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Modeling Cognitive Dysfunction in Neurofibromatosis-1

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Abstract

Cognitive dysfunction, including significant impairments in learning, behavior, and attention, is found in over 10% of children in the general population. However, in the common inherited cancer predisposition syndrome, Neurofibromatosis type 1 (NF1), the prevalence of these cognitive deficits approaches 70%. As a monogenic disorder, NF1 provides a unique genetic tool to identify and mechanistically dissect the molecular and cellular bases underlying cognitive dysfunction. In this review, we discuss *Nf1* fly and mouse systems that mimic many of the cognitive abnormalities seen in children with NF1. Further, we describe discoveries from these models that have uncovered defects in the regulation of Ras activity, cAMP generation, and dopamine homeostasis as key mechanisms important for cognitive dysfunction in children with NF1.

Keywords

neurofibromin; cyclic AMP; dopamine; NF1; learning; attention deficits

Introduction

Neurofibromatosis type 1 (NF1) is one of the most common neurogenetic disorders affecting the nervous system. The hallmark of NF1 is the development of tumors involving the central and peripheral nervous systems. In this condition, affected individuals are prone to the formation of peripheral nerve sheath tumors (ie. neurofibromas, plexiform neurofibromas, and malignant peripheral nerve sheath tumors) and brain tumors (optic pathway gliomas and malignant gliomas). Moreover, 50–70% of children with NF1 manifest specific cognitive impairments, including difficulties with attention, executive function, language, visual perception and learning [1–4]. While most children exhibit some form of cognitive deficit that negatively impacts their scholastic performance, the specific cognitive abnormality present (ie. attention deficit, spatial memory impairment, fine motor delay) and the severity of the deficit varies greatly from child to child.

In concert with clinical studies characterizing the spectrum of learning, behavioral and motor delays in children with NF1, laboratory investigations have begun to define the molecular and cellular etiologies for these common problems. Using *Nf1* genetically-engineered strains of mice and flies, investigators have successfully modeled many of the cognitive and behavioral deficits seen in children with NF1, and employed these model

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systems to better define the role of the *NF1* protein (neurofibromin) in normal central nervous system (CNS) neuronal function. This review will highlight the basic neurobiological insights that have derived from the use of these robust preclinical strains as well as their importance for the identification and validation of new therapeutic drug targets relevant to the treatment of children with NF1.

Clinical features of NF1

Neurofibromatosis-1 (NF1) is a common nervous system disorder, affecting 1 in 3500 people globally [5]. NF1 is inherited in an autosomal dominant manner, although ~50% of patients present with *de novo* mutations, and represent the first member of their family with NF1 [6]. While *NF1* genetic testing is available for select individuals, the diagnosis of NF1 is most often established on clinical grounds (Table 1). To be given the diagnosis of NF1, individuals must have at least two features of the condition, including greater than 6 café-au-lait macules (birthmarks), skinfold (underarm or groin) freckling, Lisch nodules (iris hamartomas), neurofibromas, an optic pathway glioma, a distinctive bone abnormality (tibial dysplasia), or a first degree relative with NF1 [7]. In addition to these features, individuals with NF1 may also manifest learning/behavioral problems, malignant gliomas, T2-hyperintensities on neuroimaging (ie. magnetic resonance imaging) studies, enlarged heads (macrocephaly), gross and fine motor delays, short stature, and other less common cancers [8–13].

Cognitive and behavioral deficits in children with NF1

Cognitive problems are the most frequently observed neurological impairments in children with NF1. The majority of children display some degree of cognitive deficits [1], which limit their full academic achievement and overall quality of life. Clinical studies examining cognitive problems in NF1 have revealed a left shift in average IQ, ranging from low-to-normal IQs, with specific learning deficits observed in 30–70% of children [1, 14, 15]. Additionally, children with NF1 exhibit poor performance on tasks of reading, spelling and mathematics, impaired expressive and receptive language skills, deficits in visuospatial and visuoperceptual skills, and defects in executive function (planning and concept formation) [1, 16, 17]. While less common, there is also an increased incidence of autistic spectrum disorder in children with NF1 [18]. Problems with attention and behavior in children with NF1 can also negatively impact school performance and social interactions [19–21]. Nearly 70% of children with NF1 report deficits in one or more of the attention system domains (sustained, selective, divided and shifting attention) [1, 22, 23], and one-third to one-half of children are diagnosed with attention-deficit hyperactivity disorder (ADHD) [1, 2]. Moreover, children with NF1 tend to be impulsive, and often have difficulty detecting and responding to social cues.

Neurofibromin structure and function

The *NF1* gene resides on chromosome 17 [5, 24] and encodes a large cytoplasmic protein (neurofibromin), encompassing 2818 amino acids and over 60 exons. Neurofibromin contains several domains, including a cysteine-rich domain (CSRD), a leucine repeat domain (LRD), and a Ras-GAP domain (GRD) (Figure 1a). The *NF1* gene also contains at least 3 alternatively spliced exons, 9a, 23a, and 48a, each with unique properties. Exon 9a-containing neurofibromin is a neuron-specific isoform [25, 26], whereas exon 48a-containing neurofibromin is expressed in muscle [27]. Exon 23a neurofibromin interrupts the normal function of the GAP domain, but has a more widespread tissue distribution [28–30]. Interestingly, mice engineered to lack exon 23a do not have an increased tumor predisposition, but manifest specific learning impairments [28]. Current studies are focused

on defining the functional consequences of *NF1* alternative splicing, especially exon 9a, on neurofibromin signaling and NF1-associated tumor and cognitive deficits.

