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DSP-4: A NOVEL COMPOUND WITH NEUROTOXIC EFFECTS ON NORADRENERGIC NEURONS OF ADULT AND DEVELOPING RATS

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SUMMARY

The pharmacological actions of the compound N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) are compatible with a specific neurotoxic effect on both peripheral and central noradrenergic neurons. The systemic injection of DSP-4 to adult rats transiently alters sympathetic neurons in the periphery but in the central nervous system the compound determines a marked and prolonged reduction of noradrenaline (NA) levels in all brain regions studied. When DSP-4 was injected systemically to rats at birth in doses ranging from 6.25 to 100 μg/g, no changes were found in peripheral sympathetic neurons 40 days later. On the contrary, in the same conditions and in relation to the dose injected, there were marked and persistent changes in the levels of NA in different regions of the brain. In the cerebral cortex and the spinal cord, the neonatal injection of DSP-4 produced a marked and long-lasting depletion of NA levels, similar to that observed after injection of the compound to adult rats. These changes were accompanied by a moderate increase in brain stem NA and a marked elevation of the amine in the cerebellum. These changes, different from the depletion observed in both regions when the compound was given to adult rats, are however similar to those observed after the neonatal injection of the neurotoxic compounds 6-hydroxydopamine or its precursor amino acid, 6-hydroxydopa. This indicates that probably central noradrenergic neurons respond in the same manner after different chemical injuries. DSP-4 crosses the placental barrier because when it was given to pregnant rats at the end of gestation, long-term changes were found in brain NA levels in their offspring, similar to those produced by the neonatal administration of the compound. This new neurotoxic compound provides a very useful tool for the study of noradrenergic neurons both in adult animals and during ontogenesis.

INTRODUCTION

During the search of tertiary haloalkylamines related to bretylium and capable, once in the brain, of cyclizing to the corresponding quaternary ammonium derivative endowed with adrenergic neuron blocking activity, a compound was identified with a very long-lasting inhibitory effect on the uptake of noradrenaline (NA) by brain slices[12]. This compound, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), that is a strong alkylating agent and shares some structural similarities with the α-adrenergic receptor blocker phenoxybenzamine, inhibits the uptake of NA by cortical homogenates for periods as long as 8 months after a single injection. Subsequent pharmacological studies led to the conclusion that most probably DSP-4 strongly interacts with the NA uptake mechanism at the level of the neuronal membrane and either permanently inactivates this process or triggers the degeneration of noradrenergic terminals. The marked depletion of brain NA as well as the decrease in the activity of the enzyme dopamine-β-hydroxylase, markers of noradrenergic neurons, support the assumption that the compound permanently impairs noradrenergic neurons in the brain[11,13]. DSP-4 produces similar changes in peripheral sympathetic neurons but in this case, a rapid and marked reduction of NA levels and of uptake sites for the amine as well as a disappearance of fluorescent noradrenergic fibers, are followed by a gradual recovery of all these parameters which becomes complete 2–4 weeks after injection[7,11].

There is a striking similarity between the changes produced by DSP 4 on noradrenergic neurons and those determined by the neurotoxic compounds 6-hydroxydopamine (6-OHDA) or its precursor amino acid 6-hydroxydopa (6-OHDOPA) (for references see refs. 10 and 16). In the course of the analysis of the interaction of the latter compounds with the development of noradrenergic neurons[6,17,18], we investigated the effects of DSP-4 during this process. It was found that DSP-4 given perinatally to rodents, does not alter the development of peripheral noradrenergic neurons like 6-OHDA, but causes permanent changes in regional brain NA levels that, as in the case of 6-OHDA, differ in some regions from those produced by the compound when given to adult rats.

MATERIAL AND METHODS

Rats of the Wistar strain were used throughout these experiments. Two groups of adult female rats weighing between 200 and 300 g, received 50 mg/kg of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4). The compound was synthesized by Dr. Richard Dahlbom and collaborators at the University of Uppsala, Sweden. A group of rats was given DSP-4 intraperitoneally while others received two intravenous injections, each of 25 mg/kg, with an interval of 60 min. The drug was dissolved immediately before use in water and control animals received an equivalent volume of the diluent. Control and treated animals were killed at different time intervals after injection.

