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Streptococcal infection-related autoimmunity and autism: crosstalk in protein functional networks

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Abstract

Autism Spectrum Disorders (ASD) is a group of neurodevelopment dysfunctions causing behavioral abnormalities in social, verbal and non-verbal communication. The precise aetiology of ASD is unknown but it is likely to be the outcome of a combination of genetic, neurological, environmental and immunological factors. Streptococcal infection can induce tick disorders and obsessive compulsive disorder (OCD) which are described as 'co morbid' ASD, however the link between the outcomes or susceptibility to the infection and ASD is not clear. In this study we aimed to establish, via data mining and convergent genomics analysis, functional interactions between the targets of auto-antigens produced during Streptococcal infection, and genes linked to ASD via genome wide association and experimental studies. The results point towards a relation of immune and, particularly, autoimmune responses caused by Streptococcal infection to functions associated with ASD disorder. Our study also highlights a need in further experimental research into an autoimmune aetiology of autism and movement disorders characteristic for Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS).

Introduction

Autism spectrum disorders (ASD) is an umbrella term for a variety of neurodevelopmental disorders that are characterised by abnormalities in verbal, nonverbal and social communication [1]. ASD affects around 700,000 people in the UK [2]. Disorders governed by this term include tic disorders, ADHD and autism. ASD is a relatively new term in medicine and there is little research into its etiopathogenesis. The multifactorial aetiology and the variance within ASD make it difficult to identify the cause. Long-term twin studies have indicated that there may be environmental factors that have a large part to play in the onset of ASD [3]. Evidence is mounting that ASD is caused by gene-environment interactions with an everyday substance such as a protein found in milk, Butyrophilin, for instance, causing potential autoimmune impact mimicking the nervous system (NS) antigen MOG (myelin oligodendrocyte glycoprotein) [4]. Studies have also tried to link prenatal factors, such as maternal medication, maternal age and birth order to the onset of ASD; despite modest indications, there is insufficient evidence to determine a definitive risk factor [5,6].

There is also known to be a genetic risk factor to ASD, where genetic perturbations may increase susceptibility to developing autism. Multiple genes have been identified as potential candidates for ASD, however none of these candidates account for more than 1-2% of ASD cases [7]. In addition, there is variety in the roles of the candidate genes, with no established archetype; this makes it very difficult to find a set cause and therapy for ASD. One candidate risk factor is *CNTNAP2*, from the neurexins superfamily, which has variants that can increase the likeliness of ASD through the alteration of transcription factor binding site [8]. Another candidate that has been found within some ASD patients is *Shank3,* which codes for a scaffolding protein located in synapses [9].

Studies have shown that *Shank3* mutations lead to altered behaviors that are similar to ASD patients, in mice [10]. Campbell, *et al.* [11] found genetic variants of *MET* to be associated with ASD; *MET* is a receptor tyrosine kinase and has roles in brain development and immune system regulation. However, studies on candidate genes so far have not been definitive due to small sample sizes, variation in phenotypes and poor controls. It may be also that non- syndromic ASD depends on certain combinations of mutations.

Transcriptomic analysis shows the post-mortem brains of ASD patients have downregulated neural genes and upregulated genes involved with an immune response [12], and perturbations in immune system of ASD patients have been reported [13-15]. Mounting evidence has found elevated levels of reactive autoantibodies (anti-GFAP (glial fibrillary glycoprotein), anti-MBP (myelin basic protein), anti-MOG and anti-NAFP (neuron axon filament protein)) in ASD patients compared to unaffected patients [16-19]. Although, there is still some disagreement with some studies suggesting that there is no evidence of autoimmune response in ASD [20].

Streptococcal (Group A β-haemolytic streptococcal [GABHS]) infection can induce autoimmune disease in humans. Pediatrics Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS) are a group of disorders caused by streptococcal infections in children and play a role in the onset of tick disorders and OCD [21]. PANDAS are associated with a group of antibodies, which are elevated during disease symptoms and may signal neuronal cells in the brain. The hypothesis is that autoantibodies produced in response to a streptococcal antigen allow cytokines

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activated in the periphery to cross the Blood Brain Barrier (BBB), and along with intrathecal antibodies (antibodies in the central nervous system) produced by peripheral B-cells, react with neural structures [22]. Two common antibodies, Antideoxyribonuclease titer-B and Antistreptolysin O titer-ASO act as described above by crossing the BBB where they cross- react with the vulnerable basal ganglia [23]. When damaged, the basal ganglia region of the brain is associated with disorders such as obsessive compulsive disorder, chorea, and the typical Tic's associated with Tourette's syndrome (TS) [24]. The similarities between PANDAS and some of the disorders that are within the ASD bracket, may suggest a similar etiology [25,26]. In literature TS and Obsessive Compulsive Disorder (OCD) have been linked to ASD as 'co morbid' conditions [25]. There is evidence linking childhood streptococcal infections to TS and OCD, however there is very little evidence confirming the exact link if any between childhood infections, and the development of ASD. The current idea, as depicted in Figure 1, is that combinations of autoantibody and cytokine actions cross talk with the NS, which leads to changes in the areas of the brain controlling behaviors [27]. It seems practical to believe that there is genetic susceptibility in individuals and ASD is triggered in them by an environmental/immunity factor such as infection.

Although the current rationale is in support of an autoimmune role in ASD, there is little work done to understand the mechanism behind it. Therefore, the aim of this study was to investigate the roles of streptococcal-autoantibody targets and to try to find a functional link between them and a network of genes suggestively involved in ASD.