The neurofibromin GRD functions in a similar fashion to other GTPase-activating proteins (GAPs), which negatively regulate the activity of the p21-Ras proto-oncogene (Figure 1b). Ras is recruited to the plasma membrane by adaptor proteins and activated by receptor tyrosine kinases (RTKs) following growth factor binding. At the membrane, guanine nucleotide exchange factors (GEFs) enable Ras to bind guanosine triphosphate (GTP) to become active and transmit growth-promoting signals. In its active state, Ras signals to several downstream effectors, including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3 kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathways. Neurofibromin acts as an inhibitor of Ras activity by catalyzing the hydrolysis of active GTP-bound Ras to inactive GDP-bound Ras, such that in cells lacking neurofibromin, there are high levels of Ras pathway (ie. MEK/MAPK and PI3K/mTOR) activity, and increased cell growth [31–34].

In addition to Ras regulation, neurofibromin is also a positive regulator of intracellular cyclic AMP (cAMP) levels. Studies initially performed in *Nf1* mutant flies revealed that *Drosophila* larval size did not result from de-regulated Ras signaling, but rather was dependent on cAMP-mediated Protein Kinase A (PKA) activity [35, 36]. Subsequent studies in mice likewise demonstrated that both *Nf1*-deficient embryonic brains and postnatal astrocyte cultures had reduced cAMP levels [37, 38]. While the precise mechanism(s) underlying neurofibromin control of cAMP homeostasis has yet to be fully elucidated, there appear to be both Ras-dependent and Ras-independent modes of regulation [36, 37, 39].

Nf1 Models of Learning, Memory, and Attention Deficits

Inspection of the predicted protein sequence of the *Drosophila* and murine *Nf1* homologs reveals 60% and greater than 98% identity to the human *NF1* gene product, respectively, supporting the use of *Nf1* mutant mouse and fly models to study many of the cognitive and behavioral features seen in the human condition [35, 40, 41]. In this regard, robust mouse and fly models have been developed and employed for preclinical discovery and validation initiatives aimed at improving clinical outcomes for children and adults with NF1 (Figure 1c).

The Role of Ras Activation in Learning and Memory

Studies pioneered in the late 1990s by Alcino Silva and colleagues employed mice harboring a germline inactivating mutation in one *Nf1* allele. These *Nf1* heterozygous (*Nf1*+/−) mice are genetically similar to children with NF1 who start life with one functional and one non-functional copy of the *NF1* gene in every cell. *Nf1*+/− mice exhibit impaired spatial learning in the Morris water maze, a behavioral task used to assess hippocampal-based learning [42, 43]. These learning defects improve with training time, supporting clinical observations that children with NF1 can learn with additional focused training and resources [3]. Concomitant with defects in spatial learning, *Nf1*+/− mice also have decreased CA1 hippocampal long-term potentiation (LTP) following theta-burst stimulation [43]. Further characterization of this LTP deficit revealed abnormalities in both early-phase and long-term LTP maintenance [43, 44]. Whereas early-phase LTP is thought to underlie immediate learning, late-phase LTP regulates long-term memory formation [45]. In this manner, abnormalities in both early-phase and late-phase LTP could contribute to the learning and memory deficits in *Nf1+/−* mice.

To better define the connection between reduced neurofibromin and impaired hippocampal function, Silva and colleagues explored the possibility that increased Ras activity was

responsible for the observed cognitive deficits [43]. Their demonstration of increased Ras and MEK activity in the brains of *Nf1*+/− mice prompted genetic rescue experiments, where *Nf1*+/− mice were crossed with mice to reduce Ras (K-Ras and N-Ras) expression ($Nf1^{+/-}$;$KRas^{+/-}$ or $Nf1^{+/-}$;$NRas^{+/-}$ mice). In these studies, performance of *Nf1*+/− mice with either reduced K-Ras or N-Ras expression was comparable to that found in wild-type control mice [43]. Similarly, pharmacological inhibition of Ras using a drug that blocks the post-translational farnesylation of Ras (ie. Lovastatin), improved performance of *Nf1*+/− mice on hippocampal-based spatial learning tests [43, 46]. Collectively, these findings provide strong evidence for a functional link between reduced brain neurofibromin expression, elevated Ras activity and cognitive deficits, and prompted clinical trials using Lovastatin-like treatments for children with NF1 [47].

The cellular and neurochemical bases for impaired LTP and learning in *Nf1*+/− mice have also been investigated. Using *Nf1* conditional knockout (CKO) mice in which one copy of the *Nf1* gene was selectively inactivated in neurons or astrocytes, the consequence of reduced neurofibromin expression in distinct cell populations on mouse learning and memory was evaluated [48]. While reduced *Nf1* expression in neurons recapitulated the learning and memory deficits observed in *Nf1*+/− mice, it had no effect in Glial fibrillary acidic protein (GFAP) positive astrocytes. Neuron subpopulation-specific Cre driver lines have also been employed to address learning deficits after selective inactivation of *Nf1*. Specifically, reduced *Nf1* expression in excitatory and inhibitory neurons (Synapsin-I-Cre mice) or GABA-containing neurons [Distal-less Homeobox 5/6 (Dlx5/6)-Cre mice], but not forebrain pyramidal neurons [ie. Ca 2+/Calmodulin-Dependent Protein Kinase II (CAMKII)-Cre mice], resulted in defects in learning and memory similar to that observed in *Nf1*+/− mice. These observations demonstrate a primary neuronal defect as a responsible cellular etiology underlying murine *Nf1* learning and memory dysfunction.

