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Novel therapeutic targets for autism

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Autism spectrum disorders (ASDs) are pervasive neurodevelopmental disorders, diagnosed in early childhood when acquired skills are lost or the acquisition of new skills becomes delayed. ASDs are associated with varying degrees of dysfunctional communication and social skills, in addition to repetitive and stereotypic behaviors. The diagnosis has increased considerably to approximately one in 180 people, but it is not clear whether this is because of a higher prevalence of the disorder, improved awareness by clinicians or a combination of both. There are no defined mechanisms of pathogenesis or curative therapy presently available. Oxidative stress, overactivation of the hypothalamic–pituitary–adrenal axis and increased gut–blood–brain-barrier permeability might be involved. The scope of this article is to integrate these findings and present the opinion that non-allergic activation of gastrointestinal and brain mast cells could contribute to many of the pathologic findings and provide unique targets for ASD therapy. We make suggestions for new research directives and possible novel therapies from readily available molecules.

Introduction

Autistic, pervasive developmental and Asperger's disorders constitute the autism spectrum disorders (ASDs), neurodevelopmental disorders diagnosed in early childhood [1]. They present with varying degrees of dysfunctional communication and social skills, repetitive and stereotypic behaviors, and attention, cognitive, learning and sensory defects [1]. Diagnostic criteria specify the onset of symptoms before 3 years of age, but there is no biochemical or genetic screening test and normal development might switch to delay in acquisition of new skills. Mental retardation or cognitive impairment can vary, with autistic disorder displaying the worst impairment and Asperger's disorder displaying the least.

The diagnosis of ASDs has increased more than tenfold in the past 20 years [1], with a prevalence of 4 in 10 000 children before 1980 to 36 in 10 000 since 2000 [2], and 53 in 10 000 (1 in 180) in 2006 [3]. This increase could reflect true prevalence, improved awareness by clinicians or because of mercury preservatives in vaccines (but recent studies disprove this) [4,5].

Here, we review mostly the immunological and endocrine findings in ASDs. We then present the opinion that a unique pluripotent cell [6], the mast cell, could be activated by a variety of triggers in the intestines and the brain to release key molecules that could disrupt the gut–blood–brain barrier and lead to neurotoxic effects.

The role of the gut–blood barrier in the pathogenesis of ASDs

The intestinal mucosa has tight junctions, comprising the gut–blood barrier, which prevent entry of unwanted molecules from the intestinal lumen into the blood. Similarly, the brain is protected from entry of blood-borne toxic molecules by the blood–brain barrier (BBB), which is made up by endothelial cells and pericytes. In both cases a bone-marrow-derived unique immune cell, the mast cell, is juxtaposed to endothelial cells [7]. Many autistic children present with abdominal pain and bowel symptoms [1,8] prompting the suggestion of some gastrointestinal (GI) pathology. However, a nested case-control study showed no association between autism and any GI diseases [9]. Moreover, a review of all publications in which intestinal biopsies from autistic children had been examined concluded there is no such entity as 'autistic enterocolitis' [10]. However, 'leaky gut', or a state of high intestinal permeability [11], might permit systemic absorption of substances that could adversely affect brain function.

In the National Survey of Children's Health, parents of autistic children reported symptoms of allergies more often than parents of children without autism, with food allergies showing the greatest difference [3]. Food intolerance [3] is present in 16% of all children [12] and in 34% of children up to the age of three, leading to substantial behavioral responses [13]. Unfortunately, the terms 'food allergy', 'food hypersensitivity' and 'food intolerance' often are used interchangeably to denote adverse reactions to foods (Table 1) even though they address different conditions; this leads to confusion [14]. The only validated test for food allergies is the 'double-blind controlled food challenge' [14], which can

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Table 1. Adverse reactions to foods[a]

Toxic
Heavy metals (e.g. iron, lead, mercury)
Non toxic
Immune mediated (food allergy)
IgE (e.g. shrimp)
Non-IgE (e.g. gluten)
Non-immune mediated (food intolerance)
Enzymatic (e.g. lactose)
Pharmacological (e.g. histamine poisoning)
Undefined[b]

