soft agar growth stimulating activity, and EGF competing activity (Fig. 1). Bone resorbing activity was again eluted from this column with a molecular weight of approximately 30,000. A peak of EGF competing activity and a peak of soft agar growth stimulating activity consistently (in four different experiments) coeluted with the bone resorbing activity. In addition, other peaks of soft agar growth stimulating activity and EGF competing activity were eluted from this column. None of these was associated with bone resorption. It is not surprising that several peaks of soft agar growth stimulating activity and EGF competing activity were found in tumor extracts, since others have shown that multiple peaks of TGF activity of similar molecular weight and bioactivity may be present in tissue extracts (*4, 16*).

The evidence that a TGF produced by the tumor cells is responsible for increased bone resorption is strong. The bone resorbing factor released by the tumor cells shares all the known chemical properties of the TGF released by the same cells: it is acid-stable, depends on disulfide bonds for activity, is trypsin-sensitive, coelutes from gel filtration columns with soft agar colony stimulating activity and mitogenic activity, and is able to prevent binding of EGF to its receptors. However, final proof that the bone resorbing factor is the same polypeptide as the TGF will not be possible until a pure homogeneous preparation of the bone resorbing factor is shown to contain all the relevant biological activities of the TGF (competing activity for EGF receptors, mitogenic activity, and promotion of anchorage-independent growth of nontransformed cells). It seems likely that only small amounts of protein are responsible for these activities, so purification to homogeneity will probably be difficult (*17*).

Rats carrying the Leydig cell tumor have other features that have been associated with the humoral hypercalcemia of malignancy seen in humans (*2, 18*). There is absence of bone metastasis, increased generation of nephrogenous adenosine 3′,5′-monophosphate (cyclic AMP), hypophosphatemia, and renal phosphate wasting. The relation of the production of a tumor-derived TGF to nephrogenous cyclic AMP generation and renal phosphate wasting is unknown. Recently, we found that medium conditioned by these tumor cells contains a factor that causes renal phosphate wasting (*19*). Clarification of the interrelations between the factor responsible for hypercalcemia and these other parameters associated with the humoral hypercalcemia of malignancy will require further study.

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Axonal Proteins of Presynaptic Neurons During Synaptogenesis

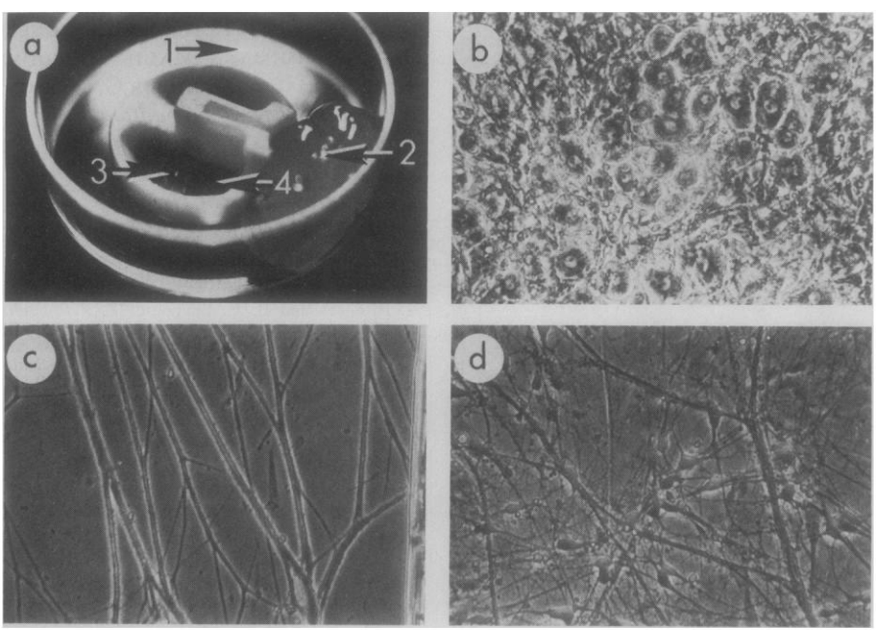
Abstract. *Changes occur in the synthesis and axonal transport of neuronal proteins in dorsal-root ganglia axons as a result of contact with cells from the spinal cord during synapse formation. Dorsal-root ganglia cells were cultured in a compartmental cell culture system that allows separate access to neuronal cell bodies and their axons. When cells from the ventral spinal cord were cultured with the dorsal-root ganglia axons, synapses were established within a few days. Metabolic labeling and two-dimensional electrophoresis revealed that four of more than 300 axonal proteins had changed in their expression by the time synapses were established. The highly selective nature of these changes suggests that the proteins involved may be important in the processes of axon growth and synapse formation and their regulation by the regional environment.*

