Original Article
https://doi.org/10.9758/cpn.2017.15.3.229
Clinical Psychopharmacology and Neuroscience 2017;15(3):229-236
pISSN 1738-1088 / eISSN 2093-4327

Low Retinal Dehydrogenase 1 (RALDH1) Level in Prepubertal Boys with Autism Spectrum Disorder: A Possible Link to Dopamine Dysfunction?

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Objective: Retinal dehydrogenase 1 (RALDH1) is a cytosolic enzyme which acts both as a source of retinoic acid (RA) and as a detoxification enzyme. RALDH1 has key functions in the midbrain dopaminergic system, which influences motivation, cognition, and social behavior. Since dopamine has been increasingly linked to autism spectrum disorder (ASD), we asked whether RALDH1 could contribute to the autistic phenotype. Therefore, we investigated for the first time the levels of RALDH1 in autistic patients. To further assess the detoxification function of RALDH1, we also explored 4-hydroxynonenal protein adducts (4-HNE PAs) and reduced glutathione (GSH) levels. Moreover, considering the effect of testosterone on RALDH1 expression, we measured the second to fourth digit ratio (2D:4D ratio) for both hands, which reflects exposure to prenatal testosterone.
Methods: Male patients with ASD (n=18; age, 62.9±4.3 months) and healthy controls (n=13; age, 78.1±4.9 months) were examined. Erythrocyte RALDH1, serum 4-HNE PAs and erythrocyte GSH levels were measured using colorimetric assays, and digit lengths were measured using digital calipers.
Results: We found significantly lower (−42.9%) RALDH1 levels in autistic patients as compared to controls (p=0.032). However, there was no difference in 4-HNE PAs levels (p=0.368), GSH levels (p=0.586), or 2D:4D ratios (p=0.246 in the left hand, p=0.584 in the right hand) between healthy controls and autistic subjects.
Conclusion: We concluded that a subset of autistic patients had a low RALDH1 level. These results suggest that low RALDH1 levels could contribute to the autistic phenotype by reflecting a dopaminergic dysfunction.

KEY WORDS: Autistic disorder; Retinal dehydrogenase; Dopamine; 4-Hydroxy-2-nonenal; Glutathione.

INTRODUCTION

Autism spectrum disorder (ASD) comprises a group of neurodevelopmental disorders characterized by persistent deficits in social communication and social interaction and restricted patterns of behavior, interests and activities.[1)] Interest in ASD has increased over time as prevalence has risen progressively over the last decades, some modern estimates putting it as high as 1 in 88 children.[2)] Intriguingly, ASD are strongly biased towards males, with a male to female ratio of 4:1.[3)] Although several theories regarding the pathogenesis of ASD have emerged, their etiology remains largely unknown.[4)] Evidence suggests that ASD is not a single disorder, but rather a set of different variants, each with its own characteristics and etiologies.[5)]

The diffuse modulatory systems of the brain represent groups of neurons which form widely dispersed connections throughout the brain, modulating vast assemblies of postsynaptic neurons. The ventral tegmental area (VTA) of the midbrain is the origin of one such diffuse modulatory system.[6)] The dopaminergic fibers arising from the VTA project to the prefrontal cortex (PFC) and to regions of the limbic system, such as the nucleus accumbens (NAcc), forming the mesocorticolimbic (MCL) circuit.[7)] This circuit is involved in high order brain functions, such as reward, emotional social behavior, motivation and cognition.[6,8)] The midbrain dopaminergic system is thought to be involved in neuropsychiatric disorders.[9)] Although scarce, evidence suggests dopaminergic dysfunction in autistic subjects.[10-12)]

