Requirement of *Drosophila* NF1 for Activation of Adenylyl Cyclase by PACAP38-Like Neuropeptides

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The human neurofibromatosis type 1 (NF1) tumor suppressor protein functions as a Ras-specific guanosine triphosphatase–activating protein, but the identity of Ras-mediated pathways modulated by NF1 remains unknown. A study of *Drosophila NF1* mutants revealed that NF1 is essential for the cellular response to the neuropeptide PACAP38 (pituitary adenylyl cyclase–activating polypeptide) at the neuromuscular junction. The peptide induced a 100-fold enhancement of potassium currents by activating the Ras-Raf and adenylyl cyclase–adenosine 3',5'-monophosphate (cAMP) pathways. This response was eliminated in *NF1* mutants. NF1 appears to regulate the *rutabaga*-encoded adenylyl cyclase rather than the Ras-Raf pathway. Moreover, the *NF1* defect was rescued by the exposure of cells to pharmacological treatment that increased concentrations of cAMP.

Mutations in the human *NF1* gene lead to a common genetic disorder that is identified by benign tumors of the peripheral nerves, hyperpigmentation, white matter lesions in the brain, learning disabilities, and many other manifestations (*1*, *2*). The NF1 protein, which contains a fragment similar to the guanosine triphosphatase (GTPase)–activating protein for Ras (Ras-GAP), stimulates the intrinsic activity of Ras-GTPase and therefore inhibits biological activation of Ras (*3*). However, NF1 may not act solely to regulate Ras but may also function as an effector that mediates signaling important for differentiation [for a review, see (*1*)]. Our study of *Drosophila NF1* mutants indicates that the activation of *rutabaga* (*rut*)-encoded adenylyl cyclase (*4*, *5*) through heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors is regulated by NF1.

The *Drosophila* homolog of NF1 is 60% identical to the human NF1 protein, neurofibromin, over its entire 2802–amino acid length (*6*). Although homozygous loss of the *Nf1* gene in mice is lethal (*7*), two viable *Drosophila* null mutations of *NF1* ($NF1^{P1}$ and $NF1^{P2}$) have been generated. No NF1 was detected by protein immunoblotting in these two mutants (*6*). $NF1^{P1}$ is a small deletion that includes the *NF1* locus and at least two adjacent genes, and $NF1^{P2}$ is a *P*-element insertion (*6*). Modulation of voltage-activated K^+ currents (*8*, *9*) induced by the neuropeptide pituitary adenylyl cyclase–activating polypeptide (PACAP38) is eliminated in these two mutant alleles.

PACAP38 [which belongs to the vasoactive intestinal polypeptide-secretin-glucagon peptide family and stimulates cAMP synthesis through G protein–coupled receptors in vertebrates (*10*)] induces a 100-fold enhancement of K^+ currents by coactivating both Rut–adenylyl cyclase–cAMP and Ras-Raf kinase pathways (*9*). Mutations in the *rut* (*4*), *Ras* (*11*), or *raf* (*12*) loci eliminate the response to PACAP38 (*9*). Activation of both cAMP and Ras-Raf pathways together, but not alone, mimics the PACAP38 response (*9*). The involvement of Ras in the PACAP38 response led us to investigate the effect of *NF1* mutations in *Drosophila*.

PACAP38-induced responses were recorded by the two-microelectrode voltage-clamp method from body wall muscle fibers of larvae at the third instar (*9*, *10*, *13*, *14*). Perfusion of PACAP38 to the neuromuscular junction induced an inward current followed by a 100-fold enhancement of K^+ currents in wild-type larvae (*8*, *9*) (Fig. 1, A and B). In $NF1^{P1}$ and $NF1^{P2}$ mutants, the inward current remained mostly intact (Fig. 1A), but the enhancement of K^+ currents was abolished (Fig. 1, B and C). Because the inward current is not affected in *NF1* mutants, it appears that PACAP38 receptors are normally activated by the peptide in these mutants.

