Mouse models of neurofibromatosis type I: bridging the GAP

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Neurofibromatosis type I (NF1) is an autosomal dominant disorder caused by mutations in the *NF1* gene, leading to a variety of abnormalities in cell growth and differentiation, and to learning disabilities. The protein encoded by *NF1*, neurofibromin, has several biochemical functions and is expressed in a variety of different cell populations. Hence, determination of the molecular and cellular mechanisms that underlie the different NF1 symptoms is difficult. However, studies using mouse models of NF1 are beginning to unravel the mechanisms that underlie the various symptoms associated with the disease. This knowledge will aid the development of treatments for the different pathological processes associated with NF1.

Neurofibromatosis type I (NF1) is a common autosomal dominant disorder, affecting approximately one in every 3500 individuals (for reviews see [1,2]), and is one of the most common single-gene disorders influencing neurological function in humans [3]. Mutations in the *NF1* gene result in abnormal cell growth and differentiation, with a variety of symptoms, typically including benign neurofibromas, hyperpigmentation of melanocytes, and hamartomas of the iris [1,2]. The benign neurofibromas can develop into malignant peripheral-nerve-sheath tumors (MPNSTs). Furthermore, in the brain, *NF1* mutations can result in astrogliosis and astrogliomas, and in learning disabilities that occur in 40–60% of patients with NF1 [4,5]. Visual-spatial function appears to be most compromised, although problems with language skills, executive function, attention and motor coordination are also common (for reviews see [4,5]).

Effects of *NF1* mutations in humans and mice

The *NF1* gene encodes a 250 kDa protein called neurofibromin, which has several known biochemical functions, including activation of the Ras GTPase [6–8], modulation of adenylyl cyclase [9,10], and microtubule association [11] (Fig. 1). The human and mouse forms of neurofibromin are highly homologous (98% sequence similarity) [12], as are the promoter sequences of the gene, suggesting that both the biochemistry of the protein and the transcriptional regulation of the gene are conserved across species [12,13]. Although investigation of all the symptoms of NF1 in a single mouse model has proven difficult, the ability to dissociate some of the symptoms from others in particular mouse models (Table 1) has often been an advantage in studies of the disease. The effects of *NF1* mutations in humans and mice show interesting parallels. For example, in mice and probably in humans, the complete loss of neurofibromin is lethal [14,15]. Also, *NF1* mutations lead to tumor formation in both humans and mice. Aged mice heterozygous for a targeted disruption of the *Nf1* gene ($Nf1^{+/-}$) have an increased incidence of phaeochromocytomas, and myeloid leukemias, two phenotypes observed in NF1 patients. Moreover, mice carrying $Nf1^{-/-}$ mutations in particular cell populations develop neurofibromas [16,17] and mice that carry linked (*cis*) mutations in *Nf1* and *p53* develop soft tissue sarcomas, namely MPNSTs [16,18]. Skin pigmentation problems have also been observed in mice and humans [19]. Chemically challenging $Nf1^{+/-}$ mice with either a skin cancer initiator or a tumor promoter results in abnormalities in skin pigmentation and proliferation [20]. In addition, *NF1* mutations result in abnormal brain function in both species. As described for NF1 patients [21], mice carrying

