METABOLIC DISORDERS ASSOCIATED WITH AUTISM SPECTRUM DISORDERS: APPROACHES FOR INTERVENTION

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METABOLIC DISORDERS ASSOCIATED WITH AUTISM SPECTRUM DISORDERS: APPROACHES FOR INTERVENTION

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Editorial: Metabolic Disorders Associated With Autism Spectrum Disorders: Approaches for Intervention

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Editorial on the Research Topic

Metabolic Disorders Associated With Autism Spectrum Disorders: Approaches for Intervention

Autism spectrum disorder (ASD) is a neurodevelopmental disease that impairs efficient responses to external sensory-stimulus and defective social interaction. ASD affects 1% of the population and has different severity degrees (Maenner et al., 2020). The pathogenesis of ASD is not completely understood, but involves a combination of genetic, environmental factors and immune dysfunction. Associated with immune system activation, ASD results from the central nervous system derangements due to chronic inflammatory reactions (Toscano et al., 2021). In addition, ASD is associated with several comorbidities, including gastrointestinal disturbances, feeding problems, selective eating behaviors, and obesity (McElhanon et al., 2014; Granich et al., 2016). In this context, metabolic, inflammatory, and brain diseases are likely mutually linked; however, a clear mechanistic understanding has remained elusive.

The major research goals of this collection were to find causes, examine developmental trajectories of the disorder, and develop new or improved time-dependent interventions or preventions to contribute to the overall health and well-being of ASD individuals. Specifically, improving early and accurate strategies for diagnosis, understanding the role of the endocrine, metabolic, immunologic pathways, and neuropathology of ASD, and testing potential dietary and behavioral interventions for ASD.

Gut peptide hormones have been found across different brain regions, and clinical studies have proposed that many of those hormones are involved in autism-related social, emotional, and cognitive deficits. Qi and Zhang reviewed and summarized major findings from clinical and animal studies showing the role of gut peptide hormones (CCK, VIP, secretin, ghrelin, and PACAP) in mediating autism-related neurological functions and their potential implications in autism pathogenesis and autism-related neurological functions (Qi and Zhang). The authors also discussed the potential use of these gut hormones as therapeutic targets to manage ASD symptoms (Qi and Zhang).

Preclinical and clinical studies have shown that ASD individuals display alterations of the gut microbiota and their metabolite production, which can be used as biomarkers. Biomarkers related to the gut-brain axis would greatly value the diagnosis, development, and follow-up of potential

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therapies for patients with ASD. The link between ASD and the gastrointestinal microbiome, their metabolites, and the use of metabolites as biomarkers (such as short-chain fatty acids, phenolic compounds, and free amino acids) was reviewed (Garcia-Gutierrez et al.). The production of metabolites and neurotransmitters by gut microbiota can stimulate the immune system and influence the central nervous system by vagal stimulation. Thus, therapies-based interventions that modulate gut microbiota (probiotics, prebiotics, and fecal microbiota transplantation therapy) have arisen as a potential treatment to ameliorate ASD symptoms (Garcia-Gutierrez et al.).

A significant number of ASD cases could be associated with various metabolic abnormalities, some of them identifiable only through untargeted metabolomic profiling, emphasizing the causal role of inborn errors of metabolism in ASD. Symptoms of ASD can be present in many inborn errors of metabolism but rarely occur in isolation. Žigman et al. give an overview of inborn errors of metabolism characterized by symptoms of ASD. The authors also proposed a diagnostic approach to assess cases in clinical practice and possible specific therapies. The authors briefly describe etiology, clinical presentation, and therapeutic principles, for several inborn errors of metabolism in differential diagnosis of ASD (disorders of the amino acid metabolism, mitochondrial disorders, cholesterol biosynthesis defects, neurotransmitter disorders, folate metabolism, lysosomal storage disorders, among others). As always, detailed medical history and clinical examination, including detailed neurological examination, should be a basis for planning focused diagnostic work-up of patients with ASD (Žigman et al.).

ASD individuals have deficiencies in motor skills. Physical activity reduces maladaptive behaviors, such as stereotypes, and positively affects social skills in young children and adolescents with autism. The association of classical pharmacological treatments with exercise and other physical activities to intervention programs with children with ASD may be beneficial. Sefen et al. reviewed the exercise-therapy as an appropriate therapeutic strategy for improving the quality of life in ASD (Sefen et al.). The authors addressed the beneficial effects of structured physical activities for children with ASD and discussed the importance of parental involvement in physical activity (Sefen et al.). The authors point out that to build a physical activity program for children with ASD, it is important to address multiple factors to decide the most appropriate elements of the program, such as individual vs. group intervention and the organization of the program. Each person with autism has a highly individualized set of symptoms and characteristics for which highly individualized physical activity programs are warranted (Sefen et al.).

Sensory hyper-responsiveness is included in the restricted interests and repetitive behaviors central to an ASD diagnosis. Studies have observed that altered γ-aminobutyric acid (GABA) neurotransmission is a central characteristic of the neurophysiology of ASD and may explain the sensory abnormalities. The group of Masakazu Ide assessed whether GABA concentrations in specific brain areas [primary visual cortex, sensorimotor cortex, supplementary motor area (SMA), and ventral premotor cortex (vPMC)] were associated with different domains of abnormal sensory experiences in individuals with ASD (Umesawa et al.). Also, using ^{1}H magnetic resonance spectroscopy, they addressed whether GABA levels were associated with abnormal sensory responses in ASD (Umesawa et al.). They observed a negative association between left vPMC GABA and the severity of sensory hyper-responsiveness. The authors suggest that reduced inhibitory neurotransmission in a higher-order motor area that integrates multiple sensory modalities may underlie sensory hyper-responsiveness in ASD (Umesawa et al.).

A final article aimed to identify the relationship between ASD and cholinergic signaling using a systems biology model. Acetylcholine plays a key role in cognitive function, memory, learning, and sensory processing signal transduction. Autism genome-wide association studies (GWAS) have provided estimates of the overlap of autism-related gene sets with those involved in sensory processing and cholinergic signal transmission. The authors evaluated the relationship between ASD and choline intake (Olson et al.). This article documented a gene set enrichment analysis results focusing on shared gene ontologies between an autism-associated gene set obtained through meta-analysis of GWAS and a gene set centering on cholinergic function (Olson et al.).

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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The Potential Role of Gut Peptide Hormones in Autism Spectrum Disorder

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Gut peptide hormones are one group of secretory factors produced from gastrointestinal endocrine cells with potent functions in modulating digestive functions. In recent decades, they have been found across different brain regions, many of which are involved in autism-related social, emotional and cognitive deficits. Clinical studies have revealed possible correlation between those hormones and autism spectrum disorder pathogenesis. In animal models, gut peptide hormones modulate neurodevelopment, synaptic transmission and neural plasticity, explaining their behavioral relevance. This review article will summarize major findings from both clinical and basic research showing the role of gut peptide hormones in mediating autism-related neurological functions, and their potential implications in autism pathogenesis. The pharmaceutical value of gut hormones in alleviating autism-associated behavioral syndromes will be discussed to provide new insights for future drug development.

Keywords: autism, cholecystokinin, ghrelin, gut peptide hormone, pituitary adenylate cyclase activating peptide, secretin, vasoactive intestinal peptide

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INTRODUCTION

Autism spectrum disorder (ASD) affects more than 1.5% of children worldwide (Christensen et al., 2016). It is estimated that genetic factors contribute to about 60% of ASD cases (Tick et al., 2016) although there are still episodic cases that cannot be fully explained by hereditary factors. Emerging evidence is suggesting gut dysfunctions in ASD, which is frequently correlated with digestive disorders. Typical gastrointestinal (GI) symptoms associated with ASD include abdominal pain, flatulence, indigestion or diarrhea (Adams et al., 2011), whose incidence were as higher as 70% in ASD children (Li and Zhou, 2016).

Gut peptide hormones refer to a group of polypeptides derived from GI tract and can exert multiple physiological functions via binding with specific receptors. Gut peptides have a long history since the identification of its first member, secretin, at almost 100 years ago. Currently dozens of molecules have been classified into this group, including cholecystokinin (CCK),

vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), peptide YY (PYY), secretin, pancreatic polypeptide, ghrelin and so on (Small and Bloom, 2004; Wren and Bloom, 2007; Neary and Batterham, 2009). Those secretory factors have been recognized for their roles in modulating GI functions and body energy metabolism. In recent 20∼30 years, their brain functions are being gradually revealed from molecular, behavioral and physiological evidence (Zhang and Chow, 2014; Donahue et al., 2016; Kingsbury and Wilson, 2016). For example, CCK and VIP are now working as markers for specific sub-population of neurons having unique morphological features of axon collaterals and firing patterns (Kawaguchi and Kondo, 2002). On the other hand, gut peptides also directly participate in the regulation of neural functions across different brain regions. Among those neurological effects, people are more familiar with the central appetite control in which gut peptides can sense the satiety and energy requirement, and mediate the activity of central feeding nuclei within arcuate nucleus (Arc) of the hypothalamus and the nucleus of the solitary tract (NTS) via vagal afferent pathway or direct effects on central nuclei (Konturek et al., 2004; Small and Bloom, 2004; Wren and Bloom, 2007; Troke et al., 2014; Roman et al., 2017).

Besides the feeding and metabolic regulation, the potential role of gut peptide hormones in other behavioral paradigms is emerging in recent decades. Notably, some gut peptides are involved in social functions. Examples include secretin, whose gene knockout in mice leads to social interaction deficits (Nishijima et al., 2006), and VIP with potent functions for social affiliation and aggression (Kingsbury and Wilson, 2016). As social deficit is one of core symptoms of ASD, one may speculate that gut peptides are related with ASD pathogenesis. Therefore, further understandings of gut peptide hormones in ASD should help to better elucidate both pathogenic mechanism and to develop potential intervention strategy of ASD.

In this mini review, for each gut peptide hormone we will start with its distribution and functions in the central nervous system, following with the evidence of its involvement in ASD. We will also discuss about the neurobiological mechanisms of those gut-brain peptides, with particular interests in mediating social and cognitive deficits as well as stereotypic behaviors, all of which are the core behavioral symptoms of ASD. Lastly, the potency of drug development targeting those gut peptides and their receptors in alleviating ASD-related symptoms will be discussed.

GUT PEPTIDE HORMONES AND ASD

Gut peptide hormones exert various neuromodulating effects as suggested by their wide distribution across different brain regions. Recent advances in neural science techniques including gene knockdown, *in vivo* recording, opto-/chemo-genetics have made it possible to delineate specific functions of those peptides within certain neural circuits including those related with ASD.

CCK

As one widely studied gut-brain peptide, CCK can be found within a certain group of inhibitory neurons and has known effects in mediating appetite and food intake (Degen et al., 2001). Previous studies have found that social isolation increases CCK mRNA expression in amygdala, hippocampus, cortex and ventral tegmental area (VTA) nuclei of rats (Del Bel and Guimaraes, 1997). Clinical assays also showed that decreased serum CCK-8 (Brambilla et al., 1997) is found in ASD children. Furthermore, in patients with Asperger's syndrome (AS), a subgroup of autistic patients, one fragment deletion has been found in CCK gene (Iourov et al., 2015). Those animal and clinical data thus suggest the possible linkage between CCK and ASD.

Some studies have already been done to investigate the potential neurological mechanisms of CCK in social behaviors. Targeted mutation of CCK receptor alters social isolation-related behaviors in female mice (Abramov et al., 2004). The selective activation of CCK-neurons (Whissell et al., 2019) and selective modulation of CCK receptors (Lemaire et al., 1992) enhances social recognition or social memory. At the upstream of CCK, endocannabinoid system plays a critical role. In hippocampal nuclei, endogenous cannabinoid receptor 1 (CB1R) suppresses CCK-positive neurons, leading to altered social behaviors (Vargish et al., 2017). Further analysis showed that CB1 modulated CCK transmission to direct social withdraw behaviors (Seillier et al., 2013). A recent study also found that CB1R in the amygdala CCK-positive afferents to nucleus accumbens modulates social defeat-induced depressive behaviors (Shen et al., 2019). The inter-correlation between endocannabinoid and CCK system may reside in the co-expression of CB1R and CCK in certain groups of interneurons (Rovira-Esteban et al., 2017). In future, more studies can be performed to elucidate the neural circuit of CCK in mediating social behaviors.

PACAP

PACAP is one polypeptide hormone sharing high sequence similarity with secretin and is also involved in social modulation. Genetic study investigated more than 1,000 ASD children with their normal siblings and identified one specific loci (rs1557299) at the downstream of PACAP gene (Nijmeijer et al., 2010) which suggested close relationship between PACAP and ASD. Furthermore, animal studies showed that PACAP is an important gut peptide in modulating social and emotional behaviors. For example, the intracerebroventricular infusion of PACAP into rat brains leads to the decrease of social behaviors (Donahue et al., 2016). On the other hand, PACAP – deficient mice presented the attenuation of depressive disorders after chronic social defeat stress (Lehmann et al., 2013). However, the knockout of PACAP type 1 receptor (PAC1) in male mice decreased social investigations after repeated exposure to the same subject, in conjunction with excessive sexual mounting and lower aggressive behaviors (Nicot et al., 2004). Those seemingly contradictory phenotypes suggest the homeostatic regulation of PACAP in maintaining normal social behaviors. The administration of PACAP also leads to prominently enhanced locomotor activity plus rearing behaviors in mice (Masuo et al., 1995; Norrholm et al., 2005), and the deficiency of PACAP in mice results in hyperactivity and jumping behaviors in a novel field (Hashimoto et al., 2001). Therefore, PACAP mediates various behavioral phenotypes that can be related with ASD.

Electrophysiological studies reveal that PACAP improves synaptic plasticity in mouse hippocampus (Cabezas-Llobet et al., 2018), and increases both frequency and amplitude of excitatory postsynaptic currents (EPSCs) of autonomic synapse (Starr and Margiotta, 2017), giving clues of how PACAP is involved in the autism-related behaviors. In addition, studies are gradually revealing the modulatory function of PACAP in neurodevelopment and neuroprotection, which might give further explanation for its involvement in ASD, which is one neurodevelopmental disorder. As further evidence, PACAP can stimulate the growth of both axons (Ogata et al., 2015) and dendritic spines (Cabezas-Llobet et al., 2018) under normal or disease conditions. Moreover, PACAP receptor PAC1 also mediates the differentiation and migration of cortical neuronal progenitors (Adnani et al., 2015).

VIP

VIP is mainly expressed in the hypothalamic nuclei such as suprachiasmatic nucleus (SCN) and tuberal hypothalamus as well as hippocampus and cerebral cortex (Acsady et al., 1996a,b), and is now commonly accepted as a neuroendocrine hormone, putative neurotransmitter and cytokine. Its biological effects are mediated by binding to the G-protein coupled receptors (GPCRs), VPAC1 and VPAC2, which have high affinity to VIP (Iourov et al., 2015). Previously, it has been shown to stimulate prolactin secretion from the pituitary (Reichlin, 1988) and catecholamine release from the adrenal medulla (Malhotra et al., 1988). It also participates in non-adrenergic, non-cholinergic relaxation of both vascular and non-vascular smooth muscle as co-transmitters with nitric oxide and carbon monoxide (Said and Rattan, 2004).

Recent evidence suggested a close relationship between VIP and ASD. Newborns with higher VIP concentrations in the cord blood were more likely to develop autism in later periods (Nelson et al., 2001). Preliminary evidence has showed that ASD phenotype is associated with VPAC2 receptor duplication (Levinson et al., 2011). The involvement of this duplication in ASD is further confirmed by a recent report observing such copy number variants in autistic child and his father with a mild autism (Firouzabadi et al., 2017). Preclinical studies support the roles of VIP in social and cognitive functions. After VIP antagonist treatment during the embryonic stage, male offspring exhibited reduced sociability and deficits in cognitive function, as assessed through cued and contextual fear conditioning (Hill et al., 2007). Male offspring of pregnant females with VIP deficiency also exhibited deficits in social approach behavior and reversal learning, with more severe and higher prevalence compared to their female littermates (Lim et al., 2008; Stack et al., 2008). VIP-deficient mice exhibited a pronounced reduction in social recall when tested 48-h or longer after primary training (Chaudhury et al., 2008). On the other hand, some early studies showed that the intracerebroventricular administration of VIP also caused a marked impairment in passive avoidance and spatial memory, together with neuronal dystrophy (Takashima et al., 1993a,b). As further supporting evidence, VIP can stimulate neurogenesis as well as cell differentiation to promote neuronal survival and regeneration probably via the induction of nerve growth factor and activity dependent neuroprotective protein (Hill et al., 2002; Giladi et al., 2007). In addition, VIP has been observed to modulate synaptic transmission efficiency via the regulation of brain-derived neurotrophic factor (BDNF) expression in cortical neurons (Pellegri et al., 1998; Ciranna and Cavallaro, 2003), providing possible mechanism for behavioral modulation.

Ghrelin

Ghrelin, a 28-amino-acid peptide hormone, is mainly produced by gastric oxyntic cells. In the central nervous system, ghrelin has been detected in the hypothalamus such as Arc, ventromedial and paraventricular nucleus areas (PVN) (Kojima et al., 1999; Lu et al., 2002; Hori et al., 2008). The hypothalamic roles of ghrelin consist of appetited regulation, glucose homeostasis, and growth hormone release from pituitary and body weight regulation (Kojima and Kangawa, 2005). Furthermore, ghrelin is found in extra-hypothalamic areas such as hippocampus, sensory-motor cortex and cingulate cortex which might be involved in higher cognitive functions (Hou et al., 2006). Ghrelin is present in two major forms: un-acylated ghrelin (des-acyl ghrelin or DAG) and acylated ghrelin (AG, C-ghrelin, or also referred to as ghrelin) which was produced by ghrelin *O*-a-cyl-transferase (GOAT) from DAG. Only AG is able to bind to the growth hormone secretagogue receptor type 1 a (GHSR1a), a G protein-coupled receptor, to execute their biological functions (Kojima et al., 1999, 2001). GHSR1a is widely distributed in the brain such as in the hypothalamus, hippocampus, substantia nigra, VTA and several thalamic and brain stem nuclei (Guan et al., 1997). Those expressional data indicate the potential neural function of ghrelin.

Recent study revealed the close relationship between ghrelin and ASD. For example, the expression level of un-acylated ghrelin acylated ghrelin in blood is lower in children with autism (Al-Zaid et al., 2014). Ghrelin has also been postulated to be one promising target in co-morbid between autism and epilepsy (Ghanizadeh, 2011). Animal studies show that ghrelin plays important roles in learning and memory, sleep and appetite, all of which are disturbed in ASD. Intracerebroventricular or intra-hippocampal injection of ghrelin increased memory acquisition and/or consolidation, but not in memory retrieval (Carlini et al., 2002, 2010). Ghrelin-knockout mice exhibited decreased hippocampal spine density and abnormal memory that can be reversed by ghrelin injection (Diano et al., 2006). In addition, GHSR1a knockout mice showed a decreased food intake after three days on restricted feeding schedule and failed to show preference to rewarding high-fat diet food (Abizaid et al., 2006), suggesting the role of ghrelin in reward circuitry modulation. In physiological terms, ghrelin was found to enhance synaptic plasticity by increasing spine formation and long term potentiation (LTP), to promote neurogenesis (Zhang et al., 2004; Moon et al., 2009), to preserve mitochondrial integrity, and to inhibit the apoptotic process and neuronal cell death (Chung et al., 2007; Lee et al., 2010). Moreover, ghrelin was recently reported to reduce mRNA expression of tumor necrosis factor-α (TNF-α) and nuclear factor kappa B (NF-κB) in lymphoblastoid

cell lines from ASD subjects (Yamashita et al., 2019), suggesting the possible involvement of neuroinflammatory modulation of ghrelin.

Secretin

Secretin is one classical gut-peptide hormone to modulate pancreatic secretion as well as body energy homeostasis. Several lines of evidence from rodent and human studies show that secretin and its receptors are widely distributed in the brain areas from cerebral cortex, hippocampus, amygdala, hypothalamus to cerebellum (Yuan et al., 2011; Wang et al., 2019). The neurological functions of secretin are also being gradually explored by molecular, physiological and behavioral approaches (Zhang and Chow, 2014; Wang et al., 2019).

In 1998, a case series reported secretin in amelioration of the symptoms in three ASD children (Horvath et al., 1998). However, later double-blind placebo-controlled clinical studies revealed that there was a lack of significant impact of secretin in the treatment of ASD symptoms (Esch and Carr, 2004; Malone et al., 2005; Krishnaswami et al., 2011; Lyra et al., 2017). In spite of the failure in clinical studies, animal obervations support the roles of secretin in some social functions. For example, secretin receptor deficient mice presented impaired social interaction (Nishijima et al., 2006), and one later study revealed the potentiation of social recognition by secretin infusion via the activation of oxytocin pathway (Takayanagi et al., 2017). One possible mechanism exists as secretin increases the release of vasopressin (Chu et al., 2009) which can regulate social behaviors (Dumais and Veenema, 2016). Besides social regulation, in a mutant mouse model with stereotypically circling locomotion, secretin infusion effectively attenuated those abnormalities (Koves et al., 2011). Altogether, these data might indicate an indirect roles played by secretin in the ASD pathogenesis and treatment.

For investigating the molecular and cellular mechanism, secretin has been found to induce the spinogenesis of hippocampal neurons (Nishijima et al., 2006), and the deficiency of secretin impairs both the induction and maintenance of LTP in hippocampal neurons (Yamagata et al., 2008). We recently found that secretin could potentiate postnatal proliferation and migration of cerebellar granular neurons (Wang et al., 2017) and potentiate inhibitory postsynaptic currents (IPSCs) of cerebellar Purkinje neurons (Yung et al., 2001), adding further knowledge to the neural plasticity modulation by secretin. As the secretin receptors mostly belong to GPCR family, the activation of cyclic AMP (cAMP)–protein kinase A (PKA) pathway in brain regions have been established for secretin (Pang et al., 2015). This also holds true for VIP and PACAP, which have high degree of sequence similarity with secretin (Jozwiak-Bebenista et al., 2015). The activation of cAMP–PKA pathway exerts pluripotent functions, among which the modulation of ion channels or neurotransmitters directly affects synaptic transmission (Wang et al., 2019). Alternatively, other downstream pathways such as cAMP-response element – binding protein (CREB) activation (Mak et al., 2019) can initiate the translation of synaptic proteins to modulate long-term plasticity and memory functions. The extracellular signal regulated kinase (ERK) pathway is also involved upon secretin stimulation (Wang et al., 2017). In sum, secretin is one possible modulator of social functions, and is worth further investigation to reveal detailed circuitry effects.

IMPLICATIONS FOR AUTISM DRUG DEVELOPMENT

Based on the inter-correlation between ASD pathogenesis and potentially neurological mechanisms in regulating social, emotional and repetitive behaviors by gut peptides, it is expected that those hormones may work as drug candidate or targets for developing novel drugs in alleviating ASD symptoms. However, failures are frequently reported when directly trying the administration of those peptides to ameliorate autistic behaviors. For example, one clinical trial failed to detect significantly improvement of social phobia by using CCK-tetrapeptide (CCK-4) (Katzman et al., 2004), and many double-blind placebo-controlled studies using secretin to alleviate autism symptoms infusion have also been failed (Esch and Carr, 2004; Malone et al., 2005; Krishnaswami et al., 2011; Lyra et al., 2017) even though the existence of one early study reporting the beneficial effects of secretin (Horvath et al., 1998). Therefore, major challenges are faced by those gut hormones probably due to their rapid turnover *in vivo* and low efficiency for penetrating the blood brain barrier (BBB). Large amounts of works are thus required to overcome those disadvantages and to generate drug candidates with satisfactory efficiency, persistency and safety.

The short half-life is one common property of gut hormones due to their peptide composition. For example, secretin has less than 5 min half-life under normal physiological conditions (Kolts and McGuigan, 1977), making it impossible to achieve stable drug concentration *in vivo*. Such limitation can be addressed by two approaches: the development of stable analog or receptor agonist, or the application of bioengineered sustained drug delivery system. The former approach is based on molecular design and screening of small molecules, or the modification of existing receptor ligands, to generate long-lasting effects as those of natural ligands. Alternatively, one can adopt nanoparticles system to chronically release peptide drugs such as those used in immunomodulation therapy for releasing autoantigens (Stabler et al., 2019). In drug development, the issue of BBB permeability should also be considered case-by-case. Current knowledge agree that VIP, secretin and PACAP27 can be transported into the brain by a non-saturable mechanism, or transmembrane diffusion, whilst PACAP38 and gastric inhibitory polypeptide (GIP) have no known routes in enter the central nervous system (Dogrukol-Ak et al., 2004). Those data thus suggest that it is necessary to develop new drug delivery system to penetrate the BBB and to chronically release effective gut peptide hormones.

CONCLUSION

Gut peptide hormones are now recognized as having prominent brain distributions and various behavioral modulatory functions. Clinical studies with ASD subjects have revealed that the

genetic polymorphisms of gut peptides such as CCK, PACAP, and VIP are related to ASD. Altered expression levels of the peptides including CCK and ghrelin are found in ASD children. Additionally, more animal studies suggest that these gut peptides are actively involved in the modulation of social, emotional and stereotypic behaviors. In future, one should perform more site-specific studies to illustrate the precise mechanisms of gut peptides in mediating social or emotional or cognitive behaviors for potential drug targets. Further drug development can be pursued to generate stable analogs that can modulate gut peptide pathways in the brain, aiming to relieve behavioral symptoms of ASD.

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GABA Concentration in the Left Ventral Premotor Cortex Associates With Sensory Hyper-Responsiveness in Autism Spectrum Disorders Without Intellectual Disability

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Individuals with autism spectrum disorder (ASD) often exhibit abnormal processing of sensory inputs from multiple modalities and higher-order cognitive/behavioral response to those inputs. Several lines of evidence suggest that altered γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, is a central characteristic of the neurophysiology of ASD. The relationship between GABA in particular brain regions and atypical sensory processing in ASD is poorly understood. We therefore employed ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) to examine whether GABA levels in brain regions critical to higher-order motor and/or multiple sensory functions were associated with abnormal sensory responses in ASD. We evaluated atypical sensory processing with a clinically-validated assessment tool. Furthermore, we measured GABA levels in four regions: one each in the primary visual cortex, the left sensorimotor cortex, the left supplementary motor area (SMA), and the left ventral premotor cortex (vPMC). The latter two regions are thought to be involved in executing and coordinating cognitive and behavioral functions in response to multisensory inputs. We found severer sensory hyper-responsiveness in ASD relative to control participants. We also found reduced GABA concentrations in the left SMA but no differences in other regions of interest between ASD and control participants. A correlation analysis revealed a negative association between left vPMC GABA and the severity of sensory hyper-responsiveness across all participants, and the independent ASD group. These findings suggest that reduced inhibitory neurotransmission (reduced GABA) in a higher-order motor area, which modulates motor commands and integrates multiple sensory modalities, may underlie sensory hyper-responsiveness in ASD.

Keywords: autism spectrum disorder, gamma-aminobutyric acid, sensory hyper-responsiveness, magnetic resonance spectroscopy, ventral premotor cortex

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INTRODUCTION

Individuals with autism spectrum disorder (ASD) often exhibit sensory abnormalities [for more, see Marco et al. (2011)]. Sensory hyper- and hypo-responsiveness are frequently observed in autistic individuals, although this is not part of the core definition of autism (APA, 2013). There is individual variation in the sensory modalities that are most disrupted in individuals with autism, and the sensory abnormalities could be seen in all sensory domains (Kientz and Dunn, 1997; Tomchek and Dunn, 2007; Lane et al., 2014). Findings from clinical contexts have revealed abnormal sensory processing in autism, not just in the sensitivity to sensory inputs, but also in later cognitive/behavioral reactivity, including passive avoiding and/or seeking external stimuli (Lane et al., 2014; Damiano-Goodwin et al., 2018; Schulz and Stevenson, 2019). Sensory processing involves registration and modulation of sensory information, as well as an internal organization of afferent inputs (Humphry, 2002). Indeed, sensory hyper-responsiveness is a key feature included in the restricted interests and repetitive behaviors central to an ASD diagnosis (APA, 2013), and some studies have further demonstrated that sensory stimuli detection sensitivity was insufficient to describe the severity of sensory hyperresponsiveness in autism (Ide et al., 2019; Schulz and Stevenson, 2019). Altered sensory processing therefore, may occur in the stream of information processing involving higher-order cognitive processing (Thye et al., 2018).

The molecular biology of autism has revealed that altered γ-aminobutyric acid (GABA)-mediated signaling within some brain circuits, may explain the sensory abnormalities seen in ASD (Rubenstein and Merzenich, 2003; Cellot and Cherubini, 2014; Braat and Kooy, 2015; Foss-Feig et al., 2017; Robertson and Baron-Cohen, 2017). Altered inhibitory GABAergic transmission may lead to an abnormal excitatory/inhibitory balance in the brain, which can alter neural signaling and information processing, as well as responding behavior (Foss-Feig et al., 2017). Recent *in vivo* studies have also revealed reduced GABA concentrations across multiple cortical areas of the autistic brain (Harada et al., 2011; Gaetz et al., 2014; Rojas et al., 2014; Puts et al., 2017; Sapey-Triomphe et al., 2019). Collectively, these findings indicate that altered GABAergic signaling may be related to the abnormal daily sensory experience of individuals with autism.

