Control of *Drosophila* perineurial glial growth by interacting neurotransmitter-mediated signaling pathways

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***Drosophila* peripheral nerves, similar structurally to the peripheral nerves of mammals, comprise a layer of axons and inner glia, surrounded by an outer perineurial glial layer. Although it is well established that intercellular communication occurs among cells within peripheral nerves, the signaling pathways used and the effects of this signaling on nerve structure and function remain incompletely understood. Here we demonstrate with genetic methods that the *Drosophila* peripheral nerve is a favorable system for the study of intercellular signaling. We show that growth of the perineurial glia is controlled by interactions among five genes: *ine*, which encodes a putative neurotransmitter transporter; *eag*, which encodes a potassium channel; *push*, which encodes a large, Zn^{2+}-finger-containing protein; *amn*, which encodes a putative neuropeptide related to the pituitary adenylate cyclase activator peptide; and *NF1*, the *Drosophila* ortholog of the human gene responsible for type 1 neurofibromatosis. In other *Drosophila* systems, *push* and *NF1* are required for signaling pathways mediated by Amn or the pituitary adenylate cyclase activator peptide. Our results support a model in which the Amn neuropeptide, acting through Push and NF1, inhibits perineurial glial growth, whereas the substrate neurotransmitter of Ine promotes perineurial glial growth. Defective intercellular signaling within peripheral nerves might underlie the formation of neurofibromas, the hallmark of neurofibromatosis.**

Both mammalian and invertebrate peripheral nerves comprise a central core of motor and sensory axons surrounded by inner glia (termed Schwann cells in mammals and peripheral glia in *Drosophila*) and outer glia (termed perineurium in mammals, and perineurial glia in *Drosophila*; refs. 1 and 2). In *Drosophila*, the peripheral glia form the "blood–brain" permeability barrier (1). The role of the perineurial glia is less clear but might serve to provide structural support for the peripheral nerves. These cells communicate extensively by the release of and response to both small molecule and peptide neurotransmitters, as well as protein factors (3). For example, acetylcholine and ATP released by the frog motor nerve terminal in response to nerve activity increase intracellular $[Ca^{2+}]$ in neighboring perisynaptic Schwann cells (4). In addition, glutamate released from the squid giant neuron activates Schwann cell potassium currents by means of increased [cAMP] (5). Finally, Schwann cells release factors such as Desert Hedge Hog that organize the surrounding perineurium, and also release factors that regulate neuronal survival, neuronal excitability, synaptic transmission, and Schwann cell survival in the absence of neurons (6–8).

One disease in humans that might reflect impaired intercellular communication within peripheral nerves is type 1 neurofibromatosis, caused by mutations in the *NF1* gene. This disease is characterized by the appearance of peripheral nerve sheath tumors called neurofibromas (reviewed in ref. 9). As is the case with other disorders involving tumor-suppressor genes, type 1 neurofibromatosis is inherited as a dominant disorder, and it is thought that tumors arise after spontaneous loss of the $NF1^+$ allele within certain cells during somatic growth. However, neurofibromas exhibit properties that are not expected for those caused by loss of a tumor suppressor. For example, neurofibromas are heterogeneous and contain cells derived from each of the cell types normally found within peripheral nerves (neurons, Schwann cells, and perineurial cells). These cells are not clonally related and thus it seems highly unlikely that the $NF1^+$ allele has been lost in each cell type. Rather, it seems more likely that phenotypically wild-type cells within these tumors overproliferate in response to factors released aberrantly from $Nf1^-$ cells. However, this possibility remains speculative and no detailed mechanism for the aberrant release of a diffusible factor has been proposed.

Here we report a genetic dissection of signaling pathways controlling the structure of the *Drosophila* peripheral nerve. We show that mutations in five distinct genes, in either single or particular double-mutant combination, increase the growth of the perineurial glial layer of the larval peripheral nerve. Two of these genes, *amnesiac* (*amn*) and *inebriated* (*ine*), are each likely to control neurotransmitter-mediated signaling pathways. *amn* encodes a putative neuropeptide related to the pituitary adenylate cyclase activator peptide (PACAP; ref. 10), whereas *ine* encodes a putative Na^+/Cl^--dependent neurotransmitter transporter and is likely to be required for reuptake of a substrate neurotransmitter (11–13). These observations suggest that perineurial glial growth is controlled by two distinct neurotransmitters. Two additional genes, *pushover* (*push*) and *NF1* (the *Drosophila* ortholog of the human *NF1* gene), have each been implicated in other systems as downstream targets of Amn or PACAP (refs. 14–16; S. Hawley, personal communication). These observations suggest that *push* and *NF1* might mediate the effects of Amn on perineurial glial growth. The fifth gene, *eag*, encodes a potassium channel that affects motor neuron excitability (17), suggesting a role for neuronal excitability in the control of perineurial glial growth. We also report the sequence of *push*, which encodes an extremely large membrane protein

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Abbreviations: PACAP, pituitary adenylate cyclase activator peptide; NT, neurotransmitter of Ine.

