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TITLE: The Role of the Neurofibromin-Syndecan-CASK Complex in the Regulation of Synaptic RAS-MAPK Signaling and Denoritic Spine Plasticity

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Neurofibromatosis type 1 (NF1) is a common dominant genetic disorder characterized by multiple benign and malignant tumors of neural origin and, often, cognitive deficits in children. How mutations in the NF1 gene lead to severe learning deficits is largely unknown. The protein encoded by NF1, neurofibromin, contains a GAP domain, known to inhibit Ras-mediated signal transduction. The objective of this proposal is to test the hypothesis that the newly identified NF1-Syndecan2-CASK signaling complex plays an essential role in the regulation of synaptic Ras-MAPK activity and dendritic spine maturation. Using several siRNAs and dominant negative constructs for NF1 GAP activity to specifically knockdown or inhibit NF1, we have obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity, and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 responsible for its Ras GAP activity. Our results have shed new lights on the molecular mechanisms that underlie some of the cognitive deficits in NF1 patients and should have important implications for developing effective treatments of this devastating disease.
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I. Introduction:

Neurofibromatosis type 1 (NF1) is one of the most common dominant genetic disorders characterized by multiple benign and malignant tumors of neural origin. About 50% of NF1 children also exhibit cognitive deficits such as spatial learning defects and reading difficulty. How mutations in a single gene lead to severe learning deficits is largely unknown. The protein encoded by NF1, neurofibromin, contains a GAP domain, known to inhibit Ras-mediated signal transduction. A recent report from Silva’s group demonstrated that the learning deficits of heterozygous null mutant (Nf1+/-) mice could be rescued by genetic and pharmacological manipulations that decrease Ras function (Costa et al., 2002), suggesting that a tightly regulated Ras activity is critical for its function in synaptic plasticity. NF1 forms a tripartite complex with CASK, a synaptic PDZ protein, and Syndecan 2, a heparan sulfate proteoglycan (HSPG) (Hsueh et al., 2001). CASK has previously been proposed to function as multi-domain scaffolding protein that organizes specific signaling complexes at contact sites, and may have a role in receptor localization. HSPGs are believed to function as co-receptors in many receptor tyrosine kinase signaling pathways, and Syndecan 2 is known to promote dendritic spine maturation. Therefore, in principle, this protein complex can function in both organizing the synaptic protein complex and mediating key signal transduction events during synaptogenesis and synaptic plasticity (Fig.1). The objective of this study is to combine structural and functional analyses in conjunction with the assessment of the underlying signal transduction mechanisms at single cell level, to better understand the precise NF1 function in neurons and how deregulation of this function leads to cognitive deficits in NF1 patients.

Fig.1. NF1 may be part of critical signaling networks underlying synapse formation and function. Model highlights the protein-protein interaction of NF1 with several key synaptic signaling molecules previously implicated in the differentiation of both presynaptic and postsynaptic structure and function. On the postsynaptic side, Ras-MAPK signaling is involved in the initiation of dendritic filopodia; however, a spatiotemporal shutting-off of its activity by NF1 may be required for stabilization and development of fully functional dendritic spines.

II. Body:

We propose to use multidisciplinary approaches, including time-lapse imaging confocal microscopy, molecular imaging with FRET, quantitative immunocytochemistry, and genetic mouse models as well as pharmacological and molecular manipulations such as dominant negative constructs and small interfering RNAs (siRNAs), to define the NF1 function in synapse formation and morphogenesis of dendritic spines. The three major tasks of this study are:

Task1: To determine if the NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

Task2: To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology
Task3: To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.

During the last 3 years supported by the NFRP, we have obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity, and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity. In the following sections, I will highlight these exciting new findings in more detail.

