

Are Molecules Involved in Neuritogenesis and Axon Guidance Related to Autism Pathogenesis?

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Abstract Autism spectrum disorder is a heterogeneous disease, and numerous alterations of gene expression come into play to attempt to explain potential molecular and pathophysiological causes. Abnormalities of brain development and connectivity associated with alterations in cytoskeletal rearrangement, neuritogenesis and elongation of axons and dendrites might represent or contribute to the structural basis of autism pathology. Slit/Robo signaling regulates cytoskeletal remodeling related to axonal and dendritic branching. Components of its signaling pathway (*ABL* and *Cdc42*) are suspected to be molecular bases of alterations of normal development. The present review describes the most important mechanisms underlying neuritogenesis, axon pathfinding and the role of GTPases in neurite outgrowth, with special emphasis on alterations associated with autism spectrum disorders. On the basis of analysis of publicly available microarray data, potential biomarkers of autism are discussed.

Keywords Autism · Neurites · Brain development · Biomarkers · Cytoskeletal remodeling

Introduction

Autism spectrum disorder is a heterogeneous disease, and numerous alterations of gene expression are necessary to hypothesize possible molecular and pathophysiological causes. Although it is still far from clear, it is becoming widely accepted that neurodevelopmental disorders, including autism, display defects in neuronal organization and plasticity. Abnormalities of brain growth and connectivity in autism are usually apparent after the first year of life (Courchesne et al. 2003; Williams and Casanova 2011), a period characterized by intensive remodelling of neuronal tissue. The organization, development and function of complex brain networks depend on numerous neuronal precursors differentiating to specific neuron or glial cell populations. Fate of neuronal progenitors during proliferation and differentiation is determined by the molecular microenvironment. Neuronal cells undergo dynamic changes in shape, size and functional specialization. Morphological changes of neurons and glial cells are based on cytoskeletal reorganization regulated by actin-binding proteins, Rho pathway signaling proteins, synaptic scaffolding proteins, adhesion molecules and locally secreted neuropeptide hormones. Especially in the last decade, intracellular mechanisms regulating changes of cytoskeletal proteins have been extensively investigated at the molecular level, resulting in discovery of complex subcellular systems. The most significant structural consequence of cytoskeletal rearrangement is neuritogenesis and elongation of axons and dendrites.

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Neurites

Neurites form functional connections among neurons via morphologically distinct cell projections and synaptic clefts. During development, initial neurites may differentiate into long-distance projections called axons or into multiple short-length dendrites. Typically, neurites which grow five to 10 times faster than other neurites develop into axons, and the remaining slower growing neurites become dendrites (Polleux and Snider 2010). Dendrites and axons differ molecularly and functionally. The many dendrites, which are highly branched, receive electrical impulses from the axons of other neurons and conduct this activity to the neuron's own axon for transmission to other cells. The cytoskeleton of dendrites is organized differently from that of axons (Dotti et al. 1988). Axons are usually myelinated, polarized and optimized for fast transmission of electrical impulses. Growth of neurites is well regulated. The fundamental structure that propels the nascent neurite forward is the growth cone. Growth cones protrude away from the neuronal cell body and extend to different proximal or distal areas of the nervous system. The concept of neurite formation was based by Ramon y Cajal, who has described fibers of the nervous system growing from neuronal soma and forming connections with neighboring cells (in Korey and Van Vactor 2000). Initial formation of a neurite depends on accumulation of microtubule bundles at the neuron periphery and subsequent growth cone attraction and repulsion driven by different mechanisms. Extracellular molecules activate membrane receptors, triggering intracellular cascades (kinases, phosphatases, GTPases) regulating changes in actin cytoskeleton (Da Silva and Dotti 2002). Coordinated control of the major cytoskeletal components, actin, microtubules and associated proteins, is important for formation of the growth cone and its direction. At the periphery of the leading tip of the growth cone are located two dynamic structures—lamellipodia and filopodia. Lamellipodia are large fan-like protrusions often located between thin, tubular filopodial extensions that can reach several cell diameters in length (Korey and Van Vactor 2000). Filopodia and lamellipodia are mainly organized from thick bundles of actin fibers, while microtubules are confined to the central region of the growth cone (Dehmelt and Halpain 2004). Actin dynamics are necessary for directed axonal outgrowth, but not necessarily for growth cone translocation, per se (Dent et al. 2011).