During the formation of a synapse, ultrastructural changes occur in the postsynaptic neuron, in the intercellular cleft, and in the presynaptic neuron (*1*). The cytoarchitectural and functional conversion of the probing growth cone to a transmitting synapse is complex, and its molecular basis is far from being understood. The proteins involved in the implementation of the structural and functional specializations of the axon and its tip are synthesized in the neuronal soma and moved to their site of destination by axonal transport. Neurons can be grown in a three-compartment cell culture system that offers separate access to cell somas and axons (*2*). We have used this cell culture technique in conjunction with metabolic labeling of proteins and two-dimensional gel electrophoresis (*3*) to provide a selective and high-resolution analysis of newly synthesized axonal proteins. We now report that a few distinct axonal proteins change in their expression when axons from dorsal-root ganglion (DRG) neurons and ventral spinal cord (VSC) neurons are cultured together under conditions in which synapse formation occurs.

Dissociated DRG cells from 10-day-old chick embryo were plated in the center compartment of the three-compartment cell culture system (Fig. 1). The outgrowing axons crossed the barrier between center and side compartments through a thin film of medium, whereas the cell somas were retained in the center compartment. After 10 days, cells from the VSC of 6-day-old chick embryos were cultured as presumptive postsynaptic cells in the two side compartments (*4*). Four days after they were

plated, the VSC cells were large enough to be impaled with high-resistance glass microelectrodes (*5*). Ongoing spontaneous activity among VSC cells was recorded. Extracellular stimulation of the DRG neurons resulted in short, constant-latency postsynaptic potentials in the VSC neurons that represented monosynaptic connections from DRG neurons to VSC neurons (Fig. 2). Other VSC neurons showed responses of longer and variable delay characteristic of polysynaptic responses. In areas where dense DRG processes entered the side compartment from the center compartment, most of the impaled VSC neurons showed monosynaptic or polysynaptic responses on extracellular stimulation of the DRG neurons (*6*).

Four days after cocultivation, when synaptic interaction could be demonstrated electrophysiologically, the pattern of synthesis of the axonal proteins of the presynaptic cells was analyzed by metabolic labeling with [^{35}]methionine (added to the somas in the center compartment for 40 hours) and subsequent two-dimensional sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*7*). In a parallel experiment, DRG axons that had no contact to VSC cells were examined. The protein pattern of DRG axons with and without the presence of VSC neurons, as revealed by the fluorographic spots on the x-ray film, proved to be similar (Fig. 3). However, four of the approximately 300 proteins observed were changed in their relative abundance after being cocultured with VSC cells. One protein with a molecular weight of about 65,000 (65K), which was among the most abundant proteins under control conditions, was markedly reduced during coculture with VSC cells. A minor protein with a molecular weight of about 50K was also reduced. These changes, although not very large, were reproducible in each of six experiments. Two proteins, each with a molecular weight of approximately 60K, were synthesized much more abundantly during coculture with VSC cells. Additional differences in the protein patterns occurred, but they either were in proteins represented by faint spots at the level of sensitivity obtained so far, their changes were not reproducible in all of the ex-