[a]Created from the classification of the European Academy of Allergy and Clinical Immunology [69].
[b]Rare diseases such as carcinoid syndrome, a GI tumor-secreting vasoactive intestinal peptide (VIP), or mastocytosis.

be performed by using either the serum RAST test or a skin prick to food antigens. Many patients actually might have abdominal cramping and diarrhea without any such positive tests due to non-immune causes such as lactose intolerance or uncooked-tuna histamine poisoning (Table 1). Even gluten sensitivity might present with systemic symptoms without definitive GI pathology [15]. Serum levels of antibodies other than immunoglobulin (Ig)E, such as IgG_4, might be useful because they could be predictors of autoimmune diseases [16].

Clinical studies of autistic children on gluten- or casein-free diets [17] have been hard to evaluate owing to the absence of appropriate control groups. Nevertheless, one study has shown significantly higher levels of IgA antibodies for casein, lactalbumin and betalactoglobulin in

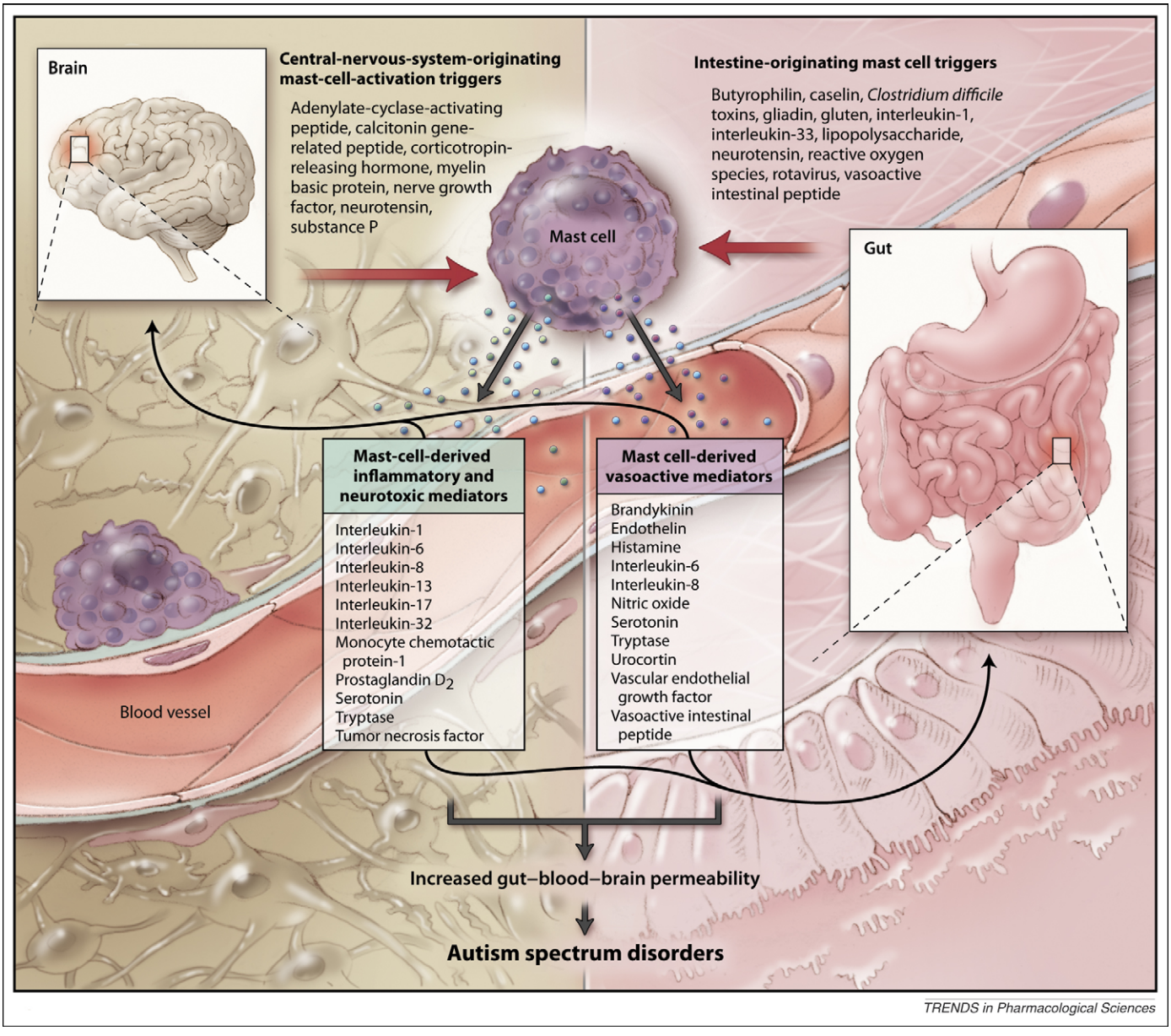

Figure 1. Schematic representation of the proposed role of non-allergic mast-cell activation in gut–blood–brain-barrier permeability and ASDs. Several non-allergic triggers could derive first from the GI tract (right-hand panel) and stimulate the release of mast-cell vasoactive, proinflammatory and neurotoxic mediators. Such molecules could increase gut–blood-barrier permeability to intestinal toxic substances that could reach the blood–brain barrier. There, such toxins or GI mast-cell-derived mediators could further stimulate brain mast cells (left-hand panel) to release molecules that increase BBB permeability and permit intestinal toxins to enter the brain. Conversely, triggers derived from the brain could be released from dorsal root ganglia locally in the intestines and activate mast cells, mediators from which can increase permeability of the gut–blood barrier and lead to GI symptoms such as those seen in ASDs.

autistic patients ($n = 36$) than controls ($n = 20$); moreover, there was marked improvement in behavioral symptoms after an 8 week elimination diet of the antigen that gave a positive skin test [18]. Serum from autistic children also was shown to contain several autoantibodies against encephalogenic peptides that crossreact with milk butyrophilin [19], indicating that the BBB was disrupted at some point.

