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Basic Science

Autism and Fragile X: Is There a Neurochemical Link?

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Abstract

BACKGROUND: Autism and Fragile X syndrome are intertwined. This study aimed at assessing Serotonin, Glutamate, and Gama Amino Butyric Acid (GABA) in autism and Fragile X syndrome patients and to detect possible neurochemical similarities between the 2 disorders that can be used as metabolic biomarkers.

DESIGN AND METHODS: Eighty subjects divided into four groups, two diseased groups (20 male patients with Autism and 20 males with Fragile X syndrome) and two control groups (20 neurotypical male controls and 20 Down syndrome male patients) were included. Estimation of Serotonin, Glutamate and GABA were done using Enzyme linked Immunosorbent Assay (ELISA), Tandem Mass Spectrometry and high-pressure liquid chromatography (HPLC), respectively.

RESULTS: Serotonin was, exclusively, significantly low in autistic children. GABA was significantly high in both autistic and Fragile X children only, but not in Down syndrome children. Glutamate was significantly high in children with autism, Fragile X and Down syndrome Children.

CONCLUSIONS: Autism and Fragile X syndrome share some neurochemical similarities with regards of high Glutamate and GABA levels while Serotonin was significantly different in the 2 disorders and may be used a unique biomarker for autism.

Introduction

Autism is a complex disorder affecting neurologic development. It is characterized by deficits in social interaction, disrupted verbal and nonverbal communication, and restricted repetitive behavior and interests [1]. It usually manifests before 3 years of age. Previously, autism was thought to be a rare disorder; however the number of reported cases rose significantly during the 1990s. According to the latest estimates from CDC's Autism and Developmental Disabilities Monitoring (ADDM) Network, about 1 in 68 children has been identified with autism spectrum disorder (ASD) (CDC, 2014) [2]. Autism heritability is estimated to be as 90% under a multifactorial threshold model (Hallmayer et al., 2011) [3]. Mendelian form of autism was identified with co morbid Intellectual Disability (ID) and epilepsy, associated with low plasma levels of branched chain amino acids (BCAAs).The mechanism by which abnormal amino acid levels may lead to autism, Intellectual Disability (ID), and epilepsy needs to be elucidated. Dietary supplementation with BCAAs reversed some of the neurological phenotypes in mice [4].

Fragile X syndrome (FXS) is considered to be the most common single gene cause of autism (McLennan et al., 2011) [5]. It is estimated to affect 1:2500 to 1:4000 individuals (See Recent reference). Recent study in 2014 has reported that the frequency of the full mutation was 1.4 (95% CI: 0.1–3.1) per 10,000 males and 0.9 (95% CI: 0.0–2.9) per 10,000 females (1:7,143 and 1:11,111, respectively) in the total population. The premutation frequency was 11.7 (95% CI: 6.0–18.7) per 10,000 males and 34.4 (95%

CI: 6.3–83.3) per 10,000 for females (1:855 and 1:291, respectively) in the total population. The prevalence of female carriers of the premutation in the normal population was 34.4 (95% CI: 8.9–60.3) per 10,000, or 1:291 (Hunter et al., 2014) [6].

Fragile X and autism are closely related. Two to seven percent of children have a mutation in Fragile X Mental Retardation 1 (FMR1**)** gene [7]. In the mean time, about 15-30% of Fragile X children have autistic features [8]. Fragile X mental retardation protein(FMRP) regulates the translation of neuroligins, neurorexins, SHANK, PTEN, CYFIP, and PSD95 proteins, which are associated with autism [7].FMR1 gene regulates the expression of other genes and affect synaptic formation and plasticity [9]. It is a neuronal and gonadal protein with key roles in neuroplasticity and neuronal translation (Soden et al., 2010) [10]. Therefore, decline or absence of FMRP, may be associated with significant alterations indifferent proteins that affect brain development, ending in developmental disorders, particularly autism (Darnell, 2011) [11].

Several studies have determined different neurotransmitters in children with autism; however, only one study in 1984 has estimated serotonin level in Fragile X syndrome patients with and without autism. Here, we evaluate 3 neurotransmitters in the 2 intertwined disorders, autism and Fragile X Syndrome. To our knowledge, this is the first study that estimates Glutamate and GABA levels in Fragile X compared to autistic, Down Syndrome (DS) and neurotypical children.

This study aimed at detection of neurobiological similarities between Autism and FXS so targeted treatment currently being studied in FXS can be applied to autistic patients. Also assessment of these neurotransmitters could be used as metabolic biomarkers for Autism or FXS.

Subjects and Methods

The present study included 80 subjects divided into 4 groups, 2 diseased groups (20 male patients with Autism and 20 males with FXS) and 2 control groups (20 neurotypical male controls and 20 Down syndrome male patients). All the children enrolled in the present study aged from 3-11 years old. Autism, FXS and DS patients were selected from The Autism Disorders Clinic, Medical Research Center of Excellence, National Research Center (NRC), Cairo, Egypt. The ethical approval of the NRC ethical committee was obtained prior to the study and a written informed consent was provided by the parents of autistic children.

