A proteomic analysis for profiling NeuroD2 related changes in N2A neuroblastoma cell line

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Abstract

Aim: NeuroD2 transcription factor is a key regulator of neurogenin-NeuroD signaling network and induces neuronal development, differentiation, neurogenesis and calcium dependent signaling. NeuroD2 regulates expression of survival and plasticity related proteins in neurons. Surprisingly, inhibition of NeuroD2 causes an increase in apoptotic cell death. Even though previous studies found out important data about NeuroD2 function, molecular interactions of NeuroD2 behind all of these impacts remains elusive. For this reason, it was aimed to shed light on the proteome profile of NeuroD2 based changes in the N2A cell line.

Materials and Methods: NeuroD2 over-expression and NeuroD2 inhibition groups were constructed via lentiviral vectors. Mouse N2A cell line was transfected with the given vectors and incubated for 6 hours. After incubation samples were prepared for proteomic analyses with Filter Aided Sample Preparation (FASP) protocol and LC-MS/MS analysis was carried out.

Results: Under conditions of overexpression and inhibition, detected proteins were filtered according to significant cut off values. The filtered proteins were further investigated to exhibit a coherent expression in each situation. Eventually, increased NeuroD2 activity was accompanied by an increase in N-alpha-acetyltransferase 25 (NAA25), and Synaptobrevin homolog (YKT6). On the other hand, when NeuroD2 was suppressed, expression of Cytoplasmic Dynein 1 Light Intermediate Chain 1, Kinesin-Like Protein (KIF-11), Leucine-tRNA Ligase (LARS1), and Ubiquitin-Associated Protein 2 (UBA2) were found to be upregulated with a reverse action.

Conclusion: Up-regulations of the proteins Cytoplasmic Dynein 1, KIF11, LARS1, and UBA2 suggested that these proteins might be controlled by inhibition of NeuroD2. In this contex it can be said that, axonal transport, neuronal signaling, and activity of PI3K/AKT pathway can be indirectly regulated by NeuroD2.

Introduction

Transcription factors are known as the main regulators of gene expression. Transcription factors which belong to NeuroD family have basic helix loop helix (bHLH) motif and function on neuronal transcription. Among the 4 members of NeuroD family, NeuroD2 is mostly known by its roles on neurogenesis, neuronal development and determination [1, 2]. Seo et al. showed that NeuroD2 is a key player in Neurogenin-NeuroD gene network [3]. Besides, it has critical regulatory activities on thalamocortical communication in brain [4, 5]. Also, over-expression of NeuroD2 was reported to induce synaptic proteins such as SNAP-25, SYT and Synaptophysin, which are related with synaptogenesis, neurogenesis and differentiation [6].

Recent studies indicate that when NeuroD2 gene expression was inhibited, levels of various neuronal proteins responsible for neuronal survival, including BDNF, decreased and accordingly apoptotic cell death increased. In addition, knock out of NeuroD2 gene causes neuronal structures such as dendrites, axons and synapses to develop abnormally. Premature death of granule neurons in hippocampus and cerebellum induced with NeuroD2 deletion proves its significant role for development of central nervous system [5]. Another work carried out by Guner

et al., showed that NeuroD2 regulates STIM1 receptors, which act as calcium sensors in cortical neurons. This finding revealed how NeuroD2 affects calcium levels, which is a universal secondary messenger molecule [7]. Regulation of calcium-dependent receptors by NeuroD2 explains that this molecule has an important role in signal transduction processes by contributing to calcium metabolism.

According to current knowledge, NeuroD2 does a significant job on neuronal activities [5, 7, 8]. The majority of previous findings focus on how NeuroD2 functions. Studies evaluating NeuroD2 activity from the perspective of molecular interactions remain limited. Therefore, there's a lack of information about molecular connections of NeuroD2 in physiological circumstances. To expand and improve our aspects about NeuroD2's functions, in this study we designed a proteomic-based analysis. Over- expression and inhibition of NeuroD2 were managed via viral vector mediated cloning. Liquid chromatography–mass spectrometry (LC-MS/MS) was performed to reveal the proteomic profile of NeuroD2 dependent changes in N2A cell line. Eventually, we detected 6 proteins changing expression in both circumstances. These proteins could possibly be interacting with NeuroD2 somehow and have a functional connection with it.

Materials and Methods

Cell culture

Mouse Neuro2A cell line was cultured with low- glucose Dulbecco's Modified Eagle Medium (DMEM) (Pan biotech, P04-01515, Germany) and supplemented with 10% Fetal Bovine Serum (FBS) (FBS-11A, Capricorn, USA) at 37°C degrees 5 % CO_2.

