Diplomarbeit

Mutations of specific neuroligins and their implications for autism spectrum disorders

eingereicht von

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zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Humangenetik

unter der Anleitung von

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Graz, am 07.01.2016

Eidesstattliche Erklärung

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Graz, am 07.01.2016 Gertrud Eveline Hofer eh

Danksagungen

Ich möchte mich an dieser Stelle bei all jenen bedanken, die mir beim Verfassen dieser Arbeit und im Studium zu Seite gestanden sind.

Ganz besonderer Dank gilt meiner Mutter, die mich stets ermutigt und bestärkt hat und ohne die diese Arbeit wohl nicht oder erst viel später entstanden wäre.

Ich möchte mich auch bei meinen Freunden bedanken, die mich im ganzen Studium unterstützt und begleitet haben.

Zu guter Letzt möchte ich meinem Betreuer, Prof. Erwin Petek, besonderen Dank aussprechen für die Unterstützung bei dieser Arbeit, von der Wahl des Themas bis zu den feineren Punkten des richtigen Zitierens, und für die interessanten und aufschlussreichen Gespräche über Autismus und Genetik.

Zusammenfassung

Autismus Spektrum Störungen (ASDs) sind sowohl in ihren Symptomen als auch in der Vererbung als heterogen und hochkomplex anzusehen. Einzelne Genfamilien wurden zwar bereits im Zusammenhang mit ASD beschrieben, eine diagnostische Anwendung im Rahmen einer humangenetischen Abklärung ist derzeit aber noch nicht möglich.

Durch Verwendung von PubMed Suchanfragen wurde systematisch versucht, eine umfassende und verbindende Arbeit zu den Neuroliginen – sowohl auf Protein- als auch auf molekulargenetischer Ebene – zu schaffen. 18 Publikationen wurden eingebunden, in denen Neuroligin-Mutationen bei ASD Patienten untersucht und erforscht wurden.

Es wurde im Rahmen dieser Diplomarbeit überdies versucht, die teilweise sehr unterschiedlichen Ergebnisse und Interpretationen dieser Publikationen auf genetischer, bevölkerungsgenetischer und pathophysiologischer Ebene in Einklang zu bringen, beziehungsweise zu erklären.

Es zeigte sich klar, dass Neuroligin-Mutationen bei einem definierten Teil der ASD-Fälle eine wesentliche Rolle spielen, allerdings nicht in jedem Fall zwingend pathogen wirksam sind.

Genauso deutlich geht aus dieser Arbeit hervor, dass weiter Forschung in vielerlei Hinsicht notwendig ist.

Abstract

Autism spectrum disorders (ASDs) are highly complex and heterogenic, both in their symptoms and in their genetics.

Utilizing mainly the PubMed research portal, I compiled this literature research to create a comprehensive source concerning the neuroligin genes and proteins as pertaining to ASDs. I found and focussed on 18 publications that investigated mutations in the relevant genes in ASD patients.

This work tries to align and explain the very different results of these publications on a (population) genetic and pathophysiological level.

It appears that neuroligin mutations are definitely relevant to a portion of ASD cases, however, they are not obligatorily pathogenetic.

It is just as apparent that further research on many levels is still necessary.

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Abbreviations

aCGH	Array-comparative genomic hybridization
ADHD	attention deficit hyperactivity disorder
ADI-R	Autism diagnostic interview - revised
ADOS	Autism diagnostic observation schedule
AGRE	Autism Genetic Resource Exchange
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARSE	arylsulfatase E
AS	Asperger syndrome
ASD	Autism spectrum disorder
BPD	Bipolar disorder
CAM	Cell adhesion molecules
CARS	Childhood autism rating scale
CDD	Childhood desintegrative disorder
CMV	Cytomegalia virus
CNV	Copy number variation
de novo	Newly occurring
DNA	Desoxyribonukleinacid
DSM	Diagnostic and Statistic Manual
DZ	Dizygotic
Fig.	Figure
FISH	Fluorescence in situ hybridization
GABA	Gamma amino butyric acid
GWAS	Genome wide association studies
HSV	Herpes simplex virus
ICD	International statistical classification of diseases and related health problems
ID	Intelectual disability
IMGSAC	International molecular genetic study of autism consortium
KAL1	Kallman syndrome
LD	Learning disorder
MMR	Measles, mumps and rubella
MR	Mental retardation
MZ	Monozygotic
NLGN	Neuroligin
NRXN	Neurexin
OR	Odds ratio
PCR	Polymerase chain reaction
PDD	Pervasive developement disorders
PDD-NOS	Pervasive developement disorders - not otherwise specified
qRT-PCR	Quantitative real time-PCR
SNPs	Single nucleotide polymorphisms
STS	Steroid sulfatase
Tab.	Table
TS	Tourette syndrome.
WHO	World Health Organization
XCI	X chromosome inactivation
XIST	X-inactive specific transcript

Introduction

The prevalence of Autism Spectrum Disorders, abbreviated ASD from now on, seems to have increased significantly in the past decades. While for a long time, estimates were as low as 4,5 in 10.000 children (Lotter, 1966), more recent surveys suggest numbers as high as 13 in 10.000 for ASD, and about 60 in 10.000 for all pervasive development disorders (PDD) (Fombonne, 2005).

Reasons for this dramatic increase range from evolving definitions to diagnostic criteria over improvement of diagnostic tools to factors like increasing survival rate of newborns (Zylstra et al., 2014)

A notable feature of ASD diagnoses is the skewed gender ratio. A greater preponderance of ASD diagnoses, excluding the Rett Syndrome, in males approximating 4:1 has been consistently reported as far back as the earliest scientific publications (Kanner, 1943). Newer studies show a far greater disparity with 7-8:1 (Whiteley et al., 2010)

What is Autism?

An short look at history

The first use of the word "autism" was not related to the syndrome at all. Rather, the psychiatrist Paul Bleuler used it to describe the self-centered and idiosyncratic thinking in schizophrenic patients. (Moskowitz et al., 2011)

This caused considerable confusion, as Kanner coined the term for a whole different syndrome, which he empathically separated from schizophrenia. His use of autism referred to difficulties in social interactions through a failure in development, rather than through regression or retreat as seen in schizophrenic patients.

While he was not the first to describe the affliction later called autism (Wolff, 2004), child psychiatrist Kanner was the first to provide a widely published, overarching clinical description (Kanner, 1943).

In his publication 'autistic disturbances of affective contact' he portrays, rather than defines, the syndrome.

Kanner noted the several features, among them "insistence on sameness", "resistance to change", repetitive movements (e.g. stereotyped hand flapping), monotonous interests, lack of social engagement and seeming *over*engagement in the non-social world, linguistic

peculiarities like echolalia or extreme literalness, as well as wildly different results on different parts of IQ tests, with high marks in nonverbal and memory based categories.
While he described that all of the parents of children he wrote about were intelligent and successful as well as rather aloof, it has to be noted that he did not assign them any responsibility for the children's condition, as the later theories ("refrigerator mom") have done. Rather, he noted that further research was required.

Based on Kanner's research, Rutter created a definition of autism emphasizing the following features (Rutter, 1978):

1. a distinctive form of social impairment (not just due to any associated intellectual disability)
2. impaired language skills (also not just due to any associated intellectual disability)
3. difficulties with change and other unusual behaviours consistent with Kanner's "insistence on sameness" like repetitive or ritualized behaviour and narrow focus of interests
4. early onset (by 30 months)

It should be noted that Hans Asperger described the same syndrome, calling it autistic psychopathy, in 1944. Unlike Kanner however his work did not receive international attention until the 1980s (Wolff, 2004).

Systematic approaches

The ephemeral link between autism and schizophrenia, as well as the later realization that the age of onset may be as late as 30 months of age, and the later reduction of essential Symptoms to just two ("extreme aloneness" and "preoccupation with the preservation of sameness") coupled with onset within the first 2 years led to a wide array of at times widely different diagnostic criteria. (Rutter, 1978)
Later, the growing interest in autism and the growing number of works on the validity and approaches to definition and diagnostic led to the inclusion in the Diagnostic and Statistic Manual as Infantile Autism under the umbrella term of Pervasive Development Disorders (PDD).
In the DSM-III the definition of Infantile Autism was mostly just that: a definition of the condition as it first presented, without regard for later development. This was addressed by including the category of Residual Autism, *where the criteria had once been met*. The

diagnostic criteria were presented monothetically (American Psychiatric Association, 1980).

Addressing these and several other concerns, the DSM-III-R saw major changes for autism, the most relevant being that rather than meeting all criteria, the diagnosis could be made with 8 out of 16 criteria.

While being far more flexible, the DSM-III-R led to a high rate of false positives. It also had major differences with the pending revision of ICD-10. (Volkmar and McPartland, 2014)

DSM-IV, DSM-V and ICD-10 – Diagnostic criteria today

The current definition of ASD is far more flexible.

For the DSM-IV, coordination with ICD-10 revision was considered important. As the table below shows, efforts were made to synchronise the diagnosis.

One of the most important changes in the DSM-V is to autism spectrum disorder (ASD).