Based on the finding that inhibitory neurons were sensitive to the effects of reduced *Nf1* gene expression, it was subsequently shown that *Nf1*+/− mice had increased hippocampal GABA inhibitory tone. In these studies, elevated MAPK-mediated synapsin-I phosphorylation and increased GABA release in hippocampal inhibitory interneurons were shown to cause impaired *Nf1*+/− mouse LTP and learning [43, 48]. Consistent with this model, treating *Nf1* mutant mice with a GABAA receptor antagonist (ie. picrotoxin) restored normal LTP in the hippocampus [43]. These findings established a direct relationship between Ras regulation and GABAergic signaling relevant to the *Nf1* learning deficits.

The notion that de-regulated Ras activation can cause cognition dysfunction is further underscored by several human disorders caused by genetic mutations that lead to increased Ras signaling. For example, patients with Legius syndrome, a NF1-like disorder that results from inactivating mutations in the *SPRED1* Ras-MEK/MAPK regulator, exhibit mild neurocognitive deficits [49–51]. Similar to *Nf1+/−* mice, defective *spred1* function in mice resulted in increased MAPK signaling [49, 52, 53], in addition to deficits in learning and memory [54]. Costello syndrome is a Ras disorder (“Ras-opathy”) caused by an activating mutation in the *HRAS* gene, leading to constitutive Ras signaling [55]. Children with Costello syndrome often have cognitive impairments, low IQs, and increased anxiety [56]. Similar to their human counterparts, $HRas^{G12V}$ knock-in mice exhibit increased anxiety behaviors and cognitive impairments [57]. Lastly, in additional models of aberrant Ras activity, pharmacological inhibition of PI3K or the MAPK kinase MEK was effective at restoring normal synaptic plasticity and learning in rodents [58–61]. Taken together, these studies demonstrate that inappropriate regulation of Ras activity can disrupt cognitive function, providing a preclinical rationale for Ras pharmacological treatments for NF1-associated cognitive deficits.

The Role of cAMP in Learning and Memory

In *Drosophila*, neurofibromin modulates cAMP by activating the adenylyl cyclase 1 (AC1) homolog, rutabaga (*rut*) [35, 36]. While the exact mechanism remains to be elucidated, activation of *rut*-AC is initiated by an interaction with the C-terminus of neurofibromin [39]. Further, this interaction is essential for learning and memory in flies [62, 63]. Using a classic Pavlovian olfactory behavioral paradigm, flies lacking *Nf1* or *rut*-AC show reduced performance, indicative of learning and memory impairments [62–66]. Restoration of neurofibromin expression using *dNf1* heat-shock transgenes or correction of the cAMP defect by ectopic protein kinase A (PKA) expression restored normal learning and memory in *Nf1* mutant flies [62]. Recent studies have revealed that distinct domains of *Drosophila* neurofibromin can modulate different components of learning and memory. Whereas mutations in the GAP domain cause defects in long-term memory, the cAMP-activating C-terminal region of neurofibromin mediates immediate memory or learning [45]. A direct link between cAMP homeostasis and cognitive defects has not been firmly established in *Nf1* mouse models, yet support for a role of cAMP in learning and memory derives from studies in other rodent models. In these reports, pharmacological and genetic manipulation of cAMP levels enhanced LTP, learning, memory, and hippocampal neurogenesis [67–71].

In *Nf1* mutant mice, some of the defects observed in CNS neurons were found to be dependent on cyclic AMP signaling. Following complete *Nf1* loss in neural stem cells (NSCs), reduced secondary somatosensory cortex thickness was observed [72]. Further examination of these *Nf1* CKO (ie. $Nf1^{BLBP}$CKO) mice revealed that this reduction in cortical thickness resulted from decreased neuronal arborization, rather than from reductions in the total number of neurons. Using *in vitro* methods, it was demonstrated that neurons differentiating from *Nf1*-deficient NSCs also had shortened neuronal processes [72]. The biochemical basis for this defect in neuronal morphology was shown to result from impaired cAMP generation, as both $Nf1^{BLBP}$CKO mouse brains and *Nf1*+/− primary neurons had lower cAMP levels *in vitro* and *in vivo* [72–74]. Re-expression of the Ras-regulatory NF1-GAP domain in $Nf1^{BLBP}$CKO mice did not correct the cortical thickness deficit. Similarly, expression of an activated K-Ras allele in NSCs both *in vitro* and *in vivo* did not alter neurite length. Instead, treatment of $Nf1^{BLBP}$CKO mice with pharmacological agents that increase cAMP levels restored normal cortical thickness *in vivo* and neurite lengths in primary *Nf1+/−* neurons *in vitro* [72–74].

Since the brains of children with NF1 are composed of neurons with reduced, not absent, *NF1* gene expression, subsequent studies have employed *Nf1*+/− CNS neurons as a relevant model (Figure 2a). Similar to *Nf1*-deficient neurons, *Nf1+/−* CNS neurons exhibited 25% and 40% reductions in neurite (axonal) length and growth cone area, respectively [72, 73]. These abnormalities resulted from impaired cAMP generation, and were corrected by treatments that elevated intracellular cAMP levels, including Rolipram (a phosphodiesterase-4 inhibitor), non-hydrolyzable cAMP analogs, and adenylyl cyclase (AC) activators (eg. Forskolin). Conversely, blocking cAMP production, using 2,3 dideoxyadenosine (DDA) to inhibit adenylyl cyclase function, was sufficient to abrogate neurite extension in wild-type neurons [73, 74]. Lastly, neurofibromin regulation of actin cytoskeletal dynamics was found to be dependent on cAMP-mediated PKA activation of Rho/ROCK/MLC signaling [74], such that correcting the defective signaling conferred by any component along this pathway normalized *Nf1*+/− axon lengths and growth cone areas *in vitro* (Figure 2b).

