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Synaptophysin Immunoreactivity in the Rat Pituitary

Alterations after 6-Hydroxydopamine Treatment

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Synaptophysin (SN) is a synaptic-vesicle-associated membrane protein whose presence is indicative of intact, functional synapses. This study examines the presence of SN in pituitary gland innervation after neurotoxin-induced denervation followed by reinnervation. Immunostaining of rat pituitary neurointermediate lobe tissue for SN reveals a pattern of dot-like densities in the intermediate lobe and intensely stained dispersed regions in the neural lobe of normal animals. In rats treated with 6-hydroxydopamine (6-OHDA), a catecholamine neurotoxin, by peripheral injection, there is a significant depletion of the SN immunostaining in the intermediate lobe, as well as a significant reduction of SN immunoreactivity in the neural lobe, in animals studied 1 wk after drug treatment, with computer analysis of the tissue sections. At 3 wk after 6-OHDA, there is a partial recovery of immunoreactivity for SN in the neural lobe in many tissue sections, and the intermediate lobe also contains only relatively sparse staining for the synaptic protein. Computer analysis revealed that at 3 wk after 6-OHDA, both lobes still had reduced SN immunoreactivity, but the difference in levels measured did not achieve statistical significance. These results contrast with the prior finding of significant recovery of immunoreactivity for GAP-43, a growth and regeneration-associated protein, in intermediate lobe innervation of rats treated with the same drug regimen. We suggest that 6-OHDA treatment damages synaptic vesicle integrity in both the intermediate and neural lobes of the pituitary, and that recovery is in progress, but not complete at 3 wk after the drug is administered.

Key Words: Pituitary; synaptophysin; 6-hydroxydopamine; reinnervation.

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Introduction

The pituitary gland of many mammals, including the rat, is innervated by nerve fibers that originate mainly from cell bodies in hypothalamic areas. Recent studies on the innervation to the intermediate lobe have found that the major dopamine-containing cell group sending axons to form synaptic connections with endocrine cells is in the periventricular nucleus (A14) *(1–3)*. Although neural regulation of endocrine function in pituitary tissue has been a major topic of interest, the modulation of the innervation itself has only recently been addressed. A model for degeneration followed by regeneration of nerve fibers to the intermediate lobe of the pituitary *(4)* makes use of the catecholamine neurotoxin, 6-hydroxydopamine (6-OHDA), to induce short-term damage to the nerve terminals, at 1 wk after drug treatment. The drug is taken up by the peripheral circulation after iv injection and gains access into nerve terminals in the pituitary via the dopamine transporter, which has been shown to be active in hypothalamic neurons *(5,6)*. Three to four weeks after administration of 6-OHDA, there is significant recovery of the terminal innervation when compared to controls. The drug is administered peripherally and affects only the nerve fibers of the pituitary, sparing the cell bodies in the hypothalamus. Earlier studies using this model have shown initial loss of immune staining in nerve fibers by histochemistry as well as in Western blots of the rat neurointermediate pituitary for the growth and regeneration-associated protein, GAP-43, at 1 wk after drug treatment, followed by recovery at 3 wk when regeneration/recovery is occurring *(7)*. The present study makes use of the 6-OHDA-induced changes in pituitary innervation to examine alterations in immunoreactive synaptophysin (SN), a synaptic vesicle-associated protein *(8)*. The presence of SN in nerve terminals is indicative of the presence of intact vesicles in functional synapses *(8)*. The hypothesis tested here is that chemical denervation of pituitary tissue acts in part by disruption of synaptic vesicle integrity, and would lead to loss or reduction in SN immunostaining. A recent previous study *(9)* has shown a reduction in SN-immunostaining in the pituitary intermediate lobe of aging rats.

