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Journal of Neuroscience Methods 103 (2000) 73–82

JOURNAL OF NEUROSCIENCE METHODS

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Neuronal lesioning with axonally transported toxins

Ronald G. Wiley [a,*], Robert H. Kline IV [b]

[a] *Departments of Neurology and Pharmacology, Vanderbilt University, VAMC Nashville, TN 37212-2637, USA*
[b] *Laboratory of Experimental Neurology, VAMC Nashville, TN 37212-2637, USA*

Received 17 March 2000; accepted 5 July 2000

Abstract

Axonally transported toxins can be used to make selective lesions of the nervous system. Collectively, these techniques are termed 'molecular neurosurgery' because they exploit the surface molecular identity of neurons to selectively destroy specific types of neurons. Suicide transport, is anatomically selective but not type-selective. The most widely used suicide transport agents are the toxic lectins (ricin, volkensin) and the immunotoxin, OX7-saporin. The toxic lectins and saporin are ribosome inactivating proteins that irreversibly inhibit protein synthesis. The toxic lectins have binding subunits but saporin requires a targeting vector to gain entrance into cells. Immunolesioning uses monoclonal anti-neuronal antibodies to deliver saporin selectively into neurons that express a particular target surface antigen. Neuropeptide–saporin conjugates selectively destroy neurons expressing the appropriate peptide receptors. Notable experimental uses of these agents include analysis of the function of the cholinergic basal forebrain (192-saporin) and pain research (anti-DBH-saporin, substance P-saporin). It is likely that more immunolesioning and neuropeptide-toxin conjugates will be developed in the near future. © 2000 Published by Elsevier Science B.V.

Keywords: Suicide transport; Immunolesioning; Saporin; Ribosome inactivating proteins; Molecular neurosurgery

Functional neuroanatomy research has long relied on analysis of the effects of lesions to infer the function of neural structures. However, conventional lesioning techniques are relatively crude compared to the complex organization of the nervous system. Since the fundamental organizational unit of the nervous system, the neuron, is generally not amenable to direct physical identification and destruction in vivo, a number of innovative techniques have been developed to destroy selected groups of neurons. Excitotoxins have been widely used to lesion cells while sparing fibers in passage, but these agents typically destroy various types of neurons at the injection site (Miettinen et al., 1995; Figueredo-Cardenas et al., 1998). Monoamine toxins, such as 6-hydroxydopamine (Kostrzewa and Jacobowitz, 1974; Lookingland et al., 1986), 5,6- dihydroxytrytophan (Jonsson, 1980; Jarrard et al., 1985), DSP-4 (Lookingland et al., 1986Riekkinen et al., 1992) and AF-64A (Hanin, 1990; Walsh and Opello, 1994), have been used to produce selective lesions of neurons based on the neurotransmitters secreted, but each of these agents has some limitations with respect to efficiency and incomplete specificity, either anatomic or cell type, particularly at doses that destroy the target neurons.

The initial impetus to develop axonally transported toxins arose from the need to selectively destroy the neurons that projected through a particular peripheral nerve branch. By analogy to the fluorescent anatomical tracing dyes, the first agents tried were low molecular weight toxins such as the fluorescent anthracycline antibiotic, doxorubicin, which produces retrograde lesions after peripheral nerve application (Koda and Van Der Kooy, 1983; Van Der Kooy et al., 1985). However, doxorubicin can be found in satellite and glial cells surrounding the perikarya of poisoned neurons suggesting diffusion of the toxin beyond the target population (Bigotte and Olsson, 1982, 1983; Kondo et al., 1987; England et al., 1988), and doxorubicin is inefficient (Cummings et al., 1988). By analogy to the anatomical tracer, wheat germ agglutinin, the next group of agents tried were the toxic lectins, ricin and abrin, which are plant proteins that bind to certain oligosaccharides on the cell surface (Olsnes and Pihl, 1982; Olsnes and

* Corresponding author. Neurology Service (127), VAMC, 1310 24th Avenue, South, Nashville, TN 37212-2637. Tel.: +1-615-327-4751-5244, fax: +1-707-202-2500.
E-mail address: ronald.g.wiley@vanderbilt.edu (R.G. Wiley).

