

# The Neurofibromatosis 1 Gene Product Neurofibromin Regulates Pituitary Adenylate Cyclase-Activating Polypeptide-Mediated Signaling in Astrocytes

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Individuals with the neurofibromatosis 1 (NF1)-inherited tumor predisposition syndrome develop low-grade astrocytomas. The *NF1* tumor suppressor gene product neurofibromin exhibits GTPase-activating activity (GAP) toward RAS, such that loss of neurofibromin expression leads to high levels of activated RAS and increased cell proliferation. Previous work has demonstrated that *Nf1* inactivation in astrocytes leads to increased cell proliferation *in vitro* and *in vivo*, accompanied by increased RAS pathway activation. Studies on *Nf1* mutant *Drosophila* have suggested that neurofibromin might also regulate cAMP signaling. Because intracellular cAMP levels have profound effects on astrocyte growth control, we sought to determine the contribution of neurofibromin to astrocyte cAMP regulation. In this report, we demonstrate that *Nf1* inactivation in astrocytes results in reduced cAMP generation in response to PACAP and attenuated calcium influx and Rap1 activation. Based on the differential effects of forskolin and dibutyryl-cAMP on *Nf1*<sup>-/-</sup> astrocytes, neurofibromin likely functions at the level of adenylyl cyclase activation. Last, the reintroduction of a fragment of neurofibromin containing residues sufficient for restoring RAS-GAP function in *Nf1*<sup>-/-</sup> cells resulted in only partial restoration of neurofibromin-mediated cAMP regulation. These results demonstrate that neurofibromin positively influences cAMP generation and activation of cAMP growth regulatory targets in astrocytes and expands the role of the *NF1* gene in astrocyte growth regulation.

**Key words:** cAMP; glia; RAS; GAP; Rap1; astrocyte

## Introduction

Neurofibromatosis 1 (NF1) is an inherited tumor predisposition syndrome in which affected individuals are prone to the development of optic nerve glioma, composed of neoplastic astrocytes (Listernick et al., 1999). NF1-associated optic nerve gliomas (astrocytomas) exhibit biallelic inactivation of the *NF1* tumor suppressor gene and loss of neurofibromin expression (Gutmann et al., 2000; Kluwe et al., 2001). Based on the presence of a 300 amino acid residue RAS GTPase-activating protein (GAP)-related domain (NF1GRD), neurofibromin growth regulation has been hypothesized to reflect its ability to function as a RAS-GAP (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). In this regard, loss of neurofibromin in NF1-associated tumors is associated with increased RAS activity, which can be ameliorated by re-expression of wild-type NF1GRD (Basu et al., 1992; DeClue et al., 1992; Bollag et al., 1996; Hiatt et al., 2001).

Although it is tempting to attribute all of neurofibromin growth regulatory function to RAS regulation, the NF1GRD represents only 10% of the entire molecule. Studies in *Drosophila* have suggested that neurofibromin also has non-RAS-GAP func-

tions that involve regulation of intracellular cAMP (Guo et al., 1997). In these studies, *Drosophila Nf1* was required for activation of adenylyl cyclase by the neuropeptide, PACAP (pituitary adenylate cyclase activating peptide), which potently increases cAMP levels in pituitary cells (for review, see Waschek, 2002). Moreover, the small size defect of *Nf1*-deficient *Drosophila* was rescued by overexpression of activated protein kinase A (PKA), but not by manipulating RAS signaling (The et al., 1997). The rescue by PKA and not RAS supports the notion that *Drosophila* neurofibromin regulates cAMP signaling and that some of the phenotypes in *Drosophila* that result from *Nf1* inactivation are RAS-independent. Similarly, neurofibromin also regulates G-protein-stimulated adenylyl cyclase activity in mammalian neurons (Tong et al., 2002).

In astrocytes, we have previously shown that increased intracellular cAMP provides a strong growth inhibitory signal, and increased intracellular cAMP leads to activation of Rap1, which in turn, inhibits Raf-1-mediated RAS signaling (Dugan et al., 1999; Apicelli et al., 2003). If neurofibromin is required for propagation of astrocyte PACAP–cAMP signaling, we predict that *Nf1*-deficient astrocytes would exhibit impaired cAMP signaling in response to PACAP, leading to increased cell proliferation. In this report, we demonstrate that *Nf1*-deficient astrocytes have impaired cAMP responses to PACAP, associated with decreased calcium influx and Rap1 activation, and that neurofibromin functions at the level of adenylyl cyclase activation. Last, using a neurofibromin fragment containing residues sufficient to restore

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RAS-GAP activity in *Nf1*<sup>-/-</sup> cells, we observed only a partial restoration of *Nf1*-mediated cAMP regulation. These results suggest that one component of neurofibromin growth regulation involves RAS-independent, cAMP-mediated, signaling, and that residues outside of the RAS-GAP domain might be responsible for this effect.