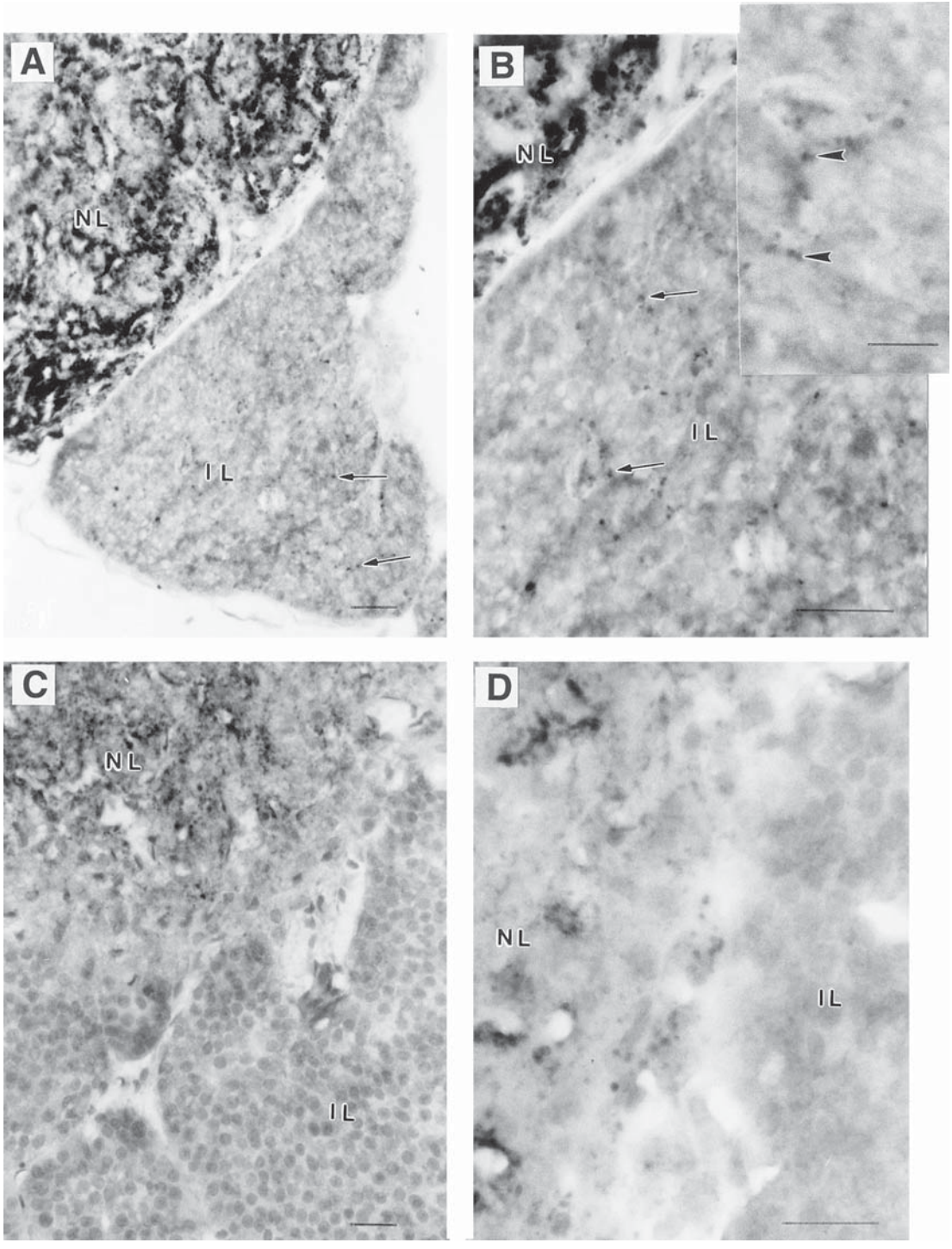

Fig. 1. (A,B) IL, Intermediate lobe; NL, Neural lobe. The marker placed on all full figures = 25 μm. Control, 1 wk. SN immunoreactive (IR) dots (arrows) are observed within the IL. In the NL, intense SN-IR areas are distributed throughout the section. Inset to (B): Enlarged area of the IL shows detail of SN-IR dots (arrowheads). Marker = 10 μm. **(C,D)** 6-OHDA, 1 wk. No SN-IR immunopositive dots can be found among the IL endocrine cells. The NL contains some SN-immunopositive areas.

Results

Immune Staining

One week after saline or 6-OHDA injections, the control intermediate lobe (IL), as expected, contained many SN-immunopositive dots among the secretory cells (Fig. 1 A,B). At the 1-wk time-point, when nerve terminals have been found to degenerate after 6-OHDA *(4)*, the IL (Fig. 1C,D) contained relatively few SN-immunoreactive dot-like regions among the endocrine cells. The neural lobe (NL) of controls (Fig. 1A,B) contained intense SN immunoreactivity, whereas the sections from the drug-treated rats retained some immunoreactive areas, although clearly