Neuronal Transport

An important factor for the outgrowth of mature axons is the microtubule-based transport of different cargoes—membranes, lipids, proteins and organelles as large as mitochondria via molecular protein motors. In general,

molecular motors utilize the energy of ATP hydrolysis to transport cargo, and various adaptor proteins play a role in binding cargo. In the neurites, transport occurs bidirectionally from the cell body to the periphery (anterograde transport) and from the periphery to the cell body (retrograde transport). This transport occurs along the axonal/dendrite microtubule bundles and is driven by dynein/dynactin and kinesin motor protein dimers/complexes (Hirokawa et al. 2010; Prokop 2013). Transport by the motor dynein requires recruiting the dynein activator dynactin, leading to the initiation of retrograde transport critical for normal neuronal function and axon maintenance (Moughamian and Holzbaur 2012; Moughamian et al. 2013). Moreover, axon growth directly depends upon microtubule stability and involves dynein motor-driven transport of microtubules to the growth cone (Dehmelt et al. 2006; Kollins et al. 2009). Anterograde neuronal transport is mediated by a large group of kinesins and actin-based motor proteins myosins, as well. Over 45 kinesins have been identified as being involved in generating forces between microtubules and cargo. Hetero-oligomeric types 1 and 2 as well as homodimeric type 3 kinesins are considered to be the most prevalent mediators of anterograde transport in axons (Prokop 2013). Moreover, kinesin motor proteins are important for the transport of ion channels across long distances in the axons of neurons (Su et al. 2013). Motor proteins either bind to the vesicles carrying ion channels or interact directly with the cytoplasmic sequences of these channels (Su et al. 2013). Essential components of some specific motor proteins are GTPases, which regulate different stages of intracellular trafficking. The GTPase family of proteins participates in distinct trafficking steps—coats for budding, motors for motility, tethers for docking and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) for fusion of vesicles (Horgan and McCaffrey 2011). Thus, structural changes of axons, together with expansion of neurites, axon growth cone membrane addition and vesicle trafficking are all under control of various GTPases.

Role of GTPases in Neurite Outgrowth

In general, small GTPases are regulators of the cytoskeletal and membrane dynamics underlying cell motility, cell polarity and cell growth (Polleux and Snider 2010). In particular, during regulation of neurite outgrowth, it is important to maintain balance between the activity of multiple GTPases to mediate growth cone collapse and axon repulsion. GTPases encompass a large group of enzymes that bind GTP (guanine triphosphate) and undergo a conformational change as GTP is hydrolyzed to GDP. Rho family proteins are the best known GTPases for their

effects on the actin cytoskeleton. The Rab family of GTPases regulates transport and docking of vesicles (Grosshans et al. 2006), while local dendrite protrusions are under strong control of Rho kinase activity. Rab GTPases recruit different motor proteins to cargo vesicles, allowing polarized addition of membrane to growing neurites (Villarreal-Campos et al. 2014). Of the large Rho GTPase family members, RhoA, Rac1 and Cdc42 have been characterized most extensively, each with specific roles in axonal and dendritic morphology. Both Ras and Rho family small GTPases have been shown to be involved in axon specification and axon growth (Hall and Lalli 2010). RhoA is involved in growth cone retraction and plays a central role in inhibition of axon outgrowth by myelin-derived inhibitors (Thies and Davenport 2003, Auer et al. 2012). Rac1 and Cdc42 regulate neurite outgrowth and the growth cone morphology of most neurons (Oblander and Brady-Kalnay 2010, Major and Brady-Kalnay 2007). Accumulating evidence indicates that another key mechanism by which Rac and Cdc42 relay signals to the actin cytoskeleton involves the Wiskott–Aldrich syndrome family of scaffolding proteins (Millard et al. 2004; Smith and Li 2004, Govek et al. 2005). The Wiskott–Aldrich syndrome protein (WASP) and its closest family member neuronal WASP (N-WASP) are regulated by Cdc42 (Rohatgi et al. 1999, Govek et al. 2005) and modulate neurite outgrowth (Pommereit and Wouters 2007).

