

Gene Expression Profiling in NF1 Knock-out Mice Suggests Link Between Neurofibromin, Kinesins, and Axonal Transport

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Abstract

The biological mechanisms by which NF1 gene mutation leads to cognitive deficits are not completely understood, although excessive Ras signaling and increased GABA mediated inhibition have been implicated.

To identify genes/proteins involved in the pathogenic process, gene expression analysis was performed comparing expression profiles in hippocampi of control and NF1^{-/-} heterozygous mice. Hippocampi were dissected from NF1^{-/-} and wild type litter mates at postnatal days 10, 15, and 20 and the total RNA expression profiled on the Affymetrix Mouse genome chip (Murine 430 2.0).

These experiments identified two G protein coupled receptors that were upregulated. At post natal day 10, a 10-fold increase was seen in the serotonin 5A receptor (5HT5A) in NF1^{-/-} mice. A second member of the G-alpha inhibitory G protein coupled receptor family, the dopamine 3 receptor (DRD3), was also dysregulated in the NF1^{-/-} mice, showing a 3 fold increase in expression. Members of the kinesin family were downregulated at P15 and P20. These trends in expression levels have been validated by qRT-PCR.

Differentially regulated genes at post-natal days 10, 15, and 20 were analyzed using GeneGo network analysis software. This network analysis identified direct interactions between NF1, kinesins, integrins, flammes, and amyloid precursor protein (APP), and between integrins and DRD3. Recently, this predicted interaction between NF1 and APP has been directly confirmed in melanocytes. Our results suggest an important link between neurofibromin and transport mechanisms in axons and dendrites.

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Methods

Tissue Dissection, RNA Preparation, and Labeling

- Hippocampus, thalamus, cerebellum, and cortex were selectively dissected from 4 NF1^{-/-} male mice and 4 wild type litter mates¹ (from Jackson Labs, www.jax.com) aged 10, 15, 20-days-old and flash frozen in liquid nitrogen.
- Animals were genotyped and sexed using PCR reactions utilizing amplification of a control gene (Musculus Pcg3), a mutant specific Cdh17, Nestin, and Cytoskeleton specific genes.
- Tissue from the hippocampus was divided to produce duplicate halves (~20 mg). Pairs of animals were combined, giving two separate samples, each with half a brain region from each mouse. Total RNA was isolated from each 40 mg tissue sample using Stratagene RT-PCR Mini-prep kit (the average yield was 15 ug RNA/40 mg tissue)
- Affymetrix labeling: cDNA was synthesized from 7ug of total RNA and subsequently *in vitro* transcribed with direct incorporation of biotin-labeled nucleotides as per manufacturer's protocols (www.affymetrix.com)

Array Hybridization and Scanning

- 15ug of each hippocampus cDNA sample was hybridized to a single Murine 430 2.0 array giving a total of 24 GeneChips (3 time points x 4 samples per condition x 2 conditions). Signal was detected after streptavidin-phycoerythrin labeling (S1 scan) as well as after signal amplification (anti-phycoerythrin)(S2 scan).

Analysis of Microarray Data

- Primary Analysis**
 - For each time point, average fold changes (relative to wildtype expression data) were calculated with error bars. Genes showing expression changes with significant p-values (p < 0.05) and fold change values of ≥ 2.0 within at least one time point were exported for functional annotation. Thereafter, the function of each gene was determined through literature searches, genes were binned into ontologic categories, and relevant biological processes and pathways identified.
- Network Development**
 - The GeneGo network building algorithms (GeneGo, Inc) were used in an iterative fashion to build gene/protein interaction pathways between known NF1^{-/-} pathway members (NF1, Ras, CABA) and proteins known to be involved in LTD. The gene expression changes with ≥ 2 fold differences (p < 0.05) were used to seed the algorithms and identified new pathway members which link the primary defect to the cognitive phenotype.

Validation of Expression Data by RT-PCR

- RT-PCR**
 - Total RNA was extracted from ~20mg of hippocampus from 3 NF1^{-/-} and 3 wild type mice using the Absolutely RNA Miniprep kit (Stratagene). Reverse transcription reactions were done using 5ug of total RNA from hippocampus, oligo dT primers, and the Super Script III First Strand cDNA synthesis kit (Invitrogen). Resulting cDNA was amplified on the Chromo4 Four-Color Real-Time System (MJ Research) using the DyNAmo HS SYBR Green qPCR Kit (Invitrogen) and gene specific primers. Standardized and optimized primers were ordered from SuperArray Bioscience Corporation.
- Fold Change Calculation**
 - The threshold for amplification was set as the number of cycles necessary to reach logarithmic fluorescence accumulation (C_T). Fold difference in cDNA concentration was calculated using the formula

$$F = 2^{(C_{T2} - C_{T1}) / (M_1 - M_2)}$$

where F = fold difference, MH = mutant housekeeping gene (GAPD) C_T, MG = mutant gene of interest C_T, WT1 = wild type housekeeping gene (GAPD) C_T, W2 = wild type gene of interest C_T. Statistical significance of the resulting fold change values was calculated with a two-tailed t-test assuming unequal variance.

Gene	10d	15d	20d
5HT5A	10	10	10
DRD3	10	10	10
KIF1A	10	10	10
KIF1B	10	10	10
KIF1C	10	10	10
KIF1D	10	10	10
KIF1E	10	10	10
KIF1G	10	10	10
KIF1H	10	10	10
KIF1I	10	10	10
KIF1J	10	10	10
KIF1K	10	10	10
KIF1L	10	10	10
KIF1M	10	10	10
KIF1N	10	10	10
KIF1O	10	10	10
KIF1P	10	10	10
KIF1Q	10	10	10
KIF1R	10	10	10
KIF1S	10	10	10
KIF1T	10	10	10
KIF1U	10	10	10
KIF1V	10	10	10
KIF1W	10	10	10
KIF1X	10	10	10
KIF1Y	10	10	10
KIF1Z	10	10	10
KIF2A	10	10	10
KIF2B	10	10	10
KIF2C	10	10	10
KIF2D	10	10	10
KIF2E	10	10	10
KIF2G	10	10	10
KIF2H	10	10	10
KIF2I	10	10	10
KIF2J	10	10	10
KIF2K	10	10	10
KIF2L	10	10	10
KIF2M	10	10	10
KIF2N	10	10	10
KIF2O	10	10	10
KIF2P	10	10	10
KIF2Q	10	10	10
KIF2R	10	10	10
KIF2S	10	10	10
KIF2T	10	10	10
KIF2U	10	10	10
KIF2V	10	10	10
KIF2W	10	10	10
KIF2X	10	10	10
KIF2Y	10	10	10
KIF2Z	10	10	10
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KIF3B	10	10	10
KIF3C	10	10	10
KIF3D	10	10	10
KIF3E	10	10	10
KIF3F	10	10	10
KIF3G	10	10	10
KIF3H	10	10	10
KIF3I	10	10	10
KIF3J	10	10	10
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KIF3L	10	10	10
KIF3M	10	10	10
KIF3N	10	10	10
KIF3O	10	10	10
KIF3P	10	10	10
KIF3Q	10	10	10
KIF3R	10	10	10
KIF3S	10	10	10
KIF3T	10	10	10
KIF3U	10	10	10
KIF3V	10	10	10
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KIF3X	10	10	10
KIF3Y	10	10	10
KIF3Z	10	10	10
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