With the DSM-IV, four separate diagnosis were possible: autistic disorder, Asperger's disorder, childhood disintegrative disorder, or the catch-all diagnosis of pervasive developmental disorder - not otherwise specified (PDD-NOS). Anyone diagnosed with one of the four PDDs from DSM-IV should still meet the criteria for the umbrella diagnosis ASD in DSM-V or another, more accurate DSM-V diagnosis (American Psychiatric Association, 2013a)

<table>
<tr><td colspan="2">ICD-10</td><td colspan="2">DSM-IV-TR</td><td colspan="2">DSM-V</td></tr>
<tr><td>F84</td><td>Pervasive developmental disorders (PDD)</td><td></td><td>Pervasive developmental disorders (PDD)</td><td>A05/ 299.00/ F84</td><td>Autism Spectrum Disorders (ASD)</td></tr>
<tr><td>F84.0</td><td>Childhood autism</td><td>299.00</td><td>Autistic Disorder</td><td colspan="2" rowspan="2">Autism Spectrum Disorder (ASD)</td></tr>
<tr><td>F84.1</td><td>Atypical autism</td><td>299.00</td><td></td></tr>
<tr><td>F84.2</td><td>Rett syndrome</td><td>299.80</td><td>Rett Syndrome</td><td colspan="2"></td></tr>
<tr><td>F84.3</td><td>Other childhood disintegrative disorder</td><td>299.10</td><td>Childhood Desintegrative Disorder (CDD)</td><td colspan="2" rowspan="2">Autism Spectrum Disorder (ASD)</td></tr>
<tr><td>F84.4</td><td>Overactive disorder associated with</td><td></td><td></td></tr>
</table>

	mental retardation and stereotyped movements			
F84.5	Asperger syndrome	299.	Asperger Disorder	
F84.8	Other pervasive developmental disorders			
F84.9	Pervasive developmental disorder, not otherwise specified (PDD-NOS)	299.80	Pervasive developmental disorder, not otherwise specified (PDD-NOS)	

Tab. 1
Diagnostic criteria in ICD-10 (World Health Organization, 2015), DSM-IV-TR (American Psychiatric Association, 2000) and DSM-V (American Psychiatric Association, 2013b)

Diagnostic criteria according to DSM-V:

A) Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history:
   1. Deficits in social-emotional reciprocity
   2. Deficits in nonverbal communicative behaviors used for social interaction
   3. Deficits in developing, maintaining, and understanding relationships

B) Restricted, repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following, currently or by history:
   1. Stereotyped or repetitive motor movements, use of objects, or speech
   2. Insistence on sameness, inflexible adherence to routines, or ritualized patterns or verbal nonverbal behavior
   3. Highly restricted, fixated interests that are abnormal in intensity or focus.
   4. Hyper- or hyporeactivity to sensory input or unusual interests in sensory aspects of the environment

C) Symptoms must be present in the early developmental period.

D) Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.

E) These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay.

A and B are graded by severity in three levels, from level 1 (‘requiring support’) to level 3 (‘requiring very substantial support’).
Language impairment and intellectual impairment as well as association with other neurodevelopmental, mental, or behavioural disorder or with other genetic conditions or environmental factors may be specified separately.

Differential diagnoses include Rett’s syndrome, selective mutism, impairment of language and social interaction, intellectual disability without ASD, Stereotypic movement disorder, Attention deficit hyperactivity disorder and schizophrenia. (American Psychiatric Association, 2013b)

Diagnostic tools and issues

Autism is an involved diagnosis.
There is a great number of rating scales, interviews and questionnaires available.
Most commonly used in research are the Autism Diagnostic Interview-Revised (ADI-R), which is a semistructured parent interview (Lord et al., 1994), and the Autism Diagnostic Observation Schedule (ADOS). In clinical setting, the Childhood Autism Rating Scale (CARS) is a widely used scale based on observation.
Other tools are the Autism Behaviour Checklist, Autism Spectrum Rating Scales, Childhood Autism Rating Scale, Gilliam Autism Rating Scale, Diagnostic Interview for Social and Communication Disorders, the Asperger’s Syndrome (and High-Functioning Autism) Diagnostic Interview, the Psychoeducational Profile and several more. (Volkmar et al., 2014, pp. 609-616).
While there is a wealth of options for the diagnosis of ASD, there are several issues: the amount of time these tools consume, the amount of meaningful information versus the ability to code it, and the vulnerability to influence by the examiner.
As can be inferred from the above paragraphs, the diagnosis of ASD is a purely clinical one. There is no single biomarker for ASD.

Aetiology and risk factors

ASD is a multifactorial disorder.
Gestational factors that could affect neurodevelopment, such as complications during pregnancy and exposure to chemicals have been suggested to increase risk of autism (Lai et al., 2014). Thalodomide and anticonvulsants (especially sodium valproate) during

pregnancy as well as foetal alcohol syndrome are associated with ASD (Zafeiriou et al., 2007).

Risk factors that have been suggested are pre- or postnatal exposure to infections (rubella, HSV, CMV) and maternal daily smoking. Exposure to heavy metals (lead, mercury and cadmium) may be either a risk factor or a secondary issue, possibly because of decreased detoxification capacity. (Zafeiriou et al., 2007),

A connection between the measles, mumps, and rubella (MMR) vaccine and autism has been claimed, however, most recent studies have shown no effect of MMR on the incidence of ASDs (Zafeiriou et al., 2007).

An important risk factor seems to be advanced paternal age (Lampi et al., 2013).

Since the Rimland first noted the high concordance rate in monozygotic twins in the 1960s, numerous twin studies followed (Zafeiriou et al., 2007), proofing a high heritability in autism.

The concordance rates of 60% in monozygotic (MZ) twins versus 0% in dizygotic (DZ) twins for classic autism attest to genetic inheritance as the predominant causative agent. The markedly increased concordance (92% in MZ and 10% in DZ) when re-evaluating for a broader autistic phenotype suggests that interactions between multiple genes cause "idiopathic" autism but that epigenetic factors and exposure to environmental modifiers may contribute to variable expression of autism-related traits (Muhle et al., 2004).

The identity and number of genes involved are subject of avid research.

Syndromic Autism

In about 15% of the overall ASD diagnoses, the cause is a known syndrome or single gene mutation.

They are generally agreed to make up about 15% of the overall ASD diagnoses. (American Psychiatric Association, 2013a; Folstein et al., 2001), several of which are single gene mutations:

Among them are fragile X syndrome, tuberous sclerosis, untreated phenylketonuria (rare in countries with established neonatal screening programme) and Smith-Lemli Opitz syndrome (Freitag et al., 2006).

Neurofibromatosis type 1, Angelman's syndrome, Prader-Willi syndrome, Cohen syndrome, Smith Magenis syndrome, mukopolysaccharidosis type III, Rett's syndrome and isodizentric 15q chromosome syndrome are others. (Hochmiller, 2014)

That, however, still leaves some 85% of ASD diagnoses that can not be explained as syndromic.

Other pathogenesis' are presumed to be polygenetic, with possibly hundreds of different gene loci contributing in some minor way. (American Psychiatric Association, 2013b)

Once ASD is diagnosed, genetic evaluation is advisable, especially if other symptoms already suggest a syndromatic cause. A specific genetic diagnosis may allow for better anticipatory guidance, more precise genetic counselling with more accurate evaluation of recurrence risks, as well as prenatal diagnosis in the future if desired. (Caronna et al., 2008)

Genetics of non-syndromic ASD, CNVs

In addition to the syndromes mentioned above, several structural chromosomal aberrations were described in the past years in patients wit ASD (Borg et al., 2002; Kroisel et al., 2004).

An until recently undervalued percentage of the genome are DNA copy number variations (CNVs) that result in gain or loss of genomic DNA and can be of a size of up to several megabases. Many CNVs of varying lengths are already registered. Through new high resolution techniques like array comparative genomic hybridization (aCGH) or next generation sequencing, more CNVs are being found rapidly. (Cook and Scherer, 2008).

Several studies show a strong association of de novo (i.e. not inherited, but newly occurring) CNVs with ASD (Sebat et al., 2007; Weiss et al., 2008).

For studying those numerous as of yet not completely understood mutations, known syndromes must be ruled out beforehand.

Array comparative genomic hybridization (aCGH)

The aCGH is a method of comparative genomic hybridization in which – unlike FISH (fluorescence in-situ hybridization) or metaphase CGH – the target DNA is not presented as metaphase chromosomes.

Rather, small fragments of reference DNA of defined size are affixed on a glass surface within a coordinate system. In this array, an equal amount of the patient's DNA is co-hybridized.

By using different colour markers (for example red for the patient's DNA and green for the reference DNA), a quantitative change (meaning a loss or gaine) in patient DNA leads to a colour shift of the fluorescence signals of single fragments.

This shift can be detected by a scanner and analyzed with appropriate software.

The aCGH permits the genome wide detection of unbalanced chromosomal aberrations and offers the possibility of recognizing far smaller aberrations in the range of less than 100 kilo bases.

This makes it the most important method of clarification for children with delayed development or mental retardation and dysmorphias. In 15-20% of all unclear dysmorphia or retardation syndromes a deletion or duplication can be proven via array-CGH. Those aberrations are usually of too small a size to be noticeable with other methods.