Abdominal pain could be misleading because it is also caused by anxiety and might actually be an 'abdominal migraine', which is often precipitated by stress. One study has shown that adults with autism ($n = 34$) were three times as anxious as controls ($n = 20$) matched for age, gender and intellectual ability, and they were significantly less likely to cope with stressful events [20]. A heightened stress state in ASDs is also indicated by elevated salivary cortisol [21] and by plasma adrenocorticotropin hormone in adults with Asperger's syndrome [22], which implies increased activity of the hypothalamic–pituitary–adrenal (HPA) axis (typically stimulated by stress). It is interesting that restraint stress for 30–120 min in rodents leads to intestinal mast-cell stimulation and flattened villi [23], similar to what is seen with food intolerance and increased gut permeability. Dysfunctional or immature development of the gut–blood barrier or BBB (Figure 1) would expose local mast cells to triggers derived from the gut and result in the release of mast-cell-derived vasoactive and inflammatory molecules that could increase intestinal permeability, in addition to disrupting the BBB. These, or other neurosensitizing molecules, could affect intestinal and brain function (Figure 1) (Table 2).

The role of mast cells in ASDs

Mast cells are crucial for allergic reactions [6], whereby they are activated by crosslinking of the high-affinity IgE receptor (FcεRI) by specific antigens, but they are also important in immunity [24,25] and inflammation [26]. Functional mast cell–neuron interactions occur in the GI and brain and could trigger neuroinflammation [26]. In addition to allergic stimulation, gut or brain mast cells also can be triggered by many non-immune molecules [6] (Table 3): immunoglobulin free light chains, bacterial and viral products, anaphylatoxins and neuropeptides [including neurotensin, substance P and vasoactive intestinal peptide (VIP)] secrete vasoactive, neurosensitizing and proinflammatory mediators (Table 4). Of these, histamine, serotonin, prostaglandins, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF) and VIP can increase vascular permeability [27]. Cerebrospinal-fluid TNF levels have been found significantly higher than corresponding serum levels in ten autistic children, indicating central nervous system production [28] that could affect cognitive functions [29]. Moreover, peripheral blood mononuclear cells only from ASD children with GI symptoms produce higher TNF-α in response to gluten gliadin, cow milk protein and soy [30]. Autistic patients also have higher urine levels of prostaglandins [31] indicating mast-cell activation (Figure 1).