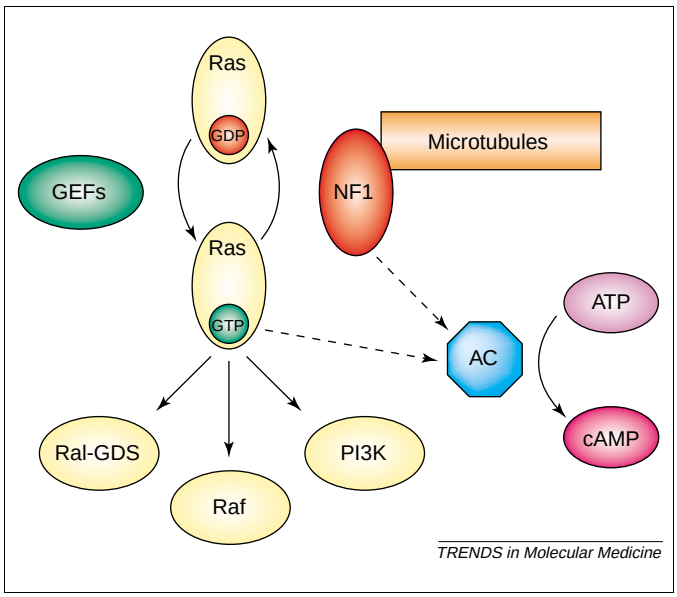

Fig. 1. The interactions of neurofibromin (NF1) with different signaling pathways. NF1 has several known biochemical functions, including activation of the Ras GTPase, modulation of adenylyl cyclase (AC), and microtubule association. Ras activates several effector molecules, including the Ral GDP-dissociation stimulator (Ral-GDS), Raf and phosphatidylinositol 3-kinase (PI3K). Abbreviation: GEF, guanine-nucleotide exchange factor.

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Table 1. Mouse models of neurofibromatosis type I (NF1)

Mouse model	Main similarities to the human disease	Main differences from human disease	Other comments
Nf1 $^{+/-}$	Increased predisposition to tumors Skin abnormalities upon challenge Astrogliosis Learning and motor deficits	No neurofibromas	−/− are lethal
Nf1 $^{23a-/-}$	Learning and motor deficits	Normal predisposition to tumors No astrogliosis	−/− are viable
Nf1 ^{Synl} knockout	Learning deficits (in the non-runty) Astrogliosis	Normal predisposition to tumors No motor deficits	Runty and cortical thickness reduced No deletion in the cerebellum
Nf1 $^{-/-}$ chimera	Plexiform neurofibromas		
Nf1 $^{+/-}$; *p53* $^{+/-}$ *cis*	Malignant peripheral-nerve-sheath tumors Astrogliosis (also *in trans*) Astrocytomas Blastogliomas		
Nf1 $^{flox/-}$; *Krox20-cre*	Plexiform neurofibromas		Haplo-insufficiency of surrounding tissue has a functional contribution
Nf1 ^{GFAP} conditional knockout	Astrogliosis	No astrocytomas	

Nf1 mutations can develop low levels of region-specific astrogliosis [22,23], and have learning deficits [24,25].

Mouse models of the cell growth and differentiation abnormalities in NF1

The *NF1* gene was cloned in 1990 [26–28] and soon afterwards mice were generated carrying a null mutation of this gene [14,15]. Homozygous *Nf1*-mutant mice display heart malformation and hyperplasia of sympathetic ganglia, and die embryonically [15]. Mice heterozygous for the null mutation (*Nf1* $^{+/-}$) are viable, but show an increasing incidence of tumor formation with age (especially phaeochromocytomas and myeloid leukemias) [14]. However, *Nf1* $^{+/-}$ mice do not develop the neurofibromas that are characteristic of NF1, which could indicate that loss of heterozygosity (LOH) is necessary for neurofibroma formation. To investigate this hypothesis, Cichowski *et al*. generated chimeric mice, composed partly of *Nf1* $^{-/-}$ cells, and these mice do develop neurofibromas [16]. However, several cell types are homozygous for mutant *Nf1* in these mice and, hence, it is difficult to determine which cell type(s) are involved in the formation of neurofibromas. A recent study has addressed this problem by showing that mice with a homozygous null mutation specifically in Schwann cells (*Nf1* $^{flox/-}$; *Krox20-cre*) can develop neurofibromas [17]. This work also suggested that, in addition to homozygosity in Schwann cells, the development of neurofibromas requires heterozygosity in the surrounding cells [17]. Neurofibromas are usually benign, but they can become malignant; for example, mice carrying linked mutations in *Nf1* and *p53* develop MPNSTs [16,18].