Gene cloning experiments

Total RNA was isolated from cultured N2A cells with Pure-Link RNA mini kit (12183018A, Thermo Fischer, USA) and cDNA synthesis was carried out through qScript cDNA Synthesis Kit (95047-100, Quanta, USA). The primers were designed based on coding sequence of NeuroD2 (NCBI ref. seq. NM_010895.3) (Forward primer: 5' AGTCAGAATTCATGCTGAC-CCGCCTGTTC 3' Reverse primer: 5'AGTCAGTC-GACTCAGTTATGGAAAAATGCGTTGAG 3'). NeuroD2 coding sequence was amplified and restricted with fast digest enzymes EcoRI (FD0275, Thermo Fisher, USA) and SalI (FD0644, Thermo Fisher, USA) together with the lentiviral vector (pLenti-CMV-GFP-2A-Puro, Applied Biological Materials). After that, gene of interest was ligated with the cloning vector by T4 DNA ligase (EL0014, Thermo Fisher, USA) and re-digested for confirmation of insert.

NeuroD2 knock down plasmids Smartvector Lentiviral Mouse hEF1α -TurboRFP (Target sequences: 5'GC-CTGCAGCTAAACTCTCGTA3' and 5'GCCTGCAGC-TAAACTCTCGTA3') and SMARTvector non-targeting mCMV-TurboRFP were purchased by Dharmacon (VSC6571, Dharmacon, UK).

Five of six well culture plates were seeded with 4 x 10^6 N2A cells. The next day transfection was carried out with lipofectamine 3000 agent (L3000008, Thermo Fischer, USA).

Two and half µg DNA, 4 µl Lipofectamine reagent and 4 µl P3000 reagent mixed in 250 µl Opti-mem (2259423, Thermo Fischer, USA) and incubated for 15 minutes according to manufactures' protocol. After transfection, cell culture plates were incubated at 37°C and 5% CO_2 for 6 hours. Following incubation, cell culture media were changed with low-Glucose DMEM [9].

Sample preparation for Liquid chromatography–mass spectrometry (LC-MS/MS)

For proteomic analyses proteins were needed to be broken down into peptides. For this aim, FASP (Filter Aided Sample Prepation) kit was used (44250, Expedon, Spain). According to the manufacturer's instructions unwanted chemicals and urine were removed from protein samples. After that, 50 µg protein samples was treated with 50:1 or 100:1 trypsin enzyme in 50 mM ammonium bicarbonate medium and incubated for 16 hours at 37°C. Next day, samples were centrifuged at 14,000 g for 10 minutes and the peptides obtained were lyophilized using a lyophilizer device. When needed the lyophilized peptides dissolved in the desired amount of 0.1% formic acid and were prepared with a final concentration of 100 ng/µL.

Liquid chromatography–mass spectrometry (LC-MS/MS) analysis

Prepared peptides were loaded onto the ACQUITY UPLC M-Class coupled to a SYNAPT G2-Si high-definition mass spectrometer (Waters, USA). The columns were set with 97% mobile phase A (H_2O for suitable for mass spectrometry containing 0.1% Formic Acid) and the temperature was 55°C. Peptides separated from the trap column (Symmetry C18, 5 µm, 180 µm i.d. × 20 mm, Waters, USA) to the analytical column by 90 minutes graduated separation from the trap column (CSH C18, 1.7 µm, 75 µm i.d. × 250 mm, Waters, USA). This separation process was carried out with acetonitrile containing 0.1% formic acid (v/v) a gradient from 4% to 40%, and a flow rate of 0,400 µl per minute. At the positive ion mode of the MS and MS/MS, scans were made in 0.7 second cycles and set to ten volts low collision energy and 30V high collision energy. The ions were separated by ion mobility separation (IMS). The wave velocity increased from 1000 to 55 m/s during the full IMS cycle. The release time for mobility was set to 500 µs, and the trap height was set to 15 V. The IMS wave delay was 1000 µs for motility discrimination after trap release. Without precursor ion preselection, all ions in the 50-1900 m/z range were fragmented in resolution mode. Progenesis QIP software was used to identify and quantify the peptides, all proteins were evaluated at least 3 unique peptide sequence and expressions ratio was calculated [9-12]. Identified protein expression were given by heat map analysis.

Statistical analysis

LC-MS/MS data were quantified in Progenesis-QI for proteomics software (Milford, Massachusetts, Waters). Then independent samples t-test was used for evaluations as the data follow a normal distribution. 1.4-fold change was considered significant between overexpression-control and

inhibition-control groups. PASW 18 (SPSS) statistical package was used for statistical analysis. Throughout the study, p-values < 0.05 were considered significant.
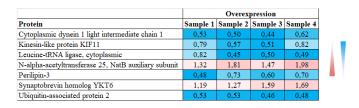
Results

For identification of NeuroD2 regulated proteins, cell culture samples were analyzed using the LC-MS/MS method and the results showed that Cytoplasmic dynein 1 light intermediate chain, Kinesin-like protein KIF11, Leucine-tRNA ligase, N-alpha-acetyltransferase 25, Perilipin-3, Synaptobrevin homolog YKT6 and Ubiquitin-associated protein 2 were expressed differentially in overexpression and inhibition groups with respect to controls (Table 1).