Mast cells are involved in the regulation of GI pathophysiology [32], especially integrating immunity, intestinal hyperpermeability and stress [33] and BBB integrity [7]. Mast cells could act as 'the immune gate to the brain' and increase BBB permeability. In fact, acute stress could activate brain mast cells and increase BBB permeability through the activation of corticotropin-releasing factor (CRF) [34]. This effect is evident only in brain areas containing mast cells, indicating a localized effect. Moreover, mediators released from mast cells could also influence CRF release (Figure 1) and stimulate the HPA axis [35].

Intradermal administration of CRF activates skin mast cells and increases vascular permeability in rodents [36] and humans [37] through activation of the CRF_1 receptor. Similar effects of acute stress have been reported for the intestines [38], and CRF increases permeability in normal human colonic biopsies through activation of subepithelial mast cells [39]. Restraint stress could disrupt both the blood–gut barrier and the BBB as shown by a higher amount of label in the blood and in the brain after intraluminal administration of ^{99}Technetium gluceptate in the mouse colon, as compared with controls (T.C.T., unpublished).

Table 2. Mastocytosis patients with ASDs[a]

Patient	Age	Sex	ASD	Mast-cell diagnosis
1	4	M	Stuttering, high functioning	Mastocytoma, solitary
2	15	M	Autism, poor speech	UP[d], asthma
3	11	M	Asperger's	UP
4	18	M[b]	Autism, dyslexia	Systemic mastocytosis
5	44	M[b]	Asperger's	UP
6	21	F	Autism, sensory defensive	UP
7	21	F	SID	Mastocytosis
8	18	M	Asperger's	Mastocytosis
9	19	F	Rett syndrome	UP
10	4.5	M	Autism	UP
11	9	M	Asperger's	Mast cell disease
12	3.5	M	Asperger's	Mastocytosis
13	46	M[c]	Asperger's	Hives, asthma
14	10	F[c]	Asperger's	Diarrhea, hives, itching
15	8	M[c]	Mood, attention problems	Mastocytosis

[a]Data obtained in response to the question listed below that was sent by the Mastocytosis Society (www.tmsforacure.org) to a database of 400 patients: ''Could you please let us know if you or any of your children have been diagnosed with autism or an autistic spectrum disorder (e.g. Asperger's disorder). Please include yours and your child's sex, current age, age at time of diagnosis, and how/where diagnosis was made.'' Five other families reported a mother with mastocytosis and one child with an ASD.
[b]Same family.
[c]Same family.
[d]Abbreviations: SID, sensory integration dysfunction; UP, urticaria pigmentosa.

Table 3. Triggers of mast-cell activation

I. Natural		II. Drugs
Allergic	**Hormones**	**Adenosine**
IgE and specific antigen	CRF	Adriamycin
Anaphylatoxins (C3a, C5a)	Estradiol (augments)	Contrast media
IgG_1	Ucn	Curare
Superallergens		Ibuprofen (high doses)
Immunologic	**Infectious**	Morphine
IL-1	LPS (TLR-4)	Taxol
IL-33	Parasites	
Immunoglobulin free light chains	Peptidoglycan (TLR-2)	
PAF	Viral antigens (TLR-3, -5, -7, -9)	
Prostaglandin E_2		
MCP-1, -2, -3		
MIP-1		
Neuropeptides and neurotransmitters	**Lipids**	
Acetylcholine	Sphingosine	
Adrenomedullin	Phosphatidylserine (augments)	
Bombesin		
CGRP		
Gastrin	**Toxins**	
Mastoparan	Fire ants	
Neurotensin	*Clostridium difficile*	
PACAP	Jelly fish	
Somatostatin	Snake venoms	
SP	Wasps	
VIP		
Growth factors	**Vascular**	
EGF	Adenosine	
Lymphopoietin	Endothelin	
NGF	Oxidized LDL	
PDGF	Reactive oxygen species	
SCF	Thrombin	
TGF-β	Urocinase	