Methods

Data mining

Online database 'Scopus' was used for literature mining. Search terms autism, autoimmune, childhood and streptococcus were used to produce an initial list of references. This was reduced to 9 by excluding papers that were not relevant, inaccessible or not in English (Figure 2). The

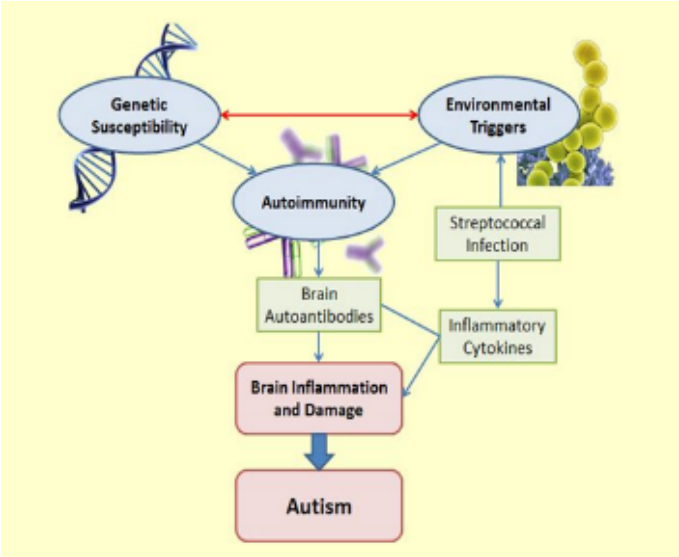

Figure 1. An overview of one hypothesis for the onset of autism. Blue circles indicate key factors thought to be involved in the onset of the disease. Genetic susceptibility and environmental triggers are thought to have an interactive relationship (indicated by red arrow). A genetic susceptibility may mean that individuals are highly reactive to environmental triggers; environmental triggers may lead to a change in gene expression that may render an individual susceptible. Both environmental and genetic factors have a role in the onset of autoimmunity. Streptococcal infections are hypothesised to trigger autoimmune-onset autism

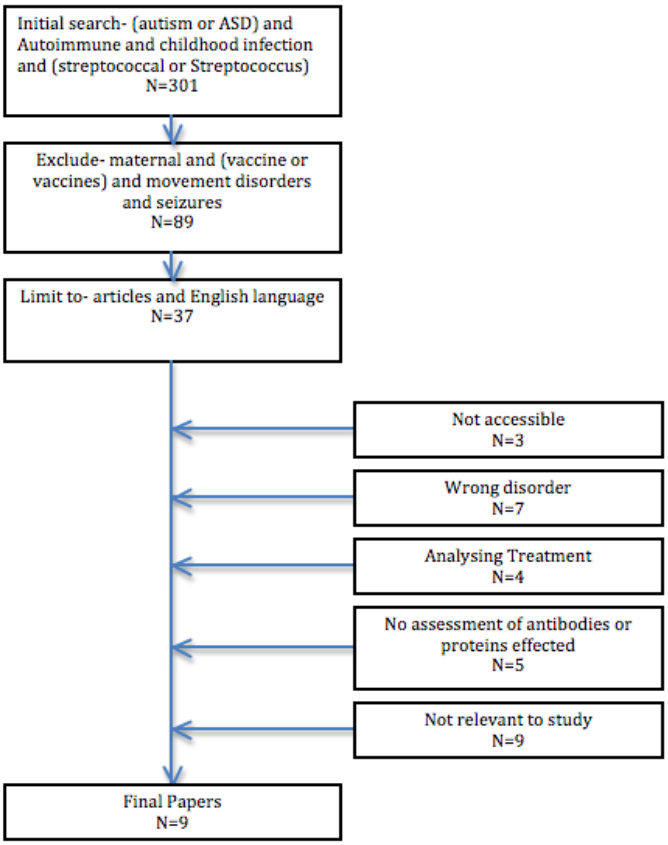

Figure 2. The order of a literature data mining on psychiatric disorders associated with autoimmunity

names (IDs) of autoantibodies that are found to be higher in autistic participants compared to the control groups were used in a further search to determine if they are produced in streptococcal infection.

Once this was confirmed the further search to identify targets of these antibodies was performed and a final list compiled (Supplementary table 1). This data was used in a reconstruction of functional networks linking the antibodies targets to genes relevant to autism (Supplementary table 2). The genes were chosen via GWAS studies by the correlation of relevant SNPs to autistic phenotype (genes) [28-31].

Reconstruction of functional networks

String software (http://string-db.org/) was used to reconstruct functional networks of the proteins identified in the above data mining process. STRING is a database of known and predicted protein interactions including direct (physical) and indirect (functional) associations derived from four sources; Genomic Context, High Throughput Experiments, Coexpression and Previous Knowledge. White nodes can be added by the software to link proteins that are inputted by the user. Functional network analysis was also performed using Ingenuity Systems (www.ingenuity.com) Ingenuity Pathway Analysis (IPA), where connections of inputted list of genes were established automatically on the basis of the Ingenuity knowledge base-database data. And a customised number of the maximum number of the linked nodes the linked nodes was set to 70.

GO enrichment analysis

Two methods were used for GO (Gene Ontology) enrichment analysis. The Database for Annotation, Visualization and Integrated

Discovery (DAVID) version 6.8 (https://david- d.ncifcrf.gov/) was used for GO enrichment analysis. DAVID uses a modified Fisher Exact P value, EASE score to measure gene annotation. String software (http://string-db.org) 'Analysis' section was used for enrichment analysis (biological functions and cellular component) of a reconstructed STRING network context.

Gene expression analysis

Genevestigator was used to analyze microarray expression meta-data for the selected genes. Both ASD-associated and streptococcal infection-associated genes were analyzed using data from the "mRNA-Seq gene level Homo sapiens" platform, with the default organism being Homo sapiens. The tissue location of expression for the individual genes was found using the 'Anatomy' tool. Expression levels were scored based on their mean score in related units in a scale between the lowest and the highest expression levels of all genes in a particular tissue, mean scores of ≥6, showed high levels of expression

Results

Our search (Figure 2) lead to 9 research articles, which described a process of autoimmunity, related to Autism, TS or OCD. The result of the search is shown in Table 1. Of the 9 papers, 6 were related to Autism, 2 to TS and one to OCD. The autoimmune antibodies that were mentioned as statistically significant to the above disease were CD19, CD8/17, Anti- Dipeptidylpeptidase IV (Anti-DDP IV), Anti-Dipeptidylpeptidase I (Anti-DDP I), Anti- Aminopeptidase N, Anti-Gliadin and Heat Shock Protein-60 peptide, Anti-Ganglioside M1, Anti-HK-I, Anti-VDAC, Anti-Nuclear and Anti-Myelin Basic Protein (Anti-MBP), Anti- Myelin Associated Glycoprotein (Anti-MAG) and TNF [1,32].