A group of pregnant rats received DSP-4 intravenously (2 $\times$ 25 mg/kg) as

indicated above on day 20 of gestation. Parturition occurred on day 21 and litters were reduced to a maximum of 8 neonatal pups. The offspring of the injected rats as well as corresponding controls, i.e. offspring of rats that received the diluent at day 20 of gestation, were weaned at 28 days and killed 60 days after birth.

In another series of experiments, newborn rats were separated into experimental and control groups and injected subcutaneously within 12 h after birth with DSP-4. Various groups received different doses of DSP 4 ranging from 6.25 to 100 μg/g in 0.1 ml water. Controls received the diluent alone. Rats were reared together, given free access to food and water, weaned at 28 days and killed at 35–50 days or at more than 100 days after birth.

Rats were decapitated, the brain was exposed and the olfactory tubercles and the pineal gland were discarded. The cerebellum was removed and the brain stem (medulla, pons, midbrain) was isolated by a coronal section between the anterior colliculi and the mammillary bodies and a section at the most caudal margin of the cerebellum. The spinal cord was isolated and the cerebral cortex was dissected from the rest of the hemispheres.

The different regions of the central nervous system as well as peripheral organs such as the superior cervical ganglia, heart, salivary gland and spleen, were homogenized in 10 ml of 0.4 N perchloric acid containing 0.2% EDTA and 0.005% $Na_2S_2O_5$ for extraction of NA. The amine was isolated by cation column exchange chromatography[3] and its content in the eluates from the columns was determined fluorimetrically[4]. Results were not corrected for recovery which was 86.5 $\pm$ 2.3%. The significance of differences between values was determined by means of Student's t-test.

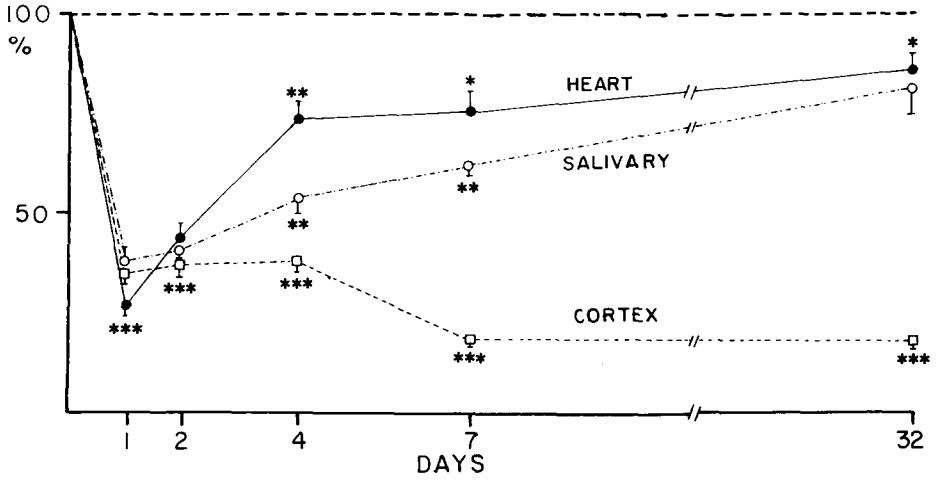

Fig. 1. Time course of DSP-4-induced modifications in the endogenous NA content of heart, salivary glands and cerebral cortex. Adult rats were injected with DSP-4 (50 mg/kg i.p.) and were killed 1, 2, 4, 7 and 32 days after injection. The results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight): heart 651 $\pm$ 31; salivary gland 1477 $\pm$ 144 and cerebral cortex 227 $\pm$ 7. Each value represents the mean $\pm$ S.E. of 3–5 groups of 5–7 rats each. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

