Neurotransmitter Plasticity at the Molecular Level

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Summary. Contrary to long-held assumptions, recent work indicates that neurons may profoundly change transmitter status during development and maturity. For example, sympathetic neurons, classically regarded as exclusively noradrenergic or cholinergic, can also express putative peptide transmitters such as substance P. This neuronal plasticity is directly related to membrane depolarization and sodium ion influx. The same molecular mechanisms and plastic responses occur in mature as well as developing neurons. Further, contrary to traditional teaching, adult primary sensory neurons may express the catecholaminergic phenotype in vivo. Transmitter plasticity is not restricted to the peripheral nervous system: ongoing studies of the brain nucleus locus ceruleus in culture indicate that specific extracellular factors elicit marked transmitter changes. Consequently, neurotransmitter expression and metabolism are dynamic, changing processes, regulated by a variety of defined factors. Transmitter plasticity adds a newly recognized dimension of flexibility to nervous system function.

By any measure, the variety of functions subserved by the nervous system is prodigious. This single organ system governs and coordinates vegetative functions, including thermoregulation, cardiorespiration, and digestion, perceives sensory stimuli, effects motor responses, governs emotions, and learns, remembers and introspects. It is reasonable to assume that performance of such extraordinary and diverse functions requires a system of profound complexity, flexibility, and mutability. It has long been assumed that the system's 10^{11} neurons, each with approximately 10^4 interconnections, potentially provided the nervous system with all of these requisite qualities. However, within the past several years we have begun to appreciate that mutability may also derive from a wholly unexpected source, neurotransmitter plasticity.

Neurotransmitters, as agents of neuronal communication, have long been the focus of intense interest. However, traditional teaching had maintained that the nervous system used only two transmitters, acetylcholine and an epinephrine-like substance. In contrast, recent work has identified at least 50 individual amine, amino acid, purine, and peptide putative transmitters, thereby vastly expanding potential diversity and complexity in the nervous system. The recent discovery that different putative transmitters are co-utilized by neurons further expanded the field of diversity and complexity [for a review, see (*1*)]. Now, ongoing studies indicate that neurons may change transmitter status during development and maturity, upsetting the tacitly assumed dogma of transmitter immutability, and adding an entirely new dimension to our appreciation of neural plasticity. It is becoming increasingly apparent that the transmitter status of a neuron represents a dynamic, changing process, influenced by multiple extracellular factors, including afferent and efferent innervation, proximate nonneuronal cells, and hormones. Consequently, neurons may respond to environmental information by altering transmitter phenotypic expression and, presumably, the signals sent to other neurons.

At this early stage in our understanding, most studies are designed to define the basic characteristics of the process of transmitter plasticity. Do neurons normally alter transmitter phenotypic characters (such as biosynthetic enzymes) in vivo? Does potential lability persist throughout life? Is transmitter plasticity restricted to certain classes of neurons? What molecular mechanisms regulate phenotypic alteration? What are the functional consequences of transmitter alteration?

These questions, in turn, derive from more fundamental issues that are of central importance for neurobiology. Do individual, postmitotic neurons normally transcribe entirely different species of messenger RNA (mRNA), coding for the elaboration and use of different transmitters, at different times in vivo? Alternatively, do apparent changes in phenotype simply reflect marked quantitative shifts in transmitters synthesized? Do neurons transcribe a variety of transmitter mRNA's continuously, but translate only selected species, producing different transmitters under different circumstances? Does the neuronal genome remain accessible for transcription leading to transmitter changes throughout life? In view of these unanswered questions, all of which are testable, the issue of "quantitative" versus "qualitative" change in transmitter phenotype is unresolved. Nevertheless, it will be useful to view the experimental results described subsequently in light of these considerations.

Plasticity During Development

Study of developing neurons, which were presumed to be maximally mutable, initially suggested that transmitter expression is a mutable, not predetermined, process. Postmitotic neonatal rat sympathetic neurons, grown in dissociated cell culture, exhibited noradrenergic or cholinergic characteristics, or both, depending on culture conditions (*2, 3*). Depolarizing stimuli elicited noradrenergic expression, whereas factors derived from nonneuronal cells evoked cholinergicity [for a review, see (*4*)]. Moreover, dual-function, noradrenergic-cholinergic neurons were identified (*3*).