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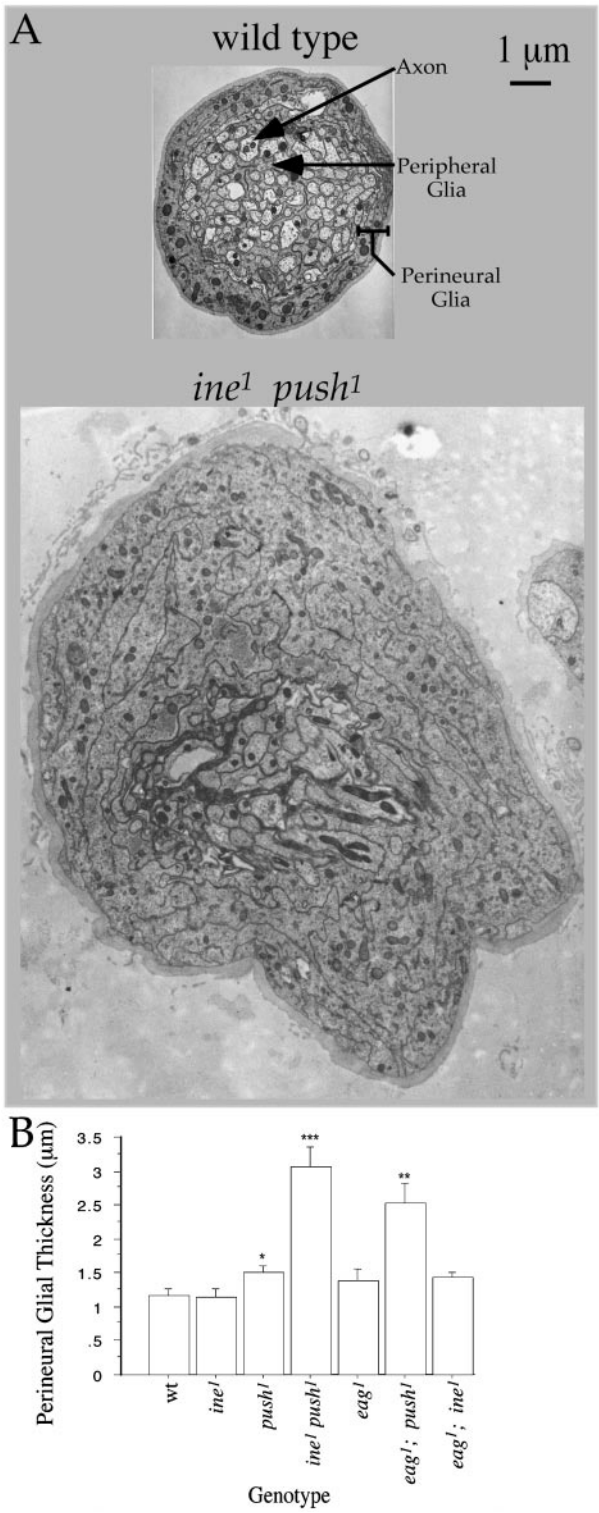

Fig. 1. Effects of indicated mutants and double mutants on perineurial glial growth. Wild type indicates the isogenic parental control line of each mutant. (*A*) Cross sections of larval peripheral nerves of wild type and *ine*^{1} *push*^{1}. Locations of the axon, peripheral glial, and perineurial glial layers are indicated. In *ine*^{1} *push*^{1}, all of the cells that make up a peripheral nerve appear to be present and contain a normal number of axons (74.8 ± 1.8, $n = 4$) that are of normal cross-sectional area. (*B*) Means and standard errors of perineurial glial thickness for the indicated genotypes. The following pairwise combinations had statistically significant differences (two-tailed unpaired *t* test) in perineurial glial thickness: wild type vs. *push*^{1} (*, $P = 0.04$), *ine*^{1} *push*^{1} vs. *ine*^{1} or *push*^{1} (***, $P < 0.0001$), and *eag*^{1}*; push*^{1} vs. *eag*^{1} or *push*^{1} (**, $P = 0.0045$ and 0.005, respectively).

containing two Zn^{2+} fingers that is conserved throughout evolution. We propose a model for the control of perineurial glial growth by two interacting neurotransmitter-mediated signaling pathways. Furthermore, we suggest that neurofibromas, the hallmark of neurofibromatosis, might result from defective receipt of neurotransmitter signals within peripheral nerves.

Materials and Methods

Electron Microscopy. Tissue sections were prepared as described (2). Because *push* mutations confer male sterility, it is not possible to construct a homozygous *push* mutant stock. Thus, to identify homozygous larvae, a stock was constructed in which the *push, eag; push* and *ine push* chromosomes were crossed into a *yellow* (*y*) background and placed in combination with the second chromosome balancer, *CyO*, that carried a P[y^+] transgene. Homozygous larvae from these stocks were identified by yellow mouth hooks before dissection for electron microscopy. Wandering third instar larvae were grown at room temperature from uncrowded half-pint bottles and collected 1 or 2 days after the first wandering larvae appeared. For experiments involving heat shock, larvae were heat-shocked daily for 1 hour at 37°C. Larvae were dissected, fixed with glutaraldehyde and paraformaldehyde, stained with both 0.5% OsO_4 and 2% uranyl acetate, and embedded in an eponate 12-araldite mixture. Ultrathin cross-sectional slices (pale gold, 75–125-nm-thick) were captured, poststained by using uranyl acetate and Reynolds lead citrate, and analyzed by using a JEOL or Hitachi (Tokyo) transmission electron microscope at either 60 or 80 kV. The thickness of the perineurial glial cell layer for a given nerve was determined by averaging the distance from the edge of the nerve to the boundary of the axon-containing lumen at 8 different positions: 12, 3, 6 and 9 o'clock and four additional measurements at points in between these positions. Measurements were not taken at positions where a perineurial glial cell nucleus was encountered. All statistical analyses were carried out by using STATVIEW v4.51 (Abacus Concepts, Berkeley, CA).