Further examination of these abnormal *Nf1*+/− CNS neurons also revealed small increases in programmed cell death. In studies examining the impact of reduced neurofibromin expression on retinal ganglion cells (RGCs), optic glioma formation in *Nf1* mutant strains was associated with increased RGC apoptosis [73, 76]. As observed *in vitro*, treatment of

Nf1 optic glioma-bearing mice with Rolipram (to raise intracellular cAMP levels) almost completely ameliorated the increased RGC programmed cell death *in vivo* [73], underscoring the importance of neurofibromin-mediated cAMP homeostasis in the maintenance of normal CNS neuronal function. Interestingly, CNS neurons with reduced *Nf1* expression had similar levels of cAMP as neurons completely lacking neurofibromin expression, emphasizing the profound effect of *Nf1* heterozygosity on CNS neuronal morphology and function [74]. Further studies using hippocampal neurons demonstrated a requirement of neurofibromin for dendritic spine formation, such that *Nf1* knockdown reduced spine formation in mature neurons *in vitro* [77]. Correspondingly, dendritic spine densities were also reduced in the brains of *Nf1+/−* mice [78]. Together, altered dendritic spine density, axonal morphological defects, and reduced neuronal survival may lead to impaired synaptic efficacy and reduced cognitive function in children with NF1.

The Role of Dopamine Homeostasis in Learning, Memory, and Attention

Due to the high prevalence of attention problems in children with NF1, stimulant medications, such as methylphenidate (MPH), have proven to be effective treatments in this affected population [2]. MPH restores dopamine homeostasis by blocking dopamine reuptake through the DA transporter (DAT), allowing for increased extracellular dopamine availability in dopaminergic neurons [79, 80]. While brain dopaminergic defects have yet to be reported in children with NF1, impaired dopamine homeostasis has been shown in an *Nf1* genetically-engineered mouse strain [81]. In these studies, *Nf1+/−* mice with total *Nf1* loss in GFAP+ astroglial cells (ie. *Nf1*+/–^{GFAP}CKO mice) were used. Similar to *Nf1*+/− mice, *Nf1*+/–^{GFAP}CKO mice had reduced exploratory behaviors and performed poorly on tests of selective and non-selective attention. Moreover, there were reduced dopamine levels and reduced post-synaptic dopamine signaling in the striatum, as measured by decreased dopamine- and cAMP-regulated phosphoprotein- 32 (DARPP32) phosphorylation. In addition, cell-autonomous decreases in the growth cone areas and neurite lengths of dopaminergic neurons *in vitro* were observed, resulting in attenuated cell projections to the striatum *in vivo*. While post-synaptic dopamine receptor expression was normal, presynaptic dopamine transporters [ie. DAT and vesicular monoamine transporter (VMAT)] and tyrosine hydroxylase (TH) expression were significantly reduced in *Nf1*+/–^{GFAP}CKO mice [81] (Figure 3). To provide a preclinical measure of reduced striatal dopamine levels, positron emission tomography (PET) imaging with ^{11}C-raclopride demonstrated increased binding in the striata of *Nf1*+/–^{GFAP}CKO mice, indicative of low endogenous dopamine [75]. Consistent with a dopamine availability deficit, and similar to children with NF1, treatment with dopamine-elevating drugs, such as MPH and L-DOPA, increased striatal dopamine levels and ameliorated attention defects in these mice [75, 81].

Dopamine system function is also critical for learning and memory in rodents, such that disruption of hippocampal dopaminergic innervations and loss of hippocampal D1/D5 dopamine receptor function result in spatial learning defects [82–84]. A recent study has explored the relationship between dopamine homeostasis and learning in *Nf1*+/–^{GFAP}CKO mice further [85]. This study found *Nf1*+/–^{GFAP}CKO mice to have reduced dopamine levels in the hippocampus, similar to the striatum. Moreover, L-DOPA administration rescued the post-acquisition Morris Water Maze probe trial deficits *in vivo* and dopamine D1 receptor agonist (ie. SKF-38393) treatment corrected the LTP abnormalities in hippocampal slice preparations *in vitro*. These findings, coupled with previous observations, establish defective dopaminergic function as a contributing factor that is important for both spatial learning/memory and attention system dysfunction in *Nf1* mutant mice.

While the precise mechanism by which neurofibromin modulates dopamine signaling has yet to be identified, these findings suggest that dopamine-targeted therapies may be useful treatments for some children with NF1-associated cognitive abnormalities. Furthermore,

these studies highlight the potential utility of raclopride PET imaging to stratify children with NF1 into subgroups most likely to respond to dopamine-elevating treatments.

Clinical heterogeneity and implications

While it is convenient to regard monogenetic disorders as homogenous medical conditions, the marked clinical variability between members of the same family with the same germline *NF1* genetic mutation argues to the contrary. Instead, it is more likely that NF1 is composed of numerous distinct diseases, each defined by factors including patient age, patient sex, the timing of *NF1* inactivation, the specific cell type, genomic modifiers, and microenvironmental influences. These factors have begun to be elucidated in cancers arising in *Nf1* genetically-engineered mice where the timing of bi-allelic *Nf1* gene inactivation [86–88], the cell type (cell of origin) [89–96], the genetic (strain) background [97–99], and the non-neoplastic cell microenvironment [93, 100–102] play critical deterministic roles.