(Murken et al., 2011, pp. 161-163)

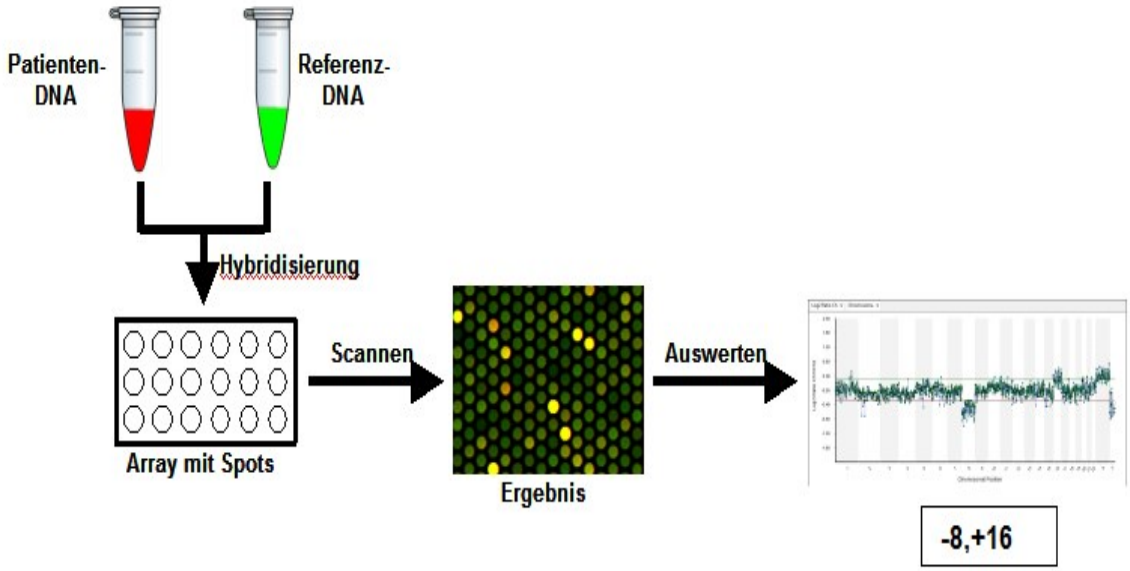

Fig. 1
Simplified schematic representation of the array-CGH method. (Reprogenetics Germany GmbH, n.d.)

Literature research

Synaptopathy and synaptic stability relevant to ASD

Synaptopathy is an increasingly popular term used to define key features of neurodegenerative and psychiatric disease. It implies that disruptions in synaptic structure and function are potentially the major determinant of such brain diseases (Brose et al., 2010).

There is significant interest in the role of synaptic dysfunction in ASD (Betancur et al., 2009) as well as other neuropsychiatric diseases (Glantz et al., 2006; Hall et al., 2009).

Both structural and functional alterations of synaptic connections, from abnormal density and morphology of dendritic spines to aberrant synaptic signalling and plasticity as well as synpse loss, have been suggested to be involved in neurological and psychiatric disorders (Blanpied et al., 2004).

It is interesting to note that many of the rare genetic variants of ASD have some relation to synaptic cell-adhesion molecule (CAM) pathways, which have roles in synapse development, function and plasticity (Betancur et al., 2009).

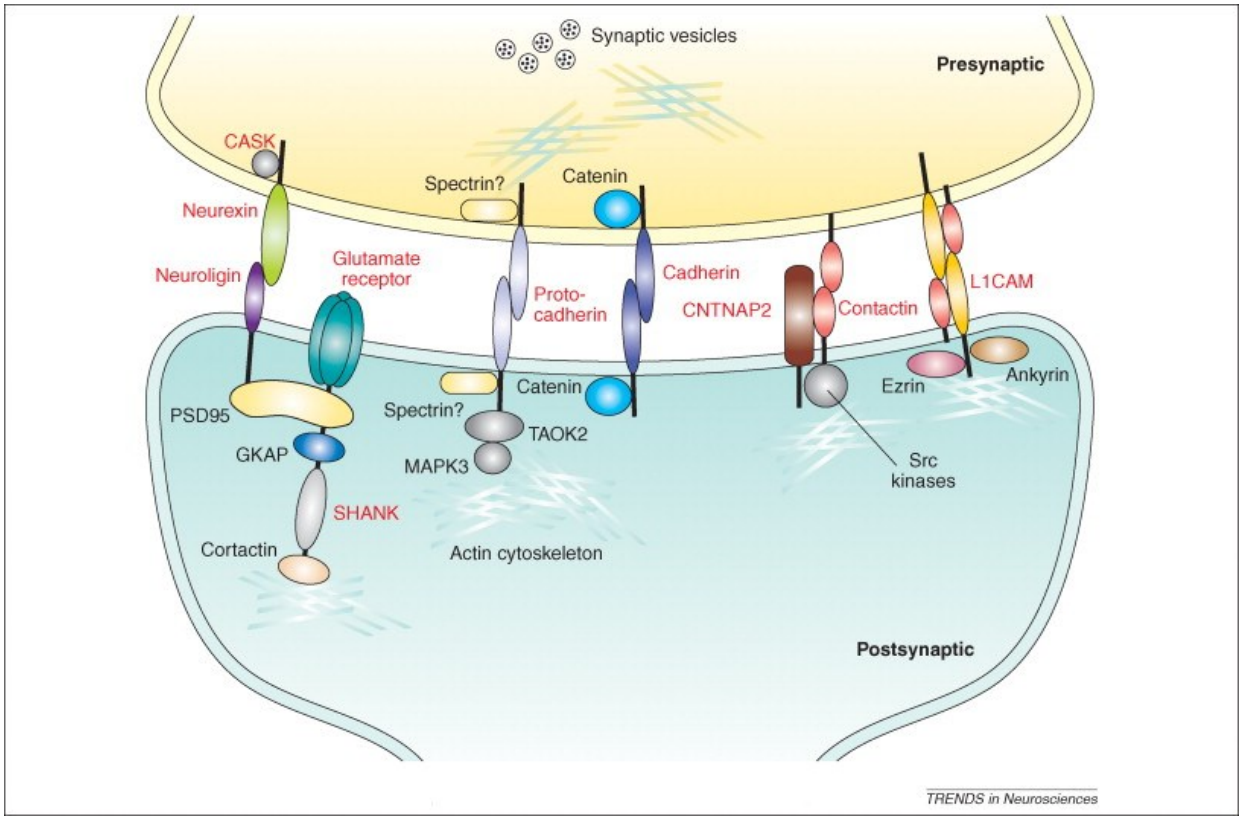

Fig. 2
Schematic of different synaptic cell adhesion molecules (CAM) pathways. Proteins whose genes have been implicated in ASD and/or ID are indicated in red. The question mark next to spectrin shows that the interaction with protocadherins is unclear. (Betancur et al., 2009)

As shown in the picture, these CAMs are involved in the initial contact between pre- and postsynaptic cell, maintaining synaptic adhesion and act as anchors for scaffolding proteins.
These scaffolding proteins assemble signalling molecules, neurotransmitter receptors and proteins in the cytoskeleton. They all work together in the development and plasticity of synapses (Okabe, 2007).

Synaptic structure

Functional synapses are highly elaborate, asymmetric neuron-neuron contacts.
The presynaptic site includes:

- the active zone, where neurotransmitter release occurs
- the cytomatrix of active zones, a network of scaffolding proteins
- a cluster of vesicles containing neurotransmitters

The postsynaptic site includes:

- an accumulation of neurotransmitter receptors, directly opposed to the active zone
- scaffolding proteins

For synapses to function, all these components must be recruited and precisely aligned across the synaptic cleft, a 20-nm-wide extra cellular space that separates two neurons at synaptic junctions.
Additionally, transmembrane proteins both pre- and postsynaptic bind each other in the extra cellular space (Dean, 2006).

Neuroligins

Neuroligins are type 1 transmembrane proteins (N-terminus located in the extra cellular space) and constitute a family of five postsynaptic cell adhesion proteins that interact with presynaptic neurexins via a large extra cellular esterase-like domain (Varoqueaux et al., 2006).
Intracellularly, neuroligins bind to several scaffolding proteins, which in turn interact with postsynaptic transmitter receptors, ion channels, and signalling proteins (Meyer et al., 2004).