## Materials and Methods

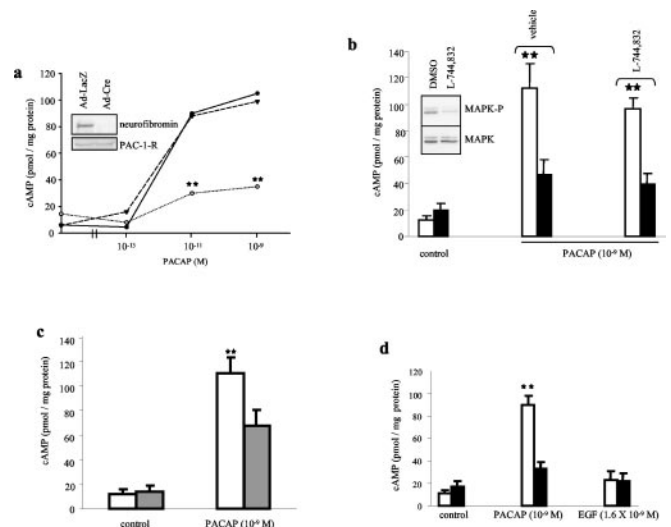
**Primary astrocyte culture and establishment of *Nf1*<sup>-/-</sup> astroglial cultures.** Murine neocortical astroglial cultures, containing >95% of GFAP-immunoreactive cells (astrocytes), were generated from postnatal day 2 *Nf1* flox/flox, RAS B8 transgenic, or *Nf1*<sup>+/-</sup> pups as previously described (Gutmann et al., 2001). To eliminate neurofibromin expression, *Nf1* flox/flox astrocytes were treated with Ad5-Cre (University of Iowa Gene Transfer Vector core, Iowa City, IA) or Ad5-lacZ (control) for 2 hr at a multiplicity of infection of 1:50, washed twice, and maintained for at least 4 d. Loss of neurofibromin was confirmed by Western blot. All experiments were performed on passage 1 (P1) or P2 cultures. Mice were used in accordance with established Animal Studies Protocols at the Washington University School of Medicine. Genotypes were determined by PCR from tail DNA.

**Measurement of intracellular cAMP.** Intracellular cAMP was measured using the cAMP Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. Briefly, equal numbers of *Nf1*<sup>+/+</sup>, *Nf1*<sup>-/-</sup> or *Nf1*<sup>+/-</sup> astrocytes were serum-starved for 48 hr and stimulated with either medium only (control), PACAP ( $10^{-13}$ ,  $10^{-11}$ , or  $10^{-9}$  M), EGF ( $1.6 \times 10^{-9}$  M), or forskolin (10  $\mu$ M) for 10 min at 37°C. For the RAS inhibition experiments, astrocytes were serum-starved for 48 hr in presence of either L-744,832 (20  $\mu$ M) or vehicle (DMSO). The concentrations of PACAP, forskolin, and L-744,832 were chosen based on previous studies in astrocytes (Tatsuno et al., 1996; Dugan et al., 1999; Feldkamp et al., 1999). Astrocytes were washed three times with ice-cold PBS, lysed in chilled ethanol, centrifuged at 3.5 K for 15 min at 4°C, and resuspended in 200  $\mu$ l of assay buffer after SpeedVac evaporation of the supernatant. All experiments were performed in triplicate or quadruplicate, and each experiment was repeated three times with identical results. Protein concentration was determined from the ethanol-insoluble pellet using the BCA reagent (Pierce, Rockford, IL).

