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Central noradrenergic lesioning using anti-DBH-saporin: anatomical findings

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Abstract

The ability to create lesions of discrete neuronal populations is an important strategy for clarifying the function of these populations. The power of this approach is critically dependent upon the selectivity of the experimental lesioning technique. Anti-neuronal immunotoxins offer an efficient way to produce highly specific neural lesions. Two previous immunotoxins have been shown to be effective in both the CNS and PNS. They are OX7-saporin, which is targeted at Thy1, and 192-saporin, which is targeted at the low affinity neurotrophin receptor, $p75^{NTR}$. In the present study, we sought to determine if an immunotoxin targeted at the neurotransmitter synthesizing enzyme, dopamine β-hydroxylase (DBH), could selectively destroy central noradrenergic neurons after intraventricular administration. This immunotoxin, which consists of a monoclonal antibody to DBH coupled by a disulfide bond to saporin (a ribosome inactivating protein), has been shown to be selectively toxic to peripheral noradrenergic sympathetic neurons in rats after systemic injection. In the present study, immunohistochemical and Cresyl violet staining showed that the noradrenergic neurons of the locus coeruleus are destroyed bilaterally after intraventricular (i.c.v.) injection of 5, 10, and 20 μg of anti-DBH-saporin (α-DBH-sap) into rats. Complete bilateral lesioning of the A5 and A7 cell groups occurred at the two higher doses. Lesions of the A1/C1 and A2/C2/C3 cell groups were incomplete at all three doses. Dopaminergic neurons of the substantia nigra and ventral tegmental area and serotonergic neurons of the raphé, all monoaminergic neurons that do not express DBH, survived all α-DBH-sap doses. The cholinergic neurons of the basal forebrain, which are selectively killed by i.c.v. injection of 192-saporin, and cerebellar Purkinje cells which are killed by OX7-saporin, were not killed by α-DBH-sap. These results show that α-DBH-sap efficiently and selectively destroys CNS noradrenergic neurons after i.c.v. injection. The preferential destruction of locus coeruleus, A5, and A7 over A1/C1 and A2/C2/C3 may be due to more efficient access of the immunotoxin to these neurons and their terminals after i.c.v. injection.

Keywords: Immunotoxin; Saporin; Noradrenaline; Locus coeruleus; Lesion; Rat

1. Introduction

The value of a lesioning technique depends upon the specificity of the produced lesion. A lesion that is highly specific for a discrete population of neurons can reveal a great deal about the function of that population. Several pharmacological methods of destroying central noradrenergic neurons have been used in the past. The agents used have included 6-hydroxydopamine (6-OHDA) [22,24], *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) [10,20], and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [8,12]. Both 6-OHDA and MPTP are not specific for noradrenergic neurons but destroy dopaminergic neurons as well [15,16,18,22]. The widespread effects of MPTP are further highlighted by the observation of [^{3}H]MPTP binding sites in the locus coeruleus (LC), raphe nuclei, cerebral gray matter [17], and arcuate nucleus of the hypothalamus [29]. An additional limitation to using 6-OHDA, MPTP, or DSP-4 to create central noradrenergic denervation is that all three of these agents seem to have more pronounced effects on axons of the LC than on the lateral tegmental noradrenergic populations (A5 and A7) when administered systemically [8,9,12,22,24]. Systemic

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administration of DSP-4 also has been shown to damage serotonergic neurons [13].

Because of these shortcomings of the current noradrenergic lesioning methods, we sought to characterize the ability of an immunotoxin targeted to the noradrenergic specific enzyme dopamine β-hydroxylase (DBH) to selectively destroy central noradrenergic neurons after intraventricular (i.c.v.) injection. This immunotoxin, dubbed α-DBH-sap, consists of a monoclonal antibody to DBH conjugated by disulfide linkage to saporin [2], a ribosome-inactivating toxin. DBH was chosen as the target of the immunotoxin because, upon noradrenaline release, the membrane-bound form of this intravesicular enzyme is exposed to the extracellular milieu before undergoing recycling by endocytosis and retrograde axonal transport [27,28]. The feasibility of this approach has been shown by the ability of α-DBH-sap to kill peripheral noradrenergic neurons of the sympathetic ganglia after intravenous infusion [25,26].

In the present study α-DBH-sap was injected into the left lateral ventricle of rats. The extent of central lesions was examined by immunohistochemical staining for the catecholamine synthesizing enzymes tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine-*n*-methyl transferase (PNMT), as well as by Cresyl violet staining of Nissl substance. The specificity of the lesions was assessed in sections stained with Cresyl violet and by immunohistochemical staining for choline acetyltransferase (ChAT).

2. Materials and methods

2.1. Synthesis of immunotoxins

The monoclonal antibody, anti-dopamine β-hydroxylase (α-DBH), was purified from ascites as previously described [5,31] from material provided by Chemicon International (MAB 308). The immunotoxin was then produced as previously described [31]. Briefly, saporin was isolated from the seeds of *Saponaria officinalis*. Antibody was coupled to saporin by a disulfide bridge introduced using the heterobifunctional cross-linker *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pharmacia). Both antibody and saporin were derivatized with SPDP separately; non-reacted SPDP was removed by dialysis. Following reduction of the SPDP-saporin with 0.1 M dithiothreitol (DTT), the reduced SPDP-saporin was separated from excess DTT by G-25 gel chromatography (Pharmacia). The freshly reduced SPDP-saporin and SPDP-antibody were allowed to react overnight at 4°C. Immunotoxin was purified by MonoS cation exchange. The final protein concentration of immunotoxin was determined by bicinchonic acid (BCA) assay (Pierce).

