Research paper

Neuregulin1 types mRNA level changes in autism spectrum disorder, and is associated with deficit in executive functions

Samane Abbasy, MSc a,b, Fazlollah Shahraki, MSc c, Arvin Haghighatfard, PhD d,e,⁎, Masoume Ghasemzadeh Qazvini, MSc a, Sahel Towfigh Rafiei, MSc f, Elnaz Noshadirad, PhD f, Mahdi Farhadi, PhD f, Horie Rezvani asl, MSc g, Atena alsadat Shiryazdi, MSc f, Rana Ghamari, BSc e, Zeinab Tabrizi, MSc h, Rashed Mehrfard, MSc l, Fereshteh Esmaeli kakhroudi, BSc e, Mahsima Azarnoosh, BSc e, Faeghe Younesi, BSc e, Narges Parsamehr, BSc e, Nooriyeh Garaei, BSc e, Sorosh Abyari, BSc e, Maede Salehi, BSc e, Maryam Gholami, BSc e, Pardis Zolfaghari, BSc e, Seyede Mahsa Bagheri, BSc e, Melika Pourmehrab, BSc e, Elham Rastegarimogaddam, MSc l, Elnaz Nobakht, MSc l, Elmira Nobakht, MSc j, Rayan Partovi, PhD f

a Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran
b Sarem Cell Research Center, Sarem Women’s Hospital, Tehran, Iran
c Department of Mind-Brain-Education, Institute for Cognitive Science Studies, Tehran, Iran
d Department of Biology, North Tehran Branch, Islamic Azad University, Tehran, Iran
e Department of Genetic, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran
f Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
g Emam Reza Hospital, Social Security Organization, Qom, Iran
h Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
i Department of Microbiology, Damghan Branch, Islamic Azad University, Damghan, Iran
j Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran

ABSTRACT

Background: Autism spectrum disorder (ASD) is a pediatric heterogeneous psychiatric and neurodevelopmental disorder with social and communication deficits, language impairment and ritualistic or repetitive behaviors. ASD has significant genetic bases but candidate genes and molecular mechanisms of disorder are not clarified. Neuregulin1 (NRG1) gene, located in 8p12 is involved in development of central nervous system and was indicated as candidate gene in schizophrenia.

Methods: mRNA level of types I, II and III of NRG1 gene were studied in peripheral blood of 1540 ASD patients (IQ > 70) and 1490 control children by quantitative Real Time PCR. Also three domains of executive functions (working memory, response inhibition and vigilance) were examined in all subjects.

Findings: All three types were significantly down regulated in ASD patients. Significant deficiencies in executive functions (EF) were found in ASD patients. EF deficiencies mostly were associated with down expression of mRNA level of types I and III. Also correlations were found between NRG1 expression with gender and severity of ASD symptoms.

Interpretations: Findings primarily have been suggested involvement of NRG1 in etiology of ASD. Also correlation of NRG1 mRNA level with EF deficiencies could shed lights on EF mechanisms and may suggest targeted treatments to improve particular executive functions.

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1. Introduction

Autism spectrum disorder (ASD) is a complex psychiatric disorder which characterized by social and communication deficits, language
Research in context

Evidence before this study

Autism spectrum disorder is heterogeneous developmental disorder with strong and complicated genetic bases. Several shared genes have been found between schizophrenia and Autism. Based on Pub-Med, Science Direct and Scopus data bases genetic variants and gene expression alterations of Neureguline1 (NRG1) had been reported in schizophrenic patients. Several studies also reported single nucleotide polymorphisms in NRG1 associated with autism. We started working on Neureguline1 (NRG1) in psychiatric disorder in 2013 by a case control study in gene expression of NRG1 types in schizophrenic patients and possible correlation with clinical symptoms. Then we continued the same approach in bipolar disorder, attention deficit hyperactivity disorder and Autism until now.