All patients were subjected to careful pedigree construction and complete diagnostic workup including medical, psychiatric, psychological and neurological evaluation. Autistic children were clinically diagnosed with Autism Diagnostic Interview Revised (ADI-R) [12] and degree of severity was assessed by Childhood autism rating scale (CARS) [13]. Nine autistic patients out of 20 were classified as having severe autism, while the other 11 were having mild to moderate autism. Fragile X syndrome patients were diagnosed clinically by Hagerman checklist [14] and in the laboratory by DNA Fragile X test. Fragile X patients were classified according to the presence of autistic features into 6 with autistic features and 14 without autistic features. Down syndrome patients were subjected to karyotyping. Exclusion criteria included neurological, metabolic, endocrine, cardiovascular, pulmonary, liver, kidney or other medical disease.

Metabolic biomarkers as Serotonin, GABA and Glutamate were estimated in the 4 groups. The aim of including DS as a control group was to see if these metabolic biomarkers are specific to certain disease as Fragile X disease and autism or it is present in other disorders associated with mental retardation as DS.Threeml of venous blood samples were taken from the children on EDTA (Ethylene Diamine Tetra Acetate) vacuum tubes. Serotonin assay was done using Serotonin ELISA kit (DIA source Immunoassay S.A. Rue de Industrie 8, 1400 Nivelles, Belgium).Calibration curve was obtained by plotting the absorbance readings against the corresponding standard concentrations. Plasma GABA levels were measured by high-performance liquid chromatography-electrochemical method [15]. Calibration curve was drawn using four standard solutions of GABA. Glutamate was assayed from dried blood spots using Mass Spectrometry/ Mass Spectrometry (MS/MS) The detector monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is recorded in the form of mass spectra [16].

Analysis of results was performed using statistical package for social science(SPSS) software(SPSS Inc., Chicago, IL, USA) ,version 15 for Microsoft Windows. Multivariate analysis was used to compare between the different groups [17].

Results

Autistic children had a significantly very low serotonin level when compared to the 3 other groups (Fragile X Syndrome, DS and neurotypical children) (Table 1). On the contrary, FXS syndrome showed significantly high levels when compared to patients with autism, DS and normal healthy children. Down syndrome cases showed lower serotonin levels compared to neurotypical children but this difference was not statistically significant.

The second neurotransmitter assayed was Glutamate (Table 2). Autism cases showed

Table 1: Serotonin levels in the different studied groups (ng/ml).

Group Mean ± SD	Group Mean ± SD	P value
Autism 79.95 ± 45.48	Normal 207.50 ± 128.37	P<0.0001**
FXS 1182.93 ± 1060.27	Normal 207.50 ± 128.37	P<0.0001**
Autism 79.95 ± 45.48	DS 186.75 ± 129.88	P<0.0001**
FXS 1182.93 ± 1060.27	DS 186.75 ± 129.88	P<0.0001**
Normal 207.50 ± 128.37	DS 186.75 ± 129.88	P>0.05
Autism 79.95 ± 45.48	FXS 1182.93 ± 1060.27	P<0.0001**

**Significant difference.

significantly high levels of Glutamate compared to other groups (FXS, DS, and normal controls). Meanwhile, cases with FXS demonstrated significantly higher glutamate levels compared to DS patients and normal healthy controls. On the other hand, when the 2 control groups were compared to each other, DS children had significantly higher Glutamate levels compared to neurotypical children.

Table 2: Glutamate levels in the different studied groups (µmol/L).

Group Mean ± SD	Group Mean ± SD	P Value
Autism 164.09 ± 33.29	Normal 67.05 ± 15.05	P<0.0001**
FXS 121.18 ± 24.19	Normal 67.05 ± 15.05	P<0.0001**
Autism 164.09 ± 33.29	DS 85.67 ± 9.40	P<0.0001**
FXS 121.18 ± 24.19	DS 85.67 ± 9.40	P<0.0001**
Normal 67.05 ± 15.05	DS 85.67 ± 9.40	P<0.0001**
Autism 164.09 ± 33.29	FXS 121.18 ± 24.19	P<0.0001**

**Significant difference.

GABA level in autism was significantly higher (117.90 ± 33.13) when compared to the neurotypical children (78.75 ± 21.63) and DS (66.40 ± 27.47) (Table 3). Also, GABA in FXS children was also significantly higher (125.35 ± 49.19) when compared to neurotypical children and DS children. Interestingly, FXS children had higher GABA levels than children with autism; however, this difference was not statistically significant. When the 2 control groups were compared to each other, DS children showed lower GABA levels (66.40 ± 27.47) than control group (78.75 ± 21.63). However, this difference was not statistically significant.