1). Task1: To determine if NF1-syndecan-CASK signaling complex is an upstream regulator of the synaptic Ras-MAP kinase pathway

Fig.2. Hyperactive basal pMAPK activity in Nf1+/- hippocampus. Hippocampus was dissected from young adult WT and Nf1 +/- brains (n=6), homogenized in hypotonic lysis buffer, and resolved by SDS-PAGE. Western blots were probed for dually-phosphorylated ERK1 (ppERK1) and ERK2 (ppERK2), total ERK1 and 2, phospho-MEK, and NF1 protein. Densitometric values were mean +/- SD. NF1 antibody was from Santa Cruz; all others were from Cell Signaling. *p<0.05; **p<0.01 by Student’s t-test.

Although the learning deficits of Nf1+/- mice can be rescued by genetic and pharmacological manipulations that decrease Ras function (Costa et al., 2002), it is somewhat surprising that there has been no evidence reported so far that the Ras-MAPK pathway is indeed altered in Nf1+/- neurons. To address this important issue, we have used both immunoblot analysis and immunohistochemistry to assay the basal and depolarization-induced MAPK activity in wild type and Nf1+/- neurons (Figs.2 & 3). Furthermore, by GST pull-down experiment (Fig.4), we have obtained evidence that Nf1+/- neurons had higher Ras GTPase activity. These novel results clearly demonstrate that as seen in many other types of cells, Nf1+/- neurons display hyperactive basal and evoked MAPK activity as compared to wild type neurons, suggesting that NF1 plays a critical role in the regulation of basal and depolarization-induced MAPK activity in neurons.

We proposed to use a FRET probe developed by the group of M. Matsuda in Japan (Mochizuki et al., 2001), and we had reported our limited success in our original application. After rigorously testing the probe as well as several other probes using similar intra-molecular link design, we have to conclude that in all of the cases, these constructs significantly interface with the normal cell signaling, probably in a dominant-negative way. Using two-photon fluorescence lifetime imaging, K. Svoboda’s group has recently developed a sensitive method to measure Ras activity in hippocampal slices (Yasuda et al., 2006). It is important for a future study to use this new approach to measure the local Ras activity in our DG explants cultures to further demonstrate that NF1 deficiency leads to elevated Ras activity.
Hippocampi were dissected from young adult WT and Nf1 +/- brains (n=8), cut into 400μm slices, then stimulated by 90mM K+ for 3min. After 10min wash, the slices were either homogenized in hypotonic lysis buffer and assayed for immunoblots, or immunostained for pERK, total ERK, and NF1 protein. Densitometric values were mean +/- SD. *<0.05 compared to WT; #<0.05 compared to WT+K+ by Student’s t-test.

Ras activity was measured using a kit obtained from Upst at Biotechnology. Hippocampus was dissected from 1 month old wild type (WT) and NF1 (+/-) brains (n=3), dounce homogenized in hypotonic lysis buffer, and 500 μg aliquots were diluted in assay buffer along with Raf-1-agarose beads to pull down active p21 Ras. Beads were washed, resuspended in SDS sample buffer, and resolved by SDS-PAGE. Western blots were probed with an antibody to p21 Ras, and densitometric values were obtained. **p<0.01, Student’s T-test.

Growing evidence suggests that Ras activates multiple parallel downstream pathways, besides the Ras-MAPK pathway, to mediate its function. Several groups recently showed that Ras-Akt-mTOR signaling (Altomare and Testa, 2005; Dasgupta et al., 2005; Johannessen et al., 2005; Yin et al., 2005) is altered in NF1 diseases models. We and Morgan Sheng’s group recently independently demonstrated that the Ras-Akt-mTOR signaling pathway plays a central role in the regulation of dendrite size and shape as well as dendritic spine morphology (Jaworski et al., 2005; Kumar et al., 2005). Interestingly, genetic defects of the Ras-PI3K-AKT-mTOR signaling pathway, either through genetic inheritance or as a spontaneous genetic mutation, are also associated with other human diseases such as tuberous sclerosis.
TSC1/2 is known to act downstream of AKT to negatively regulate mTOR. Remarkably, Bernardo Sabatini’s group recently showed that TSC1/2 also plays important role in regulation dendritic morphology (Tavazoie et al., 2005). Taken together, these new findings raise the interesting possibility that a common cause of the neurological symptoms seen in these diseases may be linked to abnormal dendrite development. Future work to further investigate a possible involvement of the Ras-Akt-mTOR signaling in NF1 and its role in the cognitive deficits in patients would be of great interest.