Cloning and Sequence Analysis of *push*. DNA flanking *push*^{3420}, a *P* element insertion that causes a hypomorphic *push* mutation (14, 18), was isolated by using a plasmid-rescue technique by transforming a dilute ligation of *Nhe*I-digested *push*^{3420} genomic DNA. The subcloned *push*^{3420} flanking DNA was used as a probe to screen both a lambda *Drosophila* genomic DNA library and a *Drosophila* testes cDNA library kindly donated by T. Hazelrigg (Columbia Univ., New York). Both of these libraries were screened according to protocols from Stratagene. A number of cDNA and genomic clones were isolated in the process of walking the entire gene. In addition, use was made of P1 clone DS002276 from the Berkeley *Drosophila* genome project, which contains the complete *push* gene. For sequencing, double-stranded DNA was prepared by using Qiagen (Hilden, Germany) plasmid purification kits, sequence reactions were performed by using the dye terminator chemistry from Perkin–Elmer Applied Biosystems (Foster City, CA), and were run on an automated DNA sequencer from the same company. Sequences were assembled by using SEQUENCHER software (Gene Codes, Ann Arbor, MI), and the completed cDNA was conceptually translated by using GCG software.

Results

Control of Perineurial Glial Growth by Interacting Pathways. Mutations in two genes that affect neuronal excitability also affect the structure of the peripheral nerve: double mutants defective in *inebriated* (*ine*), which encodes a member of the Na^+/Cl^--dependent neurotransmitter transporter family (11–13), and *pushover* (*push*) exhibit an extremely thickened nerve, which is a phenotype that is clearly visible with the dissecting microscope. To understand the cellular basis for this phenotype, we per-