Received: June 28, 2016 / **Revised:** August 23, 2016
Accepted: September 5, 2016
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The midbrain dopaminergic system relies on the function of retinal dehydrogenase 1 (RALDH1). RALDH1 is a ubiquitously distributed cytosolic enzyme which plays key roles in the dopaminergic neurons both as a source of retinoic acid (RA) and as a detoxification enzyme.[13,14] RALDH1 catalyzes the oxidation of retinal to RA, which is essential for the differentiation, development and survival of dopaminergic neurons, both in the developing and in the adult brain.[15,16] Moreover, RALDH1 plays a role in the cellular defense against oxidative stress, through oxidation of the aldehydes derived from lipid peroxidation (LPO), such as 4-hydroxynonenal (4-HNE).[17] In the dopaminergic neurons, RALDH1 catalyzes the oxidation of 3, 4-dihydroxyphenylacetaldehyde (DOPAL), a metabolite of dopamine which renders such neurons susceptible to oxidative stress.[18] RALDH1 is also involved in the degradation of dopamine itself.[19] Although low levels of RALDH1 have been reported in neuropsychiatric conditions with hypothesized dopaminergic involvement, this aspect has not been studied in autistic subjects so far.[13,20]

Considered a reliable marker of oxidative stress, 4-HNE is a reactive aldehyde derived from LPO which yields general cytotoxicity, thought to be involved in various pathological conditions.[21] Due to its reactivity, 4-HNE readily forms stable adducts with various proteins (4-HNE protein adducts, 4-HNE PAs), and consequently affects those biological function.[22,23] 4-HNE has direct dopaminergic neurotoxicity and dopaminergic functional effects, such as modulation of dopamine receptors and of dopamine uptake through the dopamine transporter protein.[24-27] Thus far, one study reported increased 4-HNE PAs levels in autistic children as compared to controls.[21]

Even though RALDH1 plays an important role in the metabolism of 4-HNE, the main enzyme involved in the 4-HNE detoxification outside the brain is glutathione-S-transferase (GST).[17,21] GST conjugates the reduced glutathione (GSH) with toxic compounds, such as 4-HNE, in order to eliminate them.[28] GSH is the main low molecular weight antioxidant synthesized in cells, its level reflecting the oxidative stress status.[29] Although not consistently, studies show that autistic subjects exhibit low GSH levels.[30]

RALDH1 gene expression has been reported to be directly regulated by androgens and estrogens.[31,32] More important, molecular pathways describing a "transfer of signaling" from sex hormones to RA through the action of RALDH1 were revealed.[33,34] Given the strong male bias, it has been suggested that ASD may arise as a result of prenatal exposure to elevated levels of testosterone, especially between weeks 8 and 24 of gestational age.[35] Considering that the direct measurement of prenatal testosterone level is technically difficult, the ratio between the length of the second and fourth digit (2D:4D ratio) was used as a proxy, given that it is negatively correlated to prenatal testosterone.[36]

This work aimed to investigate the erythrocyte RALDH1 level in autistic patients as a peripheral indicator of the RALDH1 level in dopaminergic neurons. This could provide information regarding the RA synthesis in the autistic midbrain dopaminergic system. Moreover, to obtain information about the detoxification function of RALDH1 as well, we evaluated the 4-HNE PAs serum levels. Given the fact that GSH is also involved in the 4-HNE detoxification, we further evaluated the GSH levels in order to minimize the confounding factors linked to the metabolism of 4-HNE. Lastly, the 2D:4D ratio was used in order to assess any potential influences linked to fetal testosterone levels.

METHODS

Study Population

In this case-control study, we studied samples from the male population aged 48 to 96 months (i.e., 4 to 8 years) old. Subjects diagnosed with any genetic, metabolic, or neurologic condition, as well as those having a history of acute or chronic inflammatory disease or taking long-term medication were excluded from the study.

Male patients with ASD (n=18), as well as sex-matched healthy controls (n=13) participated in the study. The autistic subjects were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) criteria for ASD.[1] Moreover, their behavior was individually evaluated through specific diagnostic tools, using the Childhood Autism Rating Scale and Autism Diagnostic Observation Schedule.[37,38] Also, the parents of each autistic subject completed a questionnaire regarding the developmental history of the child.

