Published in final edited form as:

Med Hypotheses. 2009 July ; 73(1): 56–59. doi:10.1016/j.mehy.2009.01.040.

GNAI2 and Regulators of G protein Signaling as a potential Noonan Syndrome mechanism

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Abstract

Noonan syndrome (NS OMIM 163950) is a relatively common autosomal dominant developmental disorder characterized by short stature, specific facial features, and congenital cardiac anomalies. Approximately 50–66% of cases have defined mutations in the K-ras/Raf/MEK/ERK pathway that lead to constitutive signaling, but a significant number remain unexplained. We hypothesize that enhanced signaling through $G\alpha_{i2}$ (from the GNAI2 gene) may also produce a NS-like phenotype. This is based on a recently described mouse model in which RGS-mediated inhibition of $G\alpha_{i2}$ is prevented by a knock-in mutation (G184S) that blocks RGS binding (Huang et al, *Mol. Cell. Biol.* 26:6870–6879, 2006). The mice have short body length, cardiac hypertrophy, a triangular face with wide-set eyes and ears, and hematologic alterations. There is a slight increase in ERK activation and a pronounced enhancement of PI3K/Akt phosphorylation in MEFs from these mice suggesting that abnormal increases in $G\alpha_{i2}$ signaling could represent a novel upstream mechanism for NS. This suggests a novel set of candidate genes for NS (GNAI2 and RGS proteins) and if validated could have important implications for therapy as well.

Introduction

Noonan syndrome (NS OMIM 163950) is a relatively common inherited disorder characterized by short stature, characteristic facial features, chest deformities, and congenital cardiac anomalies (1). The genetics of NS are complex. Most families exhibit an autosomal dominant inheritance pattern due to gain of function mutations in various steps in the K-ras signaling pathway (2,3). Approximately 30–50% of cases (termed NS1) involve activating mutations in PTPN11 which encodes for Shp2 – a tyrosine phosphatase that positively regulates ras signaling (3,4). The second most common cause (~15% of cases, termed NS4) have mutations in SOS1 – a guanine nucleotide exchange factor (GEF) which activates ras (5). An additional 2–5% of cases have activating mutations in K-ras itself (NS3) (6) and Raf1 (NS5) has also been found mutated in 5–15% of cases (7). The neurofibromatosis gene, NF1, which is involved in deactivation of ras, shows mutations that cause a combined NS and neurofibromatosis phenotype. A small number of families with autosomal recessive NS (NS2) have also been reported but the underlying molecular defect has not been identified (8). Consequently, about half to two-thirds of the patients with NS have a defined molecular abnormality linked to Ras

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signaling pathways (2,3,9) but a significant fraction of NS cases (30–50%) have not been defined molecularly.

We recently described a mouse model in which signaling by the heterotrimeric G protein $G\alpha_{i2}$ is overactive (10) and several features of these mice are suggestive of a NS-like phenotype. Consequently, we hypothesize that G protein coupled receptor and heterotrimeric G protein signaling pathways may also produce a NS phenotype. It is well-known that GPCRs can activate ERK and/or ras and that they employ both G protein-dependent and G protein-independent mechanisms (11–13). Of the four G protein families (Gs, Gi, Gq, and G12), at least three (Gs, Gi, and Gq) are clearly implicated in ERK activation (13). A variety of molecular mechanisms have been proposed which involve both intracellular and extracellular processes. In this report, we focus on the Gi family of G proteins which generally activates downstream signals through release of their associated $\beta\gamma$ subunits. ERK activation by Gi signaling usually involves the $\beta\gamma$ subunit but the $G\alpha_i$ subunit may also play a role (14). One mechanism for Gi-mediated ERK activation includes transactivation of tyrosine kinase receptors through matrix metalloproteinases that release ligands such as heparin-binding epidermal growth factor (11–13).