Axon Pathfinding

Neuronal growth cones in the developing nervous system are guided to their targets by attractive and repulsive guidance molecules, which include members of the netrin, semaphorin, ephrin and Slit protein families. Axon pathfinding is mainly regulated by the Slit/Robo cell signaling pathway. The Slit protein family includes three Slit glycoproteins (Slit1–Slit3). The molecular target for all three Slit proteins is a repulsive guidance transmembrane receptor known as the Robo (roundabout) receptor. Four Robo receptors (Robo1–Robo4) have been described in mammals (Goldberg et al. 2013). Slit1–Slit3 have not been shown to have a clear preference for any particular Robo receptor (Ypsilanti et al. 2010). Together, Slit/Robo regulates cytoskeletal remodeling related to axonal and dendritic branching. *In vitro* studies have shown that Slit1 contributes to axon repulsion (Murray et al. 2010). *In vivo* studies using both mice and zebrafish carrying mutants for either Slit or Robo proteins have shown that they control neuronal projections during early development (Hammond et al. 2005). Moreover, there are experimental studies describing abnormal migration of neurons in mice with mutations in Slit/Robo proteins (Wang et al. 2013).

Appropriate changes in cytoskeletal dynamics are regulated by vasodilator-stimulated phosphoprotein (VASP). Another protein, EnaH (enabled homolog), interacts with Robo receptors to transduce part of Robo's repulsive signal. Moreover, the EnaH/VASP proteins are implicated in integrating guidance signals into appropriate changes in cytoskeletal dynamics and are key regulators of filopodia formation and dynamics (Drees and Gertler 2008). EnaH/VASP activity regulates the assembly and geometry of actin networks within cellular lamellipodia. In growth cones, EnaH/VASP proteins are concentrated at filopodia tips (Lebrand et al. 2004). EnaH/VASP complex is also required for dendrite growth (Tasaka et al. 2012). EnaH/VASP proteins at the tips of lamellipodia and filopodia accelerate actin polymerization by their anti-capping and bundling activity (Krause et al. 2004), and neurons lacking these proteins fail to form neurites (Kwiatkowski et al. 2007).

Alterations in Neuritogenesis in Autism

Recently, new genetic factors affecting neuritogenesis, growth cone changes, and vesicle and membrane trafficking have been identified as related to autism (Grice and Buxbaum 2006; Castermans et al. 2010; Volders et al. 2011; van Maldergem et al. 2013). At least in a subgroup of patients, alterations in genes associated with neuronal vesicle trafficking (e.g., secretory carrier membrane protein 5—SCAMP5) coincide with the elaboration of mature synapses and may represent functional candidates for autism pathology (Castermans et al. 2010). Changes in synaptogenesis, synaptic vesicle formation and regulation of neurotransmitter release represent a family of processes also considered to play a role in a manifestation of autism spectrum disorders (Paemka et al. 2013). Some of cytoskeletal components are suspected to be involved at the molecular level in alterations of normal development, in particular affecting the number of neurites, their length and functional synaptic connectivity. Structural abnormalities in developing neurons may lead to deficiencies in axonal wiring and synaptic formation during brain maturation, causing a loss of the proper function in neuronal tissue. Alterations in control of neuritogenesis are always complex, and they may be associated with manifestation of certain symptoms of neurodevelopmental diseases. Post-mortem studies of brains of autistic subjects have linked neurite outgrowth to the pathophysiology of autism (Lepagnol-Bestel et al. 2008). Moreover, in experimental models, autism-related behavioral deficits were identified in knockout mice lacking the genes coding for neurexins, a family of cell adhesion molecules (Peñagarikano et al. 2011). Furthermore, length and branching of neurites were