Based on these observations, we propose a model of NF1-associated cognitive abnormalities in which the wide range of clinical features represent an admixture of distinct cellular and molecular etiologies. As such, there is no single molecular abnormality that underlies all of the cognitive dysfunction observed in children with NF1. Rather, the specific collection of cognitive and behavioral deficits observed in any given child with NF1 reflects the relative contributions of multiple cellular and molecular defects (Figure 4). Emerging evidence implicates specific neuronal populations, several molecular defects, and other contributing factors; as such, we envision a model of cognitive dysfunction in which the resulting clinical phenotype reflects the interplay of all of these potential abnormalities.

First, genomic factors likely influence the specific constellation of cognitive and behavioral deficits observed in children with NF1. In this regard, attention deficits are more common in boys in the general population, but are equally represented in both sexes in children with NF1 [1]. This finding suggests that sexual dimorphic genomic factors may contribute to the phenotypes observed in pre-pubescent children, as have been reported for other CNS abnormalities in mice [103–105]. Second, not all CNS neuronal populations appear to be equally affected by reduced *Nf1* gene expression. The use of conditional knockout mouse strains has already revealed that *Nf1*+/− forebrain pyramidal neurons do not contribute to the abnormal learning and memory [48]. It is possible that distinct neuronal populations differentially respond to reduced neurofibromin expression, and contribute in unique fashions to the learning, memory, and attention deficits that characterize mice and people with a germline inactivating *Nf1* gene mutation. Third, the specific germline *NF1* gene mutation may create different abnormalities in CNS neurons that reflect the degree of neurofibromin reduction or a specific impairment in cAMP or Ras signaling. While there are few studies examining the effect of specific *NF1* gene mutations on neurofibromin expression and function, *Nf1*+/− mice maintained on different genetic backgrounds exhibit a wide spectrum of reduced *Nf1* mRNA expression [99]. Fourth, it is possible that some of the more severe neurocognitive abnormalities occasionally seen in children with NF1 may reflect total loss of neurofibromin expression in select populations of CNS neurons. Recent evidence demonstrates gross brain abnormalities following *Nf1* biallelic inactivation in developing neural stem cells [106]. Further study will be required to formally evaluate this intriguing possibility. Fifth, *Nf1* mutant mouse strains have been instructive in elucidating the major neurochemical defects responsible for learning, memory and attention abnormalities associated with NF1. Intelligent use of these preclinical mouse strains may provide insights into potential treatments that reflect the predominant signaling defect (e.g., cAMP, Ras, dopamine) in individuals with NF1.

Finally, it is possible that non-neuronal cell types, including *NF1*+/− oligodendrocytes, astrocytes, and microglia, may contribute to neuronal dysfunction in the brains of children and adults with NF1. In this regard, astrocytes are important for glutamate homeostasis in one tuberous sclerosis complex (TSC) mouse model of CNS disease, such that impaired astrocyte glutamate transport is partly responsible for the seizures and synaptic plasticity defects in *Tsc1* conditional knockout mice [107, 108]. Similarly, oligodendrocytes can maintain neuronal integrity and promote axonal signal propagation. Previous studies revealed that spinal cord oligodendrocyte precursor differentiation is regulated by neurofibromin [109], such that two-fold more NG2+ progenitor cells reside in the spinal cords of *Nf1*+/− mice. Lastly, microglia can regulate synaptic plasticity and pruning [110, 111]. Increased numbers of microglia have been observed in *Nf1*+/− mouse brains [100], where they represent critical modulators of optic glioma growth [101, 102]. Their roles in NF1-associated learning, memory, and attention deficits have not been explored.

Concluding Remarks

In this review, we have highlighted the roles of Ras, cyclic AMP, and dopamine as key molecular targets that modulate NF1-associated cognitive dysfunction. Each of these factors represents a potential therapeutic target; however, the efficacy of any particular treatment regimen will rely heavily on the specific constellation of molecular and cellular abnormalities present in a given child with NF1 (Figure 5). This idea is underscored by the outcomes of several recent NF1 clinical trials. While treatment with the Lovastatin analog, Simvastatin, did not improve cognitive function in the group as a whole, it did enhance performance on some features of learning and memory in a subgroup of patients [47]. A more recent phase I Lovastatin trial demonstrated some improvement in verbal and nonverbal memory, without any effect on attention system dysfunction [112]. Similarly, some reports have shown that MPH can improve working memory in children with ADHD [113] as well as learning in NF1 patients [2]. By considering the interplay between each of these cellular and molecular abnormalities, future approaches to managing the learning, memory, and attention system deficits in children with NF1 may consider integrating targeted therapies specific to the biochemical and neurochemical abnormalities unique to each child (Box 1).

Box 1

Outstanding questions

- How does neurofibromin regulate cAMP homeostasis in CNS neurons?
- How does neurofibromin regulate dopamine homeostasis in the brain?
- What is the relationship between neurofibromin cAMP, Ras, and dopamine regulation in CNS neurons?
- Does reduced neurofibromin function in distinct CNS neuronal populations differentially contribute to the cognitive and behavioral abnormalities observed in children with NF1?
- How do non-neuronal cells (such as microglia, oligodendrocytes, and astrocytes) influence the cognitive and behavioral abnormalities in children with NF1?
- How do combinations of molecular and cellular abnormalities result in specific cognitive profiles in children with NF1?

