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GABA and Glutamate Imbalance in Autism and Their Reversal as Novel Hypothesis for Effective Treatment Strategy

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by reduced social communication and repetitive behaviors. The etiological mechanisms of ASD are still unknown; however, the GABAergic system has received considerable attention due to its potential as a therapeutic target. Based on the fact that individuals with autism demonstrate altered gene expression concomitant with impaired blood brain barrier (BBB), and gut barrier integrities, so increased glutamate levels in the blood and platelets of ASD patients can be related to lower numbers of cerebellar GABAergic neurons, less active GABA-synthesizing enzymes, and decreased brain GABA levels. Excitotoxic levels of released glutamate trigger a cascade of deleterious cellular events leading to delayed neuronal death. According to our understanding of glutamate excitotoxicity, GABA supplementation could theoretically be useful to treat certain autistic phenotypes. While there is still no effective and safe medication for glutamate-related cell damage and death, combined efforts will hopefully develop better treatment options. Here I hypothesize that an integrated treatment strategy with GABA supplements, regulation of chloride (Cl-) and magnesium (Mg2+) levels, vitamin D supplements, probiotics to enhance GABAA receptor and glutamate decarboxylase (GAD) expression, and memantine to activate glutamate transporters and inhibit NMDA receptors, could collectively reduce glutamate levels, maintain functional GABA receptors and thus treat repetitive behavior, impaired social behavior, and seizure activity in individuals with autism.

Keywords: autism; glutamate excitotoxicity; gamma-aminobutyric acid; vitamin D; gut microbiota

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ГАМК, дефициты нейротрансмиттера глутамата при аутизме и их нейтрализация как новая гипотеза эффективной стратегии лечения

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Расстройства аутистического спектра (РАС) — это нарушения психического развития, характеризующиеся снижением социального взаимодействия и повторяющимся поведением. Механизмы этиологии РАС все еще неизвестны; однако ГАМК-эргической системе уделяется большое внимание в связи с тем, что она обладает потенциалом терапевтической мишени. Основываясь на том факте, что у людей с аутизмом отмечена измененная экспрессия генов, сопутствующая нарушению гематоэнцефалического барьера (ГЭБ) и целостности кишечного барьера, повышенный уровень глутамата в крови и тромбоцитах пациентов с РАС может быть связан с меньшим количеством мозжечковых ГАМК-ергических нейронов, менее активными ГАМК-синтезирующими ферментами и сниженным уровнем ГАМК в мозге. Эксайтотоксичные уровни высвобождения глутамата запускают каскад разрушительных клеточных событий, приводящих к отсроченной гибели нейронов. В соответствии с нашим пониманием эксайтотоксичности глутамата добавки ГАМК теоретически могут быть полезны при лечении определенных фенотипов аутизма. Хотя эффективных и безопасных препаратов, предотвращающих вызванные глутаматом повреждение и гибель клеток, до сих пор не существует, мы надеемся, что благодаря совместным усилиям нам удастся разработать лучшие варианты лечения. В данной статье я выдвинула гипотезу о том, что использование интегрированной стратегии лечения с применением добавок ГАМК, регуляцией уровня хлорида (Cl-) и магния (Mg2+), добавками витамина D, пробиотиков для усиления экспрессии рецепторов ГАМК-А и глутаматдекарбоксилазы (GAD), а также мемантина для активации транспортеров глутамата и ингибирования NMDA рецепторов, может привести к снижению уровней глутамата, поддержать функционирование рецепторов ГАМК и тем самым воздействовать на повторяющееся поведение, нарушения социального взаимодействия и судорожные припадки у людей с аутизмом.

Ключевые слова: аутизм; эксайтотоксичность глутамата; гамма-аминомасляная кислота; витамин Д; кишечная микробиота.

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Introduction

Autism spectrum disorder (ASD) is a highly heterogeneous, lifelong neurodevelopmental disorder that clinically presented as social interaction and communication impairments together with restricted interests and stereotyped behaviors [34]. Its prevalence is growing; therefore, there is a need to determine the key etiological mechanisms that would facilitate the identification of predictive and diagnostic markers that might help develop treatments. There are multiple of etiopathological mechanisms attributed to autism; the most known being systemic immune activation and excitotoxicity. It is commonly believed that chronic inflammation is the hallmark of many neurodevelopmental disorders [81; 93] and research on autistic patients has uncovered a number of immune dysfunctions [37; 87]. Neuroinflammation has been described in postmortem brain specimens of both young and old individuals with autism [56; 93; 122].

Progress is being made based on accumulating evidence that genetic and environmental risk factors for autism converge to disturb the balance between glutamate-mediated excitatory and γ-aminobutyric acid (GABA)-mediated inhibitory neurotransmission; and this may help to identify treatment targets for the disorder [18; 89; 104; 107].

The hypothesis that GABA can be targeted as an appropriate means of treating autism is supported by the relatively high incidence of epilepsy in patients suffering from autism, as well as the high frequency of epileptiform activity observed in the electroencephalograms (EEG) of autistic patients [101].

Moreover, electrophysiological and behavioral autism-like features have been observed in mice after blockade of maternal oxytocin signaling, which is important for the early postnatal excitatory-to-inhibitory shift of GABAergic signaling [119; 120].

Based on this information, the model of excitation-inhibition imbalance is one of the leading hypotheses to explain the etiology of autism. There-

fore, a precise and integrated adjustment of brain metabolism through regulation of glutamic acid and GABA could be used as treatment strategy for autism.

We do not yet have a clear mechanism of action regarding GABA supplementation and we have yet to fully understand the role of GABA's behavioral effects, as well as its blood-brain barrier (BBB) permeability in humans. However, evidence from numerous clinical studies strongly supports the therapeutic effects of GABA in the brain [16; 52; 72; 99].