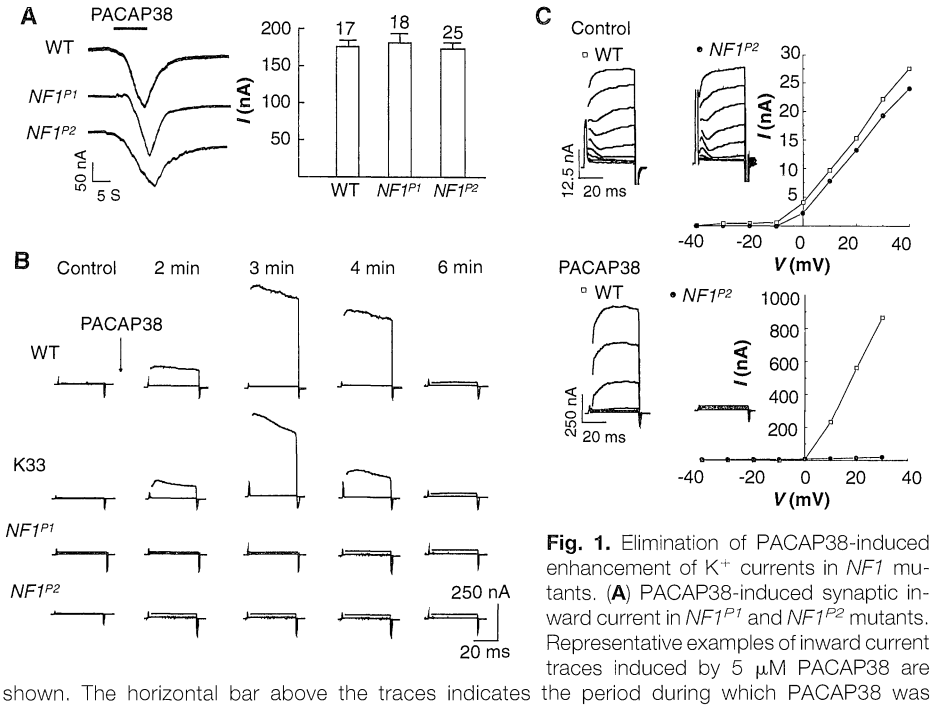

Fig. 1. Elimination of PACAP38-induced enhancement of K^+ currents in *NF1* mutants. (**A**) PACAP38-induced synaptic inward current in $NF1^{P1}$ and $NF1^{P2}$ mutants. Representative examples of inward current traces induced by 5 μM PACAP38 are shown. The horizontal bar above the traces indicates the period during which PACAP38 was perfused. The histogram compares the average peak amplitude ± SEM of the inward current among different genotypes. The number of muscle fibers recorded is shown over the error bar. WT, wild type. (**B**) Failure of PACAP38 to enhance voltage-activated K^+ currents in *NF1* mutants. K33 is the parental line from which *NF1* mutants were generated (*6*). The arrow indicates focal application of PACAP38 (5 μM). In all figures, the time in minutes after pressure-ejection of PACAP38 is indicated at the top of the current traces. The number of muscle fibers/the number of larvae recorded (*N*/*n*) = 31/21, 4/3, 8/5, and 25/18 for wild-type, K33, $NF1^{P1}$, and $NF1^{P2}$, respectively. (**C**) Current-voltage (*I-V*) relations before or 2 min after application of PACAP38 (5 μM). All wild-type and K33 but none of mutant muscle fibers responded. Data in this and all other figures (except Fig. 2B) were recorded with extracellular saline containing 1 mM Ca^{2+}.

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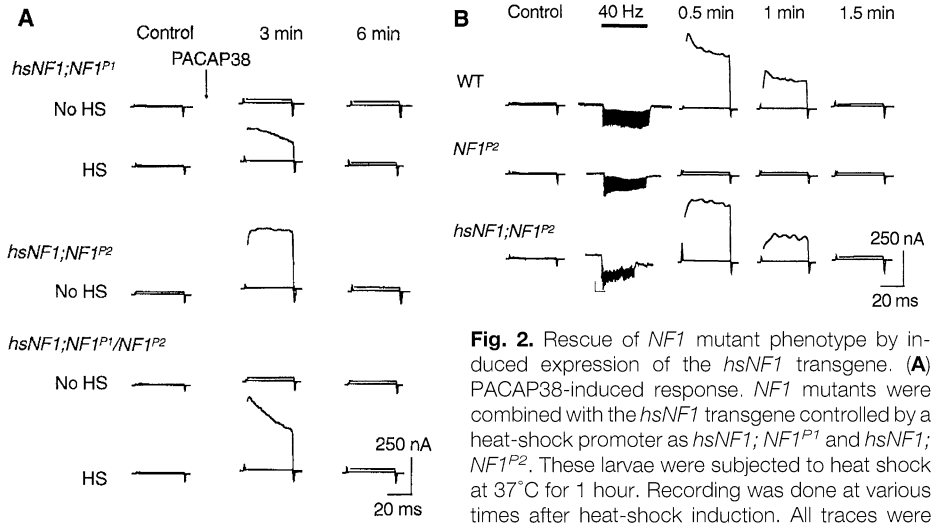