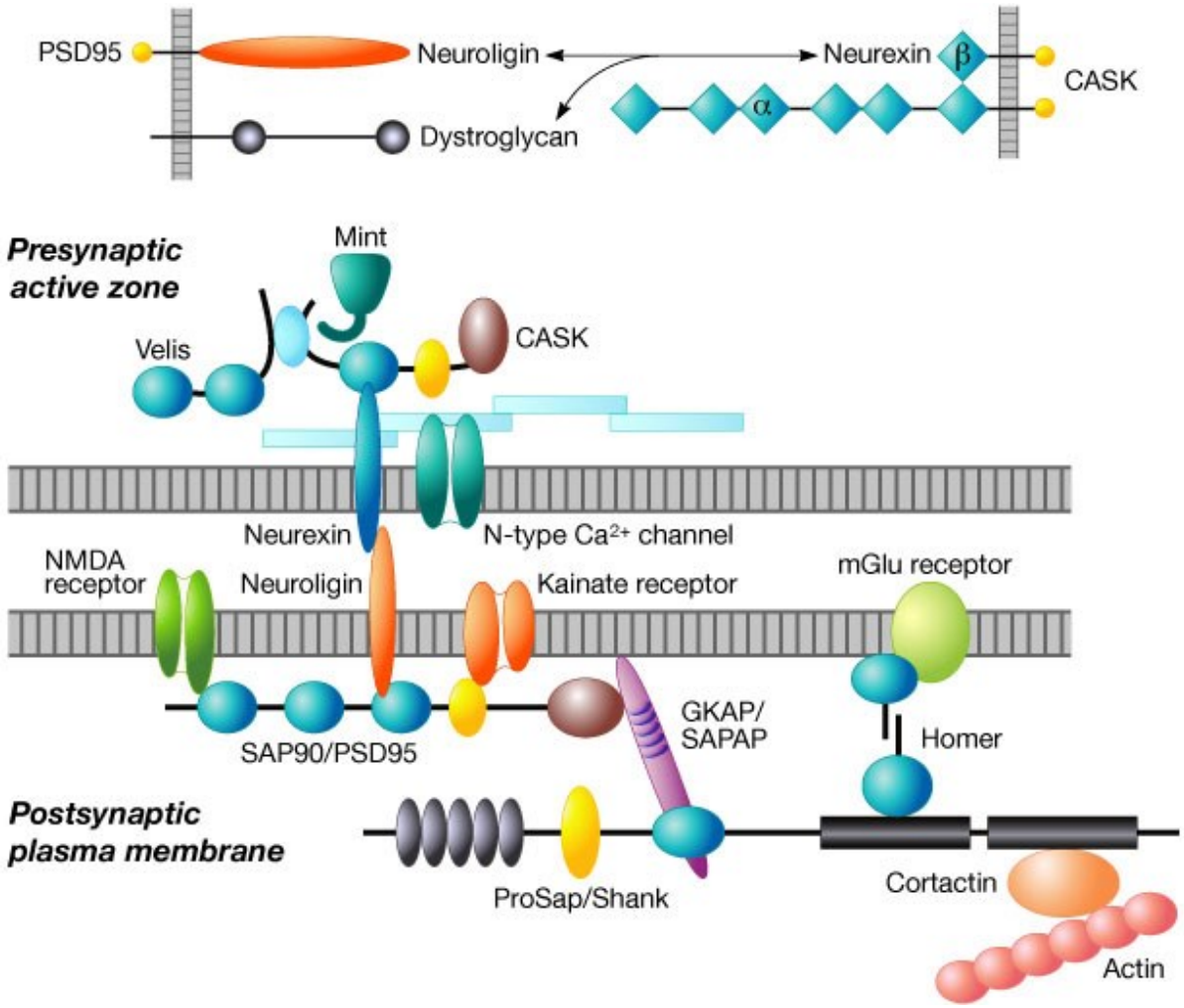

Fig. 3
Pre- and postsynaptic binding partners of neuroligin (Max-Planck-Gesellschaft, 2006)

Neurolign in CNS synapses

The neuroligin family has five proteins: NLGN-1, NLGN-2, NLGN-3, NLGN-4 (or NLGN-4X) and NLGN-4Y (sometimes referred to as NLGN-5). As NLGN-4Y is greater than 97% indentical in protein sequence to NLGN-4X, they are often discussed together as NLGN-4 (Bemben, 2015).

Neuroligin is a significant part of synaptic structuring in GABAergic and glutamatergic synapses.

Gene	Location	Protein	number of amino acids	Primarily active at these synapses
NLGN-1	3q26	Neuroligin 1	840/ 823	Positive regulation of excitatory postsynaptic potential

NLGN-2	17p13	Neuroligin 2	835	Positive regulation of excitatory postsynaptic potential
NLGN-3	Xq13	Neuroligin 3	848/ 828/ 808	Positive and negative regulation of excitatory postsynaptic potential
NLGN-4X	Xp22.3	Neuroligin 4	816/ 836	Negative regulation of excitatory postsynaptic potential
NLGN-4Y	Yq11.2	Neuroligin 4	816/ 648/ 134/ 256	Negative regulation of excitatory postsynaptic potential

Tab. 2
Overview of the NLGN family in CNS synapses (UniProtKB, 2015)
Note that while NLGNs are found in different cells and synapses, only the ones relevant ASD are shown here.

Function

Across the synapse exists a fundamental physical interaction mediated by synaptic cell adhesion molecules (CAMs). It is among the earliest and most indispensable of molecular events occurring during synaptogenesis (Bemben et al., 2015).

Neuroligins are part of a larger superfamily of postsynaptic adhesion molecules.

Their primary binding partner on the presynaptic side are the neurexins, which have several subtypes and a significant number of splice variants (Bemben et al., 2015).

The junction formed by neuroligins and neurexins resembles the architecture of tight junctions, but differs from them in that it is asymmetric in all of its components (Südhof, 2008).

Neuroligins are generally present as oligomers. Both homodimerization and and heterodimerization have been repeatedly confirmed for neuroligins 1, 2 and 3, yet there neuroligin 4 remains completely untested. Dimerization seems necessary for both trafficking of neuroligin and a requirement for synaptogenic properties of neuroligin (Poulopoulos et al., 2012; Shipman et al., 2012), but the actual mechanisms are not yet fully explained.

Post-translational modifications such as phosphorylation have been postulated as possibly responsible for isoform-specific regulation, but once again, the exact mechanism still needs more research (Bemben et al., 2015).
N-glycosylation has been shown to be of influence in the neurexin binding preferences of NLGN1 and its ability to potentiate excitatory synapses (Comoletti et al., 2003).

Role in Synapse development

Neuroligins have a role both in the formation of synaptic contacts and their maturation (Levinson et al., 2005).
Studies have shown that expression of neuroligins in non-neuronal cells induces presynaptic specializations in contacting axons (Scheiffele et al., 2000), and over-expression in cultured neurons increases the number of synapses formed, whereas knockdown of neuroligin expression leads to a reduction in synapse density and inhibits postsynaptic maturation (Chih et al., 2005).
This leads to the conclusion that the transsynaptic neuroligin/neurexin link is of key importance both in the induction phase of synaptogenesis and in synapse maturation, where they recruit scaffolding proteins, postsynaptic receptors and signalling proteins to nascent synapses (Varoqueaux et al., 2006).
Interestingly, it has to be noted that the formation of synaptic contacts does happen without neuroligin, as knock-out mice do exhibit a relatively normal number of synapses. They do, however, display a severe impairment of synaptic transmission and die at birth (Varoqueaux et al., 2006).
The regulation of adhesion molecules and their interactions with other synaptic proteins by neuroligins likely affect not only on synapse formation but also on ongoing synaptic function (Bemben et al., 2015).
Neuroligins influence the balance between glutamatergic and GABAergic/glycinergic transmission without affecting the total number of synapses. (Varoqueaux et al., 2006)
That neuroligins are also essential for proper brain function in humans is shown by the severe consequences of loss-of-function mutations in the NLGN3 and NLNG4 genes that result in autism and/or metal retardation (Jamain et al., 2003; Laumonnier et al., 2004; Talebizadeh et al., 2006).
In summary it can be said that neuroligins, by binding either neurexins or other ligands, trigger pre- and postsynaptic signal transduction events that activate synaptic function and specify synaptic properties. While synapses assemble without this activation, they do not

work properly. Clearly, this activation is not a simple yes-or-no switch. The precise details are not yet known (Südhof, 2008).

Relevant publications about NLGN-3 and NLGN-4 mutations

Considering the gender distribution of ASD, NLGN-3 and NLGN-4X are of special interest and the primary research parameter employed for this search.

Screened for	Number of screened	Number of controls	Country	Evidence relating to NLGN3/4x	number of mutants	Phenotype	Inheritance	Reference
	8 females with deletion of Xp distal to DXS1224			NLGN-4X deletion	3 females, unrelated	ASD	unknown	Thomas et al., 1999
NLGN3, NLGN4, NLNG4Y	36 sibling pairs, 122 trios with autism or Asperger syndrome (140 males, 18 females)	350	Sweden	frameshift mutation (1186insT), -> premature termination before the transmembrane (D396X)	1 male	ASD	de novo mutation of the mother	Jamain et al., 2003
					1 male, brother	Asperger syndrome		
			Sweden	C->T transition, R451C in Nlgn3: reduces membrane trafficking	1 male	ASD	maternal ineritance	
					1 male, brother	Asperger syndrome		
					1 male, brother	none		
NLGN4	Large extended family with 13 affected male subjects	200	France	2–base-pair deletion, leading to a frame shift and a premature stop codon	All related, 10 males	nonspecific MR	yes	Laumonnier et al., 2004
					2 males	ASD + nonspecific MR		
					1 male	PDD-NOS		
NLGN3:exon 6 and NLGN4: exon 5	67 unrelated with ASD, 41 females and 26 males		USA: 63 white, 3 black, 1 other	none	none	none		Talebizadeh et al., 2004
NLGN3, NLGN4	196 unrelated with ASD (149 male, 47 female)		UK, Canada, AGRE	none	none	none		Vincent et al., 2004
NLGN3, NLGN4	96 unrelated with ASD		Canada	none	none	none		Gauthier et al., 2005

NLGN1, NLGN3, NLGN4, NLGN4Y	27 with ASD, 3 with AS	85	Finland	NLGN3: 222C>T (silent mutation)	1	ASD	yes	Ylisaukko-oja et al., 2005
NLGN1, NLGN2, NLGN3, NLGN4, NLGN4Y	194 (148 unrelated with ASD, 24 with ADHD, 24 with BPD)	288	Midwest US Caucasian, Portuguese	NLGN4: NT.change: 759 G>A; A.A.change: G99S	1 female	ASD, LD	Mother is heterozygous	Yan et al., 2005
					1 male, brother	language disability		
					1 female, mother (heterozygous)	LD		
				NLGN4: NT.change: 1597 A>G; A.A.change: K378R	1 male	Mild ASD and normal IQ	Mother is carrier	
				NLGN4: NT.change: 1671 G>A, A.A.change: V403M	1 male	PDD	Mother is carrier	
					2 males, brothers	none		
				NLGN4: NT.change: 2574 C>T; A.A.change: R704C	1 male	ASD	Mother is carrier	
					1 female, sister	PDD		
					1 female, sister	none		
NLGN3, NLGN4	10 unrelated females with ASD	30		NLGN4: exon 4 deleted in transcript only (SNP)	1 female	ASD	unknown	Talebizadeh et al., 2006
				NLGN3: truncated isoform of the transcript (exon 7 missing)	9 females, unrelated	ASD	unknown	
					30 unrelated	none, controls	unknown	
FISH, PCR, X-inactivation assay	13 related			terminal deletion Xp22.2-22.3 (26 genes, including NLGN4)	1 male	ASD, congenital nystagm, retinal dystrophy,alternating strabismus	yes	Chocholska et al., 2006
					1 female, mother	none		
					1 female, maternal aunt	MR		
					1 female, half-sister	severe MR, akathisia, one-sided hearing loss		
					1 female, grandmother	severe strabism		