**Rap1 activity assay.** Rap1 activity was assayed as previously described (Apicelli et al., 2003). Briefly, equal numbers of *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes were serum-starved for 48 hr and incubated with either medium alone (control), PACAP ( $10^{-11}$  and  $10^{-9}$  M), or dibutyl-cAMP (db-cAMP) (300  $\mu$ M) for 10 min at 37°C. Cells were washed with cold PBS, lysed in cold MAPK (mitogen-activated protein kinase) buffer, cleared by centrifugation, and incubated with glutathione beads containing 30  $\mu$ g of glutathione S-transferase (GST)-tagged RalGDS (a gift from J. L. Bos, University Medical Center, Utrecht, The Netherlands) at 4°C for 1 hr. The beads were washed four times in cold MAPK buffer, and bound proteins were eluted by boiling in 2 $\times$  Laemmli buffer, followed by SDS-PAGE separation and Western blotting. Each experiment was performed at least three times with identical results.

**Western blot analysis.** Western blots were performed as previously described (Apicelli et al., 2003) after transfer to polyvinylidene difluoride membranes (Immobilon; Fisher Scientific, Dallas, TX). Neurofibromin and the NF1GRD were detected using anti-NF1GRP-D (sc67, 1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-KT3 antibodies (MMS-125P, 1:1000 dilution; Babco, Richmond, CA), respectively. Other antibodies used include Rap1 (sc65, 1:500 dilution; Santa Cruz Biotechnology), MAPK and phospho-MAPK (9102 and 9106, 1:1000 dilution; Cell Signaling Technology, Beverly, MA), and PAC-1-R (1:1000 dilution; provided by Dr. Akira Arimura, Tulane University, New Orleans, LA). Appropriate HRP-conjugated secondary rabbit or mouse antibodies (Sigma, St. Louis, MO) were used for detection by enhanced chemiluminescence (Amersham, Piscataway, NJ).

**Calcium influx assay.** Calcium uptake assay was performed as previously described (Dugan et al., 1996) with minor modifications. Equal numbers of astrocytes were grown in 35 mm dishes, serum-starved for 48 hr, and incubated with medium only (control), PACAP ( $10^{-11}$  M), or



**Figure 1.** PACAP-induced cAMP generation is impaired in both *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/-</sup> astrocytes and is independent of RAS activation. *a*, *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/-</sup> astrocytes exhibit a marked decrease in cAMP generation in response to various concentrations of PACAP. cAMP produced by *Nf1*<sup>+/+</sup> (solid line), *Nf1*<sup>-/-</sup> (dotted), and RAS transgenic B8 astrocytes (dashed) are shown. The inset illustrates neurofibromin and PAC-1-R expression in *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes after adenoviral transduction. *b*, Pretreatment of either *Nf1*<sup>+/+</sup> (white bar) or *Nf1*<sup>-/-</sup> astrocytes (black bar) with the RAS farnesyltransferase inhibitor (L-744,832) did not significantly alter PACAP-mediated cAMP generation in *Nf1*<sup>-/-</sup> or wild-type astrocytes. The inset demonstrates that L-744,832 treatment resulted in inhibition of MAPK activity (phospho-MAPK) without altering total MAPK (MAPK) expression. *c*, cAMP generation in response to PACAP stimulation in also impaired in *Nf1*<sup>+/-</sup> astrocytes (gray bar) compared with *Nf1*<sup>+/+</sup> (white bar) astrocytes. *d*, Whereas differences in cAMP levels were observed in *Nf1*<sup>+/+</sup> (white bar) versus *Nf1*<sup>-/-</sup> (black bar) astrocytes in response to PACAP treatment, no differences were observed in response to EGF treatment. Each error bar represents mean  $\pm$  SD. Asterisks denote statistically significant differences ( $p < 0.004$ ).

with db-cAMP (300  $\mu$ M; Dugan et al., 1999) in the presence of 45 Ca<sup>2+</sup> (4  $\mu$ Ci/culture dish; Perkin-Elmer, Boston, MA) at 37°C for 30 min. Cells were washed three times with cold PBS, lysed in 500  $\mu$ l of 0.2% SDS for 10 min, and 45 Ca<sup>2+</sup> incorporation was determined by scintillation counting. Parallel plates of *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes were used for cell counting and protein determination to demonstrate equal number of astrocytes per condition. Each experiment was performed at least three times with identical results.

**Retroviral transduction of astrocytes.** Retroviral transduction was performed as previously reported (Hiatt et al., 2001) with minor modifications. *Nf1* flox/flox astrocyte cultures containing equal numbers of astrocytes were infected twice, first for 4 hr and then overnight with either murine stem cell virus (MSCV)-Pac or MSCV-NF1GRD-Pac in the presence of 10  $\mu$ g/ml polybrene (Specialty Media, Phillipsburg, NJ). Forty-eight hours after infection, transduced astrocytes were selected in medium containing 2  $\mu$ g/ml puromycin for an additional 48 hr. After expression of NF1GRD was confirmed by Western blot, the same plates were used for adenoviral infection with either Ad5-Cre to inactivate *Nf1* or Ad5-LacZ (control).

