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14. ABSTRACT

Learning disabilities severely deteriorate the life of many NF1 children by limiting their academic achievement, higher education and career choice. However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. This study was proposed to identify genes that are dysregulated in the hippocampus of the Nf1+/- mouse model by DNA microarray analysis. Characterization of these NF1-affected genes will dramatically improve our understanding of the molecular pathogenesis underlying NF1-associated learning deficits.

15. SUBJECT TERMS
NF1, hippocampus, learning disabilities, LTP, microarray, gene

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Final report

Introduction

Neurofibromatosis 1 (NF1) is a common single-gene disorder that causes learning impairments in patients. Learning disabilities severely deteriorate the life of many NF1 children by limiting their academic achievement, higher education and career choice (1). However, the pathogenic process for NF1-associated learning disabilities has not been fully understood and an effective therapy is not available. Drs Silva's and Zhong's laboratories have demonstrated that *Nf1* mutations lead to the development of learning deficits in mouse and *Drosophila*, respectively (2-4). Their work suggests that *Nf1* mutations cause learning deficits by disturbing the Ras/MAPK and/or cAMP signaling. Despite these significant progresses, NF1-affected downstream genes that directly contribute to deficits in synaptic plasticity and learning are largely unknown. In this project, we aimed to identify the genes and molecular pathways that are dysregulated in the hippocampus of the $Nf1^{+/-}$ mouse model. Because of the importance of the hippocampus in learning and memory, identification of these NF1-affected genes and pathways are expected to dramatically improve our understanding of the molecular pathogenesis of NF1-associated learning deficits.

Body

During the entire period of this project (2004-2008), we carried out the following proposed research activities:

Task 1. Identifying genes that are misregulated in the NF1 mouse hippocampus:

a. Establish the breeding colony for NF1 mice.
b. Purify hippocampal RNA from wild-type control and NF1 (Nf1+/-) mice at various developmental stages.
c. Prepare cRNA targets.
d. Perform hybridization on oligonucleotide microarrays.
e. Perform statistical analysis to identify genes that are abnormally expressed in the NF1 hippocampus.
f. Perform clustering and bioinformatic analyses to annotate the functions of genes and to identify the biological pathways that are affected in the NF1 hippocampus.

Task 2. Identifying genes that are misregulated during LTP expression in the NF1 hippocampu:

a. Perform LTP experiments on wild-type control and NF1 hippocampal slices.
b. Purify RNA from CA1 mini-slices after various time of LTP expression.
c. Prepare cRNA targets.
d. Perform microarray hybridization.
e. Perform statistical analysis to identify genes that are abnormally expressed at various stages of LTP expression in the NF1 hippocampal slices.

f. Perform clustering and bioinformatic analyses to annotate the functions of genes and to identify the biological pathways that are specifically affected during LTP expression in the NF1 hippocampus.

Key Research Accomplishments

With the successful completion of the proposed DNA microarray analysis supported by this award, we have:

a. Identified many genes that are dysregulated in the NF1(+/-) hippocampus.
b. Identified NF1-affected genes and molecular pathways in synaptic plasticity.
c. Revealed potential molecular processes contributing to NF1-associated learning disabilities.
d. Found that lovastatin altered the expression of a large number of genes, including those disturbed by NF1 mutations.
e. Provided a genome-wide overview of the molecular abnormalities in the NF1(+/-) hippocampus.

Reportable Outcomes

We have described the detailed findings of this study in our published research article, 'Aberrant expression of synaptic plasticity-related genes in the NF1+/- mouse hippocampus', in the Journal of Neuroscience Research (6).

Conclusions

With the successful completion of the proposed DNA microarray analysis supported by this award, we have revealed that genes involved in a wide spectrum of biological processes are dysregulated in the NF1(+/-) hippocampus. Many of the NF1-affected genes play critical roles in synaptic plasticity, such as Rabs, synaptotagmins, NMDAR1, CaMKII, and CREB1. This new knowledge will facilitate the investigation of the molecular pathways that are disturbed in the NF1 hippocampus and probably contribute to the pathogenesis of NF1-associated learning disabilities. Because NF1-associated learning disabilities can be reversed by lovastatin, we also determined the effect of lovastatin treatment on genome-wide expression patterns of the NF1(+/-) hippocampus. We found that lovastatin altered the expression of a large number of genes, including those disturbed by NF1 mutations. Overall, our results provide a genome-wide overview of the molecular abnormalities in the NF1(+/-) hippocampus and should be useful for further identifying the novel molecular pathways that cause NF1 learning deficits. These findings may facilitate the development of therapeutic approaches.

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Aberrant Expression of Synaptic Plasticity-Related Genes in the NF1$^{+/-}$ Mouse Hippocampus

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Neurofibromatosis 1 (NF1) is a common single-gene disorder that causes learning impairments in patients. Neurofibromin encoded by the NF1 causal gene regulates Ras/MAPK and cAMP signaling pathways. These signaling pathways play critical roles in controlling gene transcription during synaptic plasticity and memory formation. We hypothesized that NF1 mutations disturb the expression of genes important for memory formation. To test this hypothesis, we performed DNA microarray analysis on the hippocampus of NF1$^{+/-}$ mice, the mouse model for NF1 learning disabilities. Our results indicated that genes involved in a wide spectrum of biological processes are dysregulated in the NF1$^{+/-}$ hippocampus. Many of the NF1-affected genes play critical roles in synaptic plasticity, such as Rabs, synaptotagmins, NMDAR1, CaMKII, and CREB1. Because NF1-associated learning disabilities can be reversed by lovastatin, we also determined the effect of lovastatin treatment on genome-wide expression patterns of the NF1$^{+/-}$ hippocampus. We found that lovastatin altered the expression of a large number of genes, including those disturbed by NF1 mutations. Our results reveal a genome-wide overview of the molecular abnormalities in the NF1$^{+/-}$ hippocampus and should be useful for further identifying the novel molecular pathways that cause NF1 learning deficits.

Key words: neurofibromatosis 1; hippocampus; synapse; memory; learning disabilities

Neurofibromatosis 1 (NF1) is a prevailing autosomal dominant genetic disorder that occurs at a rate of approximately 1 in 3,500 and is caused by mutations in a single gene, *Nf1* (Eliason, 1988; Cichowski and Jacks, 2001). In addition to a variety of physical manifestations, including benign and malignant brain tumors, NF1 patients may also experience cognitive difficulties that are often considered hallmarks of this disease (Hofman et al., 1994; North, 2000; Cichowski and Jacks, 2001). Among cognitive impairments associated with NF1, the learning disabilities (LD) manifested in 30–65% of NF1 children are especially problematic (Eliason, 1988; North, 1993; North et al., 1995; Ferner et al., 1996; Kayl and Moore, 2000).

Heterozygous mice with an *Nf1* null mutation (*Nf1*$^{+/-}$) develop multiple behavioral phenotypes that bear striking similarities to learning disabilities seen in NF1 patients (Silva et al., 1997; Costa and Silva, 2003). For example, as in NF1 patients, *Nf1*$^{+/-}$ mice have spatial learning deficits, as suggested by the Morris water maze test (Silva et al., 1997), a learning task that is sensitive to hippocampal lesions. Importantly, similar to NF1 patients, the learning deficits of *Nf1*$^{+/-}$ mice can also be compensated by extended training (Silva et al., 1997). However, simple associative learning is not affected in either NF1 patients or *Nf1*$^{+/-}$ mice (Silva et al., 1997). Similarly to NF1 patients, some *Nf1*$^{+/-}$ mice do not experience learning impairments (Silva et al., 1997). Interestingly, the homozygous knockout mice, which carry the *Nf1*$^{23a-l-}$ mutant genes lacking exon 23a, also develop learning deficits even though they are developmentally normal and are without an increased tumor predisposition (Costa et al., 2001). This observation indicates that *NF1*-associated learning impairments are specific phenotypes that can be dissociated from developmental and other physical defects. Sweveral studies have suggested that long-term potentiation (LTP) is an important cellular substrate for learning and memory (Grimwood et al., 2001). Consistently with this notion, the hippocampus from *Nf1*$^{+/-}$ mice also displays deficits in LTP expression (Costa et al., 2002).