Fig. 2. Rescue of *NF1* mutant phenotype by induced expression of the *hsNF1* transgene. (**A**) PACAP38-induced response. *NF1* mutants were combined with the *hsNF1* transgene controlled by a heat-shock promoter as *hsNF1; NF1*^{P1} and *hsNF1; NF1*^{P2}. These larvae were subjected to heat shock at 37°C for 1 hour. Recording was done at various times after heat-shock induction. All traces were recorded about 4 to 6 hours after the heat shock. The PACAP38 response was observed as early as 1.5 hours after heat shock but with a smaller amplitude of enhancement. *N/n* = 8/6, 4/3, 4/3, 5/3, and 4/3 for panels from top to bottom, respectively. (**B**) Electrically evoked endogenous PACAP38-like enhancement of K^+ currents rescued by induced expression of the *hsNF1* transgene. Stimulation at 40-Hz of wild-type motor axons for 3 s induced a PACAP38-like enhancement of K^+ currents in 2 mM Ca^{2+} (*9*). Control currents were recorded before stimulation. During stimulation, muscle fibers were clamped to −80 mV. Evoked excitatory junctional current traces are shown under "40 Hz stimulation." Then, the same voltage paradigm for eliciting K^+ currents as that used in the control was repeated at various times (indicated at the top of the current traces) after stimulation. Scale bar, horizontal/vertical: 5 s/50 nA.

To rule out potential developmental effects of the *NF1* mutation, we studied transgenic flies carrying an inducible normal *NF1* gene. The *hsNF1* transgene was expressed after heat shock in the transgenic *NF1* mutants *hsNF1*; *NF1*^{P1} and *hsNF1*; *NF1*^{P2}. PACAP38-induced enhancement of K^+ currents was observed in *hsNF1*; *NF1*^{P1} larvae subjected to heat shock (37°C for 1 hour) but not in those without heat shock (Fig. 2A). The *hsNF1*; *NF1*^{P2} larvae, however, showed a normal response to PACAP38 even in larvae not subjected to heat shock. This was probably the result of constitutive expression of the *hsNF1* transgene because a large amount of NF1 protein was detected in these flies (*6*). To reduce the amount of *hsNF1* expression, we selected *hsNF1*; *NF1*^{P2}/+; *NF1*^{P2} larvae in which only one copy of the *hsNF1* transgene was present. In these larvae, the PACAP38 response was only observed after heat shock (Fig. 2A). The PACAP38-induced enhancement was fully rescued 4 hours after heat shock but was observed with a smaller enhancement as early as 1.5 hours after heat shock. Such a time course suggests that all other components in the PACAP38 signaling pathways remain intact so that the preparation resumes PACAP38 responsiveness as soon as enough NF1 is synthesized.

Because PACAP38 is a vertebrate peptide (*10*), we tested the response induced by endogenous PACAP38-like neuropeptide (*8*, *9*). High-frequency stimulation (40 Hz) applied to motor axons through a suction pipette increased K^+ currents, presumably by causing the release of PACAP38-like peptides (*8*). This evoked PACAP38-like response was also eliminated in *NF1* mutants and rescued by the expression of the *hsNF1* transgene (Fig. 2B).

Because NF1 acts as a Ras-GAP (*3*), we examined two null alleles of the *Drosophila* gene *Gap1*: *rl*^{533B1} and *rl*^{533PB}. Flies carrying the mutations have disrupted eye development that results from increased Ras activity (*15*). PACAP38 induced a normal enhancement of K^+ currents (Fig. 3A) in both *Gap1* mutants. Moreover, recordings from transgenic larvae showed that induced expression of constitutively active Ras (Ras^{Val12}) (*16*) or active Raf protein kinase (Raf^{gof}) (*17*) neither blocked nor mimicked the PACAP38 response (Fig. 3B) (*9*). These results suggest that failure to negatively regulate Ras-Raf signaling does not explain the defective PACAP38 response in *NF1* mutants.