## Results

### Neurofibromin is required for PACAP-induced cAMP generation

To determine whether neurofibromin is required for PACAP-mediated astrocyte cAMP production, we disrupted neurofibromin expression in *Nf1* flox/flox neonatal cortical astrocytes using Cre recombinase (Fig. 1*a*, inset). No changes in PACAP receptor (PAC-1-R) expression were observed at the RNA (data not shown) or protein level (Fig. 1*a*, inset) after *Nf1* gene inactivation. Serum-starved *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes were stimulated with PACAP at several concentrations, and intracellular

cAMP levels were measured. Intracellular cAMP levels were significantly decreased in *Nf1*<sup>-/-</sup> compared with *Nf1*<sup>+/+</sup> astrocytes in response to both 10<sup>-9</sup> and 10<sup>-11</sup> M PACAP (66% reduction; *p* = 0.001) (Fig. 1*a*). Because neurofibromin functions as a GAP for RAS, we next determined whether activated Ha-RAS expression resulted in decreased cAMP levels in response to PACAP. In contrast to *Nf1*<sup>-/-</sup> cells, we observed no effect of activated RAS expression on baseline or PACAP-induced cAMP generation (B8 RAS transgenic astrocytes) (Fig. 1*a*). In addition, RAS inhibition using the farnesyltransferase inhibitor L-744,832 did not significantly alter cAMP generation in either wild-type or *Nf1*<sup>-/-</sup> astrocytes (14.1 vs 15.6% reduction, respectively) (Fig. 1*b*). At this concentration, RAS pathway activation is significantly inhibited, as reflected in reduced activated phospho-MAPK (Fig. 1*b*, inset). Collectively, these results suggest that neurofibromin-regulated cAMP activity is independent of RAS activation.

To demonstrate a direct relationship between neurofibromin expression and cAMP regulation, we examined cAMP generation in *Nf1*<sup>+/-</sup> astrocytes, with 50% wild-type neurofibromin expression (Bajenaru et al., 2001). We found reduced PACAP-induced cAMP production in *Nf1*<sup>+/-</sup> astrocytes compared with *Nf1*<sup>+/+</sup> astrocytes (38.4% reduction; *p* = 0.004), supporting a dose-dependent effect of neurofibromin expression on cAMP generation (Fig. 1*c*).

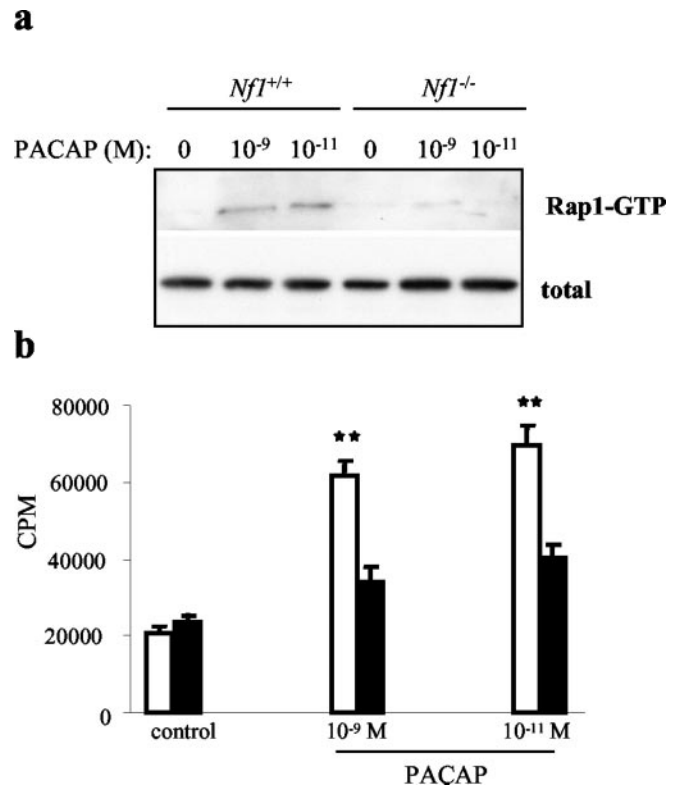
Because PACAP functions through PAC-1 and PAC-2 G-protein-coupled receptors, we examined neurofibromin-mediated cAMP regulation in response to epidermal growth factor (EGF). In contrast to PACAP stimulation, at doses sufficient to activate RAS (1.6 × 10<sup>-9</sup> M EGF; data not shown), we observed no effect of EGF on cAMP production in either *Nf1*<sup>+/+</sup> or *Nf1*<sup>-/-</sup> astrocytes (Fig. 1*d*).

