Trends in Neurosciences

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Review

Neurodevelopmental Disorders: From Genetics to Functional Pathways

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Neurodevelopmental disorders (NDDs) are a class of disorders affecting brain development and function and are characterized by wide genetic and clinical variability. In this review, we discuss the multiple factors that influence the clinical presentation of NDDs, with particular attention to gene vulnerability, mutational load, and the two-hit model. Despite the complex architecture of mutational events associated with NDDs, the various proteins involved appear to converge on common pathways, such as synaptic plasticity/function, chromatin remodelers and the mammalian target of rapamycin (mTOR) pathway. A thorough understanding of the mechanisms behind these pathways will hopefully lead to the identification of candidates that could be targeted for treatment approaches.

Highlights

NDDs are caused by the disruption of essential neurodevelopmental processes. Many genes and mutations are associated with NDDs, pointing to a heterogeneous origin of these disorders.

Genotype–phenotype correlations are difficult to establish due to the existence of multiple genetic as well as environmental factors that influence the phenotypical outcome. The two-hit model and the existence of multiple molecular diagnoses are important factors that should be taken into account when addressing NDDs.

Most of the known NDDs genes belong to few common frequently affected molecular pathways. Functional and molecular studies elucidating how different mutations can disturb the converging pathways can lead to the identification of potential targets, thereby opening perspectives for future treatment.

Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) are characterized by an inability to reach cognitive, emotional, and motor developmental milestones. Typically, NDDs are associated with the disruption of the tightly coordinated events that lead to brain development. NDDs constitute a serious health problem in our society, affecting >3% of children worldwide [1]. They have a heterogeneous etiology and lead to impaired cognition, communication, adaptive behavior, and psychomotor skills. NDDs include autism spectrum disorder (ASD), intellectual disability (ID), attention deficit hyperactivity disorder, and epilepsy [2,3]. Many studies have suggested that shared molecular pathways could account for the multiple clinical signs that characterize NDDs [4,5]. Accordingly, **comorbidity** (see Glossary) of two or more of these disorders is frequently observed. For instance, a combination of ID, ASD, and epilepsy is commonly reported in individual patients [6,7]. Identification of the shared pathogenic mechanisms of the different NDDs will help to explain the aforementioned comorbidity and eventually lead to effective treatment.

In terms of genetics, different types of mutation have been associated with NDDs, including chromosomal rearrangements, copy number variations, small indels, and point mutations. Thus, the identification of a potential underlying mutational event, known as **molecular diagnosis**, is a challenging task that needs to overcome the heterogeneity of this complex array of genetic variations. Some of the technologies currently used for the molecular diagnosis of NDDs are summarized in Box 1.

These challenges notwithstanding, recognition of NDD-causing genes is crucial for accurate **genetic counseling** and patient management, and represents an essential first step toward a better understanding of the molecular pathways affected by these disorders.

This review focuses on the molecular etiology of NDDs, starting from genetics and moving to the functional level. First, we discuss how the study of familial cases improved our understanding of the complex genetics of NDDs. Second, we consider the genetic factors that determine and influence the phenotype, such as gene vulnerability, mutational load, and multiple molecular

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Box 1. Evolution of the Diagnostic Flowchart of NDDs

Early molecular diagnosis of patients with NDD is essential for genetic counseling, patient management, and medical intervention.

Previously, G banded karyotype and FMR1 trinucleotide repeat analysis were recommended as a first-tier test for patients with unexplained NDDs. However, the yield in patients was low [111]. The breakthrough of next-generation sequencing technologies has led to significant advancements in the identification of the genetic causes of NDDs [1,7,112]. To date, >900 genes responsible for X-linked, autosomal dominant, or autosomal recessive NDDs have been reported [113,114]. Due to the correlation of genetic disorders with mutations in protein-coding genes, the cheaper and quicker whole-exome sequencing (WES) is preferred as a diagnostic tool to the more informative whole-genome sequencing [115,116]. Different studies highlighted the efficiency of exome sequencing as a diagnostic tool, having a diagnostic yield up to >40% in patients with NDDs, especially when both biological parents are considered [111] Still, mutations could also occur in noncoding regions, such as regulatory elements, and alter gene expression levels [111]. DNA microarrays are also frequently used to detect gross chromosomal aberrations otherwise not detectable with conventional WES [117,118]. The expected diagnostic yield of chromosomal microarray testing is estimated ~10–20% in patients with distinct NDDs [111]. Epigenetic alterations, also escaping WES detection, are frequently observed in the presence of NDDs. Therefore, various additional methods can be used to detect epigenetic changes, such as PCR, tandem mass spectrometry, and southern blot.

diagnoses. We also highlight the relevance of the two-hit model in the context of understanding the genetics of NDDs. Finally, we debate whether the identification of frequently affected cellular pathways allows circumventing the issue of the wide genetic variability of NDDs and whether the identification of such pathways could open perspectives for future treatments.

