# Immunotoxins and Neuropeptide-Toxin Conjugates Experimental Applications

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**Abstract:** The use of targeted toxins in research applications has recently grown considerably. The ability to remove a few specific cells, even when surrounded by different populations, has given scientists a powerful tool for the understanding of systems biology. The use of targeted toxins in research is rich and varied; here we limit ourselves to describe some of those exciting results that researchers have made in the neurosciences.

**Keywords:** Immunotoxin, Ligand-Toxin, Peptide-Toxin, Saporin, Retrograde Transport, Behavior, Molecular Surgery, Molecular Neurosurgery.

## IMMUNOTOXINS

This chapter focuses on the use of immunotoxins to make highly selective neural lesions for experimental and potentially therapeutic purposes. The promising new neuropeptide-toxin conjugates also are included. This application of targeted cytotoxins to neuroscience has recently been termed molecular neurosurgery. While targeted toxins have been used for years to find a Magic Bullet for therapeutic purposes, Enzo Stripe has often stated to us that, in his opinion, the use of targeted toxins in research applications is an area that could be extremely important by yielding important research, and certainly could be put into action almost the moment that the experimenter thought of the idea. Aspects of molecular neurosurgery have been the subject of several previous reviews [1-10]. The development of immunotoxin techniques for use in neuroscience began with the introduction of ricin as a suicide transport agent [11]. Suicide transport refers to the use of an axonally-transported cytotoxin to produce anatomically selective neural lesions (Fig. (**1**)). Although ricin was effective and continues to be used, some limitations were readily evident. The most striking limitation was that ricin was ineffective as a suicide transport agent within the central nervous system [12]. Subsequently, other toxic lectins, such as volkensin and modeccin, were shown effective within the CNS [13]. Of course, the remarkable systemic toxicity of ricin, modeccin and volkensin is another drawback to both experimenters and experimental animals. Systemic administration of anti-ricin antiserum was shown to protect animals without affecting the suicide transport lesion [14], but experimenters still had be concerned about the risk to themselves from handling toxic lectins.

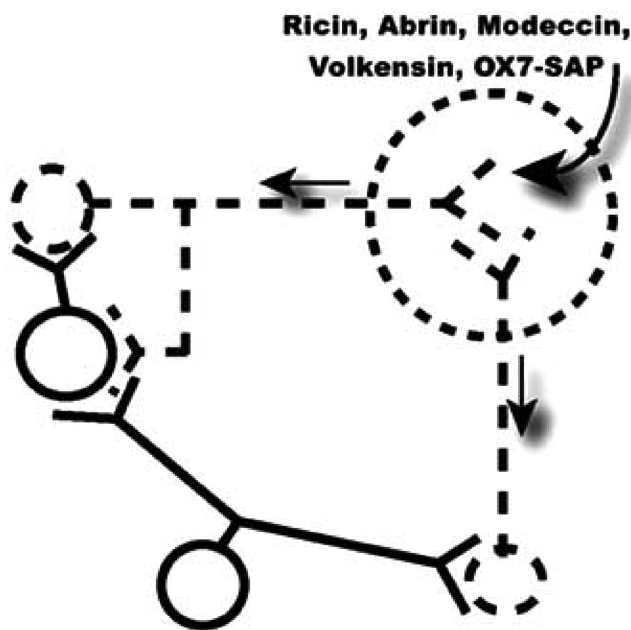


**Fig. (1). Suicide transport.** A non-specific toxic lectin or OX7-SAP is injected in the vicinity of axons (subepineurally into peripheral nerve) or axon terminals (muscle, CNS nucleus). The toxin is taken up by endocytosis and retrogradely transported to the cell body by fast axoplasmic transport (small arrows). Once in the perikaryon, the ribosome-inactivating moiety escapes endosomes via the trans-Golgi and attacks ribosomes resulting in death of cells projecting to or through the injection site (dashed cells).

It was in this setting that immunotoxins were introduced for use as suicide transport agents. The first such agent was OX7-SAP. This immunotoxin recognizes the rat and mouse. Thy-1 membrane protein that is ubiquitously expressed on all neurons and some thymic-derived lymphocytes [15]. *In vivo* injections of unconjugated OX7 antibody were shown to undergo retrograde axonal transport [16]. Vagal nerve injections of OX7-SAP destroy sensory and motor neurons projecting through the injected nerve [17; Fig. (**2**)]. Similarly, injection of OX7-Saporin into the striatum of rats destroys neurons projecting into the striatum from the substantia nigra and intralaminar thalamus. Based on these results and on superior *in vivo* activity of OX7-SAP compared to OX7-ricin A chain against AKR-A lymphoma cells in nude mice [15], the success of OX7-SAP as a suicide transport agent led directly to development of the first neuron type-selective immunotoxin, 192-Saporin.

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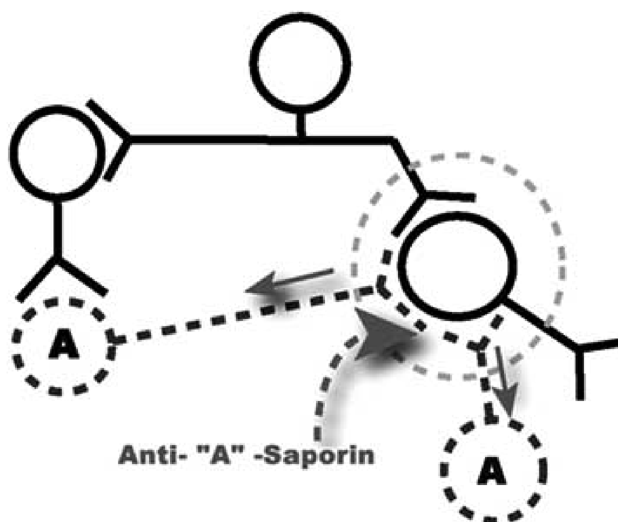


**Fig. (2). Immunolesioning by retrograde axonal transport.** Immunotoxin consisting of monoclonal antibody to a specific cell surface antigen ("A") is injected into the vicinity of axon terminals, specifically taken up by endocytosis into cells expressing the "A" antigen (A/dashed cells) and retrogradely transported to cell bodies (small arrows). Once in the perikarya, saporin escapes endosomes and attacks ribosomes leading to death of cells expressing the "A" antigen. Neurons that do not express the "A" antigen (solid cells) are unaffected by the immunotoxin. Examples of anti-neuronal immunotoxins include 192-Saporin, anti-DBH-SAP and anti-DAT-SAP that specifically target neurons expressing $p75^{NTR}$, dopamine beta-hydroxylase or the dopamine transporter, respectively.

