Norepinephrine Facilitates the Development of the Murine Sweat Response But Is Not Essential

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During development, the sympathetic neurons innervating sweat glands undergo a neurotransmitter switch from noradrenergic to cholinergic between postnatal day (P) 4, when the sympathetic neurons first contact the sweat glands, and P21. Several *in vitro* experiments suggest that norepinephrine (NE), produced by sympathetic neurons, stimulates sweat glands to produce a factor that then induces the phenotypic switch. We tested this hypothesis *in vivo* using dopamine β-hydroxylase-deficient mice (DBH −/−), which are unable to synthesize NE and epinephrine, and tyrosine hydroxylase-deficient mice (TH −/−), which are unable to synthesize any catecholamines. The cholinergic agonist pilocarpine and electrostimulation of the sciatic nerve both elicited a sweat response in adult DBH −/− mice that was indistinguishable from the response of controls, and the cholinergic antagonist atropine effectively blocked these responses. We did note, however, a 1- to 2-week delay in the acquisition of the sweat response in DBH −/− mice. Although diminished in magnitude, a sweat response to pilocarpine was also noted in TH −/− mice at P21. Immunohistochemistry demonstrated that TH and vasoactive intestinal peptide were detectable at P14 and increased to adult levels by P21 in DBH +/− and DBH −/− mice. These observations indicate that NE is not essential for the acquisition of the cholinergic phenotype, but it may facilitate its postnatal development.

Key words: norepinephrine; acetylcholine; sweat gland factor; mouse; sweating; dopamine β-hydroxylase; tyrosine hydroxylase; vasoactive intestinal peptide

Rodent sweat glands are coiled tubules found primarily on the digits and footpads of the paws (Kennedy et al., 1984). They mature during the second and third postnatal weeks and are thought to provide enhanced tactile sensitivity rather than thermoregulation (Wechsler and Fisher, 1968). Although the glands develop a normal morphology without innervation (Yodlowski et al., 1984), a sweat response depends on innervation by the sympathetic nervous system (Stevens and Landis, 1988). The sympathetic neurons that initially make contact with the developing sweat glands at P4 are noradrenergic: they express tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) and produce norepinephrine (NE) (Landis and Keefe, 1983). Beginning at about postnatal day (P) 11 and continuing to P21, however, there is a gradual loss of these noradrenergic markers and the acquisition of the cholinergic markers choline acetyltransferase and acetylcholinesterase, as well as the neuropeptides, vasoactive intestinal peptide (VIP), and calcitonin gene-related peptide (Landis and Keefe, 1983; Leblanc and Landis, 1986; Stevens and Landis, 1987; Landis et al., 1988). Sweating in the adult (P21 and older) is elicited by cholinergic agonists and blocked by cholinergic antagonists. Noradrenergic agonists do not elicit sweating nor do noradrenergic antagonists block sweating (Stevens and Landis, 1987).

The switch from noradrenergic to cholinergic phenotype depends on the interaction of the neurons with the target tissue. Schotzinger and Landis (1988) showed that when a rat footpad was transplanted to the flank of a neonatal rat, the sympathetic neurons that would normally remain noradrenergic become cholinergic after contact with the transplanted footpads. The converse experiment showed that when a normal noradrenergic target was transplanted to the foot, the neurons maintained their noradrenergic phenotype (Schotzinger and Landis, 1990). *In vitro* experiments indicated that a soluble factor called sweat gland cholinergic differentiation factor (SGF) is released from footpads to induce switching of the neuronal phenotype (Habecker and Landis, 1994). The production of SGF by the footpads is thought to depend on innervation (Habecker et al., 1995). A logical candidate for the factor derived from the noradrenergic neurons is NE. Consistent with this idea, Habecker and Landis (1994) showed that noradrenergic antagonists for either α_1- or β-receptors blocked neuronal induction of SGF in neuron/footpad co-cultures and that forskolin, a direct activator of adenylyl cyclase, stimulated the production of SGF in footpads cultured without neurons.

Innervation is also necessary for cholinergic agonists to elicit a sweat response (Grant et al., 1991). Cholinergic receptors, of the muscarinic subtype m_3, appear in normal abundance and have normal affinity for cholinergic agonists, but in the absence of innervation they cannot elicit a sweat response. Thus, some stimulus from the nerves is also responsible for activation of the signal transduction pathway linking m_3 with sweating. This factor may be acetylcholine, given the ability of cholinergic agonists to maintain secretory responsiveness in denervated glands (Grant et al., 1995).

We generated mice that cannot make NE because of the inactivation of the DBH gene, which is essential for the conver-

sion of dopamine (DA) to NE (Thomas et al., 1995). Although most DBH $-/-$ mice die during gestation, they can be rescued pharmacologically until birth. After birth, they develop into viable adults without further intervention (Thomas et al., 1995). The DBH $-/-$ mice have almost no NE beginning at P2, which is when the sweat gland primordia begin to develop and before innervation by noradrenergic fibers. Thus, if NE is necessary to induce SGF expression, which in turn causes switching, then the switch should not occur in DBH $-/-$ mice, and they should not sweat in response to cholinergic agonists.