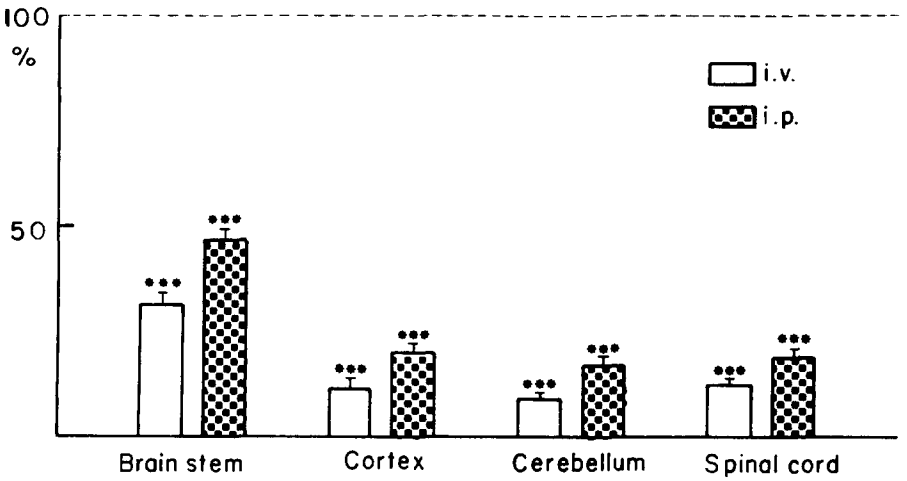

Fig. 2. Modifications in the content of endogenous NA in the brain stem, cerebral cortex, cerebellum and spinal cord of adult rats injected with DSP-4 (50 mg/kg) either i.v. or i.p. The animals were killed 40–50 days after injection. Results are expressed as percentage of untreated control values. The significance of differences between values obtained in groups receiving DSP-4 i.v. and i.p. is given in the text. Absolute control values for NA (ng/g weight): brain stem 508 ± 13; cortex 306 ± 24; cerebellum 184 ± 3 and spinal cord 258 ± 11. Each value represents the mean ± S.E. of 3–5 groups of 5–7 rats each. ***$P < 0.001$.

RESULTS

DSP-4 effects on central and peripheral noradrenergic neurons of adult rats

To determine the effects of DSP-4 on central and peripheral noradrenergic neurons in adult rats, a single i.p. injection of 50 mg/kg DSP-4 was given and the animals were killed at different time intervals after treatment. As shown in Fig. 1, in organs which received a rich sympathetic nerve supply such as the heart and the salivary glands, NA levels were reduced to 35% of those of controls during the initial 24 h after treatment. There was a gradual recovery of NA concentration in both organs, although 7 days after injection heart and salivary gland NA were significantly reduced by 24% and 38% respectively when compared with control values. At 32 days however, the depletion of NA was significant only in the heart.

In the cerebral cortex there was also a marked and rapid reduction of NA levels. In this case however, NA depletion persisted throughout the period studied, i.e. it was 82% even 32 days after the injection of DSP-4, a time at which peripheral stores of NA were almost completely recovered.

To further characterize the effects of DSP-4 on central noradrenergic neurons in adult rats, the content of NA was studied in different regions of the brain, 40 days after a single dose of 50 mg/kg given either intraperitoneally or intravenously to adult rats. Fig. 2 shows that DSP-4 markedly reduced NA levels in all the regions of the brain studied. In the brain stem, NA was reduced by 53% after i.p. administration of DSP-4 and by 70% when the compound was given i.v., the difference between both values being significant ($P < 0.01$). The depleting effect was even more pronounced not only in the cortex as already described but also in the cerebellum and the spinal cord where