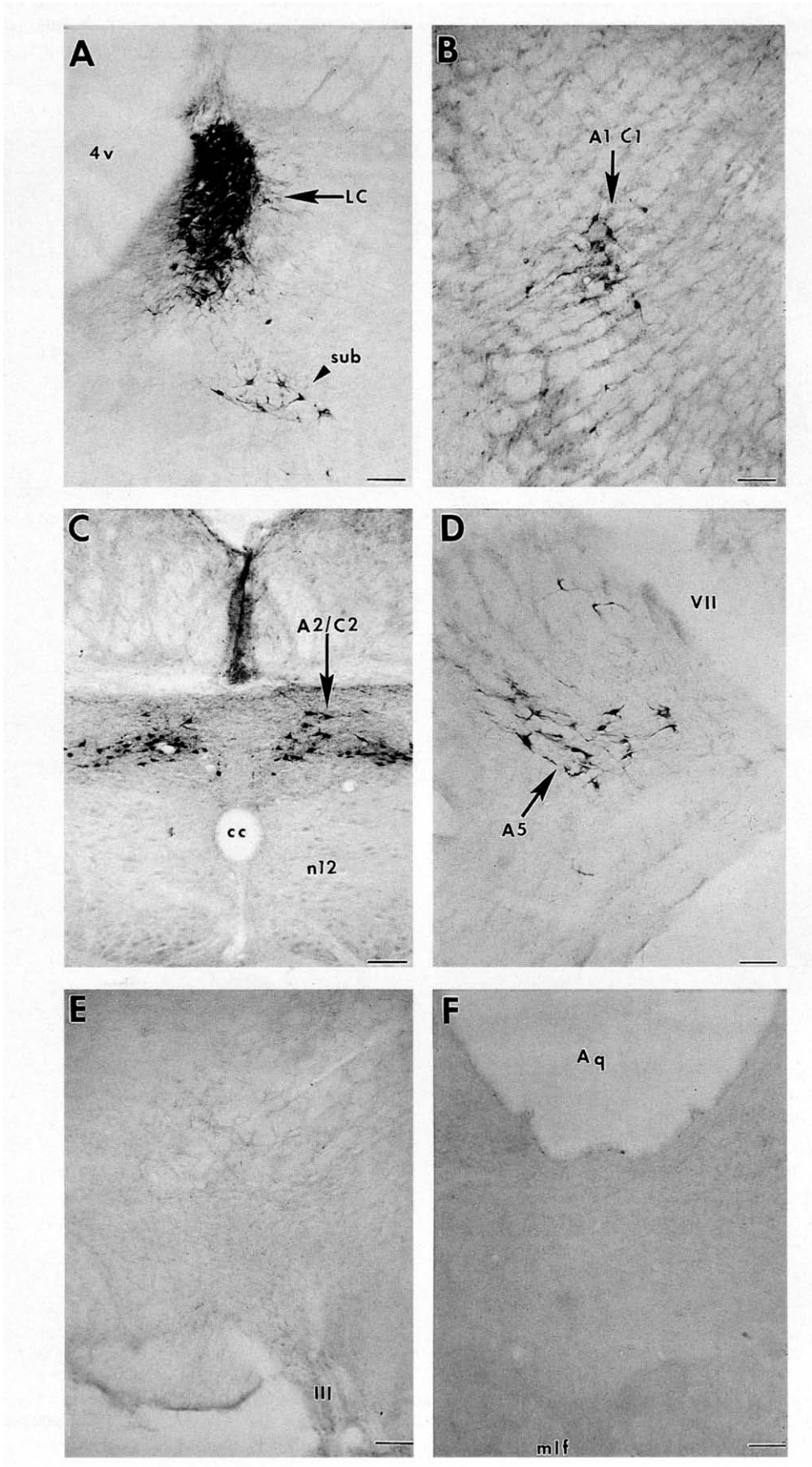
2.2. Intraventricular injection

A total of 20 Sprague–Dawley rats, 375–425 g, were used in this study. The rats were anaesthetized by intraperitoneal (i.p.) injection of a mixture containing ketamine, 150 mg/kg, and acepromazine, 3 mg/kg, titrated to achieve and maintain insensitivity to pain. Stereotactic injections were performed into the left lateral ventricle. The co-ordinates were 1.2 mm anterior to bregma, 1.0 mm lateral from midline, and 5.0 mm below the dura mater. Four rats were injected with ascites fluid containing only α-DBH. The doses of α-DBH-sap injected were 5 μg ($n = 3$), 10 μg ($n = 5$), 20 μg ($n = 3$), and 40 μg ($n = 1$). Four rats were prepared as sham injected controls by lowering the syringe into the left ventricle without injecting any toxin. After each injection the syringe was allowed to sit in place for 10–15 min in order to allow adequate diffusion and prevent leakage upon withdrawal.

2.3. Immunohistochemistry

Two weeks after surgery all rats (except for one control animal, two 10 μg injected animals, and the 40 μg injected animal) were injected i.p. with 75 mg/kg of sodium pentobarbital and perfused (transcardiac) first with phosphate-buffered saline (PBS) containing heparin (1000 U/l) and sodium nitrite (1 g/l) followed by 4% paraformaldehyde (1000 ml). After fixation, brains were removed and left in 30% sucrose at 4°C until equilibrated. Sections (40 μm) were made on a freezing, sliding microtome and stored at −20°C in groups of 6 in an anti-freeze solution consisting of phosphate buffer, ethylene glycol, and glycerol. One in 6 series of sections from the rats injected with ascites fluid containing only unconjugated α-DβH were stained immunohistochemically using rat adsorbed goat anti-mouse IgG (Cappel) and standard anti-goat IgG biotin-avidin peroxidase technique (ABC kits, Vector Labs). Sections from rats that were injected with α-DβH-sap were stained similarly using rabbit anti-tyrosine hydroxylase (Eugene Tech), rabbit anti-phenylethanolamine-*n*-methyl transferase (Incstar), or rabbit anti-dopamine β-hydroxylase (Eugene Tech) as the pri-