MATERIALS AND METHODS

Animals. The mice were fourth and fifth generation hybrids of C57BL/6J and 129/SvCPJ. DBH $+/-$ females were mated with DBH $-/-$ males and treated with 10 μg/ml each of phenylephrine and isoproterenol (Sigma, St. Louis, MO) from embryonic day (E) 9.5–14.5, and 1 mg/ml L-threo-3,4-dihydroxyphenylserine (DOPS; a generous gift from Sumitomo Pharmaceuticals, Osaka, Japan) from E14.5 to birth in the maternal drinking water. TH $+/-$ males and females were mated, and the females were treated with 1 mg/ml DOPS from E 9.5 to birth. The pups were genotyped by PCR at 3–4 weeks of age. DBH $+/-$ mice were used as controls because NE levels are normal in these mice (see Fig. 1), and the number of glands responding to pilocarpine did not differ between DBH $+/+$ and DBH $+/-$ adult mice. Adults used in these experiments ranged in age from 2 to 4 months.

Catecholamine measurements. The methods described in Thomas et al. (1995) were used. Briefly, footpads and neonates were frozen on dry ice and stored at -70°C. Footpads were sonicated; neonates were first homogenized in 4.5 ml of perchloric acid buffer/gram and then 0.4 ml was sonicated. All samples were maintained at 4°C until injection into the HPLC. Sonicates were centrifuged for 10 min at 18,700 $\times$ *g*. A 200 μl aliquot of supernatant was added to 20 mg of acid-washed alumina and 800 μl of Tris/EDTA buffer and rotated overnight. The alumina was washed twice with 1 ml of distilled water, and then catecholamines were extracted with perchloric acid buffer. The HPLC was run at a flow rate of 1 ml/min, and an injection volume of 20 μl was used. The working potential of the electrochemical detector (BAS LC-4C) was $+0.8$ V, and the full scale sensitivity was 5 nA. Quantification of catecholamines was performed by comparing the peak heights of unknowns to those of known quantities of catecholamine standards using an HP 3393A integrator. The lower limit of detection was 20 pg. Protein was determined using Bio-Rad protein microassay (Bio-Rad, Hercules, CA).

Agonist-induced sweat response. The agonist-induced sweat response was obtained by first anesthetizing the animal with a ketamine (Fort Dodge Laboratories, Fort Dodge, KA)/xylazine (Phoenix Pharmaceutical) mixture (0.66 ml ketamine/0.22 ml xylazine in 10 ml PBS; dose 0.025

ml/gm). The left hind paw was then painted with an iodine (Sigma) solution (5 mg/100 ml ethanol) followed by a coat of starch solution (5 gm/10 ml mineral oil). Pilocarpine (Sigma) was administered subcutaneously into the foot (50 μg), and 2 min after injection a photograph was taken of the sweat response.

Nerve-evoked sweat response. The nerve-evoked sweat response was obtained by anesthetizing the animal with the ketamine/xylazine mix and then isolating the sciatic nerve in the left hind leg. The foot was painted with the iodine and starch solutions. The nerve was then placed on a bipolar stimulating electrode (provided by A. Berger, University of Washington) for 2–3 min and stimulated at 15 V, 5.5 Hz (Stevens and Landis, 1987). A picture of the nerve-evoked sweating was taken, and then atropine (Sigma) was administered intraperitoneally at 0.125 gm/kg. The foot was wiped clean, and the iodine/starch solutions were reapplied. The nerve was then stimulated a second time at the same conditions for 3–4 min, and another picture was taken to quantitate the sweat response after administration of atropine.

Immunohistochemistry. The footpads were fixed in 10% neutral buffered formalin (Richard-Allen, Richland, MI). They were embedded in paraffin blocks and cut into 10 μm sections. Before immunohistochemistry, the sections were deparaffinized and hydrated, and endogenous peroxidases were inactivated in 0.6% H_2O_2 for 15 min. Nonspecific binding was blocked with 1% BSA/3% goat serum for 1 hr (Vector Labs, Burlingame, CA). The primary antibodies against VIP (Incstar, Stillwater, MN) and TH (Eugene Tech, Ridgefield Park, NJ) were both generated in rabbits. The sections were incubated at 4°C with the primary antibodies TH (1:1000) or VIP (1:1000) in incubation buffer (1% BSA/ 0.5% Triton X-100, 1% goat serum in PBS) for 16–20 hr. The slides were rinsed with incubation buffer and then incubated with the secondary biotinylated goat-anti-rabbit antibody (Vector Labs, Burlingame, CA) diluted 1:200 in the same buffer for 1 hr. The sections were rinsed again with buffer and incubated 15–30 min with streptavidin–horseradish peroxidase (Zymed, San Francisco, CA) at 1:20 dilution. After two more rinses, the immunoreactivity was visualized using an aminoethyl carbazole substrate kit (Zymed). The sections were mounted in Aquamount (Lerner Labs, Pittsburgh, PA). Adjacent sections were treated as above except that incubation with the primary antibody was not performed. None of these control sections exhibited peroxidase immunoreactivity.