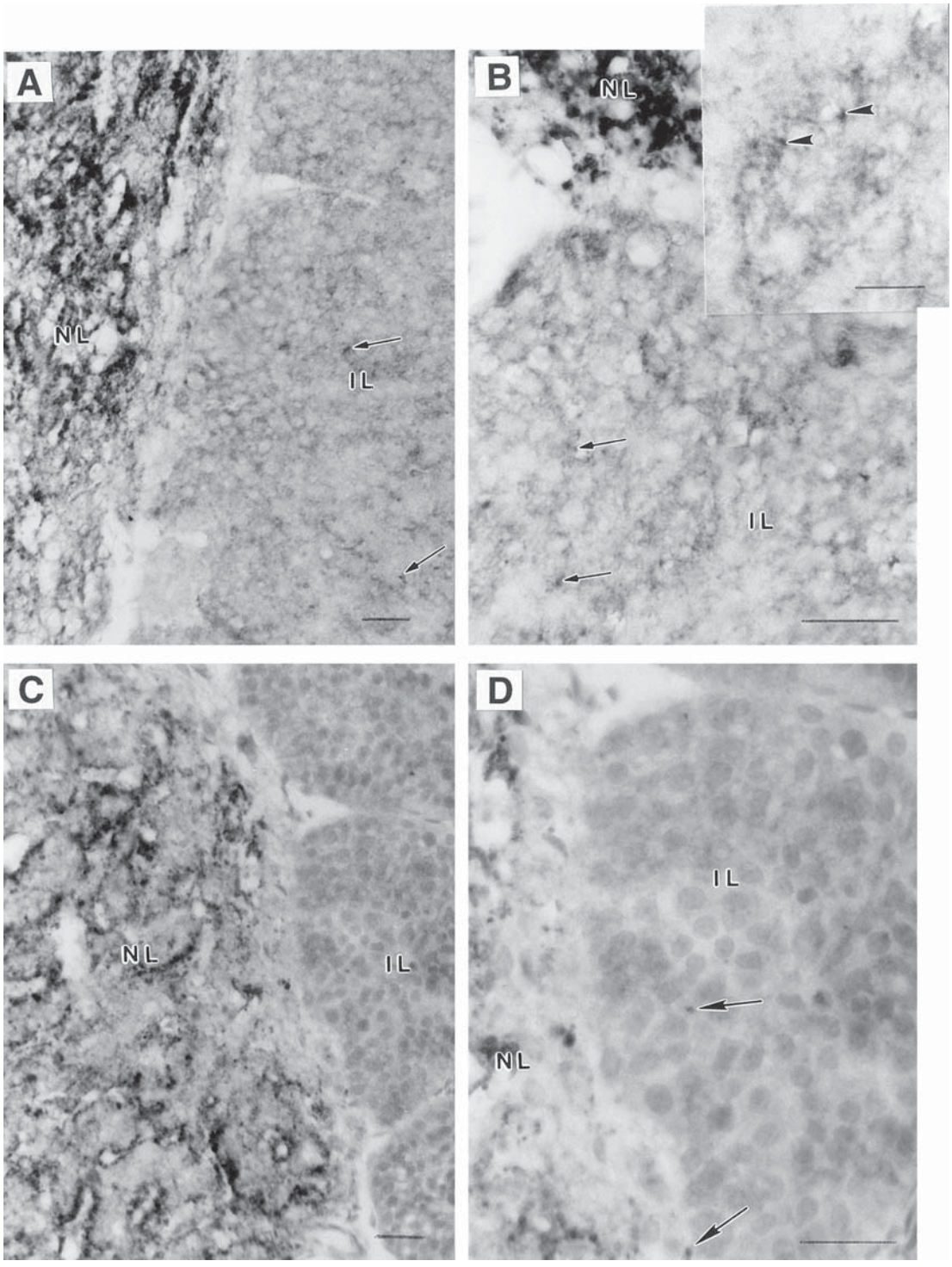

Fig. 2. (A,B) IL, Intermediate lobe; NL, Neural lobe. The marker placed on all full figures = 25 μm. Control, 3 wk. Numerous SN-IR dots (arrows) are present in the IL, as well as intense SN immunostaining in the NL. Inset to (B): Enlarged area of IL illustrates detail of SN-IR dots (arrowheads). Marker = 10 μm. **(C,D)** 6-OHDA, 3 wk. A few SN-IR dots (arrows) can be seen among the IL cells. The NL contains dispersed areas of SN immunoreactivity.