Fig. 1. Uptake of α-DBH by noradrenergic neurons after i.c.v. injection. Rats were injected into the left lateral ventricle with ascites fluid containing α-DBH. Coronal sections of rat brains were stained immunohistochemically for mouse IgG. Note the lack of stain in E and F. A: locus coeruleus; B: A1/C1 cell group; C: A2/C2 cell group; D: A5 cell group; E: substantia nigra/VTA; F: raphe; 4v: 4th ventricle; LC: locus coeruleus; sub: subcoeruleus; cc: central canal; n12: hypoglossal nucleus; VII: facial nerve; III: oculomotor nerve; Aq: cerebral aqueduct; mlf: medial longitudinal fasciculus; bars = 100 μm.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mary antibody and the appropriate ABC kit. In all cases, the chromogen was Ni^+ enhanced diaminobenzidine (DAB). One series of sections from each animal also was stained for Nissl substance using acidic Cresyl violet. The remaining control animal and two 10 μg dosed animals were allowed to survive for nine months and sections from those animals were immunohistochemically stained for TH. The 40 μg dosed animal died 12 days post-op pre-

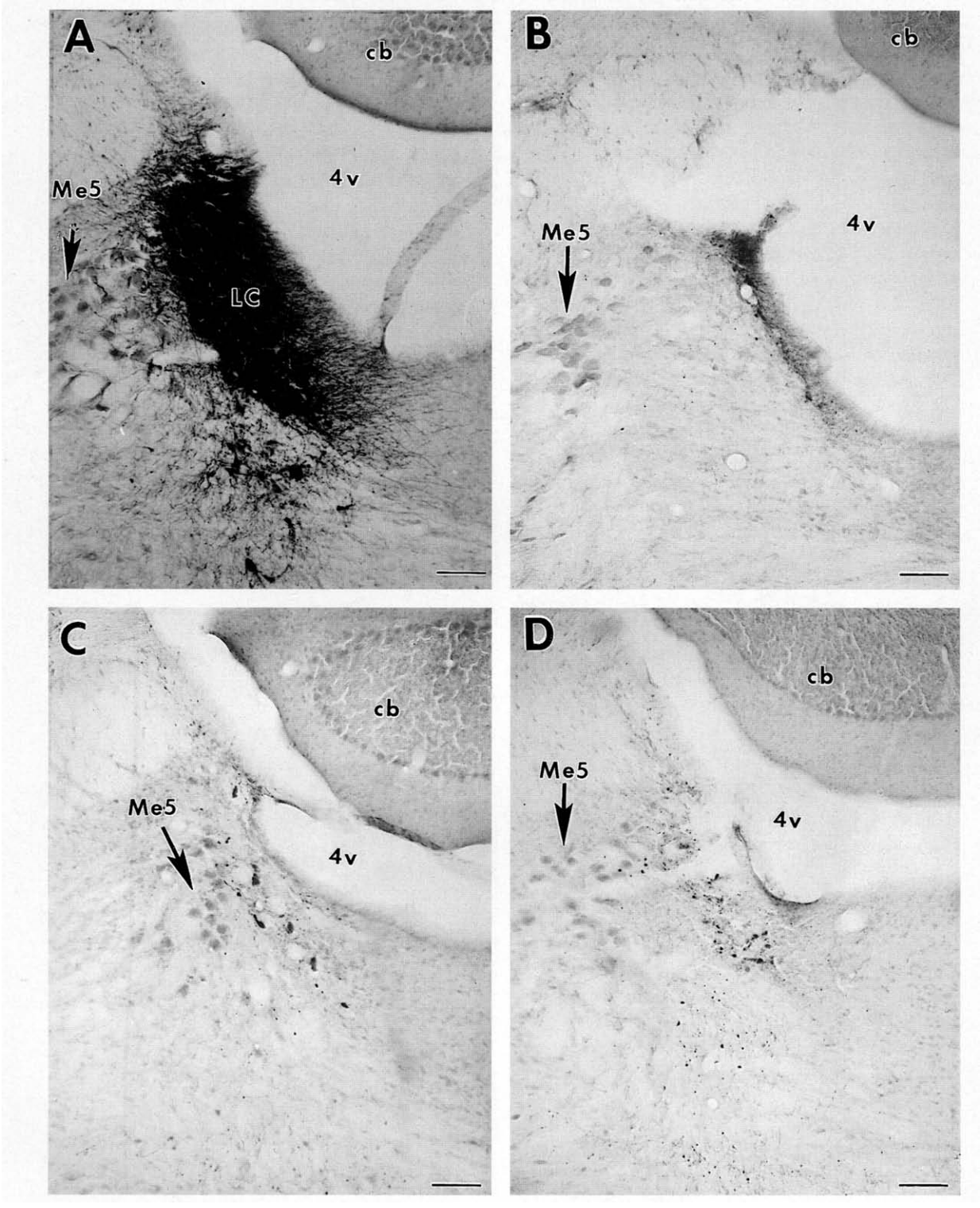

Fig. 2. Immunohistochemical staining for tyrosine hydroxylase of rat brains showing the loss of noradrenergic neurons after i.c.v. injection of α-DBH-sap. Panels A–D are of coronal sections through the rostral pons at the level of the locus coeruleus. A: control; B: 5 μg α-DβH-sap; C: 10 μg α-DBH-sap; D: 20 μg α-DBH-sap; 4v: 4th ventricle; cb: cerebellum; LC: locus coeruleus; Me5: mesencephalic nucleus of trigeminal nerve; bars = 100 μm.

Table 1
Cell counts of A1/C1 neurons

	Sham	5 μg	10 μg	20 μg
TH + cells/section/rat	36.25 ± 6.67	17.04 ± 1.83 *	12.27 ± 8.46 *	7.65 ± 0.16 *
DBH + cells/section/rat	25.90 ± 3.98	5.58 ± 1.47 *	2.30 ± 1.49 *	1.57 ± 0.57 *
PNMT + cells/section/rat	37.78 ± 4.82	20.77 ± 6.57 *	12.21 ± 9.14 *	6.77 ± 1.72 *

Neurons were counted as described under Section 2, Materials and methods. Data is expressed as the mean ± standard deviation. * denotes a statistically significant difference compared to the sham-operated group as detected by the Student-Newman-Keuls post hoc test ($P < 0.05$).

cluding us from obtaining histological data from that animal.