0165-0270/00/$ - see front matter © 2000 Published by Elsevier Science B.V.
PII: S0165-0270(00)00297-1

Sandvig, 1985). After binding, the toxic lectins undergo endocytosis followed by intracellular routing that eventually delivers some of the toxin to the *trans*-Golgi and endoplasmic reticulum where the A subunit can enter the cytosol (Eiklid et al., 1980). The A subunit is an enzyme that acts on ribosomes at a specific site to eliminate binding affinity for elongation factor-2 (Endo et al., 1987). Ribosomes attacked in this fashion cannot support protein synthesis. Inactivation of about 10% of the ribosomes in a cell probably is sufficient to kill the cell. It is likely that only one molecule of ricin or abrin free in the cytosol is sufficient to inactivate enough ribosomes to kill the cell, although it may require 10 000 or more molecules of ricin bound to a cell for one to get free in the cytosol. Also, binding is rapid and inhibition of protein synthesis follows in minutes or a couple of hours, but the cell may continue to function for some time after protein synthesis has stopped.

When axonally transported toxic lectins or immunotoxins that contain ribosome inactivating proteins are used to make neural lesions, several factors contribute to the lag in development of a lesion including (Wiley and Lappi, 1994):

1. Extracellular diffusion and binding. Injection of toxin into a nerve or CNS site is followed by diffusion of toxin to the neuronal surfaces and binding to target molecules on the plasma membrane that leads to endocytosis. This process likely occurs over a few minutes.
2. Axonal transport time. These agents undergo fast axonal transport that can be blocked by vincristine or colchicine (Wiley and Lappi, 1994). So, delivery of toxin to the perikaryon may require several hours depending on the length of the axon involved.
3. Intracellular processing of toxin. At normal body temperature, delivery of toxin via the Golgi apparatus to the endoplasmic reticulum and escape into the cytosol probably occurs rapidly.
4. Inhibition of protein synthesis. The time necessary to inflict a lethal biochemical lesion is somewhat uncertain, but probably minutes to hours at body temperature, depending on dose of toxin internalized.
5. Failure of neuronal function. The inhibition of protein synthesis does not result in immediate failure of a neuron to function (action potentials, neurotransmitter secretion). Vagal neurons ceased to respond to electrical stimulation of the nerve trunk between 36 and 48 h after toxin injection into the cervical vagus nerve low in the neck. Although, protein synthesis was completely inhibited within 18 h (Wiley and Lappi, 1994). Injection of the immunotoxin, 192-saporin, into the septum produced a decline in the amplitude of hippocampal theta activity beginning on the third day post injection and reaching a stable plateau by the seventh day (Lee et al., 1994).
6. Anatomical dissolution. After failure of the neuron to function as a neuron, disintegration of poisoned neurons requires several days. From about the fifth to the tenth day after ricin injection into a peripheral nerve, mononuclear infiltrates (microglia, macrophages) are present among the dying neurons in sensory ganglia and around motor neurons in the CNS. During this time, the poisoned neurons appear progressively more abnormal but staining for cellular antigens may persist. After two weeks, the cellular infiltrate and any sign of the poisoned neurons disappear (Wiley and Lappi, 1994). Similar results have been observed after intraventricular injections of the immunotoxin, 192-saporin (Book et al., 1995).

Knowledge of the above time sequence of events is essential to the appropriate use of these toxins.

1. Suicide transport

This term, coined by D.J. Reis, refers to the uptake and axonal transport of toxin that produces a neural lesion afferent to the site of toxin injection. In the peripheral nervous system, the toxic lectins ricin, abrin, modeccin and volkensin, have been used to ablate neurons projecting through a particular nerve (Wiley et al., 1982; Wiley and Stirpe, 1987). This approach has been used for several experimental purposes in the peripheral nervous system:

1. To determine the cellular localization of neurotransmitter receptors (Hodes et al., 1983; Helke et al., 1985, 1986).
2. To study plasticity of sensory pathways (Wall et al., 1988; Cusick et al., 1990) or motor systems (de la Cruz et al., 1991, 1994a,b) after loss of a well defined group of neurons.
3. To assess the role of primary sensory neurons in autotomy behavior (Wiesenfeld-Hallin et al., 1987; Blumenkopf and Lipman, 1991).