Table 3: GABA levels in the different studied groups (ng/ml).

Group Mean± SD	Group Mean± SD	P Value
Autism 117.90 ± 33.13	Normal 78.75 ± 21.63	P<0.0001**
FXS 125.35 ± 49.19	Normal 78.75 ± 21.63	P<0.0001**
Autism 117.90 ± 33.13	DS 66.40 ± 27.47	P<0.0001**
FXS 125.35 ± 49.19	DS 66.40 ± 27.47	P<0.0001**
Normal 78.75 ± 21.63	DS 66.40 ± 27.47	P>0.05
Autism 117.90 ± 33.13	FXS 125.35 ± 49.19	P>0.05

**Significant difference.

The twenty autistic children were also classified according to the Childhood Autism Rating Scale into 9 with severe autism and 11 with mild to moderate autism. Children with severe autism showed higher but non-significant levels of glutamate and serotonin when compared to mild-moderate autism (Table 4). Meanwhile, GABA levels showed non-significantly lower levels in severe autism compared to mild-moderate autism. Also Fragile X children with autistic features had non-significantly higher levels of Glutamate and Serotonin compared to Fragile X children without autistic features (Table 5).

Table 4: Neurotransmitters in mild-moderate and severe autism.

Neurotransmitters	Mild-Moderate Autism	Severe Autism	P Value
Serotonin (ng/ml)	73.11 ± 40.87	85.54± 50.18	P>0.05
Glutamate (µmol/L)	1.48 ± 43.94	1.76 ± 13.21	P>0.05
GABA (ng/ml)	1.35 ± 24.94	1.03 ± 32.84	P>0.05

Table 5: Neurotransmitters in FXS with and without autistic features.

Neurotransmitters	FXS with autistic features	FXS without autistic features	P Value
Serotonin (ng/ml)	1563.66 ± 1168	1019 ± 1010.96	P>0.05
Glutamate (µmol/L)	125.46 ± 19.76	119 ± 26.33	P>0.05
GABA (ng/ml)	112.50 ± 60.7	130 ± 44.8	P>0.05

Discussion

The present study evaluated 3 neurotransmitters in autism compared to Fragile X Syndrome to assess the possibility of neurochemical similarities between the 2 disorders. Autism and Fragile X syndrome have an overlap at the molecular level [18]. Proteins that regulate the balance of activity in brain glutamate and GABA systems, including PSD95 and Arc were found to be defective. These proteins are directly regulated by FMRP [18].

Previous studies have estimated different neurotransmitters in autism compared either to their relatives or normal healthy children. To our knowledge, this is the first study that evaluated Serotonin, Glutamate and GABA in autism compared to Fragile X syndrome; also it is first study to evaluate Glutamate and GABA in Fragile X Syndrome.

The most studied neurotransmitter in autism is Serotonin which is related to many human behaviors including mood, sleep, appetite and memory. An early study in 1961 showed an increased levels of endogenous 5-HT in whole blood in 26% of autistic children [19]. These results have also been confirmed by other studies [20].

In the present study, significantly low levels of plasma serotonin were detected in autism when compared to the other 2 groups (Fragile X Syndrome, and neurotypical children).These results are in agreement with those of Spivak et al., [21] who estimated plasma serotonin in 10 autistic children compared to 12 neurotypical children and concluded

that low plasma serotonin levels may have a role in the pathophysiology and symptomatology of autism, However another study in Saudi Arabia [22] detected high plasma serotonin levels in 16 Saudi autistic children compared to 16 neurotypical controls and concluded that high lead level is associated with remarkable high levels of serotonin.

The detection of low plasma serotonin levels in our study can be explained by the possibility of enhanced serotonin accumulation in platelet [23] that subsequently associated with increased platelet serotonin levels and parallel-diminished free plasma serotonin levels. Also low cerebrospinal fluid levels of 5- hydroxyindoleaceticacid [24] and low serotonin urinary excretion rate in autism [25] supports our findings.

High significant levels of serotonin in Fragile X were detected in present study compared to autism, neurotypical children and D.S. Another study reported normal serotonin levels in 12 fragile autistic children out of 13 and only one had low serotonin levels [26].

High serum glutamate levels were reported in autism compared to normal controls [27, 28]. Plasma amino acid analyses were carried out on a recent cohort of 138 autistic children and 138 normal controls using reverse-phase HPLC, it concluded that elevated levels of excitatory amino acids (glutamate and asparagine), decreased essential amino acids (phenylalanine, tryptophan and methionine) and decreased precursors of neurotransmitters (tyrosine and tryptophan) are the distinct characteristics of plasma amino acid profile of autistic children (Naushad et al., 2013) [29].