The proteins listed in Table 1 was the shared proteins in both conditions (overexpression and inhibition) that showed different pattern of expression in association with the activity of NeuroD2. The extent of increase and decrease in expression level against control groups was given as a heat map. Figure1 indicates the changes related with upregulation of NeuroD2 while Figure 2 indicates the changes related with downregulation of NeuroD2. The numbers represent the fold changes. Blue highlights demonstrate an increase whereas pink ones demonstrate a decrease in amount. All the analysis was held on 4 samples as 4 experimental repeats. (The raw data was shared as a Supplementary Materials). There was a consistent pattern of expression for all proteins but Perilipin-3 under

Table 1. List of differentially expressed proteins between overexpression-control and inhibition-control groups via liquid chromatography mass spectroscopy (LC-MS/MS). A total of 7 different proteins were significantly regulated ($p < 0.05$ and >1.4-fold change) in both groups when compared to their controls depending on the expression of NeuroD2.

Accession number	Protein
Q8R1Q8	Cytoplasmic dynein 1 light intermediate chain 1
Q6P9P6	Kinesin-like protein
Q8BMJ2	Leucine-tRNA ligase, cytoplasmic
Q8BWZ3	N-alpha-acetyltransferase 25, NatB auxiliary subunit
Q9DBG5	Perilipin-3
Q9CQW1	Synaptobrevin homolog YKT6
Q91VX2	Ubiquitin-associated protein 2

	Overexpression			
Protein	Sample 1	Sample 2	Sample 3	Sample 4
Cytoplasmic dynein 1 light intermediate chain 1	0,53	0,50	0,44	0,62
Kinesin-like protein KIF11	0,79	0,57	0,51	0,82
Leucine-tRNA ligase, cytoplasmic	0,82	0,45	0,50	0,49
N-alpha-acetyltransferase 25, NatB auxiliary subunit	1,32	1,81	1,47	1,98
Perilipin-3	0,48	0,73	0,60	0,70
Synaptobrevin homolog YKT6	1,19	1,27	1,59	1,69
Ubiquitin-associated protein 2	0,53	0,53	0,46	0,48

Figure 1. Fold changes of proteins in overexpression group. The sample size was 4 and the data was given as a relative value in comparison with the control group. Pink colored cells show upregulations while blue ones show downregulations.

	Inhibition			
Protein	Sample 1	Sample 2	Sample 3	Sample 4
Cytoplasmic dynein 1 light intermediate chain 1	2,65	2,02	1,76	2,40
Kinesin-like protein KIF11	1,70	1,81	1,92	1,59
Leucine-tRNA ligase, cytoplasmic	1,69	1,97	1,97	1,84
N-alpha-acetyltransferase 25, NatB auxiliary subunit	0,46	0,55	0,74	0,76
Perilipin-3	0,62	0,48	0,76	0,61
Synaptobrevin homolog YKT6	0,57	0,51	0,80	0,69
Ubiquitin-associated protein 2	1,69	1,36	1,28	1,60

Figure 2. Fold changes of proteins in inhibition group. The sample size was 4 and the data was given as a relative value in comparison with the control group. Pink colored cells show upregulations while blue ones show downregulations.

overexpression and inhibition conditions. Perilipin-3 was found to reduce in each case. The increase in N-alpha-acetyltransferase 25, NatB auxiliary subunit and Synaptobrevin homolog YKT6 were prominent in accordance with increased NeuroD2 activity. On the other hand, Cytoplasmic dynein 1 light intermediate chain 1, Kinesin-like protein, Leucine-tRNA ligases, cytoplasmic and Ubiquitin-associated protein 2 were observed to be abundantly expressed when NeuroD2 was inhibited.

The raw data is available as supplementary file.

Discussion

The accumulating evidence claim that NeuroD2 is important for neuronal development, differentiation, neuroplasticity and calcium mediated signaling [5, 7, 8]. Studies in literature mostly focus on the function of NeuroD2 however; the approach presented here was rather investigating the interactive action of NeuroD2. By this means, the proteomic analysis of NeuroD2 shed some light on the unknown molecular interactions of NeuroD2.