Skin pigmentation problems and epithelial carcinogenesis are commonly associated with NF1 [2]. Interestingly, *Nf1* $^{+/-}$ mice have been shown to have abnormal responses to skin wounding [19]. To determine if these mice are also more susceptible to pigmentation abnormalities or skin tumorigenesis, *Nf1* $^{+/-}$ mice in the C57/B6 genetic background, known for its resistance to chemical carcinogens, were challenged with either a skin-cancer initiator or a tumor promoter; even in the resistant genetic background, *Nf1* $^{+/-}$ mice developed skin pigmentation abnormalities and papillomas [20]. Furthermore, all of the papillomas had mutations in the H-*Ras* gene, suggesting that the decreased Ras GTPase activity in *Nf1* $^{+/-}$ mice had conferred increased susceptibility to carcinogenesis.

Astrogliosis is another symptom observed in NF1 patients [21]. *Nf1* $^{+/-}$ mice also develop astrogliosis in several brain regions, such as the nucleus accumbens, the periaqueductal gray, and the hippocampus. Interestingly, astrogliosis in the hippocampus (which is involved in spatial learning) only occurs in ~60% of the *Nf1* $^{+/-}$ mice [22]. This is consistent with the incidence of the spatial learning deficits in these mice [24], suggesting that the astrogliosis and the learning deficits could be associated. However, mice lacking exon 23a of *Nf1* (*Nf1* $^{23a-/-}$) do not have astrogliosis, but do show learning deficits [25]. Interestingly, complete deletion of *Nf1* specifically in glia [*Nf1* ^{GFAP} conditional knockout (CKO)] [29], or specifically in neurons [*Nf1* ^{SynI} knockout (KO)] [23], is sufficient to cause astrogliosis, indicating that disruption of neurofibromin in either cell type can contribute to this problem. Also, heterozygosity for *Nf1* and *p53*, or *Nf1* and *Rb*, confers a significant growth advantage to astrocytes (2.5-fold) [30]. Additionally, loss of both *Nf1* and p53 result in blastoglioma formation [31]. Surprisingly, complete deletion of *Nf1* specifically in astrocytes (*Nf1* ^{GFAP} CKO) [29] is not sufficient to produce astrogliomas, a frequently observed benign tumor type in NF1 patients.

Mouse models of the learning disabilities associated with NF1

Despite the obvious differences between mice and humans, molecular and cellular cognitive studies have revealed remarkable similarities in the learning deficits caused by *NF1* mutations in the two species. First, the NF1 mutation seems to affect some brain functions more than others. For example, visual-spatial learning [24], attention and motor coordination are all impaired, whereas various forms of learning, such as classical conditioning, seem to be intact. Second, only 40–60% of carriers of the mutation are affected, and remedial training can alleviate the learning deficits [4,24]. Third, the severity of the NF1 phenotype is affected by genetic variation, which exacerbates the

problem in NF1 patients without having a noticeable impact on normal siblings [24,32,33]. Consistent with this, it was shown that a heterozygous mutation of the *N*-Methyl-D-Aspartate receptor (NMDAR) increases the severity of the learning deficits in *Nf1* $^{+/-}$ mice without affecting learning in littermate controls [24]. Also, genetic background impacts on the incidence of learning disabilities in *Nf1* $^{+/-}$ mice [34]. These similarities suggest that mouse models could be useful for investigating the mechanisms that underlie the learning deficits associated with NF1.

The range of neurological symptoms associated with NF1 is vast and, hence, it is not clear whether the learning deficits associated with NF1 are caused by adult brain dysfunction, undetected tumors or neuro-developmental problems. However, results from recent work using mouse models have helped to clarify this matter. An interesting indication comes from studies of mice that carry a homozygous deletion of the alternatively spliced exon 23a of *Nf1* (*Nf1* $^{23a-/-}$) [25]. This exon encodes 63 base pairs (21 amino acids) within the GTPase activating protein (GAP)-related domain. Exclusion of exon 23a produces the type-I isoform of Nf1, whereas inclusion of this exon yields type II. The type-II isoform has a greater affinity for Ras, but lower GAP activity, than type I [35,36]. *Nf1* $^{23a-/-}$ mice are physically normal, and do not show any neural-crest development abnormality or increased tumor predisposition [25]. However, these mice do have learning impairments in spatial tasks (water maze and contextual discrimination) [25], much like the heterozygous null mutants [24,32]. These results show that the learning deficits can occur in the absence of tumor predisposition or neuro-developmental problems. Furthermore, mice lacking neurofibromin solely in neurons (*Nf1* ^{SynI} KO without runty phenotype) show no increase in predisposition to tumor formation but do have learning deficits (R.M. Costa, PhD thesis, University of Oporto, 2002).