2.4. Cell counts

Neurons of the A1/C1 and A2/C2/C3 populations were counted bilaterally in order to quantify the extent of the α-DBH-sap induced lesion of these neurons. Neurons stained for TH, PNMT, or DBH were counted by eye at a magnification of 100× under a Leitz light microscope. The experimenter was blinded to the identity of the sections until counting was completed. Counting included sections from the caudal extent of the hypoglossal nucleus and continued rostrally until the facial nucleus was reached. For each animal, the number of positively stained neurons was expressed as number of neurons per section. These values were used to calculate group (sham, 5 μg, 10 μg, and 20 μg) means which were compared statistically using analysis of variance (ANOVA). When group means differed significantly, the Student-Newman-Keuls post hoc test was used to detect pairwise differences.

3. Results

3.1. Uptake of α-DβH

Immunohistochemical staining for mouse IgG showed that α-DBH monoclonal antibody was taken up by central noradrenergic neurons after i.c.v. injection. The positively stained neurons included the known adrenergic and noradrenergic cell groups which are the LC, the A5 cell group, the A7 cell group, the A1/C1 cell group, and the A2/C2 cell group (Fig. 1A–D, A7 not shown). Other monoaminergic populations (which do not express DBH) such as the substantia nigra/VTA and the raphé did not take up the antibody (Fig. 1E,F). Injection of this antibody had no apparent effect on the body weights of the animals nor did it change their general appearance.

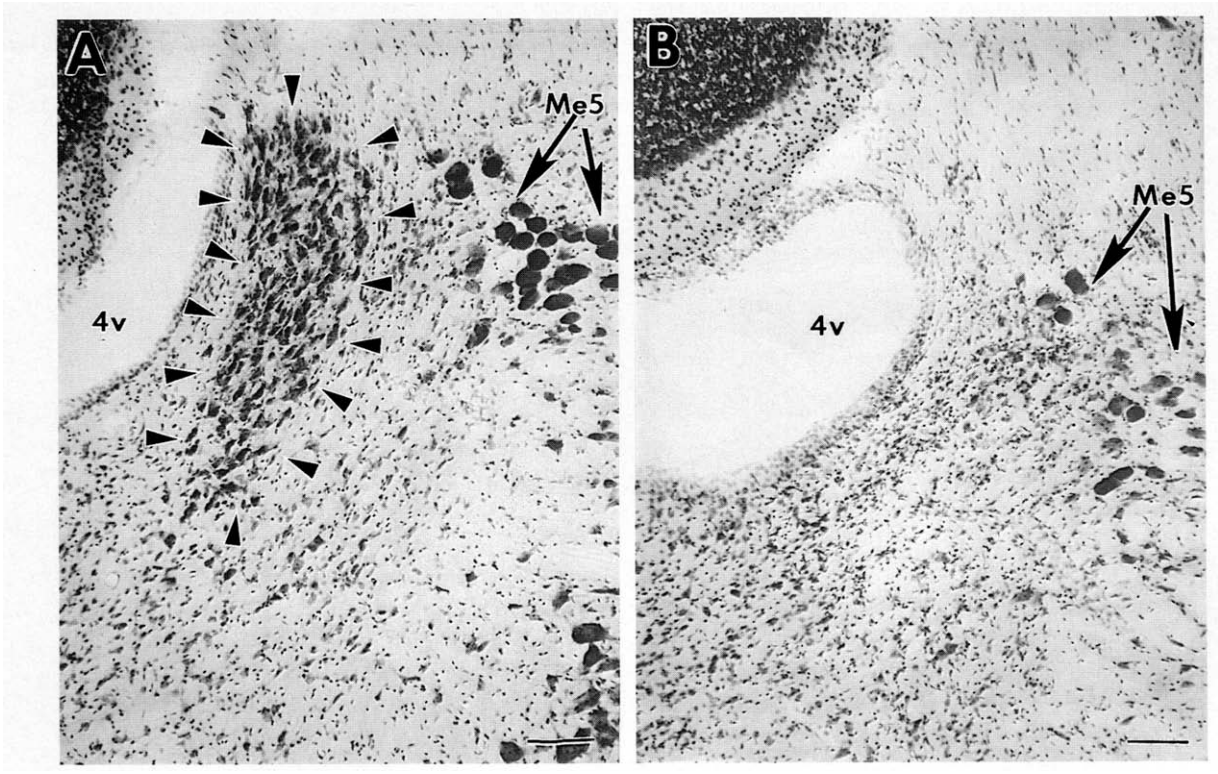

Fig. 3. Cresyl violet staining of rat brain after i.c.v. injection of α-DBH-sap. The sections are from the rostral pons at the level of the locus coeruleus. A: control; B: 5 μg α-DBH-sap. Arrows surround the locus neurons in A. Note the absence of locus coeruleus neurons in B; 4v: 4th ventricle; Me5: mesencephalic nucleus of trigeminal nerve; bars = 100 μm.