Considering the data of Lc-MS/MS based proteomic analysis we examined protein profile changes in NeuroD2 overexpression and inhibition groups. The findings pointed out some key proteins listed in Table 1 as showing a common alteration in both groups. Expressions of Cytoplasmic dynein 1 light intermediate chain 1, Kinesin-like protein KIF11, Leucine-tRNA ligase, N-alpha- acetyltransferase 25, Perilipin 3, Synaptobrevin homolog YKT6 and Ubiquitin-associated protein 2 were regulated in association with NeuroD2 in either over expression and inhibition conditions.

Our data demonstrated that, N-alpha- acetyltransferase 25 and Synaptobrevin homolog YKT6 were modulated in the same mode of expression with NeuroD2. Especially in overexpression group, the increase was remarkable. This parallelism can be explained with synergistic activities of them.

Function of N-alpha acetyltransferase 25 (NAA25) is a mystery for researchers. Bozpolat et al. showed that a critical mutation in NAA25 gene might cause a risk factor for ischemia in adults [13]. It was also reported that NAA25 could balance the cell survival and apoptosis in cell [14]. In this manner NAA25 can function as a neuroprotector substance under the control of NeuroD2. Synaptobrevin homolog YKT6 protein is a part of SNAP-Receptor Family (SNARE) which are short proteins kept bound on

the surface of the cellular vesicles and plasma membrane. SNARE proteins have functions on the regulation of membrane components and hence neuronal secretion [15]. In motor and sensory neurons, YKT6 protein expression is related with axonal transportation, neuronal stimulation and survival [16]. In this perspective, in order to coordinate the vesicular transport neurons can adjust the secretory pathways. This regulation can accelerate storage of neurotransmitters in the axon terminals and reorganize the neuronal communication. Although the other pieces of the puzzle are not clear yet, the mechanism of how NeuroD2 transcription factor activates expression of these proteins can be an important fact for its multifaceted function.

On the other hand, proteins Cytoplasmic dynein 1 light intermediate chain 1, Kinesin like protein KIF-11, Leucine t-RNA ligase and Ubiquitin- associated were negatively arranged by NeuroD2 expression. Upregulation of the given proteins were prominent particularly in the inhibition group. Cytoplasmic dynein 1 light intermediate chain 1 is a unique subunit for cytoplasmic form of dynein which is a common protein for cells having functions in many cellular processes including neuronal migration, signaling and axonal transport [17]. Kinesin like proteins are members of the kinesin superfamily, another motor protein responsible for the transport of various cargo molecules. Similar to dynein counterpart, it takes place in several crucial cellular mechanisms. Studies showed that inhibition of KIF-11 protein causes sympathetic neurons to become thinner [18]. Reduction in the amount of these motor proteins in overexpression group highlights the importance of NeuroD2 in axonal transport and signaling. This may be part of neuroplasticity related reorganization and buildup of neuronal networks according to the needs.

Leucine t-RNA ligase (LARS1) is also known as leucine tRNA synthetase which catalyzes the transesterification reaction to attach the leucine amino acid on corresponding tRNA molecule. A recent study revealed a notable role of LARS1 for regulation of Mammalian Target of Rapamycin Complex 1 (mTORC1) dependent cell growth, protein synthesis and autophagy. LARS1 may act as a leucine sensor for mTORC1 for gaining it a GTPase activating function [19]. In the light of this piece of information, increase in the expression of LARS1 when NeuroD2 was inhibited can show a complicated mechanisms behind.

Also, Ubiquitin associated protein 2 (UBA2) is a member of common ubiquitin-proteasome pathway which is involved in apoptosis, cell cycle control, cell adhesion and protein degradation [20]. Proteomic analysis indicated a negative interaction between UBA2 protein and NeuroD2. It is clear that, activity of transcription factor NeuroD2 can be controlled by ubiquitin modifications.

NeuroD2 related changes on the given proteins were more dramatic when NeuroD2 was inhibited. Loss of NeuroD2 function possibly triggers several complex outputs by positive and negative regulations of the molecular networks. The exact roles of NeuroD2 beyond its neuroprotective activities remain elusive. These findings will be of great interest to future studies to reveal the cellular connections and complete the puzzle.

Conclusion

In conclusion, up-regulations of Cytoplasmic Dynein 1, KIF11, LARS1 and UBA2 proteins indicated that these proteins could be regulated with NeuroD2 inhibition. Expression level of NeuroD2 can induce PI3K/AKT pathway activation indirectly and can contribute to axonal transport and signaling. Increasing expression of YKT6 and NAA25 proteins showed that these proteins are positively regulated by NeuroD2. However, to enlighten the interaction between NeuroD2, YKT6 and NAA25 proteins as well as the functions of them further investigations are needed.

Ethics approval

Since we did not include any data on any living organism in the draft, it is a study that does not require ethical approval.

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