RESULTS

Agonist-induced sweating and the development of the sweat response

Because most mice lacking NE (DBH $-/-$ or TH $-/-$) die *in utero* (Thomas et al., 1995; Zhou et al., 1995), all homozygotes studied were rescued pharmacologically by supplying either adrenergic agonists or the NE precursor DOPS in the maternal
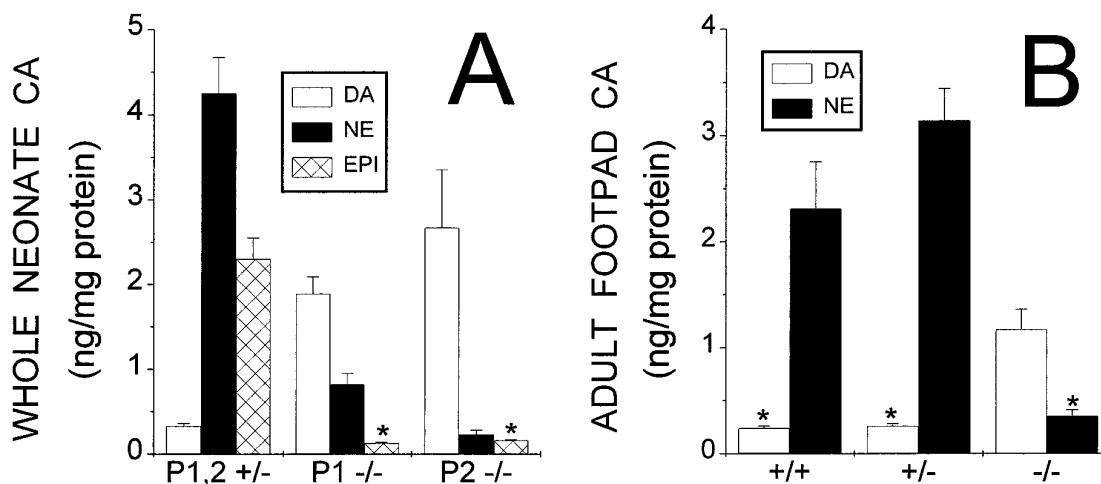

Figure 1. Catecholamine (CA) levels. *A,* Whole homogenized neonates either on the day of birth (*P1*) or the next day (*P2*). Results were combined for DBH $+/-$ neonates because they did not differ between the days. Levels are the mean $\pm$ SEM of 4–6 pups for each condition. *B,* Footpads from DBH $+/+$, DBH $+/-$, and DBH $-/-$ adult mice. Levels are the mean $\pm$ SEM of 5–6 footpads for each genotype. *Asterisks* indicate that the levels were below the limit of detection.
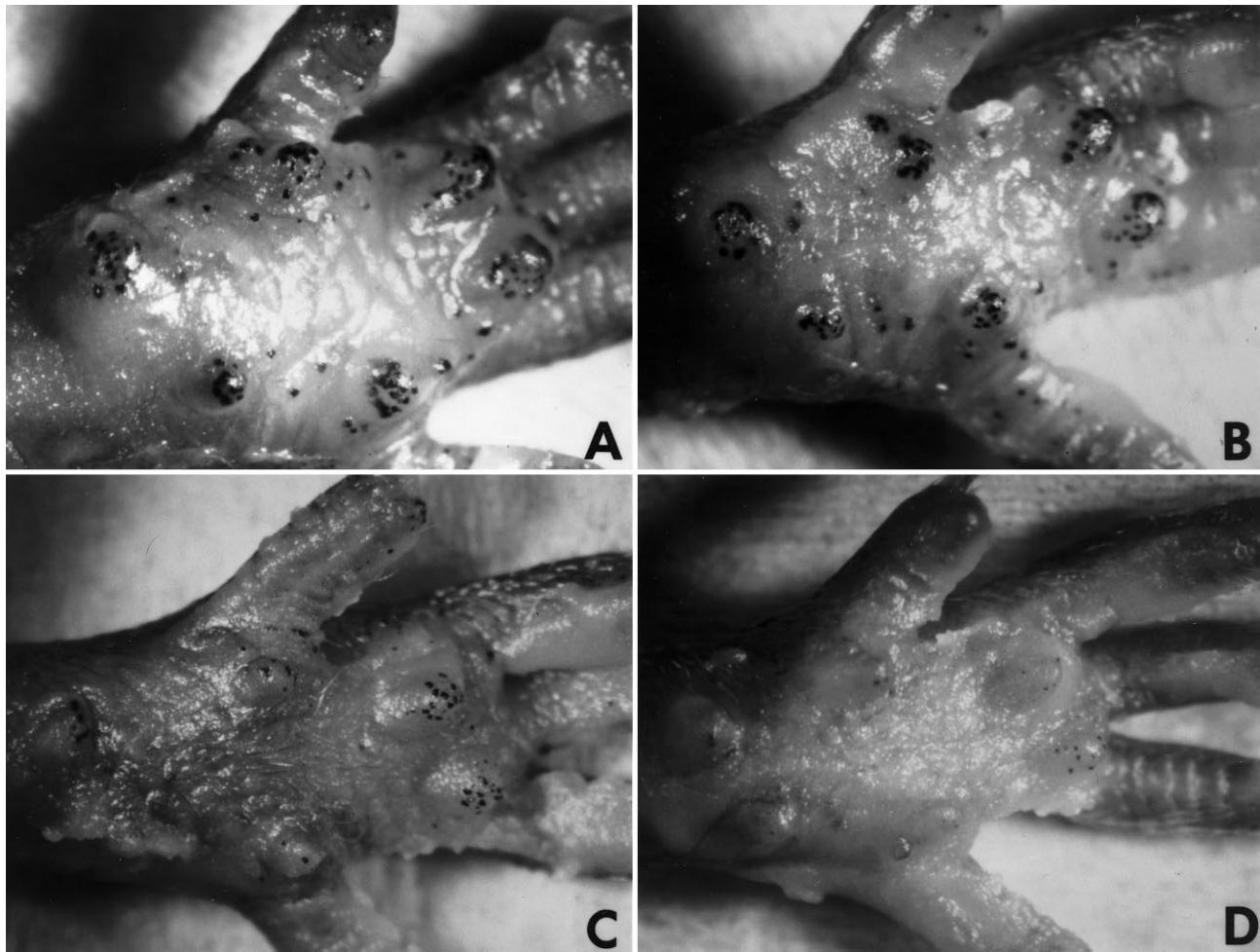
Figure 2. Pilocarpine-induced sweat response. *A*, Adult DBH +/− mouse. *B*, Adult DBH −/− mouse. *C*, P21 DBH +/− mouse. *D*, P21 DBH −/− mouse. Iodine/starch solutions were painted onto the left hind paw, the foot was injected with 50 μg pilocarpine, and the sweat response was photographed 2 min after injection. The *black dots* are the sweat response of individual glands.