Additional Supporting Information may be found in the online version of this article.

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The neurofibromin protein encoded by the *Nf1* gene is a tumor suppressor related to Ras GTPase-activating proteins (GAP; Cichowski and Jacks, 2001; Zhu and Parada, 2001b; Weeber and Sweatt, 2002). As a Ras GAP-related protein, neurofibromin functions as a negative regulator of Ras function, by accelerating the hydrolysis of GTP to GDP that leads to inactivation of Ras (Cichowski and Jacks, 2001; Zhu and Parada, 2001b; Weeber and Sweatt, 2002). Previous studies indicated that the abnormal up-regulation of the Ras activity after loss-of-function of neurofibromin is a crucial step leading to learning impairments in $Nf1^{+/-}$ mice (Costa et al., 2002). Down-regulation of Ras activities in $Nf1^{+/-}$ mice by genetic and pharmacological approaches rescued learning deficits and LTP deficits (Costa et al., 2002). *Drosophila* neurofibromin not only is a Ras-GAP (Williams et al., 2001) but also regulates the cAMP signaling (Guo et al., 1997, 2000; The et al., 1997; Tong et al., 2002). Mutations of *Drosophila Nf1* likely impair learning by inhibiting the cAMP/PKA signaling pathway (Guo et al., 2000). A role of mammalian neurofibromin in regulating adenylyl cyclase has also been reported (Tong et al., 2002). As both Ras/MAPK and cAMP signaling cascades play important roles in regulation of gene expression during synaptic plasticity and memory formation, their disturbance likely dysregulates gene expression in specific brain regions such as the hippocampal formation, where neurofibromin is highly expressed (Nordlund et al., 1995; Zhu and Parada, 2001a). However, NF1-affected genes that contribute to the pathogenesis of learning deficits have not been identified.

We used DNA microarrays to identify genes whose expression was altered in the hippocampus of $Nf1^{+/-}$ mice. A comprehensive pool of NF1-affected hippocampal genes that function in a wide spectrum of biological pathways was identified, many of which are known to be involved in synaptic plasticity and memory formation. Because lovastatin is able to reverse learning deficits of NF1 mice (Li et al., 2005), we also characterized genes that respond to lovastatin treatment. These findings provide a genome-wide overview of the molecular pathogenic abnormalities that are likely to be relevant to learning deficits in NF1 patients.

MATERIALS AND METHODS

$Nf1^{+/-}$ Mouse Breeding and Genotyping

$Nf1^{+/-}$ mice (NCI) were bred with wild-type C57BL/6 mice and genotyped at 4 weeks using the REDExtract-N-Amp tissue PCR kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. PCR primers used were NII 5′-GGT ATT GAA TTG AAG CAC-3′, NIII 5′-ATT CGC CAA TGA CAA GAC-3′ and 3′5′ 5′-TTC AAT ACC TGC CCA AGG-3′. PCR cycles were 3 min at 94°C and a cycle of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C repeated 35 times, and 10 min at 72°C.

Contextual Discrimination

Eight-week-old mice were used for the contextual discrimination behavioral tests, which were performed as described by Frankland et al. (1998). All experiments were carried out blind with respect to genotypes. Two contextually different chambers, one for training with mild electric shocks (S) and another for control (C), were used in these behavioral tests. Chambers S and C were located in the same room and housed in separate sound-attenuated boxes. Three days prior to training, animals were handled daily for 5 min and allowed to explore both chambers S and C freely for 5 min (preexposure). On training day, in chamber S, animals were allowed to explore for 2.5 min before a 2-sec 0.8 mA foot shock was delivered. Mice were then observed for an additional 30 sec before being returned to their home cages. In chamber C, mice were allowed to explore for 3 min, without shocks, and then returned to their home cages. Contextual fear memory was measured by scoring freezing behaviors during the first 2.5 min of exploration immediately after the mouse was placed in a chamber. A mouse was scored freezing when it remains in a motionless posture, with only respiratory movement. Freezing was measured with a sampling method, in which two observers independently reviewed the video recording of an animal in each chamber and 2-sec observations were taken every 5 sec. If the animal remained motionless for the entire 2-sec observation, then it was scored as freezing. Freezing data are presented as the percentage of time spent freezing during the training or testing period.

Lovastatin Treatment

Lovastatin (Mevinolin; Sigma-Aldrich) in the lactone form was dissolved in 55°C ethanol and then NaOH was added (1 M). The solution was left at room temperature for 30 min to complete the conversion of lovastatin to the sodium salt. The final lovastatin solution (4 mg/ml) was adjusted to pH 7.5 with HCl. Mice were injected with 10 mg/kg lovastatin or the vehicle control subcutaneously once per day for 4 days. On the fourth day, 6 hr following the final injection, mice were sacrificed to collect the hippocampus for RNA extraction.

Tissue Collection/RNA Extraction

Whole hippocampi were dissected and collected, with one hippocampus immediately used for RNA extraction and the other flash-frozen and stored at −80°C for additional RNA extraction or other assays. Total RNA was extracted from fresh, whole hippocampus using the RNeasy kit (Qiagen, Valencia, CA). The hippocampus was first homogenized in lysis buffer (supplied with the RNeasy kit) for 30 sec using a mortar and pestle. The lysate was then centrifuged through a QIAshredder column (Qiagen) to homogenize the tissue further. RNA was extracted by using the RNeasy kit, following the manufacturer's directions. The extracted total RNA was flash-frozen and stored at −80°C.

Microarray Analysis

Microarray processing and hybridization were performed by the University of California, Irvine, DNA Core Facility.

Prior to processing for microarray hybridization, the quality of each RNA sample was verified with the Agilent 2100 Bioanalyzer. Total RNA was reverse transcribed into cDNA, converted to biotin-labeled cRNA, and then hybridized onto individual Affymetrix Mouse Genome 430 2.0 Array GeneChip (MG430v2) arraysm, following the manufacturer's instructions.

Two sets of normalized gene expression values were obtained using GCOS 1.4 (Affymetrix) and dCHIP. Although the GCOS algorithm is suitable for determining high-level expression values, for low-level expression values the model-based expression index (MBEI) analysis performed in dChip improves the accuracy of the expression values by reducing the variability of low-expression targets. Pair-wise statistical comparisons of microarray data from *Nf1*$^{+/-}$ mice with WT control mice were carried out using CyberT, which performs *t*-tests that incorporate a Bayesian estimate of the variance of the microarray expression data to compensate for a low number of experimental replicates (http://visitor.ics.uci.edu/genex/cybert/). Genes that were differentially expressed between *Nf1*$^{+/-}$ and WT control mice at statistically significant levels ($P < 0.05$) were identified and compiled in a list for further analysis. Hierarchical clustering of expression profiles of *Nf1*$^{+/-}$ and WT control mice was performed using dChip and GeneSpring (Silicon Genetics).

