

# Autism and the gut - brain axis.

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## A U T I S M

*Persons with autism may possess the following characteristics in various combinations and in varying degrees of severity.*



Inappropriate laughing or giggling



No real fear of dangers



Apparent insensitivity to pain



May not want cuddling



Sustained unusual or repetitive play; Uneven physical or verbal skills



May avoid eye contact



May prefer to be alone



Difficulty in expressing needs; May use gestures



Inappropriate attachments to objects



Insistence on sameness



Echoes words or phrases



Inappropriate response or no response to sound



Spins objects or self



Difficulty in interacting with others

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## Summary.

Autism is a disorder of neural development for which modern medicine has no cure. As many autism subjects also suffer from Gastro-Intestinal (GI) problems, gut-brain communication may be part of the underlying etiology in autism. However, autism, or in its broader sense Autism Spectrum Disorder (ASD), is a multifactorial disorder. Genetics, immunology, hormonal and environmental effects all have proven to be relevant. Brain areas involved in autism consistently show morphological aberrations. This indicates that autism is not a dysfunction of an otherwise normal brain. Many autism subjects however seem to benefit from dietary intervention though this is hard to prove in a statistically rigorous manner. It is concluded that, although the gut-brain axis does influence functioning of the autism brain in some ways, it is not its main etiology. As it presently is the easiest accessible route available for intervention, it is worthwhile however to investigate the gut-brain axis' relation with autism further.

## Introduction.

There is much current research devoted to information exchange between the gut and the brain in humans. The question has arisen whether “functional foods” like probiotics may improve mental function or decrease malfunction. This matter is of high economic interest to food companies that seek a positive label to attach to their products. As more and more is known about detrimental effects of food additives, hormones, preservatives and artificial flavours (“E-numbers”) concern has arisen for possible harmful effects on health in general and brain function in particular (e.g. ADHD, autism).

In the field of human mental dysfunction Autistic Spectrum Disorder (ASD) receives a lot of interest. Some evidence hints towards an increase in ASD in western human society. Modern medicine does not offer any cure. In search for therapy two functional diets stand out: the Gluten Free Casein Free Diet (GFCF) and the Specific Carbohydrate Diet (SCD). Both diets appear to score positive results (Gotschall 2004, Mulloy 2009).

Is there really an environmentally induced increase in the ASD’s and does manipulation of the gut-brain axis provide an effective means to improve mental function in ASD individuals? Investigation of the recent literature on the probable causes of ASD does indicate towards a gut-brain axis component, but this is only part of the story.

## Autism.

Autism, first described by Leo Kanner in 1943, is characterized by a triade of human mental dysfunction:

- qualitative impairment in reciprocal social interaction,
- qualitative delays in early language and communication,
- presence of repetitive stereotyped behaviour and/or restricted interests.

These dysfunctions usually appear before the age of three and persist for life. They are often co-morbid with intellectual impairment, seizures and anxiety. There is no known cure. Approximately 30 % of ASD subjects suffer from seizures (Bosl 2011). Allergy prevalence is higher in ASD subjects than in control groups (Angelidou 2011, Theoharides 2009). Gastro-intestinal disorders may also be more common in ASD individuals but this is not certain (Ibrahim 2009). Diagnosis presents special challenges in individuals that have difficulties in communication or have other problematic behaviour.

Autism has a strong genetic basis but may it also be induced by environmental effects? It is likely that autism is not a single type of disease with a single cause. Hence it is useful to speak of Autism Spectrum Disorders. Besides autistic disorder ASD also comprises Asperger syndrome, as first described by Hans Asperger (1906-1980), which is a high-functioning form of autism that lacks retardation in cognitive development, and Pervasive Development Disorder-Not Otherwise Specified (PDD-NOS) which is diagnosed when the full criteria for autism or Asperger syndrome are not met (DSM-IV-TR, 2000). The definition of autism that is mostly used is found in the Diagnostic and Statistical Manual of Mental Disorders version IV Text Revised (DSM-IV-TR) as published in 2000. The fifth edition (DSM-V) is currently in consultation, planning and preparation, and is due for publication in May 2013. This will result in a change of definition, the most striking one being the deletion of the 3<sup>rd</sup> year age limit. See Appendix 1 for actual DSM-IV-TR and proposed DSM-V definitions of autism.

There is a peculiar dichotomy in the onset of autism. Some children show autistic behaviour within the first year of life. Others (25-40 %, Amaral and colleagues 2008) initially show normal behaviour and development until 18-20 months of age after which they suddenly regress. This may often involve complete loss of speech and social skills. Children that have regressed are subsequently indistinguishable from cases of early-onset autism (Stefanatos 2008).

The prevalence of autism is about 1-2 persons per 1000, for ASD it is 6 per 1000, with significantly more males than females affected (Fombonne 2005). This gender bias may be in the order of a factor 4 to 10, depending on the definition used. Concordance rates are high (60% in monozygotic twins) making autism one of the most heritable neuropsychiatric disorders. Modern times, as from 1990 on, have seen a dramatic increase in the number of people diagnosed with autism. This may however be (partly) due to diagnostic practice and increased healthcare, which results in more cases of autism being reported which previously went unnoticed. (Fombonne 2005). Study of epidemiology of autism is severely hampered by the difference in diagnostic criteria and diagnostic practice used. The introduction of DSM-V will again add complication to this matter.

Lange and colleagues (2010) devised a fMRI protocol named “ Atypical Diffusion Tensor Imaging” that investigates white matter structure in the Superior Temporal Gyrus and Temporal Stem. Both these areas (in the temporal lobe) are involved in language, emotion and social cognition, the problem arena in ASD. This procedure supplies a highly specific and reliable tool to assist in autism diagnosis. However, it does require a state-of-the-art and hence expensive MRI scanner, and performing fMRI is difficult on ASD subjects. An EEG is easier to obtain, recently Bosl and colleagues (2011) have designed software to predict autism in 6 month old subjects.

### **Morphology of the ASD brain.**

Extensive research has been performed on the morphology of the autistic brain, not only to investigate the cause of autism but also in order to provide a reliable and reproducible means of diagnosis. Most research is aimed at the specific brain areas

known to be involved in higher mental functions such as social skills, language and face recognition. Post mortem tissue of ASD individuals is available in a tissue databank of the US Autism Tissue Program. Researchers may receive tissue samples on request. Several morphological changes have been found in autistic brains, ranging from the smallest to the largest scale of structure:

-Hutsler and Zhan (2010) report increased dendritic spine densities in ASD subjects, predominantly of pyramidal cells in layer II of all cortical areas and layer V of the temporal lobe areas. Dendritic spines are important in brain plasticity. The number of dendritic spines increases during certain periods in lifetime, to make more neuronal connections thus extending neural networks. In later periods the number of dendritic spines is reduced, but some neural plasticity remains even during adulthood.

Note: Gerald M. Edelman introduced the concept of Neuronal Darwinism in 1978. According to his population theory of the nervous system, neurons and synapses compete and are selected according to their relevance for prevailing signals. Thus networks may be reinforced or reduced, providing a secondary developmental process overlying the genetically laid down morphology of brain systems. This concept has gained considerable support, see Edelman 1993 for a review.