Added value of this study

Findings of present study help to clarify the role of NRG1 in etiology of Autism and also its role in executive functions not just in autistic children also in normal children as well. In the other hand our study brings new evidence about disruption in excitation/inhibition (E/I) balance in autism. In addition sex effect and correlation of each types of NRG1 with severity of symptoms in patients showed more detail about pathophysiology of autism.

Implications of all the available evidence

Present study may help to understand about the role of epidermal growth factors such as NRG1 in etiology of autism. Also study of each types of NRG1 could clarify the role of each molecular pathway in neurons and neuroglia in severity of autism symptoms and executive functions deficits. Future studies may consider the expression of NRG1 expression in neuron made by induced pluripotent stem cells from children with autism. Also future study may consider drug therapy to increase the expression of NRG1 that based on our findings may reduce the severity of autism symptoms and also improve the executive functions ability in children.

2. Material and methods

2.1. Subject selection

The study was included 1540 unrelated ASD patients (1020 male, 520 female) aged 7 to 11 years diagnosed by two independent senior psychiatrists by unstructured or semi-structured behavioral observations in patients and interviews with patients, their parents and teachers based on DSM-V criteria and have been confirmed by exceeding threshold of Autism Diagnostic Observation Schedule (ADOS) score [2]. Higher score in ADOS is referring to high severity symptoms in ASD patients. Control group was included 1490 unrelated control children (970 male, 520 female) aged 7 to 11 years. Patients with IQ score (Wechsler Abbreviated Intelligence Scale, 1999) lower than 70 were excluded from study. All of the ASD samples were early diagnosed and did not start treatment when participated in study. Patients have been recruited from the outpatient psychiatric clinics of 18 provinces (from 31 provinces) of Iran. The subjects of control group were recruited from children who received regular medical checkups for school enrolment in local medical centers. The control children were recruited with matched sex, age, race, socioeconomic situation and familial situation to patients group and had no history of any psychological or somatic problem.
Supplementary Table 1 showed matching statistical data. Statistical data of 24 patients with combined ASD and ADHD were excluded from study and finally 1540 patients included to the study. None of subjects or their parents had current or history of severe medical condition, neurological disorder, history of head trauma with loss of consciousness, any psycho-stimulant or opioid drug abuse and alcohol or nicotine dependence. All subjects’ parents were given an explanation on the purpose of the study and next, written informed consent has been provided. Study was approved by central ethical committee of Islamic Azad University.

### 2.1.2. Neuropsychological tests

Four neuropsychological tests (as listed below) were performed to analyze response inhibition, vigilance and working memory as main executive functions (EF) which their deficiency had reported in ASD patients.

#### 2.1.2.1. Integrated visual and auditory (IVA) continuous performance test (CPT).

The integrated visual and auditory (IVA) continuous performance test (CPT) was designed as screening and symptoms severity test of ADHD, but also using for several neurodevelopmental psychiatric disorders including ASD. The IVA associates vigilance and impulsivity in a balanced design by visual and auditory modalities. The visual response control quotient (VRQC) and auditory response control quotient (ARQC) are the primary dependent variables [20,21].

#### 2.1.2.2. IVA visual attention quotient (VAQ) and Auditory attention quotient (AAQ).

We used VAQ and AAQ as primary dependent variables in this measure to examine the vigilance. Tests were performed based on previous studies method [21,22]. The VRQC, ARQC, VAQ and AAQ are all part of IVA-CPT test.

#### 2.1.2.3. Spatial span (SSP) of CANTABexpedio.

Spatial Working Memory (SWM) measures the ability to maintain spatial information and to subsequently manipulate the presented items in working memory. This test uses boxes and colors and is suitable for children. Method of performing test had followed based on previous study [23].