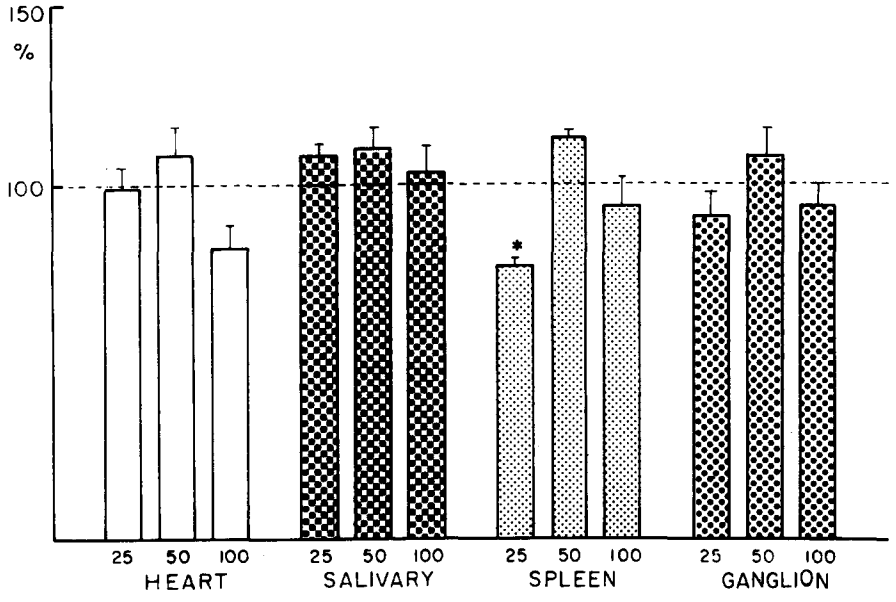

Fig. 3. Modifications in the content of endogenous NA in peripheral organs of adult rats injected at birth with DSP-4 (25 μg/g, 50 μg/g or 100 μg/g). The animals were killed at 35–50 days of age. Results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight): heart 558 $\pm$ 32 and salivary glands 874 $\pm$ 53; (ng NA/organ) spleen 106 $\pm$ 5 and superior cervical ganglion 10 $\pm$ 0.6. Each value represents the mean $\pm$ S.E. of 3–5 groups of 6–8 rats each. *$P < 0.05$.

NA levels were reduced between 80 and 91 %. In all cases, the depletion of NA was significantly more important after i.v. treatment than after i.p. administration ($P <$ 0.01).

DSP-4 given neonatally and the development of central and peripheral noradrenergic neurons

To study the effects of DSP-4 on the ontogenesis of central and peripheral noradrenergic neurons, different doses of the compound, ranging from 6.25 to 100 μg/g were given s.c. to newborn rats. Fig. 3 shows that the administration of 25, 50 or 100 μg/g of DSP-4 did not modify the content of NA in the heart, the salivary gland, the spleen or the superior cervical ganglion of 35–50-day-old rats injected neonatally. The lack of effect of the compound in the content of NA per ganglion, indicates that it does not reduce the number of sympathetic cell bodies[2,5].

In the brain, however, the neonatal administration of DSP-4 produced marked regional changes in the concentration of NA when this was analyzed 35–50 days later. Fig. 4 shows that in the brain stem, NA levels were not modified by 6.25, 12.5 or 100 μg/g but were significantly elevated in the groups treated with 25 or 50 μg/g. Cortical NA was reduced by all the doses of DSP-4 injected and with 50 and 100 μg/g, cortical NA was reduced by 60 %. In the cerebellum, marked elevations of NA were observed after doses of DSP-4 ranging from 6.25 to 50 μg/g; 100 μg/g DSP-4 did not modify cerebellar NA. Finally, in the spinal cord, 6.25 or 12.5 μg/g of DSP-4 did not change

