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CHANGES IN GENES EXPRESSION ASSOCIATED WITH CELL STRESS IN THE PATIENT WITH CHRONIC PERSEVERATIVE STUTTERING

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SUMMARY

Background: The aim of this study was to assess the expression of genes associated with cell stress response in the patient with chronic stuttering.

Case study: We examined genes expression in a 26-year-old patient (D.K.) with chronic perseverative stuttering (CPS) syndrome, the symptoms of which intensified after repeated failure to find a job and break-up with his fiancée. The symptoms of stuttering were tested using standard neurologopedic tools and the patient met Diagnostics and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria for CPS. RNA was isolated from 2 ml of blood collected from radial vein according to methods described by Chomczyński and Sachi (1987). Gene expression was determined by real time polymerase chain reaction (RT-PCR) (Applied Biosystem Step One, Lifetechnologies, Department Poland). To calculate gene expression, the $\Delta\Delta Ct$ method described by Schmittgen and Livak was used (Schmittgen & Livak, 2008). Results of standard neurologopedic testing confirmed the severity of speech hesitancy and symptoms of logophobia. Clinical findings were consistent with the genetic findings. A lower level of expression of stress-related genes was observed in the patient's leukocytes compared to a control group.

Conclusions: It is possible that cellular protection under stress conditions was reduced in this patient with chronic stuttering. Low cellular tolerance to stressors does not provide sufficient protection to cells. In our opinion, such findings may be related to the exacerbation of chronic perseverative stuttering (CPS) syndrome.

Key words: stress, RNA, fluency of speech, logophobia, apoptosis

INTRODUCTION

Stuttering is a speech disorder in which the fluency of speech is disrupted by involuntary repetitions and prolongations of sounds, syllables, words, or phrases as well as involuntary silent pauses or blocks in which the person who stutters is unable to produce sounds (Cooper 1993a). Chronic perseverative stuttering (CPS) may refer to a condition or disease that is persistent or otherwise long-lasting in its effects.

CPS is most commonly associated with involuntary sound repetition, but also encompasses abnormal hesitation or pausing before speech, referred to by people who stutter as *blocks*, and the prolongation of certain sounds, usually vowels or semivowels (Ham 1993). At present, the literature describes stuttering as a disorder of selection, initiation, and execution of motor sequences necessary for fluent speech production. For many people who stutter, repetition is the primary problem because it disturbs the process of communication (Watkins et al. 2008).

Review of the development of Cooper's thinking regarding fluency disorders over a 30-year period suggests that the study of any single aspect of stuttering must include consideration of the interactive effects of the disorder's affective, behavioral, and cognitive components present at the time of assessment (see: Pąchalska, Kaczmarek i Kropotov 2014). To stimulate the development of more comprehensive yet communicable frameworks from which to view fluency disorders, a definition is proffered of stuttering as a clinical syndrome with three subtypes (developmental, remediable, and chronic perseverative) comprised of characteristic affective, behavioral, and cognitive components (Pąchalska et al 2012).

In recent years, the neurodevelopmental hypothesis of fluency of speech has become most popular, and suggests that the main symptoms in stuttering disorders are the result of integrated genetic, developmental, neurological, and social factors (Pąchalska, Kaczmarek & Kropotov 2014; Pąchalska et al 2015). This hypothesis is articulated in the assessment of how genes relate to behavior, and should be considered in studies of endophenotypes and neuromarkers (see Fig. 1).