### Neurofibromin loss leads to decreased calcium influx and impaired Rap1 activation in response to PACAP

cAMP generation in many cell types results in enhanced calcium influx (Kamp and Hell, 2000) and activation of the small GTPase molecule Rap1 (Schmitt and Stork, 2002; Rangarajan et al., 2003). To determine whether neurofibromin loss impaired downstream effects of PACAP-stimulated cAMP production, we measured calcium influx and Rap1 activation in *Nf1*<sup>-/-</sup> astrocytes in response to PACAP stimulation (10<sup>-9</sup> and 10<sup>-11</sup> M). We observed increased Rap1 activation at both PACAP concentrations in *Nf1*<sup>+/+</sup> astrocytes, using the GST-RalGDS pull-down assay. In contrast, PACAP-stimulated Rap1 activation was significantly reduced in *Nf1*<sup>-/-</sup> astrocytes (*p* = 0.001) (Fig. 2*a*). Scanning densitometric values for Rap1-GTP in the *Nf1*<sup>+/+</sup> astrocytes were 17.66 and 15.5 after normalization to total Rap1, respectively, in response to 10<sup>-9</sup> and 10<sup>-11</sup> M PACAP. The corresponding values for the *Nf1*<sup>-/-</sup> astrocytes were 2.8 and 1.98 after normalization. Similarly, we observed a significant reduction in calcium influx in *Nf1*<sup>-/-</sup> astrocytes compared with *Nf1*<sup>+/+</sup> astrocytes in response to PACAP (10<sup>-9</sup> and 10<sup>-11</sup> M) stimulation (*p* = 0.001) (Fig. 2*b*). Collectively, these results demonstrate that neurofibromin loss not only affects cAMP generation, but also impairs downstream signaling mediated by cAMP in astrocytes.

### Neurofibromin mediates PACAP-induced cAMP generation at the level of adenylyl cyclase

To determine the level at which neurofibromin regulates PACAP-induced cAMP generation, we treated cells with forskolin (10 μM) to activate adenylyl cyclase or the cAMP analog db-