Interlinked Metabolism of Glutamate and GABA

It is well known that glutamate and GABA metabolism is strongly interlinked, so a change in any of the intermediate metabolite can affect both neurotransmitters. Glutaminase as an enzyme catalyzes the conversion of glutamate to glutamine, allowing glutamine to be either stored in astrocytes or converted to glutamate in both glutamatergic and GABAergic neurons [103]. Between synaptic events, normal levels of glutamate and GABA are kept low via permanent transporter-mediated turnover through the plasma membrane [17; 19; 28; 111]. These transporters terminate synaptic neurotransmission, enabling the reuptake of the neurotransmitters. In excitatory neurons, glutamate is transported into vesicles through vesicular glutamate transporters, whereas in inhibitory neurons, glutamate is first transformed to GABA by glutamic acid decarboxylase (GAD), and then transported to vesicles via vesicular GABA transporters. Upon release, both neurotransmitters are taken up by high affinity transporters and returned into neurons and surrounding glia to be reused. Thus, GABA, glutamate, and glutamine are in constant flux. In autism however, the levels of the enzymes controlling glutamine-glutamate-GABA cycles are altered and thus, glutamine-glutamate-GABA metabolism is likely to be unusual in the ASD brain [46; 128].

Among clinical samples, high concentrations of glutamate and glutamine (Glx), and GABA in the temporal lobe, and high levels of glutamate in the auditory cortex have been directly related to the severity of autistic phenotypes [22]. Increased anterior cingulate glutamate/creatine and Glx have been associated with severe social interaction and communication impairments [38; 117]. The hyper-glutamatergic hypothesis of autism reported by Fatemi [47] suggests that lower levels of the GAD enzyme, and increased numbers of astrocytes, which take up synaptic glutamate to resynthesize glutamine and glutamate, leads to excess cortical glutamate in autistic patients. The remarkably lower levels of the 65 and 67 kDa GAD isoforms in individuals with autism may account for the reported increases of glutamate in blood and platelets of autistic subjects [42]. GAD deficiency may be due to, or associated with, abnormalities in levels of glutamate/GABA, or transporter/receptor density in the autistic brain.

Conversion of glutamate to glutamine by glutamine synthetase requires ammonia, which help to clear both molecules. It is well known that patients with liver dysfunction and children with urea cycle disorders cannot efficiently detoxify ammonia, inducing high levels of ammonia and glutamine in the brain [48]. Liu et al. [75] reported that autistic children had high brain ammonia but low levels of glutamine, showing that their serum glutamine levels were low and they had impaired glutamate transporters. This was supported Saleem et al [107] who recorded increased levels of ammonia, a marked reduction in urea concentration, and significant increases in the glutamate/glutamine ratio in the plasma of patients with autism compared with controls, suggesting that the glutamate/glutamine cycle was greatly impaired in these patients. Additionally, Liu [75] identified a panel of 7 urinary amino acid indicators that could discriminate between urine samples from ASD and healthy control children. Collectively, they find a possible imbalance between excitatory and inhibitory amino acid metabolism in ASD children. The significantly altered urinary amino acid indicators could therefore be potential diagnostic biomarkers for ASD.

Noticing high plasma ammonia and high GABA in blood and urine of an autistic boy, Cohen [29] noted plasma GABA levels are positively correlated with those of plasma ammonia. Dhossche et al [33] found high plasma GABA in autistic children aged between 5 and 15 years old. It is also very interesting to note that fever increases CSF taurine but decreases GABA [75].

It has been suggested that ammonia produced by the gut yeast *Candida albicans* forms a metabolite that works like GABA in autistic brains [24] Wakefield et al. [126] suspected that gut bacteria

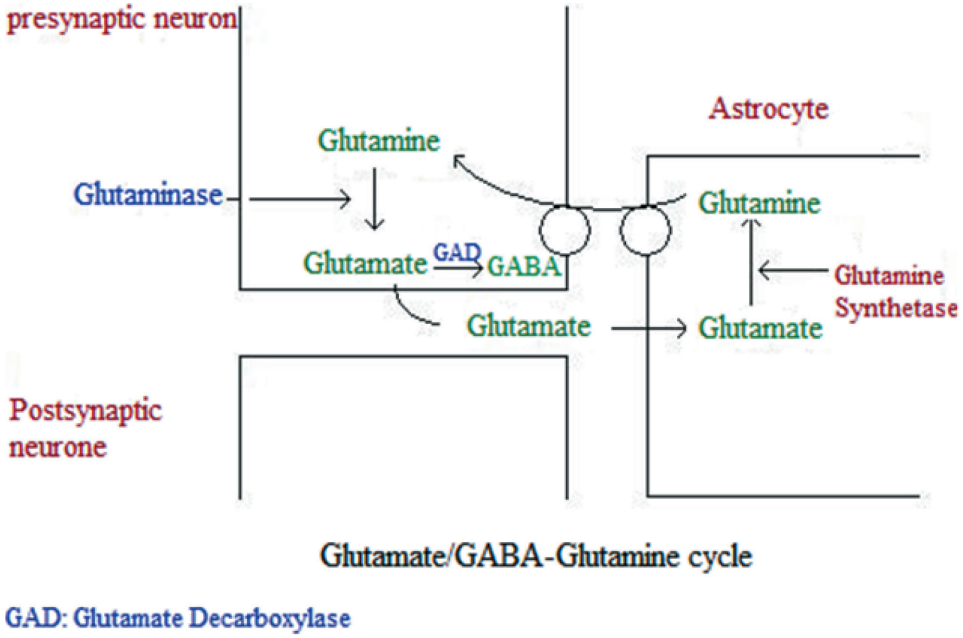

Fig. 1. Glutamate-GABA-Glutamine cycle

in children with autism generate much higher ammonia than their impaired liver can detoxify. This might happen, for example, if an oral antibiotic was given and induces overgrowth of pathogenic bacteria. Another pathogenic consequence is interrelated to glutamate is vaccination. Hoernlein [63] noted that vaccines, specifically those against measles, mumps, rubella (MMR), are usually enclosed in hydrolyzed gelatin, as a rich source of glutamate to reserve the viruses. In the presence of low-affinity glutamate transporter that exchange cystine and glutamate between the interior and exterior of the cell in a 1:1 ratio, extracellular glutamate usually accumulates, obstructs the cystine-glutamate exchange, and resulting in the reduction of cell stores of cystine as a major precursor of sulfur amino acids and glutathione. This may explain the association between glutamate excitotoxicity and oxidative stress as two etiological mechanisms of autism [39].