The aim of the present study was to examine the relationship between subjectively evaluated atypical sensory processing and GABA concentrations in primary sensory and motor areas and cortical regions involved in higher-order cognitive and behavioral functions. While higher-order motor related areas have been implicated in action responses and multimodal cognitive processes (Rizzolatti et al., 2014), whether GABA concentrations in those areas correlate with abnormal sensory processing in ASD remains unknown. To assess this, we measured GABA concentrations in multiple brain regions using ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) in the present study.

We analyzed two major higher-order motor regions-the ventral premotor cortex (vPMC) and the supplementary motor area (SMA), which have been demonstrated to have tight neural connections with sensorimotor cortex in humans and primates (Luppino et al., 1993; Yeo et al., 2011). Previous studies have suggested that the vPMC is involved in multiple sensory processing (Iacoboni and Dapretto, 2006; Bekrater-Bodmann et al., 2011; Ide et al., 2020), especially for response modulation or inhibition to sensory signals when a change of the reaction patterns is needed (Buch et al., 2010). As the execution of motor sequences and imitation of actions involved in higher-order motor areas further lateralizes to the left hemisphere (Hlustík et al., 2002; Vingerhoets et al., 2013; Reader and Holmes, 2018), we hypothesized that weakened inhibition in the left vPMC would associate with atypical sensory processing observed in ASD. The SMA is known to be involved in voluntary motor execution, motor planning, and coordinated body movements (Roland et al., 1980; Tanji et al., 1988; Sumner and Husain, 2008) rather than functions in the sensory domains. We further assessed two additional regions [the primary visual cortex (V1) (Robertson et al., 2016) and the sensorimotor cortex (SMC) (Puts et al., 2017)] in which GABA levels and perceptual performance may be related and abnormal in ASD.

MATERIALS AND METHODS

Participants

Seventeen adolescent and adult participants with ASD (12 males) and 18 typically developing (TD) participants (11 males) were recruited. Demographic data for both groups are summarized in **Table 1**. Individuals with a clinical diagnosis of ASD were recruited from parent groups of children with developmental disorders and the Department of Child Psychiatry at the National Rehabilitation Center for Persons with Disabilities. We recruited all the participants by random sampling, regardless of their genetic background and diagnosis of abnormal sensory processing. It should also be noted that we have no information regarding any motor disabilities in each individual. None of the participants recruited in this study were excluded from the analysis. To assess the validity of diagnostic group differences, we used the Japanese version of the Autism Quotient (AQ) scale (Baron-Cohen et al., 2001; Wakabayashi et al., 2004), in

TABLE 1 | Demographic information and differences between groups.

	ASD group	TD group
Sex (M:F)	12:5 (*N* = 17)	11:7 (*N* = 18)
Age, mean years (range)	21.5 ± 3.2	22.7 ± 6.0
LQ, mean (range)	68.9 ± 36.9	82.1 ± 33.3
AQ, mean (range)**	32.6 ± 8.1	20.1 ± 6.2
VIQ, mean (range)	111.8 ± 16.0	115.5 ± 12.1
PIQ, mean (range)	105.4 ± 17.8	112.4 ± 13.5
FIQ, mean (range)	109.4 ± 14.2	115.6 ± 11.3

***p < 0.01. M, male; F, female; ASD, autism spectrum disorder; TD, typically developing; LQ, laterality quotient; AQ, Autism spectrum Quotient; VIQ, verbal intelligence quotient; PIQ, performance intelligence quotient; FIQ, full-scale intelligence quotient. The AQ score was evaluated by the Autism spectrum Quotient (AQ) scale. The LQ score was assessed using the Edinburgh Handedness Inventory (Oldfield, 1971). The intellectual quotients (IQs) were assessed by the Wechsler Adult Intelligence-Third Edition (WAIS-III). Asterisk indicates significant difference between groups found by two-tailed t test.*

which higher scores indicate stronger autistic traits. None of the TD participants had AQ scores above the threshold (cut-off: 33) and a two-tailed t test revealed significantly higher AQ scores in ASD participants than in TD controls (t_{33} = 5.162, $p < 0.01$, Cohen's d = 1.75). One female ASD participant (age 23), who did not receive a clinical diagnosis, was included in the ASD group because of her AQ score of 37, which exceeded the diagnostic threshold. We further used the Wechsler Adult Intelligence Scale-Third Edition (WAIS-III) to assess participant Intelligence Quotients (IQs). No participants had full-scale IQs below 75. All participants and their parents gave written informed consent for study participation after all study procedures were fully explained. The present study was approved by the Ethics committee of the National Rehabilitation Center for Persons with Disabilities. The present experiment adhered to institutional safety procedures for human brain imaging. Note that the participants and their ^{1}H-MRS data of the left SMC and SMA were partially overlapped with those employed in Umesawa et al. (2020); 14 ASD (three females) and 11 TD (five females) participants.

Adolescent/Adult Sensory Profile

We evaluated individual sensory responsiveness using the Japanese version of the Adolescent/Adult Sensory Profile (AASP) (Brown et al., 2001), which originated from Dunn's model of sensory processing disorders (Dunn, 1997) and is based on Ayres' theory of sensory integration (Ayres, 1979). The AASP is broadly accepted for the characterization of altered sensation in individuals with ASD and is a subjective questionnaire which consists of 60 items classified into four subscales (normal range): low registration (23–38), sensation seeking (30–47), sensory sensitivity (25–42), and sensation avoiding (25–41). Low registration reflects how easily an individual misses sensory information, while sensation seeking indicates a tendency to seek out sensory stimulation. Sensory sensitivity indicates a heightened awareness of sensory stimuli and sensory avoiding reflects a tendency to withdraw from strong sensory input. The first two scales indicate the severity of sensory hypo-responsiveness and the others represent hyper-responsiveness (Dunn, 2001).

MR Acquisition

We acquired magnetic resonance imaging (MRI) data on a 3T Siemens Skyra scanner (Siemens, Erlangen, Germany) with a 64-channel head coil. We ran two sessions with a sequence designed to obtain anatomical images and two sequences for ^{1}H-MRS within a day (i.e., each participant underwent four sessions total). First, we obtained a high-resolution *T1*-weighted anatomical image using a magnetization-prepared rapid acquisition by gradient echo sequence [number of slices = 224, slice thickness = 1 mm, repetition time (TR) = 2300 ms, echo time (TE) = 2.98 ms, flip angle = 9°] to set regions of interest (ROIs) with a voxel size of 20 mm × 20 mm × 20 mm (Nakai and Okanoya, 2016). Based on this anatomical image, we manually determined different ROIs (see section "Regions of Interest (ROI)") across multiple sessions.

Regions of Interest (ROI)

Recent studies have found that specific perceptual functions, are associated with an atypical role for GABA in several ASD brain regions. We set two ROIs, the bilateral V1 and the left SMC based on those previous knowledges, in addition to the left SMA and vPMC (referred to as PMC: **Figure 1**).

Typically developing individuals with higher GABA concentrations in the visual cortex exhibited increased suppression of visual perception, but this association was absent in ASD participants (Robertson et al., 2016). Autistic children with higher GABA levels in the sensorimotor cortex had lower sensitivity to vibrotactile input amplitude after adaptation to it (Puts et al., 2017). While neurotypical children with higher GABA levels in that region exhibited greater sensitivity to the frequency of a given stimuli, children with autism didn't exhibit this (Puts et al., 2017). Another study examined associations between subjective individual difficulties in sensory processing, a psychophysical index, and somatosensory cortex GABA levels (Sapey-Triomphe et al., 2019). This study reported higher GABA levels and higher frequencies of atypical tactile experiences (as per a self-reported questionnaire) in individuals with ASD.

The anatomical definitions of ROIs were as follows; the V1 ROI was midline of the occipital cortex (Muthukumaraswamy et al., 2012). The SMC ROI included the "hand-knob" of the

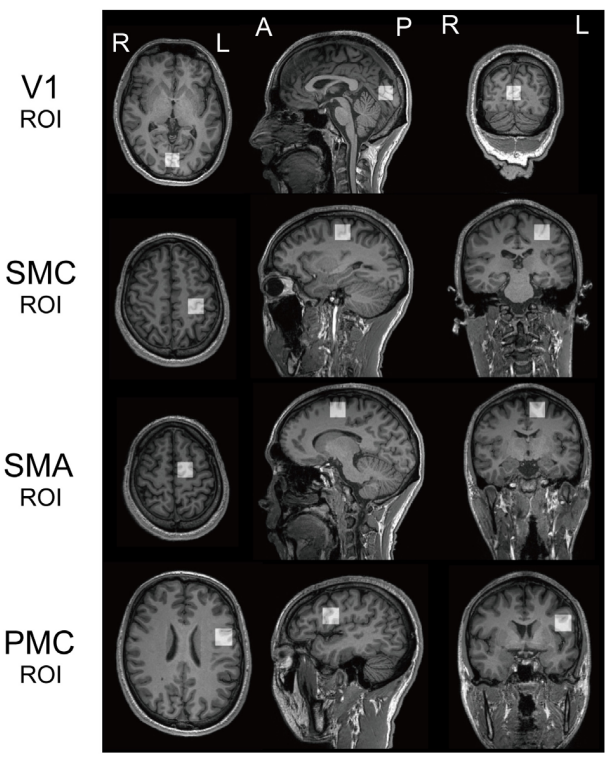

FIGURE 1 | Regions of interest. Regions of interest (ROI) for ^{1}H magnetic resonance spectroscopy for both populations. V1, the bilateral primary visual cortices; SMC, the left sensory motor cortex; SMA, the left supplementary motor area; PMC, the left ventral premotor cortex.

left central sulcus (Yousry et al., 1997). The SMA ROI was the superior and medial part of Brodmann area (BA) 6, with its inferior face anterior to the cingulate sulcus and extending to the dorsal premotor cortex. The PMC ROI included the lower and lateral parts of BA6, with its inferior face anterior to the lateral sulcus [mainly including the ventral PMC (vPMC)]. We used a MEGA-PRESS sequence for GABA-edited MRS (Mescher et al., 1998) to quantify GABA in each ROI (TR = 2000 ms; TE = 70 ms; 128 averages; 20 mm × 20 mm × 20 mm). We used LCModel (Provencher, 2001) to quantify resultant spectra and calculated a ratio of GABA+ (reflecting GABA+ co-edited macro-molecules) to N-acetyl aspartate acid (NAA) to quantify the GABA concentration in each ROI (Harada et al., 2011; Gaetz et al., 2014).

RESULTS

AASP Scores

Comparisons of AASP scores by two-tailed t test revealed that ASD participants had significantly greater low registration (t_{33} = 3.01, p = 0.005, d = 1.02), sensory sensitivity (t_{33} = 2.03, p = 0.05, d = 0.69), and sensation avoiding (t_{33} = 3.09, p = 0.004, d = 1.04) scores, but not sensation seeking (t_{33} = −0.77, p = 0.45, d = −0.26; **Figure 2**) scores, than TD controls.

GABA+ Concentrations

The mean GABA+ concentrations across four ROIs in each group are shown in **Figure 3**. The mean GABA+ /NAA ratio in the left SMA of ASD participants was significantly lower than that of TD controls (two-tailed t test: t_{33} = −2.74, p = 0.01, d = −0.93). No other regions had significant group-wise differences (V1: t_{33} = 1.35, p = 0.19, d = 0.46; PMC: t_{33} = −0.65, p = 0.52, d = −0.22; SMC: t_{33} = −0.64, p = 0.52, d = −0.22).

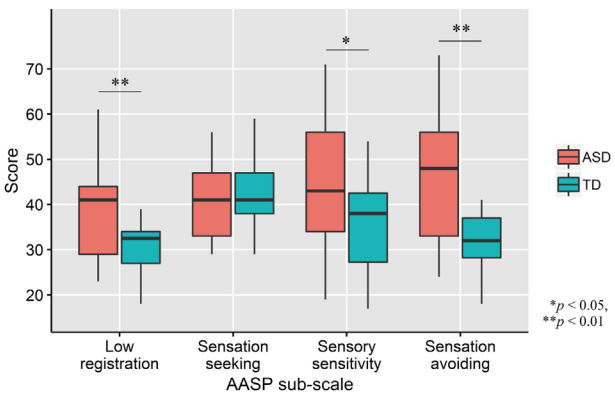

FIGURE 2 | Distribution of AASP scores in each group. The upper and lower boundaries of the standard boxplots represent the 25th and 75th percentiles. The horizontal line across the box marks the median of the distribution. The ends of vertical lines below and above the box represent the minimum and maximum values, respectively. Asterisks represent significant difference by two-tailed t test.

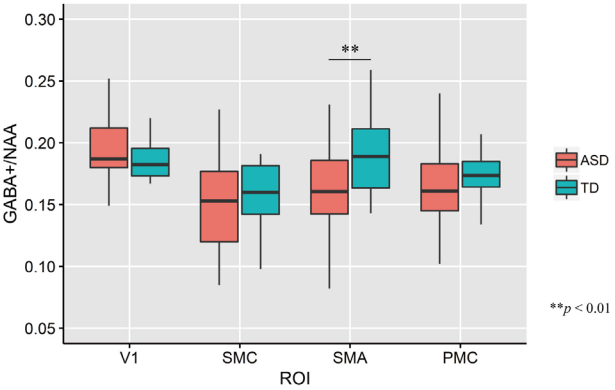

FIGURE 3 | Distribution of GABA+/NAA ratio of every ROIs in each group. The upper and lower boundaries of the standard boxplots represent the 25th and 75th percentiles. The horizontal line across the box marks the median of the distribution. The ends of vertical lines below and above the box represent the minimum and maximum values, respectively. Asterisks represent significant difference by two-tailed t test. V1, the bilateral primary visual cortices; SMC, the left sensory motor cortex; SMA, the left supplementary motor area; PMC, the left ventral premotor cortex.

Correlation Analyses

Figure 4 shows associations between individual GABA+ /NAA ratios and each AASP subscale score across the ROIs. Correlation analyses across all participants (N = 35) revealed negative correlations between GABA+ /NAA ratios and sensory sensitivity (Pearson's correlation coefficient r = −0.43, p = 0.01, 95% confidence interval (CI) = [−0.67, −0.11]) and sensation avoiding scores (r = −0.41, p = 0.013, CI = [−0.66, −0.09]) in the PMC. Some associations were significant in ASD participants, including those for sensory sensitivity (r = −0.63, p = 0.007, CI = [−0.80, −0.38]) and sensation avoiding (r = −0.59, p = 0.014, CI = [−0.77, −0.31]), but not in the TD group (p > 0.7 for both). Furthermore, there was a significant positive correlation between GABA+ /NAA ratio in the left SMC and sensation seeking in the TD group (r = 0.56, p = 0.015, CI = [0.28, 0.76]), but not in the ASD group (p > 0.6). No other subscales were significantly associated with GABA+ levels in either all participants or independently in either of the two groups.

DISCUSSION

The present study assessed whether GABA+ concentrations in specific brain areas were associated with different domains of abnormal sensory experiences in individuals with ASD. An analysis of sensory processing patterns, as assessed by a self-report questionnaire, revealed that participants with ASD had increased low registration, sensory sensitivity, and sensation avoiding subscale scores. Although we found a difference in GABA+ relative to NAA concentrations only in the left SMA between the ASD and TD groups, the other ROIs, the bilateral V1, the left SMC, and the left vPMC did not differ in this measure. Correlation analyses demonstrated that

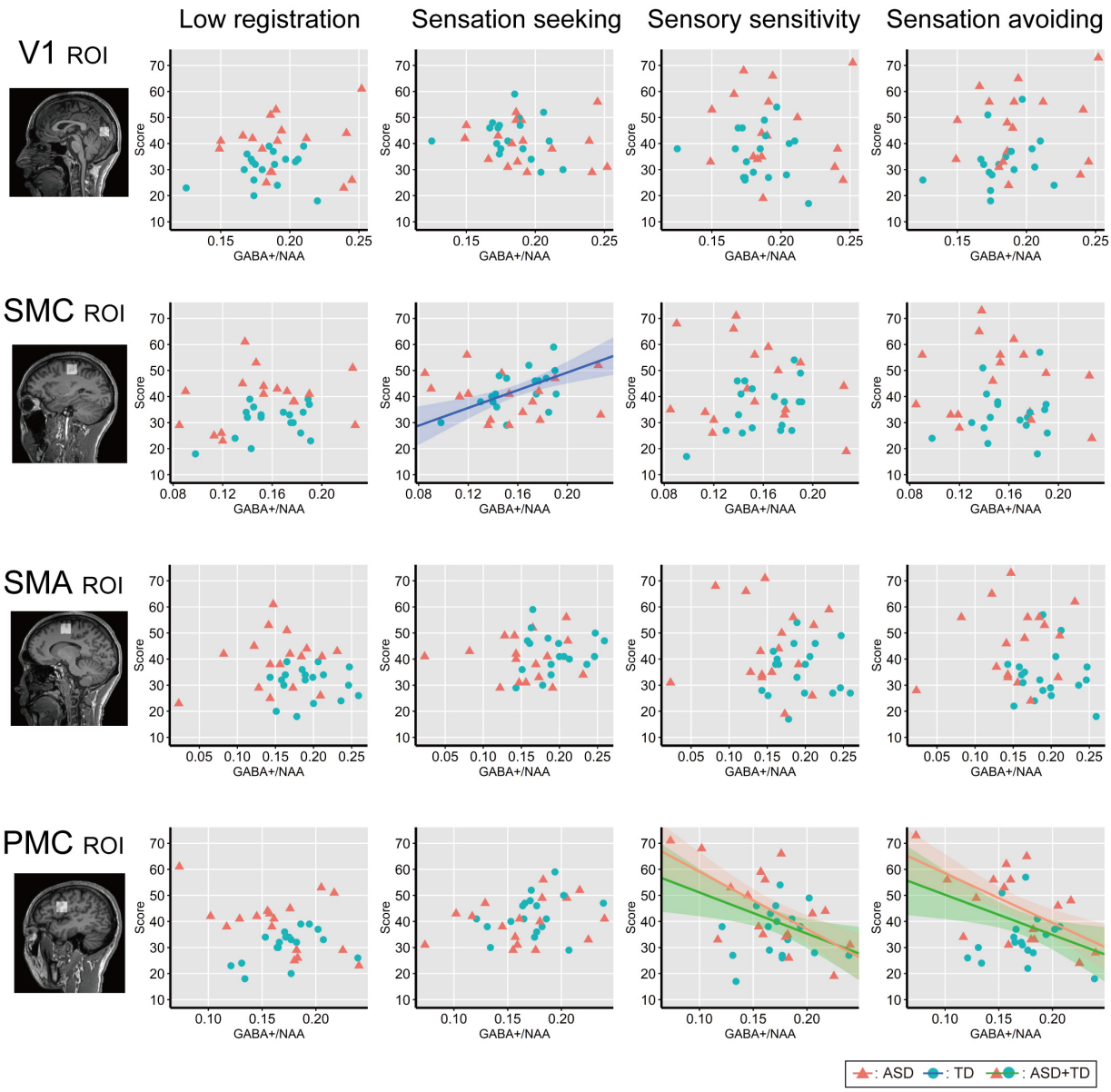

FIGURE 4 | Correlation analysis between individual AASP and GABA+ level. Individual sub-scale scores are plotted against individual GABA+ /NAA ratio in each ROI. Red triangles indicate ASD individuals and blue circles indicate TD individuals. Shaded bands represent 95% confidence intervals across individuals for each group (green: all participants, red: ASD group, blue: TD group). V1, the bilateral primary visual cortices; SMC, the left sensory motor cortex; SMA, the left supplementary motor area; PMC, the left ventral premotor cortex.

individuals with lower GABA+ levels in the left vPMC had increased sensory hyper-responsiveness (i.e., higher scores in the sensory sensitivity and sensation avoiding subscales of the AASP). This trend was obvious in ASD but not in TD participants. Recent studies in transgenic mice with deletions of autism-related genes have further revealed that reduced GABA-mediated inhibitory signals can induce hyper-responsiveness to sensory stimuli (Orefice et al., 2016, 2019; He et al., 2017). Our finding suggest that reduced inhibitory neurotransmission, caused by reduced GABA in the higher-order motor areas that modulate motor commands and integrate sensory information across multiple modalities, is related to increased sensory hyperresponsiveness in ASD.

Atypical sensory processing in people with ASD would involve behavioral patterns in extraordinary response to sensory inputs and not just restricted in sensitivity (e.g., low threshold of perception: Ide et al., 2019; Schulz and Stevenson, 2019). Two major higher-order motor-related areas, the SMA and vPMC have connections with sensorimotor cortices in human and non-human primates (Luppino et al., 1993; Yeo et al., 2011). Previous studies have suggested that the vPMC is involved in multiple sensory processing, especially for response modulation

or inhibition to sensory signals when a change of the reaction patterns is needed (Buch et al., 2010). The vPMC is involved in low-level sensory encoding and motor functions, such as goal-directed behavior in response to multiple sensory information (Rizzolatti et al., 2002). For instance, in hand grasping, this area is critical in using visual information to for appropriately shaping hands (Rizzolatti et al., 2002; Davare et al., 2008; Prabhu et al., 2009). Furthermore, inhibition of M1 activity by the vPMC is critical for rapid behavioral modulation based on action plan changes (Buch et al., 2010). Neurons in the primate vPMC respond to multiple sensory inputs, especially to tactile stimuli and partially to visual (Gentilucci et al., 1988) and auditory stimuli (Graziano et al., 1999). A human functional MRI study demonstrated that the left vPMC activated during tactile orientation judgment (Zhang et al., 2005). Previous work has shown that the secondary motor (M2) area in mice, which is functionally homologous to the human PMC, has synchronized neural activity with the primary somatosensory area and is crucial for tactile texture discrimination (Manita et al., 2015). Given those and the present result, atypical neural modulation of earlier cortical regions by the vPMC may lead to sensory and motor processing dysfunction.

Previous accounts of highly cognitive domains, such as temporal processing of stimulus, in ASD may also implicate links between the left vPMC and sensory hyper-responsiveness given reduced inhibitory cortical neuron activity (Poole et al., 2017; Ide et al., 2019). Our previous study elucidated that individuals with ASD who showed high resolution of tactile stimulus temporal order tended to have severer sensory hyper-responsiveness (Yaguchi et al., 2020). Functional MRI studies have demonstrated that multiple cortical regions are involved in temporal order judgment of multisensory stimuli, including the left vPMC, which likely plays a key role (Takahashi et al., 2013; Binder, 2015; Miyazaki et al., 2016; Ide et al., 2020). The vPMC is also involved in bodily awareness, which may derive from the integration of visual and somatosensory information (Bekrater-Bodmann et al., 2011; Brozzoli et al., 2012). The vPMC is also involved in sensation and decision-making during auditory discrimination (Lemus et al., 2009). Considering the findings presented here, the vPMC may additional be involved in the awareness of multiple sensory stimuli, leading to later action responses to these inputs. Because of its integrative function, reduced inhibitory neurotransmission in the left vPMC in ASD may induce increased reactivity across multiple stages of sensory processing in ASD (Dunn, 1997; Schulz and Stevenson, 2019).

The data presented here are somewhat inconsistent with a previous study which reported greater GABA levels in the sensorimotor cortex and a higher frequency of subjective atypical tactile experiences in individuals with ASD (Sapey-Triomphe et al., 2019). In this previous work, however, the researchers evaluated sensory hypersensitivity and hyposensitivity using the same index and by extracting only tactile domain features. The difference between our own work and this prior study may be due to a focus on modality-dependent predictability of ordinary stimuli (Sapey-Triomphe et al., 2019) and the GABA in the corresponding primary sensory region. In the present study, AASP was used, which differentiates between hypersensitivity and hyposensitivity using Dunn's model (Dunn, 1997). Additionally, given the multisensory processing role of the vPMC, this region is likely more closely related to domain-general atypical sensory hyper-responsiveness, as reported previously in functional associations between GABA and psychophysical measurements in autism (Robertson et al., 2016; Puts et al., 2017).

Despite early reports of reduced GABA across brain areas, we found significant reductions only in the left SMA and no difference in other regions. Most children with ASD have co-morbid developmental coordination disorder, which reflects dysfunction in coordinated body movements (Green et al., 2009). The SMA is thought to be essential for coordinated body movements (Roland et al., 1980; Tanji et al., 1988; Sumner and Husain, 2008). In agreement with our recent report, the present study's finding indicates that reduced GABA+ in the SMA may reflect complicated motor disability in ASD (Umesawa et al., 2020). Prior work has found that GABA concentrations in V1 did not differ between adults with ASD and controls, but rather were associated with functional measures that characterized that population (Robertson et al., 2016). Additional studies have reported reduced GABA in the sensorimotor cortex in autistic children (Gaetz et al., 2014; Puts et al., 2017), though only one study has reported this in autistic adults (Sapey-Triomphe et al., 2019). At present, little is known about GABA concentrations in frontal areas, including higher-order motor regions, in individuals with ASD. One study demonstrated significant reductions in frontal lobe GABA in children with autism compared to controls and no changes in striatal GABA levels (Harada et al., 2011). Another study of adults with ASD revealed no differences in GABA concentrations in either the medial prefrontal cortex or the striatum (Horder et al., 2018). Critically, GABA concentration may also change with age (Clement et al., 1987; McQuail et al., 2015; Rowland et al., 2016). Our findings in adolescents and adults reveal that increased variation of cerebral GABA concentration across the participants by age might reduce the clear between-group difference.

In the present study, GABA+ levels in the left SMC in TD participants were positively correlated with their sensation seeking index scores, which measures one's preference for behaviors being proximal to stimuli to create a sensation (Brown et al., 2001; Dunn, 2001). Previous work in an autism-unrelated mouse strain demonstrated that GABAergic parvalbumin neurons in the primary motor cortex are essential for the inhibition of sensory-triggered motor reaction behaviors (Estebanez et al., 2017). The present study suggests that individual variation in sensorimotor GABA+ levels modulates subjective impulsivity and associated responses to external stimuli, but not in individuals with ASD. Previous work in autistic adults has reported that sensation seeking in ASD individuals differed from the other three scales (Crane et al., 2009). Our sample did not replicate sensation seeking abnormalities in participants with ASD, but this cognitive/behavioral aspects of ASD may reflect another potential association between the neurobiological and pathognomonic traits of autism.

We should note that the findings of our study from a small cohort has limitations to be extended to the larger population. We had no information regarding genetic backgrounds of each individual. Some autism-related genes have been considered to relate to GABAergic inhibition (Orefice et al., 2016, 2019; He et al., 2017). We also did not screen for any motor disabilities of the participants. Thus, whether these uncontrolled factors had any influence on our present results is unknown.

To date, a number of studies in ASD patients have examined modality-dependent atypical sensory processing and respective neural correlations. Although individual variability in sensory modality abnormalities and later behavioral response in ASD are well known, associations between clinically-validated sensory assessments and brain metabolites are less well understood. The present study is the first to comprehensively analyze the relationship between GABA+ levels in multiple brain regions and multiple aspects of sensory processing deficits in ASD. As discussed above, the left vPMC may be involved in the processing of multiple sensory information, though its specific function which accounts for sensory processing disorder in autism remains unknown. Future work should examine whether a specific cognitive capacity which the left vPMC is involved in, such as temporal processing of stimuli (Takahashi et al., 2013; Binder, 2015; Miyazaki et al., 2016; Ide et al., 2020), bodily-awareness (Bekrater-Bodmann et al., 2011; Brozzoli et al., 2012), and decision-making (Lemus et al., 2009), mediates the association between GABA and atypical sensory processing. Furthermore, whether there is an altered role for GABA in the vPMC in individuals with autism should be evaluated by whole-brain functional and anatomical connectivity (Ameis et al., 2016; Yahata et al., 2016). Findings from those studies may allow us to evaluate the possibility of GABA levels in the left vPMC as a significant biomarker and therapeutic target for autistic sensory processing disorder.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of the National Rehabilitation Center for Persons with Disabilities. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YU, TA, MC, and MI conceived the study. YU, TA, and MI conducted the experiments. YU and TA analyzed the data. All authors interpreted the results read the manuscript, gave relevant inputs, and approved the final version of the same. YU, TA, and MI wrote the manuscript.

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REVIEW
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Autism Spectrum Disorder Associated With Gut Microbiota at Immune, Metabolomic, and Neuroactive Level

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There is increasing evidence suggesting a link between the autism spectrum disorder (ASD) and the gastrointestinal (GI) microbiome. Experimental and clinical studies have shown that patients diagnosed with ASD display alterations of the gut microbiota. These alterations do not only extend to the gut microbiota composition but also to the metabolites they produce, as a result of its connections with diet and the bidirectional interaction with the host. Thus, production of metabolites and neurotransmitters stimulate the immune system and influence the central nervous system (CNS) by stimulation of the vagal nerve, as an example of the gut-brain axis pathway. In this review we compose an overview of the interconnectivity of the different GI-related elements that have been associated with the development and severity of the ASD in patients and animal models. We review potential biomarkers to be used in future studies to unlock further connections and interventions in the treatment of ASD.