Genetics of NDDs

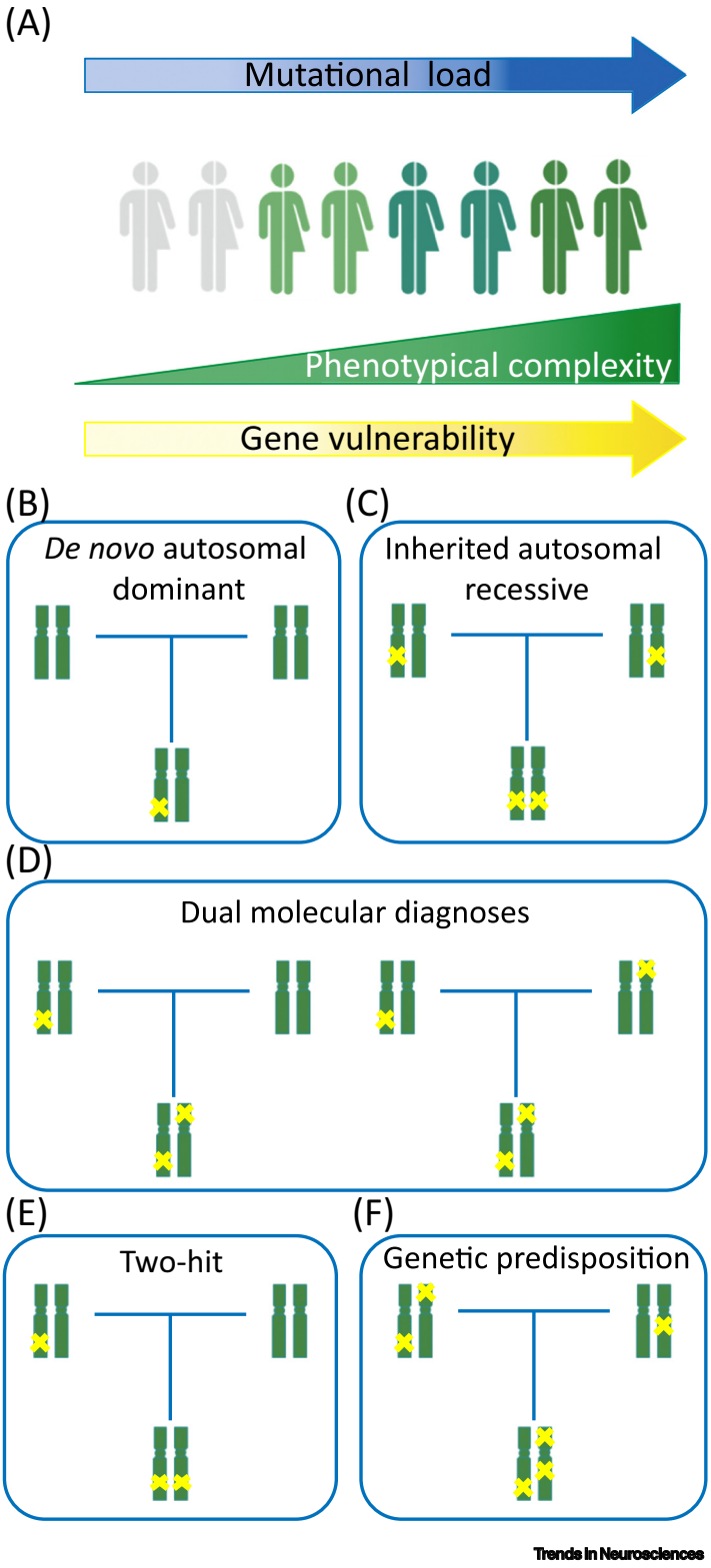
The identification of the potential genetic causes of NDDs is vital for understanding the molecular mechanisms responsible for the onset of these disorders and for the delineation of a genotype–phenotype correlation that could help to monitor the progress of the disorder and to foresee future complications. Despite the numerous NDD-causative genes identified, many individuals with NDDs still do not receive a molecular diagnosis. Additionally, phenotype–genotype correlation studies have brought to light that the number and severity of clinical signs can vary substantially among patients with overlapping genetic etiology [8,9]. Thus, missing heritability and phenotypical variability point to a **multifactorial and/or polygenic nature of NDDs**.

Familial NDDs represent a useful paradigm for dissecting the contribution of genetic and nongenetic factors to the pathogenesis of these disorders in the presence of a shared genetic background. For this reason, numerous studies have been conducted on monozygotic twins with discordant phenotypes [10–12] or on pedigrees where incomplete penetrance and phenotypical variability are observed in the multiple affected offspring [13]. This line of research has tremendous potential not only for the mere identification of the molecular causes of the disease, but also for the recognition of risk factors and protective factors. Furthermore, it has the potential for establishing more accurate genotype–phenotype correlations. Thanks to the study of inherited NDDs, it has emerged that the phenotypical outcome essentially revolves around two main principles: **gene vulnerability** and **mutational load** (Figure 1A).

Gene vulnerability can be defined as the capability of a given gene to tolerate disruptive variants: the lower the tolerance towards mutations, the higher the level of vulnerability. Some genes associated with NDDs are haploinsufficient genes characterized by a striking dosage sensitivity. These particular genes fall within the category of highly vulnerable genes, and mutations affecting these genes are associated with significant disease risk. Examples of highly vulnerable genes include *DEPDC5*, *CACNA1A*, and *SCN8A*, which are discussed later in this section. Disruption of one of these genes has a high probability of inducing the onset of a disease phenotype also