- What underlies the clinical heterogeneity in children with NF1-associated cognitive and behavioral abnormalities (genomic modifiers, environmental factors, patient sex, etc.)? What are the implications for treatment?
- How can we stratify children with NF1-associated cognitive and behavioral abnormalities based on their unique molecular and cellular defects? What are the implications for personalized therapy?

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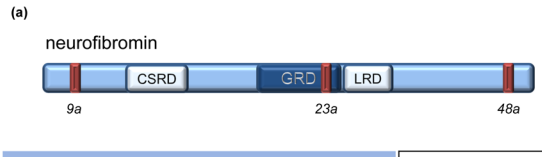

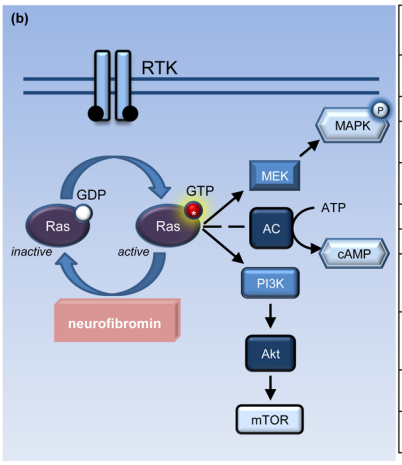

(c) **Modeling NF1-associated Cognitive Deficits**

	Nf1 mutation	Cognitive abnormality	Refs
Flies			
hs*Nf1*/+; *Nf1*^{P2}	heterozygote	olfactory associative learning	[67]
Nf1^{P1} and ^{P2}	null mutant	olfactory learning memory	[67, 68]
Nf1^{c00617}	null mutant	memory acquisition	[68]
Mice			
Nf1+/- (129/C57 hybrid)	heterozygote	spatial learning memory attention	[45, 49, 51]
Nf1$^{flox/-}$ GFAP-Cre	heterozygosity in neurons	spatial learning memory attention	[81, 87]
Nf1$^{flox/+}$ Syn1-Cre	heterozygosity in neuronal subset	spatial learning memory	[51]
Nf1$^{flox/+}$ Dlx5/6-Cre	heterozygosity in interneurons	spatial learning memory	[51]

Figure 1. Neurofibromin structure and function

(**a**) Neurofibromin is a 2818 amino acid protein that contains multiple alternatively spliced exons (9a, 23a, and 48a shown as red bars) and encodes several distinct functional domains, including a cysteine-rich domain (CSRD), a Ras-GAP domain (GRD), and a leucine repeat domain (LRD) [5, 24–30]. (**b**) Neurofibromin serves as a negative regulator of Ras by accelerating the hydrolysis of the GTP-bound active Ras, producing inactive GDP-bound Ras [31–34]. Upon receptor tyrosine kinase (RTK) activation, Ras guanosine exchange (GDP to GTP) promotes Ras activity. Activated Ras, in turn, stimulates its downstream effectors, including MEK/MAPK and PI3K/Akt/mTOR. Ras can also positively regulate adenylate cyclase (AC) activity in some cell types. (**c**) Current fly and mouse models of NF1-associated learning and attention abnormalities. Abbreviations: GFAP, glial fibrillary acidic protein; Syn1, synapsin 1; Dlx5/6, distal-less homeobox 5 and 6.

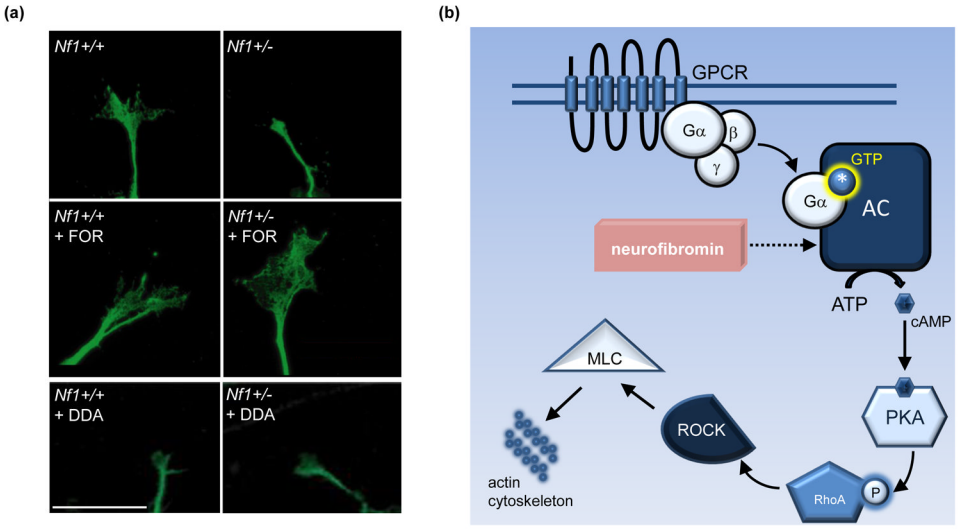

Figure 2. Neurofibromin regulation of neuronal morphology is cAMP-mediated

(**a**) Neuronal morphology (growth cone areas and neurite length) is attenuated by reduced neurofibromin expression and cAMP levels. Top panel: Primary cultured *Nf1+/–* hippocampal neurons have decreased neurite lengths (not shown) and growth cone areas as a result of reduced cAMP generation [73]. Middle panel: Stimulating adenylyl cyclase (AC) with Forskolin (FOR) elevates cAMP levels and rescues growth cone areas in *Nf1+/–* hippocampal neurons. Bottom panel: Decreasing cAMP levels in wild-type neurons by blocking adenylyl cyclase activity with the AC inhibitor 2,3-dideoxyadenosine (DDA) blunts growth cone areas, comparably to *Nf1+/–* neurons. Scale bar = 20μm. Adapted, with permission, from [73]. (**b**) Schematic diagram illustrating mechanism of cAMP-mediated morphological changes. In neurons, low cAMP generation due to reduced *Nf1* expression, in turn, leads to decreased PKA activation, RhoA/ROCK activity, and MLC phosphorylation. These changes contribute to impaired actin cytoskeletal dynamics, resulting in smaller growth cones and shortened axons [74]. Abbreviations: GPCR, G protein-coupled receptor; MLC, myosin light chain; PKA, protein kinase A; ROCK, Rho-associated protein kinase.