Observations in vivo suggest that transmitter plasticity also occurs during normal development in the animal. Sympathetic neurons innervating rat eccrine sweat glands appear to convert from noradrenergic to cholinergic postnatally, retaining, however, the high-affinity uptake system for α-CH_3-norepinephrine (*5*), a characteristic of noradrenergic neurons. Small granular vesicles, which store catecholamines, disappear in these salivary terminals, and immunoreactivity to tyrosine hydroxylase (TH) and dopamine-β-hydroxylase, noradrenergic

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biosynthetic enzymes, decreases. Finally, treatment of neonates with 6-hydroxydopamine, which destroys catecholaminergic neurons, results in loss of TH, dopamine-β-hydroxylase, and the putative peptide transmitter VIP (vasoactive intestinal polypeptide) in the adult fibers. Consequently, these neurons may convert from catecholaminergic to cholinergic and peptidergic during development [for a review, see (*5*)].

Increasing evidence suggests that phenotypic alteration occurs during normal development in the embryo as well. A number of populations in the autonomic-adrenal axis appear to change neurohumoral products. Adrenomedullary chromaffin cells provide a model that has been analyzed in some detail. During development the adrenomedullary progenitors migrate from the caudal thoracic area to the adrenal anlage, and within the adrenal convert from the noradrenergic to adrenergic phenotype. This switch from the predominance of norepinephrine to epinephrine involves the de novo appearance of the enzyme PNMT (phenylethanolamine *N*-methyltransferase), which methylates norepinephrine to form epinephrine (*6*). While mechanisms governing the initial appearance of PNMT have not yet been defined, the subsequent developmental increase is associated with a dramatic rise of mRNA coding for the enzyme, suggesting that regulation occurs at the transcriptional level (*7*). The pituitary-adrenal axis, in turn, through the mediation of glucocorticoid hormones, is necessary for the ontogenetic increase in PNMT activity and molecule number (*6*). In summary, in this instance, phenotypic alteration may be mediated, at least in part, by specific hormones and alteration in the processing of specific species of mRNA. Nevertheless, mechanisms regulating the initial appearance of PNMT remain to be elucidated; it is not yet clear whether the de novo appearance of the enzyme simply reflects initial transcription of PNMT message.

In another example, a population of presumptive neurons in the embryonic rat gut transiently expresses a number of noradrenergic traits, including TH, dopamine-β-hydroxylase, and catecholamines (*8*). These traits are detectable on embryonic day 11.5 (E-11.5) but have disappeared by E-14.5. However, the specific, high-affinity uptake system for norepinephrine selectively appears in these cells at E-12.5 and persists (*8*), permitting cellular identification after loss of other endogenous characters (*8*). Although these cells apparently do not simply die, their mature transmitter status remains to be defined. Nevertheless, these observations suggest that this population may represent another instance of phenotypic plasticity in the embryo.

Current work indicates that transient expression of transmitter traits is not restricted to gut cells, but also occurs in cranial nerve ganglia of the embryonic rat (*9*). For example, TH, the rate-limiting enzyme in catecholamine biosynthesis, transiently appears in some trigeminal ganglion bipolar cells, which extend processes into the primitive brainstem. Further, TH also transiently appears in some cells of the petrosal, nodose, and dorsal root sensory ganglia during embryogenesis. Since some trigeminal neurons are derived from the neural crest, whereas nodose neurons are epibranchial placode derivatives, heterogeneous populations, differing embryologically, geographically, and functionally, may exhibit transient catecholaminergic expression during development.

Transmitter Plasticity During Maturity

Is transmitter plasticity strictly a developmental phenomenon or, alternatively, does it persist through adulthood, playing a role in normal function of the mature nervous system? Several lines of evidence favor the latter alternative.