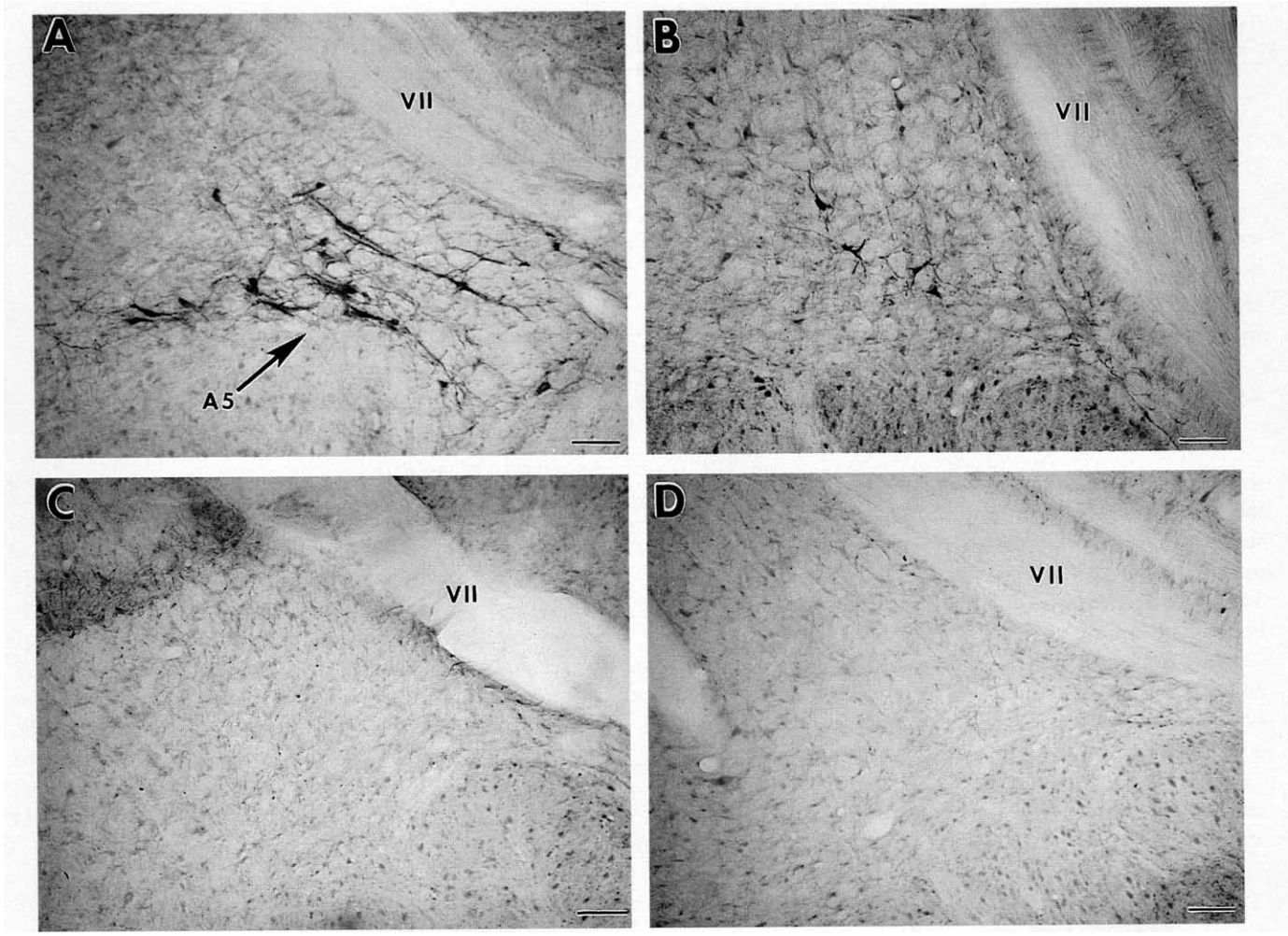

Fig. 4. A5 lesion after i.c.v. injection of α-DBH-sap. The sections are from the pons at the level of the facial nerve. The neurons are stained for TH. A5 was completely lesioned only at 10 and 20 μg of α-DBH-sap in all animals. A: control; B: 5 μg α-DBH-sap; C: 10 μg α-DBH-sap; D: 20 μg α-DBH-sap; VII: facial nerve; bars = 100 μm.

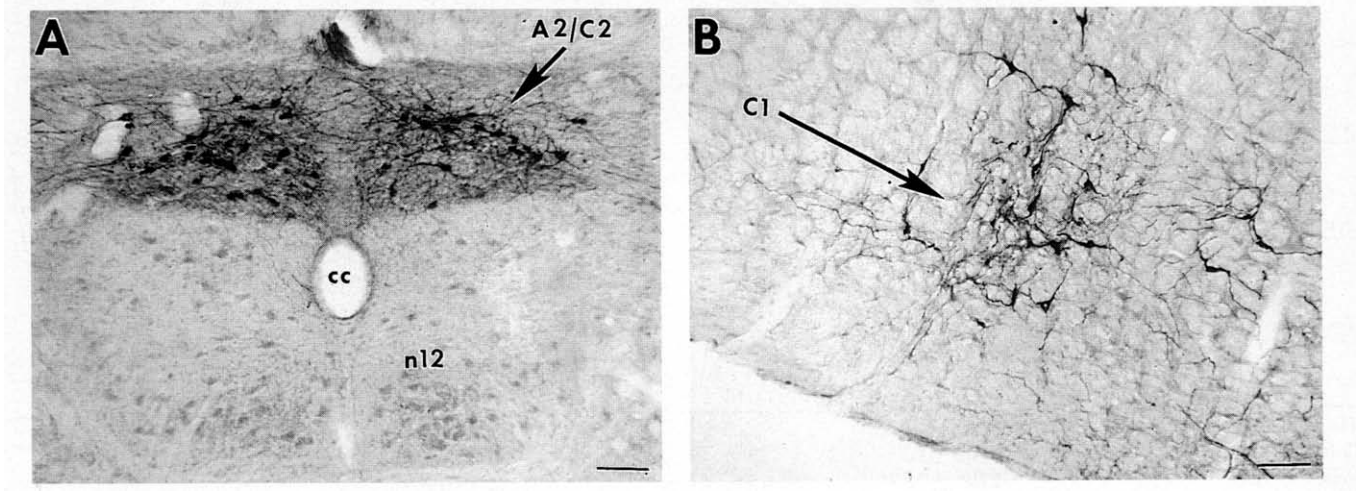

Fig. 5. Representative sections used in the cell counts of A1/C1 and A2/C2/C3. A: section from the medulla of a control animal showing A2/C2 population stained for TH; B: section from the medulla of a control animal showing C1 stained for PNMT; cc: central canal; n12: hypoglossal nucleus; bars = 100 μm.