found to be dependent on the neural cell adhesion molecules called contactins (Mercati et al. 2013). Impairment in neurite outgrowth, including both dendrites and axons, was associated with mutation in the gene for the purinergic receptor and was manifested by intellectual disability within an autism spectrum disorder (van Maldergem et al. 2013). Another study associated gene encoding enzyme monoamine oxidase B and proteins involved in regulation of neurite outgrowth with autism pathology (Piton et al. 2011). On the basis of this accumulated evidence, structural remodeling of nervous tissue, predominantly related to alterations in neuritogenesis, might be associated with brain connectivity deficits in autism.

Functional Connectivity Deficits in Autism

Molecular and imaging studies on the neurobiological foundations of autism suggest that lower activation and interconnection of specific brain areas underlie the manifestation of neurodevelopmental disorders (Just et al. 2004; Zhan et al. 2014). Very few studies deal with alterations in the connectivity among different brain areas and an aggressive phenotype (Márquez et al. 2013), although various subtypes of aggression were identified in children with autism spectrum disorder (Carroll et al. 2014). Bilateral aberrant long-range corticocortical connectivity was demonstrated in some subjects with autism (Jou et al. 2011). Reduced connectivity was suggested in short- and long-range tracts in the brains of autistic subjects (Schaer et al. 2013). Several studies directly revealed that patients with autism spectrum disorders have deficits in long-distance connections in the brain (Billeci et al. 2012; Maximo et al. 2014). Moreover, in this context, a large body of evidence implicates abnormalities in commissural fibers, specifically in the corpus callosum, as being associated with autism (Edwards et al. 2014). Thus, systemic and local underlying mechanisms in the

connectivity of the brain are likely to be related to autism pathology. In the developing brain, altered brain connectivity and a premature decrease in neuronal plasticity might result in severe neurobiological and behavioral consequences. Although brain connectivity changes during life, defects in the early stages of development are likely to remain very important throughout the lifetime. Early changes in locally released factors in the brain related to neurite outgrowth, axon guidance, axonal transport and dendritic sprouting might be related to altered brain connectivity. In view of all the factors involved in neuritogenesis, it is becoming useful to search in this field for most substantial biomarkers of autism, as they are known to contribute to neurodevelopmental disorders.

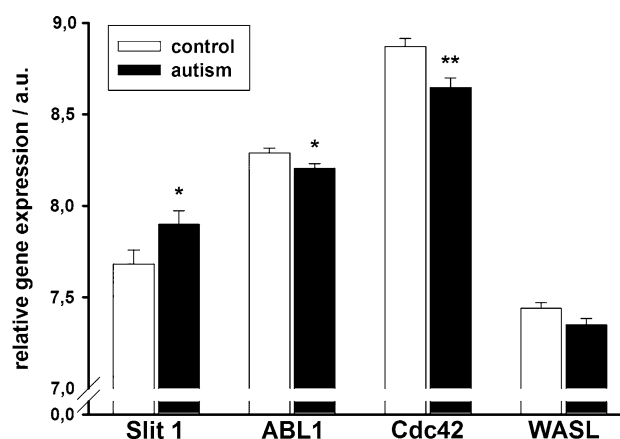


Fig. 1 Gene expression of selected genes in control and autistic subjects. Data from gene expression microarray were obtained from the study of Alter et al. (2011) and analyzed by PLIER16 algorithm using GeneSpring software. Fold changes of expression are shown on the y-axis. Out of 17 genes (29 probes) selected as related to neurite outgrowth, analysis using unpaired *t* test revealed significantly altered expression in three genes in autistic subjects: increased expression of *Slit1* and decreased expression of *ABL1* and *Cdc42*. A borderline significant increase was found in *WASL* gene expression ($P = 0.054$). Means are represented as bars \pm SEM (autistic patients, $n = 82$; controls, $n = 64$). Significant changes * $p < 0.05$, ** $p < 0.01$