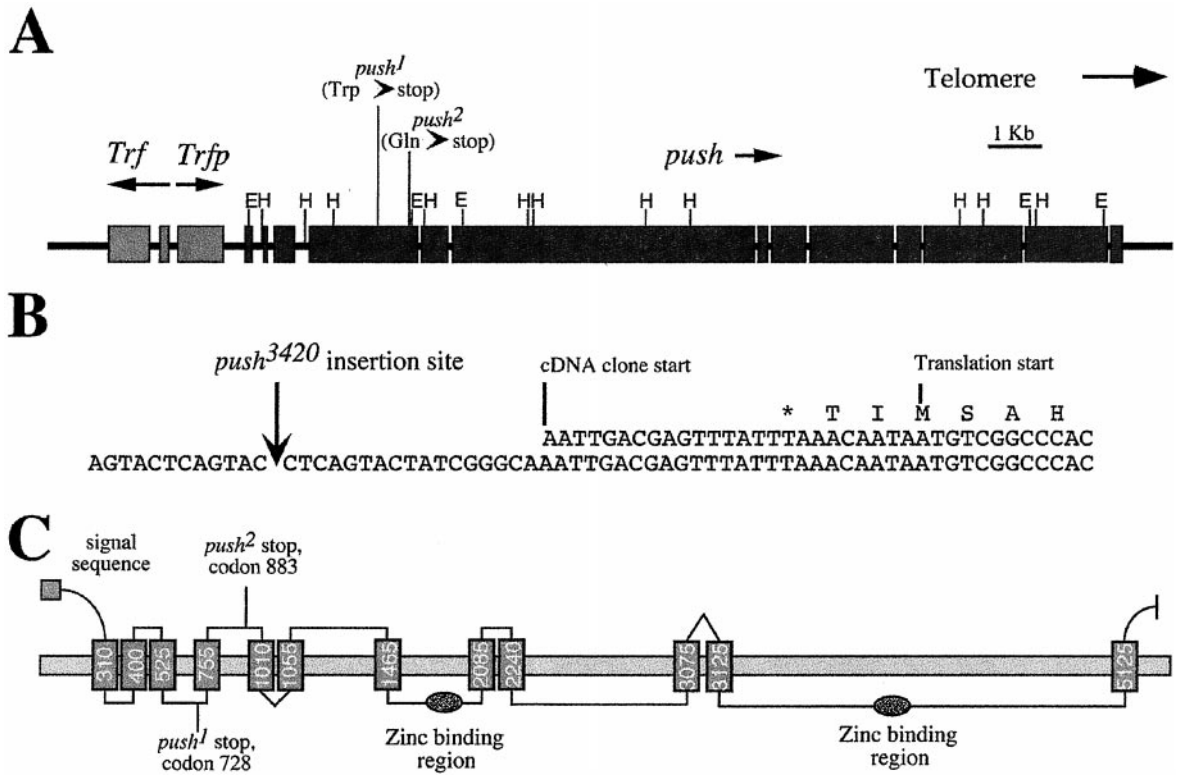

Fig. 2. Structure of the *push* gene. (*A*) Scale diagram of the *push* locus including the positions of the *Trf* and *Trfp* genes. Boxes indicate exons, arrows indicate the direction of transcription of the genes, E indicates an *Eco*RI restriction enzyme site, and H indicates a *Hin*dIII restriction enzyme site. (*B*) Sequence around the $push^{3420}$ *P* element insertion site showing the start of the most 5′ cDNA clone, the start of the *push* ORF, and the first methionine in that ORF (labeled ''translation start''). (*C*) Hypothetical transmembrane structure of Push as predicted by TM PRED (34). The horizontal box indicates the membrane bilayer, and the vertical boxes indicate putative transmembrane helices. The number on each helix represents the amino acid number at the approximate center of the helix. Positions of a putative signal sequence, the two nonsense mutations, and two putative zinc-binding regions are also indicated.

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formed transmission electron microscopy on cross-sections of peripheral nerves. This analysis demonstrated that the $push^1$ and ine^1 $push^1$ double mutants exhibited a normal axon and peripheral glial layer, but a thickened perineurial glial layer. This increased perineurial thickness was expressed only moderately in $push^1$ but very strongly in the ine^1 $push^1$ double mutant (Fig. 1 and Fig. 5, which is published as supplemental data on the PNAS web site, www.pnas.org). This increase in thickness was accompanied by an increase in the number of mitochondria within perineurial glial thin sections (130 ± 19 in ine^1 $push^1$ vs. 30 ± 7 in wild type; $P = 0.0001$), suggesting that an increase in cell material accompanies this increased thickness. The ine^1 $push^1$ phenotype was significantly rescued in transgenic larvae expressing the 943-aa Ine isoform called Ine-P1 under the transcriptional control of the heat-shock promoter (13). In particular, perineurial glial thickness in ine^1 $push^1$; *hs-ine-P1* larvae, even in the absence of heat shock, was reduced to 2.0 ± 0.2 μm ($n = 11$) from 3.1 ± 0.3 in ine^1 $push^1$ ($n = 14$, P value vs. ine^1 $push^1$; *hs-ine-P1* = 0.008). The observed synergistic interaction between *ine* and *push* mutations suggests that each gene controls perineurial glial growth through partially redundant pathways.

In certain respects, mutations in *ine* confer phenotypes similar to mutations in the K^+ channel structural gene *eag*. In particular, both *eag* and *ine* mutations interact synergistically with mutations in the K^+ channel encoded by *Shaker* to cause a characteristic ''indented thorax and down-turned wings'' phenotype, which is not exhibited by any of the single mutants (19). Because of this phenotypic similarity, we tested the possibility that *eag* mutations might also affect perineurial glial thickness. We found that eag^1 resembles ine^1 in the control of perineurial glial growth: eag^1; $push^1$ double mutants, but not the eag^1 single mutant, exhibited strongly potentiated perineurial glial growth. This increased growth was similar to, but less extreme than, what was observed in ine^1 $push^1$ (Figs. 1 and 5). eag^1; $push^2$ double mutants also exhibited a thickened perineurial glial layer (1.88 ± 0.15 μm, $n = 14$, P value vs. eag^1 and $push^1$ mutants = 0.04). In contrast, *eag* and *ine* mutations fail to display a comparable synergistic interaction: perineurial glial thickness in the eag^1; ine^1 double mutant was 1.43 ± 0.08 μm, which is not significantly different from the values of the eag^1 or ine^1 single mutants ($P = 0.8$ and 0.06, respectively).

Cloning and Sequence Analysis of *push*. It was previously shown that a *P* element insertion termed $push^{3420}$ caused a partial loss of function in *push* (14, 18). We used this *P* element as a starting point for the isolation of *push* DNA. We found that the *P* element lies between two ORFs, a centromere-proximal ORF called *Trfp*, and a novel centromere-distal ORF (20). To determine which ORF corresponded to *push*, we sequenced each ORF in the ethyl methanesulfonate (EMS)-induced $push^1$ and $push^2$ mutants and in the isogenic wild-type strain. The *Trfp* gene exhibited no sequence alterations in either *push* mutant. However, both *push* mutations were found in the novel ORF, and thus we assign this ORF as *push*; $push^1$ and $push^2$ mutations are both nonsense mutations (at codons 728 and 883 for $push^1$ and $push^2$, respectively). A putative TATA box is observed 260 bp upstream of the $push^{3420}$ insertion site. Thus, it is possible that our most 5′ cDNA is incomplete at the 5′ end, and the $push^{3420}$ *P* element is inserted in the 5′ untranslated region (UTR) of the *push* transcript. We found that *push* encodes an enormous 5,322-aa protein containing two potential Zn^{2+} binding domains and 12 potential transmembrane domains (Fig. 2).