**2). Task 2:** To assess the role of the NF1-syndecan-CASK signaling complex in regulation of dendritic spine morphology

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**Fig. 5. Use of siRNAs to Knockdown NF1.** (A) Schematic drawing shows the structure of NF1 and the target positions of some of the siRNAs. (B) Western blot shows effective suppression of the NF1 by 4 different siRNAs for NF1. Note that typically ~60% cells were transfected in our Hela cell cultures; therefore, the actual inhibition may be larger than the values showed here. As one of the controls, the siRNA for EGFP has no effect (not shown). The same Western blots re-probed for other endogenous or overexpression of non-related proteins (such as CaMKII) showed no significant non-specific reduction of these proteins (not shown). (C) siRNAs for NF1 induced immature dendritic phenotypes. The DG explant was first transfected with DsRed along with a control vector at 6 DIV (red cell). Two days later, the explant was transfected with GFP along with siRNA_{NF1-A}. The explant was then fixed and imaged at 17DIV. Arrow, cluster of immature filopodia reminiscence of those seen in Ras+ expressing cells (Fig. 5C). Similar immature spine phenotypes were seen in cells expressing other siRNAs for NF1 (not shown). (D) Immature spine phenotype revealed by expressing actin-GFP. Note the long filopodia and more diffuse actin-GFP signals in the cell transfected with siRNA_{NF1-l} (imaged at 17DIV and transfected at 9DIV).

Small interfering RNAs (siRNAs, 21- to 22-Nucleotide) have recently emerged as a powerful tool to suppress gene expression through a process known as RNA interference or RNAi. We have put significant
efforts into making this powerful tool for our use. Dr. Ming-Xiang Zhang has tested several available RNAi systems (including psilencer3.1 with H1 or U6 promoter (from Ambion); pSuperRNAi and the latest version EGFPpSuperRNAi (from Oligoengine)). Based on our experience, the pSuperRNAi system works best in neurons. We have constructed more than 10 different pSuperRNAi constructs for NF1. Western blot data showed that about half of them specifically reduced the NF1 protein level to an extent greater than 50% (Figs. 5 & 6). Non-related siRNA constructs or a siRNA for EGFP had no effect on NF1 expression at 48 hrs after transfection.

Fig. 6. Specificity of NF1 Knockdown and hyperactive pMAPK.
Hela cells were co-transfected with different constructs for 48hrs and assayed for immunoblotting, as detailed in Fig.5. Note that overexpression of NF1GRDI, a central domain of NF1 about ~360 residues responsible for its Ras GAP activity, was able to largely rescue the pMAPK level in cells co-expressing the siRNA_{NF1-K} with NF1GRDI. Cells co-expressing the siRNA_{NF1-I} with NF1GRDI still showed elevated hyperactive pMAPK. The slightly reduced pMAPK activity was due to incomplete knockdown of NF1GRDI by siRNA_{NF1-I}. * p<0.05, ** p<0.01; n=3; one-way ANOVA.

With these useful tools and reagents, we have obtained compelling evidence showing that specific knockdown of NF1 leads to abnormal dendritic spine morphology. We have been focusing on two of the siRNAs that target regions in the NF1GRDI (siRNA_{NF1-I}) or outside (siRNA_{NF1-K}) of the NF1GRDI, respectively. Both constructs significantly reduced the level of NF1 to a similar level (more than 50%) when tested in HeLa cells; and as expected, pMAPK activity was significantly increased in cells expressing both constructs (Fig. 6). Remarkably, overexpression of NF1GRDI was able to largely rescue the pMAPK level in cells co-expressing the siRNA_{NF1-K} with NF1GRDI. Cells co-expressing the siRNA_{NF1-I} with NF1GRDI still showed elevated hyperactive pMAPK and the slightly reduced pMAPK activity was due to incomplete knockdown of NF1GRDI by siRNA_{NF1-I}. As shown in Fig.7, knockdown of NF1 produces immature spine phenotypes, and co-expression of siRNA_{NF1-I} with NF1GRDI but not co-expression of siRNA_{NF1-I} with NF1GRDI largely rescued the immature spine phenotype, strongly supporting that these observed immature phenotypes indeed were due to loss of NF1 function.