Application of the membrane-permeable cAMP analogs dibutyryl cAMP or 8-bromo-cAMP to the larval neuromuscular preparation was insufficient to produce the PACAP38-like enhancement of K^+ currents (*9*, *18*) and appeared not to disrupt the PACAP38 response in wild-type larvae (Fig. 4A). This implies that cAMP may not cause inhibition of Raf activity as reported in other preparations (*19*). Application of these cAMP analogs to *NF1* mutants did restore the normal response to PACAP38 (Fig. 4A). Both *NF1*^{P2} homozygotes and heteroallelic *NF1*^{P1}/*NF1*^{P2} larvae showed enhanced K^+ currents. *NF1*^{P1} larvae also responded, but with a smaller amplitude of response (Fig. 4A), which may be a nonspecific effect of their genetic background because the response of *NF1*^{P1}/*NF1*^{P2} heterozygotes to PACAP38 was fully restored by treatment with cAMP analogs.

The cAMP analogs were effective if applied any time before or within 2 min after application of PACAP38 (examples of 10 min before and 30 s and 2 min after are shown in Fig. 4B). After 2 min, cAMP analogs failed to enhance the response of *NF1*^{P2} mutants to PACAP38. This time course is consistent with a model whereby in *NF1* mutants the Ras-Raf pathway is normally activated in response to PACAP38 for

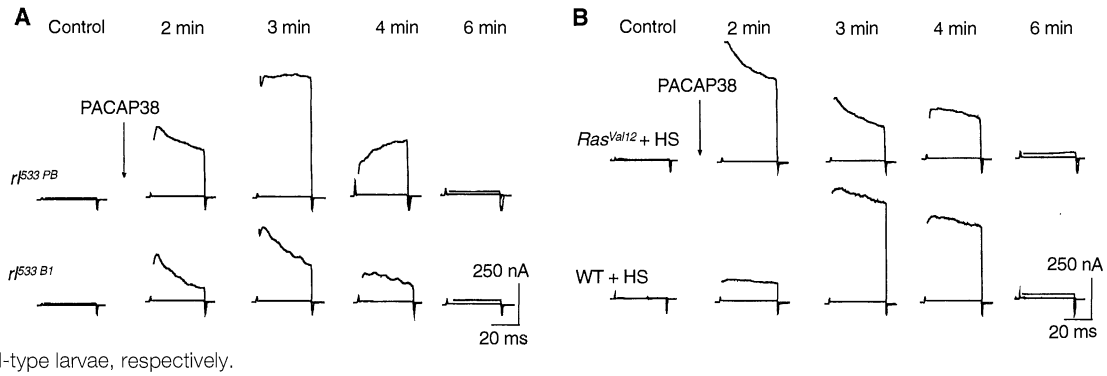

Fig. 3. Normal PACAP38 response in GAP1 mutants and in transgenic larvae expressing active Ras. (**A**) Normal PACAP38 response in mutants carrying *Gap1* null alleles *rl*^{533PB} and *rl*^{533B1}. *N/n* = 6/4 and 4/3, respectively. (**B**) Normal PACAP38 response in transgenic larvae expressing inducible active Ras (Ras^{Val12}) after heat-shock induction (37°C, 1 hour). *N/n* = 7/5 and 3/2 for heat-shocked Ras^{Val12} and wild-type larvae, respectively.