drinking water beginning at E9.5 and continuing until birth. With this paradigm, whole pup NE levels were 19% of normal on the day of birth (P1) and very close to the limit of detection for NE 1 d later (<5% of normal) (Fig. 1*A*). Epinephrine was below the limit of detection in DBH −/− neonates (P1 or P2), whereas DA was significantly elevated to about half the level of NE found in controls. Thus NE falls to below the limit of detection within 48 hr of birth in DBH −/− neonates. Adult footpads had similar catecholamine levels. NE levels did not differ between DBH +/+ and DBH +/− footpads, whereas NE was below the limit of detection in DBH −/− footpads (Fig. 1*B*). DA was present in DBH −/− footpads at ~40% of the normal NE level, whereas DA was below the limit of detection in DBH +/+ and DBH +/− footpads. DBH +/− mice were routinely used as controls, because in addition to having normal levels of NE their sweat response did not differ from adult wild-type mice.

To ascertain whether NE-deficient mice could develop a normal sweat response, adult DBH −/− mice and heterozygous controls were injected with pilocarpine, a cholinergic agonist, and the response was measured by counting the number of glands that secreted amylase using the iodine-starch method (Wada and Takagaki, 1948). The hind foot was washed and painted with an iodine solution followed by a starch solution. Then a local injection of pilocarpine was administered, and a picture of the hind

foot was taken 2 min later. Pilocarpine elicited a sweat response in mice of both genotypes (Fig. 2). The number of black spots, each representing the secretion from a single gland, was determined. Although sweat responses were obtained on the digits as well as footpads, we focused on the six footpads for quantitation. There was no significant difference between adults of the two genotypes in the number of responsive glands (Fig. 3).