Note: Recently, neuronal plasticity has been related to the mTOR signalling pathway (see figure 1). Mammalian Target of Rapamycin (mTOR) is a protein kinase that is the central component in two protein complexes (mTORC1 and mTORC2). mTOR senses a cell's state and regulates translation of large clusters of genes, amongst which are several genes involved in neuronal plasticity. Disregulation of mTOR signalling appears to be common in human neurological disorders, including autism (Hoeffler and Klann 2009).

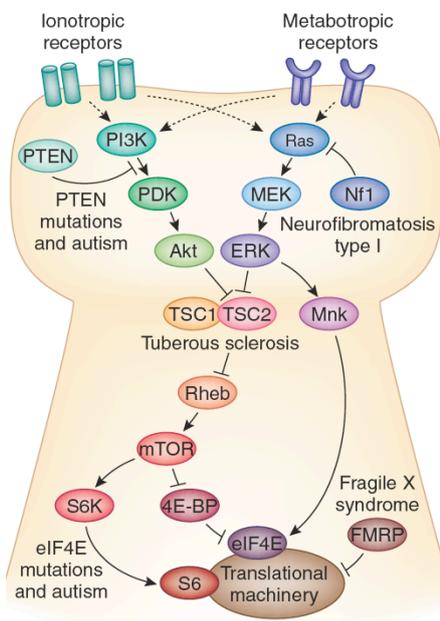


Figure 1. Various autism-related mutations affect genes encoding signaling molecules that link cell surface receptors with the protein synthesis machinery. This simplified schematic illustrates autism-associated translational regulatory pathways. PI3K, phosphoinositide-3-kinase; PDK, phosphoinositide-dependent protein kinase; Akt, serine/threonine protein kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; Nf1, neurofibromin; Mnk, mitogen-activated protein kinase-integrating kinase; Rheb, Ras homologue enriched in brain; S6K, S6 kinase; S6, ribosomal protein S6; eIF4E, eukaryotic translation initiation factor 4E; 4E-BP, eIF4E binding protein.

-Casanova and colleagues (2006) have discovered that there is perturbation of the fundamental mini-columnar organisation in the ASD brain. In human cortex this columnar organisation is an essential feature of neuronal organisation, columns forming in effect the “pixels” of cortical maps.

-Meguid and colleagues (2010) reported altered cortical thickness in bilateral medial prefrontal cortex and left anterior cingulate cortex in ASD subjects. The cortex appeared to be thicker but contained less cells in total. This could imply less processing capacity.

-Hadjikhani and colleagues (2005) report cortical thinning and local decrease in grey matter in cortical areas belonging to the mirror-neuron system. The mirror-neuron system is supposed to be required for anticipating another person’s actions and perceptions, processes that are apparently comprised in ASD subjects (They score badly in Theory of Mind Tests). The mirror-neuron system may be more of a functional concept than a physical/morphological one. Cortical thinning however was also evident in areas involved in emotion recognition and social cognition.

-Amarall and colleagues (2008) point at frontal lobes, amygdala and cerebellum as pathological in autism. Gray matter to white matter ratio is slightly different. Total brain volume is smaller at birth but starts to increase at age approximately 12 months. At 4 years of age this results in 5 to 10 % enlargement of the brain.

-Herbert and colleagues (2004) found white matter enlargement to be largely responsible for increase in brain volume in autism. This increase in white matter occurs postnatally. This implicates that possibly environmental agents influence the temporally modulated process of myelination, related to signal travel between brain areas. Signal travel between brain areas is likely to be involved in cross-modal information processing.

-Hoppenbrouwers and colleagues (2008) state that cerebellar degeneration is the most consistent neuro-anatomical abnormality found in ASD brains. The number of Purkinje cells (gray matter) is reduced, white matter is increased. The cerebellum, though better known as a center for motion coordination, has an important role in affect and emotion (Schmahmann 2000).

### **Functionality of the ASD brain.**

The amygdala plays a central role in social perception and is also involved in the first stage of face recognition, which is processed further in the fusiform face area (ventral temporal lobe). fMRI studies have shown under-activation of both these structures in subjects with ASD (Schultz 2005). Van Kooten and colleagues (2008) showed this to correlate with reduction of neuron volume and density in layers III, IV and VI of the fusiform gyrus, which could not be found in primary visual cortex or any other cortical area of the same subject.

Gervais and colleagues (2004) provided fMRI data indicating that individuals with autism failed to activate Superior Temporal Sulcus regions that are dedicated to vocal

sound receiving and processing. This is indicative of abnormal cortical processing of (in this case auditory) information in autism.

Spencer and colleagues (2000) reported a difference in visual motion perception related to dorsal stream efficiency in ASD subjects, whereas ventral stream processing is normal. This translates into ASD subjects performing well on visual recognition tasks, but scoring bad in performing motor tasks requiring visual feedback (resulting in clumsiness and lack of coordination). Bertone and colleagues (2005) elucidate on this, indicating enhanced performance on static spatial tasks and reduced performance on dynamic tasks, suggesting a lower level of information processing in autism.

This all shows that in the ASD brain we find morphological changes on all physical scales: From whole brain (brain enlargement, increased white matter) to the cellular level (cortical thickness, minicolumnar organization) and cell-structure (dendritic spines). This indicates that autism is not a dysfunction of an otherwise normal brain. Morphological changes have occurred during embryogenesis and/or during early childhood. Is this "divergence" induced by environmental factors or is it under genetic control? Inheritance patterns of ASD point toward genetic factors. Let us investigate the genetics of ASD next.

### **Genetics of the ASD brain.**

Brain morphology is consistently altered in ASD and multiple brain structures are involved in this disorder. Recurrence of autism between siblings is about 45 times greater than in the general population (Jamain and colleagues 2007). Twin studies indicate a higher concordance rate in monozygotic twins than in dizygotic twins. Male/female ratio is estimated to range from 4:1 to 10:1 This demonstrates a strong genetic basis for autism. Many genes have been identified that have correlation with ASD. Abraham and Geschwindt (2010) provide an overview. Up-to-date information is available on the National Institute of Health's website OMIM (Online Mendelian Inheritance in Man) which is regularly updated. Autism related genes are scattered over all chromosomes. Appendix 2 provides an overview of OMIM's first 10 hits for "Autism" as per 12 may 2011 (Total number of hits is 162). Only a few of autism related genes are X-linked, one special case, NLGN4Y (Neurologin), is located on the Y-chromosome, Yq11.2 (included in Appendix 2). Only few autism related genes being X-linked implies that simple mendelian inheritance alone cannot account for the skewed gender ratio. (Of the approximately 1500 genes on the X-chromosome, some 30-40 % is expressed in the brain).

Note: Rett's syndrome is X-linked, Xq22, and occurs only in girls - it is lethal in boys. Rett's syndrome is excluded from autism in DSM-IV, but not anymore in the proposed DSM V.