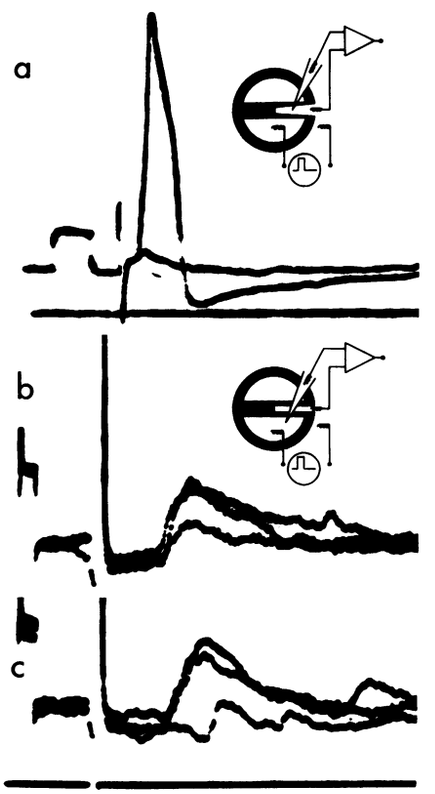


Fig. 1 (left). Modification of the compartmental cell culture system for metabolic labeling studies of axonal proteins in the presence of cocultured cells in the side compartment. Morphological aspects of DRG axons with and without cocultured VSC cells. (a) Modified three-compartment cell culture system. The compartmental cell culture system was prepared as described in detail by Campenot (*2*). However, two measures of precaution were necessary in order to obtain contamination-free representation of axonal proteins of the DRG neurons in the presence of cocultured cells. The volume of the nonradioactive medium in the side compartments had to be increased in order to dilute the radioactive amino acid diffusing through the film of medium from the center compartment, and the number of the cocultured cells had to be reduced. The volume of the side compartment medium during the time of labeling was increased by cutting the outer rim of the standard Teflon inset to about one-third of its original height, except in the area delineating the center compartment. For labeling, the opening of the center compartment was sealed with a grease plug (arrowhead 2), and medium was added until the fluid of the side compartment and the rest of the dish (arrowhead 1) were confluent. This extended the side compartment volume from about 0.5 to 3.5 ml. The number of cells in the side compartment was reduced without changing the cell density by putting a grease wall in the middle of the side compartment (arrowhead 3). Cells to be cocultured were plated only in the area next to the barrier, where axons grew in from the center compartment (arrowhead 4). (b) Phase-contrast micrograph of DRG cells in the center compartment after 14 days in culture (*11*). (c) Phase-contrast micrograph of DRG axons in the side compartment after 14 days in culture. (d) Phase-contrast micrograph of DRG axons with cocultured cells from VSC (*4*). The VSC cells include both neurons and nonneuronal cells. Fig. 2 (right). Electrophysiological recordings demonstrating synaptic connections between DRG and VSC neurons. (a) Intracellular recording from the soma of a DRG neuron during extracellular stimulation of the DRG axons extending from the center compartment to the side compartment (*5*). The stimulation and recording situation is illustrated in the inset. The upper oscilloscope trace shows a somal action potential as evoked by extracellular stimulation of the DRG axon. The lower trace probably represents an axonal action potential, which has been electronically conducted to the soma. Calibration pulse, 10 mV per 2 msec. Extracellular stimulation pulse 1 V per 0.5 msec. (b and c) Extracellular stimulation of the presynaptic DRG axons and intracellular recording of postsynaptic potentials from the VSC neurons, as schematically represented in the inset. The oscilloscope traces show an intracellular recording from a VSC neuron in a side compartment during repetitive extracellular stimulations of DRG axons. Short and constant latency responses can be seen as well as long and variable latency responses. Calibration pulse, 5 mV for 5 msec. Extracellular stimulations 1 V for 0.5 msec immediately followed by a pulse of approximately 0.5 V for 0.5 msec to shorten transients on the recording trace.

periments, or the differences were too subtle to be identified with certainty.

Changes occur in cell surface proteins in neurons of the superior cervical ganglion when the environmental conditions of the cell culture medium are changed in a way that induces a change in the neurotransmitter secreted (*8*). The changes we report do not seem to result from conditioning of the cell culture medium by the cocultured cells. None of the observed changes occurred when the VSC cells were cocultured outside the rim of the side compartment (arrowhead 4 in Fig. 1a) but shared the same medium with the DRG axons in the side compartments (Fig. 3c). This indicates that the VSC cells act on the DRG axons either by cell-surface contact or by a secreted agent with limited distance of action, possibly because of a short half-life. However, as our cocultured VSC cells are a heterogeneous population of cells, including various types of neurons and nonneuronal cells such as astrocytes, oligodendrocytes, and fibroblasts, the present data do not allow us to identify the subset of VSC cells that is responsible for the induction of the observed changes.