Glossary

BRG1/BRM-associated factor (BAF) chromatin remodeling complex: a chromatin modifier family that uses ATP energy to alter nucleosomal units in chromatin structures.
CLIP cells: human-specific caudal late interneuron progenitors characterized in organoids by a late midgestational origin and expression of genes associated with the caudal ganglionic eminence (i.e., *COUP-TFII*, *PROX1*, and *EGFR*).
Comorbidity: presence of one or more conditions together with a primary medical condition. In the context of NDDs, this refers to the occurrence of two or more NDD phenotypes in individual patients.
Epistasis: physical and/or functional interaction between gene products responsible for the onset of a given phenotype.
Gene vulnerability: capability of a given gene to tolerate potentially disruptive mutations.
Genetic counseling: process aimed at advising families with one or more individuals affected by a genetic condition. It helps to understand the genetic contributions to the disease and to calculate the risk of recurrence of the disease in the offspring.
Molecular diagnosis: identification of the mutational event responsible for the onset of a disease phenotype. To be distinguished from clinical diagnosis, which represents instead the recognition of a specific disease affecting a patient based on the interpretation of the observed clinical signs.
mTORopathies: spectrum of dysfunctional cortical development characterized by altered cortical architecture, abnormal neuronal/glial morphology, and intractable seizures as a consequence of deregulation of mTOR signaling.
Multifactorial/polygenic disorder: disorder characterized by a combination of genetic and nongenetic factors or by the combination of different mutational events.
Multipathway loop: in neurons, changes in synaptic or neuronal physiology are subserved by alterations in dendritic and nuclear events, operating via cellular feedback mechanisms. Mutations affecting one pathway may alter the correct functionality of different pathways, thus perturbing the homeostasis of the entire system.



Mutational load: genetic burden given by the total number of disruptive mutations.

Phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway: a highly conserved signaling pathway ubiquitously expressed in eukaryotic cells. This pathway controls cell survival, proliferation, migration, and metabolism.

(See figure legend at the bottom of the next page.)

in the absence of other causative events, thus resulting in monogenic forms of NDDs [14]. For this reason, mutations affecting these genes are normally subject to a strong negative selective pressure. Hence, population studies have recognized a reduced number of disruptive variants in vulnerable genes compared with other genomic loci [14]. In other words, mutations in highly vulnerable genes can be categorized as rare variants associated with significant disease risk and high penetrance.

The other end of the vulnerability spectrum comprises those genes that are less sensitive to disruptive mutations. Variants in these genes are not under negative selective pressure and are frequently transmitted in families for generations [2,14]. Since single disruptive events affecting nonvulnerable genes are not disease causing per se, they fall within the category of common variants with low disease risk. Nonetheless, recent studies have demonstrated that a significant portion of NDDs with polygenic nature can be attributed to common genetic variants [2,15]. In fact, the additive effects of these mutational events could result in a disease phenotype [2,15,16]. In these cases, however, the phenotypical outcome depends not only on the sum of the effects of the single mutations, but also the physical and/or functional interactions between the affected genes (i.e., **epistasis)** [17,18]. Epistatic interactions and dosage sensitivity strongly correlate with the concept of mutational load, which argues that the penetrance and complexity of a disease phenotype are influenced by the number of disruptive events. For example, loss-of-function monoallelic mutations in the sodium channels *CACNA1A* and *SCN8A* are commonly associated with a variety of clinical features including movement disorder, ID, ASD, and benign familial infantile seizures (Figure 1B) [19,20]. In accordance with the aforementioned criteria of dosage sensitivity and mutational load, inherited germline biallelic mutations of *CACNA1A* and *SCN8A* are associated with more severe phenotypes compared with monoallelic changes (Figure 1C) [19,20]. The recently reported *CACNA1A* and *SCN8A* compound heterozygous probands are characterized by the presence of epileptic encephalopathy, while the heterozygous parents and siblings only exhibit mild cognitive impairment without seizure [19,20].

In other cases, a higher mutational load might be determined by a combination of germline and somatic events, a mechanism known as the two-hit model. In the classic two-hit hypothesis, a constitutive inherited mutation generates a vulnerable genetic background. A subsequent somatic hit occurring later during development will then be responsible for the onset of a disease phenotype or the expansion of already present clinical features (Figure 1E). One example of a two-hit model comes from mutations in *DEPDC5*. Germline heterozygous loss-of-function mutations affecting *DEPDC5* are a major cause of familial refractory focal epilepsies [21]. A second somatic variant causing biallelic inactivation of *DEPDC5* was found to be responsible for the additional development of focal cortical dysplasia in patients with a severe phenotype [21,22].

Primary and secondary variants can also occur at genomic loci different from each other, thus expanding the classic two-hit hypothesis (Figure 1D). Several studies have unveiled the significant

Figure 1. Schematic Representation of the Genetic Mechanisms of Neurodevelopment Disorders (NDDs). (A) Mutational load and degree of vulnerability of the disrupted genes influence the phenotypical outcome. In general, the higher each of these factors is, the more complex the phenotype will be. (B) Most genetic causes of NDDs (if one excludes cases of consanguineous marriages) involve mutations arising *de novo* in the offspring of unaffected parents. (C) Although less frequently, an autosomal recessive mode inheritance is also observed. In these cases, the proband inherits a defective allele from each unaffected or mildly affected parent. (D) An increasing number of patients is reported to harbor dual molecular diagnoses. The different mutations of these patients can arise *de novo* or be inherited from one or both parents. (E) In the two-hit hypothesis, an initial inherited variant predisposes the proband to a disease state that reaches its full phenotypical outcome upon somatic inactivation of the second allele. (F) The cumulative load of inherited common genetic variants can make the proband more vulnerable to the onset of NDDs.

contribution of multiple molecular diagnoses in the context of NDDs [13,23–25]. In line with the notion of mutational load, genotype–phenotype correlation analyses have established that individuals with mutations in multiple genes are more likely to be affected [23] and that the number of disrupting events positively correlates with the number and severity of the clinical signs observed [13,24]. For instance, the contribution of different mutational events was recently dissected in two families characterized by intrafamilial clinical variability. In both families, the additional clinical features of the probands were explained by mutations at additional loci that were not present in the less severely affected siblings [13].