Bi-allelic expression of autosomal genes and mono-allelic expression of genes on sex chromosomes are the norm for humans. Both hypomethylation and hypermethylation are observed, resulting in increase respectively decrease of gene-expression (Grant 2007, Jones 2008). Chromosomal deletions or trisomies are uniformly associated with mental defects (Abrahams and Geschwind 2010). Bi-allelic expression is not the

case for all autosomal genes however. Certain genes found in clusters on several autosomes were found to be mono-allelic. The second gene, though present, was silenced. The expressed gene was consistently derived from the same parent, and the silenced gene from the other. This is called "imprinting", it adds another layer of complexity to genetic information.

The website [geneimprint.org](http://geneimprint.org) was checked to investigate whether the autism related genes are known to be imprinted, but no correlation was found here. Of the genes in appendix 2, none was known to be imprinted. Loci 7q (a region) and 15q11-13 are a special case and are known to show correlation between imprinting and some autistic traits.

Note: Prader-Willi and Angelman syndrome, both involving mental retardation, find their origin in imprinting of locus 15q11-13. In Prader-Willi syndrome the maternal gene is silenced by imprinting while the paternal gene is damaged; in Angelman syndrome it is the other way around. These syndromes have autistic traits, but subjects do not fit the full ASD definition of DSM-IV-TR. Chromosome 7q contains MET, which is discussed below (Campbell and colleagues 2010).

Jones and colleagues (2008) hypothesize that dysregulation of methylation of brain-expressed genes correlates with ASD. This may provide a mechanism for environmental factors to influence genetic make-up. Failure to completely erase methylation in early postmeiotic cells in males may result in mosaic state of methylated/unmethylated genes in cells. According to Jones: "gene mutations as well as maternal diet, maternal behaviour and postnatal experiences and exposure have the ability to modify DNA methylation, providing plausible links between genetic and environmental influences on autism". This points towards imprinting mainly on the mother's side. In a peculiar study however Sasanfar (2010) finds correlation between autistic traits and the age of the father in an Iranian population. Jones suggest other epigenetic controls that may suffer from environmental effects: Acetylation of histones, methylation of histones, antisense RNA expression, exogenous insults on DNA repair mechanisms may all be under influence of external factors.

Campbell and colleagues (2009) reported variants of promoter of Mesenchymal Epithelial Transition factor (MET) to associate with both Gastro-Intestinal (GI) and ASD symptoms. MET expression is decreased in temporal cortex in ASD subjects. MET is pleiotropic (meaning a single gene influences multiple phenotypic traits) and functions in brain development as well as in gastro-intestinal repair. The cell-surface receptor MET (7q31) is a proto-oncogene, also known as AUTS 9 in OMIM. The C-G transition in the MET promoter C-allele resulted in half the expression of the MET gene. But how this leads to ASD is not yet discovered. (Note: Campbell's reasoning here is purely statistical, based on a sample of 120 individuals with both ASD and GI symptoms. That both ASD and GI correlate with MET does not indicate that GI correlates with ASD).

### **Immunology of the ASD brain.**

Many children with ASD have allergy symptoms, food allergies being the most prevalent. When mother had a diagnosis of allergies or asthma during the second trimester of pregnancy there occurred a two-fold rise of ASD in offspring

(Theoharides 2009). In allergy, immunoglobulins type E (IgE, prevails only in mammals, mediates the most powerful immune reaction and is directed against multicellular parasites like worms and single-celled organisms like *P. falciparum*) are involved, starting a cascade that eventually results in triggering of mast cells. In the gut, the subsequent inflammatory action can result in increased intestinal permeability. This is exactly the pathway that is indicated in Wakefield's concept of autistic enterocolitis (Wakefield and colleagues 2002). Mast cells are not only important in innate and acquired immunity, but also functional mast cell – neuron interactions are observed in both the brain and the GI tract (Theoharides 2009). Mast cells are also known to secrete numerous molecules that increase permeability of the Blood-Brain-Barrier (BBB). Casein and gluten amongst others are intestine originating mast cell triggers. Oxidative stress can augment mast cell activation; increased oxidative stress and immune dysregulation are apparent in ASD (Theoharides 2009).

Note: Thiomersal/Thiomersal, a mercury containing vaccine preservative that is now widely banned, also in the Netherlands, increases oxidative stress and subsequently compromises immune system regulation. Was thiomersal a compound of the MMR vaccine that was the target of Wakefield's quest ?

Disruption of the BBB is apparent in ASD subjects. Some mothers (11%) with ASD children had IgG antibodies against fetal but not adult brain proteins (Braunschweig and colleagues 2008). IgG passes placenta and is a mechanism for fetal immune priming and protection. IgG is transported through placenta by a specific organelle in epithelial cells that express the IgG receptor Fc $\gamma$ RIIb and receptor/transporter FcRn into fetal circulation. Braunschweig and colleagues do not know what these 37 kDa and 73 kDa proteins are (both protein bands occurred together in 11% of ASD subjects; they never occurred in isolation) and did not investigate levels in next offspring. Maternal IgG is detectable in fetal circulation from week 18. By week 38 fetal IgG level is equal to maternal levels. At birth fetal levels exceed maternal levels and this persists to 6 months after birth.

In other studies it was found that serum of autism patients contained auto-antibodies against a number of brain proteins or peptides. This was especially the case in cerebellum and cingulate gyrus and three hypothalamic proteins (Theoharides 2009).

In ASD subjects:

-Tumor Necrosis Factor (TNF) in cerebrospinal fluid is significantly higher than in controls (Theoharides 2009).

-TNF receptor II is elevated in ASD subjects (Theoharides 2009).

-IL-6 and IL-8 levels are increased in ASD subjects. IL-6 is pro- as well as anti-inflammatory and is secreted by T-cells. IL-8 is pro-inflammatory, is produced by macrophages and chemically attracts neutrophils that phagocytose antigen. Macrophages can cross BBB. IL-8 is relevant to fever in many diseases (Theoharides 2009).

- Acetylserotonin Methyltransferase (ASMT) has a partial duplication in 6% of children with ASD, as compared to 2 % in controls. (Devlin 2005). A 3-fold increase

is high for a sole factor correlated with ASD, most single factors found are in the lower range. ASMT catalyses the final step in the synthesis of melatonin and is expressed in B-lymphocytes, Th-lymphocytes, cytotoxic T-cells, and NK-lymphocytes. The gene is situated in the pseudoautosomal region at the end of the short arm of the X chromosome, Xpter p22-23 and has an identical copy on Y p11,2 (see OMIM). This relates this ASD correlated factor to gender, which is interesting.

This may indicate towards a disrupted BBB in ASD, but whether ASD subjects generally have a disrupted BBB is not known.