NLGN3/4	124	250	IMGSAC	NLGN3: Thr632Ala	2 siblings	ASD	Mother is carrier	Blasi et al., 2006
					1male, 4 females	none, controls	unknown	
				NLGN3: 2 intronic changes	5	ASD	unknown	
				NLGN4x: five different sequence changes, all known polymorphisms		ASD	unknown	
Deletion analysis, NLGN4	3 males, related	0		4.5Mb deletion Xp22.3 (9 genes, including NLGN4)	1 male	ichthyosis, hypogonadotropic hypogonadism, unilateral renal aplasia, bimanual synkinesia	mother and grandmother are carrier	Macarov et al., 2007
					1 male, maternal uncle	ichthyosis, hypogonadism, synkinesia, no ID		
					1 male, maternal grandmother's brother	ichthyosis, hypogonadism, synkinesia, mild ID		
NLGN4	2 females, 2 males	96	Great Britain	deletion encompassing exons 4, 5, and 6, 756 797 bp	1 male	ASD	yes	Lawson-Yuen et al., 2008
					1 male, brother	Tourette Syndrome + ADHD + ID		
					1 female, mother	LD+Depression+Anxiety		
					1 female, grandmother	Anxiety		
NLGN3, NLGN4X	107 unrelated with Asperger syndrome, high-functioning autism and atypical autism			four previously known SNPs: rs2290488, rs7049300, rs3747333, rs3747334, no amino acid substitution	8 unrelated males with one or more of the SNPs	five AS, two HFA	unknown	Wermter et al., 2008
				one novel synonymous variant (A558) in the NLGN4X	1 female	ASD	unknown	

genome-wide assessment for structural abnormalities via SNP microarrays and karyotyping	427 unrelated with ASD	500		Xp22.33-p22.31 loss (21 genes + NLGN4)	1 female	ASD	de novo	Marshall et al., 2008
coding and regulatory sequence of NLGN4	96 unrelated with ASD (80 male, 16 female)		France	upstream from NLGN4x: −335G>A, leading to a 2.5-fold increase in the NLGN4X transcript level	1 male	ASD, nonsyndromic profound MR	de novo	Daoud et al., 2009
500K SNP-microarray, FRAXA, NLGN3, 5 coding exons in NLGN4 in both directions	2 brothers	300	UK	single amino-acid substitution (R87W) in the NL4 gene, hemizyguos	2 brothers	ASD	maternal germ-line mosaicism	Zhang et al., 2009
Exon 1 the NLGN4 gene in both directions, X-inactivation assay	Mother			mildly skewed X-inactivation pattern (75:25%)	1 female, mother	none		
Exon 1 the NLGN4 gene in both directions, NLGN4Y	father			none	1 male, father	none		
NLGN3, NLGN4	229 with ASD	184	Han Chinese	intronic SNP rs4844285 in NLGN3	1	ASD	unknown	Yu et al., 2011
				3-marker haplotype block (rs11795613-rs4844285-rs4844286)	1	ASD	unknown	
NLGN3, NLGN4	285 with ASD (246 males, 39 females)	384	Han Chinese	rs7049300 (Thr311Thr)	1	ASD	unknown	Liu et al., 2013
				SNPs in noncoding regions of NLGN4	6	ASD	unknown	

Tab. 3
Evidence of the role of mutations in NLGN3/NLGN4 in ASD and other neuropsychiatric disorders and relevant publications.
Abbreviations: AGRE – autism genetic resource exchange, ADHD – attention deficit hyperactivity disorder, BPD – bipolar disorder, FISH – fluorescence in situ hybridization, IMGSAC - International Molecular Genetic Study of Autism Consortium, LD – learning disorder, MR – mental retardation, PCR – polymerase chain reaction, PDD – pervasive development disorder, PDD-NOS – pervasive development disorder not otherwise specified, SNPs – single nucleotide polymorphisms

Thomas et al. (1999) were the first to publish a report of small deletions in Xp22.3 in 8 females, 3 of which had ASD, suggesting that a critical region for autism may be related there. They hypothesised that either loss of function of a specific gene within the deleted region or functional nullisomy resulting from X-inactivation of the normal X chromosome may be the reason.

Following this, Jamain et al. (2003) screened for NLGN3 and NLGN4/4Y mutations in 36 ASP and 122 trios with autism or AS (140 males and 18 females).
They found a frameshift mutation (1186insT) in one Swedish family with two affected brothers, one with typical autism and the other with AS. This mutation creates a stop codon at position 396, leading to premature termination of the protein before the transmembrane domain. The mutation is present in the mother but absent in the maternal grandmother and two maternal aunts. The mutation was not found in the unaffected brother and in 350 unrelated controls (250 females and 100 males).
In a second Swedish family with two affected brothers, one with typical autism and the other with AS, they identified a C to T transition in NLGN3 inherited from the mother and changing a highly conserved arginine residue into cysteine (R451C) within the esterase domain. R451 is located in a predicted EF-hand domain conserved in all known neuroligins and in all sequenced esterases from mammals, fish and birds. EF-hand domains are known to confer structural integrity and Ca2+ dependent functional properties. R451C may therefore modify the binding of neuroligins to neurexins since binding is only observed in the presence of Ca2+ 15. This mutation was absent in 200 controls (100 females and 100 males).

Laumonnier et al. (2004) examined a large extended family with 13 males whose diagnoses ranged from non-specific mental retardation (MR) to ASD with and without MR. They found a 2–base-pair deletion, leading to a frame shift and a premature stop codon in the middle of the sequence of the normal protein and is thought to suppress the transmembrane domain and sequences important for the dimerization of neuroligins that are required for proper cell-cell interaction through binding to β-neurexins.
As the neuroligins are mostly enriched at excitatory synapses, these results suggest that a defect in synaptogenesis may lead to deficits in cognitive development and communication processes. The fact that the deletion was present in both autistic and non-autistic mentally retarded males suggests that the NLGN4 gene is not only involved in autism but also in

mental retardation, indicating that some types of autistic disorder and mental retardation may have common genetic origins.

In a letter to the editor, Talebizadeh et al. (2004) screened specifically for the mutations reported by Jamain (2003) and Laumonnier (2004): exon 6 in NLGN3 and exon 5 in NLGN4) in 67 ASD patients. They found none.

Vincent et al. (2004), screening a larger sample of 196 autism probands, also failed to identify any mutations that would affect the coding regions of these genes.

Similarly, Gauthier et al. (2005) screened 96 unrelated ASD patients for mutations in NLGN3 and NLGN4, finding no mutation of any sort in those genes.

In 2005, Ylisaukko-oja et al. published a detailed molecular genetic analysis of NLGN 1, NLGN3, NLGN4, and NLGN4Y in 30 Finnish probands with ASD selected from families producing linkage evidence for Xq13 and/or 3q26 loci. They revealed several polymorphisms, none of which seemed to be functional.
One rare variant was found in NLGN3: a silent Y74Y (222C>T) mutation in the first protein coding exon present in one affected male, that consegregated completely with the phenotype in the family.
None was found in NLGN4/4Y.
They concluded that neuroligin mutations most probably represent rare causes of autism and that it is unlikely that the allelic variants in these genes would be major risk factors for autism.

Yan et al. (2005) scanned the coding regions and associated splice junctions of the NLGN4 gene for mutations in 148 unrelated patients with autism, 24 Midwest US Caucasian patients with attention deficit hyperactivity disorder (ADHD) and 24 UK Caucasian patients with DSM-IV Bipolar I Disorder (BPD).
Putative missense mutations were identified once each in the NLGN4 gene in four separate autistic patients: G99S and K378R were found in unrelated Portuguese patients. V403M and R704C were found in unrelated Midwest patients. G99, K378 and V403 are located in the esterase domain and R704 is located in the cytoplasmic domain. Three of the structural changes, K378R, V403M and R704C, occur in asymptomatic mothers, while G99S occurs in a mother with learning disability.

This study suggests that missense changes in neuroligin 4 may contribute to autism susceptibility as well as the protein-truncating mutations reported by Jamain et al. (2003) and Laumonnier et al. (2004).

Talebizadeh et al. (2006) studied 10 young autistic females and 30 non-autistic subjects for alterations in NLGN3 and NLGN4.
A novel NLGN4 isoform lacking exon 4, which occurred de novo on the paternal allele, was identified in one of the autistic females.
Monoallelic expression of NLGN4 was seen in this subject and in 11 of 14 informative autistic and non-autistic females using a single nucleotide polymorphism found at 3′ UTR.
Additionally, the NLGN3 transcript was present in two isoforms (with and without exon 7) in nine of 10 autistic females and in 30 non-autistic subjects, including parents of the autistic female having only the complete transcript with exon 7, and from the whole brain of a control.
It was speculated that the novel truncated NLGN3 product may have a regulatory role.
They concluded that splice variants may lead to potentially abnormal neuroligins in the causation of autism spectrum disorders.