The cumulative load of common genetic variants might also represent the first hit that makes the genetic background more vulnerable to subsequent pathological events (Figure 1F). In fact, it was recently reported that the burden resulting from the combination of common variants in families with a history of NDDs positively correlated with genetic predisposition to lower educational attainment and ID [2].

Thus, the data currently available in the literature suggest that purely monogenic forms of NDDs are an exception rather than the rule. Most NDDs cases most likely have a multifactorial and/or polygenic nature, hence confirming the broad heterogeneity of these disorders at both the clinical and molecular level. Importantly, the clinical outcome might also be influenced at various levels by nongenetic factors, although discussion of environmental factors is beyond the scope of the current review.

What Has Been Learned from Genetic Profiling in NDDs?

The implementation of next-generation sequencing (NGS) technologies in the diagnostic flowchart of NDDs has dramatically increased the percentage of patients who receive a molecular diagnosis. The identification of the genetic etiology of the disease has important ramifications for genetic counseling and patient management, since it can lead to a better assessment of the recurrence risk and gives the possibility to foresee future medical complications. The advances in the field of genetics have also served as a roadmap for the development of functional genomic studies aimed at understanding the pathogenic mechanisms associated with the reported mutations. As discussed next, this line of research has elucidated some of the biological pathways important for the onset of NDDs. The recognition of these networks also offers an opportunity to overcome the complexities associated with the wide genetic variability, and to develop targeted therapeutic approaches.

Principal Molecular Pathways Affected in NDDs

Functional studies performed during the past decade have shown that most rare and common variants associated with NDDs affect genes that have a role in a few conserved pathways [26–28]. 'The Psychiatric Cell Map Initiative' was established a few years ago to understand the molecular pathophysiology of NDDs and to define the key biological pathways along temporal and spatial axes [29]. Along these lines, it emerged that both common and rare variants result in the perturbation of the homeostatic equilibrium at different levels (i.e., at a cellular, circuit, or whole brain level) (reviewed in [30]). In this review, we classify the numerous genetic variants based on their effects on a discrete number of functional molecular pathways. The pathways that are examined in detail comprise: (i) protein synthesis; (ii) transcriptional or epigenetic regulation; and (iii) synaptic signaling (Figure 2). Importantly, many mutations appear to be ultimately connected in a **multipathway loop** (reviewed in [30]). In this context, second-hit mutations in highly vulnerable genes or the accumulation of common variants can affect the entire loop, hence showing the importance of the aforementioned pathways for the onset of the disorder.

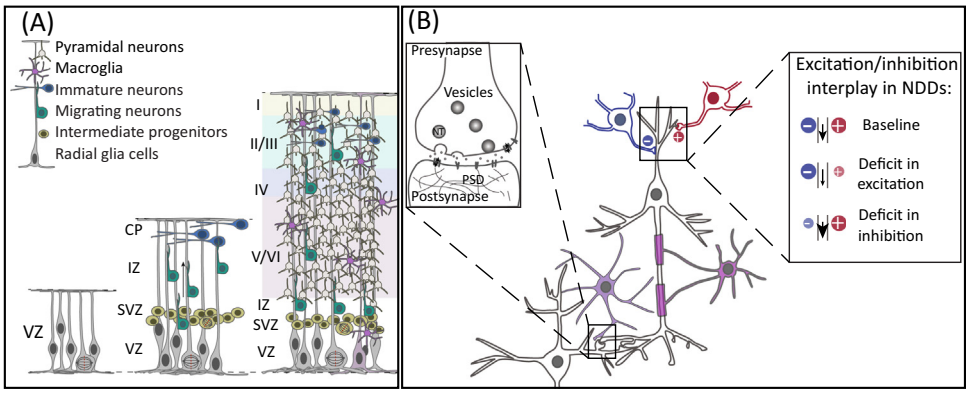


Figure 2. Processes Affected in Neurodevelopment Disorders (NDDs) in the Developing and Mature Brain. NDD risk variants have been found in genes with a role primarily in three critical processes: (i) regulation of protein synthesis; (ii) transcriptional and epigenetic regulation; and (iii) synaptic signaling, particularly when associated with synaptic maturation. Homeostasis of these processes can be disturbed during neurogenesis, migration of neurons, and their differentiation [cf. (A)] in prenatal brain development, or synaptic maturation and proper emergence of inhibitory/excitatory balance in postnatal development [cf. (B)]. The different NDD-associated variants indicate which molecular pathways and processes are fundamental to control this exquisite homeostatic equilibrium. Abbreviations: AP, Action potential; CP, cortical plate; IZ, inner subventricular zone; PSD, postsynaptic density; SVZ, subventricular zone; VZ, ventricular zone. Figure reproduced courtesy of Jasmin Morandell.