Abbreviations: CGRP, calcitonin gene-related peptide; CRF, corticotropin-releasing factor; EGF, epidermal growth factor; MCP, monocyte chemotactic protein; MIP, monocyte inflammatory peptide; NGF, nerve growth factor; PACAP, pituitary adenylate-cyclase-activating peptide; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; SCF, stem cell factor; SP, substance P; TGF-β, transforming growth factor-β; TLR, toll-like receptor; Ucn, urocortin; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

Stress can worsen many diseases through the activation of mast cells [26], which are located close to sensory nerve endings [40]. Physiological and psychological stress also worsens GI and behavioral problems. A case in point is mastocytosis, which represents a model of how mast-cell mediators can induce clinical symptoms that mimic ASDs, because many patients have a low attention span, difficulty concentrating, forgetfulness, depression, poor motivation, confusion, irritability and anxiety. Mastocytosis represents a wide spectrum of disorders involving proliferation and activation of mast cells in the GI, skin and other organs [41], the most common manifestation of which is urticaria pigmentosa (UP), small brown-red maculopapules on the skin [42]. A recent survey sent to 400 mastocytosis patients resulted in 15 responses from patients with both mastocytosis and ASDs – which translates to an incidence of 3.75 in 100 or 6.75 times that reported for the general population (i.e. 1 in 180); five other families reported a mother with mastocytosis and at least one child with an ASD (T.C.T., unpublished; Table 2). Given that both ASDs and mastocytosis are rare diseases, this association is impressive. It should be noted that the reported incidence of ASD-related symptoms (both in adults and children) in mastocytosis seems to be even higher than the actual diagnosis of ASDs; as a consequence, treatment of such symptoms in mastocytosis with new targeted therapies could constitute a good model with possible future application for the treatment of ASDs.

New aspects of mast-cell pathophysiology

Given the crucial role of mast cells in allergic reactions, some association between allergies and ASDs might be expected. In one study of families of autistic patients and 46 healthy controls, 46% of autistics had 2–3 family members with autoimmune diseases (as compared with 26% of controls), but none with allergies [43]. A nested case-control study of 407 ASD children and 2095 controls (born between 1995 and 1999) showed that only psoriasis in the mother before birth was associated with ASDs, but there was a greater than twofold risk of ASDs in mothers who were diagnosed with allergies or asthma during the second trimester of pregnancy [44]. In one study of six ASD children, even though there was at least one manifestation of immediate hypersensitivity in all patients (and more than two in 50%), there were no elevations of serum histamine or IgE, prompting the conclusion of non-IgE-dependent mast-cell activation [45]. In a recent study, 30 autistic children and age-matched neurologic controls from the same hospital were investigated for serum IgE, IgG, IgA and IgM levels and were subjected to skin prick tests to 12 common antigens; although 30% of autistic children had a family history of atopy as compared with 2.5% of controls, there was no difference in objective measurements [46]. Consequently, mast cells could be involved in ASDs mostly through their unique ability to respond to non-IgE triggers and release some mediators 'differentially' or 'selectively' without the degranulation typical of allergic or anaphylac-