The monoclonal antibody, 192-IgG, was originally made against a nerve growth factor binding protein [18]. Initial attempts to make an immunotoxin with 192-IgG used ricin A chain. 192-ricin A was effective against PC12 cells (derived from a rat pheochromocytoma) *in vitro* but it was inactive *in vivo* [19]. However, arming 192-IgG with saporin produced an immunotoxin that was highly effective, both *in vitro* and *in vivo* against neurons expressing $p75^{NTR}$, the low affinity neurotrophin receptor [20]. 192-Saporin was the first agent for immunolesioning. Immunolesioning refers to the use of anti-neuronal antibodies armed with cytotoxin to selectively destroy specific types of neurons [1,8]. The rest of this chapter will review some of the specific ways in which anti-neuronal immunotoxins have been used experimentally and the exciting neuropeptide-toxin conjugates that may have therapeutic potential.

### Systemic Immunotoxin Injections

Systemic injection of immunotoxins can produce selective neural lesions. The first immunolesioning agent designed specifically for systemic use was anti-DBH-SAP. Anti-DBH antibody is directed at the enzyme, dopamine beta-hydroxylase (DBH), which converts dopamine to norepinephrine. DBH is contained in synaptic vesicles and becomes exposed on the nerve terminal surface membrane after exocytosis of neurotransmitter. The decision to make anti-DBH-SAP was based on several reports that documented specific anti-DBH antibody uptake, retrograde axonal transport and accumulation in the perikarya of noradrenergic neurons after systemic [21] or local injections [22-24]. Others also had shown that injections of anti-DBH antibody along with complement will produce selective destruction of noradrenergic neurons *in vivo* [25-29]. Armed with this evidence for selective targeting by anti-DBH antibody, Picklo *et al.* made anti-DBH-SAP for use in producing sympathectomies by intravenous immunotoxin injection [30, 31]. At maximally tolerated doses, intravenous anti-DBH-SAP produces substantial, but not complete, destruction of peripheral sympathetic neurons with minimal effect on adrenal medulla. The only other anti-neuronal immunotoxin specifically studied by intravenous injection has been 192-Saporin. Intravenous injections of 192-Saporin will destroy some primary afferent neurons in sensory ganglia and postganglionic sympathetic neurons [20, 31]. Unfortunately, in phase I clinical trials, some anti-cancer immunotoxins also turned out to target neurons and/or Schwann cells resulting in significant peripheral nerve damage [32-34].

The above experiences indicate that successful use of systemic injections of anti-neuronal immunotoxins requires a high degree of targeting precision and efficiency (precise, selective cell kill at low toxin concentrations) since many different tissues are exposed to the immunotoxin after systemic injections. Slight cross-reactivity of the antibody with antigen on non-target cell populations or low immunotoxin potency will prevent success in producing the desired neural lesion with systemic injections. Dose-limiting, non-specific systemic effects of intravenous immunotoxin have been observed [32, 34-39]. Lastly, systemic injections generally require large quantities of immunotoxin that can become quite expensive and the resulting lesions are often incomplete.

### Intrathecal and Intracerebroventricular Injections

Administration of immunotoxin into the cerebrospinal fluid has been pioneered by Youle and colleagues as a way to treat neoplastic meningitis and primary brain tumors [40-42]. However, as they discovered, cerebellar Purkinje cells can be a problem when using intraventricular or intrathecal toxin injections [33,43]. This predilection for Purkinje cell damage may reflect the propensity of these neurons to extract substances from the CSF [44]. Taking advantage of this property of Purkinje cells, Davis and others have shown that intraventricular injection of OX7-SAP can produce a model of Purkinje cell degeneration [45-48]. The success of intraventricular OX7-SAP in targeting Purkinje cells is likely due to the above mentioned propensity for uptake of substances from the CSF and the high level of expression of the OX7 target moiety, Thy-1, by these neurons [49].

The most extensively studied intrathecal immunotoxin is 192-Saporin. Literature searching reveals well over 200 journal articles in the past 12 years using 192-Saporin, of which, more than 100 report results with intraventricular injections to destroy the cholinergic neurons of the basal forebrain (CBF). Development of this immunotoxin was originally based on work by Schweitzer and co-workers that showed intraventricular injections of 192-IgG antibody alone resulted in selective accumulation of the antibody in the CBF [50-52]. Intraventricular injection of 192-Saporin selectively destroys the $p75^{NTR}$-expressing neurons of the

CBF and cerebellar Purkinje cells (about 50% of Purkinje cells express $p75^{NTR}$) [20]. Subsequent studies have reported that Purkinje cell lesions may affect some behavioral tests of CBF function [47] but not others [48]. In order to avoid concomitant Purkinje cell loss in experiments designed to study CBF function, many investigators have used direct intraparenchymal or intracortical injections of 192-Saporin (see below).

The exact route followed by 192-Saporin going from intraventricular CSF to perikarya in the CBF has not been defined. However, inhibition of axonal transport by co-injecting colchicine along with 192-Saporin can prevent a CBF lesion, presumably by preventing delivery of saporin from superficial axon terminals to CBF cell bodies [53]. Fig. (**3**) shows the typical early effect of intrathecally-delivered 192-Saporin that has undergone retrograde transport to the cell body and will result in death of the affected neurons. Clearly, both anti-neuronal antibodies show retrograde transport and immunotoxins exhibit retrograde axonal transport. The peptide-toxins, such as SP-SAP, the potent conjugate between substance P and saporin, apparently are unable to undergo retrograde transport, even if the receptors they target are able to undergo retrograde transport after binding by antibodies.