Plasticity in mature sympathetic neurons. Recent work from this laboratory indicated that neonatal rat sympathetic neurons were capable of expressing the putative peptide transmitter, substance P, in vivo and in vitro under appropriate conditions (*10*). Consequently, principal sympathetic neurons, traditionally regarded as exclusively noradrenergic or cholinergic, are also capable of expressing a peptidergic transmitter phenotype during development. Does this plasticity persist into maturity? To approach this issue, adult neurons were examined in vivo and in culture. Denervation (decentralization) of the sympathetic superior cervical ganglion in adult rats resulted in nearly a twofold increase in ganglion substance P content (*11*). Moreover, treatment with chlorisondamine, a ganglionic blocking agent that prevents depolarization by competing with acetylcholine for postsynaptic nicotinic receptors, reproduced the effects of denervation (*11*). Consequently, transsynaptic impulses, through the mediation of acetylcholine and postsynaptic sympathetic depolarization, decreased substance P in the adult ganglion. This conclusion was supported by the observation that phenoxybenzamine treatment, which reflexly increases sympathetic impulse flow, decreased ganglion substance P, as predicted (*11*).

To analyze this plasticity in adult neurons in greater detail, we studied explanted superior cervical ganglia from rats aged 6 months or 1 year. In culture, the (now denervated) adult ganglia exhibited a tenfold rise in substance P, mimicking the increase in neonatal ganglia (*12*). To define mechanisms underlying the increase in substance P, adult explants were exposed to the depolarizing agent, veratridine. Depolarization with veratridine completely blocked the increase of substance P (Fig. 1) (*12*). Further, tetrodotoxin, which prevents Na^+ influx elicited by veratridine, prevented the effects of depolarization (Fig. 1). Viewed in conjunction with the studies in vivo, our observations suggest that nicotinic receptor stimulation, with consequent depolarization or attendant transmembrane Na^+ influx, or both, decrease substance P in mature sympathetic neurons, as in neonatal neurons (Fig. 2). This is of particular interest, since it has been known for some time that a similar sequence of events—(i) transsynaptic impulse flow, (ii) acetylcholine release, (iii) nicotinic receptor stimulation, and (iv) Na^+ influx—biochemically induce TH and dopamine-β-hydroxylase, with increased norepinephrine biosynthesis in mature sympathetic neurons (*13*). Consequently, the same or a similar sequence of mechanisms appears to have opposite effects on norepinephrine and substance P metabolism in mature sympathetic neurons (Fig. 2).

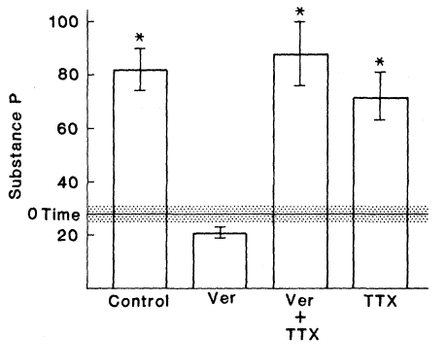

Fig. 1. Effect of membrane depolarization on adult ganglia. Superior cervical ganglia from 6-month-old rats were cultured in serum-supplemented medium in the presence of veratridine ($5 \times 10^{-5} M$), tetrodotoxin ($10^{-7} M$), or both. After 48 hours, the ganglia were examined for substance P content, which is expressed as mean picograms per ganglion ± standard error (S.E.) for eight ganglia. Line and stippled bar represent mean substance P content ± S.E. for freshly dissected ganglia. Ver, veratridine; TTX, tetrodotoxin. *Differs from zero time and veratridine at $P < 0.01$ [one-way analysis of variance; Newman-Keuls test; data from (*12*)].

