Noradrenergic Regulation of Cholinergic Differentiation

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When the sympathetic nerves that innervate rat sweat glands reach their targets, they are induced to switch from using norepinephrine as their neurotransmitter to acetylcholine. Catecholamines (such as norepinephrine) released by nerves growing to the sweat gland induce this phenotypic conversion by stimulating production of a cholinergic differentiation factor [sweat gland factor (SGF)] by gland cells. Here, culture of gland cells with sympathetic, but not sensory, neurons induced SGF production. Blockage of α_1- or β-adrenergic receptors prevented acquisition of the cholinergic phenotype in sympathetic neurons co-cultured with sweat glands, and sweat glands from sympathectomized animals lacked SGF. Thus, reciprocal instructive interactions, mediated in part by small molecule neurotransmitters, direct the development of this synapse.

Neurons communicate with target tissues at synapses by releasing neurotransmitters. Each class of neurons has a characteristic repertoire consisting of a small molecule transmitter and a neuropeptide (or neuropeptides), and subpopulations of neurons within a class share a distinctive complement of transmitter and peptide. Our understanding of how this diversity arises during development is incomplete. In peripheral neurons, environmental factors influence this aspect of neuronal development. Thus, sympathetic neurons dissociated from superior cervical ganglia (SCG) of newborn rats are initially noradrenergic, and when grown in the absence of non-neuronal cells, they continue to differentiate as noradrenergic neurons. When cultured in the presence of certain non-neuronal cells or of differentiation factors such as ciliary neurotrophic factor (CNTF) or leukemia inhibitory factor (LIF), however, their noradrenergic properties are reduced and they acquire a cholinergic phenotype instead (*1–3*). The sympathetic innervation of sweat glands in the developing rat footpad undergoes an analogous switch in neurotransmitter properties during development (*4, 5*). The sympathetic axons innervating rat sweat glands are initially noradrenergic, but as the sweat gland innervation matures, noradrenergic markers decrease and cholinergic and peptidergic properties are acquired (*6–8*), so that the mature sweat gland innervation is functionally cholinergic (*9*). This postnatal switch depends on the target sweat glands (*10, 11*).

To elucidate the molecular mechanisms of this switch, investigators have characterized sweat gland–neuron interactions in vitro. Treatment of cultured sympathetic neurons with soluble extracts of rat footpad tissue reproduces the neurotransmitter changes seen in vivo (*12–14*), which indicates that these extracts contain a differentiation factor [sweat gland factor (SGF)] that induces cholinergic and reduces noradrenergic properties in the sweat gland innervation. In primary cultures of sweat gland cells, production of SGF required neurons (*15*), an unexpected result. Thus, sweat gland cells grown alone do not produce the differentiation factor, but culture of gland cells with dissociated sympathetic neurons induces cholinergic properties in those neurons, and conditioned medium (CM) from co-cultures causes a similar change in naïve sympathetic neurons. These observations suggest that SGF production by developing sweat glands is induced by innervation. Here we examine the mechanism by which sweat gland innervation regulates SGF expression.

To further characterize the interactions between neurons and sweat glands, we used two variations of the co-culture procedure. Because morphological studies have indicated that signaling between sweat gland innervation and developing glands is mediated by secreted factors (6), we used transfilter co-cultures to test whether soluble molecules regulate production of SGF in co-cultures. Although a 0.45-μm filter prevented contact between neurons and gland cells, it did not alter the cholinergic differentiation of the neurons (Fig. 1), which indicates that the neuronal signal that induces secretion of the differentiation factor is soluble rather than membrane-associated. In addition, we determined whether other peripheral neurons could induce factor expression by gland cells or if this ability was restricted to sympathetic neurons. Gland cells were cultured with dissociated dorsal root ganglion (DRG) sensory neurons, a population of neurons that innervate non-gland targets in the footpad, and CM from these co-cultures was tested for cholinergic differentiation activity. In contrast to culture with sympathetic neurons, culture of sweat gland cells with sensory neurons failed to induce release of SGF into the CM (Fig. 2). Therefore, sensory neurons lack a component that is critical for signaling to gland cells.