A key control mechanism for turning off heterotrimeric G protein signals involves the regulator of G protein signaling (RGS) family which includes 20 or more members (15,16). These RGS proteins bind to the GTP-bound active $G\alpha$ subunit and speed the rate of GTP hydrolysis, serving as GTPase activator proteins (GAPs) acting in a manner similar to the effect NF1 on ras. RGS proteins act primarily on the Gi and Gq families of G proteins and in purified biochemical systems there is only limited specificity for RGS proteins on the different family members (15,16). Originally, it was thought that RGS proteins primarily regulated the kinetics of G protein deactivation and possibly activation (17) but there is now ample evidence that RGS proteins reduce the magnitude of GPCR signals (10,18) or more often reduce the potency of agonists (19,20). Thus, loss of RGS function would be expected to increase signals by GPCR agonists during both physiological and pharmacological receptor activation.

To address the physiological roles of RGS proteins there have been a number of reports of RGS knockout mouse models (21–26). They exhibit a variety of phenotypes but most are relatively subtle and require a pharmacological or pathological intervention to bring out the major effects. We recently described a mouse model in which the action of all RGS proteins was disrupted by a point mutation in a $G\alpha$ subunit producing a pronounced, spontaneous phenotype (10). This was based on a glycine to serine mutation in the switch 1 region of $G\alpha$ subunits identified by Dohlman and colleagues in a yeast screen (27). This mutation blocks the binding and inhibitory actions of RGS proteins. We demonstrated that the analogous mutation at the RGS binding site in mammalian Gi-family proteins similarly disrupts RGS action (19,28) but does not affect $G\alpha$ subunit expression or its interactions with $G\beta\gamma$, receptor, or effector (10,29). Creating a knock-in mouse model with this $G\alpha_{i2}$ G184S mutation produces a system in which receptor-mediated $G\alpha_{i2}$ signaling is enhanced through loss of the negative regulatory actions of RGS proteins (10).

The Hypothesis

Our hypothesis is that enhanced activation of $G\alpha_{i2}$ (genes *GNAI2* or *Gnai2*) may represent a novel mechanism for NS.

This hypothesis is based on the surprising observation that many of the phenotypic characteristics of our RGS-insensitive $G\alpha_{i2}$ mouse model ($G\alpha_{i2}^{G184S/G184S}$ knock-in) are similar to features both of human NS and of the *Ptpn11* mutant mouse model that mimics NS (30). We had previously reported that the homozygous $G\alpha_{i2}^{G184S/G184S}$ mice showed short

stature (Table 1), cardiac hypertrophy, enlarged spleens, and increased myelomonocytic cell counts (10). These are all features seen in certain cases of human NS.

Empirical Data

All animal studies were done with approval of and in compliance with guidelines of the University of Michigan UCUCA Committee. We now report that these mice also have markedly altered skull and facial morphology (Figure 1 and Table 1) a canonical key feature of NS. The shape of the face was clearly different from that of wild-type littermates with a wide-based triangular appearance and wider-set eyes (Figure 1). This was borne out by measurements of facial parameters with a highly significant increase by 2-way ANOVA in the width between the eyes compared to skull length and even more so for the width between the ears and skull length (Table 1). Although the length of the skull (measured from the tip of the nose to the occipital condyle) was somewhat smaller for the $G\alpha_{i2}^{G184S/G184S}$ mice this parameter by itself was not significantly different but the ratio of eye and ear separation to skull length was significantly increased in the mutants. These measurements are quite similar to those for the Ptpn11 mutant mouse model of NS (30).

Evaluation of the Hypothesis

The molecular basis of about half to two thirds of NS cases has been established but a significant fraction remains unexplained. Some of the known gene mutations are present in a relatively small fraction of patients (1.7% for K-RAS and 3.2% for SOS1) suggesting that there may be a number of other low abundance causative genes. While heterotrimeric G proteins and GPCRs are not intrinsic components of the ras pathway, they are well known to activate ras or ERK indirectly (11–13). The phenotypic features of our $G\alpha_{i2}^{G184S/G184S}$ mice are strikingly similar to those of human NS. The cardiac enlargement seen in our mice (ca. 20% increase in heart weight/body weight ratios) is notable because only a subset of NS patients exhibit isolated hypertrophic cardiomyopathy. In contrast, valvular abnormalities are common in patients with the more common PTPN11 and SOS1 mutations but hypertrophic cardiomyopathy is more frequent in patients with RAF1 mutations or in those without defined mutations (3,7). Consequently, we predict that enhancements in $G\alpha_i$ signaling – especially $G\alpha_{i2}$ signaling – may lead to a NS-like syndrome in humans, perhaps with hypertrophic cardiomyopathy rather than valvular abnormalities.