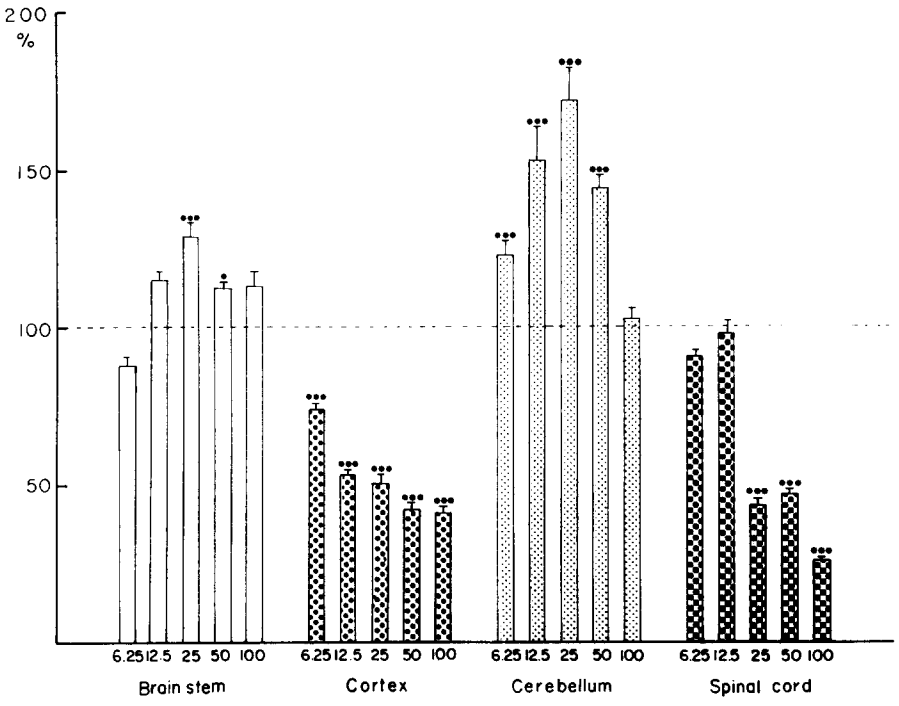

Fig. 4. Modifications in the concentration of endogenous NA in several brain regions of adult rats injected with DSP-4 at birth in doses ranging from 6.25 to 100 μg/g. The animals were killed at 35–50 days of age. Results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight) brain stem 504 $\pm$ 18; cortex 192 $\pm$ 7; cerebellum 194 $\pm$ 4 and spinal cord 290 $\pm$ 11. Each value represents the mean $\pm$ S.E. of 3–5 groups of 6–8 rats each. $*P < 0.05$; $***P < 0.001$.

NA levels but these were reduced by 60–70% after doses ranging from 25 to 100 μg/g.

To determine the persistence of these changes, NA levels in different brain regions were compared in groups of animals 35–50 days and more than 120 days after the neonatal injection of 25 or 50 μg/g of DSP-4. Fig. 5 shows that at both time intervals, brain stem and cortical NA levels were almost similar in groups that received 25 or 50 μg/g DSP-4. In the cerebellum of rats treated with 25 μg/g at birth, the elevation of NA was significantly greater at 40 days of age than at 120 days ($P < 0.01$), while no differences were apparent between cerebellar NA in rats that received 50 μg/g. A striking finding was that while NA levels were reduced by 57% in the spinal cord of rats treated 35–50 days previously with 25 μg/g, the content of NA in the spinal cord was normal in these animals 120 days after neonatal treatment. The difference between both values was significant ($P < 0.001$). In the group of rats that received 50 μg/g at birth, no difference was found in spinal cord NA between rats killed at 40 days and those killed at 160 days.

Prenatal administration of DSP-4 and development of central noradrenergic neurons

To establish if DSP-4 is capable of crossing the placental barrier and thus lesioning the central noradrenergic neurons of developing rats 'in utero', the com-

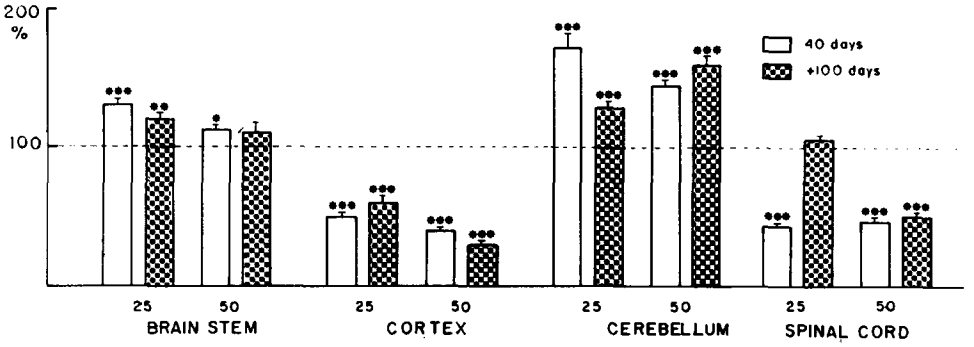

Fig. 5. Modifications in the content of endogenous NA in several brain regions of adult rats injected at birth with DSP-4 (25 or 50 μg/g). Rats were killed at the ages of 40 days (both groups) and of more than 100 days (120 days for group 25 μg/g and 160 days for group 50 μg/g). Results are expressed as percentages of corresponding untreated control values. Absolute values for NA are given in the legend of Fig. 4 for the animals killed at 40 days while those corresponding to control rats of more than 100 days of age did not differ significantly from them. The significance of the differences between the values found in treated groups at both time intervals is given in the text. Each value represents the mean $\pm$ S.E. of 3–5 groups of 6–8 animals each. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