What, then, are the molecular alterations that underlie these learning disabilities? Neurofibromin has several known biochemical functions. For example, it has a GAP domain that accelerates the inactivation of Ras by stimulating its GTPase activity [6–8]. In addition, studies in *Drosophila melanogaster* and in mice have shown that neurofibromin modulates the activity of the rutabaga-encoded adenylyl cyclase (AC) [10,37], an enzyme crucial for cAMP signaling. Neurofibromin has also been shown to associate with microtubules [11], suggesting that it is involved in the regulation of multiple signaling pathways in the brain. All of these biochemical pathways are important for brain function, and have been implicated in synaptic plasticity and in learning and memory. However, the question remains as to which of these signaling pathways are crucial for the cognitive deficits associated with NF1.

Studies in *D. melanogaster* showed that homozygosity for mutation of *Nf1* causes associative learning deficits and that these deficits are dependent on the rutabaga-encoded AC [10]. Furthermore, the cAMP pathway is crucial for memory formation in a variety of vertebrate and invertebrate species [38], and AC activity is also impaired in cells from (lethal) homozygous mutant-*Nf1* mice [37]. However, in cells from heterozygous null mice, which have learning deficits, AC activity is indistinguishable from that of wild-type animals [37], whereas the Ras-controlled mitogen-activated protein kinase (MAPK) pathway is upregulated ([39], R.M. Costa, PhD thesis, University of Oporto, 2002). These data suggest that the ability of neurofibromin to regulate AC might be important for symptoms that require a loss of heterozygosity, whereas the Ras-GAP activity of neurofibromin might be crucial for phenotypes that are manifest in heterozygosity, such as learning deficits. Interestingly, however, the circadian phenotype of *Nf1* homozygous null mutants in *Drosophila* can be rescued by Ras mutations [40].

In humans, a mutation in the *NF1* gene that specifically abolishes the Ras-GAP function of neurofibromin, without affecting its ability to bind Ras, was shown to cause multiple NF1 symptoms, including cognitive dysfunction [41], suggesting that loss of the GAP function underlies the learning deficits.

In mice, recent studies showed that the spatial learning impairments in *Nf1* $^{+/-}$ mutants can be rescued by two different genetic manipulations that decrease Ras levels, indicating that increased Ras activity is the cause for the learning deficits [42]. In agreement, the learning deficits of *Nf1* $^{+/-}$ mice can also be reversed pharmacologically, using a farnesyl-transferase inhibitor (FTI), which indirectly reduces the amount of active Ras [42]. This FTI experiment also indicates that the learning deficits of *Nf1* mutant mice can be reversed in the adult, a finding of crucial importance for the development of treatments for the learning disabilities associated with NF1. In addition, *Nf1* $^{23a-/-}$ mice, which lack the alternatively spliced exon 23 (which modulates the GAP activity of Nf1) have learning disabilities [25].

Several other genetic and pharmacological studies have substantiated the role of Ras signaling in synaptic plasticity, and learning and memory [43–45].

Taken together, these studies suggest that either abnormally high or low Ras activity can disrupt learning, indicating that precise Ras modulation by neurofibromin is crucial for learning and memory. It will be important to determine which Ras effectors (Fig. 1) are crucial for the learning deficits associated with NF1. Also, in light of the *Drosophila* studies described above [10,37], it will be important to determine whether, in mammals, regulation of the cAMP pathway by neurofibromin plays a role in the occurrence of learning disabilities. If so, it would be interesting to observe whether different behavioral impairments can be attributed to abnormalities in either the cAMP or the Ras pathways, or whether these two pathways interact during learning in a manner regulated by neurofibromin.