Push has many additional functions in *Drosophila* in addition

to controlling neuronal excitability and perineurial glial growth. First, mutations in *push* cause male sterility (14). A *P* element insertion called *purity of essence* (*poe*), which also causes male sterility by means of the inhibition of sperm individuation, is found at the identical location to $push^{3420}$, raising the possibility that *push* and *poe* are identical genes (21, 22). We found that *push* is transcribed in adult testes as well as the embryonic central nervous system (data not shown), which is consistent with this possibility. We confirmed this possibility by demonstrating that *push* and *poe* mutations fail to complement for the male sterility phenotype (data not shown). Second, *push* might have a role in eye development or function: an incomplete cDNA of *push*, encoding 4,100 amino acids and truncated at the N terminus, was identified previously under the name "Calossin." This protein was identified on the basis of calmodulin binding and expression in the photoreceptor (23). Finally, genes similar to *push* are present in several multicellular organisms including *Caenorhabditis elegans* (GenBank accession no. AF003140), *Arabidopsis*, and humans (GenBank accession no. AB007931). However, the sequence of *push* provides few additional clues as to its function.

Mutations in *push* were identified independently on the basis of defective segregation of nonrecombinant chromosomes in the female meiosis (24). *push* was implicated in this process as an intermediate in a signaling pathway mediated by the PACAP-like neuropeptide encoded by *amn* (S. Hawley, personal communication). This observation raised the possibility that *push* likewise affects perineurial glial growth by acting as an intermediate from an Amn signal. Consistent with this hypothesis, we found that the amn^{X8} deletion mutation (25) increased perineurial glial thickness, and that this increase was significantly rescued in transgenic flies expressing amn^{+} (Fig. 3 and Fig. 6, which is published as supplemental data on the PNAS web site).

A second signaling pathway mediated by a PACAP-like neuropeptide was previously identified in *Drosophila*. In this pathway, the larval muscle responds to application of PACAP by activating a voltage-gated potassium channel (26). This activation requires *NF1*, the ortholog of the human gene responsible for type 1 neurofibromatosis (16). We tested the possibility that *NF1* might affect perineurial glial growth. We found that the $NF1^{P2}$-null mutant (15) exhibited strong potentiation of perineurial glial thickness in combination with ine^{1}. This thickness was much greater than the thickness observed in ine^{1} mutants carrying *K33*, the $NF1^{+}$ parent chromosome of $NF1^{P2}$. The increased glial thickness of ine^{1}; $NF1^{P2}$ was fully rescued by heat-shock-induced expression of the $NF1^{+}$ transgene (Figs. 3 and 6). However, unlike *push*, the phenotype of $NF1^{P2}$ was potentiated only moderately by the eag^{1} mutation (not shown). In contrast, perineurial glial thickness in the $push^{1}$; $NF1^{P2}$ double mutant was 2.1 ± 0.15 μm, which is significantly thicker than either $push^{1}$ or $NF1^{P2}$ ($P = 0.002$ and 0.0005, respectively), but not significantly different from amn^{X8} ($P = 0.33$). These results are consistent with the possibility that *push* and *NF1* mediate the *amn* signal through parallel partially redundant pathways.