The healthy controls were selected during routine health checks. Parents of healthy individuals that requested routine check-ups were asked to participate in the study on a voluntary basis. During their routine visit to the Pediatrics Department of the Iuliu Haţieganu University of Medicine and Pharmacy, the children underwent digit length measurements in addition to the standard clinical examination. Blood samples were obtained from participants that opted to have samples drawn as part of their check-ups. The full set of biological samples and appropriate digit lentgh measurements were eventually ob-

tained from a total of 13 healthy males.

We chose to include only males in this study because ASD is more prevalent among males. Given the assumptions (as detailed in the Discussion section) about hormonal influences on RALDH1 levels (and therefore, the possibility of these differences acting on ASD pathogenesis), choosing to include only males would limit confounding factors related to sex and internal hormonal environment. The age group was further carefully selected so as to reduce confounding levels of hormonal activity to a minimum (as detailed in the Discussion section).

Ethical committee approval from the Iuliu Haţieganu University of Medicine and Pharmacy and informed consent from parents of all participating subjects' were obtained.

Sample Collection and Preparation

Venous blood samples were obtained from each subject and collected into 5 ml vacutainers containing clot activator or K2EDTA, in order to obtain serum and erythrocytes, respectively. The samples were processed as soon as possible in order to avoid alterations.

Serum: The vacutainers containing clot activator were centrifuged for 5 minutes at 3,500 rpm, and then the serum was collected, aliquoted and stored at −80°C until analysis.

Washed erythrocytes: After mixing the whole blood by gentle inversion of the vacutainer, an amount of 1 ml of whole blood was aspirated and centrifuged for 3 minutes at 3,500 rpm, and then the plasma and the buffy coat on the erythrocyte sediment were removed. Then the erythrocytes were washed three times with 1 ml of 0.9% NaCl solution and centrifuged for 3 minutes at 3,500 rpm after each wash. Next, the erythrocytes were resuspended in cold 0.9% NaCl solution in order to obtain the initial volume (1 ml) and the mixture was aliquoted and stored at −80°C.

Erythrocyte lysate: Prior to the analysis, the frozen suspensions of erythrocytes (in 0.9% NaCl solution) were thawed and subjected to another freeze-thaw cycle in order to break up the cell membranes. Then the erythrocyte lysates were centrifuged for 5 minutes at 5,000 *g*, 2°C to 8°C. The supernatant was collected and assayed immediately.

Instruments, Biochemical Tests, and Calculation of Results

For the colorimetric enzyme-linked immunosorbent assays (ELISA) a microplate reader (Tecan Sunrise™; Tecan, Männedorf, Switzerland) was used. For the quantitative colorimetric assays, a double beam ultraviolet-visible spectrophotometer (JASCO V-530; JASCO Inc., Easton, MD, USA) was used. In order to calculate the sample concentrations, standard curves were constructed using Curve Expert 1.4 (Daniel Hyams, Madison, AL, USA), software capable of generating a four-parameter logistic curve fit.

RALDH1 concentration: RALDH1 level in the erythrocyte lysates was determined with a quantitative sandwich enzyme immunoassay technique using the sandwich ELISA kit from Cusabio Biotech Co., Ltd. (CSB-EL001565HU; Hubei, China) according to the manufacturer's instructions.

4HNE-PAs concentration: 4HNE-PAs level in the serum was determined using competitive ELISA kit from Cell Biolabs, Inc. (STA-838; San Diego, CA, USA), according to the manufacturer's instructions.

GSH concentration: GSH level in the erythrocyte lysates was estimated according to the method described by Akerboom and Sies.[39]

The 2D:4D Ratio

To determine 2D:4D ratio, digital calipers were used to measure digit length either directly on the subjects' hands or from photocopies of the palmar surface of the hand. The lengths of the second and fourth digits from each hand were measured from the proximal crease at the base of the finger to the tip of the finger, using a standard method.[40]

Statistical Analysis

Data were analyzed using the IBM SPSS Statistics (version 20.0.0; IBM Co., Armonk, NY, USA), and two-tailed p values of less than 0.05 were considered significant. All variables were tested for normal distribution using the Shapiro-Wilk test. Normally distributed data were presented as mean±standard deviation. For asymmetric data, the median was chosen as a measure of central tendency. To assess homogeneity of variance for a given variable, Levene's test was used. Data between groups were compared using the independent-samples t test (for normally distributed data) or the Mann-Whitney U test (for asymmetric data). Correlations between groups were assessed using Pearson's correlations (for normally distributed data) or Spearman's rank correlations (for asymmetric data). Linear regression was used in order to assess the relationship between RALDH1 and the participants' age.