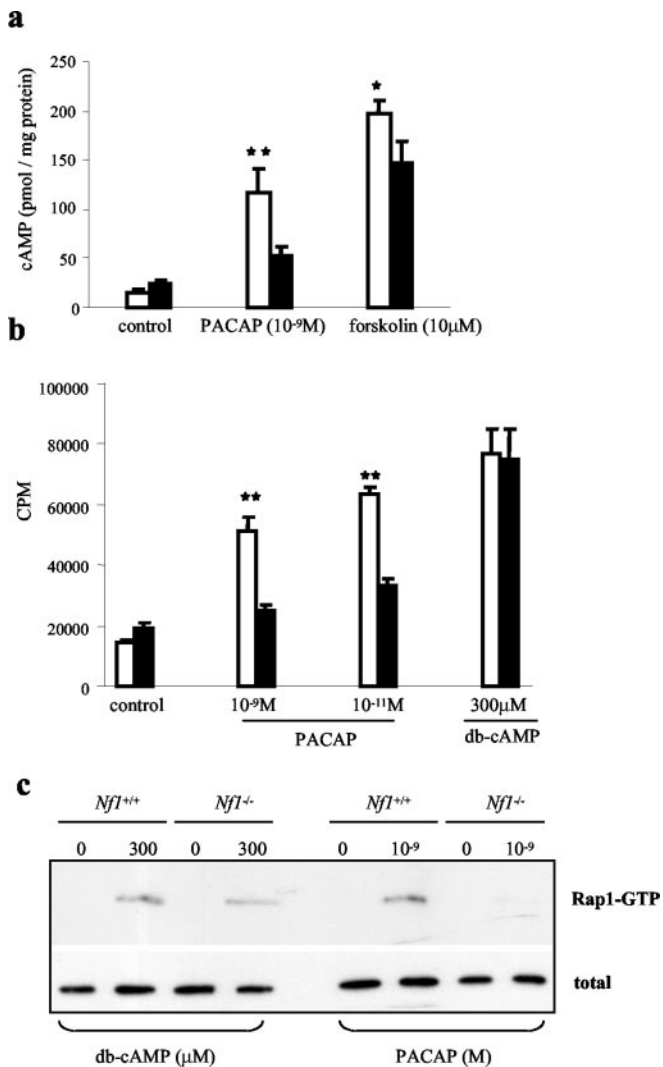


**Figure 2.** PACAP activation of Rap1 and induction of calcium influx is impaired in *Nf1*<sup>-/-</sup> astrocytes. *a*, Serum-starved *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes stimulated with PACAP were analyzed for Rap1 activation. The top panel denotes active Rap1 (Rap1-GTP bound to GST-RalGDS), whereas bottom panel demonstrates the total Rap1 in each sample. Significantly reduced Rap1 activation is observed in the *Nf1*<sup>-/-</sup> compared with *Nf1*<sup>+/+</sup> astrocytes. *b*, Serum-starved *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes stimulated with PACAP in presence of 45 Ca<sup>2+</sup> demonstrate decreased calcium influx in *Nf1*<sup>-/-</sup> (black bar) compared with *Nf1*<sup>+/+</sup> (white bar) astrocytes. Each error bar represents mean ± SD. Asterisks denote statistically significant differences (*p* = 0.001).

cAMP. We observed a reduction in forskolin-stimulated cAMP generation in *Nf1*<sup>-/-</sup> compared with *Nf1*<sup>+/+</sup> astrocytes (*p* = 0.007) (Fig. 3*a*). In contrast, *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes treated with 300 μM db-cAMP exhibited similar elevations in intracellular calcium (*p* = 0.001) (Fig. 3*b*). Similarly, we observed nearly identical levels of Rap1 activation in *Nf1*<sup>-/-</sup> compared with *Nf1*<sup>+/+</sup> astrocytes in response to db-cAMP treatment (Fig. 3*c*). By scanning densitometry, the levels of active Rap1, after normalization to total Rap1, were identical in *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/+</sup> astrocytes (19.66 and 20.02 for *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes, respectively). These results demonstrate that neurofibromin functions at the level of adenylyl cyclase activation.

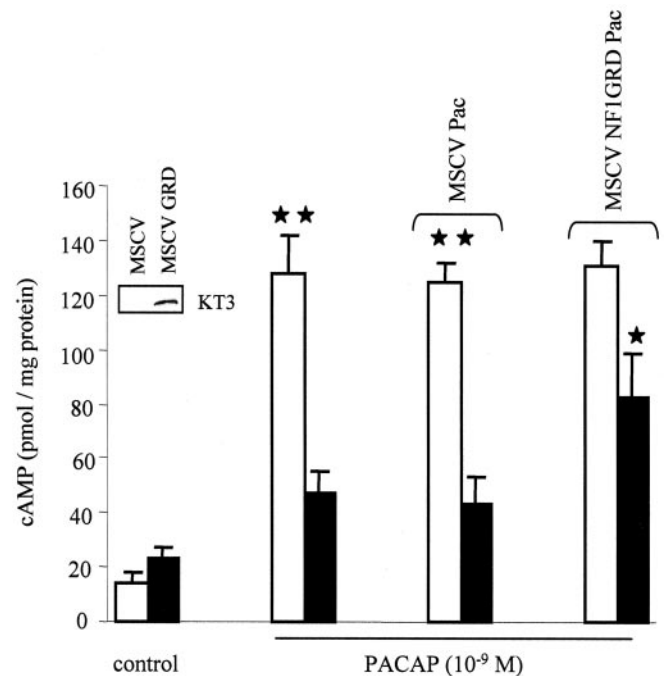
### Ectopic expression of the NF1-GAP-related domain partially rescues the cAMP response to PACAP in *Nf1*<sup>-/-</sup> astrocytes

To determine whether a neurofibromin fragment sufficient to restore neurofibromin RAS-GAP function (Hiatt et al., 2001) could also restore neurofibromin-mediated cAMP responses to PACAP, we transduced *Nf1* flox/flox astrocytes with MSCV containing either empty vector (MSCV-Pac) or the human NF1GRD fragment (MSCV-NF1GRD-Pac) before *Nf1* gene inactivation by Ad5-Cre. The NF1GRD was tagged with the KT3 epitope to facilitate detection (Fig. 4, inset), but was also detected using neurofibromin-specific antibodies (data not shown). After PACAP (10<sup>-9</sup> M) stimulation, cAMP levels increased signifi-