Keywords: autism spectrum disorder, gut microbiome, gut-brain axis, biomarker, neurotransmitter, GABA, serotonin

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INTRODUCTION

Autism spectrum disorder (ASD) is a group of brain developmental disorders characterized by stereotyped behavior and deficits in communication and social interaction. Initially, it was believed that ASD had an environmental origin. However, at the moment it is accepted that ASD development is the result of multiple factors, including environmental, genetics, and neurodevelopmental (Rylaarsdam and Guemez-Gamboa, 2019). The prevalence of ASD in the development of children and on society constitutes an economic burden for families, where the main costs are associated to special education and the loss of productivity of the parents (Buescher et al., 2014; Christensen et al., 2018). Additionally, it has been reported that over the last decades, there is an increasing prevalence of ASD, reaching 1 in 132 globally (Matson and Kozlowski, 2011; Baxter et al., 2015; Hansen et al., 2015). Therefore, there is a need to develop and implement effective interventions. However, there is no defined etiology and pathology for ASD, and this limits the development of specific therapies (Rossignol and Frye, 2012). Previous studies have shown that there are several factors that might have an influence on development and prognosis of ASD, such as genetics, immunological, inflammatory, environmental, and more recently, the gut microbiota (Fakhoury, 2015). Genetic factors thought to be involved in processes such as synapse formation, transcriptional regulation or pathways for chromatin-remodeling are

listed in **Figure 1** (Rylaarsdam and Guemez-Gamboa, 2019). However, genetic factors in ASD development are not the focus of this review and this subject is reviewed elsewhere (Chaste and Leboyer, 2012; Huguet and Bourgeron, 2016; Rylaarsdam and Guemez-Gamboa, 2019).

The gut harbors millions of microorganisms linked by complex ecological relationships between them and the host, often mediated by the production of metabolites. The gut microbiota has been proposed as a key element involved in many conditions, such as obesity, colorectal cancer, irritable bowel syndrome (IBS), diabetes type 2, rheumatoid arthritis, Parkinson's disease, and Alzheimer's disease and also with cognitive conditions such as anxiety, depression, and autism (Ceppa et al., 2019). The gut-brain axis theory, now well-established and accepted, states that the gut and the brain communicate and influence each other (Bienenstock et al., 2015; Mayer et al., 2015; Cryan et al., 2019). The gut-brain axis theory has its origin on the observation of the improvement of patients diagnosed with hepatic encephalopathy, after antibiotic treatment (Carabotti et al., 2015). Moreover, IBS and its gut microbiota alterations have been linked to anxiety and depression (Simpson et al., 2020). There is even recent evidence suggesting that human personality traits might be associated with the gut microbiome (Johnson, 2020).

Increasing evidence shows that gastrointestinal (GI) symptoms, such as gastrointestinal disruption, abdominal pain, diarrhea, constipation, and flatulence, has been characterized as a common comorbidity in patients with ASD, ranging between 9 and 84% depending on the studies being retrospective or prospective (Wasilewska and Klukowski, 2015), and are linked to the severity of ASD symptoms (Adams et al., 2011; Gorrindo et al., 2012; Chaidez et al., 2014). However, cause-effect relationship between GI symptoms and ASD has not been proven yet. In fact, it has been suggested that GI symptoms should be considered as part of the ASD phenotype, like the behavioral symptoms (Niesler and Rappold, 2020). On the other hand, there are studies that have demonstrated that the administration of a single strain, such as human commensal *Bacteroides fragilis*, is able to ameliorate social deficit in a mice model (Hsiao et al., 2013). Moreover, *B. fragilis* corrected gut permeability and altered microbial composition. Additionally, treatments such as Microbiota Transfer Therapy (MTT), focused on gut microbiota regulation, have shown promising results by improving ASD-related symptoms in patients that were sustained after finishing the treatment (Kang et al., 2019). These improvements were reported hand-in-hand with an increment in bacterial diversity and relative abundances of *Bifidobacterium* and *Prevotella*. Overall, these evidences suggest a potential correlation between these factors and communicative defects and stereotypic behavior associated to ASD that needs to be further explored. The validation of biomarkers related to the gut-brain axis would be of great value in the diagnosis, the development, and the follow-up of potential therapies for patients with ASD. This review will focus on the role of the gut microbiota in the pathology of ASD via the gut-brain axis and the related biomarkers that have been described in the literature.

THE MICROBIOTA IN THE GUT

It has been reported that the human gut carries more bacterial cells than human cells are in the entire body, and that the metagenome of the gut microbiota encodes approximately eight million genes, in contrast to the approximately 23,000 genes encoded in the human genome (Ceppa et al., 2019). The gut ecosystem comprises the bacteria, archaea, viruses, fungi, yeast, and eukarya (Ceppa et al., 2019). Gut microbiota is not uniformly distributed across the GI tract. The distribution depends on the combination of factors such as pH, water activity or gas composition that fluctuate through the gut and have been reviewed previously (Lozupone et al., 2012; Donaldson et al., 2016; Garcia-Gutierrez et al., 2018). The gut microbiota composition also varies over life span. At the moment, there is some controversy on how sterile the placenta is and whether the meconium of healthy new-borns contain traces of microbiota (DiGiulio et al., 2008; Jiménez et al., 2008; Moles et al., 2013; Aagaard et al., 2014; Rodríguez et al., 2015; de Goffau et al., 2019). However, the process of a succession of bacterial colonization events in the gut begins at birth, via the microbiome of the maternal vagina during the delivery (or the skin in the case of a C-section), and introduction of new species in the human gut through feeding (human milk first and solid food after weaning), after the delivery (Dominguez-Bello et al., 2010; Fernandez et al., 2013; Bokulich et al., 2016; Yassour et al., 2016). This changing composition stabilizes after the third year of life and it is maintained during the adult life. There is evidence of gut microbiota changes during senescence and these changes might be related to the conditions developed over this period, such as cognitive impairment or elderly malnutrition (Nagpal et al., 2018; Xu C. et al., 2019). As a general feature, gut microbiota is usually resilient and recovers after acute changes, like consumption of antibiotics. However, structural composition of the gut microbiota is determined by sustained factors like lifestyle or diet (Conlon and Bird, 2015; Singh et al., 2017; Ceppa et al., 2019).

There are five major bacterial phyla in the gut, Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, and Verrucomicrobia (Donaldson et al., 2016). The different conditions, such as presence of bile acids, oxygen, or nutrient availability, across the GI tract result in different distribution of these groups. Thus, the small intestine is colonized by representatives of groups of facultative anaerobes of Firmicutes (lactobacilli) and Proteobacteria (enterobacteria), while the colon is colonized mainly by fermentative organisms from *Bacteroidaceae*, *Prevotellaceae,* and *Rikenellaceae* families (Bacteroidetes) and *Lachnospiraceae* and *Ruminococcaceae* families (Firmicutes) (Donaldson et al., 2016). The characterization of the gut microbiota has been mainly conducted by analyzing fecal samples, however, this might provide a false image of the proportion and diversity of the gut microbiota composition (Rodríguez et al., 2015). Regardless of the potential artifact of the compositional information, functionality is the key factor for a balanced microbiota. Despite that it is not possible to define a healthy gut microbiota in terms of taxonomical composition, it has been suggested that metabolic functionality of pathways

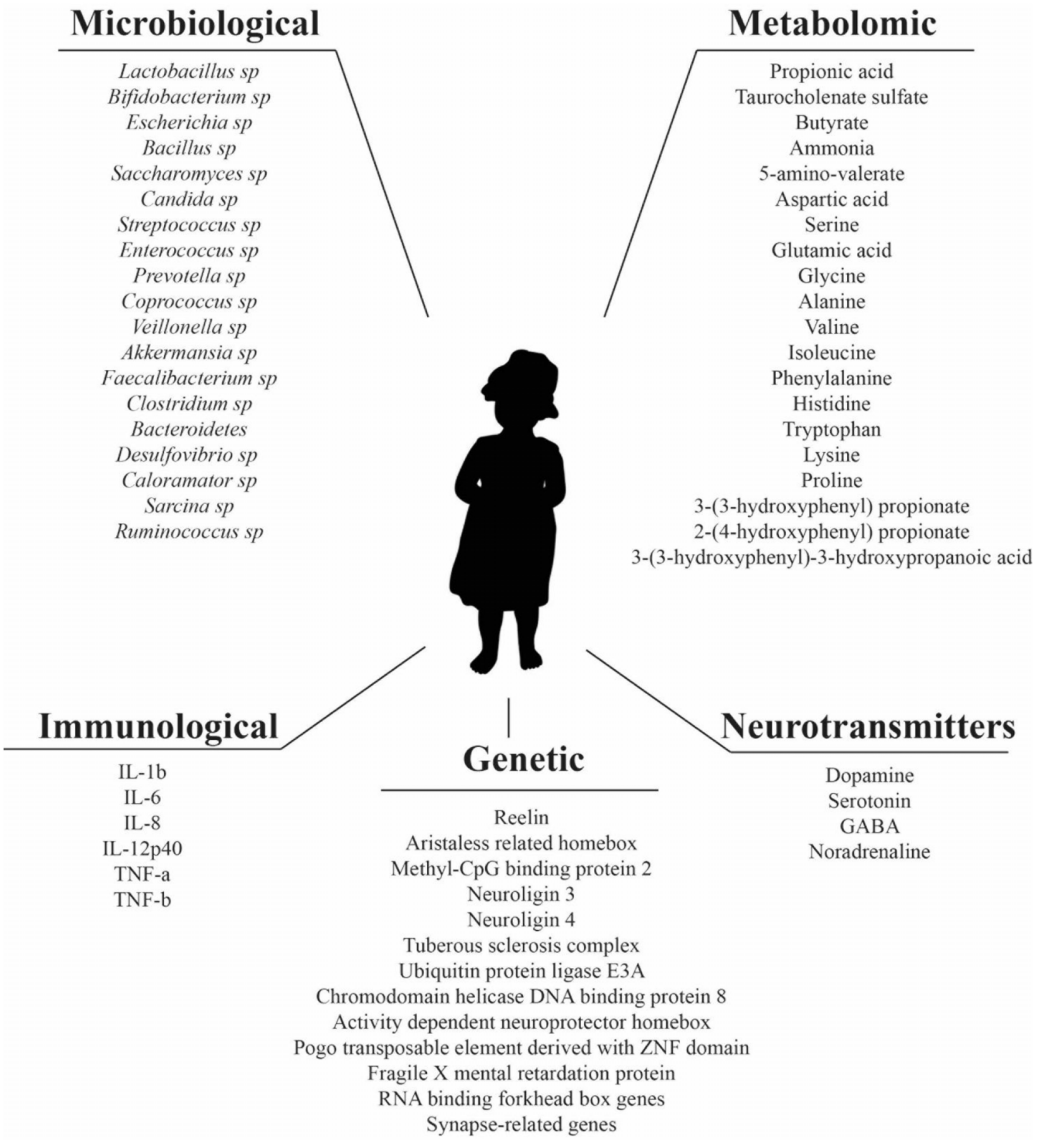

FIGURE 1 | Microbiological, metabolomic, immunological, genetic factors, and neurotransmitters reported in literature as altered in ASD patients.

remains redundant in the gut microbiome, and it is the source of a balanced equilibrium and resilience after acute perturbances (Lozupone et al., 2012). The identification of biomarkers as gene functions associated to a balanced gut health and specific pathologies will favor and improve the development of efficient microbiota-associated treatments in the future.

THE MICROBIOTA-GUT-BRAIN AXIS

The gut–brain axis is considered a bidirectional pathway for the communication between the gut and the brain. However, this concept can be expanded to include also the microbiota as a key element in this triangle (Cryan et al., 2019). The importance of the microbiota in this relationship has been established via different routes. Studies conducted in germ-free animals have provided evidence that the brain was affected when the gut microbiota was not present (Diaz Heijtz et al., 2011; Cryan et al., 2019). Other studies have shown that alterations in behavior in animals were induced by providing specific strains of bacteria, and those observations were sustained in human studies afterwards, e.g., *Bifidobacterium longum* strains 1714 and NCC3001 (Allen et al., 2016; Pinto-Sanchez et al., 2017). Exposition to infections showed alteration in gut-brain symptoms, and immune activation, and the use of antibiotics affected the central nervous system (CNS) and the enteric nervous system (ENS). In the reverse situation, hepatic encephalopathy has been successfully treated with microbiota-targeting antibiotics (Collins, 2016). Extensive work with mice models has shown that there are several processes in the nervous system that are linked to the regulatory effect

of the gut microbiota, including neurogenesis in hippocampus, the amygdala, myelination, the length, and spine density, the synaptic connections, the microglia and the permeability of the blood-brain-barrier (BBB) (Cryan et al., 2019). Another process where microbiota is involved is in the synaptic and neuronal plasticity. Studies with germ-free mice indicated low levels of expression of brain-derived neurotrophic factor (BDNF) in the cortex and hippocampus. BDNF is associated with brain plasticity and has a regulatory function on neural growth (Leung and Thuret, 2015). BDNF is involved in many learning and behavioral processes, especially the ones associated with hippocampal learning and working memory (Gareau et al., 2011). The receptors for N-methyl-D-aspartate (NMDAR) are also closely involved in the synaptic plasticity and cognitive function and the production of NMDAR is connected to the levels of BDNF (Maqsood and Stone, 2016). Low levels of BDNF in germ-free mice successively lowers the NMDAR production, which also affects γ-aminobutyric acid (GABA) inhibitory interneurons and ultimately this translates into cognitive deficits (Maqsood and Stone, 2016). Gut microbiota exerts its action over BDNF by alterations in neurotransmitter and modulatory pathways, such as the kynurenine, involved in tryptophan metabolism and by the action of the short chain fatty acids (SCFAs) (Cryan et al., 2019). Studies in mice have shown that depleted BDNF levels could be recovered by the direct administration of a strain of *B. longum* subsp. *infantis* (Bercik et al., 2012) and other probiotics, prebiotics, and antimicrobials that increase the proportion of lactobacilli, Firmicutes, and Actinobacteria and decrease the Proteobacteria and Bacteroidetes levels in the gut, suggesting potential interventions to target key regulatory elements in the CNS (Maqsood and Stone, 2016).

The microbiota in the gut and the brain can communicate through a variety of routes that involve neuroendocrine, neuroimmune, and autonomic nervous systems pathways (Grenham et al., 2011; Mayer, 2011). The immune system is particularly important (Carlessi et al., 2019) where cytokines components of the immune system communicate directly with the brain via the vagal nerve inducing changes in the BBB (Quan, 2008). It also affects the hypothalamic pituitary adrenal (HPA) axis, that centralizes the stress response system, stimulated by physical or psychological situations (Scriven et al., 2018). HPA axis alterations have been reported in post-traumatic stress or depression. A number of bacterial strains (e.g., *Lactobacillus farmicinis*) can modify such changes via impacting the gut permeability or balancing levels of adrenocorticotropic hormone (ACTH), corticosterone and BDNF (*Bifidobacterium infantis*) (Desbonnet et al., 2008; Ait-Belgnaoui et al., 2012). The vagus nerve is responsible for many anti-inflammatory effects through the contact with the HPA axis and other pathways, such as the cholinergic anti-inflammatory and the splenic sympathetic anti-inflammatory (Forsythe et al., 2014). The vagus nerve interacts with bacteria via SCFAs that cross the gut wall, and it can even differentiate between pathogenic and non-pathogenic bacteria (Bonaz et al., 2018). The function of many probiotics is also determined by their interactions with the vagus nerve. Bacteria can also produce and secrete neurotransmitters. Some representatives of the genera *Lactobacillus* and *Bifidobacterium* can produce GABA, while representatives of *Escherichia*, *Bacillus,* and *Saccharomyces* can produce noradrenaline (Barrett et al., 2012). Serotonin (5-hydroxytryptamine, 5-HT) is a product of some species of *Candida*, *Streptococcus*, *Escherichia,* and *Enterococcus* and it is mediated by tryptophan (Scriven et al., 2018). Other neurotransmitters produced by bacteria are dopamine (*Bacillus*) and acetylcholine (*Lactobacillus*) (Dinan et al., 2015). Gut microbiota is the key element that controls tryptophan catabolism via the kynurenine pathway, the primary pathway for tryptophan catabolism (Ceppa et al., 2019). Changes in the serotonergic system have been associated with depression and IBS (Owens and Nemeroff, 1994). Gut microbiota also produce metabolites as a result of the fermentative process during the food digestion in the gut. Metabolites are the result of the breakdown of carbohydrates, polyphenols, lipids, and proteins, together with gasses (carbon dioxide, hydrogen, and methane) and the production of energy. The diet composition will result in different types of SCFA that communicate with the brain through the vagus nerve, producing different effects on the nervous system (Silva et al., 2020). Butyric acid has been associated with satiety, and high levels of propionic acid (PAA) have been linked with ASD (Shultz and MacFabe, 2014; Abdelli et al., 2019). The role of PAA is particularly interesting after the observations that ASD behavioral effects in children worsen after the consumption of high levels of PPA (Meeking et al., 2020). Moreover, supplementation with PPA in animal models led to behavioral effects similar to ASD while metabolic impairment of glutathione, carnitine, and fatty acids (FA) has been observed in the serum of ASD patients receiving PPA (Frye et al., 2013). PPA can accumulate in the cells and alter neuronal communication by its impact on neurotransmitter release, gap junctions and intracellular calcium release, among other effects that will be discussed later. Overall, these studies also suggest that dietary components can play a major role in the selection of bacteria and the production of metabolites, that ultimately will affect the gut-brain axis.

Despite that many studies have established these pivotal connections between the gut and the brain, translational human studies are particularly needed to understand the mechanisms underlying the microbiota-gut-brain axis. This will be key to design microbial-based interventions and therapeutic strategies to target neuropsychiatric disorders.

POTENTIAL RELATIONSHIPS BETWEEN THE MICROBIOTA AND ASD

There are a variety of factors that seem to connect gut microbiota with ASD symptoms. Early life events, such as delivery mode, have a huge impact on the composition of the microbial communities. Infants delivered by C-section showed a higher probability of developing ASD in comparison to the children delivered vaginally (odds ratio of 1.23) (Curran et al., 2015). Other prenatal factors, such as gestational diabetes or maternal obesity during pregnancy, can modify the gut microbiota (Connolly et al., 2016). In a mice model, when mothers where fed

a high-fat diet, it induced dysbiosis and autism-like phenotypes (Buffington et al., 2016). Additionally, in children with diagnosed ASD an increased use of antibiotics was reported in comparison with controls (Atladottir et al., 2012). An explanation might be the effect of antibiotics on the gut microbiota (Bokulich et al., 2016). The effect of antibiotics on the gut microbiota has been extensively studied in recent times. Antibiotics not only target pathogens, they also affect commensal bacteria that contribute to the gut homeostasis (Mu and Zhu, 2019; Sun et al., 2019). Sometimes, the impairment produced by the use of antibiotics, depending on factors such as the type of antibiotic, length of treatment or age of the host, can be overcome and the balanced restored in the gut microbiome communities (Langdon et al., 2016). However, in other cases, the use of antibiotics leads to the loss of key species in the microbiome, producing lifelong phenotype alterations, such as obesity (Wang et al., 2017). The effects of antibiotics on the gut microbiota of children can be more detrimental. Thus, the composition of the microbiota of children of less than 3 years who were treated with antibiotics was less diverse (Yassour et al., 2016). Even the antibiotics used during pregnancy seem to be correlated with a higher risk factor for the development of ASD (Atladottir et al., 2010). Another important factor is the early feeding pattern. Infants fed with formula milk showed higher levels of *Clostridium difficile* in comparison with infants who were breast fed (Azad et al., 2013). Additionally, breastfeeding over 6 months has been associated with a lower risk of ASD development (Schultz et al., 2006) and ASD-related GI symptoms (Penn et al., 2016).

GI symptoms are a comorbidity reported in 9–84% of ASD patients (Wasilewska and Klukowski, 2015). These include constipation (20%) and diarrhea (19%), which is more frequent in children with ASD than in their unaffected brothers or sisters (42 vs. 23%, respectively) (Wang et al., 2011). The evidence linking directly or indirectly the gut microbiota with ASD symptoms shows that this might happen partially by its influence on the host metabolism and the immune system (de Angelis et al., 2015; Mead and Ashwood, 2015).

The "leaky gut" or increased permeability of the intestinal epithelium, is one of the conditions reported in ASD patients (Quigley, 2016), where 36.7% of ASD patients and their relatives (21.2%) showed higher percentage of abnormal intestinal permeability in comparison to the control group (4.8%) (de Magistris et al., 2010). As a result of increased permeability, toxins and bacterial products can get into the bloodstream, ultimately affecting brain function and impairing social behavioral scores (Emanuele et al., 2010; Onore et al., 2012; Hsiao et al., 2013). There are a few elements that are used to measure the integrity of both the gut barrier and the BBB, like claudin (CLDN)-5, CLDN-12, CLDN-3, and MMP-9, increased in the ASD-patients' brain, and the intestinal tight junction components (CLDN-1, OCLN, TRIC), decreased in ASD patients (Fiorentino et al., 2016). The lactulose: mannitol test is used to measure intestinal permeability, and it is increased in ASD patients when compared with healthy controls (de Magistris et al., 2010). On the other hand, bacterial products such as acetate and propionate may enhance the integrity of the BBB (Braniste et al., 2014). The leaky gut also increases the antigenic load from the gastrointestinal tract. Thus, lymphocytes and ASD-associated cytokines, like interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), circulate and cross the BBB. IL-1β and TNF-α are responsible for inducing immune responses in the brain by binding to the brain endothelial cells (de Theije et al., 2011).

One of the common changes observed in ASD patients and animal models relates to the composition of the gut microbiota and their metabolic products (de Magistris et al., 2010; Borre et al., 2014; Kushak et al., 2016). It was found that the gut microbiota of children with ASD was less diverse and exhibited lower levels of *Bifidobacterium* and Firmicutes and higher levels of Bacteroidetes, *Lactobacillus, Clostridium, Desulfovibrio, Caloramator,* and *Sarcina*, than that of children without ASD (de Angelis et al., 2013). ASD children with GI symptoms had lower abundances of *Prevotella, Coprococcus*, and unclassified *Veillonellaceae*, than symptom-free neurotypical children (Kang et al., 2013). A recent systematic review and meta-analysis identified approximately 431 studies conducted in ASD patients that involved analysis of the gut microbiota, although many of these studies did not provide quantitative data (Xu M. et al., 2019). The meta-analysis revealed significant differences between gut bacterial groups. Thus, ASD patients had a lower percentage of *Akkermansia* and *Bacteroides* when compared to controls. *Bacteroides* are known for inducing anti-inflammatory effects (Bolte, 1998). Another important group traditionally associated with beneficial effects in the human gut is *Bifidobacterium,* with significantly lower abundance in ASD patients (Xu M. et al., 2019). On the other hand, the analysis of five studies showed that the percentage of sequences of the genus *Faecalibacterium* was significantly higher among ASD patients (Finegold et al., 2010; de Angelis et al., 2013; Kang et al., 2013; Inoue et al., 2016; Strati et al., 2017; Xu M. et al., 2019). Higher relative abundance of lactobacilli (generally considered to be beneficial bacteria) has been observed in children diagnosed with ASD although it may reflect an effect of the diet (e.g., a high consumption of yogurt and yogurt-like fermented milks). Several studies highlighted the relevance of other bacterial groups, like the *Clostridium histolyticum* group (*Clostridium* clusters II and I), which were present in higher levels in fecal samples of ASD children (Parracho et al., 2005). *Clostridium* is known for producing neurotoxins that might have systemic effects (Parracho et al., 2005). It was observed that reductions of this *Clostridium* group brought significant improvements in children with ASD-like symptoms (Sandler et al., 2000). *Ruminococcus* is another genus associated with ASD symptoms and functional GI disorder (Joossens et al., 2011; Xu M. et al., 2019).

Despite many different studies demonstrate alterations of the gut microbiota in ASD patients, others have not described this association. To illustrate this, a study comprising 59 ASD individuals and 44 normal siblings found no significant difference between them in relative abundances of total Bacteroidetes, *Sutterella* or *Prevotella* (Son et al., 2015). Additionally, there is a lack of studies that evaluate the role of gut mycobiome and gut virome in ASD. Increasing evidence suggests that mycobiome might be a key element in maintaining a gut-brain axis balanced dynamics due to its close interaction with the gut bacteria (Huseyin et al., 2017a,b). An increased abundance of *Candida* in

the gut mycobiome composition of ASD patients was reported for the first time recently (Strati et al., 2017; Enaud et al., 2018). It was hypothesized that its interaction with other microbes, such as lactobacilli, might have an effect on the immune system via pro-inflammatory effectors and prevent the recovery of the balanced gut microbiota (Enaud et al., 2018). In any case, the interactions between bacteria and other members of the gut microbiota could bring valuable information about their role in ASD condition.

GUT MICROBIOTA-MEDIATED METABOLITES AS BIOMARKERS

Gut microbiota products include a variety of metabolites, such as SCFAs, phenolic compounds and free amino acids (FAA), that affect the behavior of ASD patients. It is believed that this effect is mediated via the vagal pathways (Macfabe, 2012).

Short chain fatty acids include acetic acid (AA), PPA, butyrate, isobutyric acid, valeric acid, and isovaleric acid, as products of the fermentation of non-digestible carbohydrates by gut bacteria (Al-Lahham et al., 2010). SCFAs have beneficial effects on the human host, like improvement in glucose metabolism, energy homeostasis, reductions in body weight and the risk of colon cancer (Chambers et al., 2015). PPA is produced mainly by Bacteroidetes, *Clostridium,* and *Desulfovibrio* and can cross the BBB. As stated before, PPA can inhibit the Na+/K+ ATPase, increase NMDA receptor sensitivity and alter mitochondrial and fatty acid metabolism. It also can trigger immune activation and changes in gene expression (Meeking et al., 2020). PPA has been linked to the development of ASD-like behaviors (MacFabe et al., 2007, 2011; Shultz et al., 2008; Ossenkopp et al., 2012). In a mice model, the administration of high doses of PPA induced some autistic-like behaviors (Thomas et al., 2012). In a rat model, the intraventricular administration of PPA induced hyperactivity and repetitive behaviors in a similar way to the behavioral changes in ASD patients (MacFabe et al., 2007). Additionally PPA led to impaired social behavior in rats, probably due to the alteration of dopamine and serotonin levels (Mitsui et al., 2005). Butyrate has shown anti-inflammatory effects and ability to modulate the synthesis of dopamine, norepinephrine, and epinephrine (Gualdi et al., 2008; Cleophas et al., 2016).

Free amino acids are derived from the hydrolysis of the proteins and peptides and their fecal levels were higher in ASD children with symptoms in comparison with healthy children and, more specifically, the levels of the amino acids Asp, Ser, Glu, Gly, Ala, Val, Ile, Phe, His, Tpr, Lys, and Pro (de Angelis et al., 2015). The levels of some of them, particularly Glu, a neurotransmitter in the CNS, is altered in other neuropsychiatric disorders (Sheldon and Robinson, 2007; Shimmura et al., 2011). Tryptophan, the precursor of GABA was increased in the urine of ASD patients and tryptophan fragments were also found in the urine of patients with depression and intellectual disability (Noto et al., 2014). Other compounds found in higher levels in the urine of ASD children were 2-(4-hydroxyphenyl) propionate and taurocholenate sulfate, while 3-(3-hydroxyphenyl) propionate and 5-amino-valerate were found in lower levels (Ming et al., 2012). A phenylalanine metabolite [3-(3-hydroxyphenyl)-3-hydroxypropanoic acid], produced by *Clostridia* spp., was increased in the urine of ASD patients and was linked to ASD-like behaviors in mice models (Shaw, 2010).

BIOMARKERS FROM THE IMMUNE SYSTEM PATHWAYS

The gut and the brain can also influence each other via immunological pathways, and microbial diversity is key to maintaining immune homeostasis, as it is linked to the development of the gut-associated lymphoid tissue (GALT) (Rodríguez et al., 2015; Ceppa et al., 2019). The GALT recognizes pathogenic microorganisms and mediates a defense response. GALT is known for producing IgA, modulating innate immune responses when bacterial cells come in contact with dendrites of the ENS (Ceppa et al., 2019). IgAs also recognizes and binds to specific undesired microorganisms to facilitate their removal in feces, while maintaining the commensal bacteria (Lebeer et al., 2010). When there is impairment of the gut microbiota balance, one of main effects is the development of inflammatory processes. A correlation has been established between inflammation and immune dysfunction in ASD patients (Fattorusso et al., 2019). In fact, a comparison between transcriptomics profiles on ileal and colonic tissues showed similarities between ASD and inflammatory bowel disease (IBD) patients (Fattorusso et al., 2019).

