Sympathetic Nerve Cell Destruction in Newborn Mammals by 6-Hydroxydopamine

P. U. Angeletti and R. Levi-Montalcini

C.N.R. Laboratory of Cell Biology, Rome, Italy, and Department of Biology, Washington University, St. Louis

Communicated October 22, 1969

Abstract. A selective and permanent destruction of sympathetic ganglia was achieved by injecting 6-hydroxydopamine in newborn animals. A lifelong chemical sympathectomy may thus be obtained. The process of cell damage and destruction is substantially different from that produced with an antiserum to a specific nerve growth factor.

Introduction. The possibility of a selective destruction of sympathetic nerve cells in newborn mammals by chemical rather than surgical procedures was achieved for the first time ten years ago with an antiserum to a specific nerve growth factor. This technique became known as immunosympathectomy. Attempts to obtain a long lasting exclusion of the sympathetic function were recently made by injecting analogs of various intermediates in catecholamine biosynthesis. Among these, 6-hydroxydopamine (6-OHDA) gave the most encouraging results. Porter et al. found that administration of this compound in mammals results in marked and long-lasting depletion of norepinephrine in sympathetic innervated organs. Subsequent studies by Tranzer and Thoenen gave clear evidence that 6-OHDA causes a dramatic degeneration of the adrenergic nerve endings in peripheral organs of treated animals. As reported by these authors, "The selective destruction affected only the distal part of the adrenergic nerves. The cell bodies revealed no morphological changes and 3 to 4 months after pretreatment with 6-OHDA regeneration of adrenergic endings could be detected in all organs examined such as heart, spleen, iris, and vas deferens."

The long experience acquired in our laboratory on developmental processes in sympathetic nerve cells, and the finding that these cells respond in a markedly different way during early or late stages in their life cycle, suggested the possibility that these cells may be more vulnerable to the action of this analog 6-OHDA during early periods of their development.

The results to be reported in the present article fully confirm this assumption. Administration to newborn mice and rats of 6-OHDA at doses which do not result in any visible detrimental effect on body growth of the treated animals, causes the selective and permanent destruction of sympathetic ganglia. Evidence was also obtained that the simultaneous injection of nerve growth factor does not prevent the lethal effects produced by 6-OHDA on sympathetic neurons.
Materials and Methods. Swiss mice and albino rats were used in all the experiments. Newborn animals (from 6 to 12 hr after birth) from the same litters were divided into control and experimental groups. Adult mice of about 25 gm of weight were also used.

Sympathetic ganglia and organs of control and experimental animals were dissected out immediately after sacrifice, with the help of a stereomicroscope, fixed and used for whole mounts and histological studies. In some cases, superior cervical ganglia of treated and control animals were fixed for 2 hr in 3% glutaraldehyde in phosphate buffer 0.1 M pH 7.4 with added CaCl₂. The specimens were washed for 2 hr in the same buffer, postfixed in osmic hydroxide 1.33% for 2 hr and finally embedded in Epon 8/12 for electronmicroscopic examination. Fresh solution of 6-OHDA, a kind gift from Dr. C. Stone of the Merck Sharp and Dohme Laboratories, was prepared every day by dissolving the powder in sterile physiological solution.

The nerve growth factor was purified from the mouse submaxillary salivary glands as previously described and was homogeneous by physicochemical and immunological criteria. The factor used in the present experiments had a specific activity of 0.01 μg/ml. The in vitro and in vivo activities were tested as indicated in previous articles.

Results. In a first series of experiments the 6-OHDA was injected into newborn mice in the amount of 50 μg/gm of body weight daily for seven days. One of the injected animals and one control of the same litter were sacrificed every other day for the first week and then at the end of the first and second week after the discontinuance of the treatment. At the gross inspection, treated and control mice did not differ from each other in body weight, somatic development, or vitality. The superior cervical ganglia, stellate, thoracic paravertebral chain ganglia, the celiac ganglion and the ganglionic complex around the vas deferens were dissected out from both groups and used for whole mounts, histological and ultrastructural studies. Brain, spinal cord, spinal ganglia, adrenal glands, thymus, spleen, and testis were also collected, sectioned, and examined. No differences were observed at the macro- and microscopic examination in brain, spinal cord, spinal ganglia, or any of the above-mentioned organs between control and experimental animals fixed three days after the beginning of the treatment. A noticeable size decrease was instead found in sympathetic ganglia of experimental animals fixed at this time. These differences between control and experimental animals become more marked in subsequent days and at the end of the treatment in seven-day old mice, the sympathetic cervical ganglia of experimental animals are reduced to such an extent as to make difficult their dissection at the stereomicroscope. The histological studies of ganglia examined at different time intervals show the impressive sequence of degenerative processes taking place in sympathetic neurons from the third to the seventh day, when all nerve cells are destroyed. Glial and other satellite cells do not appear directly affected at these early stages (Figs. 1–4).