less than controls (Fig. 1C,D). Anterior lobe secretory cells contained SN-positive areas in their cytoplasm in both control and 6-OHDA-treated animals (not shown), but there were no dot-like areas between the cells as was observed in the IL. The IL cells did not contain SN immunoreactivity in the cytoplasm. Three weeks after treatment, control sections (Fig. 2A,B) appeared similar to the 1-wk controls for SN immunoreactivity in both the IL and the NL. In the sections from 6-OHDA-injected animals, the NL showed some increased intensity of staining for SN, which was comparable to controls in some sections, but less so in others (Fig. 2C,D). The staining for SN in the IL was variable at

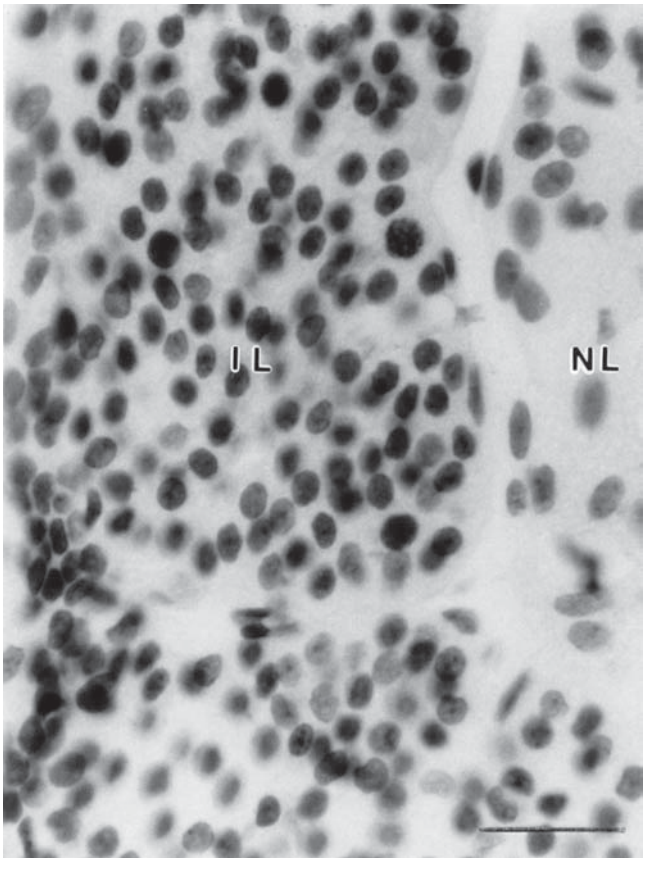

Fig. 3. IL, Intermediate lobe; NL, Neural lobe. The marker placed on all full figures = 25 μm. Control section in which the primary antibody for SN was omitted from the staining procedure. No specific immunostaining is visible in either the IL or the NL. Toluidine blue staining is seen in the nuclei of cells in both pituitary lobes.

3 wk after 6-OHDA, with some sections from individual animals containing only a few immunopositive dots and others with larger numbers (Fig. 2C,D). Omission of the primary antibody from the staining procedure showed no SN immunoreactivity (Fig. 3).

Quantitative Analysis of SN Immunoreactive Sections

At the 1-wk time-point, sections of 6-OHDA-treated tissue contained significantly fewer SN-immunopositive dots in the IL than in controls. Likewise, the area occupied by SN immunoreactivity in the NL was significantly less in the 6-OHDA-treated tissue sections than in the controls at 1 wk. At the 3-wk time-point, analysis of sections from both the IL and NL showed reduced absolute levels of SN-immunopositive staining as compared to controls, but the differences were just outside statistical significance. The latter finding confirms the variability observed in examination of the tissue sections from the 6-OHDA-treated rats at the 3-wk time. Table 1 summarizes the computer image analysis of the data for the IL and NL.

Discussion

The dopaminergic innervation of the rat pituitary is significantly affected by treatment with the neurotoxin, 6-OHDA, to induce degenerative changes in the nerve terminals. Quantitative analysis of nerve terminals using electron microscopy demonstrated that the most extensive damage occurs at 1 wk postinjection, with recovery at 3–4 wk *(4)*. In the latter study, <15% of nerve terminals in the intermediate lobe appeared normal at 1 wk after drug injection. Here, we observed significant reduction of SN-IR at 1 wk in the IL, and also in the NL, which is susceptible to 6-OHDA effects. However, many SN-immunopositive areas remained in the NL, and are suggested to be in membranes of larger, presumably peptide-containing vesicles, which may not be affected by 6-OHDA, since they are less likely to contain the dopamine transporter. At 3 wk, SN immunostaining of both the IL and the NL of drug-treated rats is not significantly different from controls using computer analysis, although there is clearly less absolute SN-immunopositive staining. The data suggest a relatively rapid, although partial, recovery of synaptic vesicle membranes in both areas. Variability in the staining pattern for SN at the 3-wk time-point in the drug-treated animals may reflect different rates of recovery for the protein within synapses.