There are different inflammatory markers for ASD that have been described in literature, but with limited consensus. For example, IgA has been found to increase in ASD patients in some studies, but not in others (Kushak et al., 2016). Levels of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and IL-12p40, have been shown to be increased in the plasma of ASD patients (Ashwood et al., 2011). Also, TNF-α and transforming growth factor (TGF-β) have been linked to the severity of the ASD symptoms. Some probiotics, including strains belonging to the species *Lactobacillus sakei*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus salivarius* and *Bifidobacterium breve*, modulate or inhibit the production of pro-inflammatory cytokines IL-8, TNF-α, and IFN-γ and increase the anti-inflammatory cytokine IL-10 (Thomas and Versalovic, 2010; Ganguli et al., 2013).

The toxins produced by the pathogenic members of the microbiota increase gut permeability, developing impaired intestinal barrier and allowing the translocation of the gut bacteria through the intestinal wall into the mesenteric lymphoid tissue, inducing the activation of the immune system (Dicksved et al., 2012). This, in turn, releases the inflammatory cytokines and activates the vagal system, regulating CNS activity (Yarandi et al., 2016). The peripheral cytokines are able to induce a behavior linked to depression via the vagal nerve (Konsman et al., 2000). Also, other metabolic compounds produced by gut microbiota, such as lipopolysaccharide (LPS), enter the blood through the impaired gut wall and activate Toll-like receptors in the ENS and CNS (Abreu, 2010). The immune response in mediated by IgE in the gut, where it raises serotonin levels and reduces 5-hydroxyindoleacetic acid (5-HIAA) ones in the gut,

which has been linked to reduced social communication and increased repetitive behavior (Li et al., 2017). Additionally, an activation of the neuroendocrine system and downregulation of the dopamine activity in the prefrontal cortex were also observed in a mice model (de Theije et al., 2014). ASD patients also have higher levels of zonulin in plasma, a protein that modulates gut permeability, and its levels seem to be associated with the severity of the ASD symptoms (Fattorusso et al., 2019).

The immune system is, therefore, closely linked to the effect of microbiota on the gut epithelial permeability connecting the gut and brain through neuroendocrine and neuroimmune pathways that ultimately modulates ASD severity.

NEUROACTIVE COMPOUNDS AS BIOMARKERS

Sensory hyper- and hypo-responsiveness are typically characteristic of autistic patients even though they are not part of the core definition of ASD. However, diet and probiotic interventions might alleviate them. A variety of neuroactive compounds that activate or inhibit central neurons are produced by gut microbiota, such as serotonin, GABA, dopamine (DA) and histamine (Eisenstein, 2016; Spiller and Major, 2016).

The first identified ASD biomarker was serotonin, proposed as a link for the gut-brain axis (Mulder et al., 2004). Serotonin is synthesized in the intestines and the brain, and is thought to be involved in the development of the CNS and the ENS (Gaspar et al., 2003). Children with ASD showed higher levels of serotonin in blood that is believed to be caused by its gastrointestinal hypersecretion (Israelyan and Margolis, 2019). It was believed that genetic factors might be the cause of this overproduction. It is believed that infections, gastrointestinal disorders, such as gut microbiome dysbiosis, and immune system impairment might also be involved in higher levels of serotonin in ASD patients (Fattorusso et al., 2019). A higher prevalence of clostridia in the gut mucosa of children with ASD and GI disorders was associated with higher levels of cytokines, serotonin, and tryptophan in biopsies (Luna et al., 2017). In addition, higher levels of tryptophan (the precursor of serotonin) in the GI tract of ASD children were associated with more severe ASD behavioral symptoms, and with a lower availability and synthesis of serotonin in the brain (Luna et al., 2017). Therefore, gut microbiota dysbiosis affects the availability of tryptophan for the host and worsens cognitive impairment. Interventions to regulate gut dysbiosis might improve the ASD symptoms. It has been observed that, in a mice model, the offspring's brains from mice exposed to valproic acid (VPA) showed ASD behavior alterations in the microbiota and lower levels of serotonin (de Theije et al., 2014). However, other strategies, such as addition of tryptophan to the diet and administration of serotonin reuptake inhibitor have not improved ASD behaviors (Muller et al., 2016).

Gamma-aminobutyric acid is the main inhibitory neurotransmitter in the brain. It has been observed that an altered GABA pattern is a key characteristic of the neurophysiology of ASD. A recent study was performed on the effect of GABA in the brain regions that are critical to sensory functions and higher-order motor, including the primary visual cortex, the left supplementary motor area (SMA), the left sensorimotor cortex, and the left ventral premotor cortex (vPMC) (Umesawa et al., 2020). Sensory processing is considered abnormal in autism at input, cognitive and behavioral reactivity levels, potentially involving processes of high cognitive processing (Thye et al., 2018). If the inhibitory GABAergic transmission is impaired in ASD patients, it may result in an abnormal balance of excitation/inhibition in the brain, alteration of neural signaling, processing of information and responding behavior (Foss-Feig et al., 2017). The reduced levels of GABA in the higher-order motor areas, integrating multiple sensory modalities, might be behind the sensory hyper-responsiveness in ASD patients (Umesawa et al., 2020). This correlates with a previous study reporting that GABA levels in processing touch areas were related to tactile hypersensitivity, frequently observed in in ASD patients (Sapey-Triomphe et al., 2019). Moreover, when mice where administered with *Lactobacillus rhamnosus* JB-1, there was a stimulation of the transcription of GABA receptors in the vagus nerve, which induced behavioral and psychological effects that were reverted after vagotomy (Bravo et al., 2011).

Other studies in animal models have shown that impaired learning and increased depression-like behaviors were observed in a mice model, after the depletion of the gut microbiota by antibiotics. This was correlated with alterations in the levels of 5-hydroxyindoleacetic acid, 5-HT, homovanillic acid, DA, and noradrenaline, and in the mRNA levels of the corticotrophin-releasing hormone receptor 1 and the glucocorticoid receptor (Hoban et al., 2016). Another type of intervention involves using epigenetic dysregulation as a pharmacological target. Thus, it was shown that sodium butyrate, acting as a histone deacetylase inhibitor, improved social and repetitive behavior in BTBRT +tf/J (BTBR) mice. The administration of sodium butyrate had an effect on the transcriptome of several neurotransmitters and regulatory genes (Kratsman et al., 2016). Overall, these studies reinforce the correlation between ASD symptoms, gut microbiota and levels of neurotransmitters, and suggest that interventions focused on neurotransmitters could have the potential to reduce ASD behavioral symptoms.

USE OF THE GUT MICROBIOTA MODULATION AS A POTENTIAL THERAPY FOR ASD PATIENTS

At the moment, there are no effective therapies for treating ASD patients. In fact, research on autism is currently focusing on strategies for alleviating symptoms of ASD patients rather that looking for a cure (Willingham, 2020). Modulation of the gut microbiota has arisen as a potential therapy through interventions using probiotics, prebiotics, fecal microbiota transplantation (FMT) and diet.

Probiotic Interventions

The use of probiotics have displayed promising results in prevention and treatment of conditions such as obesity, colorectal cancer, IBD, IBS, or depression in human studies and

animal models (Walsh et al., 2014; Sharma and Shukla, 2016; Valsecchi et al., 2016). One of the investigated areas is the prevention of inflammation by regulating the barrier function, including the expression of tight junction proteins. Some studies have shown alleviation of GI symptoms and immunomodulation of cytokines using *B. longum* subsp. *infantis* 35624 (O'Mahony et al., 2005; Whorwell et al., 2006), *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (Messaoudi et al., 2011), *Lactobacillus casei* Shirota (Rao et al., 2009), *L. plantarum* WCFS1 (Karczewski et al., 2010) or *Lactobacillus rhamnosus* GG (Patel et al., 2012).

Bacteroides fragilis was used in a treatment that reduced ASD-like behavior in a rodent model of ASD (Hsiao et al., 2013). This bacterium reduced gut permeability and modulated the gut microbiota composition, suggesting that the key factors for the treatment of relieving ASD-like behaviors in patients should aim to balance gut microbiota and enhance the gut barrier. Another study used oral supplementation with a *L. acidophilus* strain and reported reduced levels of D-arabinitol in the urine of ASD children, improving the ability to follow directions (Kaluzna-Czaplinska and Blaszczyk, 2012). In a case study that used VSL#3, a mixture of 10 probiotic strains, it was reported to relieve and improve GI symptoms and other characteristics of ASD (Grossi et al., 2016). However, despite the overall positive outcome of probiotics in the treatment of symptoms of ASD patients, large randomized controlled studies are missing.

Prebiotic Interventions

Prebiotics are non-digestible compounds that are degraded by the bacteria in the GI tract and enhance the growth of endogenous beneficial bacteria, particularly lactobacilli and bifidobacteria. Generally, bacterial fermentation of prebiotics result in production of SCFAs that can be linked to their beneficial effects (Davani-Davari et al., 2019). Some examples of prebiotics are inulin, starch, pectin, galacto-oligosaccharides, and fructo-oligosaccharides. Although the use of prebiotics is well-established and health benefits are reported from their use, studies conducted with prebiotics in ASD patients are very few and the evidence provided is limited and non-conclusive (Grimaldi et al., 2017; Fattorusso et al., 2019).

Fecal Microbiota Transplantation and Microbiota Transfer Therapy

Fecal microbiota transplantations (FMT) and MTT are two effective strategies for treating ASD symptoms. FMT is designed to alter the entire microbiome by transferring fecal material containing microbiota from a healthy donor to another person with an impaired gut microbiota. It has proved to be very successful in the treatment of recurrent *C. difficile* infections (CDI) (Kellingray et al., 2018) and is being developed for IBD and IBS treatments (Aroniadis and Brandt, 2013; Rossen et al., 2015) and other microbiota associated disorders. Therefore, it has attracted attention of researchers as potential treatment for children with ASD and currently FMT clinical trials are in progress. However, it requires careful development and consideration since some side effects are reported, including diarrhea, abdominal cramps, abdominal distress, and low fever (Kelly et al., 2015). Also, we cannot be certain about the long-term effect of FMT. The MTT is similar to FMT but comprises 14 days of antibiotic treatment and a process of bowel cleansing. There is also the administration of a standardized human gut microbiota (SHGM) for 7–8 weeks with an initial high dose. This technique has shown improvement of both GI and ASD-related symptoms, and normalized the microbiota of ASD patients (Kang et al., 2017).

Dietary Interventions

One of the characteristics of children with ASD is the narrow diet, with a refusal of foods, based on its presentation or utensil use, and a limited food repertoire (Schreck and Williams, 2006; Bandini et al., 2010). The intake of fruits, vegetables, and proteins is less than in children with typical development and ASD children also ingest lower daily levels of potassium, copper, folate, and calcium when compared with controls (Sharp et al., 2013; Malhi et al., 2017). Diet is one of the most effective regulators of the gut microbiota and metabolite levels (Wu et al., 2011), and therefore, these behaviors are associated with lower levels of *Roseburia* spp. and *Eubacterium rectale*, linked to a lower intake of carbohydrates (Duncan et al., 2007; Wu et al., 2011; Tremaroli and Backhed, 2012). ASD patients who were treated with omega-3 FA for 12 weeks improved significantly their social behavior (Ooi et al., 2015). Another double-blind, placebo-controlled study showed that a treatment of levocarnitine for 3 months also improved ASD symptoms (Geier et al., 2011).

ANIMAL MODELS FOR THE STUDY OF THE RELATIONSHIP BETWEEN GUT MICROBIOTA AND ASD

Animal models can potentially play an important role in ASD research (Patel et al., 2018). There are many genetic models for the study of autistic disorders (Patel et al., 2018). However, there are no animal models that exhibit all the symptoms of human neurodevelopmental impairment. Experiments for the study of ASD have been conducted in zebrafish, monkeys, and songbirds, but mainly in rodents bred in laboratories, such as rats or mice (Hrabovska and Salyha, 2016). Rodents are suitable for the study of ASD because their behavior is well studied and there are a number of well-established techniques to manipulate their nervous system. Moreover, rats and mice are social animals and their relationships for parental, sexual or territorial behaviors, among others, are well-established. Initial ASD studies were conducted in rats, as their social behavior is clearly displayed. However, as mice are cheaper, their use for ASD study has been increasing. As a general rule, social behavior is measured by a series of tests such as the Morris water task, the three-chambered social interaction, swimming tests or simply by evaluating the explorative behaviors (Roullet and Crawley, 2011).

Despite the many ASD genetic models, the animal models used for the study of the gut microbiota-ASD relationship are more limited and are mostly inbred (e.g., BTBR) or environmental models (e.g., VPA, MIA) (Patel et al., 2018). Among the inbred mice model, BTBR mouse strain shows

phenotypic traits of ASD symptoms and has been used extensively (Kratsman et al., 2016). It shows a consistent replication of ASD phenotype in different laboratories, and has been used in numerous studies assessing the effects of gut microbiota products and drugs on ASD-related outcomes (Kratsman et al., 2016). Another mice strain used for ASD studies is C57Bl/6J. However, this strain has been described as less impulsive and more motivated in comparison to BTBR (McTighe et al., 2013). Despite this, C57Bl/6J has been used in studies that have highlighted the alleviating effect of a probiotic strain on ASD behavior (Hsiao et al., 2013). During administration of *B. fragilis*, pregnant females of C57Bl/6J were also included as a maternal immune activation (MIA) mouse model. MIA during gestation has been shown to increase the risk of development of neurodevelopmental psychiatric disorders (Conway and Brown, 2019; Kreitz et al., 2020). This is of particular interest in ASD, as GI barrier impairment can lead to inflammatory processes that ultimately might affect the neurodevelopment of the offspring. Both BTBR and C57Bl/6J have been used simultaneously in different studies. One such study found that ketogenic diet modified the gut microbiota of BTBR mice, rebalancing the ratio of Firmicutes to Bacteroidetes and reduced *Akkermansia* levels (Newell et al., 2016). They were also used in a recent study that showed that such mice were able to develop autistic behavior after FMT using fecal material from human ASD (Sharon et al., 2019). BTBR mice were tested using four behavioral tests: open field testing, marble burying, three chamber sociability test, and ultrasonic vocalization, based on interactions in male-female context. The study showed that *Lachnospiraceae*, *Bacteroides*, and *Parabacteroides* were different between the ASD group and the typical development group. Moreover, the metabolite profiles were different between the two groups, especially in the case of 5-aminovaleric acid (5AV), a GABA receptor agonist produced by gut microbiota, that was significantly depleted. Administration of 5AV and taurine to BTBR mice restored excitability levels of pyramidal neurons, highlighting that these models can be used to monitor electrical alterations in the nervous system derived from the effect of the gut microbiota.

Long-Evans rats as ASD model were utilized for studies on PPA (Meeking et al., 2020). A study using Sprague Dawley rats, where VPA was delivered to pregnant rats to assess its effect on the gut microbial richness and diversity of the offspring, concluded that VPA induced microbiome traits for ASD and also the behavioral changes (Liu et al., 2018). Overall, the development of accurate models will be critical for the study of the gut microbiota traits in ASD.

CONCLUDING REMARKS

The prevalence of ASD indicates that there is an urgent need to find new more effective treatments. Most of the research conducted so far has focused on alleviating ASD symptoms. The evidence suggesting a link between ASD and the gut microbiota, via the gut-brain axis, is now well-established. There are a number of pathways that are used in the microbiota-gut-brain axis connection. Understanding this connection opens the door to treatments and interventions that will improve the quality of life of patients and their families. It is likely that these interventions might not improve ASD-like symptoms when there are underlying genetic and environmental reasons, but they might help if the symptoms are gut microbiota-associated. At the moment, many clinical studies have shown that treatments for regulation of the gut microbiota provide improvements in ASD symptoms. However, biomarkers related to the gut microbiota activity have not been identified until recently. There is a need of more clinical and well-designed studies that include more patients, to provide more robust evidence that supports the use of probiotics, dietary and supplement treatments. In order to improve our understanding and design better studies, it is pivotal to identify robust gut microbiota-associated biomarkers.

AUTHOR CONTRIBUTIONS

EG-G, JR, and AN designed the manuscript. EG-G wrote the manuscript. JR and AN critically revised the manuscript and approved the final version. All authors contributed to the article and approved the submitted version.

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frontiers in Behavioral Neuroscience

MINI REVIEW
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Beneficial Use and Potential Effectiveness of Physical Activity in Managing Autism Spectrum Disorder

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Autism Spectrum Disorder (ASD) is a neurodevelopmental condition characterized by poor social and communication skills. Therapeutic interventions are behavioral and educational—normally delivered as structured programs. Several well-established programs exist and most of them do not incorporate physical activity and exercise as core elements. Deficiencies in motor skills are associated with ASD and physical activity has been shown to reduce maladaptive behaviors with autistics. However, the notion of exercise being employed to manage autism is controversial. Meta-analysis and systematic reviews have concluded that physical activity has positive effects on social skills and behavior in young children and adolescents with autism. Activities such as martial arts have been singled out as being particularly beneficial. Established programs such as TEACCH have been successfully modified, as research trials, to be more physical activity-based and have shown positive results. Studies have also reinforced the importance of the role of parental involvement in delivering programs based on physical activity. There is a paucity of research evidence about the long-term effects of physical activity-based interventions. There is also disparity over the detailed nature of the activities and exercises that compose an effective program. Each person with autism has a highly individualized set of symptoms and characteristics for which highly individualized programs are warranted. This is especially true for physical activity programs.

Keywords: early intervention, autism, autism spectral disorder (ASD), autism spectrum-disorder, physical activity, neurodevelopment, children with ASD, parental involvement

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INTRODUCTION

Autism spectrum disorders (ASD) are neurodevelopmental conditions characterized by poor social communication and social interaction, as well as restricted repetitive patterns of behavior, interests, and activities (Levy et al., 2009). ASD is typically first recognized in early childhood (Christensen et al., 2016) and many of the available treatments are implemented as early interventions initiated during the pre-school years. There is currently no established curative treatment for ASD, and interventional approaches are behavioral and educational (Kaplan and McCracken, 2012; Medavarapu et al., 2019), and are normally delivered as structured programs.

Currently, there are two broad classes of ASD interventions; *comprehensive treatment models* which are structured programs, and *focused intervention practices* that only treat narrow behavioral symptoms (Wong et al., 2015). These focused single medication strategies include pharmaceuticals, nutritional supplements/special diets (e.g., melatonin, gluten-free, casein-free, vitamins, and minerals), and alternative or complementary medicine (e.g., chelation, neurofeedback, hyperbaric oxygen therapy, acupuncture, and stem cell therapy; Wong et al., 2015; Siniscalco et al., 2018; Medavarapu et al., 2019). Psychotropic drugs may also play a role in the management of some behaviors commonly associated with ASD, such as aggression, severe irritability, and hyperactivity. However, limited improvements have been reported with the use of psychopharmacologic agents to treat the core features of ASD (Kaplan and McCracken, 2012; DeFilippis and Wagner, 2016).

Early intervention programs address the broader aspects of communication and social skills. These are often delivered by multiple professionals including pediatricians, child psychiatrists, occupational therapists, speech therapists, psychologists, specialist-teachers, and parents. These teams contribute to educational and therapeutic services. Various treatment programs have become established which include Applied Behavioral Analysis (ABA), Early Intensive Behavioral Intervention (EIBI), Verbal Behavioral Intervention (VBI), Treatment and Education of Autistic and Related Communication-handicapped Children (TEACCH), Picture Exchange Communication System (PECS), speech therapy, and sensory integration therapy. Also added to this list are less conventional approaches such as horseback riding therapy, dolphin-assisted therapy, music therapy, and art therapy. This vast array of treatments, therapies, and programs have varying degrees of empirical support for their evidence base (Wong et al., 2015).

The use of physical exertion as a means of decreasing problem behaviors or increasing appropriate behaviors (exercise-therapy) is considered an evidence-based practice (Wong et al., 2015). Furthermore, research has revealed that disturbances and deficiencies in motor skills are commonly associated with ASD (Leary and Hill, 1996; Ghaziuddin and Butler, 1998; Ozonoff et al., 2008; Green et al., 2009). Given this evidence, exercise-therapy would seem an appropriate therapeutic strategy for improving the quality of life in ASD. This review discusses the benefit Physical Activity has on individuals with ASD and its potential effectiveness when employed as a tool to manage ASD. There is a paucity of studies that have examined PA among adults with ASD (Hillier et al., 2020), therefore the focal area of attention here is children and adolescents with ASD.

THE BENEFITS OF PHYSICAL ACTIVITY IN ASD

Physical activity (PA) is important for maintaining health within the general population. It reduces blood pressure, improves sleep quality and insulin sensitivity, and consequently lowers the risk of certain chronic conditions (Williamson and Pahor, 2010). Exercise induces the release of endorphins and monoamine neurotransmitters in the brain, thus mimicking the effects of antidepressants and making PA a viable alternative to drug treatments (Zhao and Chen, 2018). PA has been shown to improve motor skills and cognition in preschool children aged 4–6 years, specifically in the areas of attention, memory, behavior, and academic achievement (Lang et al., 2010; Zeng et al., 2017).

The patent health benefits of PA are also applicable to children with ASD. However, PA levels in children with ASD are lower than their typically developing (TD) counterparts (Macdonald et al., 2011; Hillier et al., 2020). This may be attributed to poor motor coordination and balance, often associated with ASD (Ghaziuddin and Butler, 1998; Green et al., 2009). These limit activity choices. Impairments in sensory, behavioral, and communication skills render participation in team activities challenging (Potvin et al., 2013). Hence, adolescents with ASD also engage in less PA relative to their TD counterparts (Srinivasan et al., 2014; Stanish et al., 2017).

Trials have been performed exploring the use of PA in ASD, many of which have endorsed the use of PA in the management of Autism. A systematic review by DeJesus et al. (2020) concluded that dance had a positive effect on ASD associated symptoms. These included improved: social involvement, behavior, communication skills, body awareness, and mental health. Meta-analysis has assessed PA interventions on youth with ASD across 29 studies and found an overall moderate positive effect (Healy et al., 2018). However, within the areas of movement (manipulative and locomotor skills, muscular strength, and endurance) moderate-to-large positive effects were found. Further, large positive improvements were observed for a social function. This being a major deficit in ASD (Healy et al., 2018).

A study conducted on 5–8-year-old children in a special school in China showed significant improvements in the social function among children on the spectrum who participated in a 12-week structured PA program (Zhao and Chen, 2018). The program consisted of twice-weekly exercise sessions both lasting 60-min. The intervention program purposely provided opportunities to enhance social interactions in a natural environment conducive to developing communication skills. Participants learned how to interrelate with others and how to express themselves. The program was evaluated quantitatively using Social Skills Improvement System Rating Scales (SISS) and Assessment of Basic Language and Learning Skills—Revised (ABLLS-R) scores as well as qualitative interviews with parents and staff. SISS assessed seven social skills subdomains and significant improvements were seen in communication, cooperation, and self-control. Improvements were also seen in ABLLS-R scores. Parents and volunteer workers gave positive feedback on the program (Zhao and Chen, 2018). Since communication and social interaction are the major deficit areas seen with ASD this study provides evidence of the appropriateness of structured PA programs in the management of ASD with children. The improvements observed could neither be solely attributed to PA itself nor the mode of delivery of the program i.e., were the improvements the result of the physical excursion or

team-based activities centered around social interaction? This may be an important area for further exploration. Although this study reported improvements in social interactions which the authors attributed to PA, it should be noted that the design and structure of the program were based upon the TEACCH model. The reported improvements may have resulted from the program used, regardless of the PA components that were incorporated as mere details into a well-established intervention. TEACCH is one of the most validated interventions used to treat children with ASD (Panerai et al., 2002) and is particularly effective in the areas of social and maladaptive behaviors (Virues-Ortega et al., 2013). The TEACCH program is based largely on activities which are normally educational or life-skills. However, these could easily be replaced by activities involving physical exercise. These activities are organized within structured elements of a specialist physical environment, predictable sequence of activities, routines with flexibility, structured work/activity systems, and visually structured activities (Myers and Johnson, 2007). While the results of this 12-week study are promising, there were no long-term assessments. Evaluations were carried after completion of the 12-week intervention only. There is a need for follow-up data and more investigations with longitudinal study-designs in this area. Other studies have shown that vigorous rather than moderate exercise is more beneficial in reducing maladaptive and stereotypic behaviors (Kern et al., 1984; Elliott et al., 1994; Celiberti et al., 1997). This suggests that children with ASD may have a higher threshold to overcome before positive effects begin to appear relative to TD children.

A systematic review (Lang et al., 2010), explored 18 PA studies involving adults and children, all of which reported improvements in either: behavior, academic performance, or physical fitness. However, the authors recommended the development of stronger experimental designs for future studies (Lang et al., 2010). This was reiterated more recently in a similar review (Ruggeri et al., 2020).

PARENTAL INVOLVEMENT IN PHYSICAL ACTIVITY

It is now recognized that parents play a paramount role in almost all treatment modalities for ASD. Parents were once considered the cause of ASD. However, they are now considered the most important resource and the most effective factor in promoting behavioral changes in the child with ASD (Schopler, 1987). The irony of this role reversal is that currently numerous therapeutic interventions for ASD are hinged upon the pivotal position of parents as therapists or co-therapists (Panerai et al., 2002). Early interventions have been shown to have a moderate-to-large effect on outcomes among children with ASD (Virués-Ortega, 2010; Virues-Ortega et al., 2013; Beaudoin et al., 2014). Interventions specifically designed to include parents have significantly improved outcomes and have shown to be more intensive (Burrell and Borrego, 2012). Meta-analysis show there to be greater improvements in language-understanding and ASD characteristics when parental involvement was incorporated into interventions (Oono et al., 2013). The equipping of parents with development-enhancing strategies while engaged with their children is considered an essential constituent of these interventions (Landa, 2018). This is concerning interventions in general. Parental support and involvement also appears to be a major component of PA-based interventions and keeping ASD individuals physically active (Nichols et al., 2019). Parental involvement has been shown to augment treatment approaches leading to more strongly positive outcomes (Mendlowitz et al., 1999; Lakin et al., 2004). Lakin et al. showed that PA delivered with family involvement resulted in better outcomes relative to therapy delivered without family involvement (Lakin et al., 2004).

Parental influence on PA with children with ASD also remains a vital area of study concerning young adults on the autism spectrum, especially those of post-secondary education age. These individuals rely heavily on either their parents or caregivers (Hewitt et al., 2017). One study suggested that the strong positive effects of parental involvement in PA were merely coincidental (Burrell and Borrego, 2012). This suggestion highlights the need for further research in the area of parent involvement in PA. Especially since PA appears to be crucial for young adults with ASD (Nichols et al., 2019). ASD children are considered relatively dependent on others for assistance in everyday life (Schall et al., 2014). ASD adolescents are more likely to have "poorer health profiles" and develop chronic diseases as compared to TD individuals. Therefore, it is imperative to identify factors, such as PA, that influence health-states to construct interventions to help young adults with ASD (Warren et al., 2012).

According to Brustad, parents who enjoyed participating in PA positively impacted their children with ASD by encouraging them to take part. Moreover, this also in turn influences ASD children's capability and therefore enhances their level of engagement (Obrusnikova and Miccinello, 2012). It has also been shown that lower activity scores correlated with having single-parent families. This suggests that two-parent families may result in better outcomes (Memari et al., 2015). We conclude that parental involvement in therapies or interventions lead to greater and more effective outcomes and should be considered whenever applicable. We suggest that this topic of PA and parental involvement should be researched further in both children and adolescents.

IMPLEMENTING PHYSICAL ACTIVITY AS AN ASD TREATMENT

Available treatments for ASD focus on making the child with ASD more independent and maximizing their quality of life. This is achieved by minimizing the core characteristics associated with ASD. Thus, the amelioration of maladaptive behaviors is a common management strategy in programs (Landa, 2007). It is apposite to perceive the role of PA as a component of wider more extensive approaches to treating ASD. This broader base involves educational and behavioral multimodal interventions represented by the *comprehensive treatment models*, such as TEACCH, and the *focused intervention practices* represented by certain pharmacological and behavioral

interventions (Wong et al., 2015). These focused interventions address the symptoms associated with impaired social skills and communication skills and repetitive and stereotypical behavior patterns and interests. PA may be categorized as one of the focused interventions and implemented as an adjunct to other focused interventions or as a constituent of a comprehensive treatment model (Smith, 2013). Several comorbid symptoms are commonly associated with ASD which include: self-injury, impulsivity, decreased attention, anxiety, depression, and sleep disruption (Levy et al., 2009). Well-established treatments exist, both pharmacological and behavioral, for some of these comorbid symptoms. Psychotropic drugs are utilized to manage irritability, hyperactivity, and repetitive behaviors (Kaplan and McCracken, 2012; DeFilippis and Wagner, 2016). PA may be co-administered with interventions used to treat the listed common comorbidities.