Another study also reported high levels of glutamate and homocystein in autism while the levels of glutamine and tryptophan are decreased (Ghanizadeh 2013) [30].

Our study replicated these results and could explain some clinical symptoms accompanied with autism as seizures and Electroencephalogram (EEG) abnormalities. Valproic acid is a mood stabilizer and was found to be effective intreanting autistic patients with or without clinical seizures but with epileptiform abnormalities [31].This explains the high rates of seizure disorders in autism that are due to high glutamate levels.

Increased glutamate levels in autism could be explained by presence of low levels of Pyridoxal Phosphate (PLP) in children with autism due to very low activity of pyridoxal kinase leading to blockage of glutamate utilization [32]. Also, alterations in the glutamate transporter or in glutaminase or glutamine synthetase activity would potentially affect glutamate levels [33].

Few studies had been conducted to assess the Glutamate in Fragile X syndrome, one of these studies was by Gruss and Braun [34] who reported unaltered Glutamate in all regions of brain in both ages (Juvenile and Adult)**.** The present study reported high significant levels of Glutamate in autism compared to neurotypical children and to D.S.

The final neurotransmitter that was assessed in this study was GABA which is known to play a crucial role in synaptic tuning and neuronal wiring in late pre and early postnatal days (Ben Ari et al.,, 2012) [35]. Studies from animal models of ASDs indicate that a dysfunction in GABAergic signaling within particular neuronal circuits may account for most of the clinical symptoms found in autistic patients. The high co-morbidity of ASDs with epilepsy (30% of cases) further confirms this issue (Frye et al., 2013) [36].

Plasma GABA was significantly high in autistic children compared to neurotypical and Down syndrome. Our results were in concordant with Dhossche et al. [37]. However, they detected significantly higher plasma GABA levels in 9 youngsters with autism than in 9 youngsters with Attention Deficit Hyperactivity Disorder (ADHD). Several studies also supported the high GABA levels in autism. A study had been conducted in Saudi Arabia showed high levels of lead and GABA in plasma [22]. Increased plasma GABA levels were found to be associated with reduced numbers of neurons expressing GABA and low brain GABA levels [38]. Divalproex is effective in treating autistic children who showed improved behavior due to elevation of brain GABA by inhibiting catabolic enzyme of GABA (GABA Transaminase) [39].

Some clinical observations have been added to support the role of GABA in autism [37]. There is increasing evidence that GABA neuronal dysfunction is implicated in various psychiatric disorders including schizophrenia, mood disorders, and anxiety disorders [40]. In some disorders, GABA dysfunction may occur in conjunction with abnormalities in reelin, a glycoprotein involved in the developmental regulation of GABAergic transmission Reductions of reelin in the cerebellar cortex of people with autism have also been reported [41]. Abnormalities on the long arm of chromosome15 have been found in a small proportion of autistic people**.** A cluster of genes coding for GABA A receptor subunits have been identified in that location (chromosome15q11-13). The GABA receptor beta-3 subunit gene has been implicated as an autism susceptibility locus in previous genetic studies, although the evidence is far from conclusive [42]. It is possible that in a subgroup of autistic people, GABA dysfunction is present in some brain areas owing to abnormalities of the assembly of GABA A receptor subunits into the GABA receptor complex. Thirdly, a few reports have suggested that benzodiazepines, i.e., positive modulators of GABA metabolism, have a negative and even paradoxical effect in autistic people.

Finally, the present study detected high significant levels of GABA in Fragile X children compared to normal healthy children and to D.S. Previous study [34] reported that GABA was significantly higher in the brainstem of FMR 1 knockout mice than in wild type.

The involvement of GABAA receptors in ASDs was provided by genetic studies that have revealed submicroscopic abnormalities known as “copy-number variations” in chromosomal loci 15q11–q13, which contains a number of genes encoding for GABAA receptor subunits Coghlan et al., 2012 [43].

It became more evident that autism and Fragile X Syndrome share a lot on the molecular and biochemical levels, so treatments that are used for FXS can also be beneficial for some autistic patients. There are large list of genes implicated in autism which can be mapped onto specific brain pathways [18]. Fragile X syndrome and autism are intertwined. Up regulation of m Glu R 5 pathways wand down regulation of GABA A pathways were reported in Fragile X children and nowadays treatment are aiming at reversing these problems [44]. These treatment can also be very useful for some children who are having autism.

In conclusion, the present study showed that low serotonin levels was exclusively and unique to autistic patients and it can be consider as a metabolic marker for autism. Also we found that autism and FXS share neurobiological similarities as GABA was significantly high in both disorders than neurotypical children, meanwhile it was specific to Autism and FXS as GABA level was not high in D.S. On the contrary DS showed lower (non significant) GABA levels than neurotypical children. With regards Glutamate, it was high in the 3 disorders but with the highest levels in Autism followed by FXS and DS, respectively.

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