Impact of Growth Factors and Amino Acid Signaling on Protein Synthesis

Rare and common NDD-causing variants frequently alter the homeostatic balance of protein synthesis during neurodevelopment. The **phosphatidylinositol 3-kinase (PI3K)-mTOR** represents a key pathway for this balance and mutations affecting this axis have been associated with several NDDs (also known as **mTORopathies**) [26,31–35].

mTOR is a highly conserved serine/threonine kinase ubiquitously expressed in eukaryotic cells. Through two different complexes (mTORC1 and mTORC2), mTOR signaling regulates cellular metabolism. During embryonic development, mTOR regulates neuronal progenitor proliferation and differentiation, and neurite outgrowth and elongation, important processes that appear to be coordinated via mRNA translation and regulation of cell cycle progression and exit [36]. In the adult brain, mTOR participates in additional key processes, such as adult neurogenesis, learning, memory, circuit refinement, and synaptic plasticity [36,37]. mTOR integrates inputs from three signaling sources: the growth factor pathway, which comprises the PI3K-AKT-TSC complex, the energy-sensing arm, which responds to low concentrations of ATP through the AMPK-TSC complex, and the amino acid-sensing arm, which is the less characterized regulator of the mTOR pathway and controls the activation of mTORC1 directly through Rag GTPases [36]. Multiple variants affecting negative regulators of the growth factor and amino acid-sensing arms (such as *TSC1*, *TSC2*, and *PTEN* or *DEPDC5*, *NPRL2*, and *NPRL3*, respectively) are known to cause hyperactivation of mTORC1 and have been reported in individuals with NDDs [38,39]. Mutations in *TSC1*, *TSC2* and signaling proteins that function upstream of the TSC complex, such as *AKT* or *PTEN*, have been observed in individuals with ASD, ID, and epilepsy. By contrast, loss-of-function mutations in components of the GAP activity toward Rags1 (GATOR1) complex, such as *DEPDC5*, have been associated with focal epilepsy. Hence, mutations in different mTOR-regulating signaling arms appear to correlate with different phenotypical outcomes. Importantly, mouse models with heterozygous mutations in the *Tsc* or *Depdc5* show variable phenotypes and do not recapitulate all clinical signs observed in humans (Table 1). However, they have been instrumental in studying the underlying pathogenic mechanisms.

Table 1. Haploinsufficient and Conditional *Tsc1/2* and *Depdc5* Mouse, Rat, and Human Cell-Based Models

Gene	Mouse or rat model	Human cell-based model	Mouse phenotype	Cellular mechanism affected	Refs
Tsc1	*Tsc1* +/– KO		Impaired memory in MWM, CFC, and social interaction	Absence of brain pathology	[40]
	L7-Cre[a]; *Tsc1* floxed		Social impairments, repetitive behaviors, and abnormal UV vocalizations (+/–), impaired memory in MWM (–/–)	Increased spine density (+/–), changes in intrinsic excitability	[46]
	CamKII-Cre[b];*Tsc1* floxed		Increase of severity of seizures, early lethality (–/–)	Changes in intrinsic excitability, reduction inhibitory synaptic transmission (–/–)	[44]
	Gbx-CreERT[c]; *Tsc1* floxed		Repetitive behavior, increase of spontaneous seizures (–/–)	Disorganization of thalamic circuit, changes in intrinsic properties, somatic hypertrophy (–/–)	[47]
	Dat-Cre[d]; *Tsc1* floxed; *Dat*-Cre;*Tsc1* KO; *Rptor* +/–		Reduction of cognitive flexibility (rescue by *Rptor* KO)	Reduction intrinsic excitability, impaired DA release (rescue by *Rptor* KO), somatic hypertrophy	[48]
	SST-Cre[e]; *Tsc1* floxed			Abnormal maturation of SST interneurons, increase PV expression (+/–, –/–), less synaptic inhibition (–/–)	[45]
Tsc2	*Tsc2* +/– KO		Impaired memory in MWM, 8 radial arm, and CFC	Changes in late phase of LTP	[41]
			Impaired UV vocalization		[42]
	NEX-Cre[f]; *Tsc2* floxed		Early lethality (–/–)	Abnormal migration and neuronal hypertrophy (–/–), increase astrogliosis (+/–, –/–)	[49]
TSC2		*TSC2* +/– Purkinje cells		Delay cerebellar neuronal maturation, somatic hypertrophy and decrease expression of FMR1	[50]
		TSC2 +/–, –/– iPSCs		Somatic hypertrophy, FMRP targets downregulated, hypersynchronicity	[51]
		TSC2 +/– organoids		Identification of human-specific interneuron progenitor (CLIP-cells) associated with TSC	[54]
TSC1, TSC2		*TSC1* +/–, *TSC2* +/– spheroids		Dysmorphic neurons (only in –/–), change in neuron:glia differentiation ratio (+/–), strong inhibition of neuronal differentiation (–/–)	[52]
Tsc2, Fmr1	*Tsc2* +/– KO, *Tsc2* +/–; *Fmr1* –/y		Impaired memory in CFC, rescued by *Fmr1* KO	Deficient mGluR-LTD (+/–), rescued by *Fmr1* KO	[43]
Depdc5	*Depdc5* +/– rat		Lethal (–/–), no seizures (+/–)	Dysmorphic neurons (+/–)	[60]
	Syn1-Cre[g];*Depdc5* floxed		Survival decrease and early onset of seizures(–/–), any changes (+/–)	Dysmorphic neurons and reactive astrogliosis (–/–)	[61,62]
	Depdc5 focal KO (IUE)		30% *Depdc5* focal KO developed spontaneous seizures	Abnormal cortical migration, dysmorphic neurons	[63]
			100% Depdc5 focal KO developed spontaneous seizures	Noncell-autonomous activation of mTOR	[64]

Mouse conditional models studied: [a]L7-Cre (Purkinje cells), [b]CamKII-Cre (forebrain mature neurons), [c]Gbx-CreERT (thalamic neurons), [d]Dat-Cre (dopamine neurons), [e]SST-Cre (somatostatin interneurons), [f]NEX-Cre (pyramidal neurons of neocortex), [g]Syn1-Cre (neuronal cells).
Abbreviations: CFC, contextual fear conditioning; LTD, long-term depression; MWM, Morris Water Maze; PV, parvalbumin interneurons; SST, somatostatin interneurons; UV, ultrasonic vocalizations.