We also examined the development of the sweat response in both groups of mice as shown in Figure 3. At P11 and P14 either no responsive sweat glands or very few responsive sweat glands were detected in either group, but by P21 there was a robust response in DBH +/− mice that was about half that observed in adults (Figs. 2, 3*A*). The NE-deficient mice showed a significant lag in the emergence of a sweat response that was significant at P21. The normal growth spurt that usually begins at ~3 weeks of age is delayed 1–2 weeks in DBH −/− mice; consequently they are ~60% of normal weight at 4 weeks of age but they ultimately grow to ~85% of normal size (Thomas et al., 1995). The delay in sweat response in DBH −/− mice might reflect either their growth retardation, or more interestingly, NE might facilitate the development of an adult sweat response. We have also plotted the development of the sweat response as a function of body weight (Fig. 3*B*), which demonstrated that the developmental lag of the DBH −/− mice was less apparent. DBH −/− mice weighing ~10
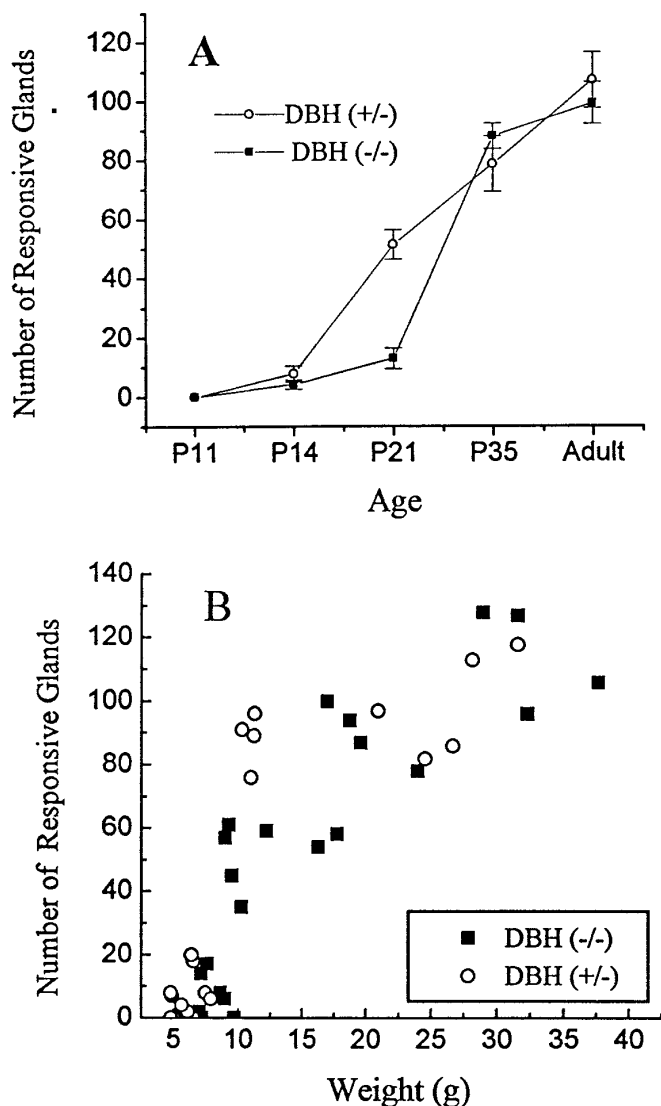
Figure 4. The pilocarpine-induced sweat response of TH −/− mice. *A*, TH +/− mice at P21 respond both on the footpads and the tips of the digits (*white arrows*). *B*, The TH −/− mice make no catecholamines yet can be induced to sweat using pilocarpine. The P21 TH −/− mice that were tested exhibited sweating mainly on the tips of the digits, and a few glands on the footpads also responded (*black arrows*).

Figure 3. Development of the sweat response in DBH +/− and DBH −/− mice. *A*, The sweat response was induced using 50 μg pilocarpine and detected with the iodine/starch method. No sweat response was detected in either DBH +/− or DBH −/− mice at *P11*, but note the fivefold difference between the genotypes at *P21* (*t* test; $p = 5 \times 10^{-4}$). These data reveal that the development of the sweat response in DBH −/− animals is delayed ~1–2 weeks. Once they reach adulthood, however, the sweat response is fully developed in both genotypes. Error bars represent SEM (*n* = 4 or 5 animals/genotype/age). *B*, The sweat response of the DBH +/− and DBH −/− mice as a function of weight, ages P11–adult. The general runting of the DBH −/− mice is not responsible for the delayed sweat response. At 10 gm, the DBH −/− mice show a significantly lower (*t* test; $p = 0.002$) number of responsive glands than DBH +/− mice. As the animals become larger (and older), both genotypes exhibit a comparable sweat response.

gm, however, still had a significantly lower number of responsive glands than the DBH ± mice at this weight, even though the DBH −/− mice were older (*t* test; $p = 0.002$).