The results of the experiments described above suggest that the learning deficits associated with NF1 result from adult brain dysfunction caused by increased Ras activity. Therefore, it is important to understand how *Nf1* mutations, and the subsequent Ras hyperactivity, alter neuronal physiology. Activity-dependent synaptic modifications in neuronal networks are thought to mediate learning. Long-term potentiation (LTP) is a widely studied

experimental model of the synaptic plasticity mechanisms thought to underlie learning and memory [46]. *Nf1* $^{+/-}$ mutant mice show learning deficits in the hidden version of the water maze, a spatial navigation task that is dependent on hippocampal function [47]. In humans, the hippocampus seems to be crucial for the acquisition of new declarative memories [48,49]. Electrophysiological studies in the CA1 region of the hippocampus of *Nf1* $^{+/-}$ mice showed that LTP is impaired when induced using a theta-burst stimulation (TBS) protocol [42], which mimics the *in vivo* activity of hippocampal neurons during exploratory behavior [50]. Further electrophysiological analysis showed that the *Nf1* $^{+/-}$ mice have increased γ-aminobutyric acid (GABA) inhibition in the hippocampus, and suggested that this increased inhibition is the cause of the LTP deficits [42]. Remarkably, as with the learning deficits, both the increased GABA inhibition and the LTP deficits of the *Nf1*-mutant mice can be reversed by manipulations that decrease Ras activity [42].

Ras has been shown to modulate chloride currents in adrenal gland cells [51]. The data described above suggest that Ras might also modulate inhibitory currents in the central nervous system. Interestingly, whereas neurofibromin affects LTP through modulation of GABA-mediated inhibition, H-Ras [52] and even other GAPs, such as Syn-GAP [53], have been implicated in LTP via their modulation of excitatory neurotransmission. Neurofibromin is also localized at excitatory synapses and interacts with the NMDAR in a large post-synaptic complex [54]. Furthermore, in excitatory synapses, Ras mediates synaptic delivery of AMPA (α-amino-3-hydroxy-5,7-methylisoxazole-4-propionic acid) receptors during LTP [55]. Hence, neurofibromin might modulate excitatory as well as inhibitory synaptic function.

The role(s) of neurofibromin and Ras at inhibitory synapses are not yet known. Pre-synaptically, Ras could modulate neurotransmitter (e.g. GABA) release through MAPK phosphorylation of synapsin I [56,57]. Post-synaptically, Ras might mediate the membrane insertion of particular GABA subunits or modulate the phosphorylation state of the receptors, as it does in excitatory synapses. Another possibility is that GABA re-uptake is altered by *Nf1* mutations. Using a combination of genetic (deletion of *Nf1* in particular cell types) and electrophysiological studies, it should be possible to test these hypotheses.

Could the increased GABA inhibition in the CA1 region of the hippocampus be the cause of the learning deficits in *Nf1* $^{+/-}$ mice? Pharmacological increase of GABA-mediated inhibition in the hippocampus has been shown to cause deficits in spatial learning and synaptic plasticity in rodents [58,59]. Furthermore, a reduction in synaptic inhibition onto CA1 pyramidal neurons follows both LTP induction [60], and acquisition of the hidden version of the water maze [61]. Inhibition has been proposed to control the discharge rate and the collective activity of CA1 pyramidal neurons, both during exploratory activity and at rest [62,63]. Therefore, it is plausible that the increase in GABA inhibition, and related synaptic plasticity deficits, could underlie the learning deficits of *Nf1* $^{+/-}$ mice. This hypothesis is supported by the observation that the spatial-learning deficits, the inhibition and the synaptic plasticity alterations, are rescued by the same Ras manipulations. Further studies in humans and in rodents will help to unravel the effects of increased Ras activity and GABA inhibition on the ensemble activity of neurons during learning.

Conclusions

Studies employing mouse models of NF1 have been crucial in investigating the pathophysiology of different aspects of the disease, such as problems with cellular differentiation, tumor formation and learning. These studies have been essential in identifying the cell types, the cellular processes and the molecular pathways that are altered by *Nf1* mutations. This knowledge might have important implications for the development of treatments for NF1.

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