**Figure 3.** Forskolin-stimulated cAMP generation is reduced in *Nf1*<sup>-/-</sup> astrocytes, whereas db-cAMP-induced calcium influx and Rap1 activation is similar in both *Nf1*<sup>-/-</sup> and *Nf1*<sup>+/+</sup> astrocytes. *a*, cAMP generation in response to PACAP and forskolin is reduced in *Nf1*<sup>-/-</sup> astrocytes (black bars) compared with *Nf1*<sup>+/+</sup> astrocytes (white bar). *b*, Calcium influx in response to PACAP is reduced in *Nf1*<sup>-/-</sup> astrocytes (black bars) compared with *Nf1*<sup>+/+</sup> astrocytes (white bar), but equivalent in response to db-cAMP stimulation. *c*, Whereas Rap1 activation (Rap1-GTP) in response to PACAP is reduced in *Nf1*<sup>-/-</sup> compared with *Nf1*<sup>+/+</sup> astrocytes, equivalent levels of Rap1 activation were observed in response to db-cAMP. Each error bar represents mean  $\pm$  SD. Asterisks denote statistically significant differences (\**p* = 0.001; \*\**p* = 0.007).

cantly in *Nf1*<sup>-/-</sup> astrocytes transduced with MSCV-NF1GRD-Pac (*p* = 0.005), compared with *Nf1*<sup>-/-</sup> astrocytes transduced with MSCV-Pac vector control (Fig. 4). The cAMP increase in *Nf1*<sup>-/-</sup> astrocytes expressing NF1GRD was intermediate between the increase observed in *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes in response to PACAP. The differential cAMP response to PACAP between *Nf1*<sup>+/+</sup> and *Nf1*<sup>-/-</sup> astrocytes was found to be unaffected by retroviral infection, because there were no differences in cAMP production between uninfected *Nf1*<sup>-/-</sup> astrocytes and *Nf1*<sup>-/-</sup> astrocytes infected with MSCV-Pac or uninfected *Nf1*<sup>+/+</sup> astrocytes and those infected with either MSCV-Pac or MSCV-NF1GRD-Pac. Although we observed a partial restoration of cAMP generation in *Nf1*<sup>-/-</sup> astrocytes expressing NF1GRD, these results suggest that NF1GRD expression at levels sufficient to completely restore RAS-GAP function are not sufficient to restore neurofibromin cAMP regulation.



**Figure 4.** Ectopic expression of NF1GRD partially rescues the impaired cAMP response to PACAP in *Nf1*<sup>-/-</sup> astrocytes. Whereas reduced cAMP levels were observed in untransduced and vector transduced *Nf1*<sup>-/-</sup> (black bars) compared with *Nf1*<sup>+/+</sup> (white bars) astrocytes, a partial restoration of this response was observed in astrocytes expressing the NF1GRD fragment. No effect on cAMP generation was observed in *Nf1*<sup>+/+</sup> astrocytes expressing the NF1GRD fragment. Expression of KT3-tagged NF1GRD in *Nf1*<sup>-/-</sup> MSCV-NF1GRD-Pac treated astrocytes is shown in the inset. Each error bar represents mean  $\pm$  SD. Asterisks denote statistically significant differences (\**p* = 0.001; \*\**p* = 0.005).

## Discussion

Previous studies on astrocyte mitogenic signaling in NF1 have focused on the role of neurofibromin in regulating RAS activity. In both human astrocytoma and *Nf1*-deficient mouse astrocytes, elevated levels of RAS and RAS effector (Akt and MAPK) activity are associated with neurofibromin loss (Lau et al., 2000; Bajenaru et al., 2002), suggesting that neurofibromin RAS regulation is a critical component of its anti-mitogenic and tumor suppressor function. However, recent studies in *Drosophila* have shown that neurofibromin loss results in attenuation of cAMP generation in response to the PACAP neuropeptide, which can be rescued using an activated PKA allele (Guo et al., 1997; The et al., 1997). Genetic complementation studies in flies have confirmed this defect by positioning *Nf1* in the amnesic (PACAP receptor) pathway (Yager et al., 2001).