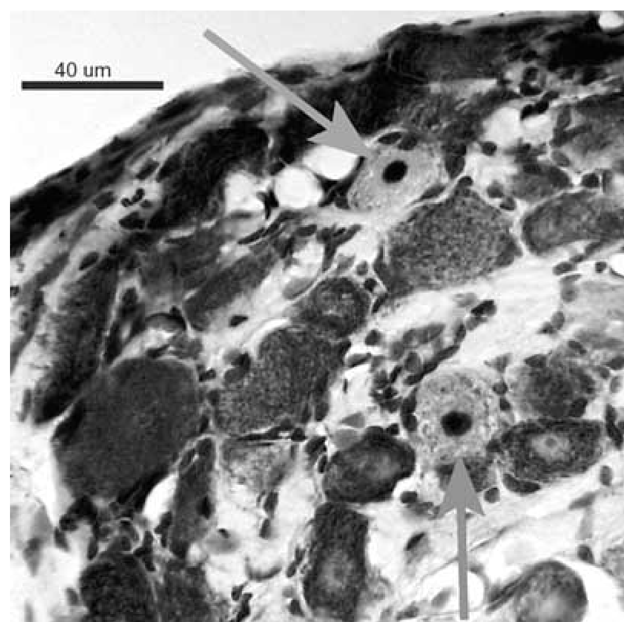


**Fig. (3).** Nissl-stained section of L5 dorsal root ganglion of a rat sacrificed 2 days after lumbar intrathecal injection of 1 µg of 192-Saporin. The arrows indicate two neurons showing the characteristic chromatolysis and karyorhexis produced by saporin.

Numerous studies have documented the effectiveness and selectivity of intraventricular 192-Saporin in destroying the CBF, see for example [54-63], but some CBF neurons that project to the amygdala do not express $p75^{NTR}$ [55,64]. In spite of these limitations, rats treated with intraventricular 192-Saporin have provided a valuable model for studying the cognitive behavioral effects of CBF lesions, thus modeling a key feature of Alzheimer's disease [65] including studies using neural transplants to reconstitute behavioral function after CBF destruction [66].

Other immunolesioning agents that have been studied by intraventricular injection include anti-DBH-SAP (brainstem noradrenergic lesion) and anti-DAT-SAP (midbrain dopaminergic lesion). Wrenn *et al.* first described the anatomic effects of intraventricular anti-DBH-SAP [67] showing dose-dependent destruction of brainstem catecholaminergic neurons. This approach has been used to examine the role of brainstem noradrenergic projections in morphine withdrawal [68] and localization of mu opioid receptors in the cingulate cortex to afferents from the locus coeruleus [69]. Spinal intrathecal injections of anti-DBH-SAP destroy spinally-projecting brainstem catecholaminergic neurons [8]. Spinal intrathecal anti-DBH-SAP has been used in studies of pain and morphine analgesia [70,71]. An interesting adaptation of the immunolesioning approach has been reported by Kobayashi *et al.* [72]. They made transgenic mice that expressed the human IL-2alpha receptor under control of the DBH promoter, which produces cell specific expression. Then, they treated mice intraventricularly with immunotoxin to the human IL-2alpha receptor (anti-Taqc(Fv)-PE40) and destroyed catecholaminergic neurons that normally express DBH. Intraventricular injection of anti-DAT-SAP destroys dopaminergic neurons in the substantia nigra, and to a lesser degree, the ventral tegmental area, thus mimicking this key feature of the neuropathology of Parkinson's disease [73].

Intraventricular injections seem to have the advantage of not producing the locally high toxin concentrations seen with intraparenchymal and intraneural injections, but as reported with 192-Saporin, results may be complicated if more than one neuronal population expresses the target antigen (i.e. CBF and Purkinje cells). Indeed, it is likely that cerebellar Purkinje cells may be affected by intrathecal immunotoxin injections even without expressing the target antigen. However, for many purposes, more restricted lesions are desired. In the CNS, direct intraparenchymal injections are often used to control toxin spread and produce very restricted lesions.

### Intraparenchymal Injections

Cell type-specific localized CNS lesions have been reported after direct intraparenchymal injections of immunotoxins in the vicinity of target cell bodies (Fig. (**4**)) or in terminal projection fields. In the basal ganglia, OX7-SAP has been used to destroy striatopallidal projection neurons by suicide transport after injection of the immunotoxin into the globus pallidus [74,75]. Corticostriatal and corticothalamic neurons can be destroyed by intraparenchymal injections of OX7-SAP into the striatum or thalamus [76,77]. Similarly, 192-Saporin destroys CBF neurons after injection of immunotoxin into hippocampus [53] or cortex [78-84] where immunotoxin comes in contact with axonal terminals of CBF neurons. Anti-DBH-SAP injected into olfactory bulb or spinal cord is effective in destroying the catecholaminergic cells of origin in the locus coeruleus [25] or brainstem [85], respectively.