Functional interactions of autoantibodies related to Streptococcal Infection

From these papers only 5 linked streptococcus infections to autoantibodies. The antibodies that were linked to Streptococcus infection were CD19 positive B cells, monoclonal antibody D8/17, Anti-DDP IV, Anti-DDPI, Anti-Aminopeptidase N, Anti-MBP, Heat shock protein-60 peptide and Anti-MAG. The remaining papers did not provide evidence that streptococcal infection caused the production of the autoantibodies.

Through the further literature mining, the target proteins of the autoantibodies where identified; these proteins and the genes that code for them are shown in Table 2.

The nodes for inputted list of genes, CD19, CHST5, DDP4, ANPEP, MBP, HSPD1 and MAG, but CTSC, where connected with

Table 1. Main results of the literature data mining on psychiatric disorders associated with autoimmunity

Paper Title	Author	Auto Antibodies	Disease Studied
D8/17 and CD19 Expression on Lymphocytes of Patients with Acute Rheumatic Fever and Tourette's Disorder	Weisz, *et al.* 2004 [33]	CD19-positive B cells	TS
Progress Toward Analysis of D8/17 Binding to B cells in Children with OCD and/or Chronic Tic Disorder	Murphy and Pichichero 2002 [26]	Monoclonal antibody D8/17	OCD/ Tic Disorders
B Lymphocyte Antigen D8/17 and Repetitive Behaviours in Autism	Hollander, *et al*. 1999 [34]	D8/17-positive cells	Autism
Heat Shock Protein and Gliadin Peptide Promote Development of Peptidase Antibodies in Children with Autism and Patients with Autoimmune Disease	Vojdani, *et al*. 2004 [35]	Anti-DPP IV Anti-DPP I Anti-Aminopeptidase N, Antigliadin Anti-HSP-60	Autism
Increased Serum Levels of Anti-Ganglioside M1 Auto-Antibodies in Autistic Children: Relation to the Disease Severity	Mostafa and AL-Ayadhi, 2011 [36]	Anti-Ganglioside M1	Autism
Serum Anti-Nuclear Antibodies as a Marker of Autoimmunity in Egyptian Autistic Children	Mostafa and Kitchener, 2009 [37]	Anti-Nuclear	Autism
Serum Anti-Myelin - Associated Glycoprotein Antibodies in Egyptian Autistic Children	Mostafa *et al*, 2008 [19]	Anti-MBP Anti-MAG	Autism
Association of the Tumour Necrosis Factor -308 A/G Promoter Polymorphism with Tourette Syndrome	Keszler *et al*, 2014 [38]	TNF -308 G-allele	TS
Antibodies Against the Voltage-Dependent Anion Channel (VDAC) and its Protective Ligand Hexokinase-I in Children with Autism	Gonzalez-Gronow *et al*, 2010 [39]	Anti-HK-I Anti-VDAC	Autism

Table 2. Autoantibodies associated with paediatric Streptococcal infection and their targets.

Paper Title	Author	Auto Antibodies	Target Protein
D8/17 and CD19 expression on lymphocytes of patients with acute rheumatic fever and Tourette's disorder	Weisz *et al*, 2004 [33]	CD19-Positive B cells	Cluster Differentiation 19 (CD19)
Murine Anti-vaccinia Virus D8 Antibodies Target Different Epitopes and Differ in Their Ability to Block D8 Binding to CSE	Matho *et al*, 2014 [40]	Monoclonal Antibody D8/17	Carbohydrate Sulfotransferase 15 (CHST15)
Dipeptidyl peptidase-4 (CD26): knowing the function before inhibiting the enzyme	Matteucci *et al*, 2009 [41]	Anti-DDP IV	Adenosine Deaminase Complexing Protein 2, (DDP4)
The Primary Structure and Tissue Distribution of Cathepsin	Kominami *et al*, 1992 [42]	Anti-DDP I	Cathepsin C (CTSC)
Contemporary challenges in autoimmunity	Shoenfeld and Gershwin, 2009 [43]	Anti-Aminopeptidase N	Alanyl Aminopeptidase (ANPEP)
Antibodies to myelin basic protein in children with autistic behavior.	Singh *et al*, 1993 [16]	Anti-MBP	Myelin Basic Protein (MBP)
Heat Shock Protein and Gliadin Peptide Promote Development of Peptidase Antibodies in Children with Autism and Patients with Autoimmune Disease	Vojdani *et al*, 2004 [35]	Anti-HSP-60	Heat Shock 60kD Protein 1 (HSPD1)
The myelin-associated glycoprotein gene: mapping to human chromosome 19 and mouse chromosome 7 and expression in quivering mice.	Barton *et al*, 1987 [44]	Anti-MAG	Myelin Associated Glycoprotein (MAG)

medium confidence (0.4) in STRING with allowed 14 additional nodes automatically added by the software to increase a network connectivity. When the network map was enriched by tissue specificity (medium confidence- 0.400, high-0.700), 8 of the nodes where linked to central nervous system and brain (Table 3). The proteins linked by biological processes are shown in Table 4. Worth mentioning here, that one protein added by string to increase network connectivity, PIK3R1, had the molecular function of neurotrophin receptor binding.

The interactions with the know autism associated genes are shown in Figure 3. One can see that there are several most connected nodes in the network, namely: GRB2 (in the centre of the network, added as a white node), SHH, PIK3R1 and HDAC2 (which are proteins from the know autism associated genes). The analysis also shows that MBP is the main antigen that is associated with the know autism associated genes in the network map and the most of CNS GO terms.

Majority of network links found within the data were green, indicating text-mining associations. ADA, APP, CUL1, FHIT, GRPEL1 and ICT1 were ASD genes that were directly associated to the streptococcal infection gene set experimentally, which may be deemed more substantial than associations by textmining. Interestingly, although some of the genes had indirect associations through homologs. GRPEL1 has a homolog that is in the same gene neighbourhood as HSPD1. ADA, ABCC1, and that are co-expressed with genes triggered by streptococcal infection.

Gene expression analysis

Table 5 shows the genes from both gene sets that were highly expressed in the nervous system (NS) and immune system (IS). Interestingly for both groups, there were genes with high expression in each of these anatomic locations. Of note, APP, BCL11A and KLC1 from the ASD gene set were highly expressed in both the NS and IS. For the streptococcal infection gene set, some of the primary genes were highly expressed in NS or IS. HSPD1, MBP, GRB2 and HSP90AA1 were highly expressed in both anatomic regions.