### **Autism and hormones.**

The androgen theory of autism proposes that ASD's are in part due to foetal testosterone levels. Interesting research has been performed by the group of S. Baron-Cohen regarding foetal testosterone levels and autistic traits in young children (Auyeung and colleagues 2010, Baron-Cohen 2002). In a group of 129 toddlers (age 18-24 months) it was confirmed that the level of testosterone in amniotic fluid (Note that this amniotic testosterone is produced by the foetus) correlates with autistic traits as measured using the Quantitative Checklist for Autism in Toddlers. This matches the Extreme Male Brain theory of autism which proposes that ASD is an exaggeration of male-typical traits (Male: systemising, Female: empathising). Testosterone levels are under genetic control, providing a possible basis for the inheritance patterns observed in ASD. The elegance of this theory is that it also may explain the sex-ratio in ASD, which other proposed autism - etiologies fail to explain. Note, however, that the children were checked for autistic traits (like reduced empathy, communication, social development) and that this group did not consist of children with a formal ASD diagnosis. (An investigation with confirmed ASD subjects would be difficult to perform as the amniotic testing, which carries some risk, can not be done in retrospect. Difficult, but not impossible). Ingudomnukul and co-workers (2007, also with S. Baron-Cohen's group) found elevated rates of testosterone related disorders in ASD women. Normal women with ASD children also showed increase in high - testosterone related disease. These results suggest concurrent androgen hormone abnormalities in both ASD women and their mothers. Foetal testosterone surely holds some key towards the etiology of ASD.

### **Environmental effects that may lead to autism.**

Both genes and environment are of critical importance in developmental processes. The search for environmental factors leading to an alleged increase of autism in modern society has led to an extensive array of hypotheses. Most notably, Dr. Andrew Wakefield linked Measles-Mumps-Rubella (MMR) vaccination to autism. This caused major public concern. Dr. Wakefield's original publication has now officially been withdrawn by Nature but his concept of entero-colonic encephalopathy still persists (Wakefield 2002). We will discuss this in the next chapter.

Other factors investigated include:

Valproate, an anti-epileptic drug,

Ethanol,

Thalidomide (“softenon”), a sedative drug,

Misoprostal, a drug that induces labour (also used for abortion),

Propionic acid, a food preservative (see Schultz 2008),

Methyl-mercury, a produce of industrial pollution (see Leslie and Koger 2011).

In animal models, prenatal exposure to Valproate (alias Valproic acid, VPA, 2-propylpentanoic acid, naturally occurring in valerian, a plant) induces neural tube defects. This results in impaired social behaviour (Kim and colleagues 2011) in a way that is comparable to autistic traits in humans. Valproate is a neuronal maturational promoter, a GABA antagonist and a histone de-acetylase inhibitor (Kim and colleagues 2011). It is used as an anti-convulsant drug and mood stabilizer in treatment of epilepsy, bipolar disorder, major depression, migraine, and schizophrenia. Children born to women taking Valproate for seizure reduction during pregnancy have an elevated risk of ASD. This may be the result of an altered time course of development of the temporal lobe in children. Valproate acts during neural tube closure of the embryo, in humans 20-24 days after gestation. At this time, most mothers are not aware of being pregnant and still take their Valproate as scheduled. (Having epileptic seizures, on the other hand, is not good for the unborn child either). Valproate action is not limited to this period however, but also influences developmental gene programs during further neuronal maturation even after birth. This happens to occur especially in temporal lobe, where the association networks subside. Temporal lobe is late in embryogenesis (it is also late in evolution). Chomiak and colleagues (2010) conclude “that early postnatal exposure to VPA at a dosage known to inhibit histone de-acetylation in vivo, can lead to synchronous developmental alterations reminiscent of ASD in rats”.

Note 1: VPA increases 5HT (serotonin) levels in the embryo. So 5HT may participate in the molecular syndrome that drives the altered developmental patterns. The gene *SLC6A4* codes for the serotonin transporter and is correlated with autism (AUTS6, see Appendix 2).

Note 2: The link of Valproate with ASD can be turned around to use Valproate to induce autism-traits in a rat autism model. (Kim et. al. 2011) Embryonic day 13 proved to be the best timing in rats. Earlier exposure leads to death, late exposure to no results. This time window coincides with the time window for closing of the neural tube, which happens in humans on embryonic day 20-24. Valproate functions as a histone acetylation inhibitor. Histone acetylation is important in running down from neuronal progenitor (stem-) cells towards differentiated neuronal cells. Valproate may thus interfere with the genetic program. It should be realized however that epigenetic controls are very diverse between mammalian species. That may impose severe limitations on animal models of ASD.

Propionic acid (PPA) is a short-chain fatty acid, a metabolic end product of some bacteria in the gut (Bercik 2011). It is commercially used as a food preservative for both animal feed and human consumption. (Wheat and dairy products both usually contain PPA). It is also produced by human skin bacteria (propionibacteria, for example *P. Acnes* which produces acne). Rats fed with PPA showed social behaviour

impairments. Histological examination of brain tissue showed astrogliosis, indicating neuro-inflammatory response. PPA is a simple molecule that readily crosses the gut-blood barrier and the blood-brain barrier. It thus can reach the CNS, where it induces acidosis. In rats this correlates with changes in social behaviour. Shultz and colleagues (2008) expand this to similar effects in humans. They used male rats as ASD is more prevalent in men than in women (which they had it done in female rats too, and see the same difference – or not).

Leslie and Koger (2011) point towards methyl-mercury as a factor in ASD etiology. Methyl-mercury is a product of industrial pollution. Efficacy is based on the induced oxidative stress. (Thimerosal contains ethyl-mercury that is supposed not to be detrimental; nevertheless thimerosal is banned as a vaccine adjuvant in many countries as a precaution). This underscores the oxidative stress model of autism as proposed by Theoharides (2009).

## Gut-Brain axis.

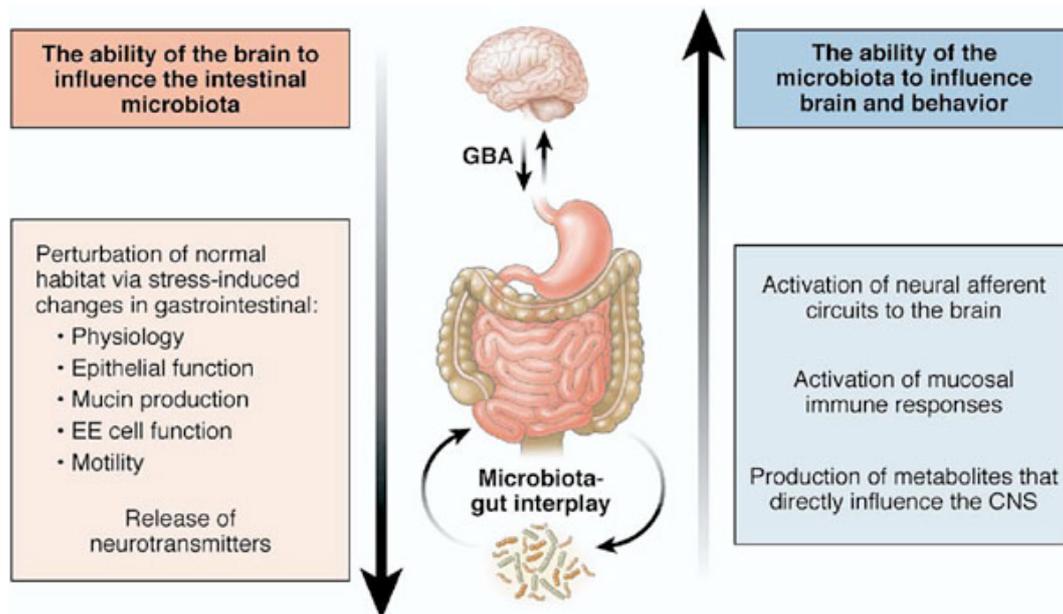


Figure 2. The Gut-Brain Axis (GBA)

There is a lot of communication between the gut and the brain (see figure 2). This communication is two-way and can be both chemical and neuronal. The enteric nervous system contains many neurons and stores a library of programs for different gut behaviours. The vagus nerve (cranial nerve X) connects the enteric nervous system with the Central Nervous System.