Many studies have used 192-Saporin injected directly into the CBF where the target cell bodies are located. Lesions made in this way have been widely used in behavior and neurochemical studies while avoiding the potentially confounding effect of Purkinje cell lesions that occur when

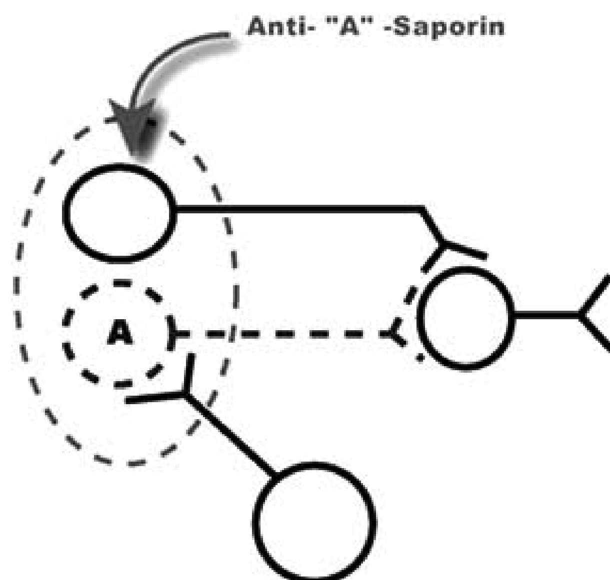


**Fig. (4). Immunolesioning applied directly to neuronal cell bodies and/or dendrites.** As in Figure **2**, immunotoxin to the "A" antigen is selectively taken up by neurons expressing the "A" antigen (A/dashed cells). Toxin is delivered to perikarya without need for axonal transport. Adjacent neurons not expressing "A" antigen (solid cells) are unaffected. In some cases, the mechanisms in Figures **2** and **4** could occur simultaneously depending on the specific anatomy and distribution of neurons expressing the target antigen.

192-Saporin is injected intraventricularly, see for example [56,86-93]. For similar purposes of studying cognitive function of the CBF in monkeys, ME20.4-SAP has been injected into the CBF [94-99]. Neurotransmitter receptor mapping also has used CBF injections of this immunotoxin in monkeys [100]. ME20.4-SAP recognizes the human $p75^{NTR}$ and is active in several species while 192-Saporin only works in rats.

Anti-DBH-SAP has been injected directly into the brainstem to study the cardiovascular consequences of destroying a specific group of catecholaminergic neurons [101]. Also, Ritter and colleagues have reported a series of studies using CNS intraparenchymal injections of anti-DBH-SAP to analyze the role of catecholaminergic neurons in metabolic control [102-104]. It seems likely that anti-DBH-SAP will find increasing use in functional studies of CNS catecholaminergic neurons.

Certainly intraparenchymal injections produce anatomically restricted lesions, but this route of administration requires very efficient targeting to prevent non-specific lesions (collateral damage) due to the high local concentrations that occur with brief pressure microinjections. Also, complete destruction of an extended cell group such as the CBF by direct injection requires multiple injection sites. Extended, high flow local infusions of dilute solutions that distribute toxin along with bulk fluid flow through brain tissue may produce better intraparenchymal lesions. This technique of extended intraparenchymal infusion has been pioneered for brain tumor therapy by Oldfield and colleagues [41].

## Intraneural Injections

Injection of immunotoxins subepineurally into peripheral nerves is the corresponding peripheral nervous system (PNS) approach to produce locally limited lesions of cells that project axons through a specific injected nerve. This approach was first used with the toxic lectins [11,105] and later adapted for use with OX7-SAP [17]. As newer agents with interesting specificities for peripheral neurons are developed, such as cholera toxin B subunit (CTB)-SAP [106] and IB4-SAP [107], more experiments with intraneural injections are likely. This approach requires that the target antigen be displayed on the axonal membrane. Heavily myelinated fibers are more difficult to lesion, presumably because the myelin sheath limits access of immunotoxin to the axonal membrane. Attempts to produce restricted PNS lesions by injecting immunotoxin directly into target organs have demonstrated suicide transport of 192-Saporin and anti-DBH-SAP with destruction of the appropriate neurons innervating the target organ. Unfortunately, the lesion often involves other PNS neurons expressing the target antigen suggesting systemic spread of the immunotoxins [3].

## Peptide Ligand Toxins

While the production of antibody-toxins, i.e., immunotoxins, was driven largely by the promise of potent and specific anti-tumor weapons, the use of ligand-toxins,

**Table 1. Immunotoxins Used in Neuroscience Research**

| Immunotoxin | Target Antigen | Target Neurons |
|---|---|---|
| OX7-SAP | (rat, mouse)Thy-1 | All |
| 192-Saporin | (rat)$p75^{NTR}$ | CNS: CBF, Purkinje<br>PNS: postganglionic sympathetic, primary afferent |
| ME20.4-SAP | (human)$p75^{NTR}$ | Same as 192-Saporin |
| Anti-DBH-SAP | dopamine beta-hydroxylase | CNS: brainstem catecholaminergic<br>PNS: postganglionic sympathetic |
| Anti-DAT-SAP | dopamine transporter | Midbrain dopaminergic (substantia nigra, VTA) |

CNS - central nervous system, PNS - peripheral nervous system, VTA - ventral tegmental area

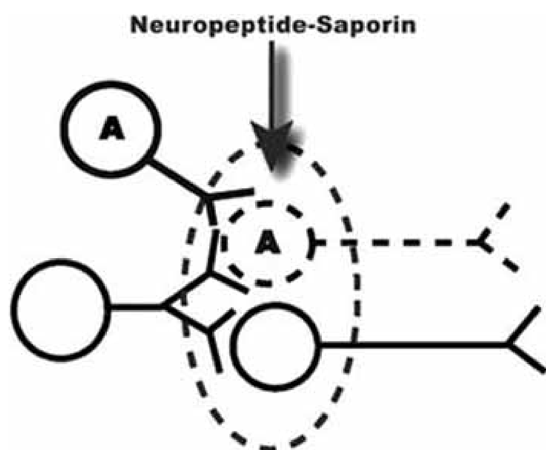


**Fig. (5). Neuropeptide toxin conjugates applied in vicinity of cell bodies/dendrites.** The A cells express the receptor for the specific neuropeptide. Target cells exposed to the neuropeptide-toxin conjugate in the vicinity of the cell body and/or dendrites (dashed cell) are destroyed, but not A cells only exposed in the vicinity of axon terminals. At this point in time, it is not apparent that neuropeptide-toxin conjugates undergo retrograde axonal transport. Cells not expressing the receptor for the specific neuropeptide (solid cells) are unaffected by the neuropeptide-toxin conjugate. Examples of neuropeptide-toxin conjugates include SP-SAP and dermorphin-SAP that bind specifically to the neurokinin-1 and mu opiate receptors, respectively.