Interestingly, ricin and abrin are not active suicide transport agents in the CNS. This is probably due to obstruction of binding by addition of a penultimate sialic acid on the oligosaccharides of CNS neuronal plasma membranes (Wiley et al., 1982). However, volkensin and modeccin are effective on some CNS pathways (Harrison et al., 1992aHarrison et al., 1992b). Examples of suicide transport experiments in the CNS include:

1. Localization of dopamine receptor subtypes on specific striatal neurons (Harrison et al., 1990Harrison et al., 1992aHarrison et al., 1992bBlack and Crossman, 1992).
2. Localization of serotonin and opiate receptors on specific neurons in the cingulate cortex (Crino et al., 1990Vogt et al., 1995).

3. Localization of neurotransmitter receptors on corticostriatal projection neurons (Pangalos et al., 1992Chessell et al., 1993a,b, 1995, 1997; Chessell, 1996).

Limitations of this approach include unavoidable, indiscriminate destruction of many cell types at the injection site and failure of volkensin to lesion some pathways such as the striatopallidal projection. However, the later problem has not been encountered with the immunotoxin, OX7-saporin. Lastly, there are times when it would be desirable to lesion only a certain type of neuron projecting to a particular injection site without producing significant injection site damage. To address these issues, immunotoxins were developed that selectively target specific types of neurons.

2. Immunolesioning

This term, coined by D.A. Lappi, refers to the use of anti-neuronal monoclonal antibodies to selectively target toxin to neurons expressing the appropriate surface antigen. Immunotoxins have been studied as cell type-specific killers for a number of years. The original impetus was to develop therapeutic agents for treatment of cancer or for immunosuppression (Uhr, 1984; Frankel, 1988). In order to achieve specific targeting, the monoclonal antibodies must be armed with a ribosome inactivating moiety devoid of intrinsic binding activity. A number of toxin moieties have been used to construct active immunotoxins with widely different activities in vivo. The first anti-neuronal immunotoxin reported was 192 IgG-ricin A chain (DiStefano et al., 1985). This agent was effective in vitro but not in vivo.

The first anti-neuronal immunotoxin effective in vivo was OX7-saporin. This agent consists of the MRC monoclonal antibody, OX7, specific for rat Thy 1 coupled to saporin, a highly active ribosome inactivating protein from *Saponaria officinalis*. The target antigen, rat Thy 1, is expressed by all adult neurons and a subset of thymically derived lymphocytes (Raff et al., 1979; Crawford and Barton, 1986). OX7-saporin is an effective suicide transport agent in rat peripheral and central nervous system (Wiley et al., 1989). It has been used in experiments to localize neurotransmitter receptors on CNS neurons that are not effectively lesioned by volkensin such as striatopallidal projection neurons (Roberts et al., 1993) and thalamocortical projection neurons (Crino et al., 1990Vogt et al., 1995). Similar to the toxic lectins, OX7-saporin can produce indiscriminate damage at CNS injection sites (Krum et al., 1997). Because OX7-saporin is safer to handle and produces little systemic toxicity, it is an excellent choice for suicide transport lesioning in the rat. The recent development of cholera toxin B subunit-saporin is a promising alternative (see Llewellyn-Smith et al., this issue).

OX7-saporin targets any and all rat neurons. The first type-selective anti-neuronal immunotoxin active in vivo is 192 IgG-saporin. The precise reason for the difference in efficacy between 192-ricin A chain and 192-saporin has not been determined. Comparison of OX7-ricin A chain and OX7-saporin has shown comparable activities in vitro but OX7-saporin was significantly more active in vivo (Thorpe et al., 1985). On the basis of this observation and our experience with OX7-saporin as a suicide transport agent, we made 192-saporin which selectively targets neurons that express $p75^{NTR}$, the low affinity neurotrophin receptor (Chandler et al., 1984). After systemic injection, 192-saporin kills postganglionic sympathetic neurons and some dorsal root ganglion neurons (Wiley et al., 1991). However, the reason for developing this toxin was to selectively kill CNS neurons that express $p75^{NTR}$. Schweitzer showed that intraventricular injection of 192 IgG resulted in selective uptake of the antibody into the cholinergic neurons of the basal forebrain (CBF) (Schweitzer, 1987, 1989). As expected, intraventricular injection of 192-saporin selectively destroys neurons of the rat cholinergic basal forebrain (Wiley et al., 1991Book et al., 1994). Because 192 IgG is specific for rat $p75^{NTR}$, 192-saporin is not active in any other species. However, a similar immunotoxin, ME 20.4-saporin, which contains a monoclonal antibody to the human receptor has been shown effective in primates (Fine et al., 1997; Ridley et al., 1999).