After three days of treatment, most of the sympathetic neurons show degenerative marks, such as vacuolization, enlarged and pale nuclei. Scattered among these cells are pyknotic cells. Only few cells still appear normal at these early stages; usually they are the smaller and less differentiated neuroblasts (Fig. 5). In subsequent days, all neurons are severely affected and no nerve cells appear intact.

Mice three weeks old, sacrificed two weeks after discontinuation of the treat-
FIG. 1.—Whole mounts of experimental (E) and control (C) superior cervical ganglia of 7-day-old mice.

Fig. 2.—Whole mounts of stellate and first two thoracic ganglia in experimental (E) and control (C) mice 7 days old.

Fig. 3.—Cross section of superior cervical ganglion of 5-day-old mouse treated since birth with 6-hydroxydopamine.

Fig. 4.—Cross section of superior cervical ganglion of 5-day-old control mouse.
ment, were also examined. The microscopic study did not reveal any change in the central nervous system, spinal ganglia, spleen, thymus, and testis. In the adrenal gland, where no degeneration was ever found during the treatment with 6-OHDA, the adrenal medulla showed at this stage clear-cut signs of hypertrophy.

![Electronmicrograph from section of superior cervical ganglion of 3-day-old mouse injected since birth with 6-hydroxydopamine. A large neuron with marked cytoplasmic lesions is shown. Two smaller less differentiated neuroblasts in lower part of the figure are apparently intact.](image)
Sympathetic ganglia—that is, the superior cervical ganglia, stellate, thoracic chain ganglia, prevertebral celiac and mesenteric ganglia—are reduced to

Fig. 6.—Electronmicrograph of section of superior cervical ganglion from a 4-day-old mouse injected since birth with 6-hydroxydopamine. Widespread lesions of neurons are apparent. Cell membranes have disappeared; altered mitochondria and lacunar spaces are scattered throughout the cytoplasmic area.
nodules of exceedingly small size. In cross sections, no nerve cells are detectable; only a very few glial and connective cells are still present.

At the ultrastructural level, after three days of treatment, marked alterations in the fine structure of nerve cells are very apparent (Figs. 5 and 6). From these preliminary observations it would appear that the cytological lesions produced by 6-OHDA differ from those caused by a specific antiserum to the nerve growth factor. A detailed analysis of the two destructive processes is now underway and will be reported elsewhere.

Results similar to those described in newborn mice were obtained in newborn rats treated with the same doses of 6-OHDA.

A few short-term experiments were performed in adult mice. For three consecutive days the animals were intravenously injected with 6-OHDA at doses of 50 μg/gm of body weight. At the end of the treatment the superior cervical ganglia were dissected out from experimental and control mice; the ganglia were fixed, sectioned and examined at the light microscope. No morphological changes were seen in sympathetic neurons of treated mice, a result which is in full agreement with those reported by Thoenen and Tranzer.

**Effects of combined treatment with the nerve growth factor and 6-OHDA:**
The unique property of the nerve growth factor to call forth a striking growth effect in sympathetic neurons has been extensively documented. It was of interest to investigate whether the administration of this specific growth factor would "protect" or in some way interfere with the biological action of 6-OHDA. This possibility was first explored in experiments in vitro. As described in previous publications, the addition of the nerve growth factor to the culture medium results in the production of a dense fibrillar halo by sensory and sympathetic ganglia cultured in this medium.

Mixture containing various proportions of the nerve growth factor and of 6-OHDA were preincubated at room temperature and then tested for biological activity in vitro. The results (see Table 1) show that the nerve growth factor effect is not decreased nor in any way altered by 6-OHDA.

**Table 1. Effects of 6-OHDA on the in vitro biological activity of NGF.**

<table>
<thead>
<tr>
<th>NGF (μg)</th>
<th>6-OHDA (μg/ml)</th>
<th>NGF activity BU/μg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>None</td>
<td>2000</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>2000</td>
</tr>
<tr>
<td>50</td>
<td>200</td>
<td>2000</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>2000</td>
</tr>
</tbody>
</table>

* Nerve growth factor. The samples were preincubated at room temperature pH 7 for 60 min and then tested in vitro.