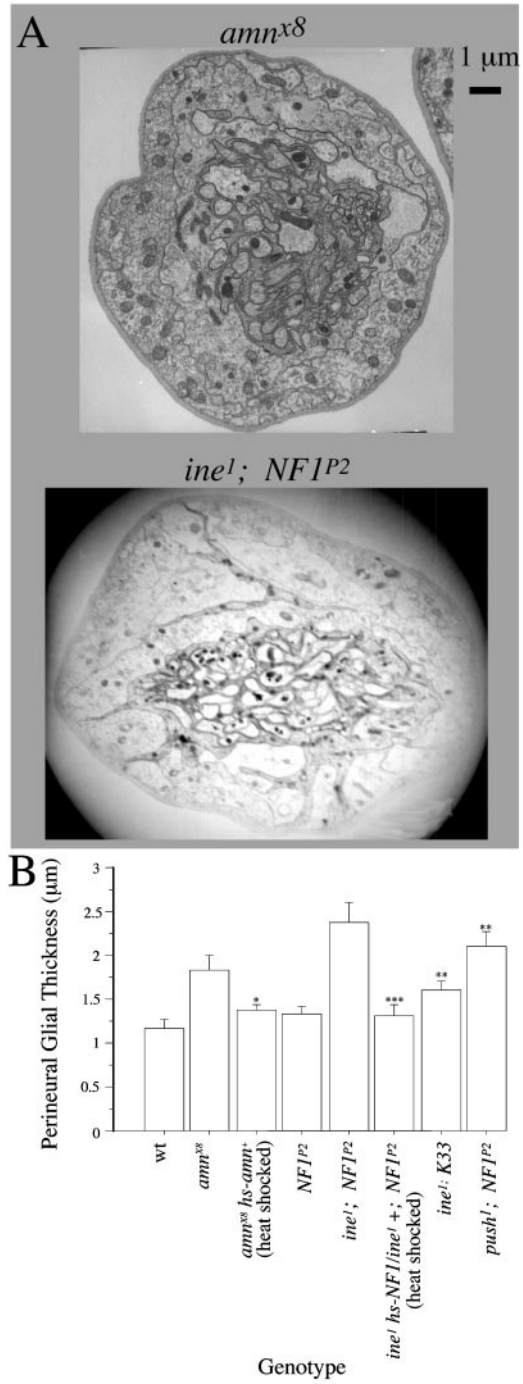

Fig. 3. Control of perineurial glial growth by *amn* and *NF1*. For both heat-shock control and heat-shock rescue experiments, lines were heat shocked daily for 1 hour at 37°C throughout development. We found that this heat-shocking protocol had no significant effect on perineurial glial thickness in either amn^{X8} or ine^{1}: $NF1^{P2}$ lacking the appropriate rescue construct. Perineurial glial thickness of amn^{X8} in the presence of heat shock was 1.82 ± 0.15 ($n = 16$) and in the absence of heat shock was 1.80 ± 0.07, $n = 24$. Perineurial glial thickness of ine^{1}; $NF1^{P2}$ in the presence of heat shock was 2.27 ± 0.17, $n = 15$, and in the absence of heat shock was 2.38 ± 0.22 ($n = 12$). (*A*) Electron micrographs of peripheral nerves of amn^{X8} and ine^{1}; $NF1^{P2}$ double mutants. (*B*) Means and standard errors of perineurial glial thickness for the indicated genotypes. The following pairwise combinations had statistically significant differences (two-tailed unpaired *t* test) in perineurial glial thickness: amn^{X8} or amn^{X8} heat shocked vs. amn^{X8} *hs-amn*$^{+}$ heat shocked (*, $P = 0.02$), ine^{1}; NF1^{P2} vs. ine^{1}; *K33* (**, $P = 0.002$), ine^{1}; $NF1^{P2}$, heat shocked vs. ine^{1} *hs-NF1*/ine^{1} +; $NF1^{P2}$, heat shocked (***, $P = 0.0003$), and $push^{1}$; $NF1^{P2}$ vs. $push^{1}$ or $NF1^{P2}$ (**, $P = 0.002$ and 0.0005, respectively).

Discussion

We have shown that mutations in five genes, in single- or double-mutant combination, increase growth of the perineurial glial layer of the *Drosophila* larval peripheral nerve. Our results are consistent with a model in which two neurotransmitter-mediated signaling pathways exert opposing effects on perineurial glial growth. One pathway, mediated by the Amn neuropeptide, inhibits perineurial glial growth. This pathway requires NF1 and Push activity. The second pathway, mediated by the substrate neurotransmitter of Ine (which we will call NT), promotes perineurial glial growth. In this pathway, mutations in *ine* or *eag* each increase signaling by NT: *ine* mutations increase NT signaling by eliminating the NT reuptake transporter thus increasing NT persistence, whereas *eag* mutations increase NT

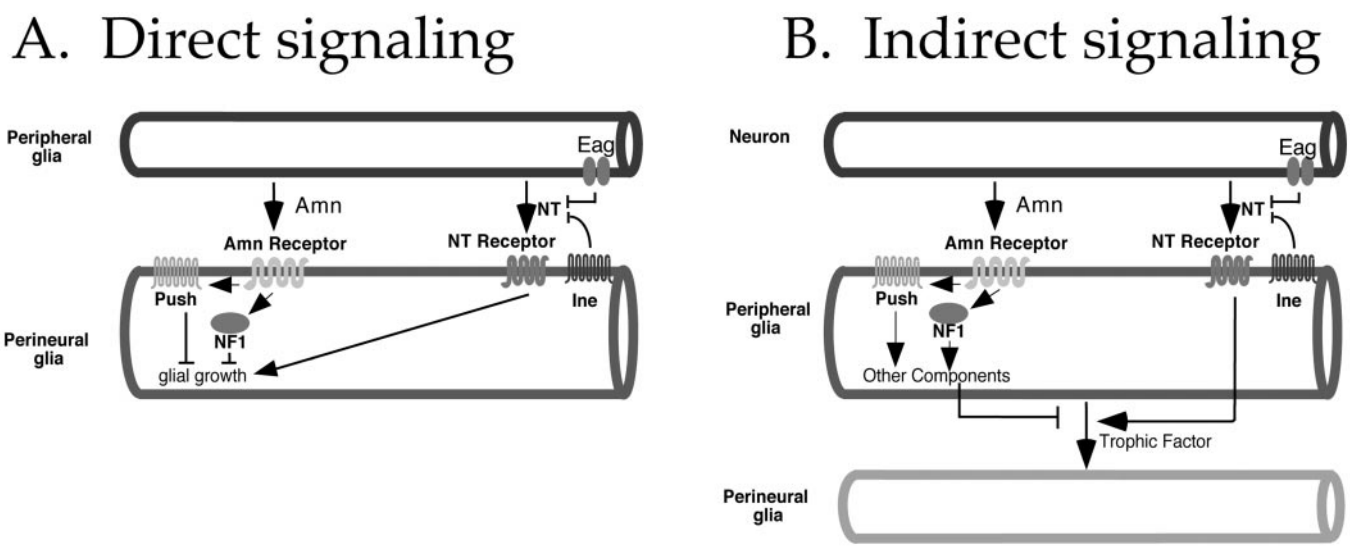

Fig. 4. Two models for the control of perineurial glial growth by neurotransmitters. (*A*) Direct signaling. The peripheral glia release both Amn and the substrate NT. The Amn neuropeptide inhibits perineurial glial growth via Push and NF1, whereas NT promotes perineurial glial growth. The Ine transporter attenuates the effects of this NT by reuptake. The Eag potassium channel, acting in peripheral glia, inhibits NT release by reducing excitability. (*B*) Indirect signaling. The peripheral glia release a trophic factor that causes increased cell growth among the perineurial glia. Release of this trophic factor is regulated by Amn and NT, each released from neurons, with opposing effects. Amn, acting through Push and Nf1, inhibits release of the trophic factor, whereas NT promotes release of the trophic factor. The placement of Push downstream of Amn is most consistent with previous studies (S. Hawley, personal communication), but we cannot rule out the possibility that Push acts in motor neurons, and its effect on perineurial glial growth results from increased release of NT or a distinct neurotransmitter. As above, Ine and Eag attenuate the effects of NT by performing reuptake and reducing release, respectively.