Morphological studies (*1*) have shown the early changes that occur when contact of a presynaptic axon with a prospective postsynaptic target is established. The metamorphosis of an axon tip from a growth cone to a presynaptic terminal is composed of two temporarily overlapping events: the disappearance of the structures of the growth cone and the appearance of the characteristics of a synaptic terminal. Thus, proteins disappearing around the time of synapse formation might be involved in some aspect of axonal growth or growth cone structure and function. Indeed, the acidic 65K protein bears a close resemblance in molecular weight and isoelectric point to data reported on the 68K subunit of the neurofilament triplet protein of the guinea pig (*9*). The proteins newly expressed around the time of synapse formation may participate in the development of the structural specialization at the presynaptic terminal. Alternatively, they may participate in the suppression of the growth cones' sprouting activity, a function that is under the influence of the postsynaptic target cells (*10*). Determining the nature of these proteins and their action during the establishment of synaptic structure, function, and specificity may help to elucidate the process of synaptogenesis at the molecular level.

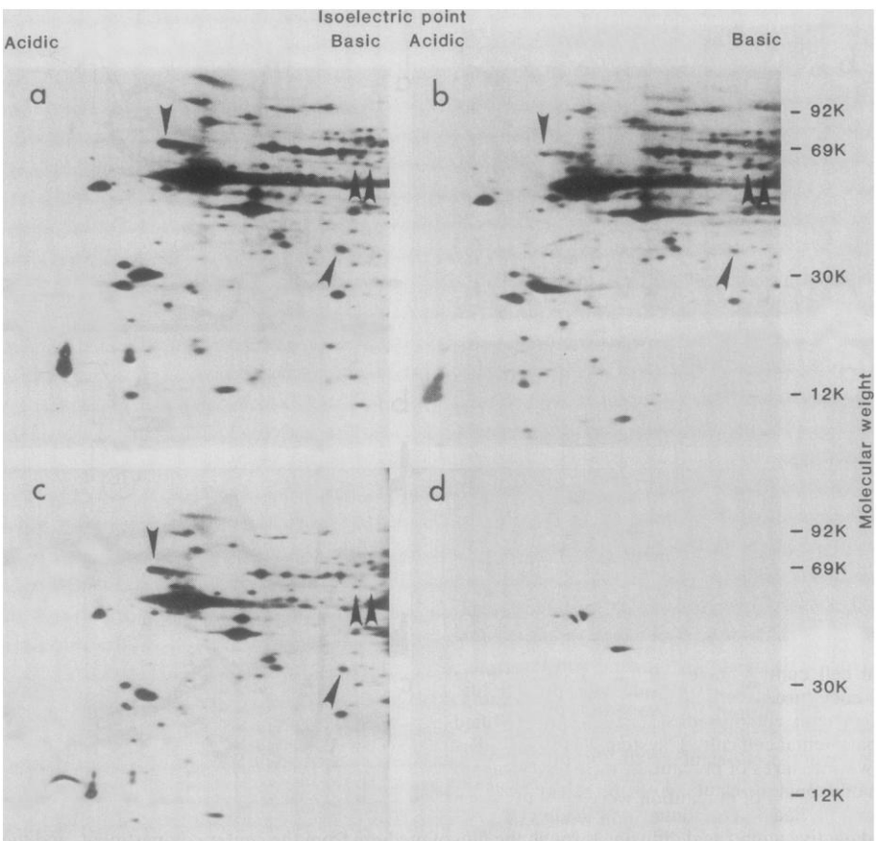


Fig. 3. Two-dimensional electropherograms of DRG axonal proteins. Samples for electrophoresis were prepared by pooling cellular material from the side compartments of three to five plates that were grown under identical conditions and in the same experiment (*7*). Samples from different experimental conditions were matched for trichloroacetic acid–precipitable material of equal radioactivity (~ 400,000 count/min per gel). (a) Axonal proteins of DRG neurons. (b) Axonal proteins of DRG neurons with cocultured VSC cells. The proteins that had changed markedly in the presence of cocultured cells are indicated by arrowheads. (c) Axonal proteins of DRG axons grown in medium constantly conditioned by VSC cells. For constant conditioning, the VSC cells were plated outside the Teflon inset (arrowhead 1 in Fig. 1a) and, after they had settled down, the medium level was raised above the peripheral rim of the side compartment. (d) Control of incorporation of radioactive label into proteins synthesized by cocultured cells in the side compartment. Side compartment medium was collected after a labeling period of 40 hours and used to incubate VSC cells, equivalent in cell number and density to the cells cocultured in the side compartments of five plates. After an incubation period of 40 hours, the radioactivity incorporated into trichloroacetic acid–precipitable material was 3000 ± 1000 count/min; no spots of labeled proteins other than tubulin and actin were observed in the two-dimensional electropherogram (*12*).