Although the route by which 192-saporin gets to the CBF has not been rigorously defined, coinjection with colchicine protects CBF neurons suggesting that fast axonal transport is required to deliver the toxin to the target cell bodies (Ohtake et al., 1997). In the forebrain of healthy, adult Sprague-Dawley rats, the only neurons that express high levels of $p75^{NTR}$ are in the CBF, but the cholinergic neurons that innervate the amygdala do not express $p75^{NTR}$ and are spared after 192-saporin injections (Heckers and Mesulam, 1994). Intraventricular injections of 192-saporin also destroy some cerebellar Purkinje cells because about half of these cells express $p75^{NTR}$. When intraventricular injections of 192-saporin have been used for behavioral experiments, controls must be included for the cerebellar damage. One such control is to use intraventricular injection of OX7-saporin which will destroy cerebellar Purkinje cells (Davis and Wiley, 1989). More restricted lesions of the CBF can be made by injecting 192-saporin directly into the CBF (Torres et al., 1994) or into a terminal field such as neocortex (Holley et al., 1994; Sachdev et al., 1998).

The next anti-neuronal immunotoxin developed was anti-dopamine beta-hydroxylase-saporin (anti-DBH-saporin). In the late 1970's, rabbit polyclonal antisera to dopamine beta-hydroxylase were reported to be taken up and concentrated in noradrenergic postganglionic

sympathetic neurons after systemic injection (Jacobowitz et al., 1975) or injection into organs innervated by noradrenergic neurons (Ziegler et al., 1976). Spinal cord injections retrogradely labeled pontine noradrenergic neurons (Westlund et al., 1981Westlund et al., 1984). Later, such injections combined with guinea pig complement were used to produce lesions of noradrenergic neurons (Blessing et al., 1977; Lewis et al., 1977; Costa et al., 1979; Geffen et al., 1982). With the development of monoclonal antibodies to DBH, it became possible to make the corresponding immunotoxin. Anti-DBH-saporin selectively destroys noradrenergic and adrenergic neurons after systemic (Picklo et al., 1994, 1995a,b), intrathecal (Wrenn et al., 1996; Rohde and Basbaum, 1998; Martin et al., 1999) or intraparenchymal (Blessing et al., 1998; Madden et al., 1999) injection. In rats, this immunotoxin is highly selective and efficient. Fig. 1 shows an example of the noradrenergic denervation obtained in rat spinal cord after lumbar intrathecal injection of anti-DBH-saporin. Fig. 2 shows that the predominate cell loss occurs in the appropriate pontine noradrenergic cell groups known to project to the cord (Westlund and Coulter, 1980Westlund et al., 1981Westlund et al., 1982, 1983Westlund et al., 1984Westlund et al., 1990; Proudfit and Clark, 1991; Clark and Proudfit, 1991, 1992, 1993; Sluka and Westlund, 1992; Yeomans et al., 1996). Preliminary experiments using lumbar intrathecal injection of anti-DBH alone showed the antibody was taken up and transported to the same pontine cells (data not shown). An immunotoxin to the dopamine transporter that is specific for midbrain dopaminergic neurons also has been reported (Wiley et al., 1996). It is likely that additional anti-neuronal immunotoxins can be developed with the requirement that the antibody targeting vector be directed against an epitope on the external surface of the neuronal plasma membrane.