Chocholska et al. (2006) described a familial interstitial deletion of 7.7-Mb involving Xp22.2-22.3. The deletion was transmitted from an asymptomatic mother to her two children with severe developmental delay, no speech development and autistic behaviour.
Assessment of the deletion boundaries by FISH and PCR analyses indicated that the deletions encompasses 27 genes, including some that are associated with known disorders, like KAL1 (Kallmann syndrome), steroid sulfatase (STS) (X-linked ichtyosis), and arylsulfatase E (ARSE) (chondrodysplasia punctata).
The deletion also includes all four VCX genes (VCX-A, VCX-B1, VCX-B, and VCX-C) and the neuroligin 4 (NLGN4) gene (VCX-A deficiency has been shown previously to be associated with mental retardation).
The phenotype associated with the Xp deletion was highly variable in female carriers, including MR, akathisia, one-sided hearing loss, severe strabism, as well as ASD in one male carrier.
For the females, this might be attributed to unfavorable X inactivation. However, all the 27 genes included in the deleted interval escape X inactivation and are expressed at variable levels from the normal X chromosome. Thus, the overall X inactivation pattern and inter-

individual expression variability of the genes in distal Xp might be determinants of the phenotype associated with the deletion.

Blasi et al. (2006) performed a mutation screening of NLGN3 and NLGN4X in a sample of 124 autism probands from the International Molecular Genetic Study of Autism Consortium (IMGSAC). They identified a new non-synonymous variant in NLGN3 (Thr632Ala), which is likely to be a rare polymorphism.
In NLGN4X, they found five different sequence changes, all corresponding to known polymorphisms.

Macarov et al. (2007) clinically and molecularly described a pedigree of three generations affected by contiguous gene syndrome that includes features of X-linked ichthyosis and Kallmann syndrome. Molecular analysis revealed the presence of an interstitial deletion spanning approximately 4.5Mb at Xp22.3.
The deletion of VCX-A and NLGN4 in this family prompted them to examine the cognitive functions of the two adult patients using comprehensive intellectual and neurocognitive assessment. Normal intellectual function was found in one patient and mild ID was revealed in the other. Neither patient met any DSM-IV criteria for a pervasive developmental disorder such as autism.
These findings suggest that deletion of VCX-A and NLGN4 can result in variable phenotypic features and that normal mental development can be achieved despite this deletion, emphasizing the importance of environmental factors and possible modifier genes.

Lawson-Yuen et al. (2008) described a family with a deletion of exons 4, 5, and 6 of NLGN4 and a variety of neuropsychiatric illnesses.
The proband is an autistic boy with a motor tic, his brother has Tourette syndrome and attention deficit hyperactivity disorder, their mother, a carrier, has a learning disorder, anxiety, and depression, and their maternal grandmother has anxiety.
This family demonstrates that NLGN4 mutations can be associated with a wide spectrum of neuropsychiatric conditions and that carriers may be affected with milder symptoms.

Wermter et al. (2008) performed a mutation screen of NLGN3 and NLGN4 in 107 probands with Asperger syndrome, high-functioning autism and atypical autism.

They identified four polymorphisms (rs2290488, rs7049300, rs3747333, rs3747334) and one novel synonymous variant (A558) in the NLGN4X. The polymorphisms rs7049300, rs3747333, and rs3747334 did not cause any amino acid substitutions in the total of the eight detected carriers.
A family-based association study for rs2290488 in 101 trios did not reveal association of this polymorphism with autistic disorders on high functioning level.
They conclude that there is no evidence for an involvement of NLGN3 and NLGN4X genetic variants with autism spectrum disorder on high functioning level in our study group. © 2008 Wiley-Liss, Inc.

Marshall et al. (2008) performed a genome-wide assessment for structural abnormalities in 427 unrelated ASD cases via single-nucleotide polymorphism microarrays and karyotyping.
With microarrays, they discovered 277 unbalanced CNVs in 44% of ASD families that were not present in 500 controls.
Karyiotyping detected additional balanced changes, most of which were inherited variants. A total of 27 were de novo alterations.
One of those was a deletion of Xp22.33-p22.31 (21 genes + NLGN4) in a female with ASD.

Daoud et al. (2009) studied 96 patients who met al.l DSM-IV criteria for autism, analyzing the entire coding sequence and the regulatory sequences of the NLGN4X gene by polymerase chain reaction and direct sequencing to gain an understanding of possible altered expression level of NLGN4X that would be caused by mutations in regulatory sequences.
They investigated by analyzing these regions in patients with ASDs and no mutation in the NLGN4X coding sequence.
They identified a de novo 1 base pair (−335G>A) substitution located in the promoter region in a patient with autism and nonsyndromic profound MR. This variation is associated with an increased level of the NLGN4X transcript in the patient compared with male control subjects as well as his father. Further in vitro luciferase reporter and electrophoretic mobility shift assays confirmed, respectively, that this mutation increases gene expression and is probably caused by altered binding of transcription factors in the mutated promoter sequence.
This suggests that the analysis of the expression level of NLGN4X might detect new cases.

Zhang et al., (2009) described two brothers with ASD who carry a single amino acid substitution in NLGN4 (R87W).

This substitution was absent from the brothers' asymptomatic parents, suggesting that it arose in the maternal germline. R87 is conserved in all NLGN isoforms, and the R87W substitution was not observed in control individuals.
At the protein level, the R87W-substitution impaired glycosylation processing of NLGN4 expressed in cell cultures, destabilized neuroligin 4, caused neuroligin 4 retention in the endoplasmic reticulum in non-neuronal cells and neurons, and blocked neuroligin 4 transport to the cell-surface.
As a result, the R87W-substitution inactivated the synapse-formation activity of neuroligin 4, and abolished its functional effect on synapse strength.
Viewed together, these observations suggest that a point mutation in NLGN4 can cause ASD by a loss-of-function mechanism.

Yu et al. (2011) screened for 7 known ASDs-related rare variants in NLGN3 and NLGN4X genes for replication of the initial findings and 12 intronic tagging single nucleotide polymorphisms (SNPs) were genotyped for case-control association analysis in a total of 229 ASD cases and 184 control individuals in a Chinese Han cohort.
They found that a common intronic variant (SNP rs4844285) in NLGN3 gene, and a specific 3-marker haplotype XA-XG-XT (rs11795613-rs4844285-rs4844286) containing this individual SNP were associated with ASDs and showed a male bias, even after correction for multiple testing (SNP allele: $P = 0.048$, haplotype: $P = 0.032$).
Simultaneously, none of the 7 known rare mutation of NLGN3 and NLGN4X genes was identified, neither in patients with ASDs nor in controls, giving further evidence that these known rare variants might be not enriched in Chinese Han cohort.
This study provides initial evidence that a common variant in NLGN3 gene may play a role in the etiology of ASDs among affected males in Chinese Han population, and further supports the hypothesis that defect of synapse might involvement in the pathophysiology of ASDs.

Liu et al. (2013) examined the copy numbers of four genes (NLGN4, NLGN3, SHANK2, and SHANK3) in 285 ASD cases using multiplex fluorescence competitive polymerase chain reaction (PCR).
They also screened the regulatory region including the promoter region and 5′/3′ untranslated regions (UTR) and the entire coding region of NLGN4 in a cohort of 285 ASD patients and 384 controls by direct sequencing of genomic DNA.

DNA copy number calculation in four genes showed no deletion or duplication in our cases. No missense mutations in NLGN4 were identified. Association analysis of 6 common SNPs in NLGN4 did not find significant difference between ASD cases and controls.

These findings showed that these genes may not be major disease genes in Chinese ASD cases.

In summary

Supporting NLGN mutations as a cause for ASD

Thomas et al. (1999), Jamain et al. (2003), Laumonnier et al. (2004), Yan et al. (2005), Talebizadeh et al. (2006), Lawson-Yuen et al. (2008), Marshall et al. (2008), Daoud et al. (2009) and Yu et al., (2011) found viable evidence linking mutations in NLGN3 and/or NLGN4/4Y or their proteins to ASD.

Laumonnier et al. (2004), Yan et al., 2005, Chocholska et al. (2006), Blasi et al. (2006), Lawson-Yuen et al. (2008) and Zhang et al., (2009) linked the genes and proteins to other phenotypes like non-specific MR, LD, language disability, Tourette syndrome, ADHD, learning disorder, depression and anxiety, or only found loose connections to ASD.

Evidence without clearly supporting NLGN mutations as a cause for ASD,

Ylisaukko-oja et al. (2005) and Talebizadeh et al. (2006) found no hard evidence, only non-coding (silent) mutations or mutations also present in healthy controls.

No evidence for NLGN mutations as a cause for ASD

Talebizadeh et al. (2004), Gauthier et al. (2004), Vincent et al. (2005), Wermter et al. (2008) and Liu et al. (2013) found no evidence linking NLGN3/4/4Y or the proteins to ASD, while Macarov et al. (2007) proved the possibility of normal cognitive development despite the deletion of NLGN4 and other nearby genes.

Discussion of mutations

Models for explaining genetic aetiology

There have been two models for explaining genetic aetiology that are used in many genetic diseases, including ASD. They are not mutually exclusive.

Name of model	Common disease-common variant	common disease – rare variant', 'multiple rare variant', 'rare alleles of major effect' model
Allelic architecture	Genetic polymorphisms found widely distributed in the population	Large number of rare or very rare variants
Risk	Modest increase in disease risk, ORs <2	Highly penetrant, ORs can be very substantial
Possible aetiology	Interaction of multiple common genetic variants and possibly other non-genetic factors	Might contribute the major part of the susceptibility

Tab. 4
Models for explaining genetic aetiology (Pritchard et al., 2002; Betancur et al., 2009)

Common disease-common variant

Common variants are genetic polymorphisms (susceptibility alleles) that can be found widely distributed in the population. They are associated with a modest increase in disease risk, with the odds ration (OR) <2, often in the range of ~1-1.3.

According to this model, a given disorder stems from the interaction and combination of multiple common genetic variants and possibly other non-genetic factors.

This model has been challenged from a population genetic perspective because, assuming any sort of selection against even moderately deleterious variants, the rates of variants would be very low (Betancur et al., 2009).