As an independent approach, we have made use of dominant negative constructs for NF1 GAP. Two arginine residues of NF1 in the catalytic arginine loop (Arg^{1276} and Arg^{1391}) in NF1-GRD, whose alterations have been found in NF1 patients with severe phenotypes, are known to be important for catalysis of NF1-GAP activity (Scheffzek et al., 1998). Mutational analysis of NF1-GRD revealed that replacing Arg^{1276} with lysine (R1276K) increases its binding affinity for Ras 1.85-fold but greatly reduces it GAP activity. The affinity of the double mutant R1276A/R1391K for the Ras-GTP form was slightly increased by 1.08 fold, and the GAP activity was decreased to 1/1080 compared to wild-type NF1-GRD (Ahmadian et al., 1997; Scheffzek et al., 1998). Therefore, these mutants are thought to act as dominant negative inhibitors for endogenous NF1 GAP activity. Indeed, Yunoue et al. (Yunoue et al., 2003) recently reported that both the R1276K and the R1276A/R1391K constructs significantly reduce the NF1
GAP activity, as well as the Ras and MAPK activity when transfected into PC12 cells and cultured primary hippocampal neurons. Interestingly, these transfected cells also displayed reduced neurite outgrowth, suggesting that NF1 may play an essential role in early neurite outgrowth. As shown in Fig.7F, similar to the siRNAs for NF1, overexpression of R1276A/R1391K also produced an immature spine phenotype and reduced spine density.

According to our working model (Fig.1), during synaptogenesis, ephrins activate EphB2, which in turn leads to clustering of NMDA receptors and phosphorylation of Syndecan-2. The phosphorylated Syndecan-2 clusters at synaptic sites and further recruits CASK and NF1. The recruitment of NF1 may function to stabilize the functional spine and limit further morphological plasticity by shutting off the Ras-MAPK signaling. One simple prediction of this model is that overexpression of CASK or Syndecan-2 should promote spine maturation as well as down-regulation of pMAPK activity during early developmental stages. Vikas Kumar, a graduate student in the lab, has carried out the overexpression experiments, and demonstrated, for the first time that overexpression of CASK in immature hippocampal neurons (8-10 DIV) promotes maturation of both the presynaptic boutons and postsynaptic dendritic spines as evidenced by increasing the numbers of the axonal varicosities (presumed synaptic boutons) and mushroom-shaped spines. In addition, he has been able to confirm Ethell and Yamaguchi’s finding (Ethell et al., 2001) that Syndecan-2 specifically promotes the maturation of dendritic spines (Data not shown). Remarkably CASK significantly inhibits the pMAPK activity induced by NMDA receptor activation. As shown in Fig.8, a 5 fold increase (5.1+/− 0.42, n=21) in CASK caused a significant reduction of MAPK activity after removal of Mg2+ blockade of the NMDA receptors for 10 min.
Fig. 8. **Overexpression of CASK in young neurons promotes spine maturation and inhibits the MAPK activation.** A. Hippocampal neurons were transfected at 6 DIV. At 8DIV the cells were stimulated by removal of Mg2+ blockade of NMDA receptors for 15 min, fixed and stained for pMAPK and CASK. Arrows indicate clustering of CASK in axons, stars indicate clustering of CASK in dendritic spines. Arrowheads indicate the soma of two CASK expressing neurons. B, C. Pooled quantification of overexpression of CASK on the pMAPK (B) and dendritic spine morphology. Values are mean +/- SEM, ** p<0.01, n>20.