Real-Time Quantitative RT-PCR Verification

Real-time quantitative RT-PCR was performed with an ABI Prism 7900HT sequence detection system and LUX fluorogenic primers (Invitrogen, Carlsbad, CA). For each target gene, fluorophore-labeled LUX forward primers and their corresponding unlabeled reverse primers were designed using LUX Designer (http://www.invitrogen.com/lux). Real-time quantitative RT-PCR was performed with 0.1 μg of total RNA (from the same samples used for the microarray analysis) and the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen) according to the manufacturer's instructions. PCR cycles were 15 min at 50°C, 2 min at 95°C, and then 50 cycles of 15 sec at 95°C and 30 sec at 60°C. The relative difference in expression levels between *Nf1*$^{+/-}$ and WT control mice for each target gene was calculated by a relative cycle threshold method ($2^{-\Delta\Delta Ct}$) using PGK1 as a reference.

RESULTS

NF1$^{+/-}$ Mice Develop Reversible Deficits in Consolidation but Not in Encoding of Fear Memories

Most of the previous studies on learning deficits of NF1 mice focused on the paradigm of the Morris water maze, but we investigated the learning performance of NF1$^{+/-}$ mice on a contextual discrimination task (Frankland et al., 1998). In this hippocampus-dependent learning task, mice were trained in two chambers; one of them was associated with electric shocks (shock chamber) and the other was not (control chamber). The expression of freezing behaviors was determined to measure fear memories (Fig. 1). On the training day, NF1$^{+/-}$ and wild-type mice were indistinguishable in both chambers (Fig. 1A). One day after training, both NF1$^{+/-}$ and wild-type mice showed a clear increase in freezing behaviors in the shock chamber but not in the control chamber, indicating formation of the contextual-shock association. At this stage, the percentage of freezing developed by NF1$^{+/-}$ mice was not significantly different from that of wild-type mice ($P > 0.05$; Fig. 1B). This observation indicates that the NF1$^{+/-}$ mice are normal for fear memory encoding and for memory consolidation processes occurring within 24 hr after training. Previous work also suggested that NF1$^{+/-}$ mice are not impaired during the acquisition phase in the Morris water maze task (Silva et al., 1997). On the other hand, memory tests on day 7 showed that the percentage of freezing of NF1$^{+/-}$ mice was significantly lower than that of wild-type mice (Fig. 1C). These observations suggest that NF1$^{+/-}$ mice may have impaired long-term memory 7 days after training. To test whether this memory deficiency of NF1$^{+/-}$ mice can be compensated by more training, we added one more session of contextual shock pairing in the shock chamber at day 1 after the initial training. Memory tests at day 7 showed that, after being reinforced training at day 1, NF1$^{+/-}$ and wild-type mice developed similar amounts of freezing (Fig. 1D). This result indicates that the deficiency in contextual fear memory of NF1$^{+/-}$ mice can be compensated by excess training. These findings, obtained with a different behavioral paradigm, support the notion that overtraining may have compensatory effects (Silva et al., 1997).

DNA Microarray Analysis of the NF1$^{+/-}$ Hippocampus

Previous results (Silva et al., 1997) together with the results described in this manuscript strongly suggest a specific impairment of NF1$^{+/-}$ mice in consolidation of hippocampus-dependent memory. To obtain insight into the molecular basis of NF1-associated memory impairments, we sought to determine the gene expression in the NF1$^{+/-}$ hippocampus. We performed DNA microarray analysis to identify genes that were aberrantly expressed in the NF1$^{+/-}$ hippocampus from male mice at 2 months of age (with age-matched wild-type littermates as controls), using an Affymetrix GeneChip (MG430v2) that contains over 39,000 probe sets. Four independent array replicates with RNAs from different mice were included. Visualization of microarray data in a distribution scatterplot revealed that the expression levels of some genes were shifted in the NF1$^{+/-}$ hippocampus (Fig. 2). *t*-Tests with Cyber-T (Hung et al., 2002; http://visitor.us.uci.edu/genex/cybert/) identified 6,418 probes that were significantly changed in the NF1$^{+/-}$ hippocampus ($P < 0.05$; Supp. Info. Table I); these probes correspond to 5,175 unique genes. We performed real-time RT-PCR analysis to confirm the expression changes of a group of genes that showed relatively large changes in microarray analysis and are impli-

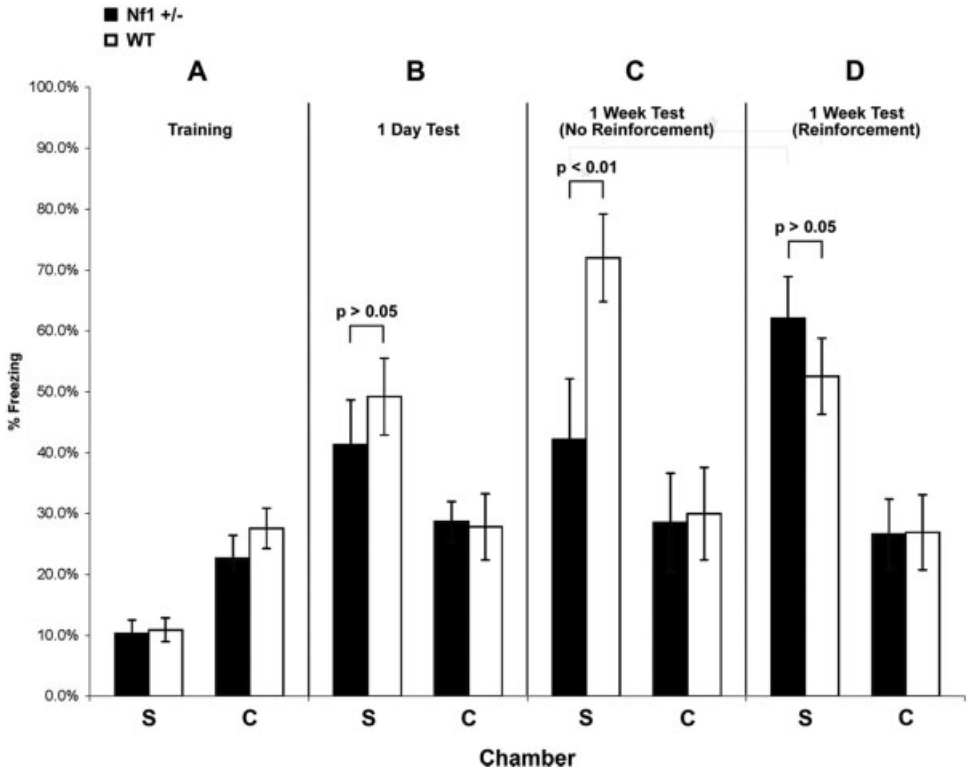

Fig. 1. $NF1^{+/-}$ mice display learning deficits in a contextual discrimination task. **A:** On the training day, age- and gender-matched wild-type and $NF1^{+/-}$ mice were placed in the training (S) or control (C) chambers to explore for 2.5 min. Mild electric shocks were then delivered in chamber S but not in chamber C. Freezing behaviors during the period before shock delivery were scored. Wild-type and $NF1^{+/-}$ mice were not different in their freezing behaviors in both chambers. **B:** One-day memory tests. One day after training, mice were exposed to chamber S or C. In chamber S, compared with the training day (A), both wild-type and $NF1^{+/-}$ mice expressed fourfold more freezing behaviors, suggesting the formation of fear memories about chamber S. Mice of both genotypes showed similar level of freezing in chamber C, similar to that from the training day (A). **C:** Seven-day memory tests after one session of training. Mice trained in A were tested 7 days after. Note that wild-type mice showed a significantly higher level of freezing than $NF1^{+/-}$ mice in chamber S. **D:** Seven-day memory tests after two sessions of training. Mice received two sessions of training on the first and second days, and memory tests were performed on day 7. Mice of both genotypes displayed similar levels of freezing in S chamber. Ten to fifteen animals were included in each group.

cated in learning and memory (Fig. 3). One of the up-regulated genes in the $NF1^{+/-}$ hippocampus is the ubiquitin ligase UBE3A, which, when mutated, is the causal gene for Angelman syndrome. -Fold changes of up- or down-regulation and *P* values associated with the changes for individual genes are given in Tables I–III and in the Supporting Information Tables.