The GABAergic System in Autism

The role of the GABAergic system in autism has received a large amount of attention due to several factors. Autism studies have reported (1) reduced numbers of cerebellar GABAergic Purkinje cells especially in the posterior lobe [8; 127]; (2) lower activity levels of key synthesizing enzymes (GAD65 and GAD67) in the cerebellum and parietal cortex [46], and decreased levels of GAD67 in Purkinje cells [128]; (3) neuropathology in the deep cerebellar nuclei, a brain region rich with GABAergic neurons, with males more affected than females [23; 65; 88]; (4) reduced density of γ-Aminobutyric acid type A (GABAA) receptors in specific areas of the hippocampus [15; 70; 123]; (5) the most common chromosomal abnormality in autism is an alteration(s) in chromosome 15q11-q13, a region that contains three GABAA receptor subunit candidate genes for autism [108; 110], including the GABAA3 subunit receptor gene [79]; and (6) elevated plasma GABA in autistic children aged between 5 and 15 years [39; 42].

GABAA receptors are heteropentameric ionotropic receptors made of 19 different subunits. The majority contain two α, two β and one γ or δ subunit [91]. GABAA receptors that contain the α5 subunit (α5GABAA α5GABAA) are important because of their limited distribution and distinctive physiological and pharmacological characteristics [28; 74]. Extrasynaptic α5GABAA receptors are highly expressed in the hippocampus and at reduced levels in the cortex, and hypothalamus. Activation of these receptors creates a stimulant inhibitory current that moderates excitability and synaptic plasticity [26; 79]. α5GABAA receptors also play a trophic role that regulates the development of neural circuits [74].

It is very interesting to note that *Gabra5–/–* knocked out mice missing the α5 subunit gene, clinically presents multiple autism-like features, such as impaired social interaction, abnormal cognitive and memory functions, and sleep disturbances [85; 131]. Actually, the most common copy number variant in autism is a duplication of the q11.2–13 region of chromosome 15 [86], which encodes for the α5, β3, and γ3 subunits of the GABAA receptor [86]. Rare variants in the gene that encodes the α5 subunit have also been identified in autistic patients [131]. These findings suggest that dysfunctional α5GABAA receptors could be contributed to glutamate excitotoxicity as etiological mechanism in autism. The role of GABAA receptors in the etiology of autism was confirmed by Han et al. [61] who showed that low doses of benzodiazepines, as a GABAA receptor agonists, increase inhibitory neurotransmission through positive allosteric modulation of postsynaptic GABAA receptors in a rodent model of autism. This was concomitant with improved social interaction, reduced repetitive behavior, much better cognitive ability. In contrast, negative allosteric modulation of GABAA receptors impaired social behavior in C57BL/6J and 129SvJ wild-type mice.

Neuronal chloride control plays a role in the dynamic regulation of GABAergic inhibition both during and after brain development. This regulation is mostly reliant on two cation chloride co-transporters (CCCs), the K^+/Cl– co-transporter KCC2, and the Na^+/K^+/Cl– co-transporter NKCC1, whose activity can decrease or increase neuronal chloride levels respectively. Ben-Ari et al. [10] observed an elevated intracellular chloride (Cl^-) levels and excitatory GABA early during gestation followed by a perinatal excitatory-to-inhibitory shift. This mechanism is found in many brain areas of different animal species which suggest that it happens early in life. It is mediated mostly by the developmentally controlled gene expression of KCC2 and NKCC1, which are exporter and importer of Cl^- respectively. In spite of the straightforward function of GABA receptors in the transmission of information from the presynaptic to the postsynaptic neurons, some other factors are involved. Among these is the difference in membrane potential between the postsynaptic dendrite against the reversal potential for chloride ions. Based on this, GABA can evoke either depolarizing (excitatory) or hyperpolarizing (inhibitory) currents. This can be also affected by the local distribution of large anions, such as glutamate. Three conditions can be observed in which the reversal potential of chloride ions is below, above, or equal to the membrane resting potential. Low intracellular chloride levels can induce the influx of more negatively charged ions is low which can enhance the inhibitory function of GABAA receptors. At higher chloride levels, its reversal potential is above the resting potential of the cell, enhancing the excitatory effects of GABA. When the reversal potential for chloride equals the resting potential of the cell, stimulation of the GABAA receptors will cause no net flux of chloride and no alteration of the membrane potential. As the excitatory potential for GABA and the resting membrane potential are in relatively close proximity, small rises in Cl– can shift the polarity of GABAA currents from inhibitory to excitatory, highlighting the importance of maintaining low Cl^- [66; 76; 98]. Studies are now beginning to examine the connection between NKCC1 and KCC2 and the etiology of autism. Mutations in the C-terminal regulatory domain of KCC2 were found to be related with autistic phenotypes [30; 63; 84]. Several models of genetic disorders strongly associated with autism have been linked to alterations in NKCC1/KCC2 ratios [35]. Exposure of rats to valproate (VPA) during gestational period has shown a significant delay in the shift of GABA from excitatory to inhibitory [62]. Oral treatment with the selective NKCC1 antagonist bumetanide in pregnant VPA- treated rats immediately before delivery has shown a remarkable restoration of the effects of elevated Cl– and excitatory GABA signaling in the new born and can reduce the behavioral problems at childhood [102].

Glutamate Excitotoxicity as an Etiological Mechanism in Autism

Glutamate excitotoxicity arises when glutamate receptors (GluRs) are overstimulated with an excessive amount of the excitatory neurotransmitter, glutamate, followed by the increase of intracellular Ca^{2+} ions, finally lead to neuronal death [90].