It is of utmost importance that each child receives an individualized treatment plan that targets their needs and allows for the participation of parents, teachers, and other caretakers (Hyman et al., 2020a,b). Tan et al. (2016) found the benefit of PA in children with ASD to be similar to those seen in TD children. There now exist guidelines that allow different sports and educational establishments to implement programs suited for individuals with ASD (Srinivasan et al., 2014). Whether or not these exercises are effective with individuals with ASD of all ages is yet to be determined. Cameron et al. suggested that movement-based interventions that are effective with older children with ASD may not be effective for preschool children (Cameron et al., 2020).

In addition to the guidelines, there are various ways of implementing PA programs. Bremer et al. (2016) considered the two best forms of physical intervention to be martial arts and horseback riding, whilst there was a limited benefit in yoga, dance, and swimming. In contrast, DeJesus et al. (2020) considered dance to positively affect children and improved many of their symptoms, such as social behaviors, communication skills, and psychological wellbeing. This disparity in interpretation may be due to a larger amount of available data for interventions such as martial arts and limited data for dance. Hence, more research is required in the area of dance as an intervention.

An individual with ASD derives benefits from PA in two main ways. Firstly, the impact of PA on weight gain and obesity and secondly reducing maladaptive behaviors. Obesity is a major concern in children with any form of developmental disabilities (Srinivasan et al., 2014). However, children with ASD are at particularly high risk for obesity (Curtin et al., 2014). It is essential to recognize obesity as it is an established comorbid in children with ASD (Pan, 2008; Sowa and Meulenbroek, 2012). This may be due to lack of structure in nutritional intake, over usage of television to calm them, and side-effects of medication (Must et al., 2017). Another contributing factor is that the ASD child might not have a supportive environment (Pan, 2008). The second perspective relates to behavioral elements such as physical condition, self-esteem, and social skills. A major focus has been placed upon improvements in stereotypical behavioral patterns and general social behavior and function (Zhao and Chen, 2018). Studies have shown that improved motor skills positively impact social skills, in addition to reducing stereotypical behaviors (Iliadis and Apteslis, 2020). Therefore, to maintain the health and wellbeing of ASD children, it is essential to include physical activities in their daily life. Several studies show that PA levels in ASD and neurotypical children are significantly different (Pan, 2008; Sowa and Meulenbroek, 2012). Bandini et al. (2013) found that 43% of TD children participated in a 60-min moderate and vigorous PA as compared to 23% of children with ASD.

To facilitate building a PA program for children with ASD, it is important to address multiple factors to decide the most appropriate elements of the program. We here address two issues: (a) *Individual vs. group intervention*; and (b) *Organization of the Program*.

INDIVIDUAL VS. GROUP INTERVENTION

When tailoring a PA program for children with ASD, an essential question should be asked; would an individual-based or a group-based intervention promote larger improvements? PA programs organized within a group situation (teammates, peers, coaches, and teachers) may enhance development among children with developmental disabilities (Rinehart et al., 2018). The implementation of group-based PA in a social context would offer opportunities for social interactions. Group-setting should facilitate social behavior and communication. However, meta-analysis compared responses to PA delivered as individual-based and group-based interventions and found greatly improved social skills and attenuated maladaptive social behaviors with the individual-based approach (Sowa and Meulenbroek, 2012). Paradoxically, social skills were less improved in a social scenario. Sowa and Mulenbroek concluded that individual-based interventions offer more specific programs and decreased stress levels due to the unpredictable events associated with group activities (Sowa and Meulenbroek, 2012). The individual approach protects the child with ASD from negative emotions arising from being misunderstood by group members (Pan, 2009), and also from tensions between teammates or opponents (Sowa and Meulenbroek, 2012). Another meta-analysis study provided evidence supporting the use of group-based PA programs. Qualitative and quantitative evaluations of group-based PA concluded that programs provided opportunities for social skill development. This study included several modes of group-based activities but only one "team sport" program. The authors suggested a future focus on team sports as a key research area (Howells et al., 2019). There are clearly advantages and disadvantages to group-based activities which must be taken into consideration for the child with ASD with very specific characteristics and behaviors. The notion of highly individualized education programs for children with ASD is now wildly accepted as each child has a unique presentation of core ASD and comorbidity symptoms. Although counterintuitive, the highly individualized program delivered in isolation of the group would seem to be advantageous with respect to social skills development for children with ASD.

ORGANIZATION OF THE PROGRAM

When structuring a program for ASD children, it is important to simplify the structure of the program to not overwhelm any participant or their teachers and parents.

Schultheis et al. (2000) modified the TEACCH program to create a recreational program. The new, modified version included three portions: (a) physical structure; (b) schedules, and (c) task organization. By keeping all three aspects in mind they were able to create simplified ways to facilitate introducing physical activities by introducing a schedule with pictures or color codes and a structure of the gymnasium with room divider boundaries. Additional equipment (timers etc.) may also be used as desired (Schultheis et al., 2000). Meta-analysis indicates that TEACCH has moderate-to-large improvements in social behavior and maladaptive behavior, which makes it an ideal program to achieve improvement within these two combined fields of ASD and PA (Virues-Ortega et al., 2013).

It is now well established that PA leads to improvements in multiple domains relevant to ASD. However, research is still needed in this field to refine and improve programs and modes of delivery of programs to maximize the benefit for individuals with ASD (Sorensen and Zarrett, 2014).

DURATION OF POSITIVE EFFECTS

PA has been shown to improve classroom behaviors and improve aspects of academic performance in neurotypical children (Álvarez-Bueno et al., 2017). It is self-evident that there are long-term benefits for any individual that achieves academically during childhood. Regarding autistic children, it has been reported that children with ASD who underwent a 14-weeks training program in karate exercise-routines *kata* (choreographed movements performed with technical precision in set sequences) showed a significant reduction in communication deficit and improvements in stereotypic behaviors compared to neurotypical controls. Interestingly, these improvements were observed for up to 1 month after completion of the intervention (Bahrami et al., 2012, 2016). This suggests that learning these karate specific exercise-routines and undergoing the physical exertion that accompanies them has long-lasting effects. These long-term effects may be related to the use of training with traditional martial art rather than PA *per se*. A study with neurotypical school students showed that participating in school-based martial arts (Taekwondo; including deep-breathing and relaxation techniques) training resulted in improved self-regulatory skills (Lakes and Hoyt, 2004). Traditional martial arts have elements of self-awareness and concentration which are not found in standard physical education classes. Adopting PA in conjunction with self-awareness in the formative years may have long-lasting effects. It has been suggested (Diamond, 2015; de Greeff et al., 2018) that cognitively and coordinately demanding physical exercises may be better at improving executive function in children rather than pure exercises and movement (Wang et al., 2020). We consider this an important area of future research for ASD; in the last decade, there has been great interest in studying the relationship of PA with cognitive functioning for neurotypical children (Lees and Hopkins, 2013; Donnelly et al., 2016).

Impaired social and communication skills associated with ASD may lead to social isolation or withdrawal (Bellini et al., 2007), and most interventions work to counter this. Any intervention that addresses social and communication skills should have long-term implications. Most organized sports activities provide environments conducive for the natural building of connections between participants. Sports and games increase opportunities for social interactions. Inherent elements of most sports and games are turn-taking, cooperative play, partnering, exchanges, and general teamwork. All these elements would practically improve both verbal and non-verbal communication skills as well as ensuring social engagement. Activities with a structured program would also have interactions between children and their teachers. A program of structure sporting activities would be a natural area in which to implement the very tenants of established ASD therapies such as TEACCH (Zhao and Chen, 2018). These therapeutic approaches all have the same goal of having a life-long influence on the child with ASD.

INNOVATIVE WAYS TO INTRODUCE PA TO CHILDREN WITH ASD

There are challenges faced by individuals with ASD regarding performing certain exercises. Therefore, research has concentrated on innovative ways of introducing PA to children and adolescents with ASD. One such innovation in the delivery of PA is *Exergaming*.

While still a new field of study, there has been a growing interest in finding different ways to include PA in the lives of autistic individuals and overcoming the challenges that exist in a normal PA program. For example, some findings have indicated that *Exergames* can be a potential tool to treat both children and adolescents with ASD. *Exergames* are defined as any game that has a combination of video games and physical interaction with participants online. Its advantages include being more enjoyable and playful, which may lead to an increase in adherence. Lima et al. (2020) have shown the benefits have been perceived in fitness perspectives only. More studies are needed in this field to cover the effect of *Exergaming* on stereotypical behaviors, social and cognitive skills.

CONCLUSIONS

PA is effective as a therapeutic strategy for the management of ASD. However, there is a paucity of research evidence about the long-term effects of interventions based upon PA. There is a difference of opinion as to the detailed nature of the activities and exercises that compose an effective PA program. There is also a need for refinements in program delivery methods, which is indicative of the actual autistic condition itself. Each person affected by ASD has a highly individualized set of symptoms and characteristics for which a highly individualized therapeutic program is warranted. The development of workable programs

requires research and evidence gathering to produce generalized guidelines. These guidelines may come to govern very specialized structured and individualized interventions for children and adolescents with ASD.

AUTHOR CONTRIBUTIONS

The abstract was written by JS and edited by SF. The introduction was written by SF, followed by the general benefits of exercise by JA. "The Benefits of Physical Activity in ASD" section was written by JS. ZS wrote the "Parental Involvement in Physical Activity" section. SA-S explained how to implement physical activity as an ASD treatment by looking at Group vs. Individual Interventions and Organization of the Program. SF has also elaborated on the duration of positive effects, and "Innovative Ways to Introduce PA to Children With ASD" section. JS also wrote the conclusion. SF has also managed references using endnote and helped JS in editing the article as well as doing the final review. The article was also reviewed by ER, who additionally contributed ideas and added suggestions to the article.

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In silico Gene Set and Pathway Enrichment Analyses Highlight Involvement of Ion Transport in Cholinergic Pathways in Autism: Rationale for Nutritional Intervention

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Food is the primary human source of choline, an essential precursor to the neurotransmitter acetylcholine, which has a central role in signaling pathways that govern sensorimotor functions. Most Americans do not consume their recommended amount of dietary choline, and populations with neurodevelopmental conditions like autism spectrum disorder (ASD) may be particularly vulnerable to consequences of choline deficiency. This study aimed to identify a relationship between ASD and cholinergic signaling through gene set enrichment analysis and interrogation of existing database evidence to produce a systems biology model. In gene set enrichment analysis, two gene ontologies were identified as overlapping for autism-related and for cholinergic pathways-related functions, both involving ion transport regulation. Subsequent modeling of ion transport intensive cholinergic signaling pathways highlighted the importance of two genes with autism-associated variants: *GABBR1*, which codes for the gamma aminobutyric acid receptor ($GABA_{B1}$), and *KCNN2*, which codes for calcium-activated, potassium ion transporting SK2 channels responsible for membrane repolarization after cholinergic binding/signal transmission events. Cholinergic signal transmission pathways related to these proteins were examined in the Pathway Studio environment. The ion transport ontological associations indicated feasibility of a dietary choline support as a low-risk therapeutic intervention capable of modulating cholinergic sensory signaling in autism. Further research at the intersection of dietary status and sensory function in autism is warranted.

Keywords: acetylcholine, dietary choline, autism, sensory processing, cholinergic, gene set enrichment analysis

INTRODUCTION

Sensory processing dysfunction, which is commonly experienced by persons diagnosed with autism spectrum disorder, extends across auditory, visual, pressure, temperature, pain, vestibulary, and interoceptive processing domains (Craig, 2002; Ashburner et al., 2008; Case-Smith et al., 2015; Boterberg and Warreyn, 2016; Crasta et al., 2016). Some features of sensory processing,

including heightened sensitivity to sound (hyperacusis), appear to be prevalent in the majority of the autistic population (Wilson et al., 2017; Williams et al., 2020). All types of sensory processing rely on sensory gating—the ligand-activated, ion-channel mediated pathways built upon cholinergic neurotransmission signals. Acetylcholine, a primary neurotransmitter ligand in these pathways, plays a key role in cognitive function, memory, learning, and sensory processing signal transduction. Acetylcholine is made in an enzymatic reaction between acetyl-CoA and choline, which is facilitated by the choline acetyltransferase (Oda, 1999). Neuronal molecules associated with acetylcholinergic signal transmission, including the nicotinic receptor family, are heavily implicated in propagation of auditory signals through respective transmission pathways (Simmons et al., 2011; Bertrand et al., 2015; Deutsch et al., 2015; Dineley et al., 2015; Sinkus et al., 2015; Gillentine et al., 2017). Research of cholinergic function in autism has led to clinical trials examining efficacy of cholinergic enhancement and/or acetylcholinesterase inhibitors—more commonly used in Alzheimer's disease—to address social and cognitive aspects of autistic behaviors (Bentley et al., 2003; Hertzman, 2003; Nicolson et al., 2006; Ghaleiha et al., 2014; Karvat and Kimchi, 2014; Rossignol and Frye, 2014; Eissa et al., 2018). However, cholinergic support to sensory processing in autism remains less examined. Recent developments in autism genome-wide association studies (GWAS) have provided estimates of an overlap of autism-related gene sets with those involved in sensory processing and in cholinergic signal transmission.

While humans can make small amounts of choline endogenously, the replenishment of the pool of acetylcholine available in the human body depends heavily upon dietary choline intake (Gibellini and Smith, 2010). In the United States, nearly nine in 10 Americans over 2 years of age do not meet recommended daily consumption levels of dietary choline. Moreover, an estimated 60–93% of children on the autism spectrum are not meeting their adequate intake (AI) level for choline either, even as experts in choline metabolism continue to emphasize its criticality for nutrition across the lifespan, particularly at critical stages of neurodevelopment (Caudill, 2010; Hamlin et al., 2013; Wallace and Fulgoni, 2016; Blusztajn et al., 2017; Ganz et al., 2017; Wallace et al., 2018, 2019; Zeisel, 2019). In rodents, choline-deficient diets are associated with decreased levels of brain acetylcholine (Cohen and Wurtman, 1976). In both rodents and humans, choline deficiency is associated with impaired sensory gating function (Wu et al., 1993; Fendt and Koch, 1999; Stevens et al., 2008; Knott et al., 2014; Swerdlow et al., 2016). Given possible risk of choline underconsumption and/or its deficiency in humans, and the known impact of choline deficiency upon brain acetylcholine and sensory function in rodents, further investigations into the potential impact of choline underconsumption upon sensory processing are warranted, particularly with respect to those on the autism spectrum. Such research may eventually open an avenue for modulation of sensory processing through nutritional interventions.

To explore the potential relationship between autism spectrum disorders (ASDs) and choline intake, we conducted an enrichment analysis of gene sets associated with ASD and cholinergic pathways, and constructed the model reflecting this relationship. Results of our study highlight the potential impact of dietary choline deficiency upon cholinergic signaling within the genetic context of autism.

MATERIALS AND METHODS

Overview of Workflow

This study has focused on gene ontologies cataloged in the Gene Ontology (GO) database for enrichment analysis of an overlap between two gene sets, one for cholinergic pathways and one for autism-associated genes. Gene ontological enrichment analysis was conducted within the Pathway Studio environment (Elsevier, Inc.)[1] between December 2019 and March 2020. The Pathway Studio database contains functional relationships and pathways of mammalian proteins, including human, mouse, and rat genes. It contains over 1.4 million entities of 14 well-defined categories, including cells, proteins, disease, and small molecules, and more than 13.4 million relationships among these entities. The database includes over 24 million PubMed abstracts and 3.5 million Elsevier and third party full-text papers. Using the natural language processing (NLP) functionality of Pathway Studio, intersections of these gene sets' pathways were rendered graphically, with connections indicating directional relationships being supported by the current scientific literature. This exploratory analysis and figure development which employed in part some of Pathway Studio's graphical capabilities took place between March 2020 and November 2020. Since many of the autism-associated genes in the GWAS study have been recently identified as autism-associated only within the past 2 years, this gene/pathway modeling effort was supported by secondary analyses, including use of predictive tools in splice site identification.

Identification of Ontologies Associated With Each Gene Set, and Shared Ontologies

To identify GO database pathways shared between cholinergic pathways and autism-associated genes, gene set enrichment analysis was conducted. Molecular Signatures Database v7.1 (MSigDB)—one of the most widely used repositories of thoroughly annotated gene sets, used in research on both neurological conditions and on cholinergic pathways, specifically—was chosen as a precurated resource for gene sets connected with cholinergic activity (Tan et al., 2010; Turcan et al., 2010; Liberzon et al., 2011; Koker et al., 2018; Schijven et al., 2018; Zhang et al., 2019). A key word search for "cholin*," identifying any word beginning with cholin- (choline, cholinergic, cholinesterase, etc.), was used to identify cholinergic-relevant gene sets encompassing a total of 345 genes, which constituted the cholinergic activity gene set for this ontological enrichment analysis. An autism gene set containing

[1] www.pathwaystudio.com

47 genes from a recent cross-trait genome-wide meta-analysis (Wu et al., 2020) was used because it identified more risk genes for ASD compared with another GWAS study for ASD, thus providing enhanced statistical power for the present study (Grove et al., 2019).

Gene ontology association analysis was conducted with each respective set of genes within Pathway Studio. The lists of ontologies assigned to each of these gene sets were trimmed at approximately the lowest 100 p-values ($p < 0.01$). A shift in the magnitude of p-values, nearest the lowest 100 p-values, served as the final determinant of cut-off for each respective gene sets' ontology lists. The overlaps were identified using the Venn diagram web tool, Venny 2.1 (Oliveros, 2007–2015).

Genes from the autism gene set were then examined for their potential upstream or downstream relationships with acetylcholine, with additional attention paid to potential roles related to the identified shared ontologies, as they were related to sensory processing and cholinergic sensory signal transmission. The identification of upstream and downstream elements and related pathways was conducted using Pathway Studio's natural language processing tools within its menu architecture, which generate indices of related entities (genes, proteins, physiological conditions, etc.) per tool sorting specifications. The main two tool specifications used in this exploratory analysis were the Pathway Studio options to view first degree connections (known in Pathway Studio as Direct Interactions) and to view the shortest path between entities within the literature (known in Pathway Studio as Shortest Path). Both of these tool specifications were the primary means of building a broader understanding and figure illustration of literature-supported cholinergic networks related to both genes within the autism gene set and to theoretical cholinergic signal disruption related to dietary choline deficiency.

Gene Set Pathway Intersection Analysis of Shared Ontologies

Shared ontologies and associated pathways were rendered graphically using Pathway Studio's NLP-driven platform. Additionally, certain elements of interest from the ontological overlap and the intersection between pathways involving genes from these sets were inserted.

Graphical models were developed to examine specific pathways or networks with key autism-associated genes, and respective genetic variants which may alter choline and/or acetylcholine levels available for neuronal sensory signal transport. Each instance of a relationship identified by NLP-based word triplet identification was graded by Pathway Studio in terms of confidence levels of 1–3, with a maximum confidence level of 3 being defined as an entity-to-entity relationship supported by at least three peer reviewed publications reflected

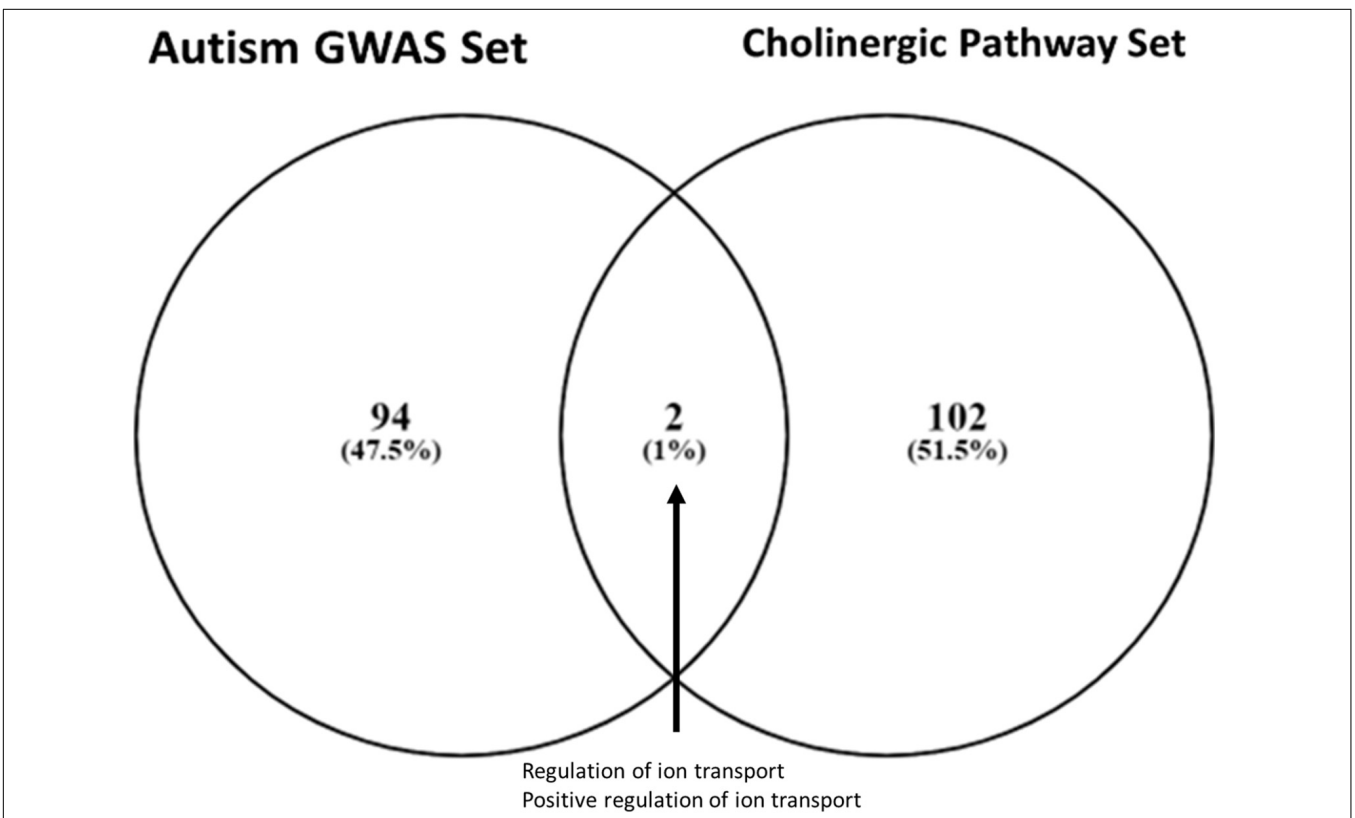

FIGURE 1 | Gene ontology overlap between autism and cholinergic metabolism gene sets. Overlap between an autism-associated genome-wide associated studies gene set (Wu et al., 2020) and cholinergic pathways gene set (MSigDB v7.1) was identified using Venny 2.1. The two shared ontologies were (1) regulation of ion transport and (2) positive regulation of ion transport, identifying ion transport functionality as a critical area of enrichment for further analysis.

by the graphical rendering. For this study and related models, the confidence threshold for including elements in graphical representation was set at 3.

Supplemental Analyses for Model Refinement

Splice site identification algorithm Alternative Splice Site Predictor (ASSP) (Wang and Marín, 2006) was used to examine potential changes in the splicing expression which may arise from intronic variants located within autism-associated genes.

RESULTS

Shared Gene Ontologies

Two ontologies were shared between the cholinergic pathway and the autism GWAS gene sets ($p < 0.05$), namely, those for ion transport regulation and positive ion transport regulation (**Figure 1**). These ion-transport-related pathways are associated with sensory processing functionality across broad spectrums of the relevant domains, including nociception, tactile response, vestibular reflex, startle response, pupillary light reflex, and sensory gating in general.

Within these two ion transport regulation ontologies, two common genes were identified as implicated in neuronal signal transmission: the gene *GABBR1*, which encodes the gamma aminobutyric acid membrane receptor $GABA_{B1}$, also known as "GABA receptor" elsewhere in the literature, and the gene *KCNN2*, which encodes potassium intermediate/small conductance calcium-activated channels of SK2 type. The latter channels are often co-located with cholinergic receptors and are capable of modulating cholinergic signaling through membrane repolarization.

Analysis of the Intersection of Autism-Related Cholinergic Gene Sets

Analysis with Pathway Studio's graphical pathway rendering platform identified regulatory and functional relationships between acetylcholine, ion transport/ion levels, *GABBR1* and *KCNN2* activity, and aspects of sensory processing. All depicted relationships are supported by experimental evidence extracted from the scientific literature (**Figure 2**).

Choline and acetylcholine were linked to ion transport function across several pathways, with the most direct implication being the neuronal influx of membrane depolarizing calcium resulting from the binding of acetylcholine to cholinergic receptors, such as the alpha-7 nicotinic receptor. This transition from chemical signal transduction (release of acetylcholine into the synapse which binds with the nicotinic receptor on the dendrite of the signal recipient neuron) to ionic signal transduction down the axon is a core aspect of sensory signal pathway functionality (**Figure 3**).

Binding of acetylcholinergic receptors is modulated through multiple means, both preemptive and *ex post facto*. The enzyme acetylcholinesterase functions by cleaving acetylcholine in the synapse, including either acetylcholine recently made available for signal transduction, or acetylcholine released

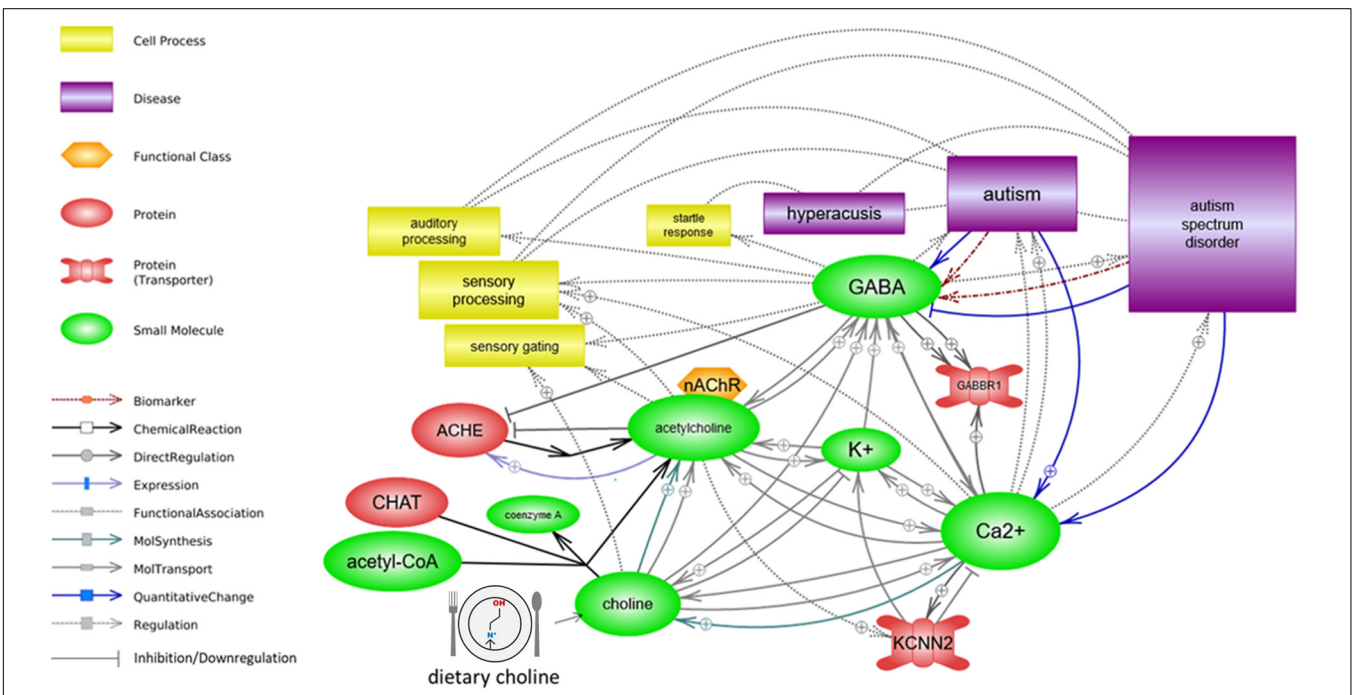

FIGURE 2 | Sensory processing is associated with autism functionally, in the context of ion transport and cholinergic signaling. Acetylcholine regulates both the ion transport and the GABA signaling. A deficiency in its precursor, dietary choline, may impact ion transport, GABA⇒*GABBR1* signaling, and *KCNN2*/SK2 channel function, either or all of which may result in altered sensory processing function.