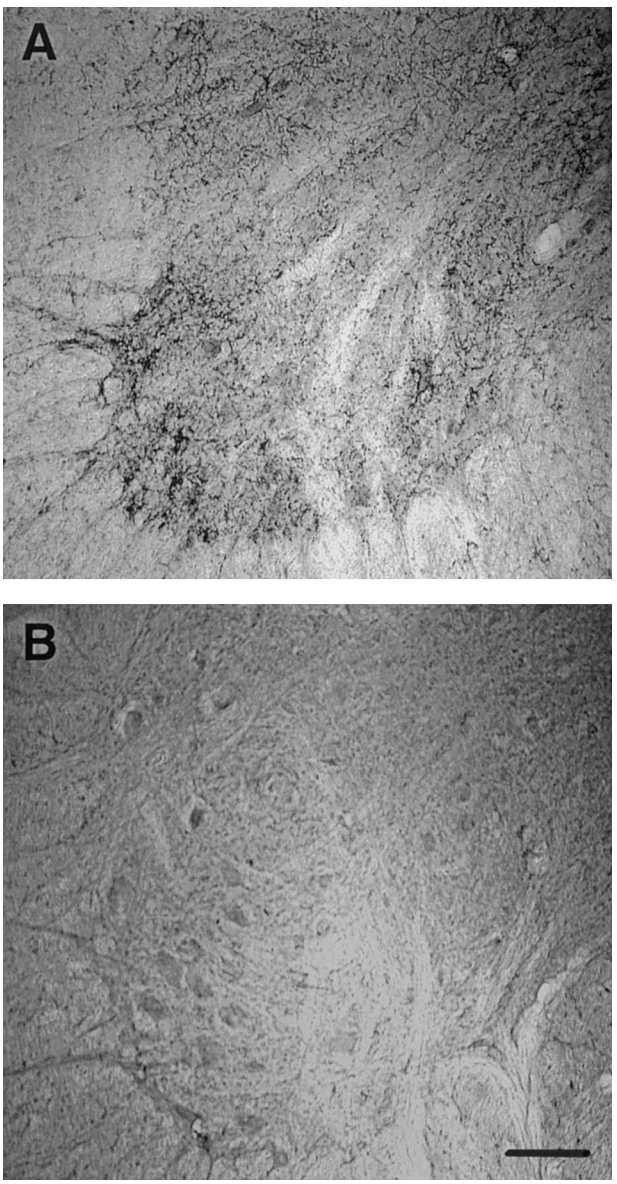

Fig. 1. Ventral horns of lumbar spinal cord sections stained for demonstration of dopamine β-hydroxylase using indirect peroxidase immunohistochemistry as previously described (Wrenn et al., 1996). Panel A is from a normal control rat sacrificed two months after lumbar intrathecal injection of vehicle (saline with 1 mg/ml bovine serum albumin and 0.1% Fast Green dye). Note the positively stained varicosities that appear as black dots. Panel B is from a rat sacrificed four months after lumber intrathecal injection of 250 ng of anti-DBH-saporin. Note the complete absence of stained varicosities consistent with complete destruction of the noradrenergic innervation to the spinal cord. The magnification bar in the right lower corner indicates 100 μm.

3. Neuropeptide-toxin conjugates

Neuropeptide conjugates were first used a number of years ago in experiments on the hypothalamic–pituitary endocrine system (Samson et al., 1992a,b, 1993, 1995; Blackburn et al., 1993, 1995a,b). Recently, Mantyh and colleagues showed that substance P is selectively internalized by neurons expressing the neurokinin-1 receptor (NK-1R) (Mantyh et al., 1995). Based on this observation, we made substance P-saporin which is selectively toxic to NK-1R both in vitro and in vivo (Wiley and Lappi, 1997). An even more efficient version using a modified analog of substance P has been described recently (Wiley and Lappi, 1999). Lumbar intrathecal injection of substance P-saporin selectively destroys neurons in lamina I of the spinal dorsal horn that express NK-1R (Mantyh et al., 1997). This lesion inhibits the development of hyperalgesia under a variety of conditions (Nichols et al., 1999) and may prove to be a useful treatment for chronic pain. However, to date, there has been no convincing evidence that substance P-saporin is axonally trans-