A possible explanation would be for susceptibility alleles to simultaneously offer some other advantage, e.g. β-globin defects that lead to sickle cell anaemia protect heterozygotes against malaria (Elguero et al., 2015), which results in selection *for* the susceptibility allele.

Multiple rare variant

The 'multiple rare variant' model assumes a very large number of rare or very rare variants of high penetrance with possibly very substantial ORs.

Unlike susceptibility alleles, these variants might contribute the major part of the susceptibility for a given individual. At the extreme, rare variants are equivalent to rare deleterious mutations (Betancur et al., 2009).

Applying the two models to ASD

Attempting to explain the complexities of the genetic aetiology of ASD with a single model would likely be a mistake.

For different aetiologies, different models may offer reasonable explanations. Some thoughts on the topic are presented in the following chapters.

CNVs and SNPs

While they are being researched more and more, the sheer number of CNVs means that research is by no means even near complete.

CNVs in general are part of the genetic variety and incredibly common. Whatever their contribution to susceptibility for a given individual, as a whole, they fall under the umbrella of the common disease – common variant model.

The same applies to SNPs, which are often non-coding mutations. Even if they do result in the substitution of an amino acid, it has to be remembered that not every coding mutation does necessarily cause pathology.

It has to be noted that while the common disease – common variant model is an appealing one in theory, the practical application is nearly impossible for the simple reason that a common variant is, by definition, common.

Following the terms dictated by this model, one would necessarily find individuals with and without the disease and with and without the mutation. Being able to actually pin down a genetic variant that only contributes a tiny amount of the overall susceptibility of an individual is very unlikely. This will likely remain the case until such a time at which the understanding of genetics is significantly improved.

Syndromic ASD and known genetic contributors

While rigorous epidemiological studies have yet to be carried out, there are reasonable estimates for the prevalence of some of the genetic contributors to ASD. The table below lists some of the more common ones, along with some of the identified rare variants (Buxbaum, 2009).

Genetic etiology	Estimated prevalence in ASD
Karyotype abnormalities	5-10%
Fragile X syndrome	2%
15q abnormalities	2%
Tuberous sclerosis	1%
16p11 deletions	1%
22q13 deletions/SHANK3 abnormalities	0,75%

22q11 abnormalities	0,50%
Rett syndrome	0,10%
c30rf58 deletions, homozygous	Rare
CNTN4 deletions	Rare
DPP10 deletions	Rare
DPP6 Deletions	Rare
NHE9 deletions, homozygous	Rare
NLGN deletions/mutations	Rare
NRXN1 deletions	Rare
PCDH10 deletions, homozygous	Rare
PCDH9 deletions	Rare
PTEN mutations	Rare

Tab. 5
Known genetic contributors to ASD (Buxbaum, 2009)

The high penetrance of these genetic contributors as well as the fact that they generally occur alone speaks to the applicability of the multiple rare variant model.

NLGNs

Over the course of researching NLGN3/4X/4Y I have found that mutations in these genes are very rare.

Combining all the publications, 4711 people were assessed. 1971 of them were ASD patients, 72 exhibited other symptoms and 2668 were healthy.

109 mutants were found, 44 of which were ASD patients, 24 exhibited other symptoms and 41 were healthy.

	ASD	Other diseases	Healthy	
Genetic anomaly	44	24	41	109
No genetic anomaly	1927	48	2627	4602
	1971	72	2668	4711

Tab. 6
Combining the publications

While the differences of the studies and publications make a statistic analysis problematic, it can be said that the number of disease-related mutations is quite small.

Since there are completely healthy, asymptomatic individuals, the mutations cannot be called ‘rare deleterious mutations’. Given the rarity of the mutations and the likely high penetrance, the multiple rare variant model likely applies.

However, attention must be drawn to the paper of Yu et al. (2011), which identified the 3-marker haplotype block (rs11795613-rs4844285-rs4844286) in NLGN3 as a possible common variant.

Generally, SNPs are part of genetic variety, and every SNP may be a common variant that is not recognized as such. In keeping with that, it should be noted that ASD patients have significantly more SNPs that the general population.

Liu et al. (2013) identified and studied six common SNPs in the promoter region and 3´UTR of NLGN4 and their relationship with ASD, as they might affect the expression of NLGN. Though they did not find any SNP which had significant frequency difference between ASD cases and controls, the validation in more samples and functional study might help to understand the potential role of these polymorphisms in ASD.

Possible pathophysiologies resulting from NLGN3/4 mutations

Südhof et al. (2008) consider ASD not a disorder of brain structure but of brain function.

Seeing as ASD is among the most heritable of cognitive diseases (~80%), one has to consider genes to be a far greater factor in it's genesis than environment.

Gender distribution and contributing factors

X-linked inheritance

X-linked inheritance is a special case of mendelian inheritance in that it is linked with the sex. This is because of the different number of X chromosomes in males (who have one) and females (who have two).

Males, having only one X-chromosome, will always have to content with the effects of a mutation or trait, whether it is recessive or dominant. Similarly, they will always pass it on to their daughters, but never their sons.

For females, on the other hand, X-linked inheritance works exactly like autosomal inheritance. Of course, a healthy second X-chromosome may compensate a mutation on the first. (Murken et al., 2011, pp. 260-267)

While ASD is associated with multiple genes and mutations, its gender distribution does suggest either direct involvement of the X-chromosome, or suggests that the penetrance of pathogenic genes is facilitated in males.

It is of note that there are a significant amount of ASD associated genes that are located on the X chromosome.

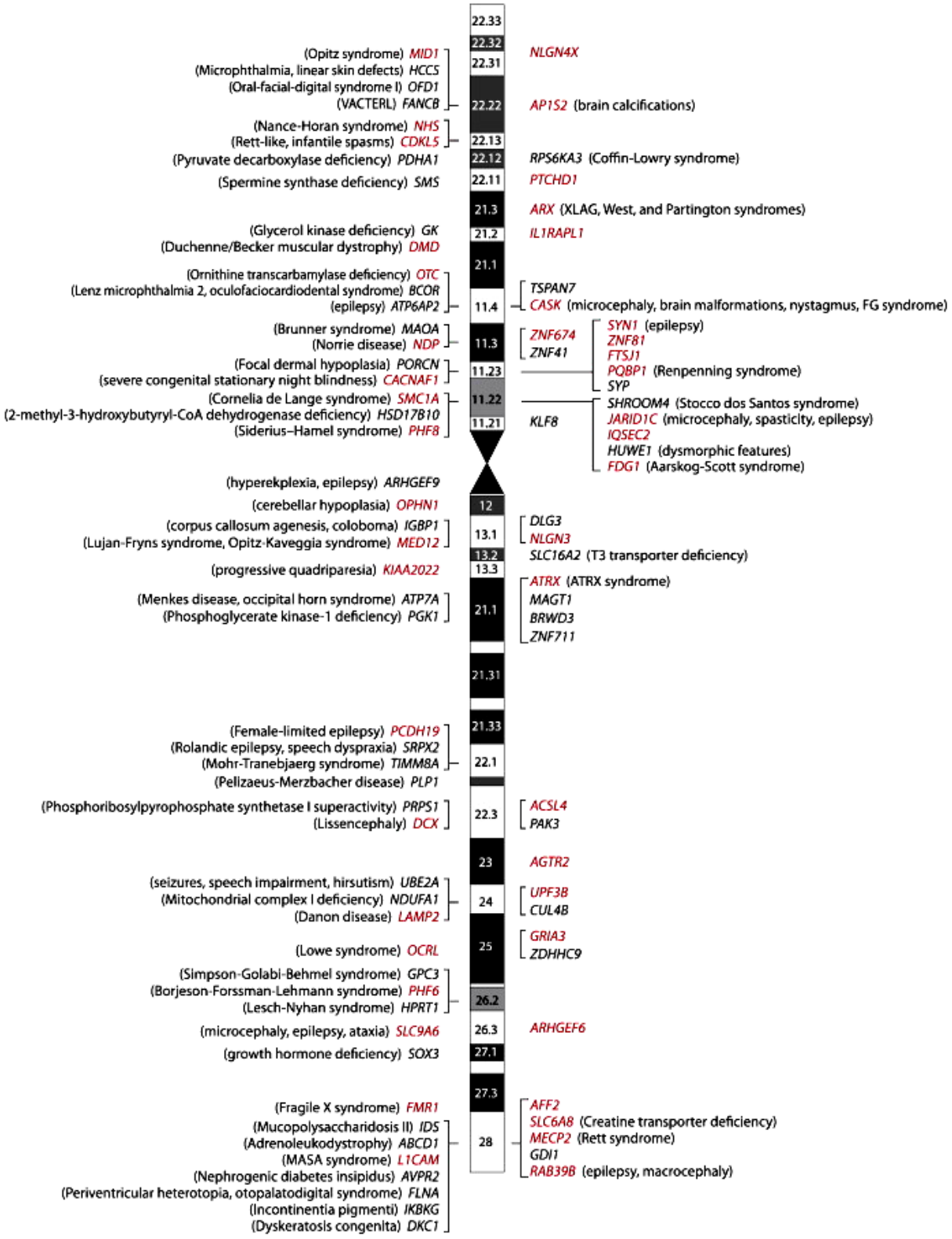

Fig. 4
Genes implicated in Syndromic and/or nonsyndromic forms of X-linked ID and their location on the X-chromosome. Genes reported to be mutated in ASD are highlighted in red. (Betancur et al., 2011)

X chromosome inactivation

Because the unequal sex ratio in autism could be explained by X chromosome gene involvement in a subset of affected females, Talebizadeh et al. (2005) studied X chromosome

inactivation (XCI) patterns in 77 autistic and control female siblings as well as their mothers from the AGRE.