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5. For electrophysiological recordings, the cultures were placed onto the stage of an inverted microscope (Zeiss) equipped with phase optics. Intracellular recordings were made with glass microelectrodes, filled with 3*M* KCl (resistance, 120 to 150 megohms), and potential signals were simultaneously displayed on an oscilloscope and a chart recorder. Current injections were made through a bridge circuit. Extracellular stimulation of the presynaptic DRG cells was obtained by pulses of 500-μsec duration from a stimulus isolation unit applied between the center compartment and one of the side compartments. The stimulation threshold, determined by recording intracellularly from the cell somas in the center compartment, was 0.4 to 0.8 V of either polarity. The assay for synaptic connections was done by intracellular recordings from the VSC neurons in the side compartments.
6. No stimulation-induced postsynaptic potentials were observed if the intracellular recordings were done in an area where no DRG axons could be seen emerging from beneath the barrier. This indicates that the stimulation of DRG axons was necessary to evoke the registered postsynaptic potentials and that antidromic stimulation of VSC neurons by axons reaching into the center compartment was not important in the generation of these synaptic responses.
7. The newly synthesized proteins were labeled by adding to the center compartment 50 μl of

labeling medium composed of methionine-free minimal essential medium, 10 percent heat-inactivated horse serum, 5 percent chicken embryo extract, nerve growth factor (10 ng/ml), 15 μ*M* unlabeled methionine, and [^{35}S]methionine (1 mCi/ml). The expanded side compartments contained 3.5 ml of the same medium, except that 4 m*M* unlabeled methionine replaced the radioactive methionine. Incubations were for 40 hours at 37°C in 10 percent CO_2. After labeling, 50 μl of medium from each compartment was aspirated and the protein precipitated by trichloroacetic acid. The free [^{35}S]methionine that remained in solution was counted in a beta counter at a counting efficiency of approximately 70 percent. This procedure provided an estimate of the leakage of radioactive label into the side compartment. After removing the rest of the supernatant media, the axons in the side compartment were washed twice with Dulbecco's phosphate-buffered saline (Gibco), and the cellular material was collected by dissolution in a solution of 2 percent SDS and 5 percent β-mercaptoethanol at a temperature of 90°C, pooled, and processed for two-dimensional polyacrylamide gel electrophoresis (*3*). The Ampholine solution of the isoelectric focusing step was composed of 1.6 percent Ampholine 5/7 (LKB) and 0.4 percent Ampholine 3/10. The second dimension was run in a 10 to 17.5 percent acrylamide gradient. The preparation of the gels for fluorography was done according to the principles worked out by W. M. Bonner and R. A. Laskey [*Eur. J. Biochem.* **46**, 83 (1974)] but with a commercially available acetic acid–based enhancer (EN^{3}HANCE, New England Nuclear). Exposure was for 4 to 5 weeks at −70°C.

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11. After tryptic dissociation, 75,000 cells were plated in the center compartment of the three-compartment system in minimal essential medium (Gibco), 10 percent heat-inactivated horse serum (Gibco), 5 percent chicken embryo extract, and nerve growth factor (25 ng/ml).
12. This minor contamination, which does not affect the outcome of the study, has to be considered as inherent to the design of this compartmental cell culture system. The plates used for all labeling studies were carefully tested for the absence of hydrostatic bulk flow between the compartments (*2*). Proteins synthesized by cells of the side compartments might be labeled by absorption of labeled amino acid or proteins secreted from DRG axons or by uptake and retrograde transport by VSC axons reaching into the center compartment. These possibilities were experimentally excluded (data not shown). Thus, we conclude that the minor buildup of radioactive amino acid in the side compartment (about 0.25 percent of the concentration of the center compartment after 40 hours) has to be the result of diffusion through the film of medium between the compartments.
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Strain Differences in Rat Brain Epinephrine Synthesis: Regulation of α-Adrenergic Receptor Number by Epinephrine