signaling by increasing NT release as a consequence of increased excitability. These pathways interact such that the most extreme effects on perineurial glial growth are observed when the NT pathway is overstimulated and the Amn pathway is disrupted simultaneously. The genetic interactions that form the basis for this interpretation require that the mutations under investigation be null. Although the *eag*^{1} mutation tested has not been characterized molecularly, the mutations in each of the other four genes that we analyzed are known to be or are strongly suspected to be null (11, 15, 25, this study). Direct neuron–perineurial glia signaling is unlikely because the peripheral glia, which form the blood–brain barrier, are expected to be an impervious barrier to intercellular traffic. Two alternative mechanisms could underlie this signaling. In the first mechanism (direct peripheral glia–perineurial glia signaling; Fig. 4*A*), the peripheral glia release each neurotransmitter, and the perineurial glia respond. In the second mechanism (indirect signaling; Fig. 4*B*), each neurotransmitter is released by neurons, and the peripheral glia respond by regulating the release of a trophic factor that acts on perineurial glia.

Although direct signaling seems to be the simplest possibility, indirect signaling is most consistent with previous studies. As described above, both invertebrate and mammalian motor neurons can release small molecule and peptide neurotransmitters that affect properties of Schwann cells. A similar motor nerve terminal-peripheral glia communication could occur in *Drosophila*, because first boutons at the larval neuromuscular junction are covered by peripheral glia (27). This observation raises the possibility that *Drosophila* peripheral glia might respond to Amn and NT released from motor nerve terminals, and propagate these signals along the length of the nerve via gap junctions. However, the alternative possibility of NT release from along the length of axons, as has been suggested in other systems (28, 29), cannot be ruled out. In addition, mammalian Schwann cells release trophic factors such as Desert Hedge Hog (Dhh) to induce growth of the surrounding perineurium (6), and astrocytes can respond to glutamate application by releasing a substance that affects blood vessels (30). Our model predicts that peripheral glia release a trophic factor that behaves similarly to Dhh. The prediction that *Drosophila NF1* acts within peripheral glia is consistent with the likelihood that mammalian *NF1* acts within Schwann cells as well (31, 32).

The possible effects of the thickened perineurial glia on motor neuron function are unclear. Mutations in four of the genes that affect perineurial glial thickness (*eag*, *NF1*, *ine*, and *push*) were each shown in previous studies to increase either neuronal or muscle membrane excitability (12–14, 16, 17, 19), which raises the possibility of a correlation between excitability and perineurial glial growth. However, we have been unable to detect any increases in neuronal excitability in the *amn* mutant or the *ine; NF1* double mutant (greater than that conferred by the *ine* mutation alone), despite the presence of greatly thickened perineurial glia in these genotypes (data not shown). It is possible that the effects on neuronal excitability of these genotypes might be subtler than our assays can detect, or that the participation of these genes in both perineurial glial growth and excitability is coincidental.

Our results are consistent with the previous observations that *push* and *NF1* act downstream of the Amn/PACAP receptor (ref. 16; S. Hawley, personal communication). However, the precise nature of the interactions among these proteins is unknown. Thus, it is possible that the interactions are direct, and that Push, the *NF1*-encoded protein Neurofibromin, and the Amn receptor bind to each other in a macromolecular complex. Alternatively, it is possible that Push and Neurofibromin mediate the effects of Amn only indirectly. In either case, the observation that the *push*^{1}*; NF1*^{P2} double mutant exhibits a perineurial glial thickness much greater than *push*^{1} or *NF1*^{P2} alone is consistent with the possibility that Push and Neurofibromin mediate the Amn signal through parallel partially redundant pathways (Fig. 4).

The indirect signaling model described in Fig. 4*B* could explain the partial cell-nonautonomy of *NF1* in neurofibroma formation. Neurofibromas most likely initiate in individuals heterozygous for *NF1* mutations by loss of the *NF1*$^{+}$ allele in Schwann cells. However, neurofibromas contain, in addition to Schwann cells,

cells derived from fibroblasts, perineurial cells, and neurons, which are thought to remain phenotypically $NF1^+$. We suggest that *NF1* mutant Schwann cells cause the overproliferation of their wild-type neighbors by oversecreting trophic factors, and that this oversecretion might ultimately occur as a consequence of defective receipt of a neurotransmitter signal from neurons.

Note Added in Proof. Phenotypes of mutants defective in BIG, the *Arabidopsis* ortholog of *push*, were recently reported (35).