In the context of genetic dysfunction in the central nervous system, blood it is generally the only source of research material and changes in genes expression in blood cells are thought to represent the systemic response of the organism (Radom-Azik et al. 2008; Neubauer et al. 2013). The expression of selected genes associated with cellular stress responses were assessed in a patient with stuttering. Analysis of the expression of genes related to stress and their signaling pathways has shown that cellular responses to stress, regardless of the stressor, results in changes in the expression of several hundred stress-related genes, associated with production of heat-shock proteins (HSPs) and interleukins (Rutkowski et al. 2005; Szołtysek et al. 2011). Multiple genes are described as stress genes, but currently only three signaling pathways regulating their expression are known (Perkins et al. 2007). The most important of these is the pathway dependent on the protein nuclear factor kappa B (NF-kB), which controls the expression of genes encoding early and late inflammatory response
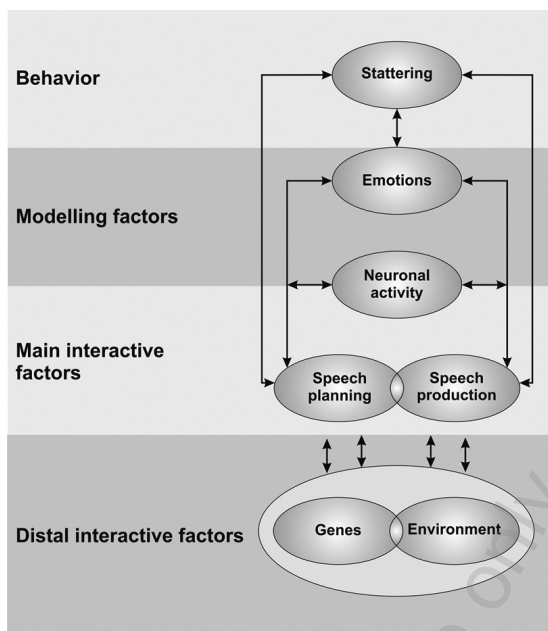
Fig. 1. From genes to behavior through the development of neuronal systems
Source: Pąchalska, Kaczmarek, Kropotov (2014), with modifications

interleukins (e.g., IL-1, IL-6, IL-8) and interferes with the two other pathways, including those dependent on heat shock factor protein 1 (HSF1) and p53 protein (Perkins et al. 2007). As an example, overexpression of *HSPA1A* is associated with cell protection from apoptosis induced by different stressors, i.e. environmental stress, high temperature, oxidative stress, and physical exercises (Dudeja et al. 2009; Feng et al. 2006; Ferat-Osorio et al. 2014).

The function of the HSP27 protein encoded by the gene *HSPB1* is degradation of proteins damaged by other stress proteins. Additionally, cellular stress also results in overexpression of genes encoding interleukins at the start of the pro-inflammatory response (e.g., IL1, IL6). However, inflammation is also accompanied by an anti-inflammatory response, such as that represented by increased expression of IL10. In the patient assessed here, it was decided to examine expression of the following genes: *HSPA1A, HSPB1, IL-1, IL6, IL10*, and *CRP* in comparison to 35 healthy subjects without diagnoses of stuttering disorders.

The neurodevelopmental hypothesis assumes that stuttering is preceded by developmental disturbances in the growth of the brain during the prenatal and perinatal period. These disturbances are provoked by environmental factors, such as puerperal complications, virus infections in the prenatal period, and fetal malnutrition. It should be stressed, however, that environmental factors lead to disturbances only in individuals who are genetically predisposed to the appearance of neurostructural, neurochemical, or neurofunctional changes in the brain (Kropotov 2009; Trystuła et al. 2015). This hypothesis also explains the fact that

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the first symptoms of schizophrenia, memory disorders, and disturbances of motor coordination can be observed during early childhood.

Anatomical studies reveal destabilization of connections between individual neurons, while neurophysiological examination points to disturbances in the working of four main functional systems: executive, sensory, emotional, and motor in patients with schizophrenia spectrum disorders.

Dysfunctions of those systems explains disorders of planning and controlling action as well as disorders of social functioning (executive system); illusions and hallucinations (sensory system); lack of motivation to act, lack or loss of feeling pleasure, and apathy (affective system); as well as mannerisms, and motor stereotypes (motor system). Moreover, neurophysiological studies indicate disruption of connections between the above systems, which means that the observed symptoms are not a result of disorder in only one system. For example, illusions are not only due to perception disorders, but to disorders of thought and consciousness as well. Faulty pathways among various brain areas may explain the variability and heterogeneity of stuttering.