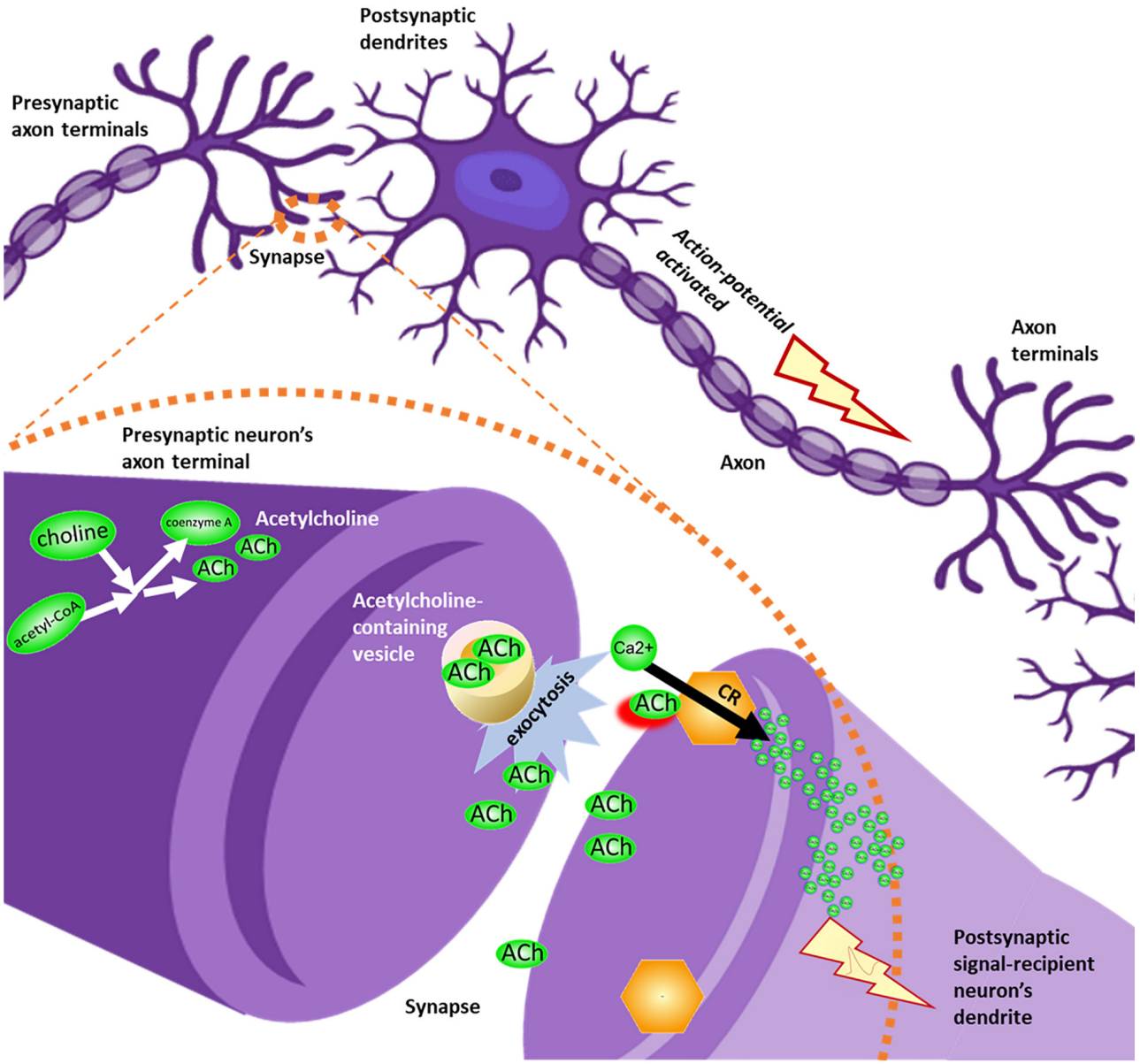

FIGURE 3 | Overview of acetylcholinergic signaling. Acetylcholine (ACh), sourced from precursors choline and acetyl-CoA, leaves the presynaptic neuron's axon terminal through vesicle-mediated exocytosis. Acetylcholine molecules diffuse through the synapse, until binding with cholinergic receptors (CR) in the membrane of the postsynaptic signal-recipient neuron's dendrite. This binding event permits influx of calcium ions into the dendrite. Ionic membrane depolarization activates an action potential, causing an electrical signal to be propagated down the length of the axon in a series of ion transport events, until the signal reaches the axon terminal and is translated again into neurotransmitter signals destined to reach the next neuron's dendrite.

from membrane-bound acetylcholinergic receptors in the signal recipient neuron (**Figure 4**).

The regulatory role of GABA-ergic binding in acetylcholinergic activity is nuanced. Typically, GABA is released from the signal-transmitting neuron synchronously with acetylcholine through separate GABA-specific vesicles, which serve as an immediate signal modulator. The subsequent cascade that arises from GABA binding to the $GABA_{B1}$ receptor suppresses further upregulation of acetylcholinesterase (ACHE) and preserves acetylcholine in the synaptic cleft from destruction. The signal propagated through the $GABA_{B1}$ receptor is, therefore, hypothesized to modulate an availability of acetylcholinesterase within the cleft. In other words, GABA-ergic suppression of ACHE upregulation may lead to decrease of synaptic acetylcholine degradation, leaving more acetylcholine available for cholinergic signal transmission. This cascade's impact upon synaptic acetylcholine levels is depicted in **Figure 5**. A hypothetical impact of genetically determined differences in GABA–$GABA_{B1}$ binding activity upon cholinergic sensory signal modulation is depicted in **Figure 6A**.

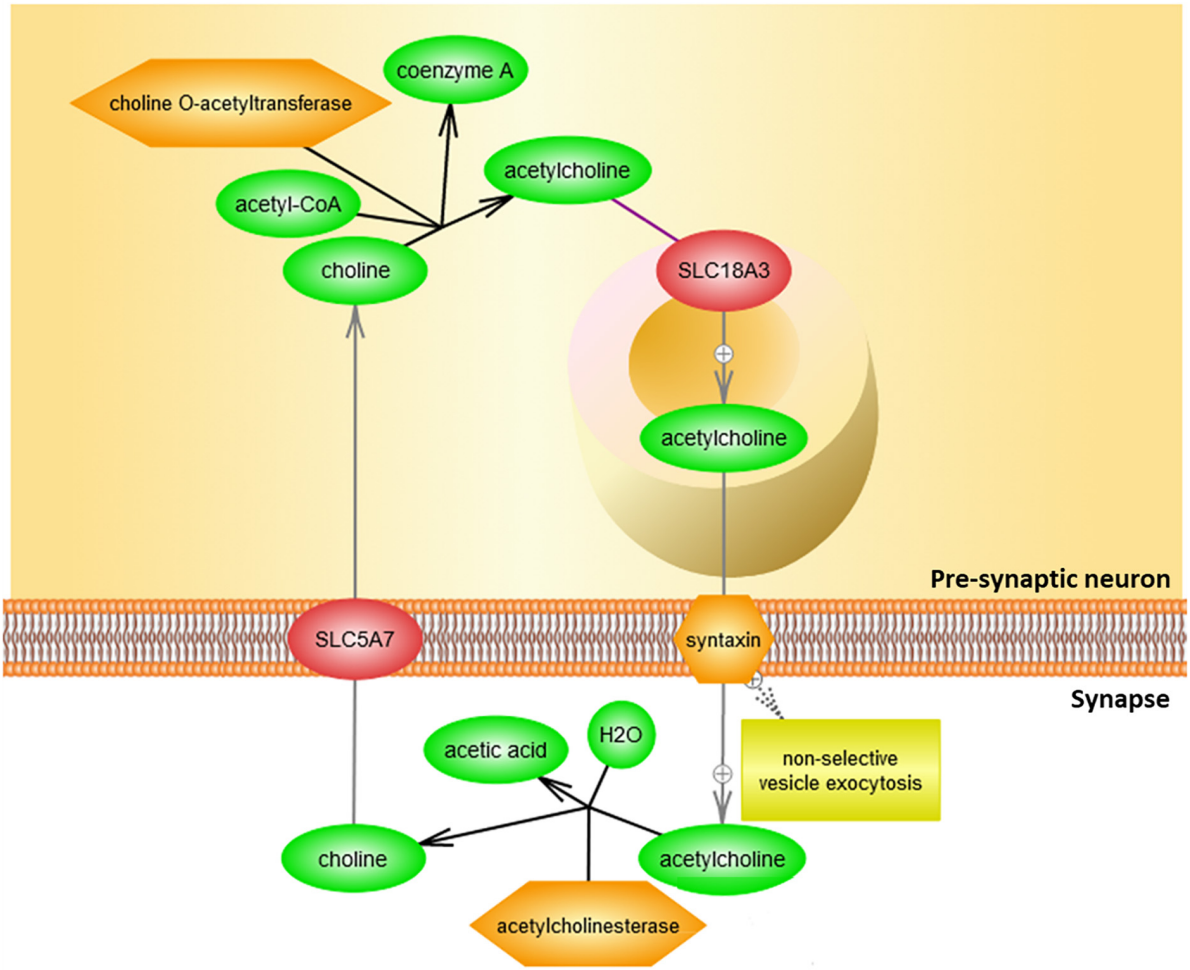

FIGURE 4 | Clearance of acetylcholine from the synapse by acetylcholinesterase. Acetylcholine (ACh) is delivered to the synapse as a result of vesicle exocytosis from pre-synaptic neuronal signaling and a release of the recycled acetylcholine from the postsynaptic neuron's cholinergic receptors. In the synaptic cleft, acetylcholinesterase (ACHE) then degrades acetylcholine. Resulting choline is then taken back by the presynaptic neuron to be recycled in order to make more acetylcholine for future signaling. This figure was extracted from Pathway Studio's curated pathways and was edited according to the needs of this study.

Other modulators of acetylcholinergic signal transmission include SK2 ion channels, which counter the membrane depolarization in the signal recipient neuron (**Figure 7A**). These types of membrane repolarizing ion channels prepare the signal recipient neuron to receive other action potential-activated sensory signals immediately after processing the preceding signal.

In part, the magnitude of the sensory signal depends upon SK2 channels' ability to effectively counter membrane depolarization in the wake of a cholinergic signal, thus, serving as an *ex post facto* modulator of cholinergic activity. The autism-associated *KCNN2* variant may either change the level of the baseline SK2 channel expression or lead to the alteration of the splicing, and the shift of the open reading frame. In either case, sensory signal stands to be impacted. These hypothetical impacts of a *KCNN2* genetic variant upon cholinergic sensory signal can be seen in **Figure 7B**.

The final modeling stage sought to evaluate and depict these hypothesized sensory signaling outcomes in light of a deficiency sufficient to reduce available acetylcholine. The intersection of dietary choline deficiency with *GABBR1*-related pathways can be seen in **Figure 6B**, and the intersection of dietary choline deficiency with *KCNN2*-related pathways can be seen in **Figure 7C**.

Supportive Evidence for KCNN2 Variant as a Modifier of Splicing

To examine a possible change in splicing patterns attributable to autism-associated variants in *GABBR1* and *KCNN2*, both the wildtype loci and the variant loci representing each of these two genes were compared using the Alternative Splice Site Predictor (ASSP) tool. While the (rs740883) SNP-related differences in predicted splicing patterns of *GABBR1* were minimal, the *KCNN2* variant rs13188074 yielded a high confidence prediction of a lowered strength within the splice site between the seventh and

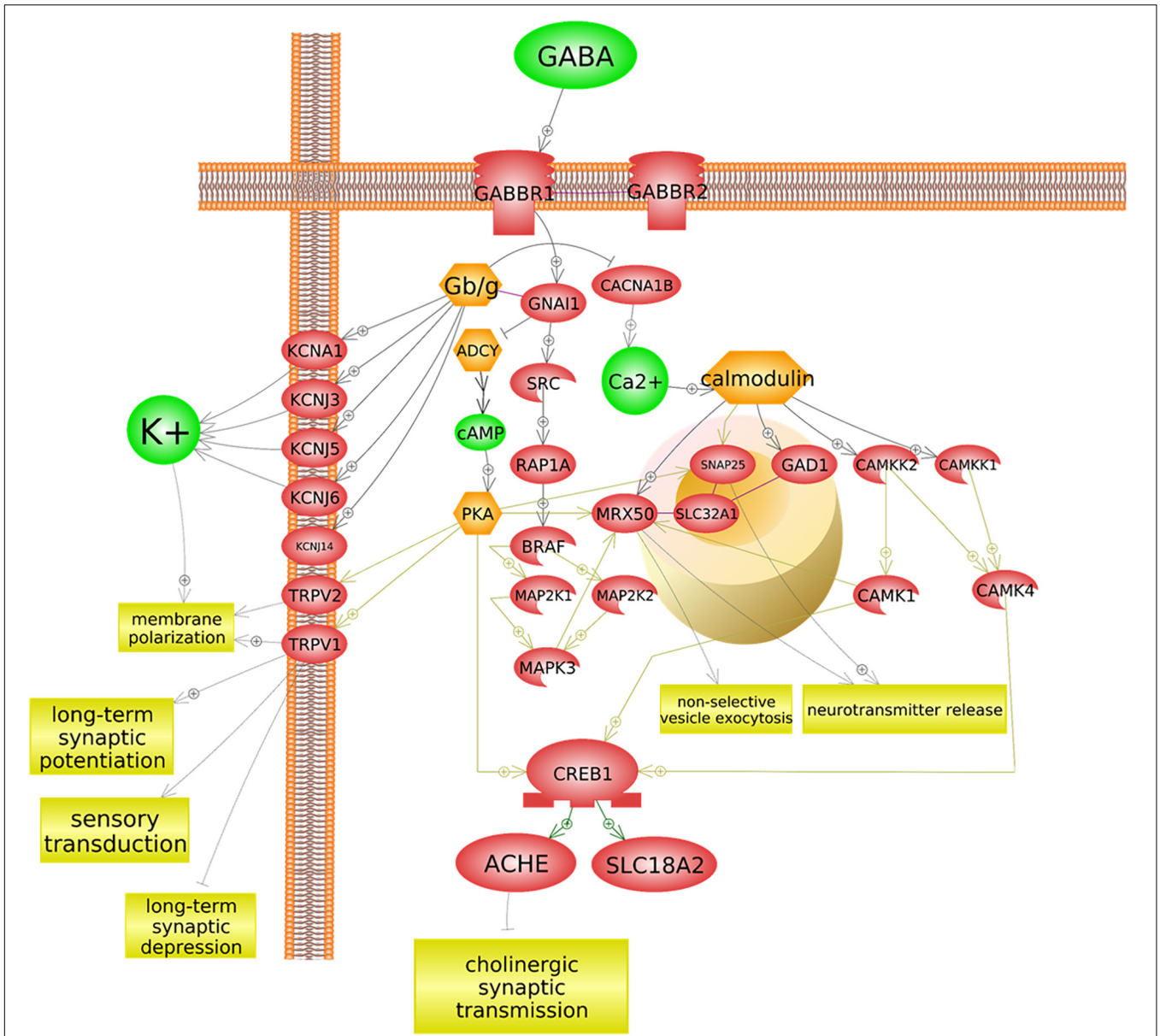

FIGURE 5 | *GABBR1* may influence acetylcholinesterase regulation. *GABBR1* is an autism-associated gene that codes for a membrane-bound protein ($GABA_{B1}$) (Wu et al., 2020). The binding of the neurotransmitter GABA to $GABA_{B1}$ has several implications for cholinergic signaling, sensory signal transduction, and ion transport, across multiple cascades. As depicted here, a typical GABA/*GABBR1* binding cascade inhibits adenylyl cyclases (ADCY), thereby preventing positive upregulation of acetylcholinesterase (ACHE). This figure was extracted from Pathway Studio's curated pathways and was edited according to the needs of this study.

eighth exons (**Table 1**), indicating the possibility of a lower rate of splicing than may occur in the wildtype version of this site.

DISCUSSION

This paper presents the results of a gene set enrichment analysis focusing on shared gene ontologies between an autism-associated gene set obtained through meta-analysis of GWAS and a gene set centering on cholinergic function, which has been collated using a combination of curated gene sets in the Molecular Signatures Database (MSigDB) (Liberzon et al., 2011; Liu et al., 2020). Our study identified two shared gene ontologies, an ion transport regulation and a positive ion transport regulation. Within these ontologies, two genes were further identified as being involved with cholinergic neuronal signal transmission, *GABBR1* and *KCNN2*. The role of ion transport in neurological signaling is complex, as it features a series of ion transport enablers, which boost ion transport and depolarize membranes, as well as counter-transporters, which work to maintain membrane hyperpolarization. These ion transport players in cholinergic and GABA-ergic pathways amplify or inhibit transmission of synaptic

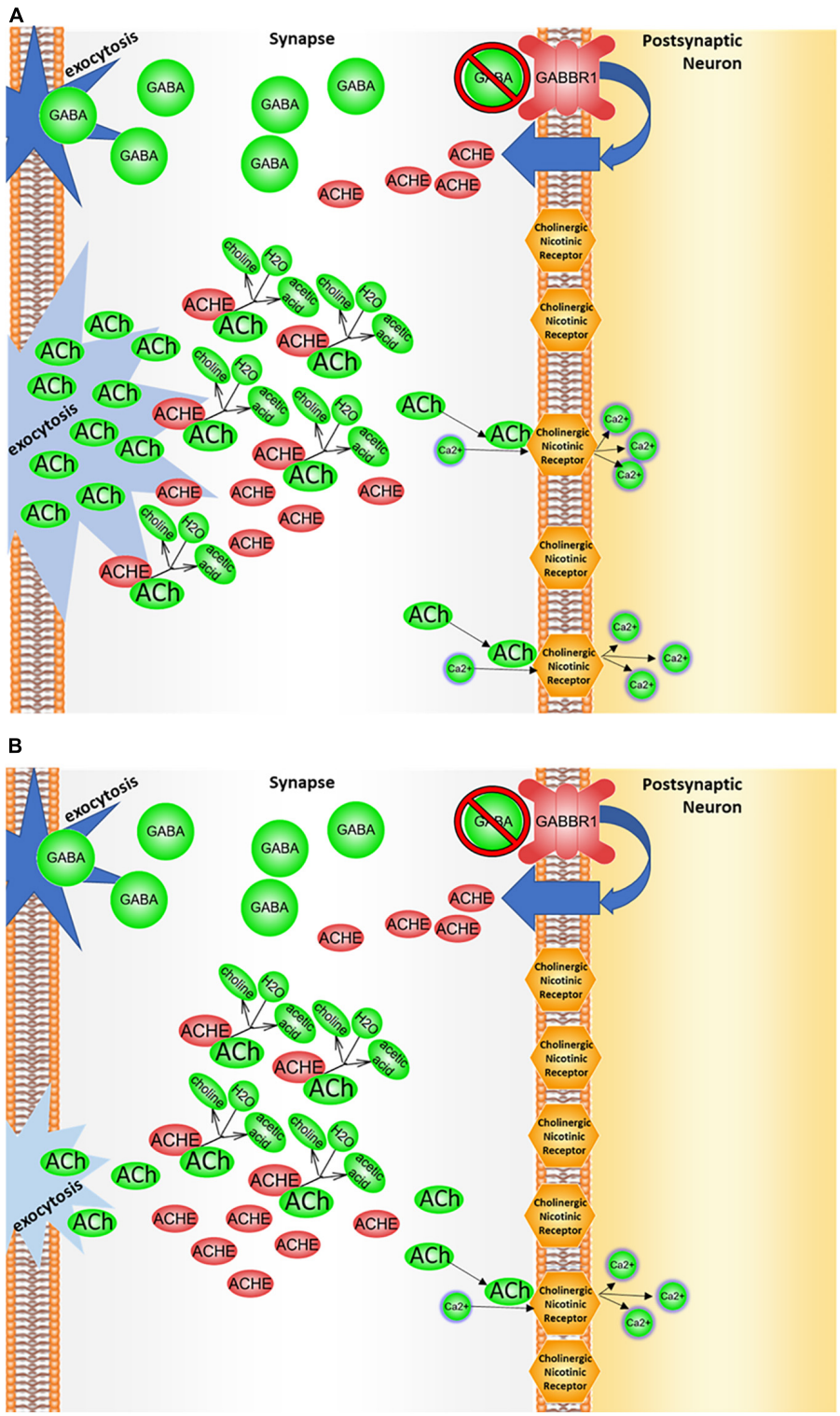

FIGURE 6 | *GABBR1*/ACHE regulation. **(A)** In the presence of *GABBR1* variant. The autism-associated *GABBR1* variant may affect levels of acetylcholinesterase (ACHE) in the synaptic cleft by decreasing either total levels of *GABBR1* expression and/or function of its gene product, the $GABA_{B1}$ receptor. Under this condition, the cascade that normally downregulates acetylcholinesterase expression may be suppressed. Because of that, larger amounts of the enzyme are produced, and the acetylcholine degrades at an elevated rate, resulting in less acetylcholine in the synaptic cleft and less signaling through the cholinergic receptor. **(B)** In the presence of *GABBR1* variant and a dietary choline deficiency. When choline levels are deficient, less acetylcholine (ACh) is available in the neurons for signaling, and a decrease in cholinergic binding/signaling attributed to *GABBR1* variant would be exacerbated.

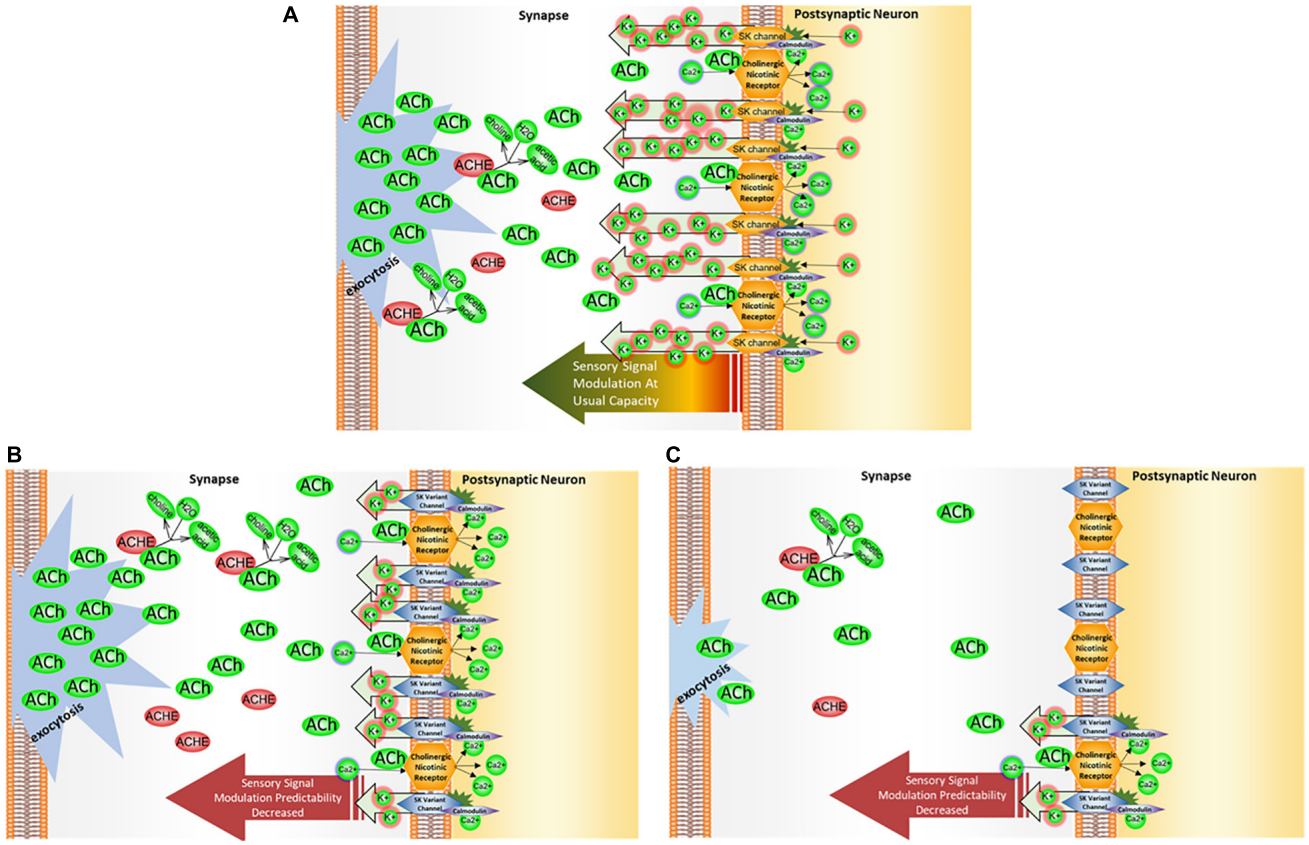

FIGURE 7 | *KCNN2*/SK2 impact on cholinergic signal modulation. **(A)** SK2 channel operation. SK2 channels operate in tandem with a propagation of cholinergic signal as they counter the membrane depolarization brought about by calcium influx through cholinergic receptors. The calcium influx activates the calmodulin domains on SK2 channels, permitting passage of potassium back into the synapse to restore membrane hyperpolarization. Thus, SK2 channels serve a critical role in helping a neuron to recover and re-hyperpolarize before receiving the next cholinergic signal. **(B)** In the presence of *KCNN2*/SK2 variant. When SK2 channel binding activity and/or functionality is altered, the sensory signaling modulation could either be *too efficient*—that is, the membrane repolarizes too soon due to overflow of K + ions back into the synapse, or *not efficient enough*, with membrane taking too long to repolarize, leaving the neuron less ready to process any subsequent signals transmitted from the presynaptic neuron. In either of these two conditions, the neuron's capacity to modulate sensory signaling through membrane repolarization may be diminished. **(C)** In the presence of *KCNN2*/SK2 variant and a dietary choline deficiency. When choline levels are deficient, less acetylcholine (ACh) is available in the neurons for signaling, and a post-signal repolarization may be less amenable to modulation by SK2 channels. When these channels are working in a suboptimal regimen due to the presence of *KCNN2* variant, modulation leeway may be curtailed even further.

TABLE 1 | The Alternative Splice Site Predictor (ASSP) tool (Wang and Marín, 2006) determined short windows containing the loci for each of the *GABBR1* and *KCNN2* autism-associated genes.

					Activations**		
Gene/Variant	**Putative splice site**	**Sequence**	**Score***	**Intron GC***	**Alt./Cryptic**	**Constitutive**	**Confidence****
GABBR1 Wildtype	Alternative isoform/cryptic acceptor	CACGCTCCAGCCATGCTG**A**A	4.170	0.614	0.847	0.127	0.827
GABBR1 Variant	Alternative isoform/cryptic acceptor	CACGCTCCAGCCATGCTG**T**A	4.170	0.614	0.888	0.106	0.880
KCNN2 Wildtype	Alternative isoform/cryptic donor	TAAGCTTATGGTA**A**AAGAGT	7.508	0.429	0.617	0.294	0.523
KCNN2 Variant	Alternative isoform/cryptic donor	TAAGCTTATGGTA**G**AAGAGT	5.195	0.443	0.778	0.160	0.795

In the case of the GABBR1 wildtype/variant comparison, no difference in scoring of splice site strength was calculated, and only a slight shift in confidence occurred of this window containing an alternative isoform/cryptic acceptor. However, the KCNN2 wildtype/variant comparison indicated a lower score for splice site strength, in its classification as an alternative isoform/cryptic donor, and this lower score attained a higher confidence rating of 0.795, compared to the 0.523 confidence rating that ASSP assigned to the splice site strength score for the window containing KCNN2 wildtype, also classified as an alternative isoform cryptic donor.

**Scores of the preprocessing models reflecting splice site strength, i.e., a PSSM for putative acceptor sites, and an MDD model for putative donor sites. Intron GC values correspond to 70 nucleotides of the neighboring intron.*

***Activations are output values of the backpropagation networks used for classification. High values for one class with low values of the other class imply a good classification. Confidence is a simple measure expressing the differences between output activations. Confidence ranges between zero (undecided) and one (perfect classification).*

Bolded letters in the sequences indicate the single nucleotide polymorphism difference between wildtype and variant.

signals, respectively. As a result of these interactions, a carefully tuned balance emerges. Potential implications of the presence of *GABBR1* and *KCNN2* variants in the context of autism and sensory processing are discussed below. Further consideration is given to how operation of these pathways may be altered during acetylcholine deficiency resulting from an inadequate supply of dietary choline.

GABBR1/$GABA_{B1}$ Implications

The *GABBR1* gene encodes for the membrane protein known as $GABA_{B1}$ (or sometimes $GABA_B$). This membrane protein, which constitutes the metabotropic class of GABA-binding membrane-bound receptors, functions at a slow, steady level, in partnership with another membrane-bound GABA-binding protein, $GABA_{B2}$. Through activation of potassium ion channels that release potassium (K^+) out of the neuron and inhibition of calcium ion (Ca^{2+}) channels which permit passage of Ca^{2+} ions into the neuron, this set of proteins brings about membrane hyperpolarization. In this circuit, GABA–$GABA_{B1}$ binding activity serves an inhibitory function, which counters the possibility of excitation of membrane depolarized neurons. This functionality helps to modulate a magnitude of incoming stimulation by inhibiting sensory signals transmitted by cholinergic membrane depolarization. Thus, activation of neuronal action potentials and a subsequent, consistent state of neuronal overexcitation are prevented (**Figure 5**).

Additionally, $GABA_{B1}$ receptor binding initiates a cascade which eventually results in the inhibition of acetylcholinesterase—the enzyme that degrades acetylcholine in the synapse. This indicates a possibility that $GABA_{B1}$ receptor binding activity may at least in part regulate the size of the acetylcholine pool available for cholinergic signaling.