There are a number of potential molecular mechanisms that could lead to increased $G\alpha_{i2}$ signaling. This could result from a mutation in the $G\alpha$ subunit like our G184S mutation leading to loss of RGS binding to the $G\alpha$ subunit. Alternatively, a mutation that leads to constitutively active $G\alpha_{i2}$ (Q205L or R179C) could occur. This has been seen as a somatic mutation in human endocrine adenomas (31,32) though such a strong activation in a germline mutation could result in embryonic lethality – as has been reported for strongly activating PTPN11 mutations (30). Since the G184S mutant does not result in constitutive activation – only reduced inactivation – a receptor stimulus is required for the enhanced signaling by the RGS-insensitive $G\alpha$ mutant. However, we still see a significantly lower number of homozygous $G\alpha_{i2}^{G184S/G184S}$ mice than expected – and even the $G\alpha_{i2}^{G184S/+}$ mice occur at slightly lower than Mendelian ratios indicating some prenatal lethality (10). Finally, loss of function of a specific RGS protein that is important for controlling $G\alpha_{i2}$ signaling in the critical tissues could result in the same effect – though this would most likely be recessive as loss of half of the RGS function is usually not sufficient to produce a detectable phenotype (24) though the $RGS2^{-/+}$ mice are hypertensive (23).

Human NS is generally an autosomal dominant disorder but a few families with recessive NS (NS2) have been described (8). The striking NS-like phenotype in our $G\alpha_{i2}^{G184S}$ mutant

mouse model is present in the homozygous mice consistent with recessive inheritance. Interestingly, like our mice, the 4 patients with recessive NS reported by van Der Burgt & Brunner all had hypertrophic cardiomyopathy and in two it was unaccompanied by other cardiac abnormalities (8). However, the $G\alpha_{i2}^{G184S/+}$ heterozygous mice do show some of the same phenotypic features of the homozygotes but to a smaller degree (10). The body weight of heterozygotes is significantly lower than wt at comparable ages; tibial lengths were smaller, spleen sizes were larger and monocyte and neutrophil counts were increased but changes in these latter parameters were not statistically significant in that study. Such subtle effects may, however, be more apparent in humans. Consequently, the phenotypes in the $G\alpha_{i2}^{G184S}$ mutant mouse system appear to be partially dominant as expected from the gain-of-function nature of the RGS-insensitive $G\alpha$ subunit. Such variable effects are consistent with the highly heterogeneous nature of human NS.

It is also worth considering the biochemical mechanisms associated with the $G\alpha_{i2}^{G184S}$ mutation. In embryo fibroblasts from $G\alpha_{i2}^{G184S/G184S}$ homozygotes or $G\alpha_{i2}^{G184S/+}$ heterozygotes there was a slightly enhanced ERK activation by the agonist lysophosphatidic acid (10). Surprisingly, a much more robust increase was seen in signaling through the PI-3-kinase/Akt pathway (10). Most of the known mutations in human NS cluster in the ras/raf/MEK/ERK pathway so the greater effect on Akt in the $G\alpha_{i2}^{G184S}$ system is perhaps a contradiction to our hypothesis. There are at least two possible explanations for this apparent discrepancy. First, the biochemical signaling mechanism in the $G\alpha_{i2}^{G184S}$ system was only evaluated in one cell type (MEFs) and with one ligand (LPA) so increased ras/ERK signaling may be more pronounced in other systems. Alternatively, PI3K/Akt signaling may also play a role in NS, as the disease-causing PTPN11 mutation results in increased integrin-mediated Akt activation as well (33).