utilizing peptide ligands as targeting vehicles, was largely driven by research curiosity. The peptide-toxins have been very successful as research tools and have been extremely useful, especially in the pain field. Perhaps the first of these, and the one that really encapsulated many of the challenges of the use of a peptide neurotransmitter as a targeting vehicle was produced by J.R. Murphy's group (Fig. (**5**)). Twenty years ago, they used thyrotropin-releasing hormone (TRH) and chemically conjugated it to a fragment of diphtheria toxin (DT) [108]. The three-amino acid peptide, pyro-glu-his-pro-amide, is small enough to make one wonder if the addition of the 45 kDa DT fragment would hamper receptor binding. Not only that, but it has both termini blocked and so only the middle amino acid is available for linkage of DT—what sort of effect on peptide binding would that have? The construct was made, and the resulting material eliminated 50% of cells from a rat pituitary cell line at 3 nM, while at 100 nM the DT fragment had no effect. The conjugate was then used to ascertain information about the mechanism of action of DT and its method of crossing the membrane barrier.

Later, researchers in search of cancer cures began to use molecules such as transforming growth factor (TGF)-$\alpha$ as a targeting agent [109]. One of the first targeted toxins approved as a pharmaceutical agent, Ontak™, utilizes interleukin-2 (IL-2) as a targeting agent; it also is a result of J.R. Murphy's work. Others entered the field and there was modest growth; Samson's group produced toxic conjugates of the natriuretic peptides and oxytocin [110-112] and Pastan's group began producing numerous ligand-toxins, mainly for anti-tumor purposes, but occasionally yielding interesting research results. The group of Nakanishi has created transgenic animals that express the IL-2 receptor under control of a specific neuronal promoter, mGluR2,

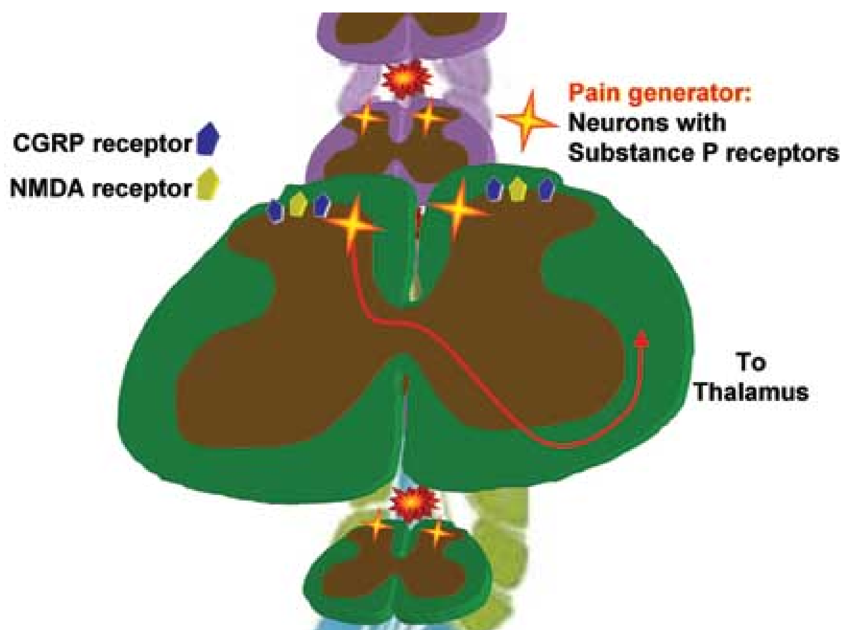


**Fig. (6). Location and Function of Substance P receptor (SPR)-positive neurons in the spinal cord.** The neuropeptide-toxin SP-SAP specifically targets and eliminates cells that express SPR. Intrathecal administration in the spinal cord kills these spinothalamic neurons that make up less than 5 percent of the neurons in the spinal cord. These SPR-positive neurons also express CGRP (calcitonin gene-related peptide) and NMDA (N-methyl-D-aspartate or glutamate) receptors. For this reason, a Substance P antagonist is unsuccessful in eliminating the pain signal; activation of either the CGRP or NMDA receptor will still start the message. These SPR-positive neurons are labeled "pain generators" and it has been shown that their elimination greatly reduces the perception of chronic pain in animal models (used with permission. Copyright 2002, Advanced Targeting Systems).

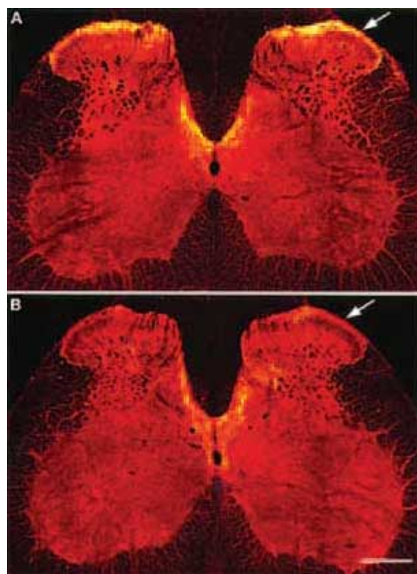


**Fig. (7). Cytotoxicity after intrathecal infusion of SP-SAP in the spinal cord.** Confocal images of SPR immunofluorescence in the spinal cord 28 days after infusion of saline (A) or SP-SAP (B), where the SPR immunofluorescence appears yellow. The only difference between saline- and SP-SAP-treated animals is the marked reduction in SPR immunofluorescence in lamina I (arrows) of the SP-SAP-treated animals. These images are 60 µm-thick tissue sections acquired with a 10X lens. Bar, 400 mm. (Used with permission. *Science* 278:275-279, 1997).

which will cause IL-2 receptor expression in different areas of the nervous systems, such as striatal cholinergic neurons [113] or starburst amacrine cells of the retina [114]. They then used an IL-2-toxin to specifically remove these cells and make conclusions as to the function of these cells.