The noradrenergic neurons of DBH −/− mice produce DA by default. Consequently, if sweat glands have appropriate DA receptors, they might be responding to DA instead of NE. Alternatively, DA might act as a weak agonist at adrenergic receptors. Because of these considerations, we also tested mice that lack TH, the first enzyme in the catecholaminergic pathway. These mice
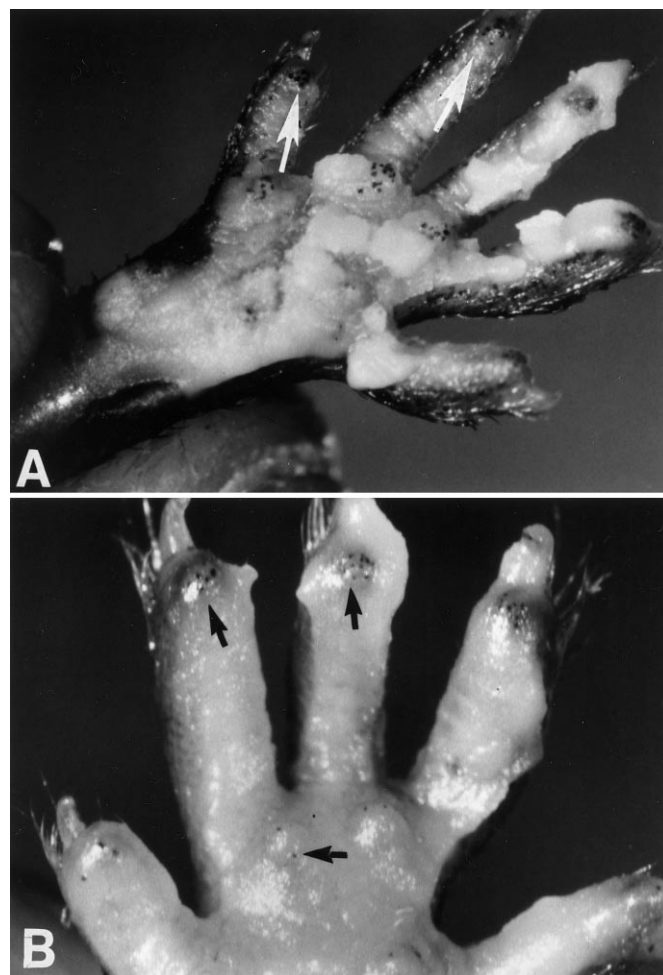
lack both DA and NE. The few that are born become runted and die a few weeks after birth (Zhou et al., 1995). We tested four TH −/− mice that survived to P21; the largest two (each 5 gm) showed a fairly normal sweat response on the digits, but only a few glands on the pads responded to pilocarpine (Fig. 4). The other two TH −/− mice did not elicit a sweat response; however, their weight (3.5 gm) was below that at which even control mice (8–10 gm at P21) elicited a response (Fig. 3*B*). Because the TH −/− mice were expected to die within several days, events not related to the absence of NE might account for the poorer sweat response as compared with DBH −/− mice at P21.

Nerve-evoked sweat response

The previous experiments demonstrated that the sweat glands of DBH −/− mice have functional cholinergic receptors because they respond to pilocarpine. To determine whether the neurons that innervate the sweat glands release acetylcholine, we examined nerve-evoked sweating. Electrostimulation of the sciatic nerve elicits sweating in cats by release of acetylcholine (Dale and Feldberg, 1934). In rats, the nerve-evoked sweat response is blocked by muscarinic cholinergic antagonists and is unaffected by adrenergic antagonists, demonstrating that it is a cholinergic event
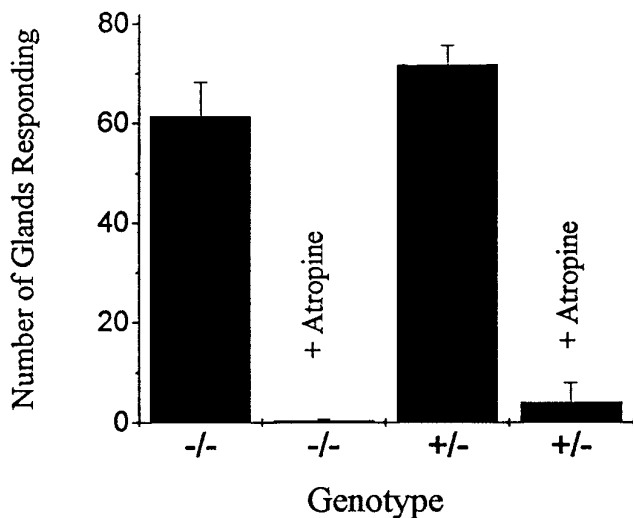
Figure 5. The nerve-evoked sweat response in adult DBH +/− and DBH −/− mice. The sciatic nerve of the left hind paw was stimulated by an electrode for 2–3 min, atropine was administered, and the nerve was stimulated a second time for 3–4 min. There were no significant differences in the number of responsive glands between the genotypes. Error bars represent SEM (*n* = 4 for each genotype).

(Stevens and Landis, 1987). The sweat response of DBH −/− mice to nerve stimulation was indistinguishable from that of DBH +/− controls (Fig. 5). Moreover, administration of atropine, a muscarinic cholinergic antagonist, after the first response blocked a subsequent response to electrostimulation in mice of both genotypes (Fig. 5). In the absence of atropine, a second response was nearly as strong as the first.

Innervation of the developing sweat glands

The morphology of the sweat glands of the DBH +/− and DBH −/− mice was indistinguishable (Fig. 6A,B). The switch from a noradrenergic to cholinergic phenotype in rats is associated with a downregulation of TH immunoreactivity and the appearance of ChAT and the neuropeptides VIP and CGRP (Leblanc and Landis, 1986; Landis et al., 1988; Stevens and Landis, 1988). To characterize the neuronal switch in mice, we performed immunocytochemistry on foot pads from mice at various ages. Our experiments revealed that there was a progressive gain in TH and VIP immunoreactivity in mice between P14 and adult (Fig. 6). VIP was faintly detectable at P14 in animals of both genotypes. At P21, VIP immunoreactivity was as strong in the DBH −/− as in the DBH +/− mice, although the sweat responses at this age were dissimilar (Fig. 3A). TH immunoreactivity was faint but detectable at P14 in DBH +/− mice, and even less apparent in the DBH −/− mice at P14. At P21, levels of immunoreactivity for TH were elevated to adult levels and similar between the genotypes.

