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Choline acetyltransferase activity increases in the brain stem of rats treated at birth with 6-hydroxydopa

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The mechanisms that control the normal development of adrenergic neurons remain largely unknown. In the peripheral nervous system, there seems to be a reciprocal influence during development between sympathetic neurons and the preganglionic cholinergic fibers which synapse with them. The integrity of these fibers is essential for the normal biochemical development of ganglionic neurons[2,21]. On the other hand, experimental situations that either promote or retard the maturation of these neurons enhance or diminish the activity of choline acetyltransferase (ChAc) in the ganglion[3,21,22]. This enzyme, responsible for acetylcholine biosynthesis, is considered as a suitable biochemical marker for cholinergic structures. These observations led to the proposal that the cholinergic system may also participate in the differentiation of central adrenergic neurons[4]. This is conceivable because some of these cells seem to receive a dense cholinergic innervation, particularly those of the nucleus locus coeruleus which originate on the main noradrenergic pathways in the brain[23]. Histochemical observations indicate that these neurons are characterized not only by a high concentration of noradrenaline (NA) but also by a high activity of acetylcholinesterase (AChE)[13]. Furthermore, the changes in the AChE staining of locus coeruleus neurons during development are compatible with the existence of a cholinergic influence in this process[11].

The neurotoxic drug 6-hydroxydopamine (6-OHDA), as well as its precursor amino acid 6-hydroxydopa (6-OHDOPA), selectively injure central noradrenergic neurons and thus may constitute useful tools to study their interaction with cholinergic systems. Attempts to demonstrate changes in the activity of ChAc produced by these compounds in adult rats have been unsuccessful (for references see ref. 10). But their administration during development, which markedly alters the maturation of adrenergic neurons, may provide some hint on the participation of the cholinergic system in this process. Profound modifications in the distribution of NA appear in the brain of adult rats that received the drug immediately after birth. In these animals, the concentration of NA in the forebrain and the spinal cord is decreased, while in the brain stem and the cerebellum NA is markedly increased[7–9,15,17–20,24–26]. These long-term changes, that seem to be relatively limited to the noradrenergic system, have been

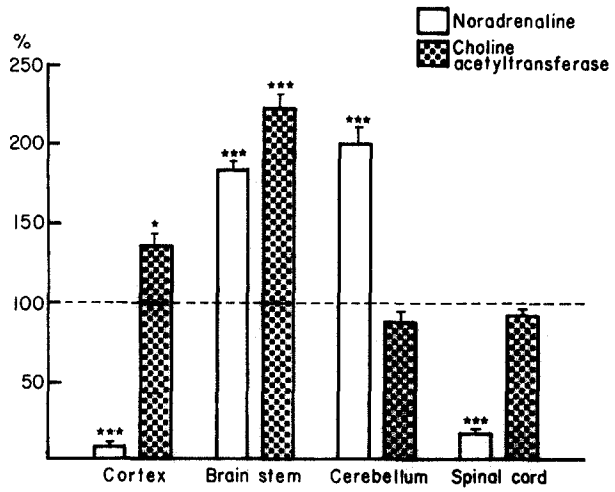

Fig. 1. Modifications in the concentration of endogenous NA and in the activity of choline acetyltransferase in different brain regions of adult rats injected on the day of birth with 6-OHDOPA (100 μg/g s.c.). Absolute control values for NA (ng/g weight): cortex 137 $\pm$ 6, brain stem 374 $\pm$ 17, cerebellum 149 $\pm$ 9 and spinal cord 247 $\pm$ 5; and for choline acetyltransferase (pmole acetylcholine/h/μg weight): cortex 0.35 $\pm$ 0.02, brain stem 0.45 $\pm$ 0.01, cerebellum 0.52 $\pm$ 0.05 and spinal cord 0.92 $\pm$ 0.05. Each value represents the mean $\pm$ S.E. of 4–6 groups of 6–8 animals each. * $P < 0.05$; *** $P < 0.001$ when comparing values of treated groups with corresponding controls.

interpreted as reflecting the destruction of NA-containing terminals in the forebrain and an outgrowth of these endings from lesioned neurons in the brain stem[8,18]. It is now clear that the administration of 6-OHDOPA can produce these permanent elevations in brain stem and cerebellar NA only when performed during a brief period of development[7,25,26].

This peculiar reaction of adrenergic neurons to the neonatal injection of 6-OHDOPA provides a suitable condition to investigate the possible interactions with the cholinergic system during their development.