Quite some people with ASD suffer from Gastro-Intestinal (GI) problems which has led to the believe that GI-problems may be a factor in the ASD's etiology. Gut-brain communication being two-way this might also be the other way around, but

apparently this line of reasoning is not popular with researchers. It should be noted however that many ASD subjects show strange and/or repetitive eating behaviours that may induce severe stress on their GI-system.

Environmental factors have also been claimed to contribute to autism via a gut-brain axis: certain foods and food-additives, infections, disease, heavymetals, chemicals/medicins. Vaccination may act more directly. Evidence however is anecdotal, most claims lacking rigorous statistical proof.

### **Autistic enterocolitis.**

The concept of autistic enterocolitis has been most profoundly advocated by Wakefield c.s. (Wakefield 2002). They theorized that Measles-Mumps Rubella vaccine (MMR) produces an enterocolitis that causes leaky gut and subsequent increased absorption of peptides with neurotoxic /neuro-active properties. This hypothesis has now been rejected, and Wakefields original publication in Nature has been withdrawn. The idea of peptides leaking through the gut (leaky gut hypothesis) was already proposed in 1979 by Jaak Panksepp (Panksepp 1979). According to Panksepp, autism is an emotional disturbance rather than a cognitive one, arising from upset of the opiate systems in the brain. Autism may be arising by endogenous overactivity of the child's own opiate systems, as opposed to Wakefield's proposed external/dietary intake.

### **Panksepp's hypothesis (1979).**

Panksepp based his theory on the behaviour of animals on a low dose of narcotics. These animals:

- do not fully experience physical pain,
- do not cry as readily,
- cling poorly to mother,
- do not seek social companionship,
- learning behaviour results in persistence of typical behaviour, even in absence of reward.

This all concurs with the symptoms of autism as originally described by Canner (1949). ASD children may thus suffer from maturational lag in change from certain brain chemistries; these are supposed to change in early childhood from early "bondage" towards independence. This maturational lag may prevent the brain from becoming appropriately tuned to the sensory and social environment. Opiate dependance subsequently results in withdrawal symptoms like in addicted adults. This opiate system's freerunning will detach the child from comfort of social acts.

In agreement with Panksepp's hypothesis, Naltrexone, an opium antagonist, has produced improvements in autism, though results have been mixed and are ultimately inconclusive (Leskovec 2008). Naltrexone is primarily used in alcohol and opioid dependence.

Wakefield did generate an awareness that GI -problems play an important role in childhood disorders. Intestinal inflammation increases intestinal permeability and that might facilitate absorption of dietary derived opioid-like peptides (exorphins) from the gut. Neuro-active compounds can permeate the intestinal mucosa, and cross the BBB. (Wakefield 2002). Wakefield points to the known Hepatic encephalopathy as an analogue to effects seen in autism. Gastro-intestinal symptoms do correlate with decreased psychophysical well-being, behavioural disturbances and impaired school performance. But what is the cause of what?

Note: Wakefield also points out that grown-up patients with short-bowel syndrome, as a result of intestinal bypass surgery, show a range of psychiatric and neurological problems. This is reversible by anti-bacterial treatment so apparently it results from changed biota in the gut. But this is in grown-ups.

Are gastro-intestinal problems really common in children with developmental disorders and specifically in subjects with ASD? In a population based study involving 124 ASD subjects, Ibrahim and colleagues (2009) found some correlation between incidence of constipation and feeding issues, but overall incidence of G.I. symptoms showed no association with ASD. They indicate this may be attributive to a behavioural etiology rather than a G.I. etiology. Indeed the perceived (anecdotal) increased occurrence of G.I. symptoms with ASD may be the result rather than the cause of ASD (correlation is not causation). Many ASD subjects have strange or problematic eating habits and food preferences.

The digestion of food proteins can produce substances that have opiate or narcotic properties. A number of peptides are created in the digestive tract, that feedback to control centers in the brain, e.g. a stop signal to the brain when enough food has been eaten is important for appetite control and to stop seeking behaviour (this signal may be defective in people with eating disorders). Especially peptides derived from milk-protein (casein) and wheat-protein (gluten) can act like the bodies own narcotics. These externally derived opioid peptides have been named exorphins (as compared to the bodies internally produced endorphins). Several are known:

|                                      |  |
|--------------------------------------|--|
| Casein from cows milk breaks down to | casomorphin, a 7 aa opioid peptide.                                  |
| Gluten from wheat                    | glutenexorphin type A5, B4, B5 and C<br>gliadorphin(=gluteomorphin). |
| Rubisco from green plants(spinach!)  | rubiscolins type 5 and 6.  |

Two diets have been designed to counteract the effects of autism: the Casein Free Gluten Free diet (CFCG) and the Specific Carbohydrate Diet (SCD). Both diets require strict adherence and result in hardship for both the patient and his parents. Nevertheless they have dedicated advocates as the results are in some cases positive. The GFCF diet is free of wheat (which contains gluten) and milk-products (which contain casein). Both are known as allergens. The GFCF diet's supposed

efficacy therefore rests on the immunological hypothesis of ASD etiology. Reports of parents and teachers show that children have been “cured” of their autism but no testable data support this. Elder and colleagues (2006) performed a study on the efficacy of the GFCF diet in a randomized double-blind trial on 15 children with autism spectrum disorder. Several parents reported improvement of their child’s behaviour after 12 weeks on the diet, but no testable findings supported this in a statistically significant way. In this investigation the sample size was small, but were the GFCF diet as good as expected, it should have shown up in the results. Elder and colleagues therefore conclude that efficacy of the GFCF diet could not be confirmed in their trial, but suggest further investigation with increased sample size. The subjective improvements as experienced by the parents may well be related to placebo-effect. The strict adherence to the GFCF diet requires a lot of effort and financial expense. In this study parents were well motivated, and in some way more or less desperate in their quest for treatment of their ASD stricken child. It is a known effect that an expensive placebo works better than a cheap one. Happy parents will result in happy children, happy children will perform better, thus closing the circle towards improvement.

Reichelt and Knivsberg (2009) investigated urine peptide levels and found that these levels were higher in ASD subjects. At least some of these peptides were of dietary origin and hinted towards increased intestinal permeability. After one year on GFCF diet urine peptide levels significantly reduced in these individuals. This led Reichelt and Knivsberg to conclude that a gut-to-brain axis is both possible and probable in ASD.

Note: the increase in cow’s milk consumption has been especially explosive in Norway, where Reichelt and Knivsberg performed their study.