SN, a vesicle-associated protein, has been considered essential for neurotransmitter secretion *(10,11)*. However, transgenic animals lacking the gene for SN were functionally normal *(12,13)*, suggesting that other molecules could substitute in the absence of the protein. SN is also present transiently in developing axons and dendrites of the rat hippocampus, perhaps indicative of functions in fiber outgrowth *(14)*. Recently, Bergmann and coauthors *(15)* found that neuronal perikarya in layers II and III of the contralateral entorhinal cortex were immunoreactive for SN between 8 and 10 d after unilateral electrolytic entorhinal lesions. They also noted labeling for the protein in axons and dendrites, and suggested that the transient labeling was related to outgrowth of nerve fibers. Bauerfeind and colleagues *(16)* have shown that neurosecretory vesicles in sympathetic neurons contain several membrane proteins, including SN and synaptobrevin in "hybrid" vesicles. The vesicles in neural lobe neurosecretory neurons have previously been found to contain significant amounts of SN *(17)* and, in the present study, appear to be less adversely affected by 6-OHDA than the intermediate lobe. The variability of SN immunostaining in the IL at 3 wk after 6-OHDA treatment suggests that for the cells innervating that region, there may be a longer time needed for recovery in production and transport of SN for incorporation into the vesicles.

In contrast to the findings within the IL, we did not observe any dot-like staining that would be indicative of SN in the areas outside of cells of the anterior lobe (AL), in any of the sections studied. These observations confirmed

Table 1
SN-IR After 6-OHDA Treatment[a]

Area	Control 1 wk	6-OHDA 1 wk	Control 3 wk	6-OHDA 3 wk
Int. lobe	77.5 ± 34.1 (4)	2.4 ± 1.1 (6)[b]	143.2 ± 38.3 (6)	36.6 ± 23.3 (4)
Neu. lobe	3472 ± 1198.3 (4)	25.6 ± 12.5 (6)[c]	2386.2 ± 899.9 (5)	192.8 ± 76.9(4)

[a]Intermediate lobe (Int. lobe) = mean number (± SEM) of immunoreactive dots per total area in microns squared measured per section. Neural lobe (Neu. lobe) = mean immunoreactive area in microns squared (± SEM) measured per total area in microns squared measured per section.

(n) = number of animals studied.

[b]$p < 0.02$, two-sample t-test, comparing int. lobe control and 6-OHDA, 1 wk.

[c]$p < 0.006$, two-sample t-test, comparing neu. lobe control and 6-OHDA, 1 wk.

our prior studies of SN immunostaining of pituitary sections from aging rats *(9)*, in which SN immunoreactivity in the AL was always within secretory cells. We did not observe any immunostaining in the AL, which could be suggestive of innervation to the endocrine cells in the latter study or in the present one. A number of investigations have shown neuropeptide *(18)* or GAP-43 *(19–21)* immunostaining in fibers to the AL, although the cell body origins of such possible innervation are not known. Areas of immunopositive staining for SN within endocrine secretory cells of the AL have been observed in other studies *(22)*, and the protein is considered to be a part of the membranes of the secretory granules within the cells. Other synaptic vesicle-associated proteins, synaptotagmin and rab3, have also been observed in the pituitary, in secretory cells of the AL, or in IL using immune staining *(23)* or *in situ* hybridization for mRNA *(24)*, or within innervation to the IL and neural lobe *(23)*.