pound was administered to pregnant rats on day 20 of gestation. As shown in Fig. 6 the content of NA in different brain regions of the 60-day-old offspring was modified as when DSP-4 was directly given to newborn animals, i.e. NA levels were slightly elevated in the brain stem, reduced in the cortex and the spinal cord and increased by 40% in the cerebellum.

DISCUSSION

The results obtained confirmed that DSP-4 administered systemically to adult rats produces a rapid and marked depletion of NA stores in peripheral organs such as the heart and the salivary glands[11]. This is a transient effect since one month after treatment, NA levels were almost completely recovered. But in the central nervous system, DSP-4 produces long-lasting modifications in the content of endogenous NA, in accordance with the loss of uptake sites for NA and the decrease of the activity of dopamine-β-hydroxylase that have been reported in comparable conditions[11,13]. NA depletion was found in all brain regions studied, being more profound in the cortex, the cerebellum and the spinal cord than in the brain stem.

The injection of DSP 4 to newborn rats does not produce long-lasting changes in the development of peripheral noradrenergic neurons. This is in contrast with the partial but permanent sympathectomy determined by the administration of 6-OHDA or 6-OHDOPA to newborn rodents[1,5,15]. DSP-4, unlike 6-OHDA[2,5], does not destroy the developing noradrenergic cell bodies in sympathetic ganglia and thus fails to alter permanently the content of NA in peripheral organs such as the heart, the salivary gland and the spleen that have a rich sympathetic innervation. This observation adds further support to the suggestion that the effects of DSP-4 might not be similar in central and in peripheral noradrenergic neurons, probably reflecting differences in

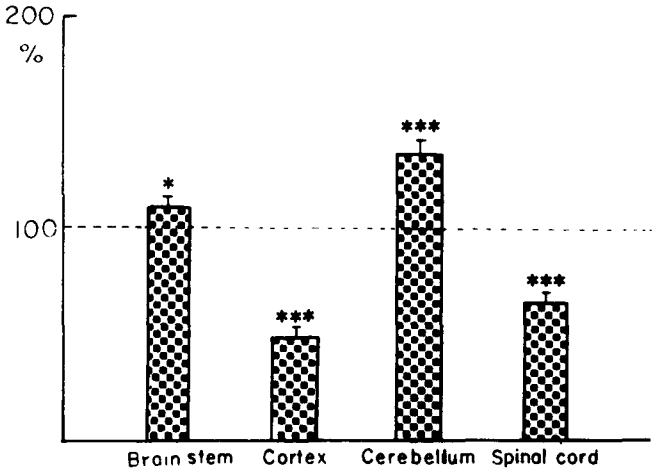

Fig. 6. Modifications in the content of endogenous NA in several brain regions of adult rats whose mothers were injected at day 20 of gestation with DSP-4 (50 mg/kg i.v.). The offspring were killed at 60 days of age. Results are expressed as percentages of untreated control values. Absolute control values for NA (ng/g weight): brain stem 510 $\pm$ 18; cortex 246 $\pm$ 12; cerebellum 205 $\pm$ 4 and spinal cord 294 $\pm$ 11. Each value represents the mean $\pm$ S.E. of 3 groups of 4 rats each. $*P < 0.05$; $***P < 0.001$.

the binding of DSP-4 to the NA transport system, responsible for the effects of the compound.