Because norepinephrine is secreted by sympathetic, but not sensory, neurons, we tested the possibility that norepinephrine released by sympathetic neurons regulates SGF production. Noradrenergic transmission was disrupted in co-cultures by addition of adrenergic receptor antagonists. Although neurons in sympathetic neuron–sweat gland co-cultures grown in control medium acquired a cholinergic phenotype, those grown with the adrenergic antagonists prazosin (α_1), propranolol (β_1, β_2), or alprenolol (β_2) lacked cholinergic function (Fig. 3). In contrast, cells grown with the antagonists yohimbine (α_2) or atropine, which are antagonists of muscarinic acetylcholine receptors, acquired cholinergic properties similar to those of cells grown in control co-cultures [choline acetyltransferase activity was measured as a percentage of the control: control co-culture, 100%; atropine, 114 ± 15%, three experiments

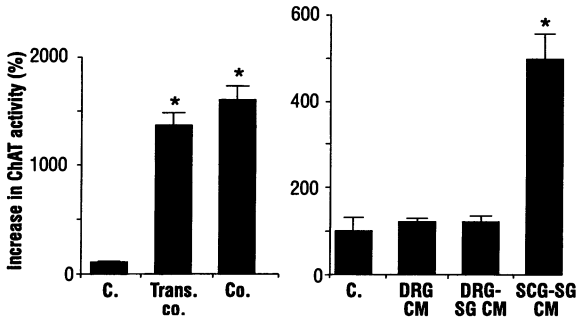

Fig. 1 (left). Soluble factors mediate signaling in neuron-gland co-cultures. Sweat gland cells were added to sympathetic neurons or to a 0.45-μm filter over neurons (*21*). Five days later, control neurons (C.), transwell co-cultures (Trans. co.), and co-cultures (Co.) were assayed in duplicate for ChAT, the synthetic enzyme for acetylcholine (*22*). Data are representative of three experiments (±SD, asterisks indicate $P < 0.05$). **Fig. 2 (right).** Sympathetic, but not sensory, neurons induce SGF production in sweat gland cells. Sweat gland cells (SG) were cultured for 6 days with either sympathetic (SCG) or sensory (DRG) neurons and conditioned media were collected (*21–23*). Sympathetic neurons were grown for 7 days in CM and triplicates were assayed for ChAT (±SD, asterisks indicate $P < 0.05$). Data are representative of three experiments.

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(±SEM); yohimbine 91 ± 7%, four experiments (±SEM)]. These observations suggest that activation of α_1- and β-adrenergic receptors is critical for production of SGF in neuron–sweat gland co-cultures and for the resulting change in neuron phenotype.

To determine if catecholamines and adrenergic antagonists act through receptors in sweat glands or neurons, we collected CM from control and propranolol-treated co-cultures and from sweat gland cells treated with forskolin to mimic activation of β receptors. Conditioned medium from control co-cultures and from forskolin-treated sweat gland cells induced cholinergic properties in sympathetic neurons, but treatment of sympathetic neurons with forskolin or adenosine 3′,5′-monophosphate analogs did not induce cholinergic function (*16*). Medium from propranolol-treated co-cultures induced little cholinergic activity in neurons (Fig. 3), although propranolol did not decrease the cholinergic activity induced by co-culture CM [choline acetyltransferase activity was measured as a percentage of the control: co-culture CM, 100%; propranolol, 99 ± 10%, two experiments (±SD)]. Therefore, catecholamines activate adrenergic receptors on sweat glands to induce SGF production, rather than affecting sympathetic neurons directly.

Culture studies indicate that adrenergic receptors are crucial for the production of differentiation factor in co-cultures and suggest that a similar mechanism regulates production in vivo. Although catecholamines are present in early sweat gland innervation [postnatal day (P) 4 to 14], adrenergic receptor expression has not been characterized in developing glands. Analysis of adrenergic receptor mRNA by reverse transcription polymerase chain reaction indicates that sweat gland cultures and gland-containing footpads from P10 rats express mRNA encoding α_1-, α_2-, and β_2-adrenergic receptors (Fig. 4). Radioligand binding studies on footpad sections from P10 rats confirm the presence of α_1 and β_2 receptors and localize their expression in developing sweat glands (Fig. 4), which indicates that gland cells bind norepinephrine secreted by the gland innervation.