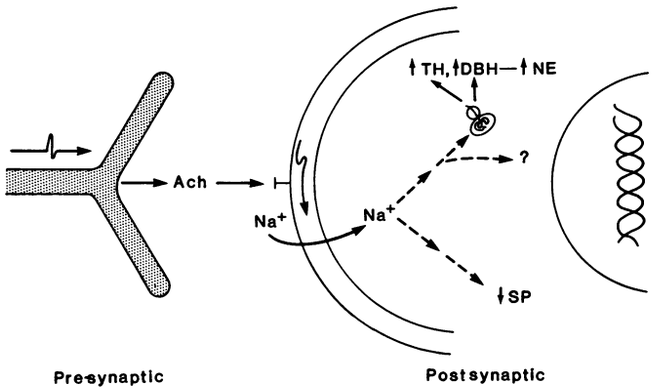

Fig. 2. Transsynaptic regulation of neurotransmitter traits in adult sympathetic neurons: schematic representation. Presynaptic impulses release acetylcholine (Ach) which interacts with postsynaptic nicotinic receptors, evoking transmembrane sodium ion (Na^+) influx. This sequence of molecular events decreases substance P (SP) and simultaneously biochemically induces TH and dopamine-β-hydroxylase (DBH). Enzyme induction elevates norepinephrine (NE) synthesis in the postsynaptic neuron.

It may be concluded that sympathetic neurons exhibit remarkable transmitter plasticity during maturity as well as development and that, at least in principle, plasticity is not restricted to ontogeny.

Catecholaminergic expression in mature sensory neurons. The foregoing studies indicated that mature sympathetic neurons, long regarded as catecholaminergic, are capable of peptidergic expression. Conversely, are mature nonsympathoadrenal neurons in the periphery capable of catecholaminergic expression? Conventional wisdom had maintained that catecholaminergic traits were restricted to the sympathoadrenal axis in the peripheral nervous system.

To approach this issue we examined primary sensory neurons of the petrosal and nodose ganglia in the normal, unmanipulated adult rat in vivo. Neurons in these ganglia had exhibited TH for a brief period in the embryo (see above). In fact, catecholaminergic expression was documented in a subpopulation of these mature, primary sensory neurons (*14*). Tyrosine hydroxylase immunocytochemical reactivity was localized to ganglion neurons which exhibited morphologic features typical of primary sensory neurons, including an initial axon glomerulus and a single bifurcating neuritic process (Fig. 3). Moreover, the immunoreactive TH was functional, as indicated by the presence of detectable catalytic activity. Further, catecholamine histofluorescence was also demonstrable in these mature sensory neurons, suggesting that the enzyme was, indeed, functioning in vivo.

The enzyme also exhibited plastic responses. Axotomy of the petrosal neurons, separating the cell bodies from peripheral targets, resulted in a precipitous decline in TH catalytic activity and disappearance of TH immunoreactivity (*14*). Catalytic activity and immunoreactivity gradually recovered over 4 weeks, coinciding with reinnervation of the target carotid body and sinus (*14, 15*). We are currently using a number of approaches in vitro and in vivo to determine whether axotomy per se or specific factors from the targets regulate TH. In either instance, it is apparent that TH may be expressed by mature, primary sensory neurons in a mutable fashion. Recent work has indicated that neurons of dorsal root sensory ganglia also express TH (*16*). Since petrosal and nodose neurons appear to be epibranchial placode and not neural crest derivatives, peripheral neurons which differ embryologically, functionally, and anatomically may express catecholaminergic traits. Finally, our observations raise the question of whether petrosal and nodose neurons that transiently express TH in the embryo reexpress this catecholaminergic trait during adulthood.

Opiate peptide regulation in adult adrenal medulla. The opiate peptides leucine- and methionine-enkephalin have recently been localized to another neural crest derivative, the adrenomedullary chromaffin cell (*17–19*). In addition, of course, these cells have long been known to contain the catecholamines norepinephrine and epinephrine. The adrenal medulla, consequently, appeared to be an excellent model in which to explore plasticity of multiple neurohumors in the adult. We used leucine-enkephalin ([Leu]enkephalin) immuno-