No multiple comparisons adjustment was used in our study since we made a small number of planned comparisons with clearly defined hypotheses pertaining to different biochemical pathways or anthropometric measurements. As such there is no universally available null hypothesis that encompasses all our parameters into one single purported theory and there has no retro-fitting of vari-

ous hypotheses after the collection of data.

RESULTS

Age

Between the autistic patients (age, 62.9±4.3 months; range, 38-102 months) and the control subjects (age, 78.1±4.9 months; range, 49-106 months) there was a significant age difference (p=0.028).

RALDH1 Level

We found significantly lower (−42.9%) erythrocyte RALDH1 level in autistic children compared to the healthy controls (p=0.032) (Fig. 1).

4HNE-PAs Level

We found no significant difference in serum 4HNE-PAs level between the ASD group and the control group (p=0.368). There was a higher (+30.5%) serum 4HNE-PAs level in the control group (Fig. 2).

GSH Level

We found no significant difference in erythrocyte GSH level between the ASD group and the control group (p=0.586) (Fig. 3).

The 2D:4D Ratios

We found no significant difference in 2D:4D ratio in the left hand (p=0.246) or the right hand (p=0.584) between ASD group and the control group (Fig. 4).

RALDH1 and 4HNE-PAs Correlation

We found a weak, non-statistically significant correlation between RALDH1 and 4HNE-PAs levels (r_s=0.182, p=0.326).

RALDH1 and 2D:4D Ratios Correlations

We found a weak, non-statistically significant correlation between RALDH1 levels and left hand 2D:4D ratio (r=0.284, p=0.179). Also, there was no correlation between RALDH1 levels and right hand 2D:4D ratio (r=−0.05, p=0.791).

DISCUSSION

Our study found significantly lower RALDH1 levels, but no difference in 4-HNE PAs level, GSH level, or 2D:4D ratios in the ASD group compared to the control group.

In dopaminergic neurons, RALDH1 plays key roles

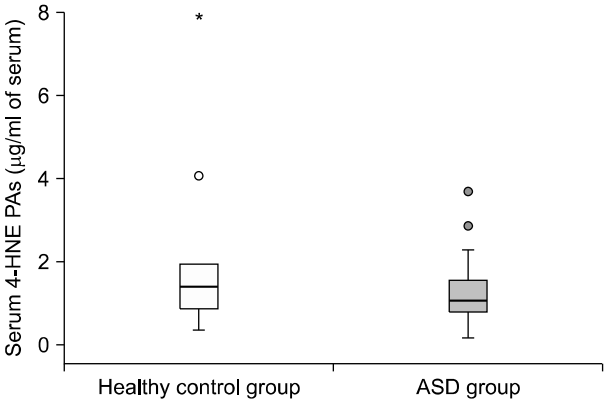

Fig. 2. Comparison of serum 4-hydroxynonenal protein adducts (4-HNE PAs) levels (μg/ml) between the control group (n=13) and the autism spectrum disorder (ASD) group (n=18). No significant differences between groups were reported.