DISCUSSION

The acquisition of the cholinergic phenotype in rats is dependent on the production of SGF, which presumably is induced by some signal from the neurons innervating the glands (Landis and Keefe, 1983; Landis et al., 1988). The secretory response of the sweat gland is dependent on the neurons innervating the glands (Grant et al., 1995), but the morphological development of the glands is not compromised by the absence of innervation (Yodlowski et al., 1984). Although muscarinic receptors are expressed at normal levels on the sweat glands even when there is no innervation

(Grant et al., 1991), innervation of the glands is the critical factor for the acquisition of the acetylcholine-mediated sweat response. Thus, it is reasonable to speculate that products released from nerves innervating the sweat glands are responsible for the induction of SGF and that NE could be a candidate. That β-adrenergic antagonists block the induction of SGF in sweat gland/sympathetic neuron cocultures is consistent with this postulation (Habecker and Landis, 1994). Also, activation of adenylyl cyclase by forskolin is sufficient to induce the production of SGF (Habecker and Landis, 1994), suggesting that NE might act by inducing cAMP signaling through β-adrenergic receptors. This same study, however, indicated that an α_1-adrenergic antagonist blocked induction of SGF, raising the question of whether these drugs were having nonspecific inhibitory effects, because β-adrenergic stimulation was expected to be sufficient.

Our results indicate that NE is not necessary for the acquisition of a mature sweat response in the mouse. Cholinergic agonists stimulate a response in the DBH −/− mice, and a nerve-evoked sweat response can be blocked with a cholinergic antagonist. Immunohistochemistry shows that TH increases dramatically between P14 and adult, as does the neuropeptide VIP. The major difference in the sweat responsiveness between the DBH −/− mice and normal mice is a developmental delay that is pronounced at P21, but this does not affect the expression of VIP or TH. The delay may be partly attributable to the general growth retardation of DBH −/− mice, but there is still an effect when the data are plotted as a function of mouse weight rather than age (Fig. 3B).

Adult DBH −/− mice have no detectable NE in footpads, as measured by electrochemical detection after HPLC (Fig. 1B). NE decays to undetectable levels within 48 hr of birth (Fig. 1A). Thus, even though the DBH −/− fetuses were rescued to birth by treating the mother with DOPS, no NE would be present after birth when the noradrenergic processes begin to innervate the developing sweat glands around P4. We cannot completely rule out the possibility, however, that NE present at the time of birth instructs the few sweat gland progenitor cells present to eventually release SGF. Sweat glands from normal rats that have presumably been exposed to circulating NE *in vivo*, however, do not produce SGF if the innervation to these glands is prevented by 6-hydroxydopamine treatment from P1 to P7 (Habecker and Landis, 1994). Of greater concern is the possibility that DA might substitute for NE, either as a weak agonist acting on adrenergic receptors or as an agonist acting on dopaminergic receptors. The amount of DA released from nerve terminals of DBH −/− mice is probably similar to the amount of NE that would normally be released in wild-type animals. The most compelling argument that switching does not require NE or DA is that some sweat glands respond in young TH −/− mice that produce neither NE nor DA. It is not clear why relatively more glands responded in the digits than the footpads of TH −/− mice (Fig. 5). Thus, we conclude that NE is not essential for induction of SGF and transmitter switching even though it does seem to facilitate the switch.