Tuberous sclerosis complex (TSC) is an autosomal NDD with variable penetrance caused by mutations in *TSC1* (hamartin) or *TSC2* (tuberin) and characterized by the presence of benign tumors (i.e., tubers) in multiple organs, including the brain. Neurological comorbidities include ASD

(observed in 50% of cases), ID (50–60%) and early-onset seizures (80–90%) [38]. Loss-of-function mutations in *TSC1* or *TSC2* result in the loss of inhibition of mTORC1. *Tsc1* or *Tsc2* haploinsufficient mouse models display hippocampal-dependent memory deficits and ASD-like phenotypes but no tumors or seizures [40–43]. Further studies point to subtle dysfunctions in heterozygous conditional mice, associated with the process of maturation of GABAergic cells [44,45] and ASD features [44,46]. By contrast, homozygous conditional models display a more severe phenotype, characterized by cognitive impairments, spontaneous seizures, and neuronal hypertrophy [44–49]. Interestingly, recent evidence using human induced pluripotent stem cell (iPSC)-derived neurons and spheroids revealed that the level of inhibition of mTOR and neuronal/glial differentiation were strongly affected by a second mutation in the complex [50–53]. Thus, a possible explanation for the lack of a clear genotype–phenotype correlation might rely on the need for a second-hit mutation. However, a recent study performed on organoids derived from samples from patients with heterozygous *TSC2* revealed that *TSC2* heterozygous mutations lead to overproliferation of one specific population of interneuron progenitors (**CLIP cells**), which the authors describe as the founder population of TSC tumors. In this model, second-hit mutations are not causative for the development of the tubers but occur during their progression. Thus, the role of these human interneurons may explain why heterozygous *Tsc1* and *Tsc2* mice do not develop a TSC phenotype, and highlight the importance of human cell-based models for the study of NDDs [54].

Germline and somatic mutations of *DEPDC5*, *NPRL2*, and *NPRL3* (Table 1) have been identified as one of the major risk factors for epilepsy [55–58]. These genes encode components of the GATOR1 complex, a trimeric complex that inhibits mTORC1 lysosomal localization and its interaction with Rheb by inactivating Rag GTPases in response to amino acid limitations [59]. Similarly to *Tsc* mouse models, haploinsufficient and conditional knockout (KO) mouse models of *Depdc5* [21,60–64] do not recapitulate the focal epileptic phenotype shown by patients [39], hence pointing to a second-hit event as the possible cause of focal epilepsy. Accordingly, recent mouse studies have shown that a second somatic mutation in *Depdc5* leads to the development of focal epilepsy and neuronal migration defects in the cortex [21,63].

Altogether, these data underscore the impact of mutational load and the two-hit model on the delineation of the phenotypical outcome and the importance of taking into account these genetic factors to fully understand the molecular mechanisms behind NDDs.

Transcriptional and Epigenetic Regulation

Numerous genes associated with NDDs belong to the category of transcriptional regulators or chromatin remodelers [65]. By regulating the transcript levels of developmental genes in the brain, this class of proteins controls the maturation of cortical inhibitory and excitatory connections during development, as well as regulatory networks that drive neuronal specification and activity-dependent responses. Examples of well-known disease-causing genes classified as chromatin remodelers or transcriptional regulators include *MECP2*, *SETD5*, *CHD8*, *ASH1L*, *ARID1B*, and *KMT2A* [66–69]. Given the multiple targets of each of these proteins, dysregulation of any one of them can show pleiotropic effects. Relatedly, the CHD protein family comprises multiple isoforms with different roles in the distinct stages of neurodevelopment (reviewed in [70]), from the early stages of migration to the maturation of synaptic connectivity [71]. Interestingly, various isoforms have been reported in association with distinct neurodevelopmental phenotypes, namely ID for *CHD1* and *CHD4*, epileptic encephalopathy for *CHD2*, and ASD for *CHD8* [72–75]. Haploinsufficient mouse models are available for most *CHD* genes. For instance, conditional KO of *Chd4,* encoding one of the core ATPase subunits of the deacetylation-dependent transcriptional repressor NuRD complex, leads to microcephaly

and altered connectivity in the cerebellar cortex [71,76]. However, patients with mutations in this gene are characterized by a marked developmental delay, ID, and macrocephaly [77]. Hence, there are some differences between the phenotype of the mouse model and the human condition. Likewise, while mutations in *CHD8* are tightly associated with ASD in humans [75], *Chd8* heterozygous mutant mice display very mild phenotypes, thus making the function of *CHD8* difficult to interpret [78,79].

Haploinsufficiency of *ARID1B*, a structural subunit essential for the assembly of the **BRG1/BRM-associated factor (BAF) chromatin remodeling complex** [80], is also recognized as one of the most frequent cause of NDDs and results in a variety of clinical signs, ranging from sporadic ASD/ID to syndromic disorders [81,82]. Studies in mouse models have uncovered a role of Arid1b and the BAF complex during interneuron migration and differentiation in early cortical development and in controlling proper neurite outgrowth and maintenance [83,84]. Interestingly, *Arid1b* heterozygous mutant mice display a normal density of pyramidal neurons, but a significant reduction of GABAergic neurons, specifically parvalbumin-positive neurons (PV) [84].