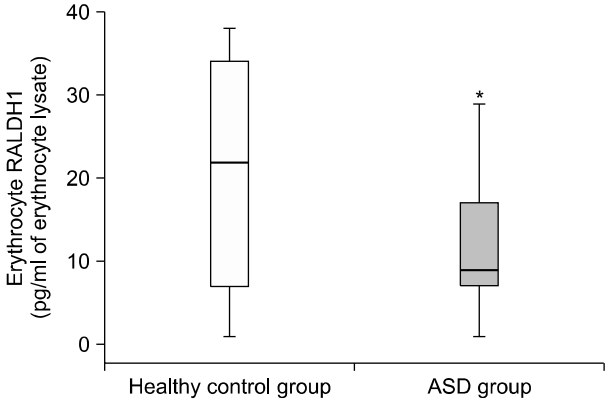

Fig. 1. Comparison of erythrocyte retinal dehydrogenase 1 (RALDH1) levels (pg/ml) between the control group (n=13) and the autism spectrum disorder (ASD) group (n=18).
*p=0.032, significantly different from healthy controls.

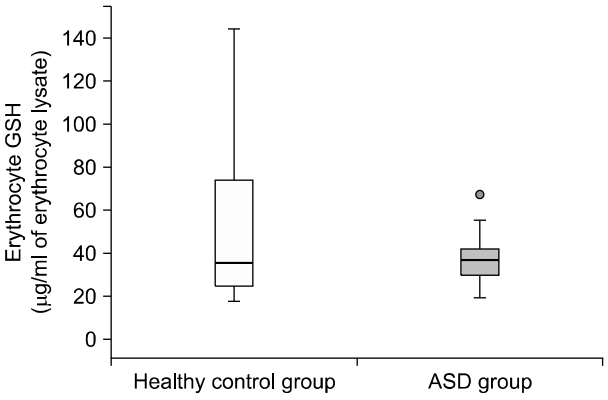

Fig. 3. Comparison of erythrocyte glutathione (GSH) levels (μg/ml) between the control group (n=13) and the autism spectrum disorder (ASD) group (n=18). No significant differences between groups were reported.

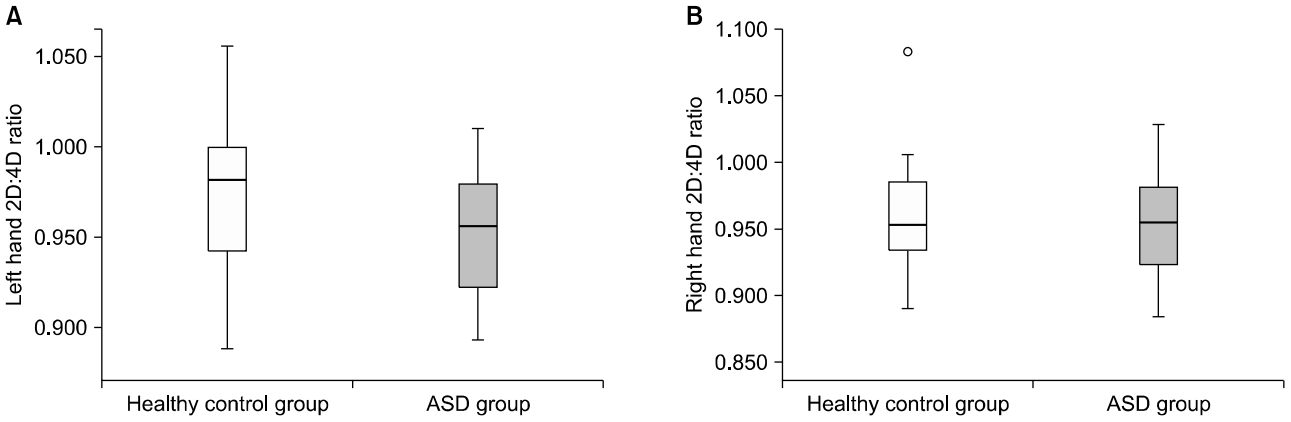

Fig. 4. Comparison of the second and fourth digit ratios (2D:4D ratios) between the control group (n=13) and the autism spectrum disorder (ASD) group (n=18). The left hand and right hand 2D:4D ratios are shown in the panel A and B, respectively. No significant differences between groups were reported.