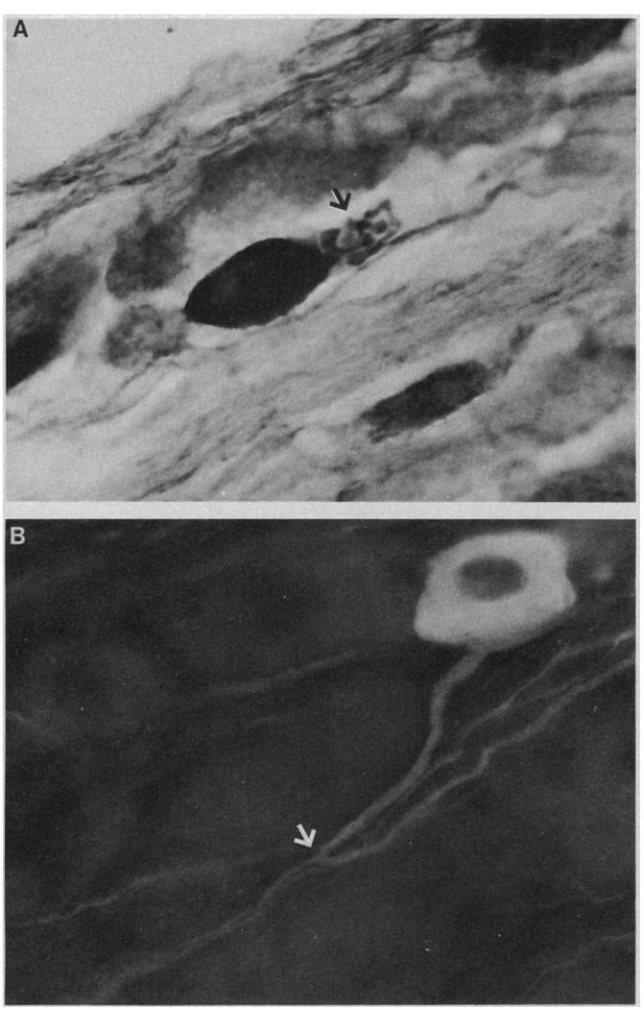

Fig. 3. (A) Brightfield photomicrograph of TH immunoreactive nodose ganglion cell, showing the initial axon glomerulus (arrow). (Peroxidase-antiperoxidase–stained preparation; ×440.) (B) Fluorescence photomicrograph of a TH - immunoreactive nodose cell, showing the bifurcating neuritic process (arrow). [Fluorescein isothiocyanate–stained preparation; ×440; from (*14*).]

reactivity to monitor opiate peptide metabolism, TH to monitor catecholamine status overall, and PNMT to follow adrenergic (epinephrine) metabolism. Surgical denervation of the medulla, known to decrease TH and PNMT, markedly increases [Leu]enkephalin in adult rat medullas in vivo (*20*). Moreover, treatment with the ganglionic blocking agent chlorisondamine reproduced this effect (*21*), implying that cholinergic, nicotinic stimulation normally decreases [Leu]enkephalin.

To begin defining molecular mechanisms in adult rat, we studied medullas grown as explants in culture (*22*). The medullary explants exhibited a 50-fold rise in [Leu]enkephalin within 4 days, after a 2-day lag period, and continued increasing through 7 days, the longest time examined. In contrast, TH activity remained constant throughout, while PNMT decreased 60 percent in the first 4 hours, maintaining a stable plateau thereafter. Our observations suggested, consequently, that opiate peptidergic and catecholaminergic traits may be differentially regulated in the adult medulla.

To characterize intracellular molecular mechanisms governing the increase in [Leu]enkephalin, we measured proenkephalin mRNA in cultured medullas using a 918-base-pair, ^{32}P nick-translated complementary DNA (cDNA) probe complementary to human pheochromocytoma proenkephalin mRNA (*23*). Dot blot analysis was performed on zero time and 4-day cultures (*24*). Autoradiography was performed for 1 and 3 days, after 24-hour hybridization. Zero time medullary proenkephalin mRNA levels did not differ from background or nonneuronal polyadenylated RNA standards. In contrast, 4-day adult explants revealed a marked increase in proenkephalin mRNA, which paralleled the rise in [Leu]enkephalin. Prompted by the striking coincidence in the rises of mRNA and [Leu]enkephalin, we are now attempting to determine whether ongoing synthesis of proenkephalin mRNA and prohormone are required for the rise in [Leu]enkephalin.