Their study suggested that X inactivation patterns in females with ASD were more often moderately (30% of the individuals) or highly (33%) skewed, while in the control group, consisting of female, healthy siblings of the ASD probands, the distribution (moderately: 34%, highly skewed: 11%) was far closer to the norm. They also found a heritability of 50%, quite unlike the general population, in which no familial consistency was reported.

Notably, they did not find any known mutation in the promoter region of the XIST (X-inactive specific transcript) gene, which is a candidate for initiation of inactivation.

Possible explanations proposed are for the observed X chromosome skewness in the study to include selective cell death after initial random X inactivation, or a selective ascertainment of individuals from the tail of a random distribution of inactivation because of an unusual or unexpected phenotype (e.g. like female carriers of Duchenne muscular dystrophy, an X-linked recessive disorder).

X-linked recessive susceptibility gene(s) for autism could explain the observation of X chromosome skewness in these females with autism (Talebizadeh, 2005).

Epigenetics

As the expression some ASD candidate genes is regulated by neuronal membrane depolarization, the hypothesis of neural activity – dependent regulation of synapse development as a mechanism in several ASD mutations is appealing.

Early brain development is driven largely by intrinsic patterns of gene independent of experience-driven synaptic activity (Sur and Rubenstein, 2005) and mutations in the genes active in early development can lead to brain malformations or sever mental retardation.

Postnatal brain development, in contrast, requires environmental input that triggers the release of neurotransmitters and promotes synaptic maturation.

During this process, the expression of hundreds of genes is altered by neural activity, and each of them vulnerable to gene dosage changes.

The connection between experience-dependent neural activity and gene expression in the postnatal period forms the basis of learning and memory, and ASD symptoms typically become noticeable during these later stages of development.

Morrow et al. (2008) noted that deletions of genes regulated by neuronal activity or regions potentially involved in regulation of gene expression in autism suggested that defects in activity-dependent gene expression may be a cause of cognitive deficits in patients with autism. They suggested that disruption of activity-regulated synaptic development may be one

mechanism common to at least a subset of seemingly heterogeneous autism-associated mutations.

Post-translational modification

Phosphorylation

While it is not yet clear how distinct neuroligins isoforms recruit different postsynaptic machineries, phosphorylation has been suggested as possibly underlying isoform-specific regulation.

It is possible that disease-associated mutations affect phosphorylation. Only a single mutation thus far has been identified: Yan et al. (2005) have described a mutation in NLGN4X at R704C.

NLGN4X is phosphorylated at T707, only a few amino acids away from the disease-associated mutation, which completely abolishes T707 phosphorylation.

Attempts to study this mutation in NLGN3 in mice described a major and selective decrease in AMPA receptor mediated synaptic transmission in pyramidal neurons of the hippocampus (Etherton et al., 2011).

Research on this topic is difficult because of all the neuroligins, NLGN4 has the least conservation between rodents and human, greatly complicating any efforts, as when studying mutations in NLGN3 instead, one cannot be certain of the applicability of the results.

Glycosylation

While a disease-related mutation directly affecting a glycosylation site in neuroligins has not yet been identified, two ASD mutations (NLGN4X: R87W and NLGN3: R451C) seem to cause differential glycosylation patterns when compared to the wild-type protein, which results in decreased surface expression.

This is likely a result of protein misfolding. Possibly, neuroligin glycosylation may be used as a marker for surface expression as a result (Bemben et al., 2015).

Excitation-Inhibition hypothesis

Approximately 30% of ASD patients have seizures, and up to 70% have sharp spike activity recorded by EEGs or magnetoencephalography.

The observation of epilepsy in patients with ASDs has fueled speculation that autism may be caused by an imbalance of excitatory vs. inhibitory synaptic transmission. (Südhof, 2008)

Hussman (2001) proposed that inhibitory GABA is suppressed in the brains of individuals with ASD, suggesting that excitatory circuity may be favoured.

Rubenstein and Merzenich (2003) formed the hypothesis that some forms of autism can be modelled by the idea that there is an increased ratio of excitation/inhibition (E/I) within critical neural systems in the ASD brain. The inherent idea behind the hypothesis is that there is increased 'noise', either due to a disproportionately high level of excitation or a disproportionately low level of inhibition. This causes an unstable cortex that is highly vulnerable to seizure activity.

Additionally, they postulated that the resulting E/I imbalance may also have delayed synapse maturation, caused abnormal myelination and/or potential problems within the local circuitry and cellular mechanisms leading to the generation of hyper excitable states.

Notably, the root cause of such an imbalance is still undetermined. The search for it is complicated by the big array of genetic, epigenetic, cellular and molecular findings, as well as the possible environmental influences.

Recently, Yizhar et al. (2011) tested the E/I hypothesis in mice. Elevation of the E/I balance in mousemedial prefrontal cortex caused a profound impairment in cellular information processing associated with behavioural impairments, and compensatory elevation of inhibition was found to partially rescue the social deficits caused by E/I elevation.

Concerning NLGNs, this hypothesis may have merit, since every NLGN positively or negatively regulates either excitatory or inhibitory postsynaptic potential.

Relevance of NLGN mutations

Overall, the description of various mutations in NLGN3 and NLGN4/4Y appear to provide compelling evidence for a role of these genes and proteins in ASD.

It should, however, be noted that there are two issues that weigh against their role in ASD.

Firstly, at least for some of the mutations, non-symptomatic carriers were found in the same families in which the patients with mutation were found. While the mutations appear almost always penetrant in males and even female carriers often have a phenotype, some do exist in non-symptomatic relatives.

Secondly, the phenotype associated with the same mutation can vary wildly, e.g. the same microdeletion in NLGN4, being present in two brothers, their mother and their grandmother, caused autism in one brother, but Tourette's syndrome in the other, learning disability,

depression and anxiety in the mother and anxiety in the grandmother (Lawson-Yuen et al., 2008).

Conversely, very different mutations may produce a similar syndrome, as shown by the quite different mutations that are associated with ASD (Südhof, 2009).

So, at present, the relation between NLGN, the neuroligin-neurexin cell-adhesion complex and ASD is not quite clear cut.

On one hand, there are observations of many mutations in familial ASD that are clearly not polymorphisms but deleterious, as evidenced not only by the phenotypes in human, but also the severe autism-like phenotypes in NLGN3/4 mutant mice (Jamain et al., 2008).

On the other hand, the nonlinear genotype/phenotype relationship in humans (evident from the only 70-80% heritability and from the occasional presence of mutations in non-symptomatic individuals) requires explanation (Südhof, 2009).

Conclusion

Clinical implications

Understanding the genetic components of ASD more completely will hopefully provide essential help for limiting the long list of environmental factors that are hypothesized to cause or contribute to ASD. Examining susceptibility to such factors in the context of known genetic vulnerability will be extremely valuable (Geschwind and Levitt, 2007).

The apparent involvement of neuroligins (and neurexins, as their binding partners) in different cognitive diseases also means that we have to consider whether 'autism', as observed in patients with mutations in NLGN3/4 is actually a truly distinct entity. To put it differently, is autism actually qualitatively different from other cognitive diseases, or is it merely a part in a continuum of cognitive disorders?

With the emerging findings on the genetics of cognitive diseases, there is hope that a molecular nosology may become possible.

Furthermore, if the exact role of neuroligins in cognitive disease is confirmed in more extensive studies, they may result in new diagnostic and therapeutic possibilities, possibly by selectively modulating the neurexin/neuroligin interaction.

Of course, there is a lot of work still to be done to explore these possibilities, but present results as well as the ever growing knowledge that is accumulated is encouraging (Südhof, 2009).

A possible application would be the implementation of more thorough screenings, possibly as a two step process: first, using aCGH for a genome wide scan (especially for gains), and second, to screen for specific genes.

Of course, the ethical implications must not be ignored. However valuable and helpful more sensitive diagnostic tools are, it has to be remembered that the more sensitive they become, the more likely they will be to give information that has not been specifically asked for, i.e. one might look for a susceptibility gene for ASD and find (instead or additionally) one for another disease. This is especially troubling when screening healthy people for whatever reason.

Especially in genetic counselling it has to be remembered that the patient does not only have the right to information but also the right *not* to know.

Questions yet to be answered

Despite great advances in research on every level of ASD, many questions yet require answers, and hypotheses require proof.

The great heterogeneity of ASD may be explained by ASDs being disorders in which complex information processing could be disturbed at different levels of development. Fundamental to this core feature of the hypothesis is the detailed hierachical (from first-order pathways to complex higher-order connections) circuit development (Hammock and Levitt, 2006).

Beyond issues of timing, differing underlying neurodevelopmental disruptions likely contribute to the heterogeneity in both symptoms and developmental course. Neurogenetic syndromes of known aetiology, such as Fragile X, Rett, Smith-Lemli-Opitz and Down syndromes, in which there is far greater incidence of ASD than in the typical population, make that reasonable.

The poor understanding of the cellular basis for dysfunctional circuits in ASD as compared to the syndromic neurogenetic disorders is in large part due to there being limited neuropathological material available for analysis. Also, structural imaging data across childhood, which provides clues regarding development, has been accumulated only recently.

While mouse models are a powerful tool for exploring synaptic or cellular physiology, ultrastructure and biochemistry, the results can hardly be completely transferred to humans (Preuss, 2000).

Since alterations in social behaviour result from distinct neural systems and environmental interactions in different species, will asocial ants help us to understand and develop treatments for the autisms (Geschwind and Levitt, 2007)?

These issues will have to be considered carefully as the genetic and environmental contributions to the autisms are uncovered and understanding of their neurobiology grows.

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