Table 4. Mast-cell mediators and their actions

Mediators	Main pathophysiologic effects
I. Pre-stored	
Biogenic Amines	
Histamine	Vasodilation, angiogenesis, mitogenesis, pain
Serotonin	Vasoconstriction, pain
Chemokines	
IL-8 (CXCL8), MCP-1 (CCl2), MCP-3 (CCl7), MCP-4 (CXCL13), RANTES (CCl5)	Chemoattraction and tissue infiltration of leukocytes
Enzymes	
Arylsulfatases	Lipid and proteoglycan hydrolysis
Carboxypeptidase A	Peptide processing
Chymase	Tissue damage, angiotensin II synthesis
Kinogenases	Synthesis of vasodilatory kinins, pain
Metalloproteinases	Tissue damage
Phospholipases	Arachidonic acid generation
Tryptase	Tissue damage, activation of PAR, inflammation, pain
Peptides and proteins	
Corticotropin-releasing factor	Inflammation, vasodilation
Bradykinin	Inflammation, pain, vasodilation
Endorphins	Analgesia
Endothelin	Sepsis
Neurotensin	Inflammation, pain
Renin	Vascular constriction
Somatostatin	Anti-secretory
Substance P	Inflammation, pain
Vasoactive intestinal peptide	Vasodilation
Urocortin	Inflammation, vasodilation
Vascular endothelial growth factor	Neovascularization, vasodilation
Proteoglycans	
Chondroitin sulfate	Cartilage synthesis, anti-inflammatory
Heparin	Angiogenesis, NGF stabilization
Hyaluronic acid	Connective tissue component
Angiogenic factors	
Adrenomedullin	Angiogenesis
Angiogenin	
Angiopoietin	
EGF	
FGFα	
b-FGF	
IL-8	
Neuropilin	
PDGF	
TGF-β	
VEGF	
II. *De novo* synthesized	
Cytokines	
IL-1, -2, -3, -4, -5, -6, -8, -10, -13, -16, -17, -32	Inflammation, leukocyte migration, pain
IFN-γ; MIF, TNF-α, TGF-β	Inflammation, leukocyte proliferation, activation
Growth Factors	
SCF, GM-CSF, b-FGF, NGF, VEGF	Cell growth
Phospholipid metabolites	
Leukotriene B_4	Leukocyte chemotaxis
Leukotriene C_4	Vasoconstriction, pain
PAF	Platelet activation, vasodilation
Prostaglandin D_2	Bronchonstriction, pain
Others	
Nitric oxide	Vasodilation
ROS	Inflammation

Abbreviations: b-FGF, basic fibroblast growth factor; GM-CSF, granulocyte monocyte colony; MIF, macrophage inflammatory factor; INFγ, interferon-γ; PAR, protease-activated receptor; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

tic reactions, as was originally shown for serotonin release [47]. It was later demonstrated that interleukin (IL)-1 can stimulate human mast cells to release IL-6 selectively [48], which could affect brain function [49], whereas CRF [50] and [51] prostaglandin E_2 could stimulate selective release of VEGF leading to disruption of the gut–blood–brain barrier [7].

The ability of bacteria or viruses to trigger mast cells through Toll-like receptors (TLRs) is particularly interesting. TLRs are important in the development of innate immunity to invading pathogens and so are mast cells. Lipopolysaccharide (LPS) induces selective release of TNF-α without degranulation through TLR-4, whereas peptidoglycan induces histamine release through TLR-2 from rodent mast cells [52,53]. Human mast cells express viral TLR-1, -3, -5, -7 and -9 [54], and activation of TLR-9 selectively produces the proinflammatory cytokine IL-6, whereas activation of TLR-3 produces interferon (IFN) in

response to double-stranded RNA [54]. These findings could be important because neuroenteric viruses are likely to affect children at the critical age of 3–5 years old [55] and possibly contribute to immune abnormalities in ASDs.

Novel therapies with available molecules

Unfortunately there are no approved treatments for the core symptoms of autism [56]; novel medications are needed for effective therapy. Mast cells, their triggers and their mediators, could be unique therapeutic targets because they are affected by CRF and seem to regulate gut–blood–brain-barrier permeability [7,36].

Serotonin receptor antagonists

In a study of six autistic children, four had high urine serotonin levels [45]. Higher platelet serotonin levels have been reported in more than 40% of patients with autism [57]. In a double-blind trial of 40 children with autism, randomized to either the antipsychotic haloperidol and cyproheptadine versus haloperidol and placebo, the combined histamine H_1 and serotonin receptor antagonist cyproheptadine was associated with significant improvement [58]. Although high platelet or serum serotonin levels might not reflect brain serotonin, they certainly could affect GI function, the neuroenteric plexus of which uses serotonin [59].