Newborn Wistar rats were injected on the day of birth with L-2,4,5-trihydroxyphenylalanine (6-OHDOPA) dissolved before use in 0.001 *N* HCl. Some animals received an s.c. injection of 100 μg/g of 6-OHDOPA while control rats received the diluent alone. Litter sizes were reduced to a maximum of 8 pups and control and treated rats were reared together and weaned at 28 days. At 30–40 days of age rats were killed by decapitation and their brains were exposed. The cerebellum was separated and the brain stem (mesencephalon, pons, medulla) was isolated from the forebrain by a coronal section between the anterior colliculi and the mammillary bodies. The concentration of endogenous NA as well as the activity of ChAc were determined in the cerebral cortex, brain stem, cerebellum and spinal cord. NA was assayed fluorometrically[6] after isolation of the amine from perchloric acid extracts by cation column exchange chromatography[1]. ChAc activity was determined in aliquots of tissue extracts with the radiometric procedure of McCaman and Hunt[12] using as substrate [^{14}C]-acetyl-coenzyme A (New England Nuclear, sp. act. 58 mCi/mmole). All assays with appropriate blanks were run in triplicate and the radioactivity of the product of the

enzymatic reaction, [^{14}C]acetylcholine, was measured. Counting efficiency was monitored by the use of internal standards.

For each experimental procedure 4–6 groups, each of 6–8 rats, were studied. The significance of differences between values was determined by Student's *t*-test.

As shown in Fig. 1, the administration of 6-OHDOPA on the day of birth produced a marked depletion of NA in the cerebral cortex and in the spinal cord while it increased NA levels in the brain stem and in the cerebellum. These changes, produced by a single injection of 6-OHDOPA given on the day of birth, are similar to those previously described following multiple injections of the drug on alternate days[7,24–26]. The only difference encountered was that while multiple injections of the drug depleted cerebellar NA, a single injection produced an increase in the level of NA in the cerebellum. The possible significance of this observation has been discussed elsewhere[7,26].

Fig. 1 also shows that the neonatal injection of 6-OHDOPA produced marked changes in the regional activity of ChAc. While in the cerebral cortex the activity of the enzyme was slightly increased, it remained unchanged in the cerebellum and in the spinal cord. On the other hand, ChAc activity was markedly increased in the brain stem. Thus, it seems that cholinergic neurons are not affected to the same extent in all brain regions since only in the brain stem was there a clear modification of ChAc activity.

The elevation of NA levels in the brain stem and in the cerebellum following neonatal 6-OHDOPA has been interpreted as resulting from the enhanced sprouting of lesioned neurons which have their cell bodies in the brain stem[8,18]. Since ChAc activity increased only in this region and not in the cerebellum, it was considered that such an increase could reflect a phenomenon taking place in the vicinity of noradrenergic cell bodies. Since the region containing the locus coeruleus can be isolated and submitted to biochemical analysis[16], it was possible to explore the hypothesis proposed by studying the level of endogenous NA and the activity of ChAc in the different portions of the noradrenergic neuron (cell body and terminal arborizations) following neonatal 6-OHDOPA. The activity of AChE, which seems to be present in the cell bodies of NA-containing neurons in the locus coeruleus[13], was also assayed because it could possibly be altered by the treatment.

For these purpose, the brain stem was divided as described by Reis and Ross[16] by a coronal section at the caudal level of the posterior colliculi and another section at the level of the facial colliculi. In this fraction, the increase of NA following neonatal 6-OHDA is higher than in the rest of the brain stem[7]. From this fraction, two zones were further separated by a horizontal section placed approximately 1.5 mm beneath the floor of the fourth ventricle. This yielded a dorsal region containing the nucleus locus coeruleus and a ventral region in which terminals of adrenergic neurons innervate several brain stem nuclei. In both regions, NA levels and ChAc activity were determined as described above while AChE activity was assayed by a colorimetric procedure using acetylthiocholine as substrate[5].