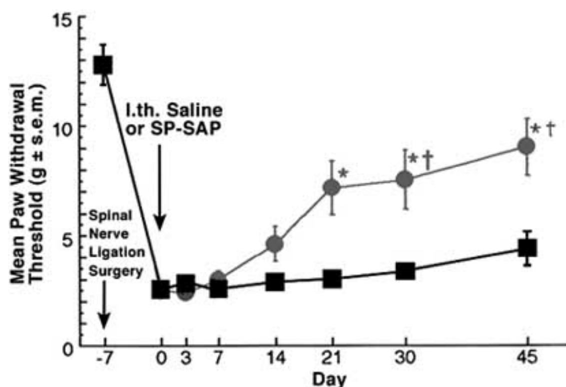


**Fig.(8). SP-SAP attenuates nerve injury (spinal nerve ligation model)-induced allodynia when administered either 30 days before or 7 days after nerve ligation.** The anti-allodynic effect of SP-SAP (red, n=14) and saline (blue, n=13) when administered after nerve ligation and development of the persistent pain state. The anti-allodynic effect of SP-SAP becomes significant 21 days after infusion. Error bars represent standard error of the mean. Asterisks represent statistical significance from baseline (P<0.05) (used with permission. *Science* 286:1558-1561, 1999).

## SP-SAP

After the success of 192-Saporin as an agent for the exploration of the role of cholinergic basal forebrain in behavioral processes, Ron Wiley suggested that the neurons in the spinal cord that express substance P (SP) and its receptor were known to be involved in pain processes, but the system was extremely complicated and poorly characterized. Imagine the spinal cord as a series of different wires coming from different areas of the body and connecting to more wires and eventually ending in different areas of the brain. How do you separate out the roles of the different wires? Ron Wiley proposed removing a few at a time to see what the behavioral effect(s) would be.

SP-SAP was synthesized and shown to be specifically cytotoxic to cells expressing the SP receptor (SPR) [115]. In short order SP-SAP had a major impact in the determination of the role of SPR-positive neurons in the outer laminae of the spinal cord. Fig. (**6**) shows a schematic representation of the positioning of these neurons in the spinal cord. Pat Mantyh injected SP-SAP into the cerebrospinal fluid surrounding the lumbar spinal cord segments; in this area, there are SPR-positive neurons in the superficial laminae of the spinal cord. These took up SP-SAP and were efficiently eliminated. Fig. (**7**) shows the removal of immunohisto-chemical staining of SPR-positive neurons in the rat spinal cord, as measured by loss of SPR immunoreactivity. The behavioral effects were profound. In a model of hyperalgesia, the irritant from the chili pepper, capsaicin is injected into the hindpaw of a rat. This causes a period of sensitivity, which can be measured by heightened response to heat and touch by a filament. Both of these measurements were inhibited in rats treated with SP-SAP [116]. Later, these studies were expanded to include several models of chronic pain, including the use of the spinal nerve ligation model [117]. This is a true neuropathic pain, and takes more than a week to become established. After establishment, SP-SAP was able to reverse the allodynia (Fig. (**8**)). In addition, the effect of SP-SAP appeared to be long-lasting without creation of central sensitization [118].

Recently, researchers have demonstrated that the very neurons that are ablated by SP-SAP are responsible for the development of central sensitization [119]. Rats treated with intrathecal SP-SAP demonstrated a lessened electrophysiological response (windup) of wide dynamic range (WDR) neurons and profoundly reduced numbers of high threshold neurons. Deeper dorsal horn neurons were not directly damaged by SP-SAP, but the loss of superficial dorsal horn neurons had a profound effect on their excitability, probably by loss of synaptic connections from lamina I neurons. Suzuki *et al*. [120] reported effects of the ablation of the superficial dorsal horn neurons on deeper neurons and showed that the inability to activate the deeper neurons was similar to the effect of inhibiting descending serotonergic projections from the brainstem. It was hypothesized that SP-SAP-driven removal of superficial neurons causes a loss of activation of brainstem neurons (to which they project) and this in turn reduces descending excitation of the deeper spinal neurons and results in a concomitant loss of central sensitization.

The results of these studies have helped to characterize the physiology of chronic pain. Pain is an important

behavioral attribute; insensitivity to pain has profound effects, often involving self-mutilation and early death [121]. But there are a number of disorders that involve excessive pain that are poorly understood: complex regional pain syndrome, phantom limb syndrome, etc. As Tony Yaksh has explained, it was unclear if these disorders were symptomatic of an increase in the system of pain transmission, or if some segment of the obviously redundant pathways had short-circuited (to return to our previous analogy about wiring), it now appears that the latter is the primary issue.

SP-SAP was then used by other researchers to discern the function of SPR-positive cells in other systems. It has been hypothesized that neurons of the pre-Bötzinger complex, a brain medullary region, are important for control of breathing rhythm [122]. A subset of these neurons were determined to be SPR-positive, and this prompted the Feldman group to study the effects of eliminating these neurons with SP-SAP [123]. The result was, as predicted, that normal regulation of respiration was likewise eliminated. Wang and co-workers used SSP-SAP, described below, to eliminate these neurons in a unilateral manner and demonstrated a 97% removal of the target neurons with no demonstrable loss of non-target neurons. These animals responded to breathing stimuli differently from normal animals, again demonstrating the importance of SPR-positive neurons in respiratory control.

It has always been difficult to produce a drug from a peptide (insulin being the dramatic counter-example), due to the brief half-life of peptide delivered systemically and/or into tissue[124]. The half-life of SP is no different; analogs have been created to decrease proteolytic degradation of peptides. We have used one of these, the $Sar^9$, $Met(O_2)^{11}$ analog of SP (SSP, for stable SP) in order to prolong the penetration through tissue and to, in a manner of speaking, cut a wider swath of elimination of SPR-positive neurons. Martin and Sloviter were unable to find significant removal of SPR neurons in the hippocampus with SP-SAP, but, with SSP-SAP achieved clear removal of target neurons, with excellent preservation of non-target neurons [125]. This allowed them to remove SPR-positive neurons in a halo around the injection site that creates a "focal epileptiform pathophysiology" due to the removal of these inhibitory SPR-positive interneurons. Upon crossing the boundary into the area to which SSP-SAP had not diffused and removed SPR-positive interneurons, normal electrophysiological responses were recorded. This was a dramatic and forceful demonstration of the ability of a peptide ligand-toxin to function after injection directly into tissue.