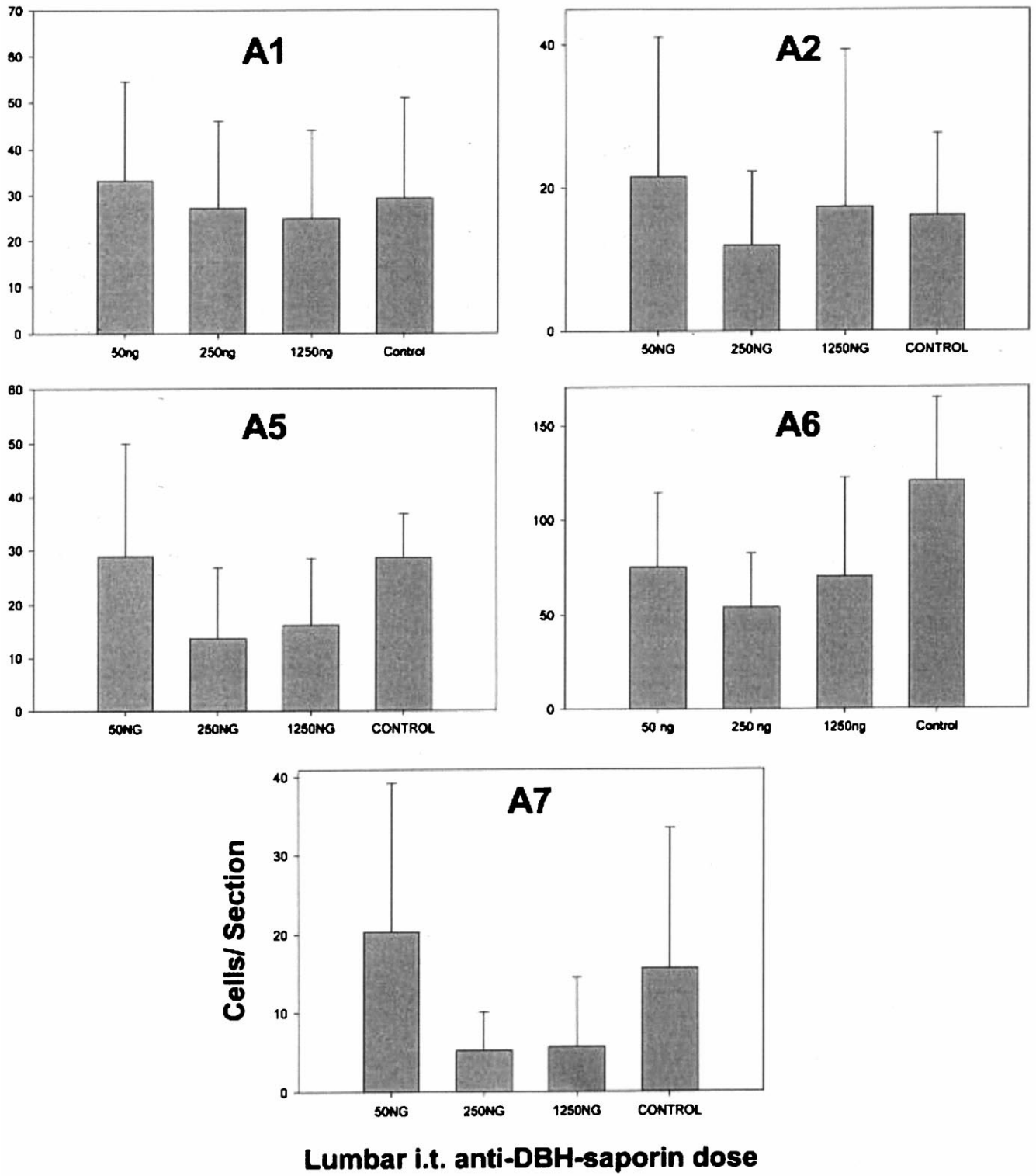

Fig. 2. Cell counts of brainstem noradrenergic neurons and the effects of lumbar intrathecal injection of anti-DBH-saporin. Two adult male Sprague-Dawley rats (Harlan) were injected at each dose of toxin and of vehicle. Two sets of one in six series of 40 μm transverse brainstem sections were stained for demonstration of dopamine β-hydroxylase using indirect immunoperoxidase technique as previously described (Wrenn et al., 1996). The number of positively stained cells per section in the sections through the normal rostrocaudal extent of the indicated cell groups were averaged to give the values indicated in each graph. In the case of the locus coeruleus (A6), cells were counted in cresyl violet (Nissl) stained sections. Note the sparing of medullary cells (A1 and A2). All three toxin doses produced the same degree of partial lesion of the A6 (locus coeruleus) and loss of A5 and A7 cells occurred only at the higher doses (250 ng and 1250 ng).

ported. All experiments have applied the toxin in the vicinity of the cell bodies. A dermorphin-saporin conjugate has been recently described that targets neurons expressing the μ opiate receptor (Wiley and Lappi, 1998).

4. Example protocols

4.1. Suicide transport

In the peripheral nervous system, ricin remains a useful agent.