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1. Edwards, J. S., Swales, L. S. & Bate, M. (1993) *J. Comp. Neurol.* **333,** 301–308.
2. Hurd, D. D. & Saxton, W. M. (1996) *Genetics* **144,** 1075–1085.
3. Jessen, K. R. & Mirsky, R. (1999) *Trends Neurosci.* **22,** 402–410.
4. Jahromi, B. S., Robitaille, F. & Charlton, M. P. (1992) *Neuron* **8,** 1069–1077.
5. Villegas, J. (1995), in *Neuron–Glia Interrelations During Phylogeny II. Plasticity and Regeneration*, eds. Vernadakis, A. & Roots, B. I. (Humana, Totowa, NJ), pp. 95–127.
6. Parmantier, E., Lynn, B., Lawson, D., Turmaine, M., Namini, S. S., Chakrabarti, L., McMahon, A. P., Jessen, K. R. & Mirsky, R. (1999) *Neuron* **23,** 713–724.
7. Pfrieger, F. W. & Barres, B. A. (1997) *Science* **277,** 1684–1687.
8. Yuan, L. L. & Ganetzky, B. (1999) *Science* **283,** 1343–1345.
9. Viskochil, D., White, R. & Cawthon, R. (1993) *Annu. Rev. Neurosci.* **16,** 183–205.
10. Feany, M. B. & Quinn, W.G (1995) *Science* **268,** 869–873.
11. Soehnge, H., Huang, X., Becker, M., Whitley, P., Conover, D. & Stern, M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13262–13267.
12. Wu, C.-F. & Wong, F. (1977) *J. Gen. Physiol.* **69,** 705–724.
13. Burg, M. G., Geng, C., Guan, Y., Koliantz, G. & Pak, W. L. (1996) *J. Neurogenet.* **11,** 59–81.
14. Richards, S., Hillman, T. & Stern, M. (1996) *Genetics* **142,** 1215–1222.
15. The, I., Hannigan, G. E., Cowley, G. S., Reginald, S., Zhong, Y., Gusella, J. F., Hariharan, I. K. & Bernards, A. (1997) *Science* **276,** 791–794.
16. Guo, H. F., The, I., Hannan, F., Bernards, A. & Zhong, Y. (1997) *Science* **276,** 795–798.
17. Warmke, J., Drysdale, R. & Ganetzky, B., (1991) *Science* **252,** 1560–1562.
18. Riesgo-Escovar, J., Woodward, C., Gaines, P. & Carlson, J. (1992) *J. Neurobiol.* **23,** 947–964.
19. Stern, M. & Ganetzky, B. (1992) *J. Neurogenet.* **8,** 157–172.
20. Crowley, T. E., Hoey, T., Liu, J. K., Jan, Y. N., Jan, L. Y. & Tjian, R. (1993) *Nature(London)* **361,** 557–561.
21. Castrillon, D. H., Gonczy, P., Alexander, S., Rawson, R., Eberhart, C. G., Viswanathan, S., DiNardo, S. & Wasserman, S. A. (1993) *Genetics* **135,** 489–505.
22. Fabrizio, J. J., Hime, G., Lemmon, S. K. & Bazinet, C. (1998) *Development (Cambridge, U.K.)* **125,** 1833–1843.
23. Xu, X. Z., Wes, P. D., Chen, H., Li, H. S., Yu, M., Morgan, S., Liu, Y. & Montell, C. (1998) *J. Biol. Chem.* **273,** 31297–31307.
24. Sekelsky, J. J., McKim, K. S., Messina, L., French, R. L., Hurley, W. D., Arbel, T., Chin, G. M., Deneen, B., Force, S. J., Hari, K. L., *et al.* (1999) *Genetics* **152,** 529–542.
25. Moore, M. S., DeZazzo, J., Luk, A. Y., Tully, T., Singh, C. M. & Heberlein, U. (1998) *Cell* **93,** 997–1007.
26. Zhong, Y. (1995) *Nature (London)* **375,** 588–592.
27. Sepp, K. J., Schulte, J. & Auld, V. J. (2000) *Glia* **30,** 122–133.
28. Kriegler, S. & Chiu, S. Y. (1993) *J. Neurosci.* **13,** 4229–4245.
29. Murphy, S., Minor, R. L., Wel, G. & Harrison, D. G. (1990) *J. Neurochem.* **55,** 349–351.
30. Lieberman, E. M. & Sanzenbacher, E. (1992) *Neuroscience* **47,** 931–939.
31. Kluwe, L., Friedrich, R. & Mautner, V. F. (1999) *Genes Chromosomes Cancer* **24,** 283–285.
32. Sherman, L. S., Atit, R., Rosenbaum, T., Cox, A. D. & Ratner, N. (2000) *J. Biol. Chem.* **275,** 30740–30745.
33. Cawthon, R. M., Weiss, R., Xu, G. F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland R., O'Connell, P., *et al.* (1990) *Cell* **62,** 193–201.
34. Hofmann, K. & Stoffel, W. (1993) *Biol. Chem. Hoppe–Seyler* **374,** 166.
35. Gil, P., Dewey, E., Friml, J., Zhao, Y., Snowden, K., Putterill, J., Palme, K., Estelle, M. & Chory, J. (2001) *Gene Dev.* **15,** 1985–1997.