On the other hand, the neonatal administration of DSP 4 produced marked changes in the content of NA in several brain regions. These changes were related to the dose of the compound injected. As has been discussed, the administration of the compound to adult rats depletes NA from all the brain areas studied, including the brain stem and the cerebellum. In contrast, the neonatal administration of DSP 4 moderately elevated NA in the brain stem and markedly increased its content in the cerebellum. This increase in brain stem and cerebellar NA after neonatal DSP-4, not observed when the compound was given to adult rats and similar to that found after the perinatal administration of 6-OHDA[8,14] and 6-OHDOPA[6,9,17,18], indicates that the processes leading to the increase of NA levels in these zones probably constitute a common response of developing noradrenergic neurons to chemical injuries. However, the effects of neonatal DSP-4 seem to be more important on the noradrenergic innervation of the cerebellum than in the brain stem where the changes are not so marked as those observed after 6-OHDA. Thus, there are some differences between the response to both compounds that could be useful to elucidate the normal process of noradrenergic neuron development. DSP-4 given neonatally also reduced markedly NA levels in the cortex and the spinal cord, the latter after doses higher than 25 μg/g. These reductions were in accordance with the effects of the compound injected to adult rats.

The modifications produced by the neonatal administration of DSP-4 persist for long periods of time and NA levels in the brain were similarly affected 40 days and more than 100 days after the neonatal injection. This suggests that the changes

observed, as in the case of those produced by neonatal 6-OHDA, have a structural correlate such as the collateral accumulation of NA and/or sprouting as well as degeneration of noradrenergic terminals in the territories where NA concentrations are respectively increased or decreased[8,14]. Two notable exceptions were found in animals that received 25 μg/g DSP 4 at birth. In this group NA levels in the cerebellum were significantly lower 120 days after injection that at 40 days. Also, the behavior of spinal cord NA was striking because while at 40 days NA levels were markedly reduced, they were found to be normal 120 days after the neonatal injection of 25 μg/g DSP-4. This puzzling observation that suggests some kind of noradrenergic reinnervation of the spinal cord, is difficult to explain and requires further careful investigation.

Finally, these experiments showed that DSP-4 given to pregnant rats at the end of gestation, like 6-OHDOPA[6,17,18], can cross the placental barrier and alter the subsequent development of central noradrenergic neurons in the offspring. In the rats that were under the influence of the compound while 'in utero', the long-term changes in brain NA levels were similar to those produced by the injection of DSP-4 at birth.

The mechanisms through which DSP-4 produces these effects are not well understood. It has been suggested that the compound has a selective affinity for the NA uptake sites to which it is bound by electrostatic attraction. There it is spontaneously cyclized to the aziridinium derivative[12], a quaternary ammonium compound that reacts covalently with an anionic component of the uptake site, probably identical to that responsible for attracting the cationic amino group of NA[11,12]. In the stability of this bond may reside the difference between the actions of the compound in peripheral and in central noradrenergic neurons. Pharmacological evidence indicates that most probably the primary action of DSP-4 is at the level of the neuronal membrane since its effects are also observed 'in vitro', are rapid in onset, independent of a functional intraneuronal storage mechanism and antagonized by blockers of the membrane uptake process such as desipramine[11,13]. Although DSP-4 can block α-adrenergic receptors, this action is of a much shorter duration than its uptake blocking activity and less potent than that of phenoxybenzamine[11,13]. The uptake inhibiting action of DSP 4 seems to be specific for NA since the compound does not alter the incorporation of dopamine or of 5-hydroxytryptamine into slices of the striatum or of the midbrain respectively[11].

The changes observed in noradrenergic neurons after DSP-4 might be due to the discussed covalent bonding of the compound to the NA uptake sites. But the long-term nature of these effects might be better explained by assuming that the initial damage to the neurons leads to their degeneration. There are pharmacological and histochemical data supporting the existence of a degenerative process whose end result would be similar to that reached after 6-OHDA, although achieved through a completely different mechanism[7,11]. Irrespective of the current ignorance about the precise mode of action of DSP-4, this compound seems to provide an interesting tool for studying the biology and pharmacology of noradrenergic neurons. It has the advantage over the widely used catecholamine neurotoxin 6-OHDA that it crosses the blood–brain barrier in adult animals to produce similar persistent and important alterations in central noradrenergic neurons. Moreover, the effects of DSP-4 seem to

be more selective for these neurons than those of 6-OHDA. The marked alterations produced by DSP-4 on developing central noradrenergic neurons now reported, also indicate that the compound constitutes a powerful probe for analyzing this process.

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