### 2.1.4. Blood sampling and quantitative real time PCR

Blood (5 ml) was collected from the cubital vein without tourniquet between 10.00 and 11.00 AM. Total RNA was extracted from peripheral blood samples immediately after samplings according to standard protocols using by RNA Purification kit (GeneJet™ RNA Purification Kit#K0732, Thermo scientific - Fermentas, Latvia). Total RNA was treated with DNase for the removal of contaminating genomic DNA using DNase Treatment & Removal Reagents (DNase I, RNase-free (#EN0521) Fermentas, Latvia), according to the manufacturers protocol. Quality of RNA was evaluated by agarose gel electrophoresis and UV-spectroscopy. The cDNA was synthesized using a Transcription First Strand cDNA Synthesis Kit (RevertAid Premium First Strand cDNA Synthesis Kit #K1652, Thermo scientific - Fermentas, Latvia) according to manufacturer’s protocol. After alignment the sequence of all three types of NRG1 gene and finding types' specific sequences, primers designed by “oligo7” software and were blasted on NCBI website. The phosphoglycerate kinase 1 (PGK1) gene was used for normalization as an endogenous reference gene. Primers were provided in Table 1. Agarose gel electrophoresis was used to verify the size of PCR amplification of genes. Standard curves for each gene were prepared using serial dilutions (1: 4) of pooled cDNA from total RNA extracted from blood samples of 10 control subjects. In each experiment, the R² value of the standard curve was >0.99 and no-template control assays was resulted in no detectable signal. Quantitative RT-PCR was performed by using SYBR green (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2x) #K0221, Thermo scientific - Fermentas, Latvia). CFX96 Touch Real-Time PCR Detection System (BIO-RAD, California, United States) used for triplicate method Quantitative Real-Time PCR. Pfafie formula used to calculate the ratio. Quantitative Real time PCR and analysis of expression data were conducted based on previous study [23].

### 3. Results

Demographic and clinical data have been presented in Table 2.

#### 3.1.1. Gene expression results

Significant down regulation of all three types of NRG1 in patient’s group in compare with control subjects were detected. No significant correlation was found between gene expression results and age, age of onset, duration of illness and intelligence quality score. Exact p values and ratio for each type have been presented in Table 3.

### Table 1. Sequences of primers used for quantitative Real time PCR.

| Genes primer | primer sequence |
|--------------|-----------------|
| PKI (forward primer) | 5' TGCTGTAATTAGACACCCAAC3' |
| PKI (reverse primer) | 5' TGGACAAATGAGATCCAGAA3' |
| NRG1 type I forward primer | 5' AAGCTTACTGAGAGGAGAT3' |
| NRG1 type I reverse primer | 5' ACTCTCTTATTATTACAAAG3' |
| NRG1 type II forward primer | 5' CCTTCTCTTGAGAAGG3' |
| NRG1 type II reverse primer | 5' GGCGATTCAAAGAAGG3' |
| NRG1 type III forward primer | 5' GCCATGCTCCAGAGAAAT3' |
| NRG1 type III reverse primer | 5' AGCAGTACTGAGAGGAGAT3' |

### Table 2. Demographic and clinical variables (Means and Standard Deviations).

| VARIABLE | ASD | Normal |
|----------|-----|--------|
| Gender | 1020 male,520 female | 970 male, 520 female |
| Age | 9.2 ± 1.6 | 9.4 ± 2.3 |
| Age of onset | 3.4 ± 1.2 | – |
| Illness time | 5.3 ± 3.4 | – |
| IQ score | 91.32 ± 23.42 | 102.4 ± 14.48 |
| VRQC | 57.21 ± 37.66 | 96.22 ± 17.28 |
| ARQC | 66.29 ± 14.62 | 97.46 ± 24.36 |
| AAQ | 66.51 ± 28.18 | 97.83 ± 20.49 |
| SSP | 3.32 ± 1.34 | 5.74 ± 0.89 |
| SWM Btwn Errors | 66.32 ± 16.11 | 42.39 ± 26.48 |
| SWM Strategy | 40.21 ± 1.15 | 31.38 ± 3.56 |