Table 1 Neurite outgrowth genes and their probes represented on the Affymetrix Human Genome U133 Plus 2.0 chip and examined in the experiment in Fig. 1

Gene symbol	Probe set ID	Gene symbol	Probe set ID	Gene symbol	Probe set ID
GAPDH	212581_x_at	ROBO1	213194_at	CLASP1	212752_at
	213453_x_at	ROBO2	226709_at		240757_at
	217398_x_at		226766_at	CLASP2	1555469_a_at
SLIT1	1557615_a_at		240425_x_at		212306_at
SLIT2	209897_s_at	ROBO3	219550_at		212308_at
SLIT3	216216_at	ROBO4	220758_s_at	CDC42	208727_s_at
ABL1	202123_s_at		226028_at		208728_s_at
ABL2	206411_s_at	WASL	205809_s_at	CDC42BPG	1564823_at
ENAH	217820_s_at		205810_s_at		1569044_at
EPHB3	1438_at		224813_at		

Potential Biomarkers in Autism

Increasing numbers of recent studies have associated specific genes with neurodevelopmental disorders, intellectual disability and autism spectrum disorders (Birnbaum et al. 2014; Corvin 2010). These range from genes for different signaling cascades, to apoptosis pathways and synaptic plasticity (Zeidán-Chuliá et al. 2014). Several studies involving gene expression microarray analysis provide evidence of abnormalities in peripheral blood leukocytes in autistic subjects that could provide a biomarker for a genetic and/or environmental predisposition to the disorder (Gregg et al. 2008; Alter et al. 2011). A large recent study revealed a significant link between paternal age, variability of gene expression and risk of autism in the offspring (Alter et al. 2011). These authors extracted RNA from peripheral blood lymphocytes and analyzed gene expression via microarrays covering greater than 47,000 unique RNA transcripts from both children with autism ($n = 82$) and age-matched control subjects ($n = 64$). This kind of gene expression profiling can help to identify markers for neurodevelopment disorders.

Nevertheless, research on gene expression in autism has previously focused on identifying variability in large groups of genes related to the disease. However, cause-related factors might predominantly depend on certain substantial factors. On the basis of the concept of alteration in neurite extension and its potential relationship to autism spectrum disorders, we assume that certain genes might represent biomarkers of the developmental diseases. We have selected 17 genes (29 probes) related to neurite outgrowth (Table 1) from a previously published, publicly accessible database of gene expression in control and autistic subjects (Alter et al. 2011). Analysis of gene expression by the PLIER16 algorithm using GeneSpring software revealed significantly increased expression of the *Slit1* gene and decreased expression of the *ABL1* and *Cdc42* genes in autistic subjects (Fig. 1). *Cdc42* belongs to the large Rho family of GTPases essential for neurite outgrowth (Kozma et al. 1997). As Rho proteins are key regulators of the cytoskeleton and ABL kinases are required at least in part for activation of *Cdc42* (Burton et al. 2005; Chang et al. 2009), there might be important link between alteration in neuritogenesis in the brain and