Consequences of the Hypothesis and Discussion

It would clearly be of interest to assess the $G\alpha_{i2}$ /RGS system for alterations in human NS. In addition to $G\alpha_{i2}$ itself, loss of RGS function could lead to a similar phenotype so any of the 20 RGS proteins could be candidate NS genes – either for the autosomal dominant or more likely for the recessive cases. Alternatively, $G\alpha_{i3}$ which is also expressed broadly in peripheral tissues and has some functions in common with $G\alpha_{i2}$ could be a candidate.

With respect to the G184S mutation in $G\alpha_{i2}$, this occurred in the yeast screen through a single base change. Given the mammalian codon for glycine¹⁸⁴ (i.e. GGG) a change from glycine to serine is not possible by a single base change – which could only result in substitution of alanine, valine, tryptophan, arginine, or glutamate. It would be useful to know if those substitutions have the same biochemical effect as that of G184S. It would not be surprising if different mutations in $G\alpha_{i2}$ could occur (G184A, R179H or C, or Q205L). In PTPN11, there are numerous mutant alleles that all have qualitatively similar – though perhaps quantitatively different effects on function (3).

If an RGS-insensitive mutation in $G\alpha_i$ or a loss of RGS expression or function were found, that could have important implications for the therapy of NS in those cases. The enhanced activity in that context remains largely dependent on an activating stimulus from a GPCR. Since GPCRs are well-established as therapeutic drug targets, the identification of the receptor(s) leading to the specific phenotype could define a novel therapeutic approach to NS. While the developmental abnormalities may not be reversed, it is conceivable that there may be some amelioration of disease features.

In summary, our hypothesis that $G\alpha_{i2}$ /RGS alterations may contribute to NS is based on a mouse model with enhanced $G\alpha_{i2}$ signaling (from the *GNAI2* or *Gnai2* gene) which has

phenotypic features typical of NS. This raises the possibility that GPCR signaling, that is not a core constituent of the canonical ras/raf/MEK/ERK mechanism could also play a role in NS.

Acknowledgments

We thank Drs. Elizabeth Petty and Michael Becker for helpful discussions and Ms. Susan Wade for assistance with the manuscript. This work was supported by National Institutes of Health Grant R01-GM39561 and in part by the Michigan Diabetes Research and Training Center (NIH P60 DK20572) and the University of Michigan Cancer Center (NIH P30 CA46592).

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Figure 1.

Table 1
Body and facial parameters for wt and Gα₁₂^{G184S} mice

Measurement	Wt			GS/GS			P value
	Mean	SD	n	Mean	SD	n	
Body Weight (g)	19.47	3.04	4	14.03	2.72	4	<0.01
Length from nose to base of the tail (mm)	93.13	3.42	4	81.63	4.15	4	<0.001
Length of the head (mm)	27.25	0.29	4	23.75	1.50	4	n.s.
Width between eyes/inside (mm)	8.13	0.48	4	8.00	0.71	4	n.s.
Width between eyes/outside (mm)	13.50	0.71	4	12.88	1.38	4	n.s.
Width between ears (mm)	13.88	0.25	4	13.38	0.48	4	n.s.
Distance between eye centers (mm)	10.81	0.43	4	10.44	0.83	4	n.s.
Distance between eye centers/head length	0.397	0.017	4	0.439	0.008	4	<0.01
Width between ears/head length	0.509	0.008	4	0.565	0.038	4	<0.001
Head length/body length	0.293	0.012	4	0.291	0.004	4	n.s.
Width between eye centers/body length	0.116	0.001	4	0.128	0.004	4	n.s.
Width between ears/body length	0.149	0.006	4	0.164	0.010	4	n.s.

Pairs of Gα₁₂^{G184S}/G184S and littermate wt mice were anesthetized with pentobarbital at 8–10 weeks of age and measurements of skull and body length and eye and ear separation were done as described (30). Eye center differences were calculated from mean of outer and inner eye measurements. Two-way ANOVA comparisons of the raw measurements and a separate 2-way ANOVA analysis of the ratios were done with Bonferroni post-tests. There were highly significant genotype differences (<0.0001) in both tests and several parameters (indicated in the table above) showed significance in the post-tests.