VRQC = Visual Response Control QuoInt, ARQC = Auditory Response Control QuoInt, AAQ = Visual Attention QuoInt, AAQ = Auditory Attention QuoInt, SSP = Spatial Span, SWM = Spatial Working Memory.
Tables 4 and 5. Summary of all correlations have been provided in

3.1.4. Severity of symptoms in ASD and gene expression

3.1.5. Sex effect analysis in ASD group

4. Discussion

NRG1 has been implicated as a susceptibility gene in schizophrenia and there are evidences that the pattern of expression of NRG1 isoforms may alter in schizophrenia. Present study is one of the first studies of NRG1 role in ASD patients and suggests NRG1 as novel potential candidate gene in etiology of ASD. Unlike the previous post-mortem genome wide expression profiling study that showed slight down expression of NRG1 in cortex tissue, strong down regulation of NRG1 detected in present cohort study [24]. Neuropsychological results and correlations suggest potential NRG1 role in executive functions specially response inhibition, vigilance and working memory which their deficits previously were reported in ASD patients. However these finding probably has been improved knowledge about NRG1 role in neuropsychiatric disorder.

NRG1 type I has a major role in neuronal plasticity which shows activity-dependent regulation, and involvement in neurotransmitter receptor expression regulations. Number of deregulations in neuronal activity such as seizures and long-term potentiation, affect type I expression. NRG1 promotes the switch from the immature form of NMDAR, which contains primarily NR2B subunits to one containing more NR2C subunits in the central nervous system. NRG1 also potentiates α7 nicotinic acetylcholine receptor transmission in hippocampal neurons, and expression of the β2 subunit of the γ-amino butyric acid receptor in cerebellar granule cells. Thus, significant decrease in type I expression in ASD patients may alter neuronal signaling of NRG1 perse, or induces abnormalities in maturation of NMDA receptors and GABA receptor signaling or may be both of them eventually in brain of ASD patients [25].

NRG1 type II (GGF) expression is essential for development of cortical neurons, and promotes the transformation and differentiation of radial glial cells. Glial cells in turn are involved in cortical neuronal cell migration and differentiation. It may make it reasonable that down regulation of type II mRNA level could lead to putative abnormalities of oligodendroglial functions including those functions which is related to communication and abilities in ASD patients [25]. Also correlation between type II and ADOS score may relate to the role of low functioning

### Table 3

| Gene          | ASD vs. Normal |
|---------------|----------------|
| NRG1 type I   | Ratio: 0.63    |
| p value: 0.001|
| NRG1 type II  | Ratio: 0.65    |
| p value: 0.002|
| NRG1 type III | Ratio: 0.47    |
| p value: 0.001|

#### 3.1.2. Neuropsychological results and correlations with gene expression

3.1.2.1. 1) IVA tests results. Significant deficiency and lower performance in ASD group in compare with control group were showed in both VRCQ (P value = 0.0002) and ARQC (P value = 0.001). Also significant lower performances were detected in ASD group in compare with control group in VAQ (P value = 0.001) and AAQ (P value = 0.0001). In ASD group, correlation analysis between NRG1 types expression with IVA tests results have showed significant correlation between down expression of type I and III with lower performance of all IVA tests (VRCQ, ARQC, VAQ and AAQ). Also down expression in type II were significantly correlated with lower performance of all IVA tests. Results presented in Tables 4 and 5.

3.1.2.2. Working memory tests results

Difference of SSP (P value = 0.002), SWM Btw Errors (P value = 0.001) and SWM strategy (P value = 0.001) were significant between ASD and control groups which indicate significant deficiency in working memory of ASD in compare with controls. Significant correlation was found between types of NRG1 expression and working memory deficiency. In ASD group, significant correlations were detected between down expression of type I and III with increase of SWM Btw Errors and SWM strategy score. Also significant correlations were detected between down expression of type I, type II and type III with decrease of SSP score. Correlations between NRG1 expression level and working memory tests in all subjects as combined group showed significant correlation between down expression of type III with increase of SWM strategy score. Summary of all correlations have been provided in Tables 4 and 5.