Diverse Molecular and Cellular Processes Are Disturbed in the $NF1^{+/-}$ Hippocampus

To obtain insights into the biological processes disturbed in the $NF1^{+/-}$ hippocampus, we performed gene ontology (GO) analysis of NF1-affected genes (http://www.affymetrix.com/analysis/index.affx; http://www.genmapp.org). The results indicate that genes disturbed in the $NF1^{+/-}$ hippocampus are associated with a variety of molecular and cellular processes, such as cell–cell communication, signal transduction, cellular transport, transcription, and the cytoskeleton (Table I). These observations indicate that a wide range of biological processes is disturbed in the $NF1^{+/-}$ hippocampus. Interestingly, previous DNA microarray studies have suggested that spatial learning in the Morris water maze regulates genes in many of these processes, including cell signaling, cell–cell interaction, transcriptional and translational regulation, and regulation of the cytoskeleton (Cavallaro et al., 2002). Thus, it is likely that there is a global disturbance of genes in cellular and molecular processes underlying memory formation in the hippocampus of $NF1^{+/-}$ mice.

Dysregulation of Synapse-Related Genes in the $NF1^{+/-}$ Hippocampus

Synapses are the structural units for information storage. We next sought to determine whether synapse-related genes are disturbed in the $NF1^{+/-}$ hippocampus. By searching the PubMed database on the NF1-affected genes ($P < 0.05$), we found that 213 were synapse

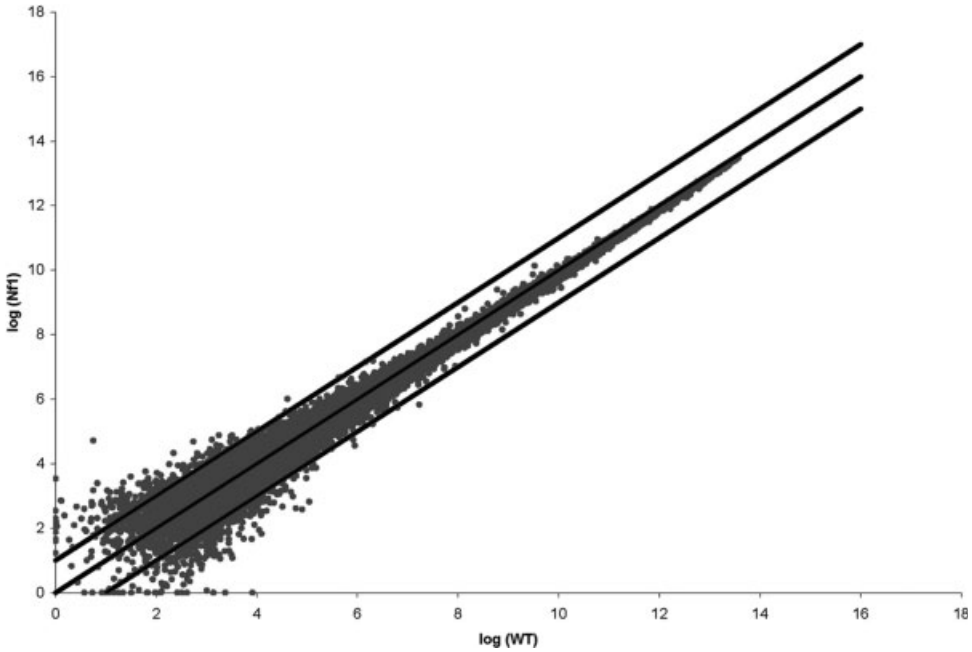

Fig. 2. Scatterplot of expression values of all genes on microarrays. Genes that were differentially expressed in the wild-type and NF1$^{+/-}$ hippocampus are indicated by dots outside the diagonally centered channel.

related (Fig. 4A). One hundred three of them were aberrantly down-regulated and 110 up-regulated. Hierarchical clustering of these synapse-related genes showed that many of the synapse-related genes were consistently dysregulated in the NF1$^{+/-}$ hippocampus from different animals (Fig. 4). Dysregulation of these genes may be a reliable biomarker of the NF1$^{+/-}$ hippocampus.

Among the synapse-related genes dysregulated in the NF1$^{+/-}$ hippocampus, many of them are involved in neurotransmitter vesicle trafficking/recycling (Table II). These include Rab and synaptotagmin proteins, such as Rab3A and synaptotagmin1 (Syt I; Table II; Supp. Info. Table I). It has been reported that Rab3A mutant mice are impaired with the learning task of cued fear conditioning (Yang et al., 2007) and that Syt I is involved in aversive learning (Liu et al., 2008). Furthermore, the expression of several synaptic receptor genes, including NMDA receptor (NMDAR) 1, AMPA receptor (AMPAR) 4, and metabotropic glutamate receptor 5 (mGluR5), was altered (Table II; Supp. Info. Table I). The important role of NMDARs, AMPARs, and mGluRs in synaptic plasticity and learning has been well established (Malenka and Bear, 2004; Kessels and Malinow, 2009). Several genes encoding synaptic structural proteins, such as neurexin1, integrin β6, integrin β7, and Ncam1, were disturbed (Table II; Supp. Info. Table I). The involvement of these genes or their related genes in synaptic plasticity and learning has been reported (Chan et al., 2003; Bukalo et al., 2004; Zeng et al., 2007). Another interesting finding is that specific important synaptic signaling proteins were dysregulated in the NF1$^{+/-}$ hippocampus (Table II). One of them was αCaMKII, which is a master regulator of synaptic plasticity and learning (Lisman et al., 2002). Together, our findings indicate that the altered expression of the genes involved in the regulation of neurotransmission and structures and signaling of the synapse may contribute to the observed impairments of synaptic function and plasticity in NF1$^{+/-}$ mice (Costa et al., 2002).

Disturbance of LTP-Regulated Genes in the NF1$^{+/-}$ Hippocampus

Long-term potentiation (LTP) is widely considered as a critical cellular mechanism underlying memory formation. We hypothesized that specific LTP-related genes are disturbed in the NF1$^{+/-}$ hippocampus. To test this idea, we compared the activity-regulated genes (ARGs) that changed their expression after LTP induction (Park et al., 2006) with those that were disturbed in the NF1$^{+/-}$ hippocampus (Supp. Info. Table I). We found that 121 ARGs were dysregulated in the NF1$^{+/-}$ hippocampus (Fig. 4B). Among these ARGs, 71 were abnormally down-regulated and 50 up-regulated in the NF1$^{+/-}$ hippocampus. This finding indicates that the LTP-related processes associated with these ARGs are disturbed in the NF1$^{+/-}$ hippocampus. The malfunction of these processes may contribute to the LTP impairments in the NF1$^{+/-}$ hippocampus (Costa et al., 2002). Among the LTP-related genes that are dysregulated in the NF1$^{+/-}$ hippocampus are αCaMKII and CREB1 (Table II; Supp. Info. Table I).