1. Dilute stock ricin solution in sterile saline with 0.1% Fast Green dye. For a typical rat peripheral nerve, a concentration of 1 μg/μl is a reasonable starting point.
2. Expose and mobilize a short segment of the nerve in an anesthetized animal.
3. Pressure microinject 1 μl of ricin solution using a glass micropipette with the tip broken back or a fine syringe needle and a Hamilton microsyringe. Insert the pipette or needle into the nerve at a shallow angle. Progress of the injection can be followed by observing the spread of the dye.
4. Allow the rat to recover for two weeks and then apply appropriate tests.
5. Sacrifice the rat by formalin perfusion fixation and stain frozen sections of the appropriate sensory ganglia (ipsilateral and contralateral to toxin injection) and/or appropriate CNS region with acidic cresyl violet to confirm loss of the target neurons.

4.2. Immunolesioning

Intracerebroventricular injection of the immunotoxin, anti-DBH-saporin will preferentially destroy pontine noradrenergic neurons in rats.

1. Dilute anti-DBH-saporin (Advanced Targeting Systems, San Diego, CA) in sterile saline with 0.1% Fast Green dye. To lesion all pontine noradrenergic neurons, a good starting concentration would be 1 μg/μl.
2. Pressure microinject into the lateral ventricle in a volume of 10 μl (total dose = 10 μg) using standard stereotactic technique. Use injection volumes of at least 10 μl to insure adequate distribution of toxin through the ventricular system.
3. Allow rats to recover for two weeks and then perform appropriate testing.
4. Sacrifice rats by formalin fixation and immunohistochemically stain frozen sections of the brain and/or spinal cord using monoclonal antibody to dopamine beta-hydroxylase (MAB308, Chemicon International, Temecula, CA) to confirm the extent of lesion.

4.3. Neuropeptide-toxin conjugate

The best studied of these agents is substance P-saporin which will destroy lamina I spinal dorsal horn neurons that express the neurokinin-1 receptor.

1. Dilute substance P-saporin (Advanced Targeting Systems, San Diego, CA) in sterile saline containing 1 mg/ml bovine serum albumin and 0.1% Fast Green dye. A good starting concentration for lumbar intrathecal injection is 30 ng/μl in a rat.
2. Pressure microinject into the lumbar subarachnoid space in a volume of 10 μl followed by a 10 μl flush of vehicle using a stretched PE-10 catheter inserted through the atlanto-occipital membrane as described by Yaksh (Yaksh and Rudy, 1977).
3. Allow rats to survive for two weeks and then perform appropriate testing.
4. Sacrifice rats by formalin perfusion and stain spinal cord sections for demonstration of neurokinin-1 receptor using rabbit polyclonal antibody (Chemicon International, Temecula, CA) to confirm extent of lesion.

5. Caveats – lessons learned the hard way

A number of considerations are important when using axonally transported toxins:

1. These toxins are proteins that must be handled appropriately including minimizing freezing and thawing, keeping aliquots sterile and cold and not foaming when mixing. Standard preservatives (i.e. azide) are incompatible with the toxins.
2. Each experiment should be preceded by pilot experiments to determine the dose and route of administration that will produce the desired lesion.
3. Anatomical studies are always needed to confirm the lesion location, selectivity and extent in each animal.
4. Control experiments may include injection of vehicle alone, saporin alone, reduced toxin (pre-treated with dithiothreitol) or an irrelevant immunotoxin. In situations where collateral damage is unavoidable, an appropriate control would involve producing the collateral damage without producing a lesion of the primary target population. In the CNS, this can be done using ricin to reproduce non-specific injection site damage because ricin is not axonally transported within the CNS (Pangalos et al., 1991Pangalos et al., 1992Francis et al., 1992Chessell et al., 1993aChessell et al., 1993bChessell et al., 1995Cevolani et al., 1995; Roberts et al., 1993, 1995; Wiley and Lappi, 1994).

6. Summary

The collection of techniques described now makes possible a variety of powerful experiments generally aimed at determining the function of a particular group of neurons by analyzing the effect of selectively destroying the neurons in question. The choice of targets is continuing to increase ever more rapidly now that the usefulness of this approach has become apparent. The challenge will be to integrate this information with the other lines of inquiry in coming to conclusions about the function of a neural system. In pharmacology, the interesting paradigm of analyzing drug action by removing populations of neurons expressing a particular receptor promises new and illuminating insight into the site(s) and cellular mechanism(s) of therapeutically important drugs. Ultimately, the delivery of non-toxic moieties, such as genes, will develop based on the same targeting principles. Wiley et al., 1983

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