3.1.4. Severity of symptoms in ASD and gene expression

Diagnosis of ASD patients, have been confirmed by ADOS tests. Also severity of symptoms and their relation with gene expression and executive functions patterns were assessed. As the patients were high functioning, age compatible module three has been used for all ASD patients. Findings revealed significant correlation between down expression of type II and higher score of ADOS (r: −0.66, P value = 0.003). In addition significant correlation was found between lower score of VRCQ and higher score of ADOS (r: −0.72, P value = 0.001).

#### 3.1.5. Sex effect analysis in ASD group

Effects of gender in severity of symptoms, executive functions and NRG1 expression had been assessed with in ASD group. Findings showed significant down expression of type III in boys in compare with girls (P value = 0.002). In the other hand significant increase of SWM strategy score (P value = 0.002) were found in males in compare with females. Also type III down expression was significantly correlated with increase of SWM strategy score in overall ASD group (P value = 0.001, r = −0.77) and in ASD girls (P value = 0.003, r = −0.62) and ASD boys (P value = 0.003, r = −0.71) as independent groups.

### Table 4

| Gene expression | Gender | Age | Age of onset | illness time | IQ score | VRCQ | ARQC | VAQ | AAQ | SSP | SWM Btw Errors | SWM Strategy |
|-----------------|--------|-----|--------------|--------------|---------|------|------|-----|-----|-----|----------------|--------------|
| NRG1 type I     | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: |
| 0.16            | 0.06   | 0.25 | 0.13         | 0.19         | 0.001   | 0.001 | 0.001 | 0.002| 0.001| 0.007|
| NRG1 type II    | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: |
| 0.08            | 0.17   | 0.12  | 0.15         | 0.15         | 0.001   | 0.016 | 0.016 | 0.003| 0.003| 0.34|
| NRG1 type III   | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: | p value: |
| 0.11            | 0.08   | 0.11  | 0.14         | 0.18         | 0.094   | 0.88  | 0.81  | 0.84 | 0.89 | 0.77 |

VRCQ = Visual Response Control Quotient, ARQC = Auditory Response Control Quotient, VAQ = Visual Attention Quotient, AAQ = Auditory Attention Quotient, SSP = Spatial Span, SWM = Spatial Working Memory.
of glial cells in severity of symptoms and cognitive disabilities in ASD patients.

NRG1 type III (SMDF) is involved with early development and survival of the Schwann cells. Previous studies were confirmed the role of type III signaling in the maintenance of corticostriatal components and in the neural circuits involved in sensorimotor gating and working memory [26]. Nicodemus et al. were reported down expression of type III in hippocampus—which is the main region of memory in schizophrenic patients [14]. Down regulation of type III mRNA level in ASD patients could explains sensorimotor abnormalities such as language impairments (as sensorimotor related symptom) in these patients [27,28].

NRG1-ErbB4 signaling regulates the function of amygdala. Inhibition of NRG1-ErbB4 signaling which is natural consequence of NRG1 down regulation, decrease GABAergic activity and disrupts the excitation/inhibition (E/I) balance. Altered E/I balance is a pathophysiological mechanism of many psychological diseases, such as autism and schizophrenia [29]. Different lines of evidence were showed reduced GABAergic signaling in the brains of autistics children. It may be related to down regulation of NRG1 as an upper hand growth factor which is involved in signal transduction of GABAergic neurons. Significant down expression of type III in boys in compare with girls may strength the evidence about association of GABAergic signaling abnormalities and repetitive behaviors (which are most sever in boys) in ASD patients.