Our prior studies using the same denervation/reinnervation model, with immunostaining for the growth and regeneration-associated protein, GAP-43, showed significant recovery of GAP-43 levels by 3 wk in both tissue sections and Western blots of extracted neurointermediate lobes *(7)*. GAP-43 is a membrane-associated protein that is not a part of synaptic vesicles, and may be more rapidly transported than synaptophysin after new synthesis, or may become associated with the membrane more rapidly during the recovery process. In addition, the recovery of GAP-43, but not SN may reflect a process of continued sprouting or reorganization at 3 wk post-6-OHDA injection. Our findings are consistent with those of Masliah and coworkers *(25)*, who found that after lesioning afferents to the dentate gyrus of the rat hippocampus, GAP-43 was increased in nerve terminals that were negative for SN at 2 wk after the lesion. Our most recent investigation of synaptophysin in aging rat pituitary innervation *(9)* found that the oldest animals retained fewer areas of SN immunoreactivity in the IL compared to younger animals (15–17 vs 8 mo old). The SN immunostaining of the neural lobes of aging rats did not appear to be as affected as the IL, with intense staining observed in all age groups. The findings here indicate an effect of 6-OHDA on SN immunostaining in nerves innervating both areas of the pituitary, but the NL may have a more rapid recovery than the IL for production of synaptic vesicles. Alternatively, the NL may be less susceptible to the toxic effects of 6-OHDA and retain more of the synaptic (and neuropeptide-containing) vesicles in an intact form than in the IL.

Methods

Animal Injections

Young adult male Sprague-Dawley rats (wt approx 100 g) were obtained from Harlan Industries (Indianapolis, IN), and housed in the Animal Resource Facility of the School of Medicine. All animal handling and protocols were reviewed and approved by the University of New Mexico Animal Use and Care Committee. Rats were briefly restrained in a Plexiglas holder and injected intravenously, via the tail vein, with 150 mg/kg 6-OHDA hydrobromide (Sigma Chemical Co., St. Louis, MO) in sterile saline containing 1 mg/mL ascorbic acid, in a total volume of 0.5 mL. Control rats were injected with the same volume of vehicle. Two injections per animal, with a day off in between, were administered. At 1 and 3 wk after the first injection, rats were anesthetized with halothane and perfused intracardially with cold phosphate-buffered saline (PBS) followed by cold 4% paraformaldehyde in 0.1 *M* phosphate buffer. Pituitary glands were removed, stored for 3 h in fresh cold fixative, and then transferred to cold 5% sucrose in phosphate buffer for storage prior to processing for paraffin embedding. The treatment protocol has been previously published in detail *(4)*.

Tissue Processing and Immunostaining

Pituitary glands were dehydrated in ethanol, processed through Histoclear, and embedded in paraffin using an automatic tissue processor. Sections (8 μm) were cut on a rotary microtome and placed on Fisher superfrost plus slides. Immunostaining of sections for SN was as described previously *(9)*. Briefly, sections were deparaffinized with Histoclear, treated with 1% hydrogen peroxide, then with trypsin (Worthington Biochemicals, Freehold, NJ), 1 mg/mL

in Tris-HCl buffer, diluted 1:3 in Tris buffer for 3 min prior to incubation in 2% normal horse serum. Sections were then incubated in monoclonal anti-SN antibody (Boehringer-Mannheim, Indianapolis, IN, dilution 1:50) for 24 h at room temperature, washed, incubated in horse antimouse IgG-biotin, and then with ABC reagent (Vector Labs, Burlingame, CA), followed by the peroxidase reaction with diaminobenzidine and hydrogen peroxide. Sections were briefly treated with osmium tetroxide vapors, lightly counterstained with toluidine blue, then dehydrated, and cover slipped with Permount. To control for background, additional sections were included in which the primary antibody was omitted, but otherwise processed with the same methods. No specific staining for SN was observed in those sections (*see* Results and Fig. 3).

Quantitative Analysis of SN Immunostaining

Sections of pituitary glands from 3 to 7 animals/group were examined using an Olympus BX-40 microscope with a color video camera (Optronics DEI-470) mounted to the microscope and attached to a DTK 486DX computer equipped with Bioquant (Nashville, TN) image analysis software. Two to three areas of the intermediate lobe of each section, from distinctly separated regions of the lobe, were selected at 40× magnification, a rectangle of fixed area (17,650 μm^2) overlaid onto the section, and dense dots of synaptophysin immunostaining were counted using a predetermined threshold density. For the neural lobe, the same threshold density was used to quantify 1 or 2 regions/section as outlined by the fixed-area rectangle, but overall density of the area was obtained, since staining of the NL was more diffuse than in the IL. Statistical analysis of the data was done with a two-tailed Student's *t*-test to compare 6-OHDA-treated vs control animals, at the 1- and 3-wk time-points, with $p < 0.05$ considered significant.

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