The *GABBR1* variant associated with autism—variant rs740883—is an intronic variant in which a thymine replaces an adenine (Liu et al., 2020). This single-nucleotide polymorphism (SNP) occurs in approximately 9–10% of individual human genomes, and is located within a 3 prime untranslated region, commonly associated with a stability of a transcript. *GABBR1*-encoded $GABA_{B1}$ receptors are expressed at lower levels in autistic brains, in both the superior frontal and parietal cortices, and in the cerebellum (Fatemi et al., 2009, 2010). Levels of related GABA-ergic biomarkers also differ in the brains of people with an autism diagnosis, when compared to levels of GABA-ergic biomarkers in control brains (Blatt and Fatemi, 2011). While the autism-associated *GABBR1* intron variant discussed here is discovered too recently to be analyzed for its relation to these epigenetic differences, examining the possibility that this variant may explain the observed autism-specific differences in GABA-ergic transcriptomic regulation described in the scientific literature is warranted.

Impact of GABA/$GABA_{B1}$ Binding on the Synaptic Supply of Acetylcholine

GABA is a common neuromediator which often functions in tandem with acetylcholine, in either inhibitory or excitatory roles that span learning, memory, sensory signal transmission, neuromuscular function, and cardiac function. In the hippocampus/somatosensory cortex, the mammalian central nervous system, and elsewhere in the body, GABA and acetylcholine may be co-released, in a relationship which is not yet fully understood (Bianchi et al., 1982; Kusunoki et al., 1984; Granger et al., 2016; Takács et al., 2018). Both of these mediators rely upon vesicle-based exocytosis to be released into the synapse, with GABA and acetylcholine transporting vesicles within the same presynaptic vesicle pools being filled independently of each other (Takács et al., 2018).

Notably, the binding of $GABA_{B1}$ to its ligand impacts available interneuronal acetylcholine supply (**Figures 5**, **6A**). More specifically, GABA–$GABA_{B1}$ binding inhibits a cascade in which the transcription factor CREB1 boosts expression of acetylcholinesterase (ACHE), an enzyme that degrades acetylcholine in the interneuronal junction, thereby lessening the magnitude of cholinergic signal. If GABA–$GABA_{B1}$ binding activity were to *decrease* as a result of a presence of a genetic variant in a *GABBR1* gene—for example, rs740883, which is strongly associated with autism—an increase in acetylcholinesterase production would ensue after being prompted by an increase in the binding of CREB1 *ACHE* promoter. In this scenario, the increased supply of acetylcholinesterase would degrade acetylcholine in the interneuronal junction faster, thus suppressing cholinergic signaling to a greater extent than in the brain with wildtype *GABBR1*. **Figure 6A** systematically depicts hypothesized impact of *GABBR1*'s autism-associated intronic variant on GABA-ergic and cholinergic signaling.

Impact of Acetylcholine Deficiency on Cholinergic Output, and Hypothesized Interplay With *GABBR1* Genetics

Both our modeling and limited experimental evidence suggest that insufficient dietary intake of a choline may contribute to a reduced acetylcholine supply in the brain (Cohen and Wurtman, 1976). The combination of a dietary choline deficiency with a *GABBR1* variant may result in cholinergic signaling being lowered further, or even markedly impaired. Moreover, given that GABA sometimes co-transmits with acetylcholine, reduced acetylcholinergic signaling may also be tied to the temporal restriction on the GABA-driven neuronal inhibition as a secondary event. Sensory processing depends upon function of both of these neurotransmitters, so it is reasonable to extrapolate that the combination of dysregulated ion transport due to the presence of *GABBR1*/$GABA_{B1}$ variants and dietary-driven acetylcholine deficiency may result in altered sensory processing for the individual in question.

KCNN2/SK2 Channel Implications

The gene *KCNN2* encodes for the membrane-bound SK2 calcium-activated potassium ion channel, which modulates the excitability of neurons and neuromuscular activity. After being activated through co-located acetylcholine nicotinic receptors, SK2 channel modulates cholinergic signals through neuronal membrane repolarization (**Figure 7A**). SK2 channels are

abundant throughout the body, including the brain and the cardiorespiratory system (Gu et al., 2018). The SK2 channel encoded by *KCNN2* is associated with sensory processing in a variety of contexts, with much research focusing on auditory processing with respect to cholinergic nicotinic receptors in the cochlear hair (Elgoyhen et al., 2001, 2009; Kong et al., 2008; Murthy et al., 2009),vestibular awareness (Wangemann, 2002), and nociception (Bahia et al., 2005; Mongan et al., 2005; Pagadala et al., 2013; Hipólito et al., 2015; Thompson et al., 2015).

Impact of Acetylcholine Supply on the Function of *KCNN2*/SK2 Channel

As discussed above in relation to the $GABA_{B1}$ receptor, dietary choline deficiency may markedly reduce the pool of acetylcholine in the brain (Cohen and Wurtman, 1976). It follows that diminished binding of the acetylcholine to cholinergic receptors may reduce activation of co-located SK2 channels. In turn, reduced acetylcholine supply in the brain may require increased sensory input, which, in practical terms, translates into sensory hyposensitivity, or sensory underresponsiveness. Reduced signaling may alter the closely-tied counterfeedback of SK2 ion transport signal modulation.

Impact of Acetylcholine Deficiency on Cholinergic Output, and Its Possible Interplay With *KCNN2* Genetics

Similar to the autism-associated epigenetic regulation of *GABBR1* observed in the literature, an autism-specific epigenetic regulation of SK2 ion transport activity was noted. In the brains of people with an autism diagnosis, cholinergic nicotinic receptors are expressed at lower levels when compared to that in neurotypical brains (Lee et al., 2002; Andersen et al., 2013). A lower level of available cholinergic receptors may translate to fewer opportunities for acetylcholine binding, and, as collateral, the normally colocalized SK2 channels may also fire less frequently, cumulatively altering the degree of membrane repolarization, and therefore, the timing of subsequent cholinergic sensory signaling.

Because SK2 signaling depends upon the signals from co-located cholinergic nicotinic receptors, diminished or altered SK2 activity arising from the presence of autism-associated *KCNN2* coding variant(s) may be compounded by the synaptic acetylcholine deficiency. Thus, dietary choline deficiency may ultimately exacerbate genetic weakness in the SK2 activity, resulting in altered sensory processing, possibly resulting in sensory over-responsiveness, under-responsiveness, or a combination of both, for a given autistic person.

rs13188074 Variant May Influence *KCNN2* Splicing Pattern

Currently, there is a little evidence of direct impact of intronic variants in *GABBR1* and *KCNN2* on the function of the respective proteins. ASSP analysis hints that, in case of *KCNN2*, the change in a strength of a splice site may influence an amount of functional mRNA encoding for full-size *KCNN2*. Experimental gauging of the impact of *KCNN2* variant upon sensory processing is warranted.

Targeting Cholinergic and GABA-ergic Signaling Pathways May Modulate Sensory Processing in Autism

The models indicated in **Figures 2**, **3** are consistent with extant literature examining the possibility of cholinergic pharmacotherapy of autism, as increasing the pool of available interneuronal acetylcholine may improve function in several domains related to cholinergic signaling activity and related ion transport dynamics. For example, acetylcholinesterase inhibitors galantamine and memantine, which are typically prescribed for Alzheimer's disease patients, are also actively explored in autism (Maire and Wurtman, 1984; Hertzman, 2003; Nicolson et al., 2006; Ghaleiha et al., 2014; Rossignol and Frye, 2014; Rahman et al., 2018). A systematic review examining the impact of these drugs in individuals with autism identified an improvement across several domains, including communication and social interaction, and a decrease in disruptive behavior, hyperactivity, inattention, and irritability (Rossignol and Frye, 2014). Many of these domains are linked to sensory processing difficulties (Ashburner et al., 2008; Sanz-Cervera et al., 2015). In the present study, we posit that variant function of *GABBR1* may lead to a smaller pool of available acetylcholine (**Figure 5**). For patients with *GABBR1* variants, including individuals with autism, prescription of acetylcholinesterase inhibitors such as galantamine or memantine may counter the disturbance in GABA/*GABBR1*/acetylcholinesterase cascade, by boosting the synaptic acetylcholine levels to match these seen in neurotypical brains.

In the context of addiction, the literature supports a relationship between acetylcholinergic transmission and genetic variation of *GABBR1*. Individuals with genetics variants of the *GABBR1* locus are more likely to develop dependency upon a major cholinergic nicotinic receptor agonist, nicotine. This observation highlights the complexity of the influence of genetic *GABBR1* variation upon acetylcholinergic function (Li et al., 2009; Li, 2018). Notably, nicotinic receptors have been of particular interest as a therapeutic target in autism, as their stimulation affects both working memory and executive functions. In a placebo-controlled trial, nicotine patches have been investigated as a mean to improve sleep and to address aggressive behavior in autistic adults (Deutsch et al., 2015; Olincy et al., 2016; Lewis et al., 2018; Deutsch and Burket, 2020).

The evidence for pharmacological intervention on $GABA_{B1}$ upon autism-related sensory domains is equivocal. $GABA_{B1}$ receptor agonists such as arbaclofen have been investigated through evaluation of their efficiency across measurable features of irritability, lethargy, and social responsiveness (Veenstra-VanderWeele et al., 2017). When examining a specific sensory subdomain—auditory processing—a subset of teenagers with autism taking arboclofen were found to exhibit improved magnetoencephalographic (MEG) measures of their auditory response (Roberts et al., 2019).

It is of certain importance that in addition to pharmacological means, cholinergic transmission may also be influenced by change in the dietary intake of the choline, one of the common nutrients abundant in milk, liver, eggs, and peanuts. As discussed, many Americans do not consume the daily AI level of choline, and indeed, recent surveys indicate that a majority of a sample of autistic children (60–93%) do not meet their choline AI level recommended respective of age (Hamlin et al., 2013; Wallace and Fulgoni, 2016). A number of autism-associated traits such as preference for routine, aversion to particular foods, as well as sensory aversions to particular smells, textures, or temperatures are known to profoundly shape the diets of people on the autism spectrum (Dunn, 1997; Baranek et al., 2006; Cermak et al., 2010; Nadon et al., 2011; Kral et al., 2013; Hubbard et al., 2014; Sanz-Cervera et al., 2015; Bogdashina and Casanova, 2016; Boterberg and Warreyn, 2016; Crasta et al., 2016; Bitsika and Sharpley, 2018; Weeden, 2019). Because of that, the availability of an adequate pool of acetylcholine for cholinergic signaling may be a legitimate concern, particularly in light of the potentially exacerbated need for proper function of sensory signaling pathways discussed above.

Dietary Choline: A Low-Risk Intervention to Support Acetylcholine Supply, Cholinergic Signaling, and Sensory Processing in Autism

The recommended dietary consumption levels for choline are set by the Food and Nutrition Board of the National Academies as assessed by measuring serum alanine aminotransferase levels reflecting overall liver function. Whether or not the choline at AI level would be sufficient to modulate sensory processing symptoms is not known. It is notable, though, that most Americans do not achieve even this level of the choline intake (Wallace and Fulgoni, 2016). For autistic individuals, who experience particular difficulty in meeting dietary guidelines for choline through diet alone, choline supplementation may provide an alternate effective means of ameliorating daily choline intake levels. As opposed to pharmacological interventions, using the dietary modifications or choline supplements is considered low risk, because neither dietary nor supplementary forms of choline are known to interact with medications. Additionally, overconsuming choline through diet or supplementation to the point of excess—which is defined by the tolerable upper limit of 3,500 mg/day—would require Americans to consume more than seven times their recently estimated mean intake levels of choline, which is currently at less than 500 mg/day for all age groups (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1988; Wallace and Fulgoni, 2016; Wallace et al., 2018).

To call dietary intervention for choline status "low risk" does not negate the challenges specific to supporting the increased consumption of choline in autistic children and adults. The research and development which is required to determine targeted, effective ways for elevating choline consumption and identifying personalized intake baselines sufficient for fulfilling physiological need for choline in this population is considerable. As with any autism research, investigations should purposefully include representatives and researchers with an autism diagnosis who can help to shape such interventions optimally for autistic children and adults (Chown et al., 2017; Hoekstra et al., 2018; Lebenhagen, 2019; Cascio et al., 2020; Hogan et al., 2020).

Future Research Directions

The autism-associated *GABBR1* and *KCNN2* genetic variants have been highlighted as such only recently, and therefore there is an opportunity to collect more definitive experimental data, which can inform the modeling of sensory processing dynamics as they relate to these variants. Studies of *GABBR1* and *KCNN2* in autism should examine possibilities such as structure/function differences in the autism-associated variant version of these membrane proteins, as well as measurement of *GABBR1* and *KCNN2* expression levels in those presenting with the respective autism-associated variants.

Because of the relative likelihood that autism-associated intron variants such as those found in *GABBR1* and *KCNN2* would result in transcriptomic changes, transcriptomics should be a primary focus of future research on these genetic variants' impact. Because of the measured difference in $GABA_{B1}$ protein expression in autism, examining a potential link between the autism-associated *GABBR1* variant and these expression differences should be a priority. Similarly, SK2 channel expression levels in autism should be examined with respect to this SK2 channel variant. Any transcriptomic differences which would be found to be associated with these genetic variants would set the foundation for more refined examination of impact upon cholinergic signal transmission and modulation.

In terms of further examination of potential alterations to autism-related variant versions of $GABA_{B1}$ and SK2 channel structure and/or function, there are several tools and methods available, which vary in scope and performance. For example, if it can be determined that a reading frame shift has occurred as a consequence of an altered splice site, basic computational homology modeling may be helpful. Without the context of a frame shift, if structural differences are still suspected for the variant version of either of these proteins, validated structural assessment through means of electron microscopy, NMR spectroscopy, and X-ray crystallography remains as resource-intensive possibilities. Additionally, new structure prediction AI presented by AlphaFold, exhibiting unprecedented accuracy in structural prediction, could be considered as a potential future tool (DeepMind, 2020). Additionally, if structural differences were uncovered, molecular dynamics modeling may provide insights into the relative binding frequencies and strength in wildtype vs. variant versions of $GABA_{B1}$ receptors and SK2 channels. If these models provide actionable information, they may merit further development in terms of broadening molecular dynamics model scope to include various elements from the cholinergic sensory signal pathways and cascades discussed in this study. Computational modeling and pathway exploration may continue to provide support and context as researchers continue to explore potential impact of these genetic variants upon sensory processing in autism.

In terms of clinical interventions, while available evidence on dietary interventions for autism remains limited, this work provides mechanistic support for further exploration of exogenous choline—through diet or supplementation—as a potential low-risk sensory processing support intervention. Future clinical interventions in humans will *de facto* require the input of multidisciplinary teams of researchers and stakeholders—including those with autism diagnoses—to effectively develop and test autism-tailored clinical interventions that carefully measure inputs through diligent nutritional assessment, and outcomes such as standardized sensory processing scores, and choline activity measured through functional magnetic resonance imaging (fMRI). Such teams may include researchers from a broad range of fields including nutrition, neuroscience, occupational therapy, bioinformatics, and computational biology. Additionally, choline-related biomarkers such as serum alanine aminotransferase may provide useful outcome measurements, particularly in cases where nutritional assessment indicates risk of deficiency.

CONCLUSION

In connecting dietary choline intake as a mediator of acetylcholinergic pathways, especially for sensory signal ion transport in the context of two autism-associated genes, GABBR1 and KCNN2, this study is consistent with the growing evidence base concerning the role of choline in pre- and postnatal nutrition and neurodevelopment. In our opinion, clinical evaluation of choline intake interventions with respect to validated sensory processing scores is warranted in future studies whose age ranges include children, teens, and adults.

Because of the physically, mentally, and logistically demanding requirements involved in collection of experimental data through clinical trials, particularly from participant individuals with autism and their families, it is incumbent upon researchers to ensure that clinical trials examining dietary status, genetic variants, and sensory processing are effective, efficient, and respectful of their participants. Advance planning and implementation for these clinical trials is therefore at a premium. Accordingly, in preparation for clinical trials, research employing computational biology, bioinformatics, and predictive modeling should also be considered as essential precursor elements in examining the intersection of dietary choline status with sensory processing in the context of autism-associated genes.

DATA AVAILABILITY STATEMENT

The autism GWAS dataset is available as a supplementary file for the original article (Wu et al., 2020).

AUTHOR CONTRIBUTIONS

AO, AB, and MS were responsible for the conception of the project and editing the final manuscript. AO carried out analyses and interpretation with input from all other authors and drafted the manuscript with feedback from all other authors. All authors contributed to the design.

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REVIEW
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Inborn Errors of Metabolism Associated With Autism Spectrum Disorders: Approaches to Intervention

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Increasing evidence suggests that the autism spectrum disorder (ASD) may be associated with inborn errors of metabolism, such as disorders of amino acid metabolism and transport [phenylketonuria, homocystinuria, S-adenosylhomocysteine hydrolase deficiency, branched-chain α-keto acid dehydrogenase kinase deficiency, urea cycle disorders (UCD), Hartnup disease], organic acidurias (propionic aciduria, L-2 hydroxyglutaric aciduria), cholesterol biosynthesis defects (Smith-Lemli-Opitz syndrome), mitochondrial disorders (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes—MELAS syndrome), neurotransmitter disorders (succinic semialdehyde dehydrogenase deficiency), disorders of purine metabolism [adenylosuccinate lyase (ADSL) deficiency, Lesch-Nyhan syndrome], cerebral creatine deficiency syndromes (CCDSs), disorders of folate transport and metabolism (cerebral folate deficiency, methylenetetrahydrofolate reductase deficiency), lysosomal storage disorders [Sanfilippo syndrome, neuronal ceroid lipofuscinoses (NCL), Niemann-Pick disease type C], cerebrotendinous xanthomatosis (CTX), disorders of copper metabolism (Wilson disease), disorders of haem biosynthesis [acute intermittent porphyria (AIP)] and brain iron accumulation diseases. In this review, we briefly describe etiology, clinical presentation, and therapeutic principles, if they exist, for these conditions. Additionally, we suggest the primary and elective laboratory work-up for their successful early diagnosis.

Keywords: autism spectrum disorder, early diagnosis, genetic testing, inborn errors of metabolism, therapeutic principles

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INTRODUCTION

Autism spectrum disorder (ASD) is a behavioral developmental disorder defined by the impairment of communication and social interaction. It usually starts before age three and children with autism can be recognized by stereotyped and repetitive patterns of behavior as well as their restricted activities and interests. ASD varies in degrees of severity, occurring in 1% of the population, and its prevalence has been increasing during the last three decades (Maenner et al., 2020). The causes of different subtypes of autism lay in the complex landscape of environmental, genetic

and epigenetic influences (Waye and Cheng, 2018; Šimić et al., 2020). Although the etiology of most cases of ASD is still unknown, numerous studies have shown that there is a strong heritable component. Change (mutation or premutation) in the *FMR1* gene sequence on the chromosome X that results in fragile X syndrome (FXS) or fragile X-premutation tremor/ataxia syndrome (FXTAS) are among the leading genetic causes of ASD (Šarac et al., 2011; Ghaziuddin and Al-Owain, 2013). Current genetic testing strategies, including the combination of molecular cariotypisation and exome sequencing, are the most successful diagnostic approaches, yielding exact diagnosis in about 20–25% of the cases. The term "non-syndromic" autism refers to patients without dysmorphic characteristics or any other disease or additional signs or symptoms than those known to be associated with ASD. On the other hand, the term "syndromic" is being used to describe individuals with clinical characteristics additional to ASD, such as epilepsy, motor deficits, developmental delay and regression, dysmorphic features, and other manifestations or diseases that can contribute to autistic phenotype (Campistol et al., 2016).

It has been recently proposed that a significant number of ASD cases could be associated with various metabolic abnormalities, some of them identifiable only through untargeted metabolomic profiling, simultaneously opening additional space for therapeutic attempts (Glinton and Elsea, 2019).

Inborn Errors of Metabolism

Clinical symptoms deriving from central nervous system (CNS) occur in more than 50% of patients with inborn errors of metabolism (IEM). In addition to neurological or somatic manifestations, psychiatric symptoms are also the presenting sign in 2−5% of IEM cases (Saudubray and García-Cazorla, 2018). Recent reports emphasized the causal role of IEMs in ASD, meaning that some IEM could be prevented, especially in population with high level of consanguinity (Celestino-Soper et al., 2012; Novarino et al., 2012). Many IEM are treatable conditions; in some of them positive effects are observed when treatments were started early in life. Phenylketonuria (PKU), the prototype of IEM successfully diagnosed by neonatal screening program and treated with diet if initiated early in life, is characterized by intellectual disability and autism (Asato et al., 2015). Possible IEM should be considered in the diagnostic approach of patients with syndromic ASD because for many of them there is a chance for intervention and improvement (Márquez-Caraveo et al., 2020). Spilioti et al. (2013) found significant proportion of patients with an IEM in patients with autism. For most of the IEM, ASD is not a single symptom, but accompanied with other neuropsychiatric and somatic disturbances (Simons et al., 2017). The data of Schiff and colleagues provide a large cohort of non-syndromic autistic subjects for whom a systematic metabolic work-up has been carried-out (Schiff et al., 2011). Their data suggest that usual metabolic workup should not be done routinely in non-syndromic ASD because the prevalence of IEM in the group of non-syndromic ASD is not higher than in general population (<0.5%). In the case of non-syndromic ASD, a precise clinical workup represents a good clinical practice (Schiff et al., 2011). In this review, we give an overview of IEM characterized by symptoms of ASD, propose diagnostic approach to assess such cases in clinical practice, and suggest possible specific therapies.

ASD ASSOCIATED WITH INBORN ERRORS OF METABOLISM

Disorders of Amino Acid Metabolism

Phenylketonuria

Phenylketonuria (PKU) is the most common IEM in the group of aminoacidopathies. It is caused by biallelic mutations in the phenylalanine hydroxylase (PAH) gene with consequent lowering of PAH activity. PAH metabolizes phenylalanine to tyrosine, a process which requires the cofactor tetrahydrobiopterin (BH4). Deficient activity of PAH results in elevated concentration of phenylalanine in the blood and toxic levels in the CNS. If untreated, PKU is accompanied with severe and progressive intellectual disability, but may also be associated with symptoms such as autism, seizures, and motor deficits. Psychiatric or psychological difficulties may become apparent if the patient is not compliant with the diet (Blau et al., 2010). Newborn screening programs successfully diagnose patients with PKU. Treatment with a low phenylalanine diet in the postneonatal period has resulted in a worldwide population of over 50 thousand PKU individuals without cognitive deficits (ten Hoedt et al., 2011). BH4, the natural cofactor of PAH, may be effective in about 50% of PKU patients who are BH4-responsive to increase phenylalanine tolerance (van Wegberg et al., 2020). Possible new treatment options include enzyme therapy with phenylalanine ammonia lyase, enzyme that metabolizes phenylalanine, and gene therapy (van Wegberg et al., 2017).

Homocystinuria (Cystathionine β-Synthase Deficiency)

Cystathionine β-synthase (CBS) deficiency, also known as classical homocystinuria (HCU), is caused by biallelic mutations in the *CBS* gene. CBS deficiency prevents the conversion of homocysteine (Hcy) to cystathionine, resulting in homocysteine increase. Subjects with classical homocystinuria may have a clinical picture of varying severity and with different age of onset. It can start in childhood as a severe multisystemic disease or stay asymptomatic until adulthood (Morris et al., 2017). Untreated patients may have various symptoms, including osteoporosis, thromboembolic events, and intraocular lens dislocation. These patients can also have a variety of CNS disturbances, including symptoms of ASD (Abbott et al., 1987; Kiykim et al., 2016). Extrapyramidal signs and seizures are frequent features too (Morris et al., 2017). Treatment options include vitamins B_6, B_{12} and folate supplementation, low-methionine diet and betaine (Kruger, 2017). Current efforts for developing novel therapies for HCU include enzyme replacement therapy with recombinant enzyme OT-58 that has been shown effective in lowering plasma and tissue homocysteine, ameliorating metabolic balance and clinical symptoms in a phase II clinical study (clinical trial

NCT03406611). Gene therapy using adenovirus or minicircle DNA is also being appraised (Bublil and Majtan, 2020).

S-Adenosylhomocysteine Hydrolase Deficiency

S-adenosylhomocysteine hydrolase deficiency (SAHHD) is a rare disorder of methionine metabolism characterized by (not constant) hypermethioninemia, elevation of S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy, Barić et al., 2004). AdoHcy is an inhibitor of different methytransferases, enzymes that transfer methyl group to various molecules such as DNA, RNA, lipids, proteins, amino acids, and others. The removal of adenosine and Hcy under physiological conditions is sufficient to direct the flux toward hydrolysis. Hydrolysis of AdoHcy plays a critically important role in the regulation of reactions of biological methylation processes (Barić et al., 2004).

Clinical presentations vary from severe perinatal to milder forms and include various combinations of myopathy with elevated creatine kinase (hyperCKemia), behavioral disturbances, developmental delay, dysmyelination, coagulopathy, strabismus, and hepatic disease. Diet with low methionine intake can decrease and sometimes normalize plasma AdoMet and AdoHcy concentrations, with positive effects on methylation and clinico-biochemical parameters. Together with low protein/methionine diet, creatine and phosphatidylcholine supplementations have been used in some individuals, although without firm evidence of clinical improvement. Myopathy is less responsive to treatment, whereas liver, coagulation and neurological abnormalities are more responsive (Barić et al., 2017). Liver transplantation in a single patient improved cognitive development, especially gross motor, language and social skills (Strauss et al., 2015).

Branched-Chain α-Keto Acid Dehydrogenase Kinase Deficiency

Biallelic mutations in the *BCKDK* gene that codes for the kinase behind the negative regulation of the branched-chain α-keto acid dehydrogenase complex (BCKD) have been associated with clinical symptoms of autism with seizures (Novarino et al., 2012). Normalization of plasma branched-chain amino acids (BCAA) improves hyperactivity, attention span, gross motor and communication skills (Burrage et al., 2014). Supplementation with high-protein diet and frequent BCAA supplement dosing throughout the day are required for normalization of BCAA plasma concentrations (Burrage et al., 2014; García-Cazorla et al., 2014).

Urea Cycle Disorders

The urea cycle is a metabolic pathway that serves to eliminate excess of nitrogen, arising primarily as ammonia. Nitrogen in small quantities is essential substance for growth and cellular equilibrium, but excessive ammonia can lead to life-threatening consequences. In its non-ionized form ammonia easily crosses the blood-brain barrier and enters the CNS. In case of a severe metabolic crisis, a great amount of ammonia can accumulate in the blood and tissues, especially in the CNS that is most vulnerable. As the acute hyperammonaemia increases extracellular glutamate and causes excitotoxic cell death it comes as no surprise that neurological disturbancies such as ataxia, tremor, and seizures may ensue (Matsumoto et al., 2019). Urea cycle disorders (UCD) comprise deficiency of one of several enzymatic steps or transporters of the urea cycle. Clinical presentation is characterized by severe hyperammonaemic crisis in the newborn/infancy period, while the late-onset form is primary characterized by neurological symptoms from infancy through adulthood with possibility of metabolic decompensating and hyperammonaemia triggered by catabolic states. Besides other neurological symptoms, ASD was described in patients with carbamoyl phosphate synthetase deficiency, ornithine-transcarbamylase (OTC) deficiency, citrullinaemia type 1, and arginase deficiency (Frye, 2015). It is of immediate urgency to reduce the plasma ammonia concentration when hyperammonaemia develops. Acute management relies on ammonia detoxification by giving ammonia scavengers (sodium-benzoate or sodium-phenylbutirate), but hemodialysis and hemodiafiltration are the most efficient treatment strategies for plasma ammonia reduction. In parallel, catabolic state is reversed by providing high amounts of intravenous glucose and lipids. Arginine-hydrochloride is given to stimulate urea cycle reactions (except in case of arginase deficiency). Chronic treatment with low-protein diet prevents a catabolic state and recurrence of hyperammonaemia. In severe neonatal forms, prognosis is unfavorable if severe metabolic crisis develops. In late-onset forms improvement of symptoms has been reported with treatment. Liver transplantation can change the unfavorable clinical course in male patients with OTC deficiency (Matsumoto et al., 2019).

Hartnup Disease

Hartnup disease is a disfunction of the B^0AT1 protein that results in overexcretion of neutral amino acids in feces and urine (Hashmi and Gupta, 2021). B^0AT1 is a transporter responsible for the absorption of neutral amino acids in small intestine and renal tubules. As tryptophan serves as a precursor for niacin, deficiency of tryptophan and niacin manifests as pellagra-like skin changes and neurological disturbancies (Orbak et al., 2010). Along with ASD, clinical presentation includes reversible and intermittent episodes of neurological and symptoms affecting the skin. Neurological symptoms include ataxia, tremor, depression, mood disorders, epilepsy and psychosis. The skin eruptions are present at areas exposed to sun and have erythematous and scaly appearance. The disease is treatable with high protein diet and nicotinamide (Hashmi and Gupta, 2021).