To further demonstrate that the tripartite NF1-syndecan-CASK signaling complex plays key role in spine maturation, we have begun to co-transfect Syndecan2 or CASK with siRNAs for NF1 or the dominant negative NF1GAP constructs (Fig.9), our results showed that knockdown of NF1 blocked the effect of CASK on spine maturation, suggesting that down-regulation of Ras activity by recruiting NF1 to the CASK-Syndecan2 signaling complex likely plays an essential role in stabilization and maturation of dendritic spines.

Fig. 9 **Knockdown of NF1 in CASK overexpressing cells produced immature dendritic phenotypes.** The DG explant was first transfected with DsRed along a control vector at 6 DIV(red cell). Two days later, the left cell was transfected with GFP along with CASK plus a RNAi construct for NF1. The explant was fixed at 17DIV and immunostained for CASK shown in blue. Arrows, immature filopodia. (C), (D) (E) are higher power views of (A) and (B). Scale bar: 20 μM for (A) and (B), 30 μM for (C) (D) and (E). Notice that INF1 did not inhibit CASK expression but induced immature spine phenotypes.

3). **Task3:** To determine if NF1-deficient cells or NF1 deficient mice have an altered capacity to undergo morphological plasticity after spaced depolarizing stimuli, and if deficits in morphology can be rescued by manipulating Ras-MAPK signaling.
This aim will complement the studies in Aim 1 & 2 on the role of NF1 in development, and determine if NF1 also plays an essential role in dendritic spine plasticity. If so, additional pharmacological interventions and genetic manipulations of the Ras-MAPK pathway will be used to rescue the morphological deficits. A similar combination of the confocal time-lapse imaging of morphological changes with the assessment of spatiotemporal activation of the Ras-MAPK pathway will further link the morphological plasticity to changes in signal transduction pathways in this activity-dependent model.

We have successfully established organotypic slice culture method in the lab. In a separate study, we have used slice culture system to demonstrate a central role of the Ras-PI3K-mTOR signaling pathway in the regulation of dendrite size and shape (Jaworski et al., 2005; Kumar et al., 2005). We have begun to investigate a possible role of NF1 in activity-dependent dendritic spine remodeling using both DG-CA3 explants and CA1/3 slice cultures. With the DG explants, we showed that Nf1 +/- also displayed some subtle immature spine phenotype, albeit much less profound than those seen in NF1 knockdown cells. As the Nf1 flox mice are now available to us at the NCI Mouse Models of Human Cancer Repository, we plan to establish the mouse line in the lab in a future study. We will cross breed the Nf1 flox line with forebrain specific CRE line driven by αCaMKII promoter. In parallel, we will transfect CRE construct in DG explants derived from the Nf1 flox brain and temporally control the deletion of Nf1. The effects on Ras-MAPK signaling and dendritic spine morphology will be examined. These additional new experiments will provide the crucial complementary evidence to further substantiate our results from siRNA studies, supporting that NF1 deficiency lead to abnormal spine morphology.

The second major goal of this aim was to pharmacologically rescue the abnormalities of dendritic spine morphology in the DG explants. We proposed to use the farnesyl-transferase inhibitors (FTI; BMS 191563) that Silva’s group used in their behavioral study (Costa et al., 2002) to rescue the spine phenotype. Unexpectedly, we found even low dose of the inhibitors produced significant toxicity in the DG explant cultures. We next sought out to see if manipulation of the Ras downstream pathways, specifically the Ras-MAPK and Ras-PI3K-Akt-mTOR pathway can rescue the morphological abnormality. We found that chronic inhibition of either the Ras-MAPK pathway by U0126 or the Ras-PI3K-Akt-mTOR pathway by mTOR specific inhibitor rapamycin reduced soma and dendrite size, and dendritic complexity, as well as density of dendritic filopodia and spines. Remarkably, a short-term inhibition (24hrs) promoted formation of mushroom-shaped spines on cells expressing constitutively active mutants of Ras, PI3K or Akt, or treated with upstream activator, BDNF (Kumar et al., 2005). These novel exciting observations should have important implications for developing therapeutic strategies to target the cognitive deficits in NF1 for future studies.