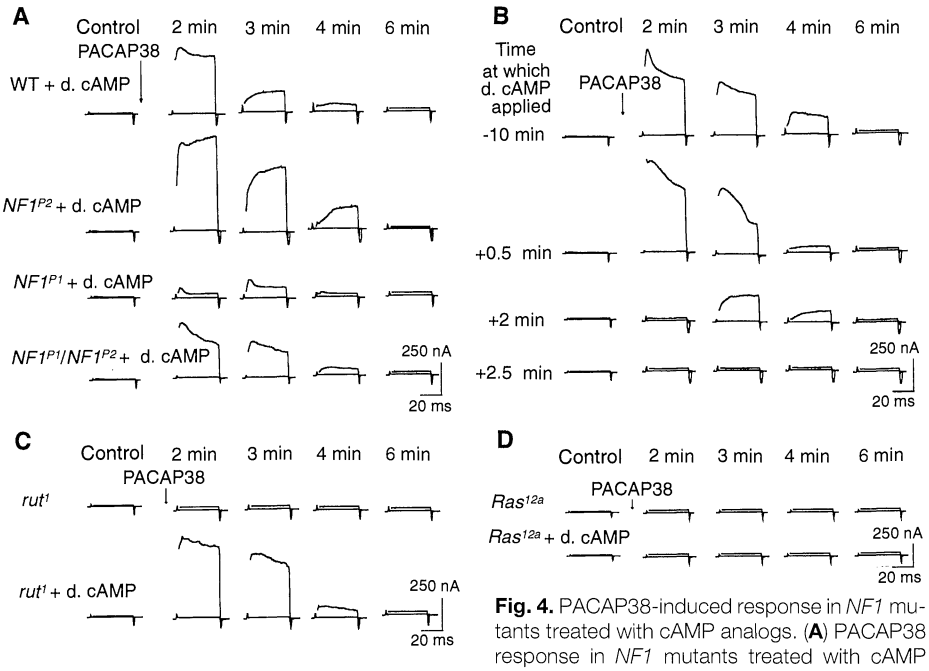

Fig. 4. PACAP38-induced response in *NF1* mutants treated with cAMP analogs. (**A**) PACAP38 response in *NF1* mutants treated with cAMP analogs. Dibutyryl cAMP (d. cAMP) (1 mM) was added before perfusion of PACAP38. *N/n* = 5/2, 19/14, 16/9, and 13/9 for wild-type, $NF1^{P2}$, $NF1^{P1}$, and $NF1^{P1}/NF1^{P2}$, respectively. (**B**) Time course of the cAMP effect. Dibutyryl cAMP was applied 10 min before or 30 s, 2 min, or 2.5 min after the perfusion of PACAP38. (**C**) PACAP38 response in rut^1 mutants treated with cAMP analogs. *N/n* = 22/15 and 5/4 for without and with the cAMP analog, respectively. (**D**) Failure of the cAMP analogs to restore PACAP38 response in *Ras* mutants. Flies with Ras^{12a}, a weak allele, were viable but showed no PACAP38-induced enhancement of K^+ currents (top panel) (*9*). *N/n* = 9/5 and 5/4 for without and with the cAMP analog, respectively.

2 min, but the cAMP pathway is blocked. Therefore, synergistic modulation of K^+ currents can be achieved if cAMP analogs are supplied during the transient activation of the Ras-Raf pathway. Addition of cAMP analogs also restored the response to PACAP38 in rut^1 mutants, but not in Ras^{12a} mutants (Fig. 4, C and D) (*4*, *5*, *9*).

To further test whether activation of cAMP signaling rescues the defective PACAP response of *NF1* mutants, we applied the drug forskolin, which stimulates G protein–coupled adenylyl cyclase activity (*20*, *21*), to the neuromuscular preparation. PACAP38 induced a normal response in $NF1^{P2}$ and $NF1^{P1}/NF1^{P2}$ mutants exposed to forskolin (Fig. 5A). This indicates that adenylyl cyclase is present but is not activated by receptors for PACAP38-like neuropeptides. Forskolin also restored the PACAP38 response in rut^1 mutants (Fig. 5B) even though the Rut–adenylyl cyclase is completely nonfunctional (*4*, *5*). It is possible that cAMP synthesized by other adenylyl cyclases upon forskolin stimulation is sufficient to modulate K^+ currents together with the Ras pathway activated by PACAP38 (*22*).

Adenylyl cyclase shows abnormal subcellular localization in yeast *IRA* (inhibitor of Ras activity) mutants (*23*). The *IRA* gene encodes proteins that are distantly related to NF1 and that are involved in mediating Ras-dependent activation of adenylyl cyclase. Although the yeast cyclase is very different from Rut–adenylyl cyclase and other cyclases in higher organisms (*24*, *25*), we examined adenylyl cyclase activity in membrane fractions (*26*). The Rut–adenylyl cyclase is the only cyclase that can be activated by Ca^{2+}-calmodulin in the tissues from fly abdomen, as indicated by the lack of the Ca^{2+}-dependent cyclase activity in rut^1 mutants (*4*, *5*) (Fig. 5C). In addition, the basal activity (*4*) and the forskolin-stimulated (*21*) activity of adenylyl cyclase were also reduced in rut^1 mutants. However, *NF1* mutations did not affect the basal activity, the Ca^{2+}-dependent activity, or the forskolin-stimulated activity of adenylyl cyclase (Fig. 5, C and D). Therefore, Rut–adenylyl cyclase is present in these membranes and can be normally activated by Ca^{2+}-calmodulin and forskolin.