TABLE I. Ontology Groups of Differentially Expressed Genes in the Nf1$^{+/-}$ Hippocampus ($P < 0.01$)*

	Probe set	Gene symbol	Gene description	Fold change	*P* value
Cell–cell communicaiton (99)	1415800_at	Gja1	Gap junction membrane channel protein alpha 1	1.137	0.040
	1420744_at	Chrnb2	Cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)	−0.047325	0.006
	1421531_at	Akap3	A kinase (PRKA) anchor protein 3	1.840	0.004
	1449206_at	Sypl2	Synaptophysin-like 2	−1.520277	0.006
	1450043_at	Fzd7	Frizzled homolog 7 (*Drosophila*)	−1.820426	0.003
Signal transduction (82)	1417605_s_at	Camk1	Calcium/calmodulin-dependent protein kinase I	1.170	0.002
	1418098_at	Adcy4	Adenylate cyclase 4	1.217	0.008
	1426233_at	Map2k4	Mitogen-activated protein kinase kinase 4	−1.058896	0.006
	1428813_a_at	Drd1ip	Dopamine receptor D1 interacting protein	−0.0120294999999999	0.003
	1435196_at	Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	1.120	0.041
Cellular transport (74)	1446431_at	Dnm3	Dynamin 3	−1.2647915	0.010
	1421990_at	Syt1	Synaptotagmin I	−0.105	0.006
	1426578_s_at	Snapap	SNAP-associated protein	1.156	0.007
	1432004_a_at	Dnm2	Dynamin 2	1.197	0.007
	1446826_at	Xpo7	Exportin 7	−0.8570615	0.000
Transcription (71)	1416149_at	Olig1	Oligodendrocyte transcription factor 1	1.140	0.015
	1418091_at	Tcfcp2l1	Transcription factor CP2-like 1	1.298	0.034
	1420811_a_at	Ctnnb1	Catenin (cadherin-associated protein), beta 1, 88kDa	0.053	0.010
	1426437_s_at	Hdac3	Histone deacetylase 3	−0.0161705	0.002
	1452529_a_at	Creb1	cAMP responsive element binding protein 1	−1.1183315	0.006
Protein modification (59)	1421340_at	Map3k5	Mitogen-activated protein kinase kinase kinase 5	−0.0122274999999999	0.003
	1424116_x_at	Ppp5c	Protein phosphatase 5, catalytic subunit	−1.0323095	0.004
	1430022_at	Uble1a	Ubiquitin-like 1 (sentrin) activating enzyme E1A	−0.4153185	0.009
	1431825_at	Stk23	Serine/threonine kinase 23	−1.3169315	0.003
	1435757_a_at	Uqcrc2	Ubiquinol cytochrome c reductase core protein 2	1.119	0.004
Cytoskeleton (17)	1422481_at	Krt2-1	Keratin complex 2, basic, gene 1	0.043	0.007
	1448554_s_at	Myh6	Myosin, heavy polypeptide 6, cardiac muscle, alpha	1.344	0.006
	1450112_a_at	Gas2	Growth arrest specific 2	1.206	0.049
	1453193_s_at	Kif12	Kinesin family member 12	−1.863316	0.006
	1456618_at	Mark4	MAP/microtubule affinity-regulating kinase 4	−1.1109675	0.026
Cell differentiation (19)	1416714_at	Irf8	Interferon regulatory factor 8	1.146	0.009
	1417419_at	Ccnd1	Cyclin D1	1.221	0.000
	1422300_at	Nog	Noggin	1.233	0.006
	1438067_at	Nf1	Neurofibromatosis 1	−1.5948515	0.000
	1446185_at	Frap1	FK506 binding protein 12-rapamycin associated protein 1	0.074	0.007740626

*Representative examples given for each group. Total number of genes is given in parentheses.

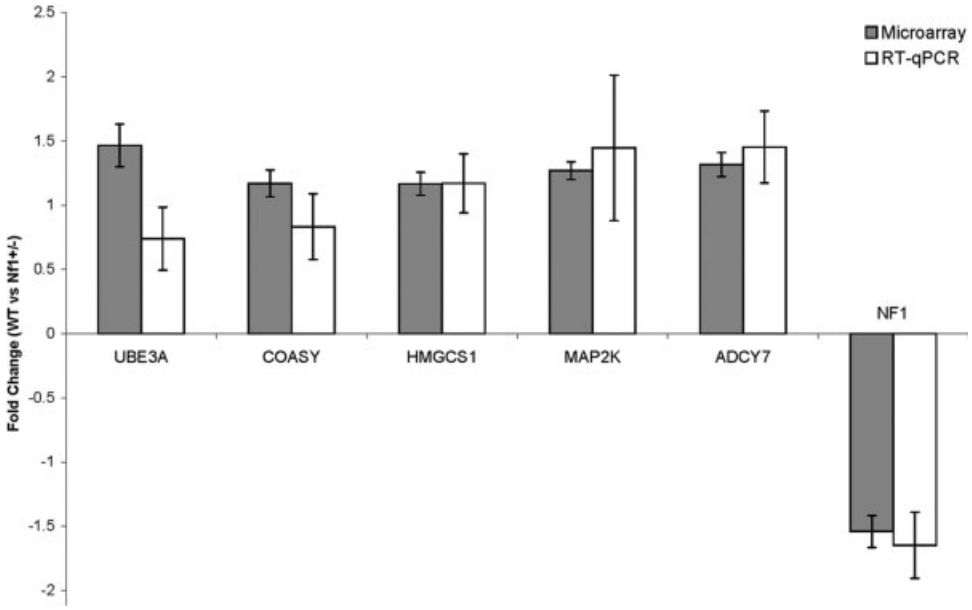

Fig. 3. Real-time RT-PCR confirmation of altered expression of a set of genes in the NF1$^{+/-}$ hippocampus. A set of genes with potential roles in learning and relatively large -fold changes (NF1 vs. WT) from the microarray analysis was chosen for RT-qPCR confirmation.

Lovastatin Treatment of NF1$^{+/-}$ Mice Changes Genomic Expression Patterns in the Hippocampus

A recent, exciting study showed that treatment with lovastatin, a specific inhibitor of three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, improved the learning performance of NF1$^{+/-}$ mice, probably by inhibiting the MAPK signaling in the brain (Li et al., 2005). Another HMG-CoA reductase inhibitor, simvastatin, has also been shown to facilitate learning and memory (Li et al., 2006; Ling Li, 2006; Lu et al., 2007). To understand the mechanism by which lovastatin rescues NF1 memory deficits, we sought to determine the effects of lovastatin treatment on the gene expression in the NF1$^{+/-}$ hippocampus. We performed DNA microarray analysis to determine gene expression in the hippocampus of NF1$^{+/-}$ mice that received lovastatin for 4 days, which was effective for rescueing memory deficits (Li et al., 2005). NF1$^{+/-}$ mice that received an equal volume of vehicle were used as controls. We found that the expression level of 2,976 probe sets was altered by lovastatin treatment, with a P value of 0.05, and 682 genes with a P value of 0.01. GO analysis indicated that the lovastatin-altered genes were involved in many biological processes, including cell–cell communication, cell signaling, transcription, and cytoskeleton dynamics (Table III). These results suggest that treatment with lovastatin affected genes involved in diverse biological processes known to be disturbed in the hippocampus of NF1$^{+/-}$ animals (Table I). Among the NF1-disturbed genes ($P < 0.05$), 377 changed their expression level after lovastatin treatment. We observed that lovastatin reversed the aberrant expression of some NF1-affected genes (Fig. 5A,B). For example, *Rabl3* and MAPK8 interacting-protein 3 (*Mapk8ip3*) were down-regulated in the NF$^{+/-}$ hippocampus; lovastatin treatment up-regulated their expression (Fig. 5A). On the other hand, *Sema6a*, which critically regulates axonal growth in the hippocampus (Suto et al., 2007), and *Cacna1c*, a gene encoding the calcium channel subunit α1c that plays important role in synaptic plasticity and spatial learning (Moosmang et al., 2005), were up-regulated in the NF1$^{+/-}$ hippocampus; lovastatin down-regulated their expression (Fig. 5B). It would be interesting for future studies to determine whether the reversal effect of lovastatin on the abnormal gene expression contributes to the learning improvement of NF1$^{+/-}$ mice following lovastatin treatment. We also observed that lovastatin treatment was able to increase the magnitude of the aberrant expression of a set of NF1-disturbed genes (Fig. 5C,D).