Table 6 shows the top 10 general biological processes for the streptococcal-associated gene list. Interestingly, multiple NS pathways are associated with the streptococcal infection gene. See (Table 7), molecules of note that reoccur in different pathways (both NS and IS) include the primary streptococcal associated genes: HSPD1, MAG, MBP, and the secondary streptococcal infection- associated genes: FGFR1, FGFR2, GRB2, HSP90AA1 and LYN.

Pathway analysis

Neural-associated diseases and functions for the streptococcal-linked gene list indicated a function in neural development (Table 8).

The listed genes are essential for normal brain formation as seen with links to familial holoprosencephaly, formation of brain cells and

Table 3. Brain function-related ontologies of streptococcal infection-associated targets of autoantibodies

Disease	Nodes	Proteins	Confidence
CNS Disease	2	MBP,HSPD1	High
Autoimmune Disease of the CNS	1	MBP	High
Brain Disease	1	HSPD1	High
Autoimmune Disease of Gastrointestinal Tract	1	CD79A	Med
Disease of Mental Health	2	SYK, CASP3	Low
ASD	1	MBP	Very Low

Table 4. Enrichment of biological processes categories in the immediate proximal network reconstructed for proteins-targets of infection-induced autoantibodies

Process	Nodes	P-value	Proteins
Positive Regulation of Developmental Process	6	0.01969	IFITM1, BTK, SYK, LYN, ADA, CCL11
Negative Regulation of Developmental Process	5	0.03849	PIK3R1, LYN, MAPK1 MBP, MAG
Organ Development	9	0.0596	CR2, PIK3R1, SYK, MBP, MAG, MAPK1, CD79A, ADA, CCL11
Substantia Nigra Development	2	0.08279	MAG,MBP
Subthalamus Development	2	0.08840	MAG, MBP
Neural Nucleus Development	2	0.1419	MAG, MBP
Generation of Neurons	5	0.2899	MAG, MBP, MAPK1,CASP3, HSP90AA1
Neuron Differentiation	3	0.2959	MAG, MBP, LYN
Diencephalon Development	2	0.3150	MAG, MBP
Neurogenesis	5	0.3469	MAG, MBP, MAPK1, CASP3, HSP90AA1
Nervous System Development	5	1	MAG, MBP, MAPK1, CASP3, HSP90AA1
CNS Development	3	1	MAG,LYN MBP
Forebrain development	2	1	MAG, MBP

Table 5. ASD and streptococcal associated genes that are highly expressed (mean score ≥6) in the nervous or immune system according to mRNAseq data

Gene list	Anatomic location	Genes
ASD	Nervous system	APP*, BCL11A*, CACNAI1I CADPS, CCDC64, CDH22, CNTNAP2, CTNNA2, DMD, DNER, DTNB, EPN2, FAM171A1, FEZF2, FGF1, FHIT, GDAP1, GFAP, GRIN1, KLC1*, MAST3, MFSD6, MYO5A, NDFIP1, NTM, PSD3, PTPRN2, RBFOX1, SH3GL3
ASD	Immune system	APP*, ATXN2L, BCL11A*, CDC37, CSDE1, DYNLT1, ETS1, FLI1, IL17RA, KLC1*, LCP2, MSN, NDRG1, PTPRE, RASGRP4, SEMA4D, SLTM, SP110, TBXAS1, TYK2
Streptococcal	Nervous system	HSPD1[1]*, MAG, MBP*, FGFR2, GRB2*, HSP90AA1*, PIK3R1
Streptococcal	Immune System	ANPEP, CHST15, CTSC, HSPD1*, MBP*, CD79A, GRB2*, HSP90AA1*, LYN, MEFV

*indicates genes that are highly expressed in both the nervous system and immune system within their own gene list [1]underlined font indicates primary streptococcal-associated genes