Truitt and Coolen used the same technique to remove SPR-positive neurons in lumbar segments of the male rat spinal cord, again resulting in specific depletion. The loss of these neurons resulted in animals that were able to mount and achieve penetration, but unable to ejaculate. These neurons were deemed to be necessary elements of an ejaculation generator [126].

The role of SPR-positive neurons has been shown to be important in several systems. The implication is that there are systems that can be defined by what is on the cell surface, e.g., the ejaculation generator is defined by not only its interesting behavior effects, but also by its biochemistry. The immunologists long ago discovered this and have spent a considerable amount of time characterizing systems by the cluster designation system, and this has been extremely useful.

The utility of SP as a targeting agent is almost despite the fact that there is more than one receptor that will bind SP; the SP receptor is considered to be the neurokinin (NK)-1 receptor. However, SP also binds, at a low but measurable level with the homologous NK-2 and NK-3 receptors [127]. This promiscuity is not unusual and must be carefully considered when using cytotoxins that are targeted with peptide-ligands. For instance, in the works cited here concerning SP as a targeting agent, in all of these cases, only the NK-1 receptor, the SP receptor, was expressed in the regions accessible by the reagent. The situation with somatostatin and its receptors is somewhat different. Of the five somatostatin receptors, four have very good affinity for the ligand, and there is co-expression of these receptors that would cause some uncertainty as to which receptor was actually receiving the ligand. Our current hypothesis is that this receptor ligand system would be best served by individual antibodies to each receptor as a targeting agent. The advantage of antibodies is that they can be uniquely specific for one receptor sub-type. Unfortunately, antibodies to extracellular domains of G protein-coupled receptors are not always available.

## Dermorphin-SAP

The mu opioid receptor (MOR) clearly has important physiological and behavioral roles, and for investigation of these, dermorphin-SAP was created. Dermorphin is a frog skin peptide that has a higher affinity for MOR than the endogenous opioid peptides and its analgesic potency is much greater than morphine [128]. In addition, it has an excellent ability to differentiate between the diverse opioid receptors [129]. We demonstrated that in the striatum, MOR-positive neurons could be removed with minimal non-specific damage [7]. Frank Porreca then began a series of experiments that demonstrated dermorphin-SAP could be useful in research studies.

Porreca's group used dermorphin-SAP for the characterization of the role of the brain in chronic pain, especially supraspinal control of neuropathic pain. In Porreca *et al.* [130], it was shown that specific ablation of MOR-expressing cells of the rostroventromedial medulla (RVM) both prevents and reverses experimental neuropathic pain. It has previously been suggested that MOR-positive cells of the RVM are the ON cells that cause increased nocifensive responsiveness [131,132]. The data produced by Porreca *et al.* showed a dramatic normalization of abnormal paw withdrawal threshold with a von Frey filament after spinal nerve ligation. Animals treated with saporin or dermorphin alone showed the usual result: a strong hypersensitivity to probing by the filament.

An aspect of Porreca's paper that is common to new technologies or the use of new reagents is the necessity of demonstrating that the effect of the targeted toxin is the removal of the target neuronal population, in this case, the MOR-expressing neurons. In this case, there was significant concern because there were no obvious co-localized markers,

and immunohistochemical staining for the MOR in the RVM proved fruitless. This necessitated the use of *in situ* hybridization for the MOR to demonstrate the absence of the receptor coupled with a pharmacologic method, the use of a MOR-specific irreversible antagonist to block the toxicity of dermorphin-SAP. These studies point out the rigor demanded with a new reagent that is claimed to be specific. In a case in which staining for MOR is possible, Tokuno *et al.* [133] were able to show degeneration of MOR-positive neurons, with the retention of other neurons at the same site. In this case, these authors used dermorphin-SAP to destroy neurons projecting to widely-distributed multiple nuclei of the basal ganglia, and to demonstrate that degenerating axons and terminals could be observed in those areas.

Porreca and colleagues continued their work to demonstrate that the input from the RVM maintains, but does not initiate, neuropathic pain [134]. Again, they utilized the spinal nerve ligation model [117] and lesioning of MOR-expressing neurons of the RVM with dermorphin-SAP. Tactile and thermal hypersensitivity was established, but reversed to baseline in dermorphin-SAP-treated animals starting at day 4 after nerve ligation.

## Orexin-SAP

Another peptide ligand-toxin that has given interesting data is the conjugate between orexin (also known as hypocretin) and saporin. Orexin, originally thought to be a peptide involved in appetite control (hence the name from the Greek word for appetite), burst on the scene as being involved in sleep after studies showed narcoleptic Dobermans have a mutation in one of the orexin receptors [135], and that orexin knockout mice have narcolepsy [136]. Peter Shiromani requested that Advanced Targeting Systems synthesize an orexin-2 conjugate with saporin and used it to deplete neurons of the hypothalamus of orexin receptor-expressing neurons. Rats treated with orexin-SAP proved to be narcoleptic. In a surprising result, neurons containing orexin were also removed, indicating that orexin-secreting neurons may be autocrine. Most of human narcolepsy involves the loss of orexin-positive neurons, rather than mutation [137], so the treatment of rats with orexin-SAP offers an efficient and cost-effective model for the human condition. Like the human disease, which has considerable variation of symptoms, low doses of orexin-SAP results in animals with milder symptoms than high doses [138]. These researchers made clever use of the distribution of orexin receptor in the medial septum. Knowing that there is a specific cytotoxin for cholinergic neurons of the medial septum, 192-Saporin, and that orexin receptor is expressed on both parvalbumin-positive GABAergic and cholinergic neurons, they injected animals with one or the other of the toxin in order to determine the source of hippocampal theta activity. While 192-Saporin had no effect on theta activity, treatment with orexin-SAP eliminated it, indicating that GABAergic neurons are responsible for the generation of theta activity [139].