Several studies have reported that glutamate excitotoxicity is one of the repeatedly recorded etiological mechanisms in autism. The GluRs are classified as ionotropic receptors, or metabotropic receptors (mGluRs) [43; 44]. Each type is composed of a variable association of subunits,

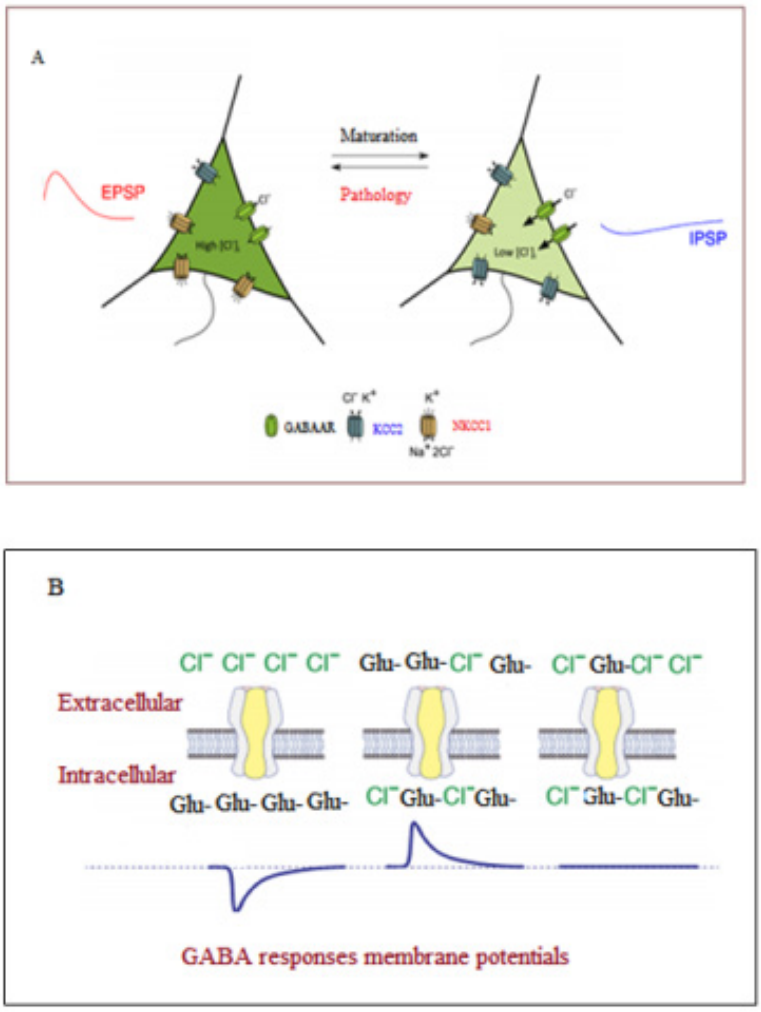

Fig. 2. A: Role of NKCC1/KCC2 ratio in GABA neurotransmission;
B: Different GABA responses membrane potentials

which determines their biophysical and physiological properties. N-methyl-D-aspartate receptor (NMDAR) is an ionotropic GluR that is classically composed of a tetrad arrangement of subtype receptor subunits — GluN1, GluN2A-D, and GluN3A and B. During development, there are significant modifications in the composition of these subunits. In the mammalian brain, functional NMDARs require a GluN1 subunit associated with one or more GluN2 subunits. Magnesium (Mg^{2+}) binding sites inside the NMDAR regulate its function, with Mg^{2+} playing a crucial role as a voltage-dependent channel blocker. Upon depolarization, the Mg^{2+} blockade is released, permitting the action potential to go on. Sensitivity to Mg^{2+} blockade varies with subunit ratio composition. Mg^{2+} ions mainly oppose Ca^{2+} ions, and Mg^{2+} deficiency induced brain seizures can be avoided by treating with NMDA-receptor antagonists [41]. Since Mg^{2+} is required for many brain enzymes, a striking decrease of Mg^{2+} in a propionic acid (PPA)-induced rodent model of autism was recently related to glutamate excitotoxicity as a persistent autistic feature in juvenile rats [41; 50; 78]. When Mg^{2+} deficiency occurs, excessive Ca^{2+} and glutamate can induce synaptic dysfunction in the brain, which can manifest as repetitive behavior, impaired social behavior, seizure activity, and hyperactivity, as previously reported [3; 31; 32].

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are ionotropic receptors composed of GluA1-4 subunits (GluR1-4 by older nomenclature). The presence of the GluA2 (GluR2) subunit in the AMPAR blocks Ca^{2+} entry into the neuron [14]. AMPAR sensitivity is regulated by whether they contain or lack a GluA2 (GluR2) subunit when they are transported to the

neuronal membrane. The latter causes the AMPAR to be Ca^{2+} permeable, and trafficking of GluA2-lacking AMPARs to the synaptic unit increases glutamate-induced neuronal activation, which is observed in long-term potentiation (LTP), plasticity, and during neurodevelopment. Under pathological conditions, such as inflammation, the activity of GluA2-lacking AMPARs can trigger excitotoxic damage [50; 71].

Edfawy et al. [36] studied the functional role of *Gprasp2*, a gene linked with neurodevelopmental disorders that encodes a protein that contributes to post-endocytic organization of G-protein-coupled receptors. They observed that *Gprasp2* deletion leads to clinically presented ASD features and changes in synaptic neurotransmission in mice. Manipulating the levels of *Gprasp2* expression reduced the surface availability of mGluR5 and produces alterations in dendritic complexity, spine density and synaptic maturation. These findings demonstrate a role for *Gprasp2* in glutamatergic synapses and suggest a possible mechanism by which this gene is linked to autism.

Astrocyte-mediated clearance of free glutamate from synaptic cleft is mostly accomplished through two astroglia-specific, high-affinity glutamate transporters called excitatory amino acid transporters 1 and 2 (EAAT1 and 2), classified in rodents as glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1), respectively. These transporters use the electrochemical gradients across the plasma membranes as motivating forces for glutamate transfer into the intracellular compartment [4]. Conventionally it was thought that following uptake into astrocytes, glutamate is converted into non-toxic glutamine by glutamine synthetase, which is then released into the intercellular fluid and taken into neurons to be used for glutamate recycling in order to replenish the neurotransmitter pool [112]. However, more recent data suggest that, on some physiological and pathological circumstances, astrocytes are capable of releasing glutamate via multiple mechanisms [25; 124]. Several mechanisms connecting astrocyte glutamate release with the excitotoxicity present in autism have been described, mainly involving microglia activation [11; 94; 115]. Based on their crucial role in neurological disorders, EAATs are targets for the development of new treatment strategies for brain diseases [59; 115] and a recent study has shown that ß-lactam antibiotics can remarkably increase the gene expression of *EAAT2/GLT-1* in a rodent model of autism [3].