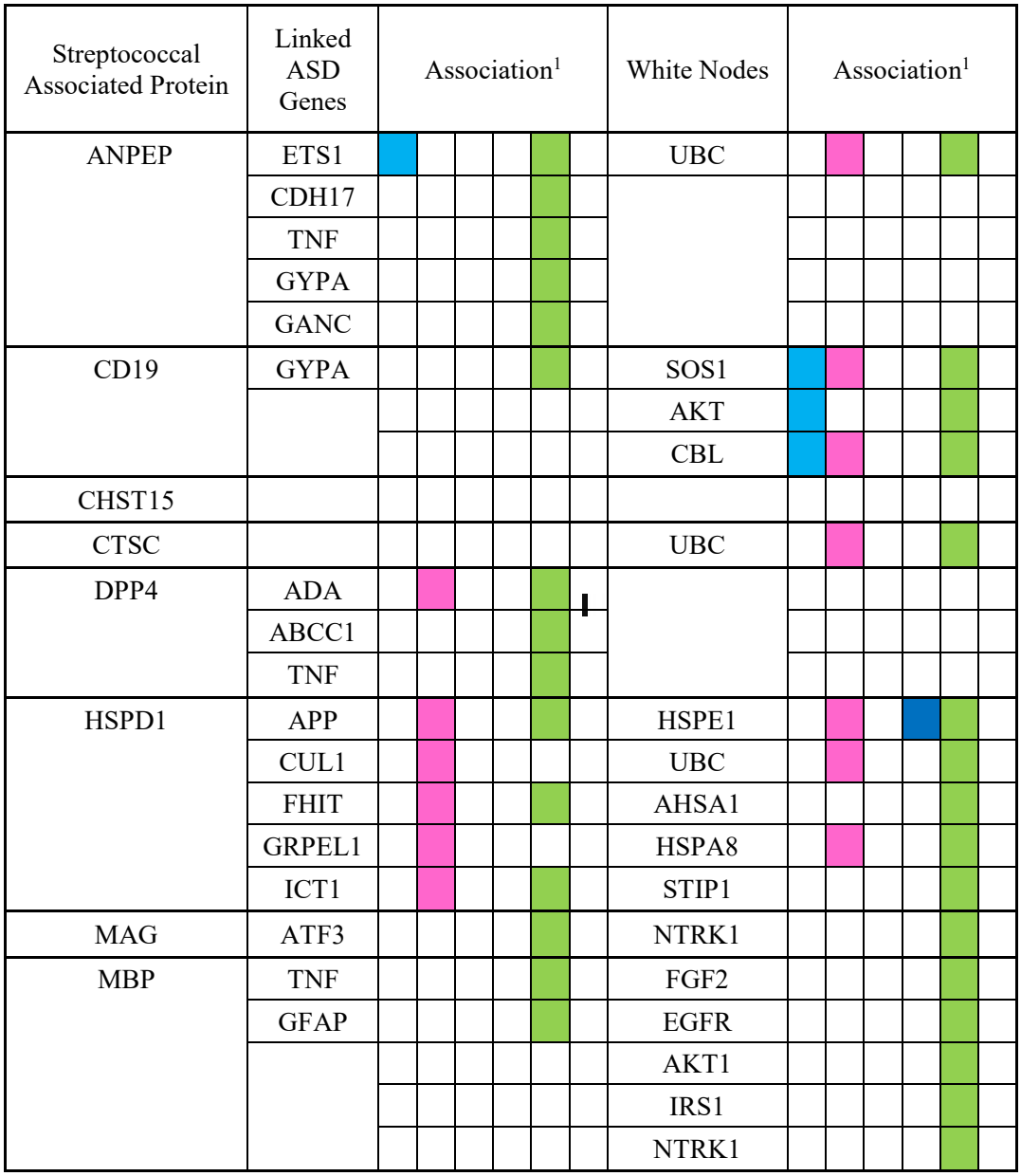

Figure 3. The different evidence found between the primary streptococcal-associated genes and ASD-associated genes at medium confidence (0.4). [1]The different coloured boxes indicate different evidence; light blue = associated in a curated database, dark green = gene neighbourhood, dark blue = gene co-occurrence, light green = text mining and black = coexpression. Striped boxes indicate that although the gene itself has no association, its homologous proteins do

Table 6. Top 10 general biological processes found from functional enrichment within the *Streptococcal* gene network using STRING

Pathway description	Molecules[1]	False discovery rate[2]
Neurotrophin TRK receptor signalling pathway	FGFR1, FGFR2, HDAC2, CASP3, MAG, CD19, GRB2, PIK3R1	1.25E-05
Fc receptor signalling pathway	FGFR1, FGFR2, PIK3R1, LYN, GRB2, HSP90AA1, CD19	5.14E-05
Regulation of T cell activation	DPP4, SHH, PIK3R1, LYN, GRB2, CASP3, HSPD1	5.95E-05
Cell morphogenesis involved in differentiation	CASP3, HSP90AA1, FGFR1, LYN,GRB2, ST8SIA2, SLITRK5, SHH, SNAI1	6.45E-05
Midbrain development	SHH, FGFR1, FGFR2, MBP, MAG	6.45E-05
Neuron projection development	HDAC2, SHH, FGFR1, FGFR2, HSP90AA1, LYN, GRB2, SLITRK5, ST8SIA2	6.45E-05
Locomotion	DPP4, ANPEP, MAG, SIX3, SHH, SNAI1, HSP90AA1, LYN, PIK3R1, GRB2, ST8SIA2	6.45E-05
Positive regulation of T cell activation	SHH, LYN, PIK3R1, GRB2, HSPD1, DPP4	6.45E-05
Fc-epsilon receptor signalling pathway	CD19, LYN, FGFR1, FGFR2, PIK3R1, GRB2	6.60E-05
Axonogenesis	SHH, FGFR1, FGFR2, HSP90AA1, LYN, GRB2, ST8SIA2, SLITRK5	8.48E-05

[1]Primary streptococcal genes are underlined.
[2]The significance is expressed as a p-value, where significance is $p \leq 0.05$.

Table 7. Top 10 neural specific biological processes found from functional enrichment within the *Streptococcal* gene network using STRING

Pathway description	Molecules[1]	False discovery rate[2]
Neurotrophin TRK receptor signalling pathway	FGFR1, FGFR2, HDAC2, CASP3, MAG, CD19, GRB2	1.25E-05
Midbrain development	SHH, FGFR1, FGFR2, MBP, MAG	6.45E-05
Neuron projection development	HDAC2, SHH, FGFR1, FGFR2, HSP90AA1, LYN, GRB2, SLITRK5, ST8SIA2	6.45E-05
Axonogenesis	SHH, FGFR1, FGFR2, HSP90AA1, LYN, GRB2, ST8SIA2, SLITRK5	8.48E-05
Axon development	SHH, HSP90AA1, FGFR1, FGFR2, LYN, GRB2, ST8SIA2, SLITRK5	1.07E-04
Cell morphogenesis involved in neuron differentiation	SHH, HSP90AA1, FGFR1, FGFR2, LYN, GRB2, ST8SIA2, SLITRK5	1.19E-04
Positive regulation of gliogenesis	HDAC2, SHH, LYN, MAG	1.48E-04
CNS development	SHH, FGFR1, FGFR2, LYN, CASP3, MBP, MAG, MOG, SLITRK5	2.30E-04
Neural precursor cell proliferation	SIX3, SHH, FGFR1, FGFR2	5.06E-04
FGF signalling receptor pathway involved in orbitofrontal cortex development	FGFR1, FGFR2	5.33E-04

[1]Primary streptococcal genes are underlined.
[2]The significance is expressed as a p-value, where significance is $p \leq 0.05$

Table 8. The top ten brain and neural-linked diseases and functions categories detected in the immediate functional connectivity proximity of the *Streptococcal* infection-associated gene set by IPA software

Diseases or Functions Annotation	Molecules[1]	*p*-value[2]
Development of central nervous system	CASP3, EXOC4, FGFR1, FGFR2, HDAC2, MAG, MBP, MOG, SHH, SIX3, SLITRK5, ST8SIA2	1.53E-10
Neuritogenesis	CASP3, FGFR1, FGFR2, HDAC2, LYN, MAG, MBP, MOG, MYO5B, SHH, SLITRK5	1.33E-09
Development of neurons	CASP3, FGFR1, FGFR2, HDAC2, LYN, MAG, MBP, MOG, MYO5B, SHH, SLITRK5, ST8SIA2	2.45E-09
Differentiation of nervous system	EXOC4, FGFR1, FGFR2, HDAC2, MAG, SHH, SIX3, SNAI1, ST8SIA2	3.35E-08
Familial holoprosencephaly	FGFR1, SHH, SIX3	1.05E-07
Hypomyelination	FGFR1, HSPD1, MAG, MBP	3.39E-07
Formation of brain cells	FGFR1, FGFR2, SHH, ST8SIA2	8.13E-07
Abnormal morphology of central nervous system	CASP3, FGFR1, FGFR2, HDAC2, MAG, MBP, SHH, ST8SIA2	9.06E-07
Dysmyelination	CD19, FGFR1, HSPD1, MAG, MOG	1.45E-06
Transformation of mesencephalon	FGFR1, FGFR2	1.59E-06

transformation of mesencephalon. The development of the central nervous system is strongly linked to the set of infections-associated genes (with a strong p-value of 1.53E-10) (Figure 4) and their effect on dysmyelination is illustrated in Figure 5. IPA was used to construct networks of the genes associated with Streptococcal infection. The links formed are predominantly of protein-protein interactions.