To analyze mechanisms underlying transsynaptic regulation, we cultured explants in the presence of depolarizing agents (*22*). Depolarizing concentrations of K^+ prevented the increase in [Leu]enkephalin, paralleling the effects of transsynaptic stimulation in vivo. Further, veratridine also prevented the rise in [Leu]enkephalin, and tetrodotoxin blocked this effect. The activities of TH and PNMT were unchanged. These observations suggest that membrane depolarization and associated transmembrane Na^+ influx decrease [Leu]enkephalin in adult medulla. These studies, viewed in conjunction with the cDNA hybridization data, raise the possibility that increased [Leu]enkephalin after decreased depolarization reflects increased synthesis of proenkephalin mRNA and prohormone. Regardless, it is apparent that enkephalins and catecholamines, which are co-localized and co-released by chromaffin cells (*17, 19, 25*), are differentially processed. Consequently, diverse physiological regulators, elaborated by the same cells, may be independently expressed and regulated in the adult, permitting a wide range of plastic responses to environmental stimuli. These observations, then, are analogous to those described above, in which substance P and catecholamines are expressed and regulated differently in adult sympathetic neurons.

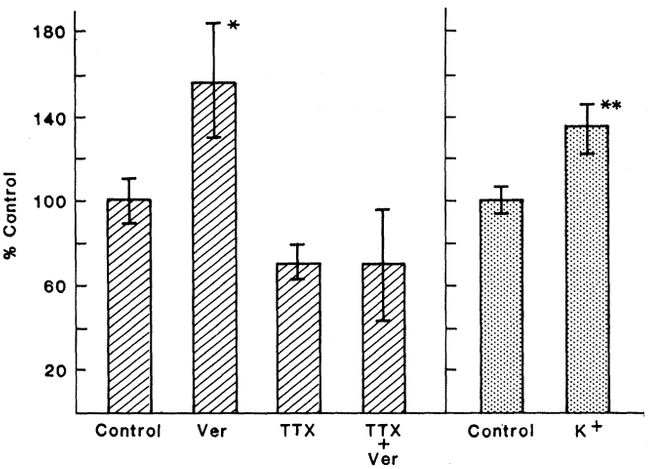

Fig. 4. Effects of depolarizing agents on TH activity in the brain nucleus locus ceruleus. E-12 cultures were grown for 1 week and then exposed to the indicated agents for 7 days. In the left panel cultures were exposed to veratridine (Ver; 1.5 μM) or tetrodotoxin (TTX; 0.1 μM), or both. In the right panel, cultures were exposed to K^+ (20 mM). Cultures were rinsed well in cold Hanks balanced salt solution and dissected free of the collagen-coated coverslips for TH assay. Each sample in each group consisted of two cultures. At least seven such samples were included in each group in the left panel, and 24 in the right panel. Results are expressed as percentages of the appropriate vehicle-exposed control. Control values were 8.9 ± 0.99 pmol of dopa per locus pair per hour (mean ± S.E.) for the left panel and 15.8 ± 1.12 for the right. *Differs from control, TTX, and TTX + Ver groups at $P < 0.01$ (one-way analysis of variance: Newman-Keuls test). **Differs from control at $P < 0.025$ by Student's t-test.

Plasticity in Brain Neurons

These studies indicate that a wide variety of peripheral neuronal and neuroendocrine cells exhibit transmitter plasticity during development and maturity. To begin examining plasticity in brain neurons we have been growing noradrenergic (norepinephrine) neurons of the pontine nucleus locus ceruleus in explant culture. Initial observations indicated that embryonic mouse locus expressed TH de novo in culture and faithfully reflected ontogeny in vivo (*26*). We used this system to determine whether the locus exhibits plastic responses to extracellular stimuli during development in vitro, and whether plasticity persists after stable, adult plateau values are attained.

