：多くの自閉症(ASD)のモデルマウスが異常なタンパク質合成を示すことが最近、示されてきた。しかし、自閉症のモデルマウスで、どのようなタンパク質の合成が影響を受けているかは十分に理解されていない。本研究では、神経刺激後において、野生型およびASDモデルマウスの脳細胞内で合成される新生タンパク質を調べるための新規なバイアスのない解析手法を用いた研究を行った。その結果、脳細胞の機能に重要なタンパク質が同定され、さらに、ASDモデルの細胞ではより多くのタンパク質が同定された。今後は、ASDに関連した遺伝子変異の組み合わせが神経刺激後のタンパク質合成に与える影響を明らかにしていくつもりである。

研究成果の概要（英文）：Recently, mouse models of many major forms of autism spectrum disorders (ASD) were shown to have abnormal protein synthesis. However, there has been no comprehensive studies to identify the proteins affected in ASD models. I have utilized a novel unbiased method to identify newly synthesized proteins after stimulation of normal and a common ASD model brain cells. Using this method, I have found that the proteins produced in response to stimulation include proteins required for chemical communication between brain cells and proteins mediating structural changes of the connections between brain cells. I have also identified that an increased number of proteins are produced by the ASD model brain cells. Interestingly, many of these were previously unknown. In future studies, I aim to examine how combination of ASD-related mutations affect proteins produced after stimulation as it was shown that two ASD-related mutations surprisingly could counteract and rescue ASD abnormalities in mice.

研究分野：　Molecular neurobiology

キーワード：　proteomics　autism spectrum disorder　translation regulation

１．研究開始当初の背景

The causes of autism spectrum disorder (ASD) remain largely unknown although linkage analysis and genome-wide association studies (GWAS) have identified a number of diseases-related mutations. It is generally believed that ASD arise from abnormal activity-dependent synaptic changes during neurodevelopment, which leads to miswiring of neural circuitry (Penzes *et al.*, 2011). *De novo* protein synthesis is known to be at least partially responsible for some of these activity-dependent synaptic changes.

At the time of application, a series of groundbreaking publications revealed a causal relationship between disrupted activity-dependent translation in neurons and ASD pathogenesis (Auerbach *et al.*, 2011; Bhattacharya *et al.*, 2012; Gkogkas *et al.*, 2013; Santini *et al.*, 2013). A few of those studies further demonstrated that ASD-like phenotypes in mutant mice could be reversed by restoring translational regulation. Interestingly, combining mutations of *Fmr1* and *Tsc2*, two disease-associated genes, surprisingly rescued the mGluR-dependent long-term depression (LTD) and fear conditioning deficits observed in *Fmr1* KO mice. This unexpected finding suggested that altered translational regulation, both in excess or reduced) could lead to defective synaptic homeostasis and ASD pathology.

However, little was known about the identity of the newly synthesized proteins triggered by neuronal activity, and whether these proteins are selectively affected by mutations of different disease-associated genes.

２．研究の目的

As stated above, while it was revealed that disrupted translational regulation and ASD pathology may be linked, the identity of the full spectrum of proteins regulated by changes in neuronal activity remained elusive.

In addition, though *Tsc2* heterozygosity rescued both LTD and fear conditioning defects in *Fmr1* KO mice, the underlying mechanism behind the rescue was unclear. For example, at least two distinct possibilities existed: **(1)** *Tsc2* heterozygosity rescued *Fmr1* KO-associated deficits by balancing the enhanced global protein translation rates observed in *Fmr1* KO neurons. **(2)** Alternatively, the two proteins regulate translation of distinct sets of proteins, such that the changes are offset when both are deficient.

The research proposal therefore aimed to address two fundamental questions:

1. What are the protein synthesized in response to neuronal activity?
2. How do ASD-related mutations like *Fmr1* and *Tsc2* alter the neuronal activity-dependent proteome?