Interactions were visualised to see if any of the genes have joined functional roles, and to aid in constructing a mechanism of etiology for ASD. Figure 6 shows a top populated network (IPA) for the streptococcal infection gene set with links to infection and inflammation categories, but also to multiple NS –associated diseases and functions. Of the primary streptococcal infection associated genes, CD19, HSPD1, MAG and MBP are heavily connected to other genes in the network, as well as to the associated diseases and functions. Of the secondary streptococcal infection associated molecules, BCR (complex), CASP3, PI3K, and CD79A are well connected to both molecules and functions. The network also includes nodes corresponding to molecules automatically added by the software, such as multiple immunoglobulins, which highlights the theme of antibodies. Interestingly, of these molecules added by IPA, are some of the ASD-associated genes such as, APP and IL1RN. One can see that there are several nodes in the network, highly connected to the seed genes: GRB2 (in the centre of the network, added as a white node), SHH, PIK3R1 and HDAC2 (which are proteins of genes know to be associated with ASD). The analysis also shows that MBP is the main antigen that is associated in networks with ASD-attributed functions and also matches to the most of network's CNS-relevant GO-terms.

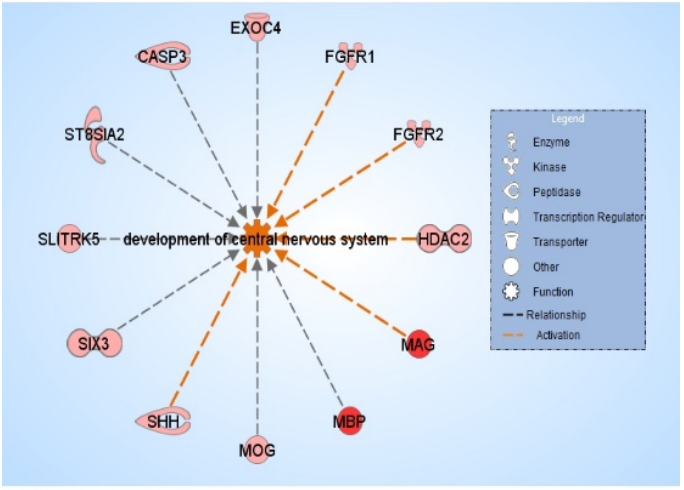

Figure 4. Visualisation of the molecule interactions for 'development of central nervous system' from Table 11, showing the streptococcal-associated genes involved. Primary streptococcal genes are indicated by red shaped whilst their directly linked genes are indicated by pink shapes. As shown in the legend orange lines indicate activation, whilst grey lines indicate a relationship that has not been fully determined.

Discussion

Roles and functions of the genes

Results from GWAS studies suggest that ASD-associated genes have a role in development and synapse maintenance, and are expressed in areas of the brain such as the frontal and temporal cortex [12,45,46].

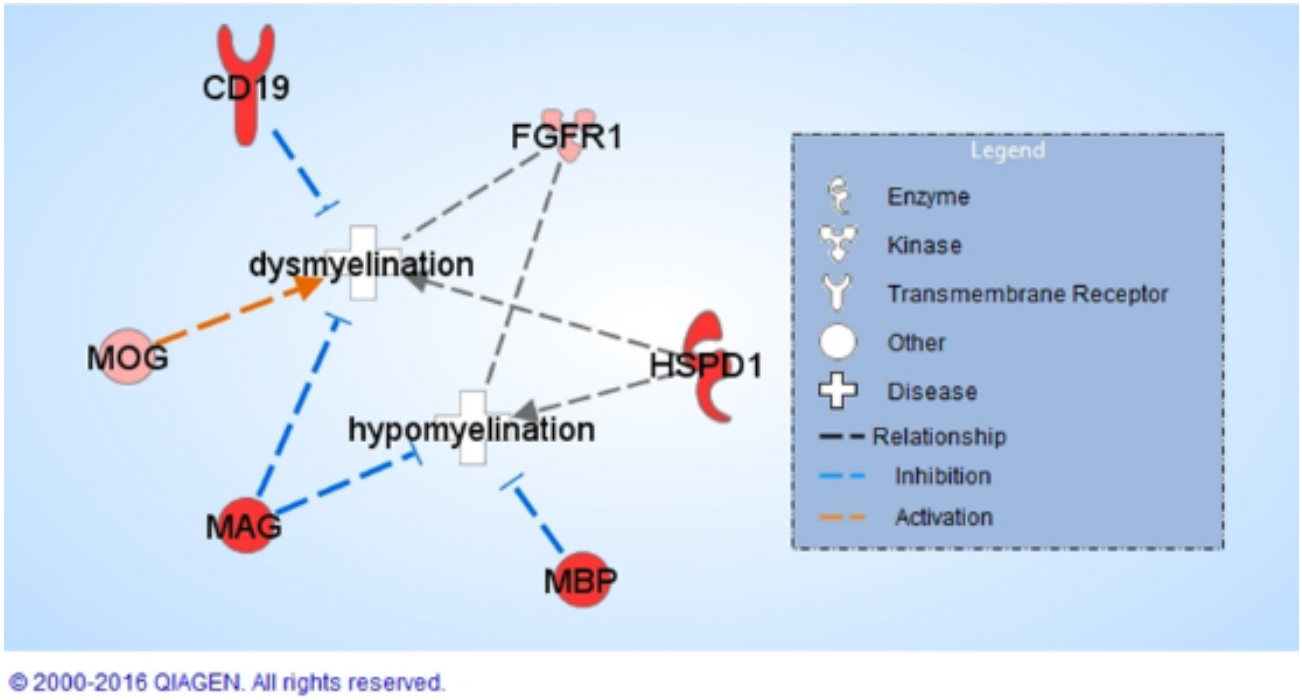

Figure 5. Visualisation of the molecule interactions for 'hypomyelination' and 'dysmyelination' from Table 8, showing the streptococcal-associated genes involved. Primary streptococcal genes are indicated by red shaped whilst secondary streptococcal genes are indicated by pink shapes. As shown in the legend orange lines indicate activation, blue lines indicate inhibition whilst grey lines indicate a relationship that has not been fully determined