To test whether sympathetic innervation is necessary for the production of SGF in developing sweat glands in vivo, we treated newborn rats with the neurotoxin 6-hydroxydopamine (6-OHDA), which specifically destroys developing sympathetic neurons (*17*). In contrast to surgical denervation (*14*), chemical sympathectomy does not disrupt sensory innervation of footpads or alter CNTF expression in Schwann cells (*18*). Extracts were prepared from footpads of sympathectomized and of control animals at P21. Extracts from vehicle-injected rats induced a cholinergic phenotype in sympathetic neuron cultures but those from sympathectomized animals did not (Fig. 5), which indicates that sweat glands deprived of noradrenergic input do not produce SGF. Sweat glands of adult rats contain SGF even though their innervation is no longer noradrenergic. It is unclear if SGF production becomes innervation-independent or if activation of muscarinic acetylcholine and of vasoactive intestinal peptide receptors by the mature innervation (*7–9*) substitutes for

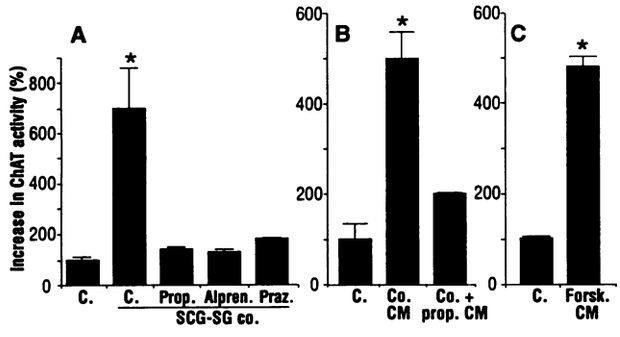

Fig. 3. (**A**) Adrenergic receptor antagonists prevent cholinergic differentiation in sympathetic neuron–sweat gland co-cultures (Co.). Co-cultures were grown in control medium (C.) or in medium containing 1 μM (−)propranolol (β_1 and β_2) (Prop.), 10 μM alprenolol (β_2) (Alpren.), or 100 nM prazosin (α_1) (Praz.) (*21*). Drugs were added every 2 days, and cells were harvested for ChAT assay after 5 to 7 days (*22*). Samples were assayed in triplicate (±SD, asterisk indicates $P < 0.05$) and are representative of four or more experiments. (**B** and **C**) Antagonists act on sweat glands. Conditioned media were collected from control and propranolol-treated co-cultures and from sweat gland cultures treated for 24 hours with 10 μM forskolin (Forsk.). Samples were concentrated (*23*) and added to sympathetic neurons 7 days before ChAT assay [$n = 2$ or 3 (±SD, asterisks indicate $P < 0.05$)]. Data are representative of three experiments.