III. Key Research Accomplishments:

- Immunoblot analysis and immunohistochemistry demonstrated that Nf1 +/- neurons display hyperactive basal and evoked MAPK activity.

- Developed several siRNAs that target regions either in or outside of the NF1GRDI to specifically knockdown NF1 expression.

- Developed two dominant negative constructs for NF1 GAP activity and their effects on Ras-MAPK activity and spine morphology are being tested.

- Demonstrated that overexpression of CASK promoted the maturation of both the presynaptic boutons and dendritic spines with concomitant reduction of MAPK activity in immature neurons.
Used GST pull-down assay to confirm that Nfl+/- neurons or cells with knockdown of NF1 display hyperactive basal Ras activity

Obtained compelling evidence showing that specific knockdown of NF1 produced hyperactive Ras-MAPK signaling and immature spine phenotype; and these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity.

Demonstrated that overexpression of CASK promoted the maturation of both the presynaptic boutons and dendritic spines with concomitant reduction of MAPK activity in immature neurons; obtained preliminary data showing that NF1 is required for mediating the effect of CASK on spine maturation.

Made mouse DG explants and CA1/3 organotypic cultures; and have begun to investigate a possible role of NF1 in activity-dependent dendritic spine remodeling.

IV. Reportable outcomes:

1). Papers


11. Long, C., Cook, L., Wu, G-Y. and Mitchell, B., Removal of FKBP12/12.6 from endothelial ryanodine receptors leads to an intracellular calcium leak and endothelial dysfunction, ATVB, in revision.


16. Long C., Kumar, V. and Wu, G-Y. Akt differentially regulates synaptic growth and strength through mTOR dependent and independent mechanism, Neuron, in preparation.


2). Presentations:

Annual Neuroscience Meeting, San Diego, 2004


Annual Neuroscience Meeting, Washington D.C., 2005


Annual Neuroscience Meeting, Atlantic, 2006


3). Patents

4). Grants

“Cell Signaling and Dendritic Spine Plasticity”  2/01/2006-11/30/2010
Type: R01 (1R01NS055339-01A2)   Agency: NIH/NINDS   $262,500
The major goals of this project are to determine whether NF1 plays an essential role in dendritic spine formation and plasticity, and whether it does so through its role as a negative regulator for Ras (and MAPK) signaling.

Type: Research Grant   Agency: Whitehall Foundation   $75,000
The goal of this project is to define the central role of the Ras-PI3K-Akt-mTOR pathway in the regulation of dendrite size and shape.

5). Ph.D. Dissertation
Jacqueline Lee Alldritt was awarded Ph.D. on June 10, 2005.
Vikas Kumar was awarded an Ph.D. on June 25, 2006

V. Conclusions
In summary, the NFRP fund has provided us the crucial support for our research on the biological function of the newly identified NF1-syndecan-CASK signaling complex. With the support, we have been able to make significant progress on several innovative approaches to manipulate the signaling complex and image the underlying signal transduction mechanisms at single cell level. With the several siRNAs for NF1 and the two dominant negative constructs for NF1 GAP activity that we generated during the three-year support by the new Investigator Award, we have now obtained compelling evidence showing that NF1 deficiency indeed leads to abnormal development of dendritic spines and hyperactive Ras-MAPK activity, and furthermore, these deficits can be rescued by overexpression of NF1 GRD I, a central domain of NF1 containing ~360 residues responsible for its Ras GAP activity. Our novel results that NF1 deficiency leads to abnormal dendritic spine maturation resulting from hyperactive Ras-MAPK signaling would have important implications for developing therapeutic strategies to target the cognitive deficits in NF1.

VI. References


VII. Appendices: None

VIII. Personnel supported by the awarded:

Gang-Yi Wu, Ph.D., PI
Cheng Long, Ph.D., Postdoc Associate
Jing Zhao, Ph.D., Postdoc Associate
Mike Swank, Ph.D., Postdoc Associate
Vikas Kumar, MD, Ph.D. student