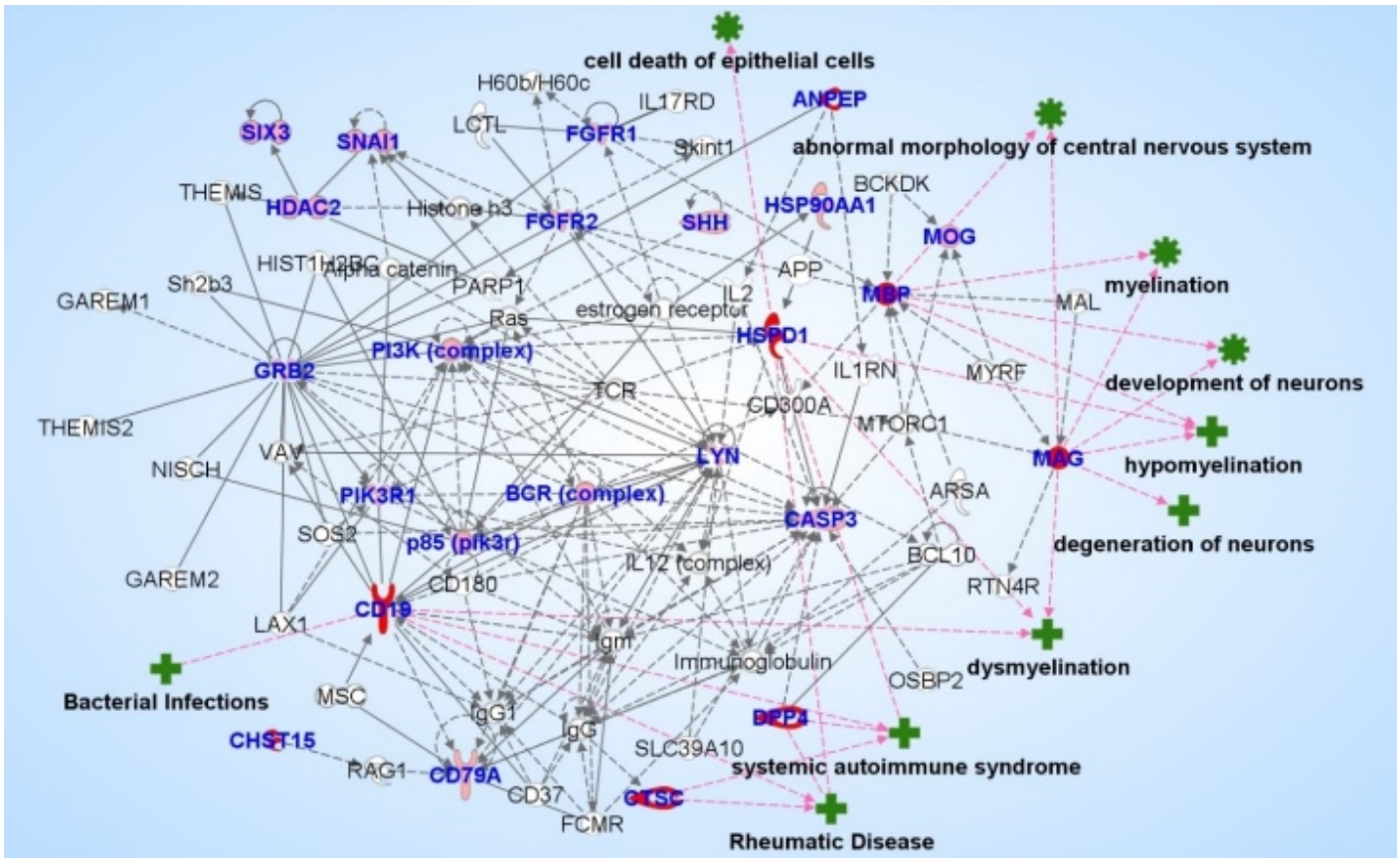

Figure 6. Network reconstructed in IPA from the streptococcal infection associated genes. All genes in lists are indicated by blue font. Shapes shaded red are for primary streptococcal genes and pink for their direct immediately connected genes. Solid lined indicate known direct interactions and dashed lines indicate indirect interactions. Diseases and functions associated with the network are linked by pink lines and are labelled in bold green font. Shapes represent molecular functions (cytokines-as for CD18, receptors-as FGFR1, proteases-as CASP3, TFs-as SIX3, other or combined functions-circles).

Interestingly, HSPD1, MBP, GRB2 and HSP90AA1 are highly expressed in both the IS and NS and may link to microglia in the brain, particularly as HSPs activate and can be expressed by microglia [47,48]. Gene enrichment analysis clearly show that the streptococcal-associated genes are linked to both the IS and NS (Table 5). The streptococcal-associated genes are well linked to biological processes such as Neurotrophin TRK receptor signalling, T cell activation, locomotion, combining both IS, and NS functions (Table 6). Of note, the genes are linked to midbrain development and CNS development (Table 7). This is interesting when we consider the paediatric onset of ASD, as these genes are likely to be active and therefore available to be targeted by streptococcal autoantibodies. The main primary streptococcal genes involved in NS development include MAG and MBP whereas most of the other genes are more associated with the IS (Table 8). MAG and MBP are also associated with abnormalities in the NS, such as Hypomyelination and Dysmyelination (Table 8 and Figure 5). These results allow us to suggest that streptococcal infections may indeed affect the brain development and function.