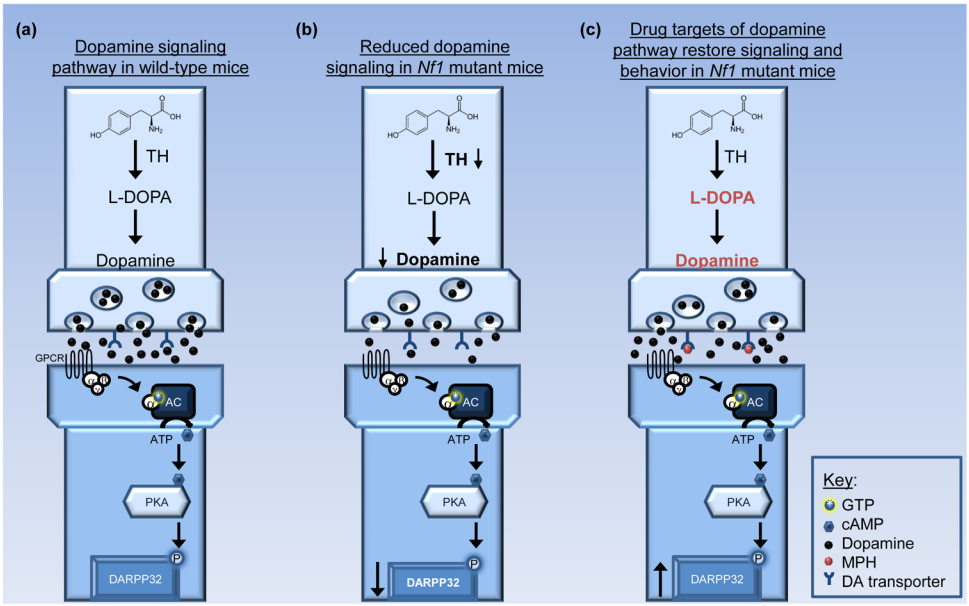

Figure 3. Role of dopamine in NF1-associated behavior

(**a**) Tyrosine hydroxylase (TH)-positive neurons in the substantia nigra project to the striatum and modulate dopamine signaling. Presynaptically, TH converts tyrosine to L-DOPA. Upon release, dopamine returns to the presynaptic dopamine pool by binding dopamine transporters or it activates adenylyl cyclase (AC) by binding post-synaptic dopamine receptors. AC activation stimulates cAMP generation and dopamine- and cAMP-regulated phosphoprotein-32 (DARPP32) phosphorylation in the striatum. (**b**) In *Nf1* mutant mice, TH expression is reduced, leading to lower striatal dopamine levels and lower effector signaling, as indicated by reduced DARPP32 phosphorylation [81]. Abnormal dopamine homeostasis also results in attention system dysfunction in the *Nf1* mutant model [75, 81]. (**c**) Treatment with drugs that increase dopamine synthesis (L-DOPA), exogenous dopamine levels (dopamine), or block dopamine reuptake [eg. methylphenidate (MPH)] restores striatal dopamine levels and reverses the attention phenotype in *Nf1* mutant mice [75, 81]. Abbreviations: GPCR, G-protein coupled receptors.