３．研究の方法

In order to address the research questions posed, a novel quantitative mass spectrometric approach was adapted for use in primary mouse neuronal cultures to isolate and identify newly synthesized proteins. This involves the combination of stable isotope labeling with amino acids in cell culture (SILAC) and biorthogonal labeling through the use of a methionine analogue (L-azidohomoalanine [AHA]) which could subsequently be linked to a biotin tag for isolation of the labeled protein, and therefore was termed quantitative noncanonical amino acid tagging (QuaNCAT) (Howden *et al.*, 2013).

In order to identify proteins synthesized in response to neuronal activity, both wild-type neurons at basal state and stimulated wild-type neurons were treated with AHA, and with basal state neurons incubated with medium lysine and arginine while stimulated neurons were treated with heavy lysine and arginine. Click chemistry was performed for the addition of the biotin tag to newly synthesized proteins with AHA incorporation. Such proteins were subsequently isolated by using streptavidin-conjugated magnetic beads, followed by washing, enzymatic digestion, and identification by mass spectrometry. Since newly synthesized proteins from basal state and stimulated neurons were labeled with medium and heavy amino acids, respectively, the relative amount in each sample could be directly compared to identified any differences in protein synthesis in response to neuronal stimulation.

In order to examine the neuronal activity-dependent proteomes in *Fmr1* KO and *Tsc2* heterozygous neurons, a similar approach was taken to label newly synthesized proteins from neurons of different genotypes for direct quantitative comparison.

４．研究成果

A number of neuronal stimulation protocols were initially tested to determine their effects on global protein translation rates through the use of surface sensing of translation (SUnSET), including BDNF, DHPG, and high K^+ treatments. It was determined that BDNF produced the most reproducible induction in global translation in treated neurons. QuaNCAT was subsequently applied to wild-type neurons to quantitatively compare the newly synthesized proteins made by BDNF- and vehicle-treated cells. Comparison of representative data from two independent experiments demonstrated high reproducibility between independent experiments (Figure 1). Proteins with $\log_2$ H/M were upregulated by BDNF treatment (top right) while those with negative $\log_2$ H/M were downregulated (bottom left). As expected, the number of upregulated proteins by BDNF treatment was greater than those downregulated by the neurotrophic factor. Positive control proteins known to be upregulated by BDNF treatment including c-Fos and BDNF receptor NT3 were identified as expected.

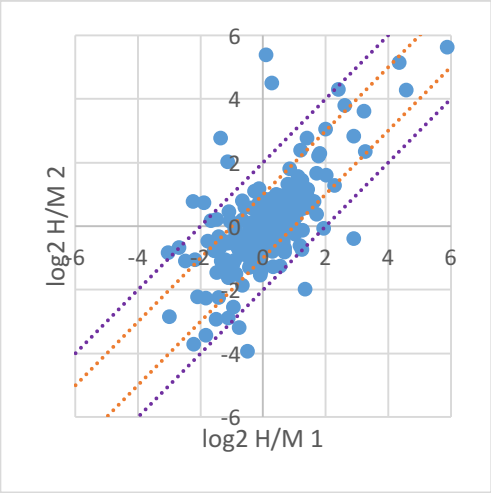

Figure 1. Data from two independent experiments examining the identity of proteins differentially induced by BDNF show high reproducibility and that consistent with expectations, neuronal stimulation by BDNF treatment increases protein synthesis.

Gene ontology (GO) enrichment analysis was performed to examine for enrichment of proteins associated with specific molecular function, cellular component, and biological process. As expected, proteins upregulated by BDNF treatment were enriched for proteins known to be involved in the regulation of actin polymerization and depolymerization (Figure 2).