Previous studies of phenotypic switching of the neurons innervating the sweat glands were performed in the rat. In that system, immunoreactivity for TH decreases as that for VIP increases. Our work in the mouse, however, demonstrates a gradual increase in the expression of both TH and VIP between P7 and P21, although the glands are functionally cholinergic. Guidry and Landis (1995) demonstrated that TH-immunoreactive fibers innervate the developing sweat glands as early as P4, and the number of TH-positive fibers increases dramatically by P14. Rao et al. (1994)
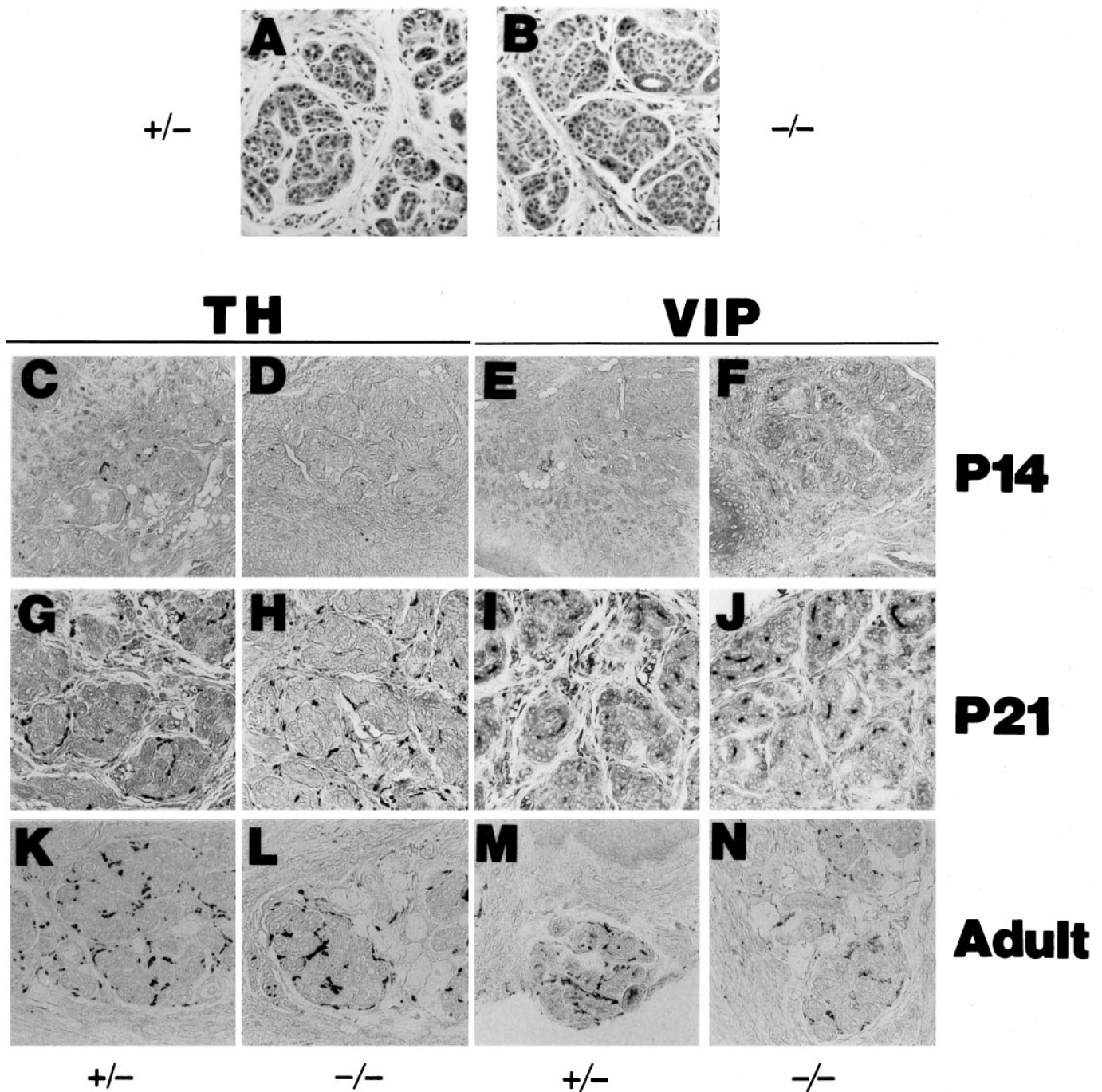
Figure 6. The development of the innervation of the sweat glands of DBH +/− and DBH −/− mice. Hematoxylin and eosin-stained adult glands show a comparable morphology. The expression of VIP and TH is similar in mice of both genotypes. Footpad sections of both DBH +/− and DBH −/− mice show a large increase in immunoreactivity for VIP and TH from age P14 to P21 and is strongest in adult sections. *A, B*, Hematoxylin and eosin-stained adult footpads; *C–F*, footpads from P14 mice stained with antisera to TH and VIP; *G–J*, footpads from P21 mice stained with antisera to TH and VIP; *K–N*, footpads from adult mice stained with antisera to TH and VIP.

demonstrated that substantial TH immunoreactivity is still present in sweat glands of adult mice; however, catecholamine histofluorescence that is present at P4 largely disappears by P21 in mice (Guidry and Landis, 1995). Thus although levels of TH remain high in mice, catecholamine synthesis is switched off as cholinergic synthesis is switched on, similar to that observed in rats (Landis and Keefe, 1983).

If NE is not essential for induction of SGF, what neuron-

derived factor might act in concert with NE and promote switching in its absence? One possibility is ATP. Catecholamines are packaged in synaptic vesicles along with ATP and they are co-released. A number of purinergic receptors have been characterized recently (Surprenant et al., 1995) and at least one of them ($P2X_4$) is expressed in epithelial tissues (Surprenant et al., 1996), but the presence of purinergic receptors in developing sweat glands has not been determined yet. Alternatively, neuropeptides

are produced and released by most sympathetic neurons in addition to NE and ATP, so they are also potential candidates. Because NE seems to potentiate development of the sweat response, our results suggest that innervation of the sweat glands may normally activate at least two pathways that act together to induce SGF. In the absence of NE, the second pathway can lead to complete switching but with a delay of up to 2 weeks. It will be interesting to determine the nature of the second signal, the signal transduction pathway that it activates, and whether it is also dispensable for switching if NE is present.

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