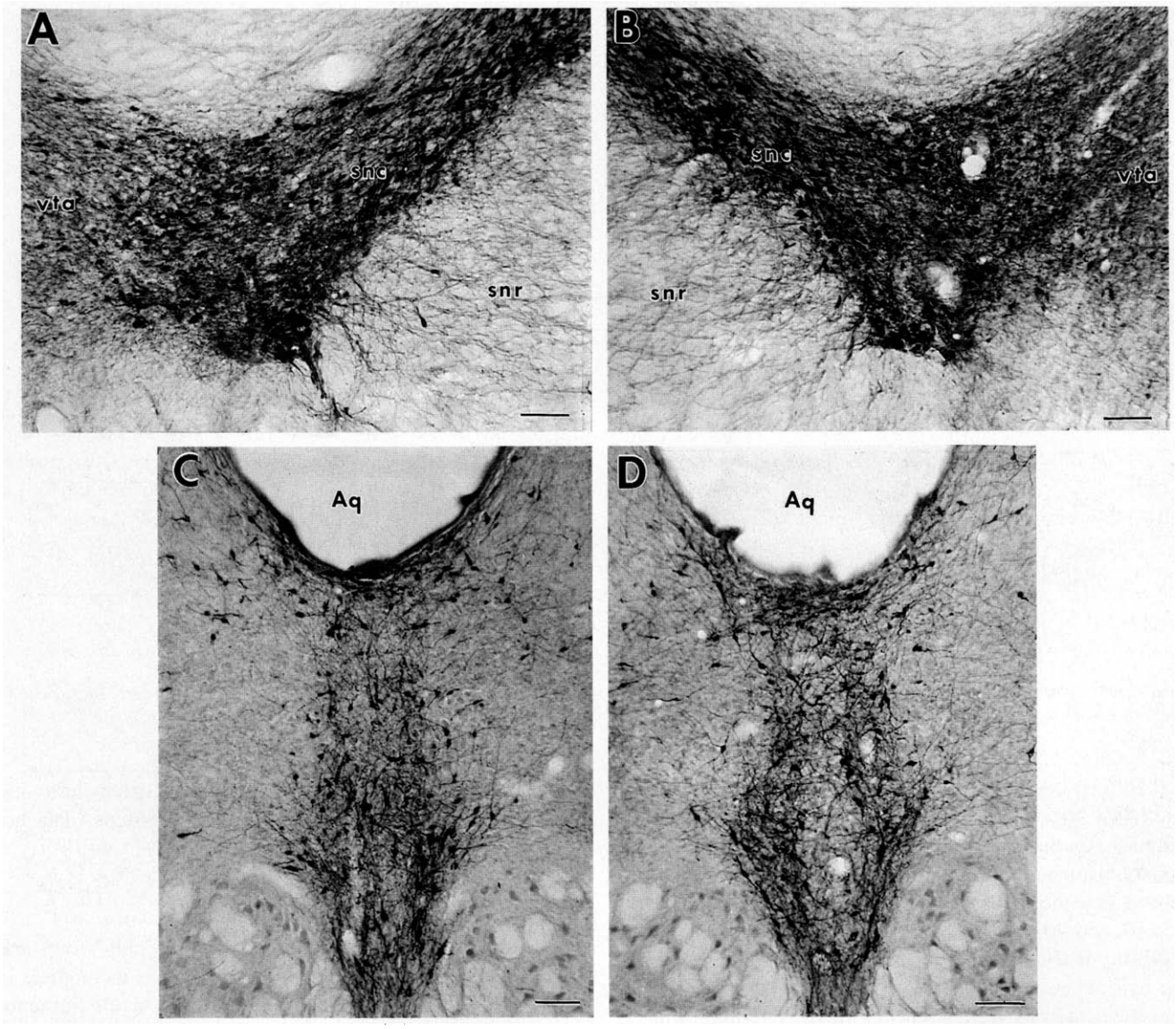

Fig. 6. Effect of α-DBH-sap on other monoaminergic populations. Neurons of the substantia nigra/ventral tegmental area (SN/VTA) and of the dorsal raphé, monoaminergic populations which do not express DBH, are spared by α-DBH-sap. Neurons are stained immunohistochemically using anti-TH. The serotonergic neurons of the raphé stained because the TH antibody cross-reacts with tryptophan hydroxylase. A: SN/VTA, control; B: SN/VTA, 20 μg i.c.v. of immunotoxin; C: raphé, control; D: raphé, 5 μg i.c.v. of immunotoxin; vta: ventral tegmental area; snr: substantia nigra pars reticularis; snc: substantia nigra pars compacta; Aq: cerebral aqueduct; bars = 100 μm.

3.2. Effect of α-DBH-sap on locus coeruleus, A5, and A7

Having established the specificity of unconjugated α-DβH uptake, the effects of i.c.v. injection of α-DβH-sap were then examined. Injection of α-DBH-sap caused weight loss that persisted over the two week survival period. The ranges of weight loss of the sham, 5 μg, 10 μg, and 20 μg dosed animals were (−1.94% to +0.87%),

Table 2
Cell counts of A2/C2/C3 neurons

	Sham	5 μg	10 μg	20 μg
TH + cells/section/rat	40.17 ± 7.77	26.03 ± 5.40 *	21.66 ± 3.60 *	18.69 ± 4.07 *
DBH + cells/section/rat	35.19 ± 6.77	29.47 ± 10.44	22.68 ± 4.31	16.21 ± 1.07 *
PNMT + cells/section/rat	27.80 ± 6.47	19.66 ± 2.37	20.81 ± 0.60	12.65 ± 2.06 *

Neurons were counted as described under Section 2, Materials and methods. Data is expressed as the mean ± standard deviation. * denotes a statistically significant difference compared to the sham-operated group as detected by the Student-Newman-Keuls post hoc test ($P < 0.05$).