In normal sleep, there are four stages of slow wave sleep (as defined by EEG readings), followed by REM sleep. The pattern lasts for about 90 minutes and then repeats. Patients with narcolepsy are afflicted with abnormal sleepiness, sleep attacks and REM sleep that occurs outside of the normal rhythm of sleep. These so-called sleep-onset REM periods (SOREMPS) occur rapidly after sleep onset or even, despite the nomenclature, directly from waking periods. Fig. (**9**) shows monitoring of sleep periods of a normal rat treated with saline compared to a rat treated with orexin-SAP that shows unusual SOREMP is seen in Fig. (**9b**). In this case, a rat falls into SOREMP with very little slow wave sleep, if any, occurring. This can happen at very inappropriate moments, for instance when the rat is eating [139]. Orexin-SAP offers a relatively rapid model for narcolepsy and a unique tool for determination of hypocretin/orexin receptor-expressing neurons.

## Neuronal Tracer-Toxins

Peptide-ligand toxins take advantage of the presence of their receptors for delivery to specific cell types. However, other methods can be used to deliver toxins to a specific cell type, such as neuronal tracers. These were first used in 1986

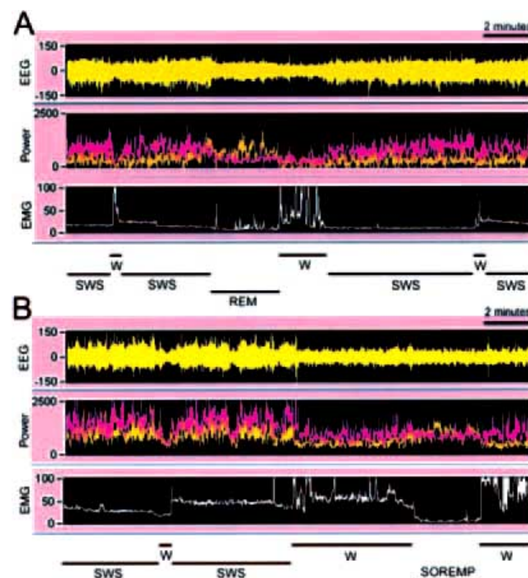


**Fig. (9). Alternation between wakefulness (W), SWS, and REM sleep in rats administered saline (A) or orexin-SAP (B) into the lateral hypothalamus.** The figure represents a 20-minute segment of a sleep-wake recording during the night (9:00 P.M.). (A) and (B) consist of a recording of the EEG, power of the EEG in the δ (0.3-4 Hz; pink) and θ (4-12 Hz; yellow) bands, and integrated activity of the nuchal muscles (EMG). The sleep-wake state determination, based on the relationship of the EEG, power, and EMG activity, is indicated at the bottom of each panel. (A) depicts a normal transition from slow-wave sleep (SWS) to rapid-eye movement (REM) sleep to wakefulness. (B) depicts a sleep-onset REM sleep period (SOREMP) exhibited by an orexin-SAP-treated rat with a 90% loss of orexin-ir neurons. The SOREMP is identified by a loss of EMG tone (near zero), by increased θ activity, by a reduction in δ activity (pink band in B), and by an EEG amplitude that is similar to wakefulness. These criteria are used to identify REM sleep, including SOREMP, and they are not present during wakefulness or SWS. Note that the first brief bout of wakefulness in (B) cannot be construed as REM sleep, because there is no θ activity and the EMG tone is rising, denoting that the rat woke up, albeit briefly (used with permission. Journal of Neuroscience 2001, 21:7273-7283).

[140], when wheat germ agglutinin was attached to ricin A chain to eliminate vagal neurons through retrograde transport after injection into the cervical vagus nerve low in the neck. The authors stated that their experiments "provide a basis for subsequent development of a variety of useful agents, based on other carrier moieties." More recent examples are with IB4, the isolectin of *Griffonia simplicifolia* that recognizes a subset of neurons in the dorsal root ganglia, and cholera toxin B, which recognizes a ganglioside and can undergo retrograde transport. Llewellyn-Smith utilized a saporin conjugate with cholera toxin B (CTB) chain, the binding moiety of the toxin, to eliminate sympathetic preganglionic cholinergic neurons that project to the superior cervical ganglion by injection into the ganglion by taking advantage of retrograde transport of CTB to the cell body [106].

Jasmin *et al.* [141] used CTB-SAP to demyelinate the rat lumbar spinal cord, remove macroglia, and produce paraplegia in order to study remyelination. Schwann cells were able to migrate into the spinal cord and cause axonal remyelination and recovery from paraplegia. Schwann cell myelination was progressively replaced by oligodendrocyte myelin without lapse in motor function. Thus, endogenous peripheral Schwann cells reversed a drastic neurological deficit due to central demyelination.

IB4-SAP utilizes the α-D-galactosyl-binding isolectin as a targeting agent. IB4 differentiates a population of primary small sensory neurons that are dependent on glial-derived neurotrophic factor and are devoid of SP and calcitonin gene-related peptide, which are contained by another set of small sensory neurons that have trkA as the useful marker [107,142]. Vulchanova *et al.* injected IB4-SAP into the sciatic nerve. It was retrogradely transported to cell bodies in the dorsal root ganglion and eliminated the IB4-positive population, which resulted in the loss of IB4-positive nerve fibers in the footpads of treated animals. These animals had a temporary decrease in nociceptive thresholds; surprisingly, the effect disappeared after 15-20 days. The authors expressed the opinion that there is a plasticity in the pain-sensing systems that could overcome this neuronal loss [107].

Internalization of a ribosome-inactivating protein by cells targeted with peptide ligands or molecules that bind to the cell surface has been a rich source of experimental information, even though the number of agents is small compared to the possibilities. This field has room for great expansion in the future, particularly since the human genome sequencing projects will reveal new and interesting targets.

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