The mouse locus was explanted at E-12, grown in basal medium for 1 week, and then exposed to veratridine depolarization for 7 days (*27*). Veratridine exposure significantly increased TH activity over cultures of the contralateral control locus, and this effect was blocked by tetrodotoxin (Fig. 4). Moreover, depolarizing concentrations of K^+ reproduced the effect of veratridine, suggesting that depolarization per se increased TH activity. Further, morphometric analysis indicated that veratridine did not significantly alter TH-positive cell number, suggesting that depolarization increased TH per neuron rather than increasing the number of neurons.

These studies were performed when basal TH is normally exhibiting a developmental rise (*26*). To ascertain whether depolarization also elevates TH after mature, plateau values are attained, older cultures were exposed to veratridine. Depolarization with veratridine after 2 weeks in culture also significantly increased TH, suggesting that mature as well as developing locus ceruleus exhibits plastic responses to depolarization.

Although we have not yet defined underlying molecular mechanisms, it now appears that brain as well as peripheral neurons exhibit transmitter trait plasticity during development and maturity. We are now examining other brain nuclei and areas to determine the prevalence of transmitter plasticity in the central nervous system.

Some Functional Considerations

It is apparent that we have only begun to explore the phenomenon of transmitter mutability in mature neurons. While it is certainly too early to rigorously define functional implications, it may be useful to delineate a number of general considerations raised by these studies. Two broad classes of questions are of immediate interest.

Although phenotypic plasticity may potentially alter neurotransmission through multiple mechanisms, two aspects of transmitter action may be particularly relevant. A change in transmitters may alter the target cell response. Alternatively, a change to different transmitters may allow the same neurons to affect different targets. In the former instance, neurons may interconvert from excitatory to inhibitory to "modulatory," by differentially affecting the postsynaptic membrane. Recent work, for example, indicates that substance P may inhibit adrenomedullary epinephrine release by interfering with cholinergic stimulation of nicotinic receptors (*28*). Thus, the effective ratio of substance P to acetylcholine at the synapse may critically alter effector responses. Additional work is now required to determine whether such modulatory interactions occur among transmitters released by the same neuron. The work described above implies that intraneuronal concentrations of excitatory and inhibitory transmitters may vary in relative concentrations over time. Functional analysis at this level requires elucidation of the modes of action of most of the recently discovered putative peptide transmitters.

In addition to altering the response of a given target cell, transmitter plasticity may, effectively, allow a neuron to interact with new targets. It is well documented that heterogeneous receptor types and subtypes are differentially distributed over different pre-, and postsynaptic membranes. For example, released norepinephrine stimulates presynaptic "autoreceptors," thereby reducing subsequent norepinephrine release; norepinephrine simultaneously, of course, exerts conventional postsynaptic effects. In contrast, stimulation of other presynaptic receptors by appropriate ligands may elicit entirely different responses, leading to finely tuned modulatory interactions. Indeed, it is possible that the multiplicity of presynaptic receptors allows a neuron to respond to small alterations in concentrations of its own transmitters. In this manner a change in transmitters may change the primary locus of actions from post- to presynaptic, depending on receptor distribution. Recent work with bullfrog sympathetic ganglia suggests another mechanism for alteration of site of transmitter action. An LHRH-like (luteinizing hormone–releasing hormone) peptide may diffuse for many micrometers before eliciting the late, slow excitatory postsynaptic potential in ganglion cells (*29*). Consequently, different transmitters may act over very different distances and thereby influence very different spectra of postsynaptic cells. Therefore, alteration of transmitter phenotype could, theoretically, alter neuronal communication without an alteration in neuronal circuitry.

Consequently, transmitter mutability may constitute a unique mechanism underlying plasticity in the nervous system. Transmitter plasticity in the adult may effectively alter neural pathways and circuits in the absence of growth of new cell assemblies, neosynaptogenesis, neurogenesis, and ongoing neuronal turnover.

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