High affinity glutamate (L-Glu) transporters facilitate L-Glu re-uptake into neurons and glial cells [20]. These transporters combine the uptake of L-Glu with the exchange of one H^+ ion, one K^+ ion, and 3 Na^+ ions, [6]. Impaired glutamate and glutamine transporters are repeatedly reported in autism [6]. Cystine is an essential amino acid for the biosynthesis of the reduced glutathione (GSH), and its uptake requires the cystine/glutamate exchanger SLC7A11 [95]. This transporter catalyzes the uptake of one molecule of cystine with the release of one molecule of L-Glu. As it is highly expressed in astrocytes but not in neurons, neurons are dependent on astrocytes for the production of GSH. Once cystine enters astrocytes, it forms the tripeptide GSH. Synthesized GSH can be released into the extracellular space followed by enzymatic catabolism, eventually leading to the formation of cysteine. Cysteine is then taken up by neurons through SLC1A1 transporter to synthesize GSH. GSH depletion as a pathological feature in autism could influence the capacity of cells to scavenge free radicals, making them susceptible to accumulate reactive oxygen species (ROS), possibly injuring of the L-Glu transporter SLC1A2, especially in motor neurons. Together with additional alterations, including activation of caspases as pro-apoptotic markers, this will finally lead to neuronal death.

Ford et al. [53] revealed that strong autistic tendencies are associated with the GABA+ concentration and an increased glutamate/GABA+ ratio in the lower right hemisphere of the brain. Previous reports also show that increased excitatory and reduced inhibitory neurotransmission is implicated across the autism spectrum, particularly in the social domain [54; 55; 68; 129]. These findings are consistent with evidence from animal studies showing that social deficits caused by increased glutamate/GABA+ ratio can be reduced by increasing in the inhibitory neurotransmission of GABAergic neurons [27; 109; 129].

To understand the role of microglia activation in excitatory/inhibitory imbalance in autism, Lieberman et al [73] proposed two different hypotheses. It is well known that the healthy developing brain initially form additional, inappropriate excitatory synapses. These unnecessary or less active synapses are pruned by microglia, and only functionally mature synapses are conserved [12]. Their first hypothesis

stated that in autism, microglia may fail to detect and prune immature synapses, which would result in the conservation of an excess number of glutamate excitatory synapses. Their second hypothesis stated that over-activated microglia may selectively prune GABAergic inhibitory synapses.

These hypotheses are consistent with the idea that disturbance of microglial activation by immune stimulation such as maternal infection during a critical period can deleteriously affect synaptogenesis [13]. Pruning by microglia is activity dependent, which suggests that excitotoxic stimulation during prenatal or early post-natal periods could also adversely affect brain architecture. Bilbo et al. have shown that activation of brain microglia early in life can have long-term consequences on brain function, even into adulthood [12; 13].

Vitamin D Deficiency and Imbalanced Excitatory/Inhibitory Neurotransmission in Autism

It is well known that autism is more prevalent in areas of relatively poor ultraviolet light exposure, such as urban areas, areas with high air pollution, and areas of high precipitation [45]. Animal studies have confirmed that severe vitamin D deficiency during pregnancy negatively affects numerous proteins which contribute to brain development, resulting in pathological alterations in neonatal animals similar to those seen in autistic patients [60; 125]. Meguid et al. [82] measured serum levels of vitamin D in Egyptian children with autism compared to healthy controls, and reported that the level of vitamin D3 was remarkably lower in autistic patients. Multiple studies have confirmed vitamin D deficiency as an etiological mechanism in autism [1; 40; 51; 57; 87; 125], whereas only two studies have shown no difference between children with autism and controls [121].

Based on this, vitamin D supplementation for autistic children with insufficient vitamin D is necessary. Saad et al. [105] reported that vitamin D supplementation can help as a treatment strategy in autism. Their cohort study of autism spectrum disorder children receiving vitamin D supplementation at a dose of 300 IU/kg/day showed that children with a final vitamin D3 serum level below 30 ng/ml demonstrated no improvements in clinical presentation, 31/102 children with final vitamin D3 serum levels between 30–39 ng/ml had decreased childhood autism rating scale scores (CARS) by 1.5–4.5 points, while those with a final serum vitamin D3 levels above 40 ng/ml had decreased CARS by 3.5 to 6.5 points. This seems to suggest that the lower limit for vitamin D levels in autism treatment is 40 ng/ml, or at least above 30 ng/ml. Feng et al [49] showed that autistic phenotypes were greatly improved after 3 months of vitamin D being either intramuscularly injected or orally supplemented, with younger patients being more responsive. Moreover, animal studies have shown that vitamin D demonstrates a protective rather than therapeutic effect on PPA-induced neurotoxicity in rats, as there was a remarkable amelioration of the impaired interferon-gamma IFN-γ, serotonin, Glutathione-s-transferase, and DNA damage [2]. Of course, additional large scale, international multi-center studies are essential to explore the relationships between the clinical response to vitamin D supplementation, long-term effect of vitamin D supplementation, and the biochemical changes in autistic patients.

Epidemiological studies have revealed that vitamin D deficiency is associated with a wide range of neuropsychiatric disorders, including autism [58; 60; 67; 69]. It is known that vitamin D deficiency significantly reduces the levels of glutamic acid decarboxylase, the key enzyme in GABAergic interneurons, and glutamate and glutamine in mouse brain tissue [60]. This suggests that glutamate excitotoxicity, and impaired glutamate-glutamine-GABA cycle in autism can be corrected through the treatment of vitamin D insufficiency or deficiency [125].

To validate this suggestion, we can consider the recent study by Krisanova et al. [69] who showed that vitamin D3 deficiency disturbs synaptic neurotransmission by affecting both Ca^{2+}-dependent and Ca^{2+}-independent processes. The Ca^{2+}-dependent action of vitamin D3 deficiency was associated with a decrease in exocytotic release of glutamate and GABA, presumably caused by malfunctioning voltage-gated Ca^{2+} channels. The Ca^{2+}-independent action of vitamin D3 deficiency was associated with a decrease in the expression of glutamate and GABA transporters that in turn result in the decrease of glutamate and GABA reuptake. Their main finding demonstrates that vitamin D3 deficiency could be and etiological mechanism in autism by contributing to an impaired glutamate/GABA transporter expressions and excitation/inhibition imbalance.