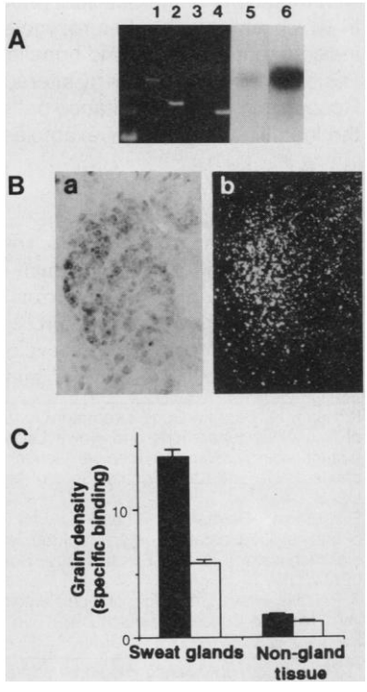

Fig. 4. Sweat glands express adrenergic receptors. (**A**) Total RNA was isolated from cultured sweat gland cells and from gland-containing footpads of P10 rats. After reverse transcription, oligonucleotides specific to α_1-, α_2-, β_1- and β_2-adrenergic receptors (*24*) were used to prime polymerase chain reactions. Unmarked lane shows 123–base pair ladder; lanes 1 to 4 show amplification products from cultured cells (α_1, α_2, β_1, and β_2); lanes 5 (α_1) and 6 (β_2) show Southern (DNA) blots of products from footpads. Rat brain cDNA was a positive control and RNA was a negative control for all primers (*18*). (B and C) Radioligand binding in footpads. Sections were incubated with either ^{125}I-labeled (2-{β-(4-hydroxy-3-[^{125}I]iodophenyl)-ethylaminomethyl}-tetralone) ±100 nM prazosin to identify α_1 receptors or with ^{125}I-labeled pindolol ±10 μM propranolol to identify β receptors (*25*). (**B**) ^{125}I-labeled pindolol identifies β-receptor localization. Shown are a bright field micrograph of a footpad section with stained glands (a) and a dark field micrograph showing highest grain density over glands (b). (**C**) α_1 (open bars) and β (filled bars) binding was quantified by assessing grain density with the Cue 4 image analysis system (Olympus, Lake Success, New York). Data shown are the average of 25 fields (±SEM) minus the 20 to 25% of total binding not blocked by an antagonist. Similar results were obtained in four assays of β binding and two of α binding.

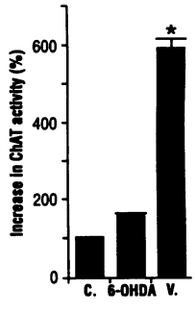

Fig. 5. Sweat gland factor activity does not appear in footpads of sympathectomized rats. Newborn rats were injected for 7 days with 100 mg per kilogram of body weight of the sympathetic neurotoxin 6-OHDA or of vehicle (V.) (0.9% NaCl and 1 mM ascorbate) (*17*). Control: C. At P21, extracts were prepared from footpads of sympathectomized and control animals and added to sympathetic neuron cultures at a concentration of 100 μg/ml. Medium was changed after 3 days, and ChAT activity was assayed after 6 days (*22*). Data shown are representative of extracts from three treated and three control animals [$n = 3$ (±SEM, asterisk indicates $P < 0.05$)].

noradrenergic receptor activation.

Our data show that catecholamines from sweat gland innervation induce production of a cholinergic differentiation factor in developing sweat glands, thereby triggering the switch to a cholinergic phenotype. Further, they indicate that the initial expression of catecholamines by cholinergic sympathetic neurons is not simply a default pathway (*4*) but is an essential step in their developmental differentiation. The requirement for target innervation, which is not necessary for production of nerve growth factor (*19*), the target-derived trophic factor for sympathetic neurons, may restrict the availability of the differentiation factor and increase the specificity of neuron-target interactions.

This study shows that both anterograde and retrograde signaling are needed for development of functional synapses between sympathetic neurons and sweat glands. Noradrenergic neurons contact the glands and stimulate production of SGF. Sweat glands release SGF, which induces cholinergic properties in the noradrenergic neurons, and acetylcholine then induces maturation of the sweat glands (*20*), which results in the establishment of a functional synapse.