In summary, signaling by the PACAP38 neuropeptide is impaired in *NF1* mutants, and the defect is apparently caused by a

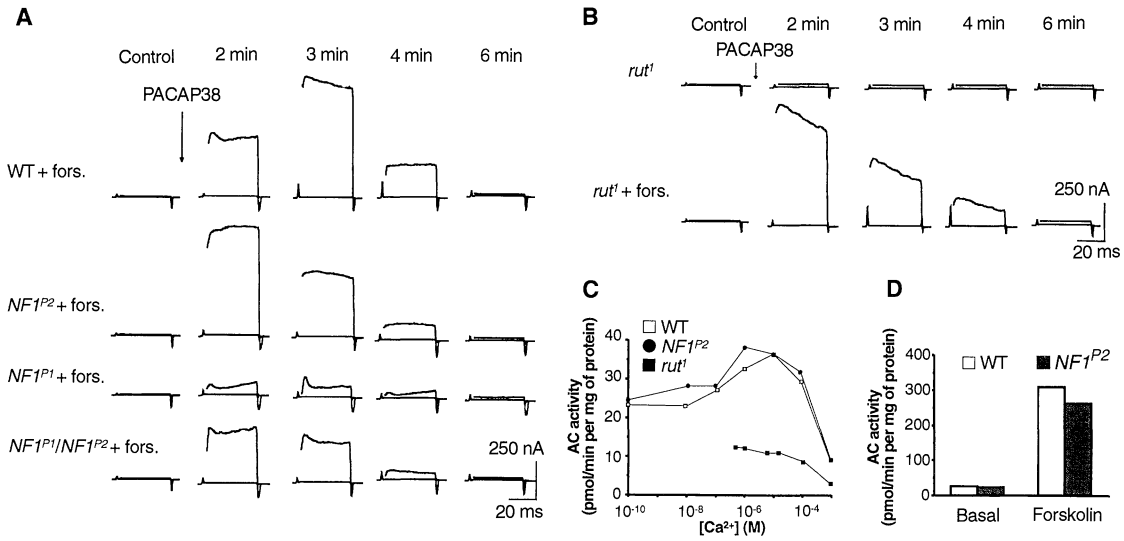

Fig. 5. PACAP38 response in *NF1* mutants treated with forskolin. (**A**) Effect of forskolin (fors.) in *NF1* mutants. Forskolin (10 μM) was applied to the extracellular solution 4 min before perfusion of PACAP38 to wild-type or mutant larvae as indicated. Forskolin at 2 μM was also effective. *N/n* = 5/4, 21/16, 10/4, and 9/4 for wild-type, $NF1^{P2}$, $NF1^{P1}$, and $NF1^{P1}/NF1^{P2}$ larvae, respectively. (**B**) Effect of forskolin in rut^1 mutants. Forskolin (10 μM) was applied 4 min before PACAP38. *N/n* = 5/4. (**C**) Adenylyl cyclase activity (*26*) in membrane fractions. The Ca^{2+} dependency of adenylyl cyclase assayed from the membrane fraction of fly abdomens is normal in $NF1^{P2}$ mutants. (**D**) Basal and forskolin-stimulated activities of adenylyl cyclase are similar in wild-type and $NF1^{P2}$ flies. Forskolin (100 μM) was added to the extracted membrane fraction.

blockade of PACAP38-stimulated activation of Rut–adenylyl cyclase. Thus, the NF1 protein not only acts as a negative regulator of Ras but also as a crucial component for activation of the cAMP pathway. The induced expression of a catalytic subunit of cAMP-dependent protein kinase rescues the developmental phenotype of small body size in $NF1^{P1}$ and $NF1^{P2}$ mutants (6), providing further support for the above conclusion. Exploration of the mechanism by which NF1 influences G protein–mediated activation of adenylyl cyclase may lead to new insights into mechanisms of G protein–mediated signal transduction and the pathogenesis, and possibly the treatment, of human type 1 neurofibromatosis.