Next, we examined whether lovastatin affected activity-regulated genes (ARGs) that are regulated by LTP induction. We reasoned that lovastatin-induced memory improvement of NF1$^{+/-}$ mice may be accompanied by the reversal of the abnormal expression of some ARGs. To this end, we compared ARGs (Park et al., 2006) with the genes that are NF1-disturbed and lovastatin-regulated and found that 210 NF1-disturbed ARGs were regulated by the lovastatin treatment ($P < 0.05$; Supp. Info. Table III). This observation indicates that some of the ARGs that are disturbed in the NF1$^{+/-}$ hippocampus are affected by lovastatin. However, lovastatin did not selectively affect NF1-disturbed ARGs ($P > 0.05$).

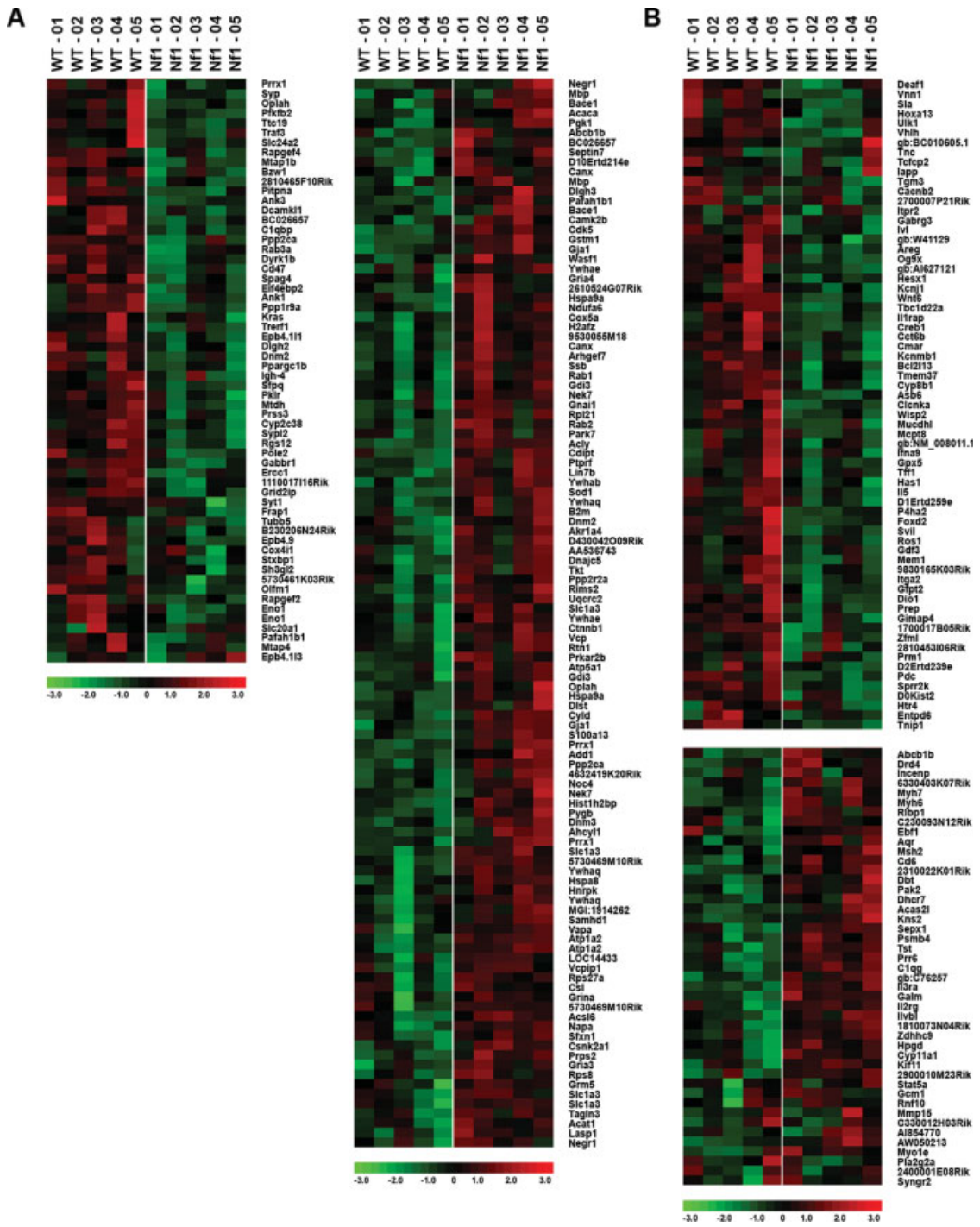

Fig. 4. Aberrant expression of synapse- and plasticity-related genes in the $NF1^{+/-}$ hippocampus. **A:** Abnormally down-regulated (left) or up-regulated (right) synapse-related genes. **B:** Abnormally down- or up-regulated ARGs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Neurofibromin negatively regulates MAPK signaling via Ras (Cichowski and Jacks, 2001). Therefore, NF1 mutations cause aberrant activation of MAPK signaling (Cichowski and Jacks, 2001). Previous studies have suggested an important role of MAPK signaling in regulating gene expression during synaptic plasticity and memory formation (English and Sweatt, 1997; Orban

TABLE II. Differentially Expressed Synapse-Related Genes in the Nf1$^{+/-}$ Hippocampus ($P < 0.05$)

	Probe set	Gene symbol	Gene description	Fold change	*P* value
Vesicle recycling	1416465_a_at	Vapa	Vesicle-associated membrane protein, associated protein A	1.111	0.005
	1418621_at	Rab2	RAB2, member RAS oncogene family	0.003	0.041
	1420506_a_at	Stxbp1	Syntaxin binding protein 1	−0.020702	0.023
	1421990_at	Syt1	Synaptotagmin I	−0.105356	0.006
	1422589_at	Rab3a	RAB3A, member RAS oncogene family	−0.045038	0.020
	1422809_at	Rims2	Regulating synaptic membrane exocytosis 2	1.273	0.009
	1443150_at	Dnm2	Dynamin 2	−1.3517645	0.039
	1446431_at	Dnm3	Dynamin 3	−1.2647915	0.010
	1446641_at	Syt7	Synaptotagmin 7	1.253	0.038
	1448210_at	Rab1	RAB1, member RAS oncogene family	1.155	0.009
	1449206_at	Sypl2	Synaptophysin-like 2	−1.520277	0.006
	1456249_x_at	Syp	Synaptophysin	−1.5221795	0.032
Synaptic receptor	1420563_at	Gria3	Glutamate receptor, ionotropic, AMPA3 (alpha 3)	0.082	0.025
	1425595_at	Gabbr1	Gamma-aminobutyric acid (GABA-B) receptor, 1	−0.047085	0.002
	1436297_a_at	Grina	Glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1	1.096	0.041
	1436772_at	Gria4	Glutamate receptor, ionotropic, AMPA4 (alpha 4)	1.276	0.027
	1450202_at	Grin1	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	0.010	0.044
	1450310_at	Grid2ip	Glutamate receptor, ionotropic, delta 2 (Grid2) interacting protein 1	−1.122938	0.010
	1455272_at	Grm5	Glutamate receptor, metabotropic 5	1.115	0.015
Synaptic signaling	1419034_at	Csnk2a1	Casein kinase II, alpha 1 polypeptide	1.190	0.018
	1420842_at	Ptprf	Protein tyrosine phosphatase, receptor type, F	1.132	0.036
	1422590_at	Cdk5	Cyclin-dependent kinase 5	1.068	0.003
	1424482_at	Arhgef7	Rho guanine nucleotide exchange factor (GEF7)	1.116	0.013
	1441734_at	Camk2a	Calcium/calmodulin-dependent protein kinase II, alpha	−1.437117	0.052
	1444875_at	Ppp2ca	Protein phosphatase 2a, catalytic subunit, alpha isoform	−1.2680235	0.026
	1446651_at	Rapgef2	Rap guanine nucleotide exchange factor (GEF) 2	−0.7789785	0.031
	1455869_at	Camk2b	Calcium/calmodulin-dependent protein kinase II, beta (Camk2b)	1.389	0.029
	1456475_s_at	Prkar2b	Protein kinase, cAMP-dependent regulatory, type II beta	1.254	0.023
Synaptic structure	1418741_at	Itgb7	Integrin beta 7	0.853	0.004
	1433428_x_at	Tgm2	Transglutaminase 2, C polypeptide	1.329	0.003
	1420811_a_at	Ctnnb1	Catenin (cadherin-associated protein), beta 1	0.053	0.010
	1421850_at	Mtap1b	Microtubule-associated protein 1 B	−1.0574625	0.016
	1423846_x_at	Tuba2	Tubulin, alpha 2	−0.00447549999999997	0.041
	1432281_a_at	Itgb6	Integrin beta 6	−1.447017	0.009
	1433413_at	Nrxn1	Neurexin I	−1.131349	0.027
	1443291_at	Ank1	Ankyrin 1, erythroid (Ank1), mRNA	0.094	0.039
	1443702_at	Mtap4	Microtubule-associated protein 4	1.211	0.021
	1448346_at	Cfl1	Cofilin 1, nonmuscle	−0.00793350000000004	0.039
	1450438_at	Ncam1	Neural cell adhesion molecule 1	0.033	0.053
	1452124_at	Ank3	Ankyrin 3, epithelial	1.083	0.020
	1454651_x_at	Mbp	Myelin basic protein	1.110	0.04472307