Abstract. *Inbred rat strains Fischer 344 (F344) and Buffalo (BUF) differ in several physiological and behavioral measures. It was found that the activity of adrenomedullary and regional brain phenylethanolamine* N*-methyltransferase is at least four times higher in F344 rats than in BUF rats; these strain-dependent differences corresponded directly with the epinephrine content of the medulla-pons and hypothalamus. Conversely, α-adrenergic receptor density in brain regions containing phenylethanolamine* N*-methyltransferase is two to three times lower in F344 rats than in BUF rats; α-receptors in frontal cortex (a brain region lacking phenylethanolamine* N*-methyltransferase activity and epinephrine) are similar in both strains. These findings suggest that strain-dependent differences in α-receptors are regulated by inherited differences in presynaptic adrenergic neuronal function in different brain regions.*

Phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that converts norepinephrine (NE) to epinephrine (EPI), is present in adrenomedullary cells and in medulla-pons and hypothalamic neurons (*1, 2*). PNMT-containing neurons may play key roles in physiological processes such as regulation of blood pressure and responses to stress (*2*). Recent studies have demonstrated the importance of genetic factors influencing regulation of catecholamine biosynthetic enzymes and, by association, catecholamine-mediated physiological functions (*3*). In this regard, two inbred rat strains, Fischer 344 (F344) and Buffalo (BUF), demonstrate differences in midbrain and cortical tyrosine hydroxylase (TH) activity, spontaneous motor activity, apomorphine-induced stereotypy, NE- and amphetamine-induced behavioral responsiveness, and NE-sensitive adenosine 3′,5′-monophosphate (cyclic AMP) accumulation in midbrain slice preparations (*4, 5*). Differences in brain receptor sensitivity to the various catecholamines may explain the behavioral and physiological strain differences (*4*); thus, Helmeste *et al.* (*5*) have demonstrated differences in brain dopamine receptor number. It is likely, however, that other neurotransmitter systems, including adrenergic ones, are involved in the many differences seen between these inbred rat strains. We observed that adrenomedullary PNMT activity was five times higher in F344 rats than in BUF rats; this observation led us to explore differences in PNMT activity and adrenergic receptors in brain regions of F344 and BUF rats.

Breeding colonies of F344 and BUF rats were established from parental stock obtained from Microbiological Associates and maintained by brother-sister mating for at least two generations. Individual rat brain regions were processed to allow simultaneous assay of TH, PNMT, catecholamines, and receptor sites (*6, 7*). Adrenal and regional brain PNMT and brain TH were assayed with modifications of published methods (*8*), and brain catecholamine levels were measured by high-performance liquid chromatography with electrochemical detection (*9*). We followed standard methods in our radioligand binding assays for adrenergic and opiate receptors (*10*) and used [^{3}H]prazosin (PRAZ), an α_1-receptor–specific antagonist; *p*-[^{3}H]-aminoclonidine (PAC), an α_2 partial agonist with selectivity for the α_2(H) state (*11*); [^{3}H]rauwolscine (RAUW), an α_2 antagonist with some selectivity for the α_2(L) state (*12*); [^{125}I]iodocyanopindolol (ICYP), a β-receptor antagonist; and [^{3}H]naloxone (NAL), an opiate antagonist (*11*).

Adrenal PNMT activity in male F344 rats (N = 41) was 6.52 ± 0.07 U per adrenal pair (mean ± standard error; U = 1.0 nmole of product per hour at 37°C), compared to 1.34 ± 0.02 U per adrenal pair in BUF (N = 34) adrenals. The same fivefold difference in adrenal PNMT activity was seen between female rats of the two strains. Similar differences were also observed in two brain regions (Table 1). Medulla-pons PNMT activity was four times higher in F344 rats than in BUF rats. Differences between strains were even more striking for hypothalamic PNMT activity. As expected, little or no PNMT activity (or EPI) was detected in cortical tissue (*12*). Strain-dependent differences in TH activity were similar to those reported previously (*4*), with F344 rats showing 30 to 40 percent greater TH activity.

Regional catecholamine levels generally reflected differences in synthetic enzyme activity. Most notably, the prominent between-strain differences in medulla-pons and hypothalamic PNMT activity were accompanied by three- to fivefold higher EPI content in F344 than in BUF rats (Table 1). Cortical, hypothalamic, and medulla-pons NE levels reflected brain TH activity differences, with NE levels up to 40 percent higher in F344 than in BUF rats.

Agonist regulation of adrenergic re-