both as a source of RA and as a detoxification enzyme.[13,18] RALDH1 is highly and specifically expressed in the VTA dopaminergic neurons, suggesting RALDH1's strong involvement in controlling the dopaminergic function and signaling in the MCL circuit.[13] The observed decrease in the RALDH1 level could reflect an insufficient synthesis of RA in the VTA. A lower RA level would deprive the MCL circuit of a critical factor for the circuits' development and function and would ultimately lead to alterations in motivation, emotional behavior and cognition.[6,8] Furthermore, considering the detoxification function of RALDH1, a low RALDH1 level would render VTA neurons more susceptible to reactive aldehydes, such as the neurotoxic DOPAL and 4-HNE. The aldehyde toxicity stemming from these compounds could also lead to the circuit's malfunction. Curiously, studies of autistic subjects reveal anomalies directly linked to MCL circuit dysfunction, such as reduced release of dopamine in prefrontal regions, decreased NAcc activation and hypoactivation of the reward circuit.[10-12] However, the RALDH1 role in the dopaminergic molecular machinery supporting these high order brain functions remains to be determined.

Intriguingly, RALDH1 is involved in the degradation of dopamine.[19] Thus, a low RALDH1 level could expose the MCL circuit to higher levels of dopamine. In fact, ASD were hypothesized to be associated with dopaminergic hyperactivity of the MCL circuit, given that dopaminergic antagonists like risperidone and aripiprazole are the only drugs approved for the control of ASD-associated behavior.[41,42] However, ASD were also hypothesized to be associated with dopaminergic hypoactivity.[43] Thus, a decrease in the RALDH1 level could be the result of a compensatory down-regulation in order to limit the dopamine degradation and prolong its half-life. Future investigations are certainly needed in order to establish the characteristics of dopaminergic signaling in ASD. Lately, authors increasingly involved dopamine in ASD, widening the perspective of finding new drugs for the control of ASD-associated behavior.[44]

Given the reports of oxidative stress in ASD and the low observed RALDH1 level, we expected to see significantly higher levels of 4-HNE PAs in the ASD group compared to the control group.[45] However, the ASD group had lower (−30.5%) levels of 4-HNE PAs, although not significantly different. Also, our data showed that there was no significant correlation between the RALDH1 level and 4-HNE PAs level. The lower 4-HNE PAs levels in autistic subjects could mean that several RALDH1-independent mechanisms account for the 4-HNE metabolism, such as detoxification by GSH. In this respect, we evaluated the GSH levels, but we did not find a significant difference between the two groups. However, the GSH values of the control group were spread on a larger interval compared to those of the ASD group. Further studies are necessary in order to establish if the 4-HNE PAs are a common finding in ASD.

Male fetuses exhibit ample testosterone secretion between weeks 8 and 24 of gestational age. This testosterone production masculinizes the developing fetus' brain. Males also produce another surge of testosterone during the perinatal period.[46,47] Considering these particularities, subjects of pre-pubertal age were selected, presuming that most of the masculinizing effect on their brain would have been exerted prenatally and/or perinatally. Moreover, the prenatal testosterone is an indicator that can be estimated from the 2D:4D ratios. Thus, to minimize confounding

factors linked to sex hormones, we aimed to study males aged 48 to 96 months (i.e., 4 to 8 years old). Apart from the direct influence on *RALDH1* expression, testosterone directly influences the functions of the MCL circuit as well.[48] The midbrain dopaminergic system has been specifically reported to be both morphologically and functionally sexually dimorphic.[8,49] As a consequence, behavioral and imaging studies revealed gender differences at the cognitive and behavioral level, specifically involving the MCL circuit.[8,50] Our results regarding the 2D:4D ratios suggested that the autistic subjects and the controls were prenatally exposed to comparable levels of testosterone. Thus, our study did not confirm the hypothesis which states that ASD are associated with increased levels of prenatal testosterone.[35] Although other studies have shown that testosterone directly influences RALDH1 expression, our data showed that there was no significant correlation between the 2D:4D ratios and the RALDH1 level.[31] It is possible that *RALDH1* expression is tightly regulated by sex hormones only during the brain development, when the "transfer of signaling" from sex hormones to RA is vital.[34]