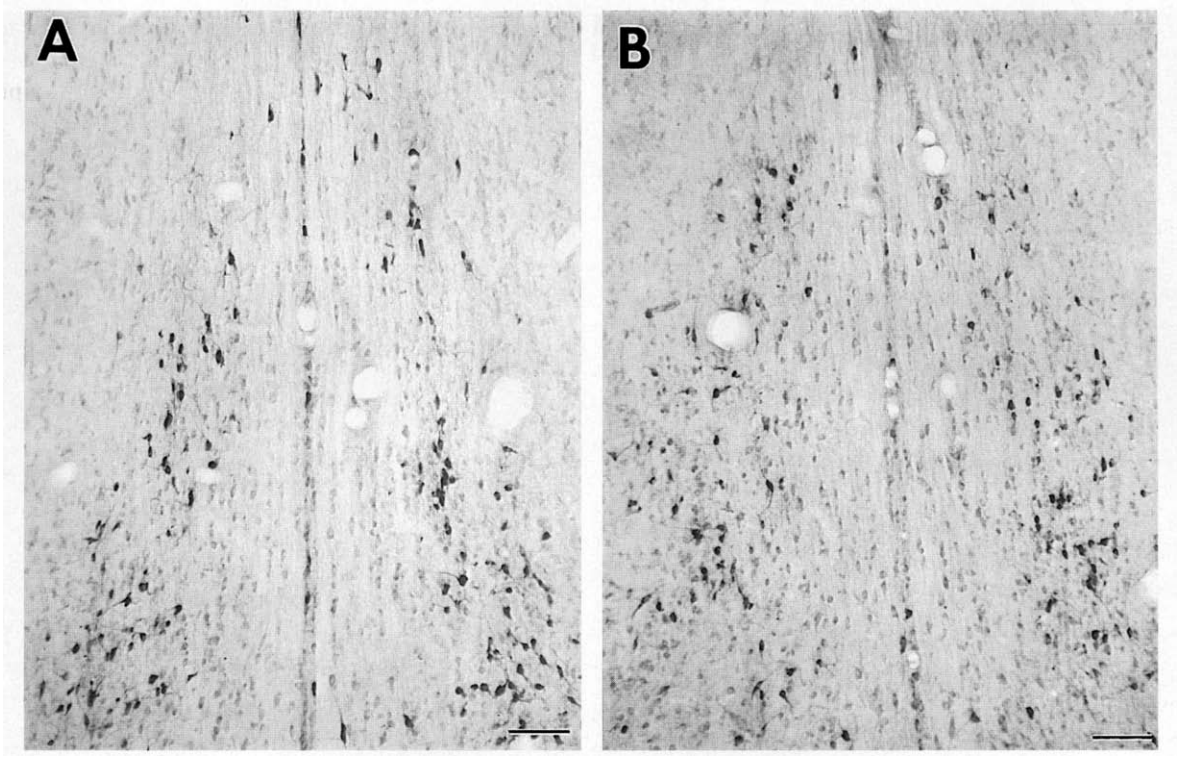

Fig. 7. Cholinergic cell survival. Photomicrographs of the medial septum stained immunohistochemically for ChAT. α-DBH-sap leaves these neurons intact. A: medial septum from a control animal; B: medial septum from an animal receiving 20 μg i.c.v. α-DBH-sap; bars = 100 μm.

(−9.16% to +0.53%), (−18.14% to −10.53%), and (−25.44% to −19.39%), respectively. The 20 μg dosed animals experienced mild diarrhea and bilateral ptosis. Immunohistochemical staining for tyrosine hydroxylase showed that the locus coeruleus was destroyed bilaterally at 5, 10, and 20 μg in all animals (Fig. 2). To rule out the possibility that these neurons merely down-regulated tyrosine hydroxylase in response to the immunotoxin, sections from the same rats were stained with Cresyl violet. Fig. 3 compares Cresyl violet staining of a control rat and of a rat that received 5 μg of the immunotoxin. Locus coeruleus neurons were absent from Cresyl violet-stained sections of immunotoxin-treated animals.

Other noradrenergic neurons were also killed bilaterally by α-DβH-sap. A few cells of the A5 cell group survived in the 5 μg dosed animals; however, no cells of this nucleus survived in the animals that received the 10 and 20 μg doses (Fig. 4). The same observations were made for the A7 cell group (data not shown). The results of immunohistochemical staining for DBH of these cell groups, as well as of the locus coeruleus, did not differ from those results obtained with the TH stain. Furthermore, DBH staining of terminal fields of the locus coeruleus such as the hippocampus and neocortex showed that noradrenergic fibers were absent from immunotoxin-treated animals (data not shown). Cresyl violet-stained sections confirmed the loss of neurons.

The TH staining of sections from the two rats which were allowed to survive for nine months after 10 μg injections also revealed complete and persistent lesioning of the locus coeruleus, A5, and A7 cell groups (data not shown).

3.3. Effect of α-DBH-sap on A1/C1 and A2/C2/C3

Lesioning of the ventrolateral A1/C1 cell group and the dorsomedial A2/C2/C3 cell group was incomplete in that some cells survived all three doses in all immunotoxin-treated animals. The pattern of cell sparing was similar on both sides of the brain stem. In order to discern to what extent these populations were lesioned, counts of TH-positive (TH +), DBH-positive (DBH +), and PNMT-positive (PNMT +) neurons were obtained using sections from the two-week survival animals. Fig. 5 provides representative examples of the immunohistochemical data used for obtaining the counts. The number of TH + , DBH + , and PNMT + neurons in both the ventrolateral (Table 1) and dorsomedial (Table 2) populations were decreased in a dose-dependent manner. The number of TH + neurons of the dorsal motor nucleus of the vagus (dmnX), which are thought to be dopaminergic [1,21], was found to not differ among the groups (data not shown). These neurons, as expected, did not stain for DBH or PNMT.