CASE STUDY

We assessed gene expression in a 26-year–old patient (D.K.) with chronic perseverative stuttering (CPS) syndrome. The patient was born via natural labor without complications, but suffered from asphyxia due to difficulties during inspiration and scream (Apgar score 8/10). The patient weighed 2890 g at birth. No motor retardation was observed. Speech development was slightly delayed (first words at the age of 13 months, first simple sentences at the age of 3 years). At 3 years of age, it was also observed that the patient did not speak fluently. He especially tended to repeat words and syllables. At the age of approximately nine the patient began to be afraid of speaking (logophobia). He was referred for logopedic therapy targeting speech fluency disorders. Therapy did not relieve symptoms, which increased during adolescence. The patient was interested in logopedic studies. He graduated with a master's degree in logopedics. Despite possessing professional qualifications, the patient is unable to find work, primarily due to his stuttering. This failure in professional life became a reason for breakup with his fiancée, whom he was to marry soon. The patient had a nervous breakdown. Stuttering intensified along with logophobia.

Logopedic examination

A neurologopedic examination performed in August 2015 after another professional failure proved that the patient's stuttering had intensified. The patient scored 7/10 points on the Questionnaire of Speech Hesitancy and Logophobia (Tarkowski, 2001) indicating a high severity of logophobia. He scored 18% on the Syllable Test for Speech Hesitancy Assessment (Kurkowski, 2003) (norm 3-5%), indicating severe speech hesitancy manifesting mainly as blocking consonants. The patient presented full symptoms of stuttering according to Diagnostics

and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria, including difficulty with initial articulation of a word, sentence or phrase, prolonging a word or the consonants in the word, a pause for some syllables, pauses during articulation of words, and adding additional words. He also presented co-movements, such as movements of the face and upper portion of the body during articulation, trembling of the lips or mandibula, fast blinking, tics of the face, and head jerks.

Genetic tests

Patient gene expression was compared to gene expression in 35 health individuals for *HSPA1A*, *HSPB1*, *IL1*, *IL6*, *IL10*, and *CRP*.

RNA isolation and reverse transcription

For RNA isolation, 2 ml of venous blood were collected from the ulnar vein. Isolation of RNA was performed via the method described by Chomczynski and Sachi (1987) using Trizol reagent (Initrogen, Lifetechnologies, Poland). Isolated RNA was purified of DNA with DNaza I (Initrogen, Lifetechnologies, Poland). RNA purity and concentration was determined spectrophotometrically (Eppendorf BioPhotometer Plus, Germany). Reverse transcription was performed using 1 µg RNA with the Transcript Me RNA kit (DNA, Gdańsk, Poland), containing oligo dT and random primers. After reverse transcription, c-DNA was purified of RNA with RNaza H.

Q-PCR

Real Time PCR was performed in two biological and technical replicates using Applied Biosystem Step One (Lifetechnologies, Poland). The reaction mixture contained: 5 µl of SensiFast polymerase (Bioline, UK), 0.4 µl each reverse and forward primers, 2 µl of cDNA, and water to a final volume of 10 µl. Thermal profile of the PCR was consistent with the manufacturer's instructions. Calculation of gene expression was conducted using the $\Delta\Delta C_t$ method described by Schmigtten and Livak (2008). *Tata box protein* (*TBP*) was measured as references gene. All calculations were performed in the same manner (sample dilution, prescribed amount of mRNA, etc.).