Several findings suggest that non-RAS functions of neurofibromin might have important implications for *Nf1*-mediated astrocyte growth regulation. First, PACAP and its receptor, PAC-1, are expressed in differentiating cells of the mouse neural tube (Waschek et al., 1998), where they have multiple actions during nervous system development and differentiation (Waschek, 2002). In E10.5 hindbrain neuroepithelial cells, PACAP potently increased cAMP levels and downregulated expression of the PKA-target gene, *gli-1*, concomitant with a decrease in DNA synthesis. Similarly, in neural stem cells, PACAP induces astrocyte differentiation from precursor cells (Vallejo and Vallejo, 2002). Second, increased intracellular cAMP results in activation of Rap1, an anti-mitogenic RAS pathway antagonist. In the absence of B-raf, which we have previously shown is not expressed at significant levels in neocortical astrocytes, Rap1 activation leads

to inhibition of Raf-1 activation (Dugan et al., 1999). In this regard, the expression of an activated Rap1 allele in transgenic mouse astrocytes results in decreased astrocyte numbers *in vivo* and decreased astrocyte proliferation *in vitro*, associated with reduced MAPK activation (Apicelli et al., 2003). Moreover, the expression of an activated Rap1 allele in astrocyte-restricted RAS transgenic mice results in a marked attenuation of astrocyte proliferation and tumor formation (Apicelli et al., 2003) (E. J. Uhlmann and D. H. Gutmann, unpublished observations).

In this report, we demonstrate a dose-dependent impairment in cAMP generation in *Nf1* mutant astrocytes, with *Nf1* +/– and *Nf1* –/– exhibiting graded reductions in intracellular cAMP levels in response to PACAP. Our results are in agreement with similar trends observed by others in mouse brain (Tong et al., 2002). We show that downstream pathways activated by cAMP in astrocytes are likewise impaired by neurofibromin loss, including intracellular calcium influx and Rap1 activation. This impairment in cAMP regulation is specific to PACAP and is not observed in response to EGF treatment or RAS activation, suggesting that specific signals are required to activate this pathway and unmask the neurofibromin-deficient phenotype in astrocytes. Interestingly, the baseline cAMP levels in *Nf1* –/– astrocytes were found to be 1.5- to 1.8-fold higher than that observed in *Nf1* +/+ astrocytes, as reported for *Nf1* –/– Schwann cells (Kim et al., 2001). These baseline differences may result from additional effects of neurofibromin on adenylyl cyclase regulation in quiescent cells that reflect the differential regulation of inhibitory and stimulatory small G-proteins on adenylyl cyclase activation. However, unlike astrocytes, in which cAMP acts as an anti-mitogenic signal, cAMP is a mitogen for Schwann cells, and neurofibromin functions to antagonize, rather than stimulate, cAMP production (Kim et al., 2001).

We further mapped the level of neurofibromin regulation to the adenylyl cyclase, based on the differential effects of forskolin and db-cAMP stimulation. db-cAMP treatment was equally as effective at inducing intracellular calcium influx and Rap1 activation in *Nf1* +/+ and *Nf1* –/– astrocytes, suggesting that neurofibromin acts upstream of cAMP in the PACAP pathway. In contrast, forskolin activation of adenylyl cyclase resulted in reduced cAMP generation in *Nf1* –/– astrocytes. Although we observed a reduction in forskolin-mediated cAMP generation, it is not reduced as dramatically as seen after PACAP treatment. Similar trends were observed in *Nf1* –/– brains (Tong et al., 2002). Collectively, these observations suggest that neurofibromin might function to facilitate adenylyl cyclase activation by PAC-1.

Last, we demonstrate that the re-expression of the *NF1*-GAP-related domain in *Nf1* –/– astrocytes can partially restore normal cAMP generation in response to PACAP. In contrast, complete restoration in *Drosophila* is seen with the full-length neurofibromin (Guo et al., 1997). These results suggest that a portion of neurofibromin-cAMP activity maps to the RAS-GAP domain, but that additional residues might also be important. Because the GAP domain used in these studies (residues 1095–1569) does not contain the IRA homology domains, it is conceivable that more complete functional restoration will be observed with *NF1*GRD constructs containing these additional sequences. Experiments are in progress to determine what residues are important for neurofibromin cAMP regulation and to define the mechanism by which neurofibromin modulates PAC-1 activation of adenylyl cyclase. A more complete understanding of this unique function of neurofibromin will provide critical insights into the mechanisms underlying the control of astroglial cell proliferation

and differentiation and offer additional targets for therapeutic drug design in patients with *NF1*-associated astrocytomas.

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