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21. Sympathetic and sensory neuron cultures were prepared essentially as described by E. Hawrot and P. Patterson [*Methods Enzymol.* **58**, 574 (1979)] and by Rao and Landis (*12*), except that dorsal root ganglia were incubated for only 40 min in dispase and collagenase. Non-neuronal cells were removed by preplating, and neurons were grown in 10 μM arabinosylcytosine for the first 2 days. Neurons were plated with 25,000 to 30,000 cells per well in 24-well plates for transfilter experiments and 3000 to 4000 cells per well in 96-well plates for other experiments. Primary rat sweat gland cultures were established by explanting tissue from hind footpads of P7 to P9 rats. Pads were treated with dispase and collagenase for 75 min at 37°C before gland-containing regions were explanted and cells were grown to near confluence in serum-free medium [D. Gruenert, C. Basbaum, J. Widdicombe, *In Vitro Cell. Dev. Biol.* **26**, 411 (1990); G. Collie, M. Buchwald, P. Harper, J. Riordan, *ibid.* **21**, 257 (1985)]. Gland cells were added to neurons after 2 days, 4×10^5 cells per well in 96-well plates, 4×10^6 cells per well in 24-well plates or in filter inserts.
22. Cholinergic function was determined by the presence of choline acetyltransferase (ChAT) activity, assayed by the method of F. Fonnum [*Biochem. J.* **115**, 465 (1969)] as modified by Rao and Landis (*12*). Data were analyzed by analysis of variance with Statview II (Abacus Concepts, Berkeley, CA).
23. Conditioned medium was collected from day 5, concentrated through a 10-kD Centricon filter for 90 min at 3000 rpm, diluted in medium, and sterilized through a 0.22-μm filter.
24. RNA was isolated by the method of P. Chomczynski and N. Sacchi [*Anal. Biochem.* **162**, 156 (1987)]. Samples were reverse-transcribed with the Promega cDNA synthesis kit and amplified by polymerase chain reaction through 35 cycles (1 min each at 94°, 52°, and 72°C) with primers encoding subtype-specific regions of receptor proteins: α_1(+)TCTAGGCGCGTGCGCTTACA, (−)GCCAGGTCGGTCGTGGTGTC (bases 1373 to 1393 and 1713 to 1733) [M. Voight, J. Kispert, H. Chin, *Nucleic Acids Res.* **18**, 1053 (1990)]; α_2 (+)CCCAAGCCTCCAAGAGAGAA, (−)CCACTGGCAACTCCCACATT (bases 1133 to 1153 and 1417 to 1437) [D. Zeng *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3102 (1990)]; β_1 (+)GCCCAGAAACAGGTGAAGAA, (−)CCTTGGACTCGGAGGAGAAG (bases 760 to 780, and 1376 to 1396) [C. Machida *et al.*, *J. Biol. Chem.* **265**, 12960 (1990)]; β_2 (+)AGAGCACAAAGCCCTCAAGA, (−)AGCCGTTCCCATAGGTTTTC (bases 804 to 824 and 1044 to 1064) [P. Buckland *et al.*, *Nucleic Acids Res.* **18**, 682 (1990)]. Hybridization was carried out in Quickhyb (Stratagene) with ^{32}P-labeled oligonucleotides [α_1, bases 1442 to 1472; β_1, bases 930 to 960 (cross-hybridizes with β_2)] that were washed in 0.1× standard saline citrate and 0.2% SDS at 45°C and exposed onto film overnight.
25. Rats at P10 were perfused with phosphate-buffered saline, and 10-μm footpad sections were mounted on gelatin-coated slides. For α_1, sections were incubated with 50 pM (2-{β(4-hydroxy-3-[^{125}I]iodophenyl)-ethylaminomethyl}-tetralone) with or without 100 nM prazosin as described [H. Glossmann, F. Lübbecke, P. Presek, *Eur. J. Pharmacol.* **75**, 149 (1981)]. For β, sections were incubated with 50 pM ^{125}I-labeled pindolol with or without 10 μM propranolol [V. Arango *et al.*, *Brain Res.* **516**, 113 (1990)]. Autoradiography and receptor quantitation were completed as described by M. Grant, S. Landis, and R. Siegel [*J. Neurosci.* **11**, 3763 (1991)]. Autoradiograms were exposed for 2 days at 4°C.

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Digenic Retinitis Pigmentosa Due to Mutations at the Unlinked Peripherin/*RDS* and *ROM1* Loci

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In spite of recent advances in identifying genes causing monogenic human disease, very little is known about the genes involved in polygenic disease. Three families were identified with mutations in the unlinked photoreceptor-specific genes *ROM1* and peripherin/*RDS*, in which only double heterozygotes develop retinitis pigmentosa (RP). These findings indicate that the allelic and nonallelic heterogeneity known to be a feature of monogenic RP is complicated further by interactions between unlinked mutations causing digenic RP. Recognition of the inheritance pattern exemplified by these three families might facilitate the identification of other examples of digenic inheritance in human disease.

Retinitis pigmentosa (RP) is the name given to a set of hereditary human diseases that cause blindness resulting from degeneration of rod and cone photoreceptors in the retina. Patients typically develop night blindness in adolescence, lose midperipheral vision in young adulthood, and are blind by middle age. In the United States alone 50,000 to 100,000 people are afflicted with RP. Oral vitamin A supplementation has been reported to slow but not stop the course of the disease (*1*). RP exhibits genetic heterogeneity and can be transmitted as an autosomal dominant, autosomal recessive, or X-linked trait. Most cases are considered to be monogenic; that is, in any given family, only one responsible locus is thought to be defective. Although polygenic inheritance could explain the variable expressivity seen in some pedigrees (*2*), no specific combinations of genes have been identified that cause or modify this disease.

We identified three families with RP

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