To amplify the genes, the following primer sequences were applied:
TBP reverse: TCTGTCGGCTCCGCTCTGAGAT
TBP forward: ACTCCCGTTGTCCCAAGGCTTC
HSPA1A reverse: TTCGGAGAGTTCTGGGATTGTA
HSPA1A forward: TGGACTGTTCTTCACTCTTGGC
HSPB1 reverse: GAGGAAACTTGGGTGGGGTCCA
HSPB1 forward: AAGGATGGCGTGGTGGAGATCA
IL6 reverse: GACATCAAGGCGCATGTGAAC
IL6 forward: TCCACGGCCTTGCTCTTGTTT
IL10 reverse: AATTCGGTACATCCTCGACGG
IL10 forward: GAATCCAGATTGGAAGCATCC
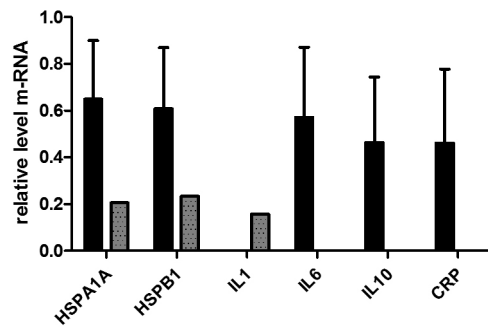*CRP* reverse: TCTTGGTCTTGACCAGCCTCT
CRP forward: TCGTTAACGGTGCTTTGAGG

Fig. 2. Genes expression (mean+/-SD) in control group (dark bars n=35) and patient (gray bars)

RESULTS

Patient gene expression results are presented in Figure 2. The lower expression in *HSPA1A, HSPB1, IL6, IL10*, and *CRP* was determined from the patient's blood leukocytes. The level of RNA for transcripts of *IL6*, *IL10*, and *CRP* was undetermined. In contrast, IL1 could be detected in the patient's sample. This pilot study suggests that the expression of genes associated with the cellular stress response were different in a patient with stuttering compared to a healthy control group.

DISCUSSION

It is well known that various stressors affect the synthesis of heat shock proteins and interleukins in leukocytes. In a situation of excessive stress, the body's immunity decreases. Overexpression of *HSPA1A* enables a cell to survive by protecting it from apoptosis (Morimoto, 1998) and the genes encoding interleukin pro- and anti-inflammatory cytokines cause mobilization of leukocytes throughout the body. There have been many publications describing the expression of stress-related genes under stress conditions, such as high temperature, oxidative stress, and physical performance (Pizurki and Polla, 1994; Jacquier-Sarlin et al. 1995; Polla and Cassarizza, 2002). Stress-related genes are also upregulated during exercise, as reported by Buttner et al. (2006). Radom-Azik et al. (2008) and Neubauer et al. (2013) reported that the upregulation of genes related to apoptosis and immune response in neutrophils represents an integrated response to various physiological dysfunctions. These findings led us to hypothesize that other dysfunctions may be associated with altered stress-related gene expression. In this pilot study assessing stress-related gene expression in a patient with CSP, the expression of almost all tested genes, with the exception of *IL1*, in patient's leukocytes were lower than in the control group. These data are difficult to interpret. Lower levels of *HSPA1A* mRNA may suggest either a lower stress load or insufficient protection of cells under stressful conditions. Similar research conducted in a larger group of indivuals with the same dysfunction is necessary to better understand these findings. It is possible that lower gene ex-

pression may result in poorer cell survival under stress conditions and this phenomenon may play a role in chronic stuttering, but this observation needs to be assessed in a larger group designed to reach statistical significance.

In summary, results of standard neurologopedic testing showed chronic stuttering with logophobia as the main symptom. Clinical testing is consistent with genetic findings and lower level of expression of stress-related genes observed in the patient's leukocytes compared to control group. It should be emphasized that the patient had a generally lower level of expression of genes associated with stress, which may result in a reduced tolerance to stressful conditions. We hypothesize that these conditions may increase the occurrence of apoptosis and fuel a „vicious cycle" of causes and effects that may also be related to the severity of chronic stuttering.

CONCLUSIONS

Preliminary gene expression data suggest that cellular protection under stress conditions was reduced in a patient with chronic stuttering. Low tolerance of cells to stressful conditions may result in insufficient protection. These findings may be associated with exacerbation of chronic perseverative stuttering (CPS) syndrome.

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