Over the past few years, loss-of-function mutations affecting the SET-domain containing 5 (*SETD5*) gene have also been recognized as one of the most frequent causes of ID and ASD [81]. SETD5 represents an important regulatory link between the transcription machinery and the activity of a chromatin-modifying transcriptional corepressor complex. For this reason, SETD5 appears to be essential for the regulation of gene expression during early development and learning. Accordingly, *Setd5* heterozygous mice show dysregulation of the dynamic expression of synaptic proteins and changes in cell fate determination during early development [85].

Despite the phenotypical differences between the mouse models and the clinical signs observed in humans, the haploinsufficient models described in this section highlight the importance of the correct dosage of chromatin remodelers and transcriptional regulators for the proper execution of crucial cellular processes during development. The pathogenic mechanism associated with this class of proteins is linked to global transcriptional disturbances in the cells. The differentially expressed genes overlap across different cellular models and gene ontology analyses reveal an enrichment in genes involved in neuronal development, chromatin dynamics, cell cycle regulation, and RNA [86,87]. Importantly, the dysregulated modules are strongly enriched for known NDD-risk genes [85–87]. The dysregulation of the NDD genes that occurs in the presence of mutations in chromatin remodelers and transcriptional regulators also contributes to cellular dysfunction and further influences the phenotype. Therefore, it is the combination of direct and indirect effects deriving from the initial mutation that probably leads to the complex pattern of symptoms observed in NDDs and that makes the genotype–phenotype correlation difficult to interpret.

Dysregulation of Synaptic Signaling, Transcriptional Changes, and Translational Perturbations

During development, two major groups of synaptic protein contribute to the activity-dependent formation of neuronal circuits: cell-adhesion molecules (CAMs), which mediate the bidirectional organization of the pre- and postsynaptic compartments through *trans*-cellular signaling [88], and scaffolding and synaptic signaling-associated proteins, located at the postsynaptic density, which form large molecular networks of receptors and actin-associated proteins [89]. Deleterious variants in genes encoding these proteins can significantly alter the course of brain development and, therefore, it is not surprising that they have been repeatedly associated with NDDs [89]. For instance, neurexins (NRXN), neuroligins (NLGN), and SHANKs, proteins with an important role in the pre- and postsynaptic compartments, have been implicated in NDDs by independent studies in patients and

mouse models [90–98]. Human neurons carrying loss-of-function mutations in *NRXN1* display a significant synaptic impairment coupled with dysregulated release of the neurotransmitter [91]. By contrast, a specific missense substitution of *NLGN4* or loss-of-function mutations of *SHANK2* have been found to induce either an increase of excitatory synapse or hyperconnectivity of excitatory neurons, respectively [92,93]. Several excellent reviews focus on the role of these and other synaptic proteins in the context of NDDs. Herein, we describe recent studies indicating the functional and bidirectional connection of these genes with NDD-risk genes categorized within the other two pathways reviewed in this article. This connection highlights the modifying role of potentially any genetic variant affecting other NDD-linked signaling cascade genes on the clinical outcome.

For example, a recent transcriptomic analysis of *SHANK2* mutant human neurons identified a significant number of Fragile-X mental retardation protein (FMRP) targets and chromatin/transcriptional regulators among the differentially expressed genes [93]. In addition, patient studies revealed that mutations in *SHANK2* can coexist with variants in other NDD genes and suggested that these alterations act as phenotypic modifiers [94]. In particular, one patient was found to carry a deletion of *CYFIP1,* a cytoplasmic interactor of FMRP that modulates cap-dependent translation of mTOR [99] as well as the inhibitory:excitatory ratio [100,101]. Therefore, various defects can result from the simultaneous dysregulation of multiple pathways. Similarly, data obtained in a human model point to dysregulation of the PI3K pathway in neurons with reduced *SHANK3* expression [95]. Additionally, *Shank3* haploinsufficient mice show an abnormal level of histone acetylation, which can be rescued by acute treatment with romidepsin, a class I histone deacetylase inhibitor, thus linking epigenetic modifications to synaptic scaffolding proteins. Importantly, the inhibition of histone deacetylase leads to robust and long-lasting rescue of social deficits without affecting locomotor and anxiety behaviors in young mice. However, this rescue is limited by the lack of chronic effects in adult mice, suggesting a time developmental window for the interconnection of synaptic and epigenetics pathways [96]. SynGAP, the synaptic Ras/Rap GTPase-activating protein, is another critical component of the postsynaptic density associated with scaffolding proteins involved in the regulation of AMPA receptors [102]. Patients with *SYNGAP1* loss-of function mutations exhibit ID and ASD. Interestingly, *SynGAP* heterozygous mice show an increase of protein synthesis due to dysregulation of the mGluR-Erk1/2 signaling pathway, which mimics the molecular pathophysiology associated with the loss of FMRP in *Fmr1* KO mice. Pharmacological manipulation of the mGluR-Erk1/2 signaling pathway rescues behavioral phenotypes in both models [103].