Antioxidants

The innate ability to neutralize free radicals might be impaired in ASDs and could be uncontrolled in response to psychological, immune or infectious stimuli. For instance, mast-cell activation is associated with production of reactive oxygen species (ROS) [60]. One study has shown that the ratio of S-adenosylhomocysteine, used as an indicator of methylation ability, was significantly reduced in autistic children ($n = 305$) as compared with controls ($n = 205$) [61]. Another study found reduced plasma levels of S-adenosylmethionine (SAMe), the most common endogenous antioxidant [62]. Both of these studies imply that autistic patients might have excessive free-radical production and could be helped by SAMe supplements.

Mast-cell-activation blockers

Recent evidence indicates that mast-cell activation might be regulated by several co-stimulatory molecules [25]. Further evidence has shown that mast cells might be blocked through their inhibitory receptor FcγRIIβ [63]. However, such inhibition is relevant only to allergic stimulation of mast cells. Several mast-cell mediators actually could inhibit mast-cell secretion. Chondrotin sulfate, which is abundant in mast-cell secretory granules, inhibits mucosal mast-cell activation [23] and the release of histamine induced by compound 48/80, unlike disodium cromoglycate (cromolyn) which showed rapid tachyphylaxis [64].

Cromolyn is a potent inhibitor of rodent mast-cell histamine secretion but a weak inhibitor of allergic activation of human mast cells [6]. Yet, in spite of this and although it is poorly absorbed orally, it seems to reduce mastocytosis symptoms including neurobehavioral problems in mastocytosis patients, indicating that GI mast cells affect the brain. The structure of cromolyn is similar to that of certain flavonoids, polyphenolic compounds present in fruit, vegetables, nuts, seeds and red wine with potent antioxidant, anti-inflammatory and mast-cell inhibitory actions [65]. Quercetin and other flavonoids can inhibit histamine, IL-6, IL-8, TNF-α and tryptase release from normal human mast cells [66], making them possible candidates for treating ASDs.

Given that mast cells are activated by CRF, CRF-receptor antagonists developed for several diseases [67] such as anxiety, neuroinflammation and irritable bowel syndrome [68] could also be useful in ASDs.

Concluding remarks

ASDs represent a serious medical and social problem; despite this, few advances have been made regarding their pathogenesis, prevention or treatment. Several biochemical findings indicate that non-allergic mast-cell activation leads to the release of mediators that could disrupt the gut–blood–brain barrier, in addition to affecting GI and brain function. This premise is reinforced by findings from mastocytosis patients in whom ASD prevalence is higher than in the general population, while even more mastocytosis patients have ASD-like symptoms.

Future studies in ASDs should investigate (i) the exact prevalence of ASDs in mastocytosis; (ii) plasma levels of neurohormonal mast-cell triggers; (iii) evidence of non-allergic mast-cell activation; (iv) markers of gut and brain permeability; (v) the association of these end points with ASD patients of 1–4 years of age, which is when the symptoms of ASDs most often appear; (vi) the development of non-invasive scan technology to identify the number of activated mast cells in target tissues; (vii) pilot clinical trials with select flavonoids with antioxidant, anti-inflammatory and mast-cell-blocking activity; (viii) a selection of such inhibitors that might mostly concentrate in the intestine for ASD patients less than 4 years old, but that might penetrate the brain for older ASD patients; (ix) the use of neurohemal mast-cell-activation blockers. These proposed aims constitute novel directions that are easily testable and likely to lead to effective therapeutic approaches.

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Disclosure statement

US patents No 6,624 148; 6,689 748; 6,984 667; 7,115 278; 10/811 825 and EPO 136577 (awarded to T.C.T.) cover methods and compositions for blocking mast-cell activation. These patents and the dietary formula NeuroProtek®, which could be used for autism, have been licensed to Algonot, LLC (www.algonot.com).

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