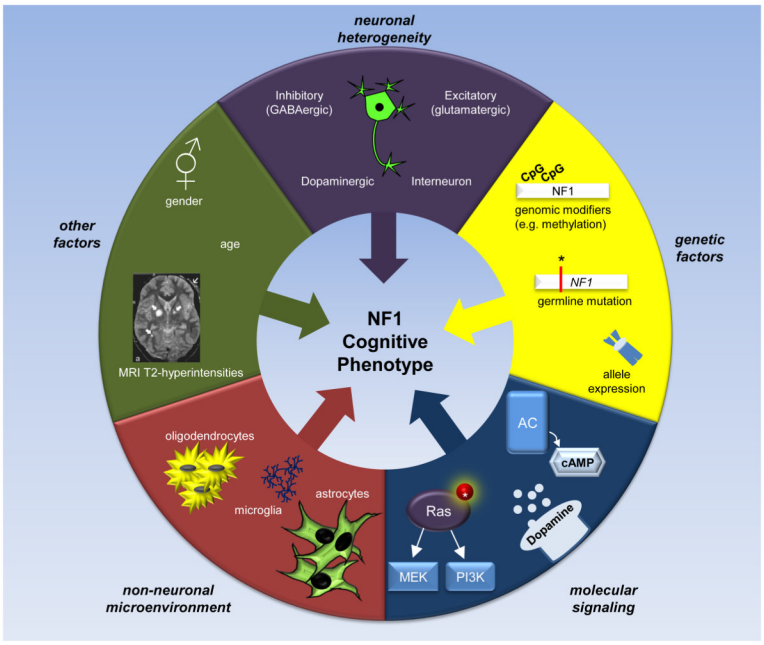

Figure 4. Factors Influencing the NF1 Cognitive Phenotype

The specific cognitive phenotype observed in any given individual with NF1 reflects the confluence of genomic, molecular, cellular, and environmental factors. The specific germline *NF1* gene mutation, allele expression, and genomic modifying events (including methylation), may influence clinical heterogeneity and create variations in neurofibromin expression in different cell types. Heterogeneity in neuronal subpopulations may also factor into the overall cognitive phenotype. In this manner, the relative contributions of defects in different populations of CNS neurons (e.g., GABA-ergic, dopaminergic) may lead to a distinct spectrum of cognitive and behavioral abnormalities. Similarly, less well-studied characteristics, such as patient sex, age, and NF1-associated brain abnormalities (e.g., T2-hyperintensities), may also contribute to the observed patient cognitive profile. In addition, the effects of abnormal signaling through specific molecular pathways (ie. signaling heterogeneity) could likewise differentially impact distinct neuronal populations and lead to unique cognitive phenotypes. While understudied to date, it is also possible that *NF1*+/− oligodendrocytes, microglia, and/or astrocytes contribute to abnormal neuronal function as a result of disrupted axonal signal propagation, impaired synaptic pruning, and/or changes in glutamate or other neurotransmitter availability.

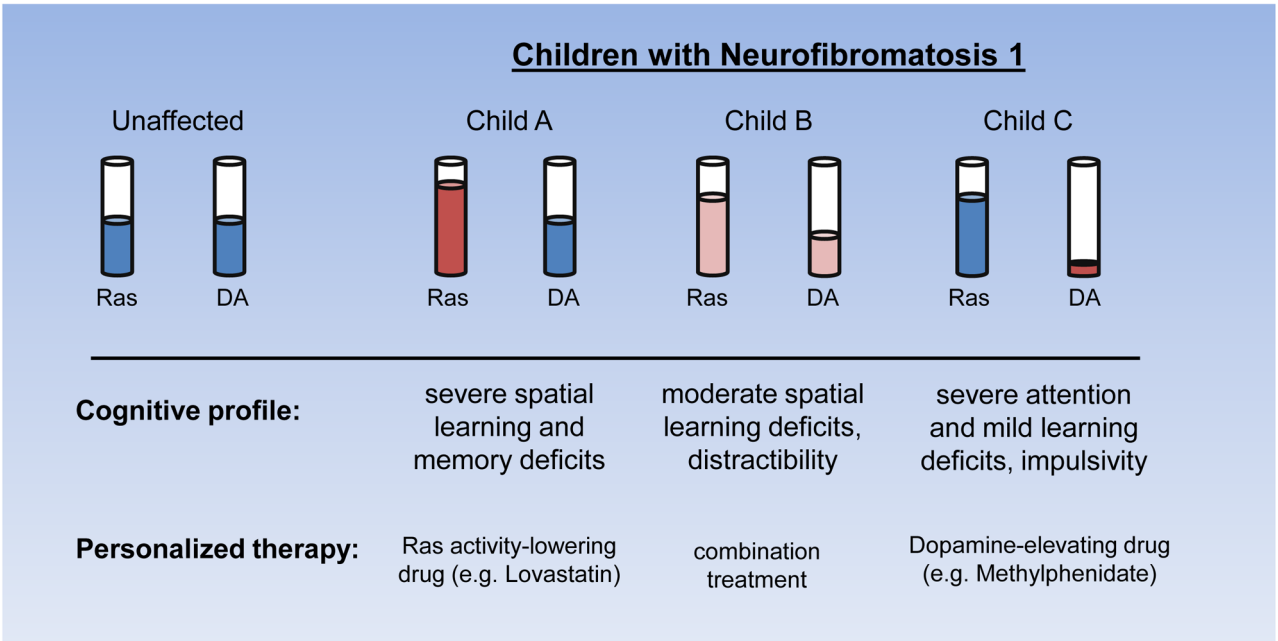

Figure 5. Model of clinical heterogeneity and implications for treatment

The cognitive profile of children with NF1 likely includes a diverse set of learning, memory, attention, and motor deficits, each linked to a distinct molecular abnormality (i.e. increased Ras, reduced cAMP, or low dopamine). In this regard, each child with NF1 has a different level of Ras, dopamine, and cAMP (not shown) signaling that collectively contribute to the overall cognitive phenotype. Identifying the primary set of cellular and molecular abnormalities may lead to individualized treatments with a higher likelihood of improving the neurocognitive deficits specific to that child or adult with NF1.

Table 1

NF1 Clinical Manifestations

Clinical Feature	Diagnostic criteria (Fulfillment of ≥2)	Prevalence [114, 115] (% of NF1 population)
Family history		50%
Neurocutaneous		
Café-au-lait spots	6 or more	99% in adults
Axillary/inguinal freckling	2 or more	85%
Lisch nodules	2 or more	95% in adults
Neurofibromas	2 or more	99% in adults
Plexiform neurofibromas	1 or more	20–45%
Skeletal		
Scoliosis	ND	5–10%
Bone dysplasia		1–5%
Pseudarthrosis		2%
Short stature	ND	30%
Macrocephaly	ND	45%
Tumors		
Optic pathway glioma		15%
Malignant peripheral nerve sheath tumor	ND	2–5%
Low-grade glioma	ND	2–3%
Malignant glioma	ND	1–2%

Note: ND=non-diagnostic; denotes NF1 clinical features associated with disease, but are not assessed for diagnosis