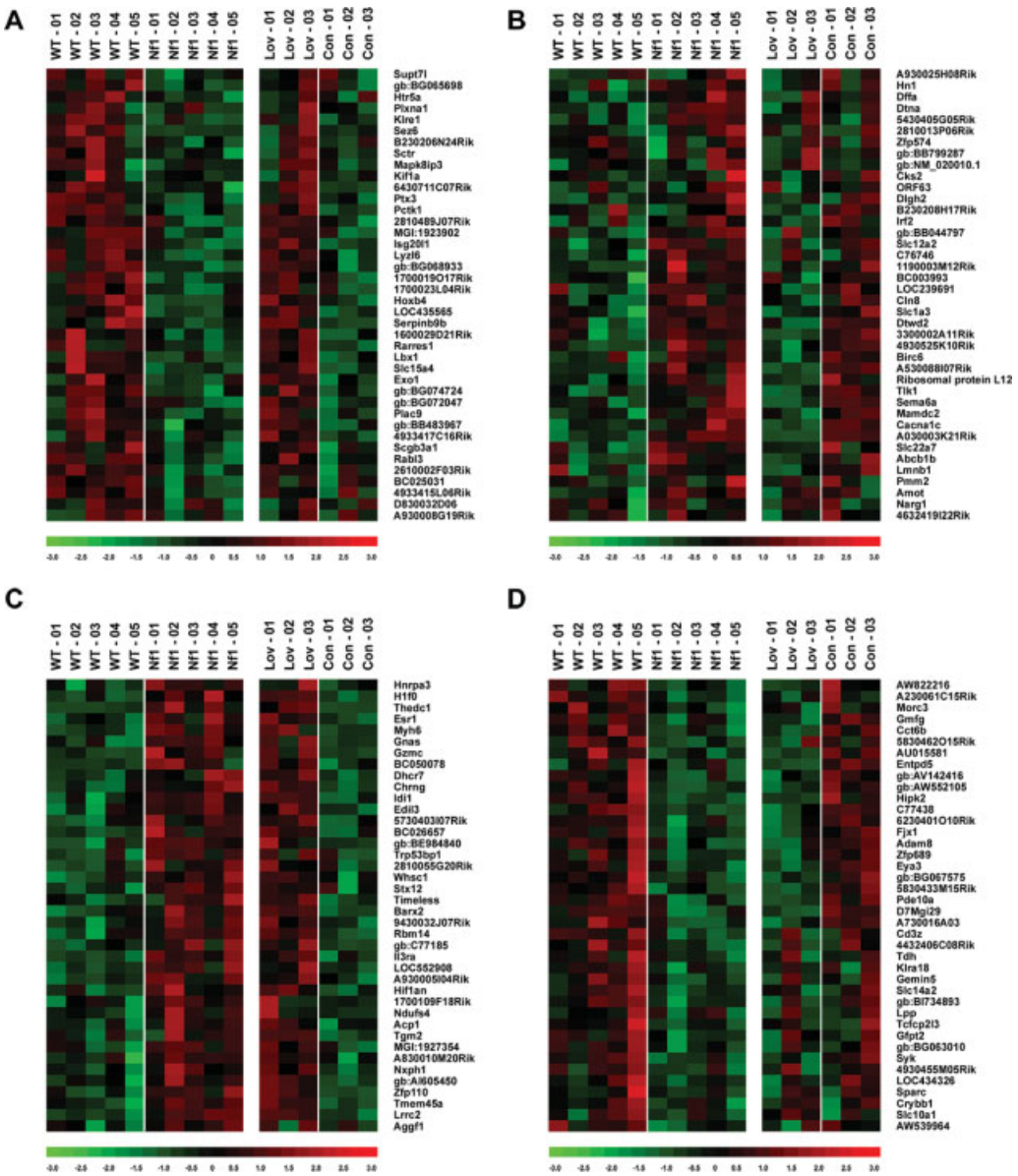

Fig. 5. Effects of lovastatin on hippocampal genomic expression of NF1$^{+/-}$ mice. **A:** Some genes that were down-regulated in the NF1$^{+/-}$ hippocampus were up-regulated by lovastatin. **B:** Some genes that were up-regulated in the NF1$^{+/-}$ hippocampus were down-regulated by lovastatin. **C:** Some genes that were up-regulated in the NF1$^{+/-}$ hippocampus were also up-regulated by lovastatin. **D:** Some genes that were down-regulated in the NF1$^{+/-}$ hippocampus were also down-regulated by lovastatin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1999; Atkins et al., 2000; Davis et al., 2000; Thomas and Huganir, 2004). Hence, it is reasonable to hypothesize that abnormally expressed genes caused by NF1-mediated MAPK signaling activation underlie NF1-associated memory impairments. Consistently with this view, our studies indicate that NF1 mice are specifically impaired in memory consolidation but not encoding (acquisition; Fig. 1). We like to point out that the current study by itself does not allow us to exclude the possibility that the aberrant MAPK signaling may cause the memory deficits secondary to subtle developmental problems, although we did not observe apparent developmental defects in NF1 mice. However, previous studies with mice (Costa et al., 2001, 2002) and *Drosophila* (Guo et al., 2000; Tong et al., 2002) clearly indicate that NF1-caused learning problem may direct result from alterations of MAPK or cAMP signaling rather than being secondary to developmental problems. Future microarray analysis may directly resolve this potential complication by compar-

TABLE III. Ontology Groups of Lovastatin-Affected Genes in the Nf1$^{+/-}$ Hippocampus ($P < 0.01$)*