Although CAMs proteins are central regulators of synapse development and specification, human loss-of-function mutations in NDD-linked cell adhesion molecules (*NRXN* and *NLGN)* are not associated with changes in transcriptional or translational regulators [91,92,97]. This discrepancy may be explained by gene redundancy. For instance, *NRXN1*, *2*, and *3* have been shown to have overlapping functions. Thus, mutations in one gene might not be enough to lead to certain phenotypic features. Similarly, alternative CAMs may overcome specific deficits in individuals with mutations in one of these genes. Since the signaling pathways activated by many of these CAMs are still unknown, a detailed molecular analysis might help to understand convergence between CAMs. In contrast, it is important to mention that mutations in genes regulating mRNA translation (e.g., 4eibp2 KO) have been associated with a specific dysregulation of neuroligins, suggesting a specific link between mTOR and neuroligins [104].

In summary, while significant research attention has focused on the role of synaptic proteins in NDDs, more recent studies have started to decipher the link between synaptic signaling and regulation of gene expression and protein synthesis. Future studies are warranted to understand how transcriptional, translational and synaptic signaling may converge at different developmental stages and how variants along these processes may functionally interact in NDDs.

Concluding Remarks and Future Perspectives

The study of inherited forms of NDDs has helped to raise awareness of the contribution of multiple genetic factors to the pathogenesis of these disorders. Despite the broad genetic heterogeneity of NDDs, the functional consequences of the different mutations appear to converge towards the disruption of highly interconnected core molecular pathways. These signaling cascades are important during different critical periods of neurodevelopment as well as in the adult brain [105]. In this review, we focused on changes in nuclear or cytoplasmic functions (i.e., transcription and translation) that can result, among other issues, in alterations of synaptic homeostasis. In this context, the severity of the phenotypical outcome may reflect the level of perturbation of these homeostatic mechanisms [30,106].

Despite these important advancements in the theoretical understanding of NDDs, the road to successful treatment is still long (see Outstanding Questions). Importantly, the functional convergence of the genetic causes raises the possibility that drugs targeting these core networks could be used to reverse some clinical features associated with NDDs. The establishment of reliable models to fully dissect the molecular mechanisms of NDDs, identify potential targets, and finally test new treatments represents a crucial step toward this possibility. Animal models aid to disentangle the complex genetic architecture of NDDs and the effects of various mutations at a molecular and phenotypical level. For example, ASD is characterized by discrete behaviors, such as impairment in social interaction, restlessness, and stereotyped behaviors. These complex behaviors can be modeled in mouse models, thus allowing behavioral analyses in the presence of disease-causing mutations. Accordingly, a detailed description of behavioral phenotypes is currently available for numerous haploinsufficient mouse models [89]. However, most functional studies in mouse models normally focus on isolated variants. Still, the phenotypical outcome is strongly influenced by mutational load, and can often be interpreted in the context of the two-hit model. In other words, the complex genetic architecture of NDDs, characterized by the presence of multiple variants, underscores the need to understand the epistatic effects of co-mutations. Additionally, it is also important to underscore the fundamental differences between animals and humans, especially during development. Some of these caveats can be addressed using models derived from human iPSCs obtained from patients with NDD. These models offer a useful complement to the analysis of postmortem human tissue, by allowing the design of *in vitro* neural circuits that recapitulate the genetic background of individuals with NDDs. Hence, they have the potential to lead to personalized treatments by predicting (at least *in vitro*) how drugs act on patient-specific molecular pathways, allowing researchers to determine the optimal readout for treatment [107–110]. One major disadvantage of models derived from human iPSCs is that systemic effects are largely neglected. Therefore, the combination of human cell-based models and animal models is crucial, especially for a better comprehension of the mechanisms leading to the onset of a disease-phenotype. In addition, CRISPR/Cas9 gene editing offers the possibility to generate multiple gene KOs in the mouse brain and study their modifying effect when acting simultaneously [21]. Furthermore, the rapid development of new bioengineering methods represents a promising tool to imitate neural circuit formation and brain development in 3D. Lastly, single-cell 'omics approaches, computational models, and bioinformatics network analysis could complement the aforementioned strategies and support our understanding of gene expression changes during neuronal development of the human brain. Altogether, these approaches will allow a more exhaustive comprehension of the molecular mechanisms underlying NDDs. This knowledge could lead to the development of targeted drugs and to a shift from the current paradigm of symptomatic treatment toward more resolutive curative treatments.

Outstanding Questions

Rare and common NDD-causing variants frequently alter the homeostatic balance of protein synthesis during neurodevelopment. Compensatory mechanisms can protect cells to some degree from the otherwise deleterious effects of some mutations. For example, changes in network activity can be compensated by neurons by controlling the strength of their synapses. Analogously, up- or downregulation of certain ion channels represents a neuronal strategy to alter intrinsic activity and adjust neuronal firing. Can we utilize these compensatory mechanisms to develop future treatments? If so, are additional consequential mechanisms being activated?

Technology is constantly improving, allowing a better understanding of the molecular etiology of NDDs. To what degree will it be possible to translate the knowledge obtained about NDDs into clinical applications in the future?

Brain development is a critical and tightly regulated process, involving multiple neurobiological pathways, which establishes the basic functions of the brain. Are there common pathways affected in different NDDs during prenatal or early postnatal stages? If so, could these common pathways serve as an early prognosis signature of the disease?

NGS can help understand the genetic architecture of NDDs and provides a substantial amount of information. However, the application and analysis of NGS data remain challenging. Which would be the most effective methods to cope with this massive amount of data in terms of analysis and database management?

Gene therapy approaches are advancing rapidly, and have already been applied clinically in a few cases of monogenic disorders. Does gene therapy represent a realistic possibility for the treatment of certain NDDs? How much of an obstacle, in terms of gene therapy, is the complex genetics existing in many of these disorders?

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