In another series of experiments, newborn mice of the same litters were divided in three groups. Control mice received daily injections of saline; a second group was injected with 6-OHDA (50 μg/gm of body weight). A third group was injected with the same dose of 6-OHDA as the second group, immediately followed by injections of the nerve growth factor in the amount of 10 μg/gm of body weight. The treatment in all cases was repeated daily for seven days. The animals were examined at the end of the treatment.
At gross inspection and under the dissecting microscope, the ganglia of the third group appear larger than controls. The histological examination of cross section of these ganglia reveals that this volume difference is due to a marked increase in number of connective cells while nerve cells are absent.

**Discussion.** The results reported in this article show that it is possible to obtain the complete and permanent destruction of sympathetic ganglia by injecting 6-OHDA in newborn animals for a few days, immediately after birth. 6-OHDA has been used for its potent and long-lasting blocking effect of sympathetic function in adult animals. Evidence was presented by Thoenen and Tranzer that the treatment with 6-OHDA leads to severe lesions of adrenergic nerve terminals. Recovery of function, some weeks after discontinuation of the treatment, coincides with regeneration of nerve terminals.

In newborn animals, as reported in this article, the effects elicited by this agent are much more dramatic. They consist of the total, selective and permanent destruction of sympathetic nerve cells. The treated animals, deprived in such a way of the sympathetic system, do not differ from their littermates in size, vitality, or in any other respect, at gross inspection.

The destructive process takes place in a few days, and from these still preliminary studies it would appear that nerve cells in all sympathetic ganglia, are equally and permanently destroyed.

The possibility that 6-OHDA might produce the destruction of sympathetic nerve cells in newborn animals by a mechanism similar to that of the antiserum to the nerve growth factor was explored by comparing the cellular lesions produced by the antiserum to the nerve growth factor and by 6-OHDA, and by examining the sympathetic ganglia of animals injected simultaneously with 6-OHDA and with the nerve growth factor. The results of these studies seem to indicate that the two processes are of an entirely different nature. At the histological level, the sequence of degenerative events caused by the antiserum and by 6-OHDA are markedly different. Lesions produced by administration of the antiserum are much more severe in neuroblasts which have not attained distinct differentiative marks. In the antiserum-treated newborn mice, the early lesions are in the nuclear and nucleolar compartments. In 6-OHDA treated animals, cytoplasmic lesions are predominant and diffuse following the administration of the drug. Immunosympathectomy never results in the total destruction of all adrenergic neurons. Celiac and mesenteric ganglia are more resistant to the antiserum than the superior cervical, stellate, and other thoracic chain ganglia. Ganglionic complexes innervating the sex organs in both sexes are resistant to the action of the antiserum. The results obtained so far with 6-OHDA indicate that the celiac and mesenteric ganglia are destroyed by this drug in the same way as all other sympathetic ganglia.

The nerve growth factor, which neutralizes the toxic effects of the antiserum on sympathetic cells in vivo as well as in vitro, does not counteract the 6-OHDA lethal effects on these cells. The volume increase in ganglia of newborn animals which are treated simultaneously with 6-OHDA and with the nerve growth factor is not due to preservation of sympathetic nerve cells in these ganglia. Examined at seven days, these ganglia appear to consist only of connective and
glial cells. A plausible explanation, which is now under test, is that the administration of the nerve growth factor, even in the presence of 6-OHDA, would induce during the first 24–48 hours of treatment an increase in the mitotic activity of potential nerve cells, thus resulting in the production of a larger population of neuroblasts. Subsequently these cells, as soon as they acquire differentiative marks, become a target of 6-OHDA and die out, while glial and other connective cells persist, at least for some time, in these ganglia voided of nerve cells.

A comparative analysis of destructive processes of sympathetic nerve cells achieved by chemical or immunological procedures is now in progress. These studies are expected to shed light on an extensively investigated but still practically unanswered question, namely, the role played by centrally and peripherally acting agents on growth and differentiation of nerve cells.

* This work was supported by grants from the USPHS (NB-03777), from the National Science Foundation (GB-7304), and from the Consiglio Nazionale delle Ricerche (Rome).

1 Levi-Montalcini, R., and B. Booker, these PROCEEDINGS, 46, 373 (1960).
2 Cohen, S., these PROCEEDINGS, 46, 302 (1960).