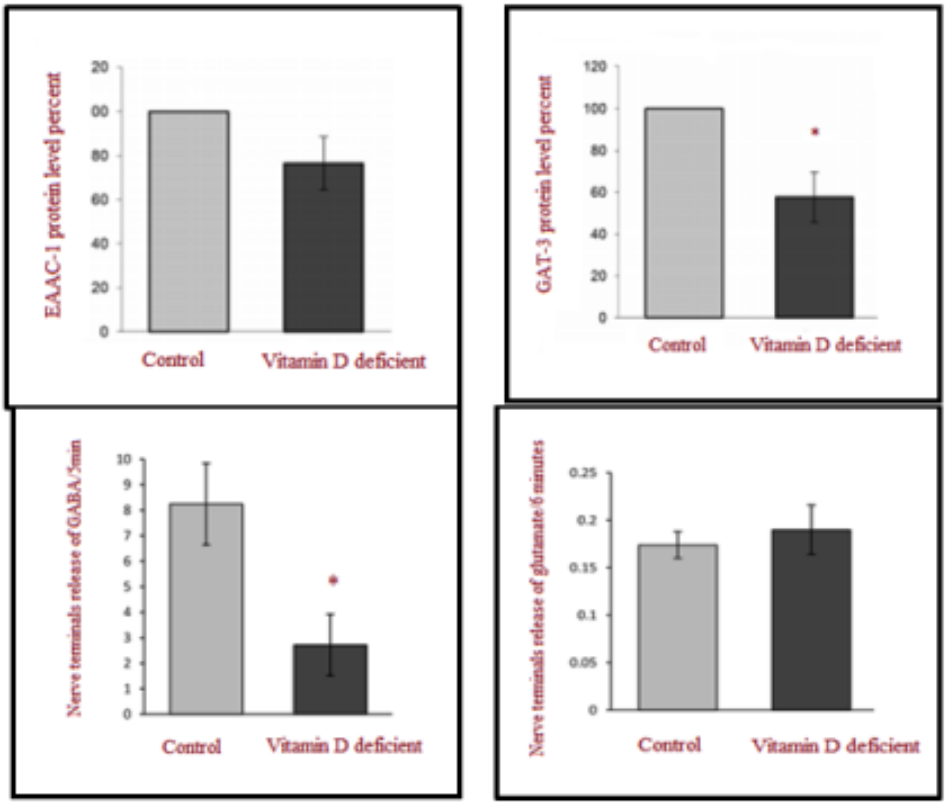

Fig. 3. Protein expression of EAAC-1 and GAT-3 transporters (UP) and release of GABA and glutamate from nerve terminals (Down) in control and vitamin D3-deficient rats (Modified from Krisanova et al., [70])

Role of Microbial Glutamate and Gaba sSignaling in Autism

Microbial endocrinology concerns the role of microorganism-produced neurochemicals such as GABA, glutamate, and serotonin, as a common language that allows crosstalk between microbe and host. Understanding the gut microbiota—brain axis in humans as a bidirectional cross talk system that links the gut with the brain through several pathways including endocrine, immune, and neural, might open the road toward the use of neurochemical-producing probiotics as a therapeutic strategy to treat autism.

The impaired methylation capacity reported in autism may be related to the imbalanced GABA/Glu ratio in autism. Impairment of the methylation pathway prevents the use of folate, which is broken down into glutamate. The Krebs cycle is critical for methylation, and can become impaired in a variety of ways, such as vitamin B deficiency, the presence of heavy metals, and toxins from bacteria or *Candida* [7]. *Candida* overgrowth is known to occur in autistic patients [64; 65]. If methylation is impaired, then it is even more important to manage glutamate levels.

Lactobacillus plantarum, *L. paracasei*, *L. lactis*, *Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, and *B. flavum* are among the bacterial strains which are capable of producing glutamate [116; 130]. A study has revealed that about 15% of lactic acid bacteria strains isolated from Asian fermented foods are glutamate producers [96]. Both Gram-positive and Gram-negative bacteria such as *E. coli* and *Pseudomonas* can produce GABA via the decarboxylation of glutamate catalyzed by GAD. This enzyme has been found to be associated with pH homeostasis and the generation of metabolic energy [9].

Amongst the microorganisms that are commonly identified as health-promoting probiotics, one *Lactobacillus* strain and four strains of *Bifidobacterium* isolated from the human intestine have been reported to be able to produce GABA [97]. Furthermore, an analysis on metagenomic data from the human microbiome project suggests that genes encoding GAD could be present in a significant proportion of human gut microbiota [21]. Real time PCR revealed decreases in both the *Lactobacillus* and *Prevotella* in both male and female *Shank3* knockout mice, a genetic model of

autism, compared to wild type mice. Moreover, examination of stool and colon microbiota of normal healthy female mice revealed a significant increase in genera and species of *Lactobacillus* compared to males. This suggests that higher levels of *Lactobacillus* in female mice could act as a protective factor and may explain the sex bias of autism towards males. Previous studies have suggested a possible connection between *Lactobacillus*, autism-related behaviors, and GABAergic function. In particular, Bravo et al. [21] determined that *Lactobacillus* may regulate GABA receptor expression in the brain through secretion of GABA. In *Shank3* knockout mice, GABA receptor expression is particularly affected in multiple brain regions, including the hippocampus, which is also one of the regions that have been shown to be affected in ASD patients. Pearson correlation analysis between levels of *L. reuteri*, *L. brevis*, *L. ruminis* and GABA receptor levels revealed specifically that the abundance of *L. reuteri* correlated significantly with expression of each of the three GABA receptor sub-units (GABARA1, GABARA2, and GABARA3).