3.4. Effect of α-DBH-sap on other monoaminergic neurons

If α-DBH-sap is truly specific for noradrenergic neurons, the immunotoxin will not kill other monoaminergic

neurons. Fig. 6 shows that the dopaminergic neurons of the substantia nigra/VTA and the serotonergic neurons of the raphe (the tyrosine hydroxylase serum cross reacts with tryptophan hydroxylase) survived i.c.v. injection of α-DBH-sap. These results are consistent with the localization of unconjugated α-DBH after i.c.v. injection.

3.5. Cholinergic cell survival

A previously developed immunotoxin, 192-saporin, has been shown to selectively kill the neurons of the cholinergic basal forebrain [3,4,14,31]. Immunohistochemical staining for choline acetyl transferase (ChAT) revealed that α-DBH-sap spares these neurons (Fig. 7).

192-saporin, as well as OX7-saporin, can kill Purkinje cells of the cerebellum after i.c.v. injection [14]. There was no apparent loss of Purkinje cells after α-DBH-sap injections as revealed by Cresyl violet stain (data not shown). This observation is consistent with the absence of any grossly observable ataxia or tremor in these animals.

4. Discussion

In this study our goal was to produce specific lesions of central noradrenergic neurons. The described data show that i.c.v. injection of the immunotoxin α-DBH-sap selectively destroys central noradrenergic neurons while it spares cholinergic, dopaminergic, and serotonergic neurons. These lesions are stable over time as shown by the animals that were sacrificed nine months post-injection.

We have relied upon immunohistochemical staining of catecholamine synthesizing enzymes to show the lesions. It could be argued that the immunotoxin did not kill these neurons, but only altered them such that they could no longer express these enzymes at detectable levels. However, the observation that LC neurons are missing from Cresyl violet-stained sections of immunotoxin-treated rats argues strongly that α-DβH-sap does indeed kill central noradrenergic neurons.

The lesioning accomplished by α-DBH-sap correlated well with the selective uptake of the unconjugated DBH antibody by all CNS noradrenergic neurons. The dose of 5 μg completely destroyed the locus coeruleus but left some survivors in the A5 and A7 populations. At doses of 10 and 20 μg the immunotoxin created complete lesions of all central noradrenergic populations with the exception of the medullary cell groups (A1, A2, C1, C2, and C3). The survival of some neurons in these nuclei could be due to relative lack of access of the immunotoxin to these neurons after i.c.v. injection, a lower threshold antigen number that does not allow sufficient saporin to enter, or a combination of these factors. In fact, cell surface target number has been shown to sometimes be important for cytotoxic activity of immunotoxins [23]. It may be possible to kill both populations completely with higher doses of α-DBH-sap; however, we observed that a 40 μg dose is lethal indicating that viability of the animals is an issue at higher doses. Local intraparenchymal injections of α-DBH-sap into the medulla may be a more efficacious approach to lesioning these neurons.

α-DBH-sap is the newest addition to the arsenal of anti-neuronal immunotoxins. Previously, the neuronotoxic effects of two other immunotoxins have been characterized. OX7-saporin, which contains an antibody to the surface glycoprotein Thy 1, was shown to destroy ipsilateral vagal motor and sensory neurons after injection into the cervical vagus; the substantia nigra, pars compacta, and intralaminar thalamic nuclei after injection into the caudate [30]; and cerebellar Purkinje cells after i.c.v. injection [6]. The success of this conjugate foreshadowed the use of antibody-saporin conjugates selective for discrete neuronal populations. The first of these highly specific anti-neuronal immunotoxins, 192-saporin, was shown to selectively kill the $p75^{NTR}$ expressing cells of the CBF [3,4,31]. 192-saporin also kills some cerebellar Purkinje neurons which express p75 [14]. The present results show that lesions limited to central noradrenergic populations also can be created by this approach and that damage to cerebellar Purkinje cells does not occur with every immunotoxin injected i.c.v. The cerebellar lesions caused by OX7-saporin and 192-saporin result from the expression of the target antigens by Purkinje neurons. The lack of cerebellar toxicity with α-DBH-sap occurs because Purkinje cells do not express DBH and because non-specific toxicity is low at the doses studied.

Several other agents have been employed to produce central noradrenergic lesions. These include DSP-4 [10,20], 6-OHDA [22,24], and MPTP [8,12]. α-DBH-sap is more selective than 6-OHDA and MPTP. We have shown here that unlike those two agents, α-DBH-sap spares the dopaminergic neurons of the substantia nigra. When administered systemically, DSP-4, 6-OHDA, and MPTP have a 'striking regional specificity' for axons of the LC with a much lesser effect on non-coerulean noradrenergic neurons [8,9,12,22,24]. α-DBH-sap, on the other hand, completely destroys locus coeruleus noradrenergic neurons as well as non-coerulean populations such as A5 and A7. Another shortcoming of 6-OHDA is that the PNMT containing neurons of the medulla resist this toxin [19]. We show in the present study that α-DBH-sap reduces the number of TH +, DBH +, and PNMT + neurons of the medulla after i.c.v. injection. α-DBH-sap is thus a very effective agent for selectively removing both central noradrenergic and adrenergic innervation.

Immunotoxins offer a powerful and efficacious method for generating highly specific neural lesions. The lesions created by α-DBH-sap can be used to model the noradrenergic degeneration known to occur in Alzheimer's disease [7] and Parkinson's disease [11]. α-DBH-sap can also be used along with the cholinergic immunotoxin 192-saporin to study the importance of the interaction of the forebrain cholinergic-noradrenergic systems in behavior.

Acknowledgements

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