The SCD diet is not as popular as the GFCF diet and was originally designed by Dr. Sydney V. Haas (1870-1964) to treat Inflammatory Bowel Disease. Its aim is to present the patient with monosacharides only, as multisacharides are more difficult to digest. In some people multisacharides remain in the gut undigested and reach the large intestine where they are fermented by an overpopulation of intestinal microbes, causing excess gas, diarrhoea and constipation, leading to inflammation. This diet has been tried on autism with varying results; see E. Gottschall (2004) for a description. The concept of microbiota to communicate with the brain and modulate behaviour is presently emerging as an important concept in health and disease (Cryan and O’Mahony 2011). Neuronal (both CNS and enteric nervous system), hormonal and immunological systems integrate bidirectional signalling between the GI tract and the brain and influence overall behaviour. This can be tested in gnotobiotic animals. These animals, without any microbiota in their gut, show several detrimental effects in their behaviour, (increased stress and anxiety) indicating towards some positive effect of gut-microbiota on brain performance (Cryan and O’Mahony 2011). Unfortunately nearly all animal research has been performed on male animals only, as female animals add severe statistical complications to an experiment because of their hormone cycle. These experiments therefore do not provide gender related information on behavioural effects, one of the striking properties of the ASD’s.

A third approach towards dietary intervention is based on Antioxidant theory. Antioxidants have been shown to influence the immune system in several ways.

Luteolin (a flavonoid) in Celery, green pepper, thyme, carrots, and olive oil is an IL-6 inhibitor. It inhibits IL-6 release and IL-8 release from astrocytes. (i.e. in brain) and IL-6 release from microglia. Quercetin (also a flavonoid) in fruits, vegetables, leaves, grains and red berries also may have anti-inflammatory properties. Both are ingredients of the dietary supplement neuroprotek® produced by Algonot and marketed by – Theoharides. See [www.Algonot.net/neuroprotek.php](http://www.Algonot.net/neuroprotek.php) for a complete motivation. (Theoharides 2009)

## Discussion.

It is a general public perception that the prevalence of Autism Spectrum Disorders has recently increased in modern societies. Allegedly ASD subjects suffer from increased Gastro-Intestinal problems. This poses suspicion that the gut-brain axis is involved in the etiology of ASD. However, these propositions are hard to prove in a statistically rigorous and reliable manner and may well be influenced by improved reporting and diagnosis of modern medicine. Note that the inverse, that ASD prevalence is not increasing in modern times is also not possible to prove. It would be interesting to see what happens to ASD prevalence in a society that has recently underwent transition towards modern western lifestyle (e.g. East-Germany).

When looking for correlations between two extensive and complex systems like the human brain and the human gut, one has to realize that one is bound to find associations. The human mind has high associative power and is extremely apt in finding correlations even if they are not there. This has resulted in public concern on vaccines, food additives, drugs, pollution and other factors on many occasions.

Nature does not design systems by an intensive and purposeful way, but more or less solves her problems like a busy handyman using whatever lies available in his cabinet. Structures, components and chemical pathways may be used and shared in many if not all of an organism's subsystems. For example, the gene DCC "Defective in Colon Cancer" (OMIM \* 120470, 18q21.3) got his name from being related to a gut problem, but also has correlation with autism as it is a cell cycle control gene also expressed in brain. Another example is the GRPR gene for Gastrin Releasing Peptide Receptor (OMIM \* 305670, Xp22.3) that was found defective in a female with autism. Most likely, these examples are a coincidence of gut – brain correlation. They indicate towards a shared component of both systems, but do point towards a causation of gut to brain interaction – or the other way around for that matter.

Extensive research has been performed on the morphology of ASD brains. Deviations from normal brains have been found on all scales from small (dendritic spines) to large (whole brain size). This proves that ASD is a constructional defect of the brain rather than a malfunctioning of an otherwise normal brain. These defects have a large genetic component; at least 162 genes are known to be correlated with ASD. Morphology however does not result from genes alone, but from the running down of a complete genetic program. This includes imprinting and hormonal control and may well be disturbed by environmental substances beyond the body's control.

Substances that are known to interfere with brain development (valproate, thalidomide) appear to do so in the 20-24 week after gestation, the period when the

neural tube of the foetus closes and the basic organisation of the brain is laid down. This is a period when a healthy condition of the mother is of extreme importance to the developing foetus, and factors like smoking and drinking have large effects on the unborn's correct development. Unfortunately, many women do not know that they are pregnant at this time.

Subsequently, a young brain has to mature by means of pruning of the appropriate brain structures during a critical period. This is especially applicable to brain structures engaged in social interaction and language acquisition, functions that are comprised in ASD. The genetic component of ASD often results in parent – child social relationships that suffer from inappropriate social interactions from parent to child, resulting in incomplete pruning of the child's social systems. ASD may thus be partly a result of inappropriate behaviour of the parent towards the child, the concept of “refrigerator mom” (Dutch: “ijskastmoeders”). This behavioural concept of heredity is seldom addressed in the literature, likely because the somewhat outdated concept of “ijskastmoeders” is not popular with the general public and researchers are reluctant to investigate this concept.

After all this building and pruning has been completed, the brain is ready to function but will only do so when appropriate conditions can be maintained. This requires perfect coordination of the providing system – gut , with the using system – brain. Communication between these systems is two-way and uses neuronal systems (the enteric nervous system contains many neurons and is connected to the CNS by the vagus nerve, cranial X), hormonal systems, and diverse biochemical control-loop arrangements for levels of several nutrients. All is guarded by a ubiquitous safety service: the immune system. In many cases of ASD, immune system aberrations are found: allergies have higher than normal prevalence in ASD and some ASD individuals have antibodies against (self) brain proteins. Oxidative stress, apart from being detrimental to various metabolic routes, induces the immune system to high activity and may result in increase of these problems.

Promising research is being performed by S. Baron-Cohen and his group. Their hypothesis on high pre-natal testosterone levels being an inducer of ASD makes much sense: not only is there an apparent correlation between foetal testosterone and ASD-like traits, but this etiology also explains the large male-female ratio observed in the ASD that other proposals fail to explain. Whether the high testosterone levels are genetically based or environmentally induced still remains to be seen.

It seems unlikely that the gut –brain axis in an unborn child can influence the developing brain's morphology as is seen in ASD. This precludes gut-brain axis aberrations as a single cause of ASD. It is however possible and probable that gut-brain interactions during life can exacerbate problematic functioning of an already compromised brain to such an extent that autism may result. Gut-brain axis is thus superimposed on genetic program run-down. This explains the efficacy of food related therapies that have become popular with many parents of ASD children:

- A diet high in anti-oxidants either from natural sources (Blueberries!) or from dietary supplements. Anti-oxidants have other positive effects as well (like reducing cancer).
- The Gluten Free Casein Free diet addresses the cow's milk protein allergy and/or gluten allergy that are observed in many ASD subjects (and in many

“normal” subjects as well for that matter. The suspicion has arisen that cow’s milk is NOT good for you)

- The Specific Carbohydrate diet, though originally designed to treat Inflammatory Bowel Disease, may exert positive effect in ASD subjects by reducing bacterial load of a compromised gut that leaks peptides with opiate properties.

The efficacy of these dietary therapies are hard to prove statistically and may to a large extent be dependent on Placebo effects. Placebo effects, however, are very real and may comprise a significant part of any therapy.