Therapeutic Targets of Glutamate Excitotoxicity as Rational Treatment Strategy

1. Glutamate transporters GLAST/GLT-1

Recently Pajarillo et al. [92] reported that GLAST/GLT-1 may be dysregulated at the genetic, epigenetic, transcriptional or translational levels, leading to high levels of extracellular glutamate and excitotoxicity. Accordingly, understanding the regulatory mechanisms of GLAST/GLT-1 has been highlighted as a means to develop therapeutic targets for the treatment of autism [3; 92]. Pharmacological agents such as β-lactam antibiotics, estrogen/selective estrogen receptor modulators (SERMs), growth factors, histone deacetylase inhibitors (HDACi), and translational activators have presented noteworthy effectiveness in increasing the expression and function of GLAST/GLT-1 and glutamate uptake both *in vitro* and *in vivo* [92]. Torrez et al. [118] reported neuroprotective effects of memantine (MN), a glutamatergic NMDAR channel blocker. The drug could prevent the increase in cerebrospinal fluid (CSF) glutamate levels and cognitive decline in treated rats. It decreased glutamate uptake in the hippocampus and increased the release of S100B protein in the CSF in response to okadaic acid (OKA)-induced neurotoxicity. This identifies a promising neuron-astrocyte coupling protective mechanism, and sheds light on astrocytes as potential targets for treating glutamate excitotoxicity.

2. Oxidative stress:

Oxidative stress and related mitochondrial dysfunction are directly related to glutamate excitotoxicity in autism. Through the use of multiple regression analysis, El-Ansary [39] showed that high levels of lipid peroxidation (LPO), a marker of oxidative stress, together with lower enzymatic and non-enzymatic antioxidants (GSH, GSH/GSSG, thioredoxin, peroxiredoxins), were related to glutamate excitotoxicity, presented as glutamate, glutamine, glutamate/glutamine ratio, and glutamate dehydrogenase. This suggests that oxidative stress could be a target to treat glutamate excitotoxicity related phenotypes in autistic patients [5; 113].

LPO products affect the electron transport chain in the inner membrane of the mitochondria, leading to the loss of the membrane potential (ΔΨm) and boosting the generation of ROS, such as superoxide anion and hydrogen peroxide [83]. Although, neurons have strong antioxidant defenses, such as peroxiredoxins, superoxide dismutase enzymes (SODs) such as Cu^{2+}/ $Zn2^{+}$-SOD (SOD1), Mn^{2+}-SOD (SOD2), glutathione peroxidase, and catalase (in low amounts), their function is greatly compromised in autism [100]. Recently, Rivero-Segura et al [100] assessed the probable antioxidant effect of prolactin (PRL), a hormone secreted by numerous cells and tissues including the mammary glands, T-lymphocytes, and hypothalamus. They proved that PRL augments the activity and amount of the antioxidant SODs and lowers LPO as marker of oxidative stress, which is repeatedly reported to be significantly higher in autistic patients. Moreover, they demonstrate that PRL prevents mitochondrial dysfunction induced by glutamate and significantly ameliorates membrane potential (ΔΨm) of dysfunctional mitochondria which help to suggest its effectiveness as treatment option. Their related findings are presented collectively in *Fig. 4*.

3. Inactivation of NMDAR and activation of mGlu receptors:

Excessive activation of NMDARs leads to the accumulation of intracellular Ca^{2+} and consequent neuronal death [131]. Many compounds, includ-

ing memantine and neurosteroids, are able to target glutamate toxicity through the modulation of NMDARs. 20-oxo-5β-pregnan-3α-yl sulfate (pregnanolone sulfate, PAS) is an endogenous neurosteroid that inhibits NMDAR currents. Glutamatergic NMDAR has also been targeted with multiple synthetic neurosteroids [80; 114]. Among the studied compounds, the 3α, 5β-pregnanolone glutamate (PAG) -like steroid compound (*Fig. 5*) demonstrated the most promising NMDAR-mediated neuroprotective effect. This suggests it could act as a therapeutic agent for improving glutamate-related autistic phenotypes by decreasing Ca^{2+} level, scavenging ROS, and preventing glutamate-induced caspase-3 activation.

It is very interesting to note that functional GABAA receptors tend to cluster; overstimulation of glutamate NMDA receptors by high glutamate concentrations leads to calcium influx and the loss of GABAA receptor-clustering, which negatively affects the inhibitory effect of GABA. Glutamate can also bind to the mGluR receptor to induce the release of internally-stored calcium into the cytosol of the neuron. This calcium can, in turn, restore the clustering of postsynaptic GABAA receptors by interacting with protein kinase C. These findings demonstrate that glutamate signaling is activated by distinct receptors and calcium signaling patterns, which oppose the control of inhibitory GABA synapses (*Fig. 6*).

4. Activation of GABAergic receptor:

The release of excitotoxic levels of glutamate triggers a cascade of events leading to neuronal death. This phenomenon involves imbalance between excitation and inhibition. Mazzone and Nistri [80] hypothesized that augmenting the inhibitory network should prevent excitotoxicity and provide

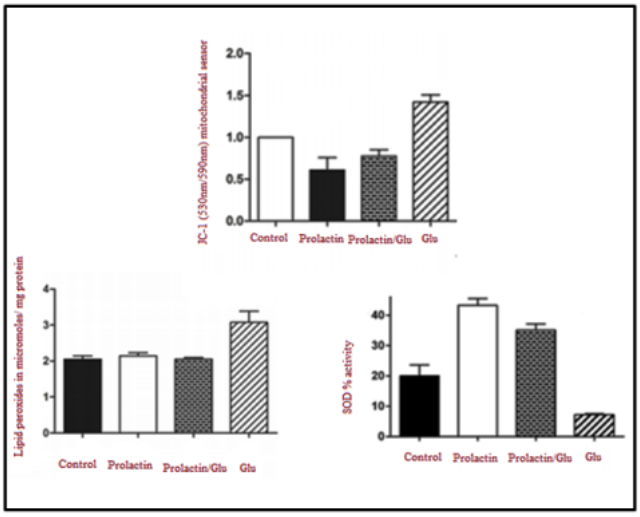

Fig. 4. Therapeutic effects of prolactin through amelioration of glutamate induced mitochondrial dysfunction, lipid peroxidation, and SOD antioxidant enzyme. JC-1 is a mitochondrial membrane potential (ΔΨm) sensor, which emits fluorescence at 590 nm when the mitochondrion is polarized (functional) and at 530 nm when mitochondrion is depolarized (dysfunctional).
(Modified from Rivero-Segura et al., 2019 [101])