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14. The larval body wall neuromuscular preparation has been described (*8, 9, 13*). The setup, saline, recording conditions, and voltage paradigms for the electrophysiological recordings were as described (*8, 9*). For recording the PACAP38-induced synaptic current, the membrane potential was clamped at −80 mV. For recording K^+ currents, command voltages were stepped from the holding potential of −80 to −50 and 20 mV, respectively. These currents include outward K^+ and inward Ca^{2+} currents, but the inward Ca^{2+} component is completely masked (*27*). PACAP38 was applied by pressure ejection through a glass electrode positioned near the voltage-clamped muscle membrane. Forskolin and cAMP analogs were applied to the solution bathing the preparation.
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26. Adenylyl cyclase activity was assayed as described (*4*) with membranes from abdomens of rut^1, $NF1^{P2}$, and wild-type flies. Calcium concentrations were calculated according to MaxChelator v1.31 (*28*). Assays were done in duplicate, and each result represents data from at least two separate experiments.
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Long-Term Responses of River-Margin Vegetation to Water-Level Regulation

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The long-term effect of water-level regulation on riparian plant communities was assessed for storage reservoirs and run-of-river impoundments. Soon after the onset of regulation, there were few species and sparse vegetation cover, regardless of whether the new water level intersected former upland or riparian vegetation. In the longer term, an impoverished vegetation was maintained by storage reservoirs, whereas in run-of-river impoundments, some community characteristics deteriorated and others recovered compared to adjacent free-flowing rivers.

Riparian ecosystems are among the most diverse systems on the world's continents (*1*) and have been suggested as sensitive indicators of environmental change (*2*). Although the majority of the world's riparian systems are affected by water regulation schemes (*3*), the long-term development of riparian communities along regulated water bodies is not known because most studies of succession after dam closure are performed on rivers which have been regulated for only a few decades (*4, 5*). Environmental impact assessments of water regulation schemes suffer from this lack of knowledge and generally provide very little information about likely postdamming development of ecological communities. Therefore, regulations are approved without considering the chance a river's ecological integrity and biodiversity will be maintained in the long term (*6*).

We used riparian vegetation along storage reservoirs and run-of-river impoundments in central and northern Sweden to assess ecosystem readjustment after regulation (*7*). These are the two major types of regulated waters in the study area, and they are consecutive along the regulated rivers; tailwater reaches are absent or scarce. Storage reservoirs have enough capacity to offset seasonal fluctuations in water flow and provide a constant supply of water throughout the year. They have their lowest water levels in spring and their seasonal highs in summer and fall. Run-of-river impoundments provide water flow through the turbines and may store a day's or week's worth of water. The water level fluctuates daily or weekly between its statutory high and low levels throughout the year.

Depending on whether water levels were raised at the onset of regulation to intersect former upland soils or overlapped with the range of pristine water-level fluctuations, succession started either with few or many riparian species at hand. To control for this effect, we distinguished between regulated sites with preupland and preriparian land.

We sampled river-margin sites along 43 storage reservoirs and 45 run-of-river impoundments, ranging between 1 and 70 years in age. Each study site encompassed a section of river margin 200 m in length between the highest and lowest water levels during the growing season. At each site, the presence of all vascular plant species and the percentage cover of trees and shrubs and of herbs and dwarf shrubs were recorded (*8*). Species richness was then transformed (*9*) to compensate for between site variation in river-margin width, and thus study site area. We also determined height of the river margin, substrate fineness, and substrate heterogeneity (*8*). In 34 of 43 storage reservoirs, water levels were raised to increase storage capacity, and preriparian sites ($n = 9$) were too few to be treated in all analyses.

Compared to similar sites along adjacent, free-flowing rivers (*10*), the storage reservoirs had fewer species per 200-m length of river margin (58 versus 83 species; $P < 0.0001$, Mann-Whitney U test) and a lower transformed species richness (57 versus 95 species, $P < 0.0001$). The corresponding figures for run-of-river impoundments were 74 versus 87 ($P = 0.0032$) and 97 versus 97 ($P = 0.71$). Thus, after nar-

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