## Conclusion.

Autism is a chronic neurodevelopmental disability for which medicine does not offer any cures. Thus complementary and alternative treatments are widely provided to children with autism by parents who are searching for any biomedical intervention that they believe may help their children. Diets excluding gluten, cow's milk, complex carbohydrates or diets high in anti-oxidants may subjectively score positive results but this is hard to prove in a statistically rigorous manner.

Subjects with Autism Spectrum Disorder invariably show aberrations in brain morphology, ranging from small scale (cell and cell-structures) to large scale (whole brain enlargement). Environmental substances that are known to influence brain development seem to do so in the 20-24 week after gestation, when the neural tube closes and most brain systems are laid down. Influence by a gut-brain axis seems unlikely in this process. Further maturation of the brain however is dependent on maintaining a proper environment and pruning of adaptive cognitive and emotional networks. These processes can easily be disturbed in a negative sense via a gut-brain axis pathway. Leaky gut may allow peptides that have opiate function to reach the brain, or other detrimental substances that are either food-ingested or produced by the gut's own microbiota.

Both gut and brain are immunologically special. Inflammation of the gut may induce the immune system to higher efficacy, having effects on the brain-immunology as well. Food allergies are especially prevailing in ASD subjects. Oxidative stress by pollutants may exacerbate the effects. None of the aforementioned processes can explain the striking gender ratio of at least 4 to 1 in ASD. The foetal testosterone model can, and the team by Baron-Cohen are doing well in investigating along this line (Auyeung and colleagues 2010).

The gut-brain axis forms at most a secondary step in the etiology of ASD. This step however is the most accessible for therapy making it very worthwhile to further investigate the brain-gut axis in relation with ASD.

Note: Just after completing this thesis exciting results were published online by Geschwind and co-workers (Voineagu 2011) on the transcriptome of the autistic brain. It is discussed in Appendix 3.

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# Appendix 1. DSM Definitions of autism.

## DSM-IV-TR

### Diagnostic criteria for 299.00 Autistic Disorder

A. A total of six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

- (1) qualitative impairment in social interaction, as manifested by at least two of the following:
  - (a) marked impairment in the use of multiple nonverbal behaviours such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction.
  - (b) failure to develop peer relationships appropriate to developmental level
  - (c) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people ( e.g., by a lack of showing, bringing, or pointing out objects of interest)
  - (d) lack of social or emotional reciprocity
- (2) qualitative impairments in communication as manifested by at least one of the following:
  - (a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
  - (b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
  - (c) stereotyped and repetitive use of language or idiosyncratic language
  - (d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level.
- (3) restricted repetitive and stereotyped patterns of behaviour, interests, and activities, as manifested by at least one of the following:
  - (a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
  - (b) apparently inflexible adherence to specific, non-functional routines or rituals

- (c) stereotyped and repetitive motor mannerisms (e.g. hand or finger flapping or twisting, or complex whole-body movements)
- (d) persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's disorder or Childhood Disintegrative Disorder.

### **DSM-5 proposal.**

Autism Spectrum Disorder

Must meet criteria A, B, C, and D:

- A. Persistent deficits in social communication and social interaction across contexts, not accounted for by general developmental delays, and manifest by all 3 of the following:
  - 1. Deficits in social-emotional reciprocity; ranging from abnormal social approach and failure of normal back and forth conversation through reduced sharing of interests, emotions, and affect and response to total lack of initiation of social interaction,
  - 2. Deficits in nonverbal communicative behaviors used for social interaction; ranging from poorly integrated- verbal and nonverbal communication, through abnormalities in eye contact and body-language, or deficits in understanding and use of nonverbal communication, to total lack of facial expression or gestures.
  - 3. Deficits in developing and maintaining relationships, appropriate to developmental level (beyond those with caregivers); ranging from difficulties adjusting behavior to suit different social contexts through difficulties in sharing imaginative play and in making friends to an apparent absence of interest in people
- B. Restricted, repetitive patterns of behavior, interests, or activities as manifested by at least two of the following:
  - 1. Stereotyped or repetitive speech, motor movements, or use of objects; (such as simple motor stereotypies, echolalia, repetitive use of objects, or idiosyncratic phrases).
  - 2. Excessive adherence to routines, ritualized patterns of verbal or nonverbal behavior, or excessive resistance to change; (such as motoric rituals, insistence on same route or food, repetitive questioning or extreme distress at small changes).
  - 3. Highly restricted, fixated interests that are abnormal in intensity or focus; (such as strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests).
  - 4. Hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of environment; (such as apparent indifference to pain/heat/cold, adverse response to specific sounds or textures, excessive smelling or touching of objects, fascination with lights or spinning objects).
- C. Symptoms must be present in early childhood (but may not become fully manifest until social demands exceed limited capacities)
- D. Symptoms together limit and impair everyday functioning.

## Appendix 2. Some autism related genes from OMIM.

Total number of OMIM hits on ‘autism’ is **162**.

|                   |              |         |  |
|-------------------|--------------|---------|--|
| AUTS1             | 7q22         | Region, | containing many genes including IBD1 (MUC3) an epithelial glycoprotein involved in Intestinal Bowel Disease.   |
| AUTS3             | 13q14.2-14.1 | region  | At least 4 genes.  |
| AUTS5             | 2q           | Region, | 9 candidate genes.   |
| AUTS6             | 17q11        | SLC6A4  | serotonin transporter.   |
| AUTS7             | 17q21        | ITGB3   | Integrin Beta, glycoprotein, subunit of platelet adhesive protein receptor.  |
| AUTS9             | 7q31         | MET     | proto-oncogene. Connects with semaphorin. Expression in mouse-forebrain confirmed, but not yet in humans. Note: FOXP2, related to speech disorder, is also in this region.                                     |
| AUTS10            | 7q36         | EN2     | Engrailed2, homeobox transcription factor.   |
| AUTS11            | 1q41-42      |         | MARK1(probably) Microtubule regulator.   |
| AUTS15            | 7q35-q36     |         | region, based on Quantitative Trait Loci. CNTNAP2 is in this region (contactin-associated protein-like 2 (CASPR2) belonging to neurexin superfamily). Transmembrane protein involved in cell-cell interaction. |
| AUTS16            | 3q24         | SLC9A9  | Solute carrier family, sodium/hydrogen exchanger, transmembrane protein.   |
| AUTS17            | 11q13.3-13.4 | SHANK2  | Synaptic scaffolding. In rat this gene is only expressed in brain.   |
| <b>X-linked :</b> |              |         |  |
| AUTSX1            | Xq13         | NLGN3   | Neurotrophin, neuronal cell-cell adhesion  |
| AUTSX2            | Xp22.23      | NLGN4   | Neurotrophin, neuronal cell-cell adhesion  |

|        |         |        |   |
|--------|---------|--------|---|
| AUTSX3 | Xq28    | MECP2  | Chromatin associated protein that can both activate and repress transcription. Binds methylated DNA (CpGs). Required for maturation of neurons. Related to Rett's disease, mental retardation, encephalopathy. Subject to X-inactivation in humans. |
| PTCHD1 | Xp22.11 | PTCHD1 | patched-domain-containing protein. Transcription inhibitor expressed in several tissues, mostly in cerebellum and spinal cord. Probable in hedgehog pathway.  |

**Y-linked:**

|                       |         |        |  |
|-----------------------|---------|--------|--|
| Neuroigin4, Y-linked. | Yq11.12 | NLGN4Y | Neuroigin Y-linked is a homologue of NLGN4(Xp22.23). Is expressed in foetal and adult brain, prostate and testis in males. Single copy X-degenerate on male-specific region of Y-chromosome. |
|-----------------------|---------|--------|--|

## Appendix 3. mTOR signalling is relevant in ASD.

Just as this thesis was completed, a very relevant and exciting publication by Voineagu and colleagues from D. Geschwind's lab appeared in nature on-line. Using full-genome micro-arrays they compared gene expression in brain samples from the US autism tissue program with brain samples from healthy controls. They found consistent differences in the transcriptome organization between the autistic and the normal brain. Whole clusters of genes appeared to be transcribed differently. Among these were several genes known to be correlated with autism.