4.1. Executive functions deficits association with NRG1

Results of neuropsychological battery of EF measures across three domains (response inhibition, vigilance and working memory) were confirmed previous studies about EF deficit in ASD patients [19,30]. Associations between schizophrenia related risk allele of NRG1 and NRG3 with memory and attention deficits had been previously reported [31,32]. In addition impacts of type I over expression on working memory abnormalities was reported in transgenic mice [33]. Present findings were showed impacts of down regulation of NRG1 types on working memory and attention deficits which may cause by abnormalities in neuronal plasticity, gelial cell functions and sensorimotor. Type III which is a sensory and motor neuron-derived factor has showed strongest correlation with all three domains.

NRG1 mRNA level increase by particular antipsychotic treatments in schizophrenic patients [34,35]. Correlation of NRG1 down regulation with executive functions deficits that were detected in ASD patients may suggest that same antipsychotic treatments might increase NRG1 mRNA level and improve executive functions such as memory and attention in ASD patients as well.

Neuroimaging assessments in addition to gene expression and genotyping examinations could provide more reliable results about role of neuregulin family and their receptors in ASD. Also evaluation of antipsychotic effects on NRG1 mRNA level in ASD patients would provide interesting data for therapists.

5. Conclusion

We primarily suggest involvement of NRG1 main types in etiology of autism spectrum disorders. Correlation of executive functions deficits with down regulation of NRG1 types has showed important role of NRG1 in orchestration of number of executive functions including response inhibition, vigilance and working memory in ASD patients.

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Declaration of interest

The authors declare that they have nothing to disclose.

Ethics committee approval

The project followed the Helsinki declaration obligations and had been approved by central ethical committee of Islamic Azad University.

Author Contributions

Samane Abasy, Fazlollah Shahraki, Masoume Ghasemzadeh Qazvini, Sahel Towfigh Rafiee, Horie Rezvani asl, Atena alasadat Shirzadi, Mehdi Farhadi, Elnaz Noshtadiar and Rana Ghamari were involved in clinical and laboratory data collection and data analysis. They have also participated in editing the manuscript. Zeinab Tabrizi, Rashid Mehrfard, Fereshteh Esmaili kakroudi, Mahsima Azarnoosh, Seyedeh Mahsa Bagheri, Faeghe Younesi, Narges Parsamehr, Nooriyeh Garaei, Soroush Abyari, Melika Pourmehrabi, Pardis Zolfaghari, Maryam Gholami, Maede Salehi, Elham Rastegarimogaddam, Elnaz Nobakht, Elmira Nobakht and Rayan Partovi were involved in laboratory data collection and data analysis. Arvin Haghighatfard was the head of the research team. He was the study designer and was involved in clinical and laboratory data collection, analysis of data and writing the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.10.022.

| Gene expression | Gender | Age | IQ score | VRCQ | ARCQ | VAQ | AAQ | SSP | SWM | Btwn Errors | SWM Strategy |
|-----------------|--------|-----|----------|------|------|-----|-----|-----|-----|-------------|--------------|
| NRG1 type I     | p value | 0.12| 0.16     | 0.23 | 0.17 | 0.44| 0.11| 0.14| 0.37| 0.2          | 0.23         |
| NRG1 type II    | r: - 0.9 | 0.09| 0.11     | 0.15 | 0.18 | 0.23| 0.19| 0.04| 0.17| r: -0.06     |              |
| NRG1 type III   | p value | 0.07| 0.07     | 0.14 | 0.07 | 0.09| 0.08| 0.07| 0.14|              |              |
|                 | r: 0.11 | 0.03| 0.16     | 0.27 | 0.2  | 0.34| 0.32 | 0.29| 0.3 | r: -0.12     |              |
|                 | 0.4     | 0.1 | 0.13     | 0.02 | 0.01 | 0.02| 0.03| 0.01| 0.03| 0.002        |              |

Table 5
Correlation of gene expression results with demographic and clinical characteristics in all subjects.

VRCQ = Visual Response Control Quotient, ARCQ = Auditory Response Control Quotient, VAQ = Visual Attention Quotient, AAQ = Auditory Attention Quotient, SSP = Spatial Span, SWM = Spatial Working Memory.
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