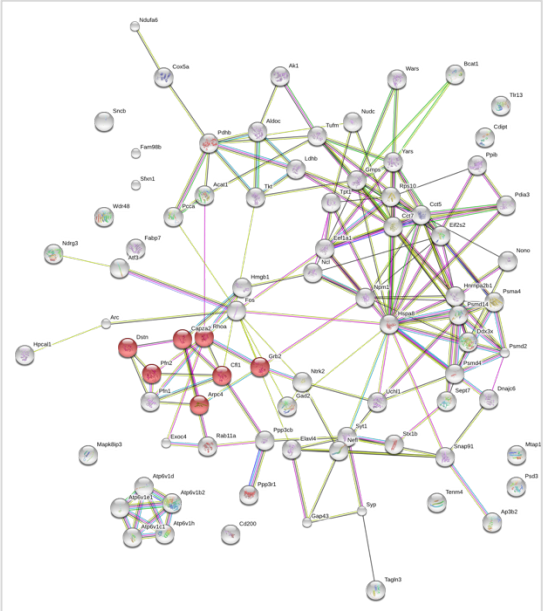

Figure 2. GO enrichment analysis of proteins upregulated by BDNF treatment in wild-type neurons showed an enrichment for proteins involved in regulation of actin polymerization and depolymerization (red nodes), processes which are required for structural synaptic changes.

Next, BDNF treatment was performed on wild-type and *Fmr1* KO with newly synthesized proteins labeled and identified via QuaNCAT. Consistent with previous studies demonstrating that *Fmr1* KO neurons have higher global translation rates, data from independent experiments showed that, relative to wild-type neurons, many proteins were differentially upregulated by BDNF treatment in *Fmr1* KO neurons (Figure 3).

Interestingly, of the differentially upregulated proteins identified in *Fmr1* KO neurons ($\log_2$ KO/WT > 0.5), only ~23% of them are known FMRP targets (Darnell *et al.*, 2011), suggesting that either the previously identified set of FMRP target mRNAs as identified by high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) was incomplete or that secondary effects in *Fmr1* KO neurons lead to changes in the translation of other proteins which are not direct FMRP targets. Further experiments will be required to validate the identity of the differentially regulated proteins in *Fmr1* KO neurons in response to BDNF treatment.

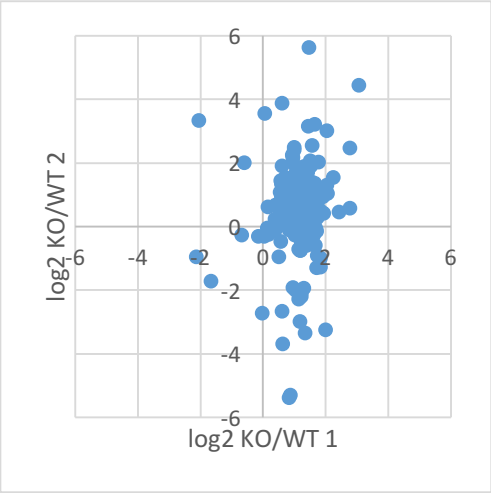

Figure 3. QuaNCAT proteomic data reveals that a number of proteins are differentially upregulated in Fmr1 KO neurons (top right), which is consistently with its known role as a translational suppressor.

As for the effect of *Tsc2* mutation on activity-dependent protein synthesis, shRNA-mediated knockdown successfully reduced Tsc2 protein level in primary mouse neuronal culture. Interestingly, despite its function as a negative mTOR regulator, the knockdown of *Tsc2* led to a reduction in global translational rates under basal conditions when assessed by SUnSET (data not shown). Additional experiments are now ongoing to identify the differentially regulated newly synthesized proteins in *Tsc2* knockdown neurons following BDNF treatment.

In summary, by using a novel unbiased quantitative mass spectrometric approach in primary mouse neuronal culture, activity-dependent newly synthesized proteins were identified in wild-type ASD mutant neurons. Overall, this project is providing a greater molecular insight into how changes in activity-dependent protein synthesis caused by ASD-associated mutations affects neuronal function and synaptic connections which ultimately lead to ASD pathology.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 0 件）

〔学会発表〕（計 0 件）

〔図書〕（計 0 件）

〔産業財産権〕
○出願状況（計 0 件）

名称：
発明者：
権利者：
種類：
番号：
出願年月日：
国内外の別：

○取得状況（計 0 件）

名称：
発明者：
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番号：
取得年月日：
国内外の別：

〔その他〕
ホームページ等

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