Genes of note

Many ASD-associated genes are particularly expressed and associated within the IS include, APP, BCL11A, KLC1, ETS1, MET, IL1RN, NDRG1, NGF, TNF and VEGF (Table 5). These genes are receptors (APP, MET, IL1RN), involved in angiogenesis (NDRG1, VEGF), cell maintenance and development (BCL11A, KLC1, NGF,) and inflammation (ETS1, TNF). APP, ETS1, TNF and VEGF may be of a particular interest. APP encodes for the amyloid beta precursor protein, which is highly expressed in neurons and glial cells [49-51]. However, APP products also have a functional role, including synaptic adhesion, neuroprotective properties and antimicrobial functions [52-54]. APP transport in the brain is mediated by subunits formed by KLC1 molecules [55]. ETS1 encodes for ETS proto-oncogene 1, a transcription factor that has roles in inflammation and chemokine and cytokine activation particularly in endothelial cells and links to reactive microglia [56,57]. ETS-1 has been shown to co-localise with VEGF, TNF and APP products in the brain [57]. Vascular endothelial growth factors (VEGF) are a family associated with endothelial cell regulation and angiogenesis, even influencing the blood-brain barrier (BBB) [58,59]. Notably, VEGF appears to be able to alter the permeability of the BBB, and can increase permeability and cause breakdown [59-61]. Tumour necrosis factor (TNF) is a proinflammatory cytokine and is implicated in numerous functions and pathologies. TNF is implicated in inflammation and can affect the permeability of the BBB [62]. Of the streptococcal-associated genes, autoantibody targets MAG and MBP are the most prominent. MAG encodes for myelin-associated glycoprotein, a membrane protein that inhibits nerve regeneration [63]. MBP encodes for myelin basic protein, which is a major protein of the myelin sheath and has a regulatory role in myelination [64].

Potential mechanism

Our vision of how streptococcal infections can cause ASD in children is presented in Figure 7. Alterations in ETS1, TNF or VEGF functions, or their combination can decrease the permeability of the BBB and leave an individual vulnerable to toxic or autoimmune components developed due to an infection. Mutations may not act directly upon these genes but may affect their expression and protein proliferation [65,66]. This may be a result of mutations in promoter regions or polymorphisms in cytokines (TNF) or may be an upstream molecule [67-69]. VEGF and ETS1 have been associated with TNF in Alzheimer's disease [57]; therefore, we can conceive that they work functionally

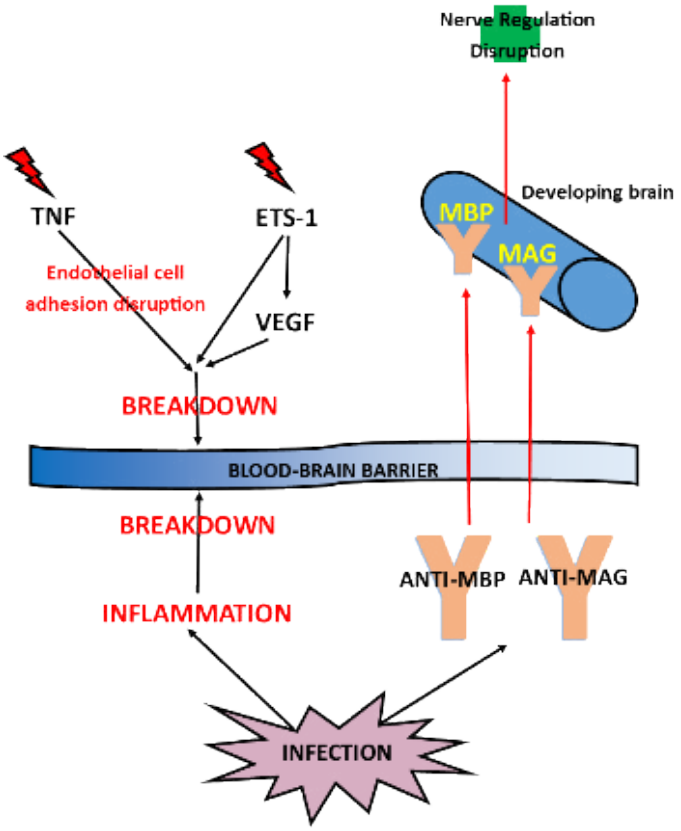

Figure 7. A proposed hypothesis for paediatric streptococcal-induced ASD, produced from the results of this study. Red bolts indicate a possible mutation affecting the gene either directly or upstream, red text indicates a negative effect, orange Y-shapes indicate antibodies and the green cross indicates pathology.

together in the brain. ETS1 and VEGF can induce microglial activity towards increased TNF production [57,70-72]. Additionally VEGF expression in astrocytes, a component of the BBB, can induce the BBB breakdown [73], supported by a streptococcal- induced inflammation. Streptococcal infections produce autoantibodies against MAG and MBP. With a weakened BBB, anti-MAG and anti-MBP can travel across and target developing neurones for destruction by immune cells able to cross the weakened BBB. In fact, thinner myelination has been reported in some areas of the brain in ASD, such as the orbitofrontal cortex (OFC), as well as a high density of thinly myelinated neurons when compared to controls [74]. OFC abnormalities have already been associated with ASD and it is speculated that a decrease in myelin may mean that longer axons that provide the cross talk for emotion-based behaviors are less efficient [75,76]. From the other hand, neurite overgrowth found in ASD patients [77,78] may be explained by MAG (inhibitor of neurite regrowth) immuno- inhibition.

Conclusions

From published and our study it is clear that there is overlap within the IS and NS functions that may be responsible for synergism in inheritable and environmental components in aetiology of a number of neuro-psychiatric disorder, and ASD, in particular. APP, BCL11A, KLC1, ETS1, MET, IL1RN, NDRG1, NGF, TNF, VEGF, HSPD1, MAG and MBP proteins are likely candidates to affect susceptibility to the environmental component. What is notable is that many of these genes were identified to have roles in the developing brain, reflecting the paediatric nature. Despite a proposed theory (Figure 7), uncertainties

remain as to how some of these genes may interact to affect ASD. We suggest that APP, HSPD1, MET and NDRG1 may be implicated in the reaction caused by the autoantibodies. Those would be ideal candidates to investigate further using experimental methods in animal models or to be tested for in cases of PANDAS or more profound ASD cases in children.

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Conflicts of interests

The authors do not have any conflicting interests relevant to this study.

Author's contributions

OV has designed and supervised the study, MH and JS made equal contributions to data generation, all authors played equal roles in the data analysis and drafting of the manuscript.

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