	Probe set	Gene symbol	Gene description	Fold change regulated by lovastatin	*P* value
Cell–cell communicaiton (46)	1419245_at	Rab14	RAB14, member RAS oncogene family	1.285	0.003
	1425549_at	Psen1	Presenilin 1	1.173	0.010
	1431050_at	Rps6ka5	Ribosomal protein S6 kinase, polypeptide 5	1.299	0.002
	1451711_at	Wnt9b	Wingless-type MMTV integration site 9B	−1.2103695	0.004
	1459256_at	Nrp2	Neuropilin 2	2.061	0.006
Transcription (37)	1417516_at	Ddit3	DNA-damage-inducible transcript 3	1.155	0.001
	1420410_at	Nr5a2	Nuclear receptor subfamily 5, group A, member 2	2.078	0.006
	1425630_at	Sin3b	Transcriptional regulator, SIN3B (yeast)	1.237	0.002
	1449566_at	Nkx2-5	NK2 transcription factor related, locus 5 (Drosophila)	1.302	0.004
	1450482_a_at	Pitx2	Paired-like homeodomain transcription factor 2	−1.516109	0.002
Signal transduction (37)	1421465_at	Wnt2b	Wingless related MMTV integration site 2b	0.859	0.009
	1436251_at	Pde1c	Phosphodiesterase 1C	1.236	0.005
	1450176_at	Ern1	Endoplasmic reticulum (ER) to nucleus signalling 1	1.251	0.005
	1451714_a_at	Map2k3	Mitogen-activated protein kinase kinase 3	0.036	0.010
	1459912_at	Map4k4	Mitogen-activated protein kinase kinase kinase kinase 4	4.755	0.007
Cellular transport (19)	1422809_at	Rims2	Regulating synaptic membrane exocytosis 2	1.104	0.013
	1452747_at	Atp13a2	ATPase type 13A2	1.120	0.006
	1454077_at	Vti1a	Vesicle transport, interaction with t-SNAREs homolog 1A	−2.315182	0.005
	1460129_at	Slc6a2	Solute carrier family 6, member 2	1.569	0.008
	1460712_s_at	Ap1g1	Adaptor protein complex AP-1, gamma 1 subunit	1.140	0.004
Protein modification (19)	1419988_at	Map3k7	Mitogen-activated protein kinase kinase kinase 7	1.182	0.006
	1426004_a_at	Tgm2	Transglutaminase 2, C polypeptide	1.352	0.002
	1447617_at	Cdk2	Cyclin-dependent kinase 2	−4.415282	0.005
	1449013_at	Eef2k	Eukaryotic elongation factor-2 kinase	1.104	0.002
	1456992_at	Ube3c	Ubiquitin protein ligase E3C	2.296	0.009
Cell differentiation (10)	1418054_at	Neurod4	Neurogenic differentiation 4	2.447	0.001
	1418420_at	Myod1	Myogenic differentiation 1	1.679	0.004
	1425264_s_at	Mbp	Myelin basic protein	1.403	0.000
	1425703_at	Ppard	Peroxisome proliferator activator receptor delta	−1.386574	0.002
	1449773_s_at	Gadd45b	Growth arrest and DNA-damage-inducible 45 beta	1.215	0.007
Cytoskeleton (9)	1420248_at	Tubg2	Tubulin, gamma 2	1.990	0.003
	1420358_at	Krtap13	Keratin-associated protein 13	1.239	0.007
	1421053_at	Kif1a	Kinesin family member 1A	1.123	0.012
	1435046_at	Myo1d	Myosin ID (Myo1d), mRNA	1.372	0.009
	1453997_a_at	Nes	Nestin	2.883	0.0005430795

*Representative examples given for each group. Total number of genes is given in parentheses.

ing the NF1 genomic patterns before and after a learning paradigm.

Functional assignment of NF1-affected hippocampal genes indicates that they are involved in a broad spectrum of molecular and cellular processes, including cell–cell communication, signal transduction, transcription, and cytoskeleton dynamics (Table I). Many genes in these biological pathways have been shown to be regulated during synaptic plasticity and memory formation (Cavallaro et al., 2002; Levenson et al., 2004; Park et al., 2006). It is not clear at this stage whether the NF1 memory deficits are caused by the disturbance of one or more of these pathways. Nonetheless, the identified NF1-affected hippocampal genes provide a basis for further characterization of the specific processes that are causally relevant to the development of NF1 memory impairments.

We found that the expression of many genes encoding proteins involved in synaptic transmission is altered in the NF1 hippocampus (Table II). These include proteins that are involved in regulation of synaptic vesicle recycling, such as Rabs, synaptotagmins, and dynamins. An interesting feature is the bidirectional regulation of different members of the same protein family. For example, Rab1 and Rab2 are down-regulated in the NF1 hippocampus, whereas Rab3A is up-regulated; synaptotagmin 1 is down-regulated, whereas synaptotagmin 7 is up-regulated; and dynamin 2 is up-regulated, whereas dynamin 3 is down-regulated. It is intriguing to think that the bidirectional regulation of different members in the same protein family may provide a compensatory mechanism for the primary defects caused by NF1 mutation. In addition, the expression of glutamate receptors is also disturbed. For instance, NMDADR1 is down-regulated, whereas AMPAR4 and mGluR5 are up-regulated. Previous studies revealed a deficit of synaptic transmission in NF1 mice, which was considered to be caused by enhanced inhibition (Costa et al., 2002). Our findings reveal a disturbance of molecular processes underlying synaptic vesicle recycling; it would be interesting to determine whether these molecular disturbances contribute to the observed impairments of synaptic transmission.

Synaptic plasticity is thought to be accompanied by synapse remodeling (Engert and Bonhoeffer, 1999; Toni et al., 1999; Yuste and Bonhoeffer, 2001; Matsuzaki et al., 2004). We observed that some cytoskeleton regulatory genes are disturbed in the $NF1^{+/-}$ hippocampus (Table I). In addition, we found that, in the $NF1^{+/-}$ hippocampus, the expression of many genes encoding synaptic structural protein genes is altered. For example, integrin β7, NCAM1, and transglutaminase2 are up-regulated, whereas integrin β6 and neurexin 1 are down-regulated (Table II). The involvement of these classes of proteins in LTP has been demonstrated (Sanes and Lichtman, 1999; Park et al., 2006). Our findings suggest that synapse remodeling underlying synaptic plasticity may be dysregulated in the $NF1^{+/-}$ hippocampus.

The expression of long-term synaptic plasticity is controlled by synaptic signaling. αCaMKII protein is one of the key signaling proteins that are enriched at synapses and critical for synaptic plasticity and memory formation (Lisman et al., 2002). Interestingly, in the $NF1^{+/-}$ hippocampus, αCaMKII gene is down-regulated (Table II). On the other hand, CaMKIIβ, which interacts with αCaMKII to form a holoenzyme, is up-regulated (Table II). Other signaling protein genes that are important for synaptic plasticity and disturbed in the $NF1^{+/-}$ hippocampus include CDK5 (Table II; Angelo et al., 2006). A major mechanism by which synaptic signaling regulates synaptic plasticity is to control gene transcription. CREB is a transcription factor that plays an important role in long-lasting synaptic plasticity and memory consolidation (Silva et al., 1998). Interestingly, CREB1 expression is decreased in the $NF1^{+/-}$ hippocampus (Table I). The down-regulation of CREB1 expression may contribute to the deficiency of memory consolidation in $NF1^{+/-}$ mice (Fig. 1).

Recent studies have demonstrated that lovastatin suppresses MAPK activity and rescues memory deficits of NF1 mice (Li et al., 2005). We found that lovastatin treatment of NF1 mice for 1 week, which was sufficient to rescue memory deficits (Li et al., 2005), changed the expression of many genes in different functional groups (Table III); some of them are synaptic plasticity-related genes. However, lovastatin-regulated genes are not significantly enriched with NF1-affected genes or ARGs. It is not clear at this stage whether lovastatin rescues NF1 memory deficits via reversing aberrant genomic expression. We cannot exclude the possibility that lovastatin may rescue the memory defects by a mechanism that does not require changes in gene expression.

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