The observations made with the histochemical fluorescence method for the demonstration of catecholamines support the hypothesis that the elevation in brain

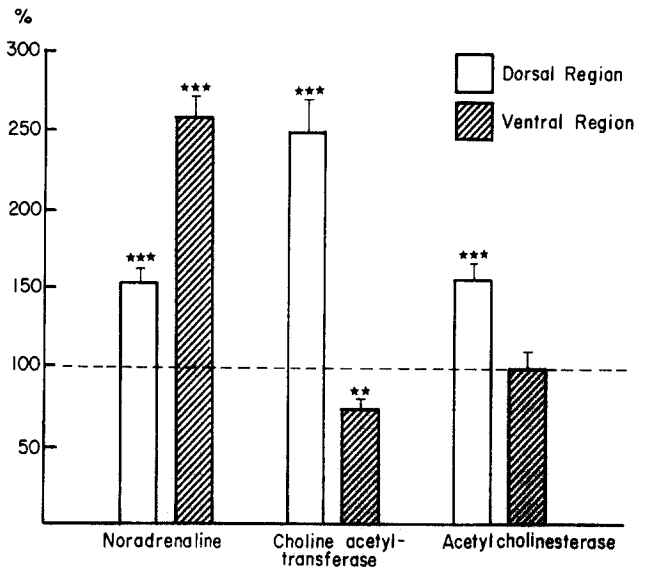

Fig. 2. Modifications in the concentration of endogenous NA and in the activities of choline acetyltransferase and acetylcholinesterase in the fraction of the brain stem containing the locus coeruleus of adult rats treated at birth with 6-OHDOPA (100 μg/g s.c.). The dorsal region contains the noradrenergic cell bodies of the locus coeruleus while the ventral region contains noradrenergic nerve terminals. Absolute control values for NA (ng/g weight): dorsal region 1629 $\pm$ 112, ventral region 2492 $\pm$ 117; and for choline acetyltransferase (pmole acetylcholine/h/μg weight): dorsal region 4.51 $\pm$ 0.23, ventral region 1.45 $\pm$ 0.07; acetylcholinesterase (pmole acetylthiocholine hydrolyzed/min/μg weight): dorsal region 14.68 $\pm$ 0.91, ventral region 10.28 $\pm$ 0.46. Each value represents the mean $\pm$ S.E. of 4–6 groups of 6–8 animals each. ** $P < 0.01$; *** $P < 0.001$ when comparing values of treated groups with corresponding controls.

stem NA is mainly due to an increase in the number of NA-containing terminals and/or in the concentration of NA in these terminals, and not to a marked modification in the content of NA in the cell bodies[8,18]. If this interpretation is correct, the biochemical analysis should demonstrate a much greater elevation of NA in the zone containing the nerve terminals than in the portion corresponding to the cell bodies. Fig. 2 shows that these were precisely the changes produced following neonatal 6-OHDOPA. NA levels, which were relatively higher in the dorsal region than in the ventral zone, were moderately increased in the dorsal zone containing the cell bodies but markedly elevated in the ventral region.

The activity of ChAc was also greater in the zone containing the cell bodies. As a result of 6-OHDOPA injection, enzymatic activity increased markedly in this dorsal region while it decreased slightly in the ventral zone (Fig. 2). It has been reported that the activity of tyrosine hydroxylase also increases in the region of the locus coeruleus following neonatal 6-OHDOPA[14]. Thus, the changes in this zone are similar to those produced in peripheral sympathetic ganglia by the administration of nerve growth factor, *i.e.*, an elevation of tyrosine hydroxylase activity accompanied by an increase of ChAc activity[22].

Finally, Fig. 2 also shows that the activity of AChE, which was higher in the

dorsal region than in the ventral zone, increased 55% after treatment with 6-OHDOPA in the zone of the cell bodies. In the ventral regions, AChE activity remained unchanged.

Central adrenergic neurons respond to 6-OHDOPA given immediately after birth in such a way that leads to an enhanced sprouting reflected in the increased concentration of endogenous NA in the structures close to their cell bodies. The elevation in the content of NA in the region of the locus coeruleus following neonatal 6-OHDOPA was of a similar magnitude to the increase in the activity of AChE in that zone. This could reflect either the presence of a greater number of perikarya or an increased content of the amine and the enzyme per cell body. In any case, although it can not be proven with absolute certainty due to the complexity of central nervous structures, the modifications of cholinergic neurons revealed by the selective increase of ChAc activity in the brain stem, and more precisely in the vicinity of adrenergic cell bodies, seem to be the consequence of a retrograde influence exerted by these cell bodies altered by 6-OHDOPA injection.

Therefore, it seems that not only sympathetic ganglion cells but also central adrenergic neurons interact during their ontogenesis with the cholinergic system. These observations also indicate that the analysis of the alterations produced by 6-OHDOPA given in the course of the development of central noradrenergic pathways may clarify the participation of different factors in this process.

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