A question now immediately comes to mind:

Are genes of the mTOR signaling pathway expressed differently in the ASD brain? This pathway is known to regulate plasticity. According to Edelman's theory of neural Darwinism that will influence the laying down of neural networks. We know from Hutzler and Zhan (2010) that dendritic spine densities are different in ASD subjects. Forming dendritic spines requires extensive transcription, meaning up-regulation of clusters of genes. That is exactly what mTOR does.

Action 1: Investigate whether genes of the mTOR pathway are regulated differently in ASD subjects. Voineagu and colleagues (2011) have made their results available on-line, this can be checked on a rainy Sunday.

Action 2: Does the mTOR pathway up- or down regulate forming of dendritic spines? This can be tested in a mouse model, human mTOR genes having homologues in all mammals (that is what the "m" stands for). All the equipment to do this is available at the Went building, University Utrecht. The mtor protein (mouse homologue of human mTOR) can be blocked by rapamycin (that is what the "r" stands for) or even simpler by ketamine (Cryan and O'Leary 2010) but then it should be noted that ketamine blocks the NMDA receptor, which may influence neural Darwinian selection. Subsequently dendritic spines can be counted in mouse prefrontal cortex using the available Leica 2-photon laser scanning microscope. Similar experiments are already being performed today. A new series of experiments will be started soon with the OBX mouse model, which also involves dendritic spine counting. The control group of the OBX mice may equally well serve as control group for our proposed mtor experiment. If the results are clear than about six mice may suffice to show our case. This can be done in a few weeks.

Action 3) As I indicated in "Conclusion" the foetal testosterone model as investigated by the team led by Baron-Cohen can explain the gender bias that is apparent in ASD. Other approaches can not. Is the mTOR pathway influenced by testosterone? Again we may use a mouse model, administering testosterone during the foetal phase, and checking expression of mtor genes (and homologues of human ASD related genes as well). We will need a few micro-arrays to do this, if the effect is clear some six micro-arrays will do. We will need six for the control group as well. Costs?

If all the scores are positive, we have demonstrated a

Testosterone - mTOR - dendritic spine - neural network

cascade towards neuronal dysfunction that may provide several possibilities towards intervention.

## Appendix 4. Tidbits.

The “male brain” model was first suggested in 1944 by Hans Asperger.

The term “refrigerator mother” was coined by Leo Kanner in 1949.

The phenomenon of genomic imprinting evolved only in mammals, before the divergence of marsupials and eutherians over 150 million years ago. Mammalian species vary markedly in their genomic imprinting repertoires (source: [geneimprint.org](http://geneimprint.org)). Indeed, imprinting may be the underlying factor in the evolution of such a large number of mammalian species. This poses a severe problem in developing suitable animal models of human disease if imprinting is involved.

“The proper study of mankind is man.” (Alexander Pope)

The gene DCC 18q21.3 “defective in colorectal carcinoma” is also expressed in the brain, see OMIM. (coincidental link between gut and brain?)

OMIM autism#54: GRPR on Xp22.3 Gastrin Releasing Peptide Receptor. (Coincidental link between gut and brain?)

Loci 7q and 15q show strong correlation between imprinting and autism. 15q is associated with Angelman syndrome, which resembles autism in many ways. Three GABA receptor units, namely GABA  $\alpha$   $\beta$   $\gamma$ , are located near the imprinted region of 15q. MET is on 7q31 (see Campbell 2007).

Autism AND DDT shows no hits in UU library. Did nobody think of that?

Honda,H, Shimuzu Y, Rutter, M: No effect of MMR withdrawal on the incidence of autism: a total population study. This study was instigated by, and to some extent concludes, the “Wakefield” hussle.

Mastocytosis (too many mast cells) patients have a 10 fold higher occurrence of autism. (Angelidou et. al. 2011).

Mast cell activation could be particularly critical during gestation, since mast cell derived mediators might act epigenetically to alter the expression of autism susceptibility genes. (Theoharides 2009)

Il-6 can disrupt the BBB as well as promote the development of Th-17 cells, which are critical for the development of autoimmune disease (e.g. in Crohn) Th-17 produce Il-17 pro-inflammatory cytokine commonly associated with allergic response.

Placebo effect is called Hawthorne effect in economics; e.g. when workers participate in an experiment regarding work environment, production goes up whatever the change in that environment.

An expensive placebo works better than a cheap one.

Oxytocine (“knuffelhormoon”) may be the mediator in the placebo effect.

Labelling is a form of placebo-effect. In Dutch: repelsteeltje effect.

Triple Reuptake Inhibitors are also available in nature: St. John's Wort (*Hypericum perforatum*, used as a herbal antidepressant) is believed to inhibit reuptake of dopamine, serotonin and norepinephrine. It works against depression – sometimes. But it reduces effect of other drugs (including oral contraception e.g.). Problem with natural herbs and the like is the dosage, which may not be consistent across batches. Most likely placebo effect is also involved here.

My “worker in the field” (I live with an “ambulant begeleider” with a lot of experience with children with autism) reports “angel face” appearance, i.e. most (male!) ASD children have pretty faces. This is apparently not addressed in literature.

Human casomorphin differs from cow's casomorphin by two amino-acids.

Did someone measure 2D-4D ratios in ASD subjects yet? Yes. Voracek, Dressler, found lack of correlation between digit ratio (2D:4D) and Baron-Cohen's “reading the mind from in the eye” test, empathy, systemising, and autism-spectrum quotients in a general population sample. They found no correlation...this may be due different timing of the two processes of digit-forming and brain-formation. Auyeung et. al. (2010) however, did mention a 2D:4D correlation with ASD.

Probiotics have potential to modulate brain and behaviour (Cryan and O'Mahony 2011) Antibiotics reduce biodiversity of gut microbiota. This has been shown to alter behaviour in mice.

Probiotics may decrease oxidative stress and also lower inflammation.

Testosterone blockers already exist and are available on the market even without prescription: herbal extract, e.g. *Serenoa repens* (saw palmetto).

mTOR pathway senses the condition of the cell (e.g. ATP – level) and subsequently stimulates transcription. It induces “lifeliness“ of the cell (The Force?). All this transcribing may run astray: increased cancer is observed in mTOR upregulated cells. Lively cells also appear to use-up their lifetime more quickly. Die early from good health. Is this the standoff modern society faces?