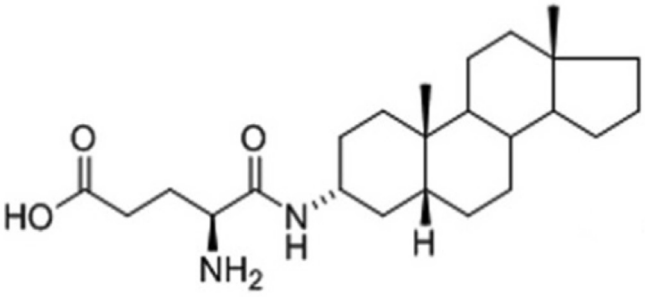

Fig. 5. Chemical structure of 3α, 5β-pregnanolone glutamate (PAG)-like steroid compound tested for a neuroprotective effect against glutamate-induced excitotoxicity

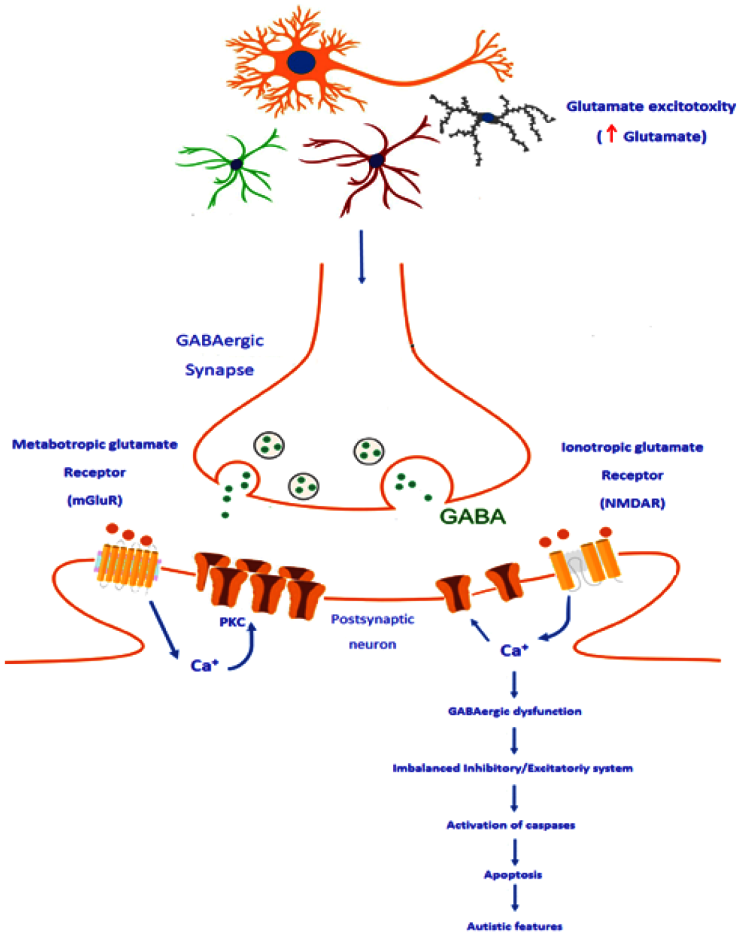

Fig. 6. A suggested mechanism of inhibitory connections dysfunction as an etiological mechanism in autism spectrum disorder through the loss of GABA receptors clustering caused by glutamate as an excitatory neurotransmitter. In the left part binding of glutamate to the mGluR receptor induces the release of internally stored calcium into the neuron's internal environment, activates protein kinase C to maintain clustering and functional GABAA receptors at the postsynaptic membrane. Under the effect of glutamate excitotoxicity, and down-regulation of mGluR receptors in ASD patients, NMDA receptor overstimulation with glutamate leads to an excess of incoming calcium, which causes the receptors to become more spread out, reducing how much GABA can inhibit the neuron. This can lead to an imbalance of the inhibitory/excitatory system, followed by activation of caspases as pro-apoptotic proteins, and finally leading to autistic features

neuroprotection. They proved that glutamate release induced by kainate was intensely decreased by the allosteric GABAA modulator midazolam (10 nM) or the GABA agonist 4,5,6,7 — tetrahydroisoxazolo [5,4-c]-pyridin-3-ol (THIP; 10 μM), leading to neuroprotection.

Conclusion

This work presents evidence of glutamate excitotoxicity's involvement in autism, making it a potentially viable treatment strategy in ASD. Correction of imbalanced Glut/ GABA ratio in individuals with autism may be tried at different levels through the use of multiple targets such as: 1) activation of GLAST/GLT-1 to increase glutamate reuptake; 2) amelioration of oxidative stress; 3) inactivation of NMDAR and activation of mGlu receptors to induce the proper function of GABA receptors; 4) supplementation of GABA; 5) use of GABA and GAD producing probiotics such as lactobacillus and bifidobacterial; and 6) activation of GABAergic receptors. Collectively, these targets can help to lower glutamate levels and thus induce the proper function of GABA receptors, which make GABA supplementation a successful and promising treatment strategy.

Future research in this direction will help to design specific drugs targeting these signaling proteins and possibly modulate the expression dynamics of glutamate NMDAR and mGlu receptors,

transporters, and GABA receptors for therapeutic application in autism.

Further studies using the optimal animal model followed by clinical trials are recommended to prove the safety and efficacy of the suggested strategy in order to improve the success of translational research and future clinical applications.

List of abbreviations:

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs); Childhood autism rating scale scores (CARS); Cation chloride co-transporters (CCCs); Cerebrospinal fluid (CSF); Electroencephalogram (EEG); Excitatory amino acid transporters 1 (EAAT1); Glutamate receptors (GluRs); Glutamate aspartate transporter (GLAST); γ-aminobutyric acid (GABA); Glutamate (Glu); Glutamic acid decarboxylase (GAD); Glutamate transporter-1 (GLT-1); Glutamine (Glx); γ-Aminobutyric acid type A (GABAA); Glutathione (GSH); Histone deacetylase inhibitors (HDACi); Interferon-gamma IFN-$^{\gamma}$; K^+/Cl^- Co-transporter (KCC2); Long-term potentiation (LTP); $Na^+/K^+/Cl^-$ co-transporter (NKCC1); N-methyl-D-aspartate receptor (NMDAR); Reactive oxygen species (ROS); Superoxide dismutase enzymes (SODs); Prolactin (PRL); Propionic acid (PPA); Valproate (VPA).

Compliance with Ethical Standards.

Competing interests:

No conflict of interests.

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