RALDH1 has not been investigated in autistic subjects, so far. RALDH1 has been previously proposed as a peripheral marker for Parkinson's disease, a condition characterized by the degeneration of another dopaminergic modulatory system of the brain originating in the midbrain's *substantia nigra*.[6,51] More importantly, a study reported low RALDH1 levels in the VTA of schizophrenic patients.[13] Schizophrenia, a neuropsychiatric disease with hypothesized dopaminergic involvement, is primarily treated with dopaminergic antagonists such as risperidone and aripiprazole, also used in the control of ASD-associated behavior.[20] Even though schizophrenia and ASD are known to share behavioral traits, dopaminergic involvement in ASD remains disputed.[52] Furthermore, although one study reported significantly elevated plasmatic 4-HNE PAs levels in autistic children,[21] our study did not reproduce these results; by contrast, we found a slightly lower 4-HNE PAs level in autistic patients compared to controls. Likewise, we found no significant difference in GSH level between the two groups, although studies reported lower GSH levels in autistic subjects compared to controls.[30]

Whereas our study has reached its aims, it had several limitations. Given that we tested a new research hypothesis, our study was conducted on small samples. Thus, we avoided spending too many resources on studying a solely hypothesized association. However, our ability to make broader generalizations from the results is limited and a larger confirmatory study is needed. Second, given the conducted testosterone inferences, only males aged 48 to 96 months old were selected. Although the ASD have a strong male bias[3] with possible sex hormones involvement, larger samples representing both genders and wider range of ages would more accurately represent the target populations. Lastly, there was a significant age difference between the autistic subjects and healthy controls. Considering that this significant age difference could have acted as a confounding factor, we further explored this possibility. Introducing the data into a linear regression model, we found a moderate correlation between age and RALDH1 level ($r=0.389$, $p=0.031$). Despite this moderate correlation, only 15.1% of the total variation of the RALDH1 levels can be explained by age ($r^2=0.151$). The literature lacks information regarding the variation of erythrocyte RALDH1 levels across age. Thus, we further determined the level of hemoglobin in each sample and considered it as an erythrocyte reference protein for the RALDH1 level. As expected, the mean hemoglobin level in the ASD group was lower than that of the control group (99.56 mg/ml compared to 117.47 mg/ml, respectively), considering the well-known variation of hemoglobin across age.[53] However, unlike the difference in RALDH1 level, the difference in hemoglobin level between groups was not statistically significant ($p=0.066$). The comparable hemoglobin levels suggest that even if age has a similar influence on RALDH1 level as it has on hemoglobin, it would not entirely explain RALDH1's significantly lower level. Therefore, there might be another factor, besides age, that can account for the significantly lower levels of RALDH1 in autistic children.

One important direction that remains to be explored is the role of RALDH1 as a source of RA in the developing brain.[54] RALDH1 is developmentally expressed in specific ventral midbrain cells corresponding to dopamine progenitors.[55] It is likely that newborn neurons are exposed to the RALDH1-derived RA in order to acquire specific dopaminergic differentiation.[56] Given that it is a dynamic and complex process which requires real-time observation, it is technically difficult to unravel the molecular machinery that supports the development and function of specific brain areas, such as VTA. The use of patient derived induced pluripotent stem cells (iPSC) recently provided an important opportunity to closely study the behavior of specific types of neurons in certain disorders. It would be of great interest to explore midbrain dopaminergic neurons derived from autistic patients, as

this type of cells has already been differentiated from iPSC from Parkinson's disease patients.[57] This model would provide a better understanding of the molecular pathways involved in the development and function of the dopaminergic neurons in autistic patients.

We conclude that RALDH1 levels are low in a subset of autistic subjects, possibly reflecting a midbrain dopaminergic dysfunction, with long-lasting effects on high order brain functions, such as motivation, emotional behavior and cognition.

■ Acknowledgments

This work was supported by the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania under Grant 1493/12/28.01.2014. The authors report no conflict of interest.

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