Loss of CLTRN protein and its functions results in a disorder of similar biochemical phenotype to Hartnup disease. *CLTRN* gene encodes the protein collectrin, a homolog of angiotensin-converting enzyme 2 (ACE2), which is involved in transportation and activation of B^0AT1 protein in the renal epithelium (Singer and Camargo, 2011; Pillai et al., 2019).

Organic Acidurias

Propionic Aciduria

Propionic aciduria (PA) is an inborn error of branched chain amino acids metabolism, defined by accumulation of propionic acid due to deficiency of enzyme propionyl-CoA

carboxylase (Baumgartner et al., 2014). Clinical features of PA may occur due to accumulation of toxic metabolites, altered mitochondrial energy metabolism, carnitine depletion, and coenzyme A sequestration. Acute illness may be presented by metabolic acidosis, acute alterations of consciousness or encephalopathy due to hyperammonaemia, anorexia, nausea, and vomiting. Chronic complications include poor growth, movement disorders, progressive spastic quadriparesis, epilepsy, cardiac dysfunction, osteopenia/osteoporosis, and functional immunodeficiency (Fraser and Venditti, 2016). Neurological complications include epilepsy, intellectual disability, and ASD (Ghaziuddin and Al-Owain, 2013). PA is treated with protein-restricted diet, precursor-free amino acid supplementation, and carnitine supplementation due to secondary carnitine deficiency. Acute treatment aims to stop catabolism and enable ammonia detoxification by giving sodium benzoate, L-arginine, and N-carbamyl-glutamate. Severe metabolic acidosis sometimes requires hemodiafiltration. Despite adequate therapy, significant number of patients have intellectual disability and psychiatric problems, thus requiring psychosocial support from childhood throughout adult life (Baumgartner et al., 2014).

L-2 Hydroxyglutaric Aciduria

L-2-hydroxyglutaric aciduria is a metabolic disorder affecting CNS and characterized by elevated concentrations of L-2-hydroxyglutaric acid in plasma, urine and cerebrospinal fluid (CSF) due to the deficiency of L-2-hydroxyglutarate dehydrogenase. Disease is defined by progressive neurological symptoms: ataxia, mental deterioration, subcortical leukoencephalopathy, and ASD (Zafeiriou et al., 2008). A few successful therapeutic trials with riboflavin and flavin adenine dinucleotide (FAD) have been reported in subjects with L-2-hydroxyglutaric aciduria. Yilmaz (2009) described the first patient treated with riboflavin in whom a partial improvement in motor and cognitive performance and significant decrease of urinary excretion of L-2-hydroxyglutarate was observed. Another approach, described by Samuraki et al. (2008) in an adult patient, is based on treatment with levocarnitine and FAD, which resulted in improvement of dystonia and tremor as well as in a significant decrease of urinary excretion of L-2-hydroxyglutarate. These examples suggest that supplementation of riboflavin improves the enzymatic activity by raising the intracellular concentration of FAD. Therefore, they may be categorized into a group of vitamin-responsive IEM (Van Schaftingen et al., 2009).

Cholesterol Biosynthesis Defects

Smith-Lemli-Opitz Syndrome

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder caused by biallelic mutations in the *DHCR7* gene, resulting in impaired biosynthesis of cholesterol. Besides dysmorphic features and intellectual disability, autistic symptoms are often a part of the SLOS behavioral phenotype (Thurm et al., 2016). SLOS is characterized by elevated 7-dehydrocholesterol and low plasma cholesterol levels. Some of the major consequences of cholesterol deficiency include abnormal formation of the CNS, face and limbs via an effect on sonic hedgehog (SHH) signaling pathways during development (Li et al., 2020) and its effect on steroidogenesis, particularly that of dihydrotestosterone. Therapeutic intervention with supplementation of cholesterol and symptomatic treatment after early diagnosis decreases mortality and improves long-term outcome (Donoghue et al., 2018). It is also important to know that several common antipsychotic, antidepressant, and anxiolytic compounds, including aripiprazole, buspirone, fluoxetine, haloperidol, nefazodone, perospirone, and trazodone may induce an *in vitro* biochemical profile similar to SLOS (Kim et al., 2016).

Mitochondrial Disorders of Energy Production

Mitochondrial dysfunction is a common metabolic disturbance observed in ASD subjects. At the same time, different clinical features and biochemical abnormalities seen in ASD subjects can be linked to mitochondrial dysfunction (Frye and Rossignol, 2011). Although it seems that mitochondrial dysfunction can unify the seemingly disparate abnormalities associated with ASD, mitochondrial failure is commonly a secondary phenomenon related to other biological processes, such as chronic immune dysfunctions and increased oxidative stress that have been observed in ASD patients (James et al., 2009; Li et al., 2009; Malik et al., 2011).

Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is one of the most frequent mitochondrial disorders, inherited maternally. Clinical criteria include: (1) stroke-like episodes before age of 40, (2) encephalopathy with seizures and/or cognitive deficits, and (3) mitochondrial myopathy accompanied by lactic acidosis and/or ragged-red fibers (El-Hattab et al., 2015). In addition to its neurological manifestations, MELAS syndrome can include a clinical picture of ASD (Ahmadabadi et al., 2020). Connolly and collaborators described kindred with MELAS, autism, cardiomyopathy, and rhabdomyolysis associated with the A3260G mtDNA mutation (Connolly et al., 2010). Treatment of MELAS syndrome with L-arginine, coenzyme Q, creatine monohydrate, and carnitine can be beneficial. Valproic acid, metformin, and dichloroacetate should be avoided (El-Hattab et al., 2015).

Neurotransmitter Disorders

Succinic Semialdehyde Dehydrogenase Deficiency

Succinic semialdehyde dehydrogenase deficiency (SSADH-D) is a genetic disease caused by disrupted metabolism of the γ-amino butyric acid (GABA). Impaired activity of the mitochondrial enzyme SSAD leads to the accumulation of γ-hydroxybutyric acid (GHB). The SSADH-D has highly heterogeneous clinical picture, with varying degrees of autism, mental retardation, hypotonia, ataxia, epilepsy, and a delay in speech development. There has been no approved curative therapy for the disease yet, but vigabatrin can be beneficial by reducing accumulation of SSA and GHB through inhibition of GABA transaminase. Inhibition with vigabatrin, however, has no effect on peripheral GABA transaminase, so the

GHB keeps rising and reaches the CNS (Pearl et al., 2016). Many therapeutic approaches are currently being studied (Didiášová et al., 2020).

Disorders of Purine Metabolism

Adenylosuccinate Lyase Deficiency

Adenylosuccinate lyase (ADSL) deficiency is a disorder of purine metabolism that reduces purine *de novo* synthesis and purine nucleotide recycling. ADSL was first described in Jaeken and Van den Berghe (1984), who discovered succinylpurines in the plasma, urine, and CSF of a few patients with severe psychomotor delay and autism. Growth and development might be normal in the newborn period (Jurecka et al., 2015), but in severe forms neurological symptoms might be evident immediately after birth (Ciardo et al., 2001). They include acute and chronic encephalopathy, behavioral abnormalities, and ASD. Treatment relies on epilepsy treatment. Intervention with D-ribose administration, which increases levels of phosporibosylpyrophosphate and stimulates purine synthesis *de novo*, has only limited success (Jurecka et al., 2015).

Lesch-Nyhan Syndrome

Lesch-Nyhan syndrome (LNS) is an X-linked disorder caused by mutations in the *HPRT1* gene, coding for hypoxanthine-guanine phosphoribosyltransferase (HPRT). Symptoms include dystonia, intellectual disability, gout, autism, and behavioral changes such as self-mutilation. Pathogenesis is unclear and it remains to be uncovered how deficits in hypoxanthine and guanine recycling lead to a severe phenotype. Upon identification of several isoforms of amyloid precursor protein (*APP*) mRNA with a deletion followed by an insertion that accounted for epigenetic control of genomic rearrangements of *APP* gene in fibroblasts of patients with LNS, Nguyen proposed a role of epistasis between mutated *HPRT1* and *APP* genes affecting the regulation of alternative *APP* pre-mRNA splicing as a possible pathophysiological mechanism of the severe neurobehavioral phenotype of LNS (Nguyen, 2019). Patients excrete large amounts of uric acid. Allopurinol prevents urate nephropathy. Treatment with allopurinol has no effect on neurological symptoms (Bell et al., 2016).

Cerebral Creatine Deficiency Syndromes

Creatine serves as a donor of high-energy phosphates for the synthesis of hydrolyzed adenosine triphosphate. The role of creatine transporter coded by the *SLC6A8* gene on chromosome X is to translocate creatine across the cell membrane in the cytoplasm of neurons and myocytes. Mutations in the *SLC6A8* gene lead to cerebral creatine deficiency syndrome (CCDS) with a spectrum of clinical manifestations, including severe intellectual delay, autism, epilepsy, and motor dysfunction. Females heterozygous for the *SLC6A8* pathogenic mutation are asymptomatic or have mild intellectual delay. The other two CCDS disorders are caused by the deficiencies of two enzymes involved in the synthesis of creatine: arginine-glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). All three conditions result in creatine depletion in the brain (Farr et al., 2020). GAMT and AGAT are treated with oral creatine monohydrate to restore cerebral creatine levels. Therapy of GAMT using ornithine supplementation and protein dietary restriction has only limited success (Mercimek-Andrews and Salomons, 2015).

Disorders of Folate Transport and Metabolism

Cerebral Folate Deficiency

Cerebral folate deficiency (CFD) is any neurological condition with a low CSF concentration of 5-methyltetrahydrofolate (5-MTHF) and normal folate plasma concentrations. This syndrome is characterized by a wide variety of clinical symptoms, from irritability and sleep disturbances to severe seizures, developmental regression, autism, progressive ataxia and extrapyramidal symptoms, including choreoathetosis (Hyland et al., 2010). CFD is a treatable condition if timely recognition is followed by treatment with folinic acid perorally. Treatment with folic acid is contraindicated as it may exacerbate the 5-MTHF deficiency in the CNS. Autoimmunity against the folate receptor (transporter of 5-MTHF into the CSF) and mutations in the folate receptor 1 (FOLR1) gene are the primary causes of CFD. Secondary CFD has been observed in other IEM, including mitochondrial diseases, serine deficiency, and pyridoxine-dependent epilepsy (Pope et al., 2019).

Methylenetetrahydrofolate Reductase Deficiency

Methylenetetrahydrofolate reductase deficiency (MTHFR-D) is the most common genetic cause of hyperhomocysteinemia. MTHFR has an important role in the conversion of Hcy to methionine. A common genetic variant in the *MTHFR* gene is the c.677C > T polymorphism (rs1801133), where the common variant is less active at higher temperatures (Dean, 2012). In comparison to controls, subjects with two copies of this variant usually have higher Hcy levels and lower serum folate levels ("TT homozygous"). Another common *MTHFR* variant, c.1298A > C, is not associated with homocysteinemia alone, but only combined heterozygosity of c.1298A > C and c.677C > T results in an outcome similar to TT genotype. Pathogenic variants in the *MTHFR* gene can cause an autosomal recessive IEM with high concentrations of Hcy in plasma and urine. This, in turn, may cause developmental delay, thrombosis, eye disorders, and osteoporosis (Dean, 2012). Importantly, the TT genotype is frequent among ASD patients and their mothers, compared to the general population. The results of the study of Sadigurschi and Golan (2019) support the view that the maternal *MTHFR* genotype is associated with increased risk for ASD in children. The study of Orenbuch et al. (2019) showed that prenatal nutritional intervention by adding folic acid and choline in the diet of pregnant MTHFR-deficient mice reduce autistic-like behaviors among offspring that are MTHFR-deficient. Further studies are needed to better define the role of methionine cycle in the pathogenesis of ASD.

Lysosomal Storage Disorders

Muccopolysaccharidosis Type III (Sanfilippo Syndrome)

Mucopolysaccharidosis type III (MPS III) is a multisystemic lysosomal storage disease characterized by progressive neurodegeneration characterized by severe developmental regression, intellectual disability, autism, and other behavioral problems. Multisystemic manifestations include hearing loss, musculoskeletal problems (contractures, joint stiffness, hip dysplasia and scoliosis), respiratory tract infections, and valvular cardiac disease (Wagner and Northrup, 2019). Patients are commonly misdiagnosed as having idiopathic developmental delay, attention deficit/hyperactivity disorder or ASD with unnecessary testing procedures and treatment attempts. Neurological decline is inevitable (Wijburg et al., 2013). Treatment is symptomatic with therapies such as enzyme replacement; however, the latest developments could push gene therapy into the mainstream (Seker Yilmaz et al., 2021).

Neuronal Ceroid Lipofuscinoses

Neuronal ceroid lipofuscinoses (NCL) are a group of autosomal recessive, progressive lysosomal storage disorders with dominantly neurological symptomatology. This group of diseases has a high genetic heterogeneity and variable clinical presentation. The most common forms are infantile NCL (INCL), late infantile NCL (LINCL), and juvenile NCL (JNCL). Common symptoms include epilepsy, developmental regression, and ophthalmologic abnormalities. Madaan et al. (2020) described a patient with autistic regression and epilepsy, along with EEG photosensitivity that is considered as an early sign of Batten disease. Cerliponase α, a recombinant human tripeptidyl peptidase 1 enzyme replacement therapy, became the first globally approved treatment for LINCL—Batten disease in 2017, signifying major therapeutic progress (Johnson et al., 2019).

Niemann-Pick Disease Type C

Niemann-Pick disease type C is an autosomal recessive disorder caused by mutations in *NPC1* (in 95% of cases) or *NPC2* (in about 5% of cases) genes. When either of two proteins, NPC1 or NPC2, is not functional, it leads to impaired cellular trafficking of endocytosed LDL cholesterol and its accumulation in the lysosomes. The clinical presentation is highly heterogeneous, with an age of onset ranging from the perinatal period to late adulthood. Visceral signs such as fetal hydrops, hepatosplenomegaly, and cholestatic jaundice in early forms usually preceed neurological and psychiatric manifestations. Manifestations of neurological forms (late-infantile and juvenile forms) are ataxia, clumsiness, handwriting difficulties, impaired attention, vertical supranuclear gaze palsy and gelastic cataplexy. Some patients develop seizures. Compared to younger-onset patients, individuals with the adolescent/adult onset more frequently develop psychiatric symptoms and cognitive impairment (Newton et al., 2018).

Oxysterols (cholestane-3β, 5α and 6β-triol and 7-ketocholesterol are elevated in most patients, but they can also be elevated in the deficiency of lysosomal acid lipase and in neonatal cholestasis in general. Gene analysis is diagnostic. Miglustat is currently the only causal treatment for neurological manifestations (Geberhiwot et al., 2018; Patterson et al., 2020).

Cerebrotendinous Xanthomatosis

Cerebrotendinous xanthomatosis (CTX) is a disorder of bile acid synthesis caused by mutations in the cytochrome P450 *CYP27A1* gene. These mutations cause dysfunction of sterol 27-hydroxylase enzyme, resulting in incomplete oxidation of the cholesterol side chain. Although CTX does not belong to the lysosomal diseases, by its clicial presentation it resembles storage diseases. Hence, it is often classified in this group. CTX is characterized by high levels of plasma cholestanol and accumulation of cholestanol and cholesterol in the CNS and tendons, which is manifested as tendon xanthomas. Typical clinical symptoms of CTX include chronic diarrhea, tendon xanthomas, bone fractures, bilateral cataracts, and neurological dysfunction. The disease is progressive, particularly due to severe neurological presentations that may include autism, intellectual disability, psychiatric and behavioral problems. Treatment with chenodeoxycholic acid is the current standard of care; if initiated early, it can stop the progression of the disease (Duell et al., 2018). At present, level of 7α-hydroxycholestenone appears to be the best biochemical marker for the evaluation of CTX treatment (Lütjohann et al., 2020).

Although Smith-Lemli-Opitz syndrome, Niemann-Pick disease type C and CTX all affect cholesterol metabolism and can present with psychiatric symptoms including autistic features, other clinical signs, metabolic background and treatment approaches do not overlap.

Disorders of Copper Metabolism

Wilson Disease

The key clinical features of Wilson disease include acute episodes of hemolysis in association with acute liver failure, cirrhosis, neuropsychiatric disturbances including autistic features, and eye manifestations such as Kayser–Fleischer ring. Psychiatric and behavioral symptoms are common and may precede other neurological or hepatic signs and symptoms. Wilson disease is a disorder of copper metabolism, caused by biallelic mutations in the *ATP7B* gene, and characterized by low serum ceruloplasmin levels and elevated daily urinary copper excretion (Ferenci, 2017). Current treatment includes avoiding copper-rich foods, reducing copper absorption with zinc, chelation therapy to remove copper from tissues, and symptomatic treatment (Mulligan and Bronstein, 2020). Early recognition is prerequisite for treatment success (Mulligan and Bronstein, 2020).

Disorders of Haem Biosynthesis

Acute Intermittent Porphyria

Mutations in hydroxymethylbilane synthase gene (*HMBS*) cause autosomal dominant acute intermittent porphyria (AIP). AIP is characterized by life-threatening neurovisceral attacks and increased risk of hepatocellular carcinoma, hypertension, and kidney failure. The only possible treatment is liver transplantation (Bustad et al., 2020). Luder et al. (2009) described a 15-year-old girl with AIP, whose first symptoms were autistic

features at the age of 4. Visceral symptoms have occurred in the later course and the diagnosis of AIP was confirmed by the finding of a known causative AIP mutation (Luder et al., 2009).

A new therapeutic approach to AIP is RNA interference-based therapy (Givosiran), which decreases aminolevulinic acid syntase mRNA levels. Another promising approach is gene therapy that targets hepatocytes harboring mutated porphobilinogen deaminase (*PBGD*) gene (Spiritos et al., 2019).

Brain Iron Accumulation Diseases

Neurodegeneration with brain iron accumulation (NBIA) is a group of genetic diseases characterized by movement disorders such as parkinsonism and dystonia, psychomotor delay, and early death. There is still no established therapy available to mitigate or stop the progression of these diseases (Di Meo and Tiranti, 2018). Two of these diseases are characterized by autistic regression as a dominant clinical symptom: panthotenate kinase-associated neurodegeneration (PKAN) and β-propeller protein-associated neurodegeneration (BPAN). Veeravigrom and colleagues described a girl diagnosed with ASD at the age of three, later confirmed to have PKAN. The brain MRI at 15 year of age showed abnormally low T2 signal intensity in the globus pallidus and posterior limb of internal capsule bilaterally due to iron accumulation ("eye of the tiger" sign), as well as cortical atrophy (Veeravigrom et al., 2014). Yogananthan and colleagues described a girl with seizures, autistic regression, intracranial calcification, and iron accumulation in the nucleus niger and globus pallidus. The diagnosis of BPAN was established by identification of pathogenic variant in WD repeat domain 45 (*WDR45*) gene encoding for β propeller protein. Current treatment is only palliative (Yoganathan et al., 2016).

DIAGNOSTIC AND THERAPEUTICAL APPROACHES

Many IEM present with syndromic ASD, most commonly as an associated symptom. Diagnostic approach includes wide neuroradiologic, metabolic, and genetic workup (Polšek et al., 2011). In this review, we briefly describe etiology, clinical presentation, and therapeutic principles, if available, for several groups of IEM in differential diagnosis of ASD. Many of them are nowadays diagnosed and successfully prevented by neonatal screening program. Based on the list of potentially treatable IEM mentioned in this paper, we suggest following laboratory work-up: ammonia and lactate concentration, acid-base balance, plasma amino acid analysis, urinary organic acid analysis, urinary amino acids, plasma or dry blood spot acylcarnitine profile, total cholesterol, total homocysteine, vitamin B_{12} concentration, and uric acid. Further, more specific laboratory tests such as purine and pyrimidine analysis, CSF analysis (amino acids, folic acid concentration), cholestanol concentration, urinary glycosaminoglycans and porphyrins, copper excretion, and ceruloplasmin concentration should be done based on additional leading symptoms or laboratory results, but should not be a part of the primary workup. All laboratory tests should be done after a detailed anamnesis and clinical examination with special attention on associated neurological abnormalities. Brain MRI with spectroscopy and electroencephalogram have their place in the primary workup of an ASD patient due to several specific findings that can be leading to a proper diagnosis (CCDSs, NBIA). Here, we do not focus on common genetic causes of ASD such as FXS, tuberous sclerosis, Rett syndrome or on rare dysmorphic syndromes and genetic diagnostic tests such as molecular karyotyping (copy number variants could be found in 10−15% of cases), PCR assay or single specific gene analyses. In the end, unspecific genomic approaches like whole exome sequencing could be useful in diagnosing rare disorders missed by proposed diagnostic workup and will reveal the etiology in about 30−40% of syndromic ASD cases (Schaefer et al., 2013).

Over the years, ample evidence has accumulated to suggest that children with ASD have different biochemical profiles compared to healthy children. For example, age-independent hyperserotonemia is present in approximately one-third to one-half of subjects with ASD (Šimić et al., 2020). Untargeted metabolomics, although still not implemented in routine clinical practice, has been shown to be a promising approach in screening of underlying biochemical abnormalities, discovering new specific biomarkers and directing treatment (Glinton and Elsea, 2019).

An interesting study was done in Greece where a large cohort of children with confirmed ASD were screened for the presence of IEM (Spilioti et al., 2013). Their data provide the evidence for a new potential biomarker—3-hydroxyisovaleric acid as well as a few novel treatment approaches for children with ASD: biotin supplementation and a ketogenic diet elicited mild to significant improvement in clinical picture (Spilioti et al., 2013).

Basic six therapeutic principles of IEM include: (1) substrate reduction therapy (as in PKU), (2) removal of toxic metabolites (e.g., urea cycle disorders), (3) product supplementation (e.g., cerebral creatine deficiency disorders), (4) stimulating of deficient enzyme activity (e.g., B_6 supplementation in CBS deficiency), (5) enzyme replacement therapy (e.g., in LINCL) or 6) organ transplantation (in some cases of UCD and AIP). As exemplified in parentheses, these principles can be also observed throughout the IEM listed above.

Methionine Cycle and its Disturbances as Well as Folic Acid Enigma Deserve Special Attention Regarding Therapeutic Trials and Opinions

Periconceptional folic acid supplementation in pregnant women along with the dietary and blood folate concentrations in children with ASD have been shown as environmental factors contributing to the incidence of autism. Moreover, children with autism have hyperhomocysteinemia compared to controls, whereas serum levels of folate and vitamin B_{12} may be diminished or normal. This can be due to lower folate intake in these subjects. Furthermore, folinic acid supplementation in ASD subjects with low 5-MTHF levels in CSF leads to the normalization of folate levels and alleviates some symptoms of ASD (Castro et al., 2016).

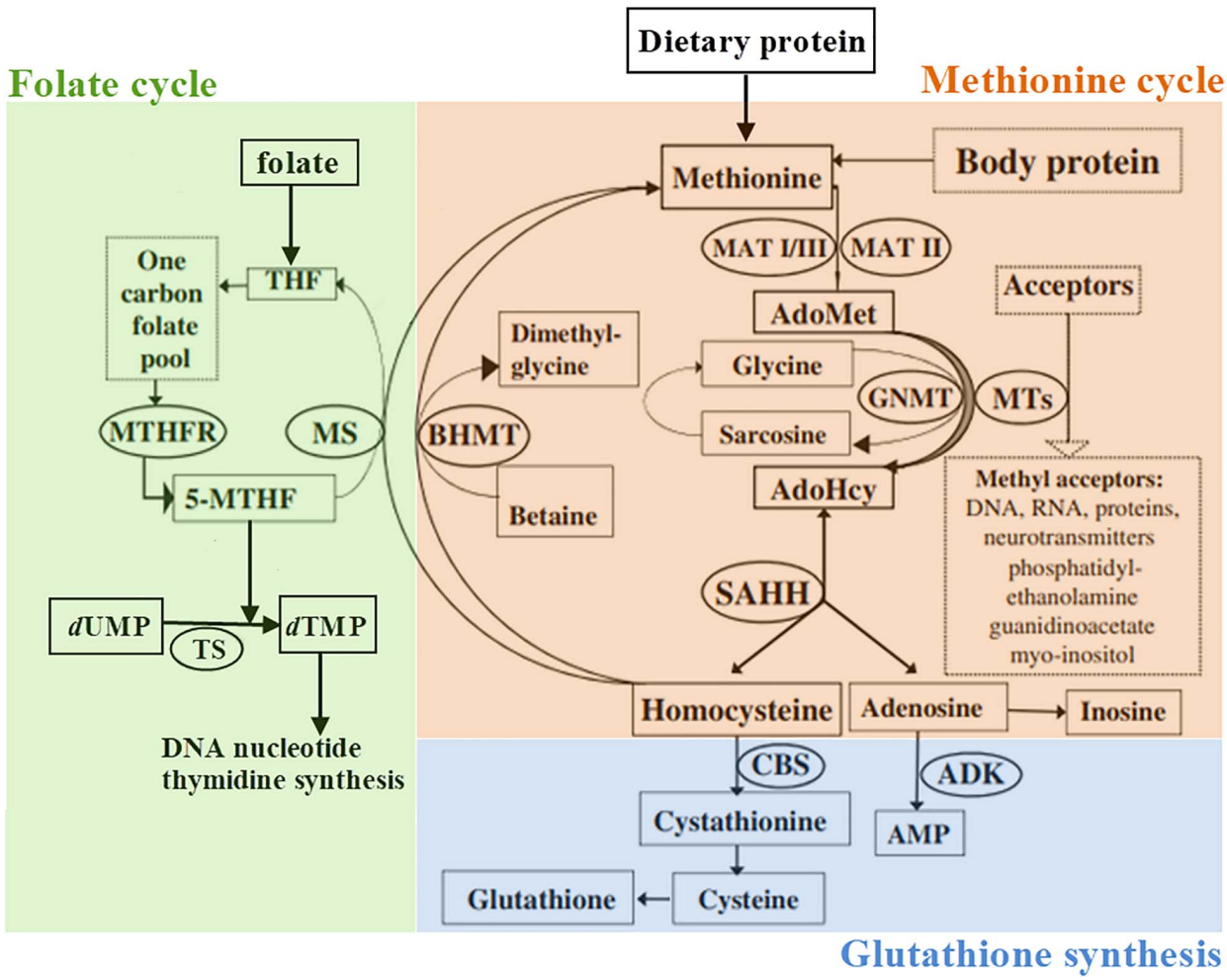

FIGURE 1 | Schematic representation of the methionine metabolism according to Barić et al. (2017), slightly modified. 5-MTHF, 5-methyltetrahydrofolate; ADK, adenosine kinase; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AMP, adenosine monophosphate; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; GNMT, glycine *N*-methyltransferase; dTMP, 2′-deoxythymidine-5′-monophosphate (thymidylate); dUMP, 2′-deoxyuridine-5′-monophosphate; MAT, methionine adenosyltransferase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; MTs, a variety of AdoMet-dependent methyltransferases; THF, tetrahydrofolate; SAHH, S-adenosylhomocysteine hydrolase; TS, thymidylate synthase.

There are several examples of enzyme deficiencies in methionine cycle (CBS, SAHHD, MTHFR) with clinical presentation of ASD. It is yet to be elucidated how subtle disturbances in this metabolic pathway affect methylation and consequent epigenetic dysregulation of numerous genes.

Malaguarnera and Cauli (2019) showed the positive clinical effects of L-carnitine administration in non-syndromic forms of autism. Several clinical trials suggest that carnitine supplementation is useful in diminishing symptoms of non-syndromic ASD. Nevertheless, future clinical trials to identify the subgroup of ASD patients that could benefit from carnitine supplementation are needed (Malaguarnera and Cauli, 2019).

Treating an autistic child is always associated with additional healthcare-associated costs, therapies, education, and family services. The associated social and emotional stresses often lead to depression, somatization, and impairment in quality of life parameters (Glinton and Elsea, 2019).

At the present time, there is not enough evidence for routine supplementation of ASD patients with folic acid, choline, or other supplements. We suggest methionine cycle (**Figure 1**) to be carefully analyzed in syndromic ASD patients (as proposed earlier, this should be performed by plasma amino acid analysis and total Hcy concentration measurement) and specific supplementation given in case of biochemically proven deficiencies.

CONCLUSION

Symptoms of ASD can be a present in many IEM, but rarely occur in isolation. Since some of IEM are treatable, physicians treating children with ASD should be aware of a long list of rare and ultra-rare disorders in the differential diagnosis. Given the enormous etiologic variability of autistic symptoms,

including those of genetic and metabolic origin, diagnostic work-up of these patients should include appropriate genetic and metabolic studies elected on the basis of leading symptoms and associated clinical signs. This review is also intended to serve as a reminder on IEM that could present with ASD as a leading or associated presentation. As always, detailed medical history and clinical examination, including detailed neurological examination, should be a basis for planning focused diagnostic work-up of patients with ASD.

AUTHOR CONTRIBUTIONS

IB conceived the manuscript. TŽ wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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