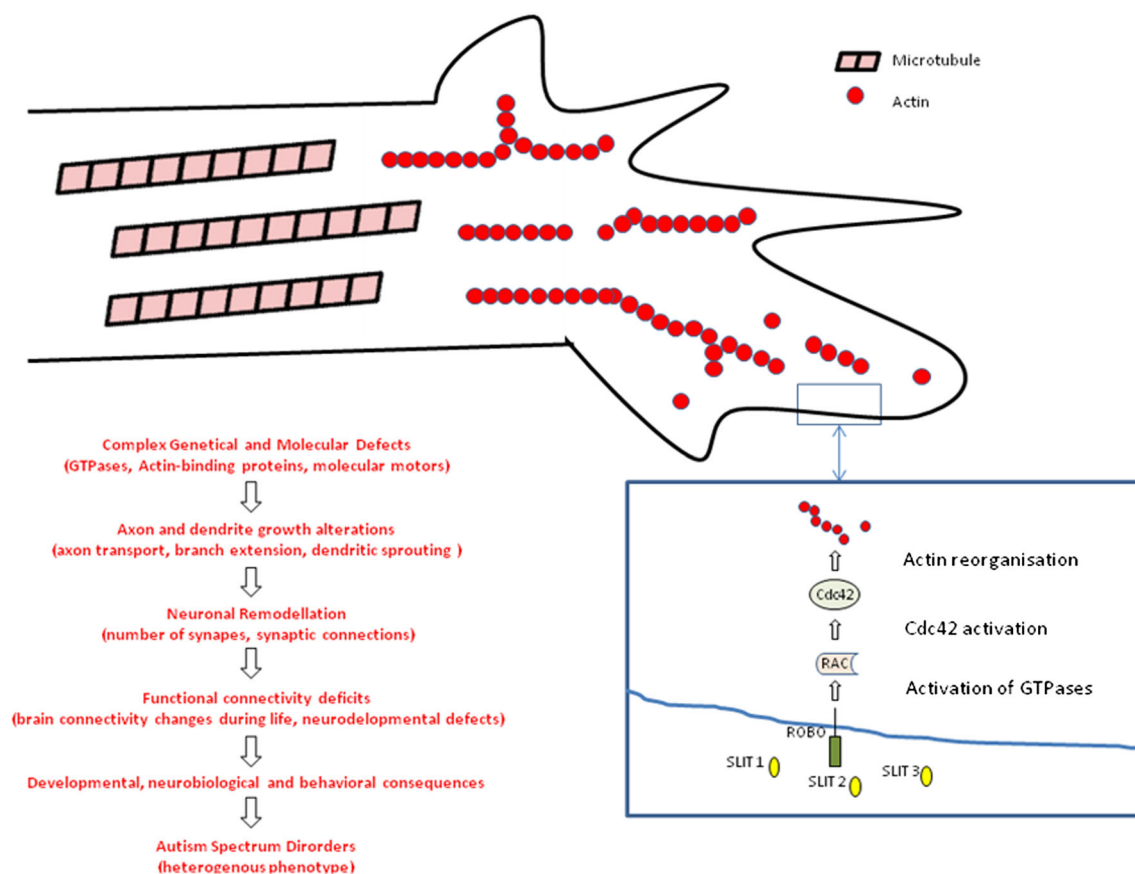


Fig. 2 Model of the role of axon growth cone changes in autism. A typical neuron is comprised of a cell body, dendrites and axon. The tip of an axon is represented, with associated microtubules and actin

filaments. The potential role of Slit/Robo signaling is indicated in the blue box. A schema of complex defects in autism presented in red to the left of the blue box (Color figure online)

peripheral blood biomarkers. Ubiquitously expressed ABL1 tyrosine kinase and Cdc42 protein might represent potential diagnostic and predictive biomarkers for autistic patients. Furthermore, our preliminary results indicate that alterations of cytoskeletal proteins in leukocytes might be accompanying phenomenon of neurodevelopmental disorders. Understanding of the molecular mechanism of neuritogenesis might be extended to other cell types sharing a common phenotype. In our view, complex genetical and molecular defects (GTPases, actin-binding proteins, molecular motors) can result in neuronal remodelling comprising neuritogenesis and functional connectivity deficits that can manifest as the heterogenous autism spectrum phenotype (Fig. 2). Alteration in Cdc42-induced actin polymerization might represent a mechanism of cytoskeletal changes in neuronal growth cone with consequences for neurite elongation and interconnection (Fig. 2). Further studies are needed to test the potential role of Slit/Robo signaling in neuritogenesis, with special emphasis on its potential to cause neurodevelopment disorders. Although many previous studies have concentrated on the early stages of development, there is a need (Wolff and Piven 2014) to expand research on brain development to biomarkers from adult subjects and associate them with mechanisms of disease.

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