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# ANTI-NOCICEPTIVE EFFECTS OF SELECTIVELY DESTROYING SUBSTANCE P RECEPTOR-EXPRESSING DORSAL HORN NEURONS USING [Sar$^9$,Met(O$_2$)$^{11}$]-SUBSTANCE P-SAPORIN: BEHAVIORAL AND ANATOMICAL ANALYSES

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**Abstract—Lumbar intrathecal injections of substance P-saporin (SP-sap) destroy dorsal horn neurons that express the neurokinin-1 receptor (NK-1R) resulting in decreased responses to a range of noxious stimuli and decreased hyperalgesia and allodynia. Forebrain injections of SP-sap produce considerable nonspecific damage raising some concern about use of this toxin *in vivo*. The more stable and selective substance P congener, [Sar$^9$,Met(O$_2$)$^{11}$]substance P coupled to saporin (SSP-sap) produces much more selective forebrain lesions at significantly lower doses. The present study sought to determine the anatomic and nocifensive behavioral effects of lumbar intrathecal injections of the more precisely targeted SSP-sap. On the basis of loss of lamina I NK-1R staining, lumbar intrathecal SSP-sap was seven times more potent than SP-sap and produced no loss of NK-1R expressing neurons in deeper laminae (III–VI or X). Transient decreases in hotplate responding occurred at 44 °C and 47 °C but not 52 °C during the first 3 weeks after SSP-sap injection with return to baseline by 4 weeks. Operant escape responses were reduced at 0.3 °C, 44 °C and 47 °C for at least 4 months. In the formalin test, SSP-sap also was about seven times more potent than SP-sap in reducing phase two behavior in both female Long Evans and male Sprague–Dawley rats. Both SSP-sap and SP-sap reduced formalin-induced FOS expression in deep and superficial laminae of the L4 dorsal horn in parallel with the reduction in phase 2 behavior. In summary, SSP-sap is highly effective in destroying lamina I NK-1R expressing neurons, without loss of deep NK-1R neurons. The behavioral effects of SSP-sap are similar to SP-sap suggesting that the antinociceptive effects of both toxins are indeed due to selective loss of NK-1R neurons in lamina I. SSP-sap is an attractive agent for possible treatment of chronic pain.** 

**Key words: molecular neurosurgery, neurokinin-1 receptor, operant, hotplate, formalin.**

Previous studies suggest substance P receptor (neurokinin-1 receptor, NK-1R)-expressing neurons in the superficial dorsal horn of the spinal cord play a role in transmission of nociceptive information to the cerebrum and brainstem (Littlewood et al., 1995; Marshall et al., 1996; Todd et al., 1998, 2000, 2002) Destruction of NK-1R-expressing dorsal horn neurons by lumbar intrathecal injection of SP-sap (disulfide conjugate of substance P to saporin) has been reported to eliminate high threshold nociception-specific dorsal horn neurons and prevent development of electrophysiological windup, central sensitization and activation of diffuse noxious inhibitory controls (DNIC) in response to noxious thermal stimuli applied to the hindpaw (Khasabov et al., 2002; Suzuki et al., 2002). In addition, lumbar SP-sap decreases FOS expression (product of the immediate early response gene c-fos) in the dorsal horn and nucleus raphe magnus in response to formalin or noxious thermal stimulation of the hind paw (Suzuki et al., 2002). Reduction of operant escape responding by rats with lesions of NK-1R-expressing dorsal horn neurons using intrathecal injection of SP-sap indicates a role for these neurons in rostral transmission of nociceptive information to the cerebrum (Vierck et al., 2003). Loss of NK-1R-expressing dorsal horn neurons has been reported not to alter baseline innate reflex nocifensive responses (tail flick, hotplate, paw withdrawal) which depend on nociceptive circuits in the spinal cord and brainstem. However, within 3 to 5 days after toxin injection, SP-sap injection has been reported to decrease reflex responses of rats with hind paw injection of capsaicin, mustard oil, carrageenan or complete Freud's adjuvant or with spinal nerve ligation (Mantyh et al., 1997; Nichols et al., 1999; Vierck et al., 2003). The reflex responses were reported to be reduced for at least 90 days after toxin injection (Nichols et al., 1999), and operant escape from nociceptive thermal stimulation has been shown to be reduced by intrathecal SP-sap for at least 6 months in rats (Vierck et al., 2003).

The above experiments have documented robust long lasting changes in sensitized reflex and operant responses to nociceptive stimulation but the unsensitized reflex responses have not been examined in detail. In other situations using highly selective targeted toxins, time course experiments have been useful in revealing primary (early, direct) and secondary (delayed, indirect) effects of destroying a specific type of neuron in the CNS (Lappi and Wiley, 2004). In some cases, the delayed secondary effects, presumably due to trans-synaptic plastic changes, have been quite robust and possibly functionally significant (de la Cruz et al., 1994a,b, 1996). Such changes may be

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*Abbreviations:* NK-1R, neurokinin-1 receptor, the principal receptor for substance P; SP-sap, disulfide conjugate of substance P to saporin; SSP, [Sar$^9$,Met (O$_2$)$^{11}$]-substance P; SSP-sap, disulfide conjugate of [Sar$^9$,Met (O$_2$)$^{11}$]-substance P to saporin.

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particularly relevant to the apparent inconsistency between reports that baseline innate reflex nocifensive responses were not altered by lumbar intrathecal SP-sap (Mantyh et al., 1997) compared with the observation of clear reduction in baseline operant escape responses to noxious thermal stimuli (Vierck et al., 2003).

Current studies of nociception are based entirely on lumbar intrathecal injections of SP-sap at a variety of doses. In some cases, the anatomical extent of the SP-sap lesion has been reported to vary over a number of months (Nichols et al., 1999) raising questions about possible effects in addition to selective destruction of lamina I NK-1R-expressing neurons. Since only limited data are currently available on the selectivity of the lesions made by intrathecal SP-sap, there is reason for some concern about selectivity of lumbar intrathecal SP-sap because of reports that SP-sap injection into other CNS sites produces significant non-specific damage in the striatum (Wiley and Lappi, 1997) and hippocampus (Martin and Sloviter, 2001). In an effort to achieve more selective lesions with intraparenchymal injections, we conjugated saporin to [Sar$^{9}$,Met$(O_2)^{11}$]-substance P (SSP). SSP is more slowly catabolized (Sakurada et al., 1994) while retaining somewhat greater selectivity for NK-1R (Regoli et al., 1988) which results in significantly greater *in vivo* biological activity (Tousignant et al., 1990). Injections of disulfide conjugate of [Sar$^{9}$,Met$(O_2)^{11}$]-substance P to saporin (SSP-sap) into the striatum (Wiley and Lappi, 1999) and hippocampus (Martin and Sloviter, 2001) produce much less non-specific damage and more robust selective lesions of NK-1R-expressing neurons at significantly lower doses than SP-sap.

The present study sought to determine in rats with lumbar intrathecal injections of SSP-sap: 1, the relationship between lumbar intrathecal doses of SSP-sap and extent of loss of dorsal horn neurons expressing NK-1R in both superficial and deep dorsal horn; 2, the early time course of any changes in innate reflex responses on the hotplate in search of evidence for any secondary (plastic) effects of the lesion; 3, the comparison of effects of the more selective SSP-sap on innate reflex nocifensive responses and operant escape response using similar thermal stimuli for comparison to previously reported effects of SP-sap in the same tests; and 4, comparison of the effects of SP-sap and SSP-sap on nocifensive responses to persistent noxious chemical stimulation in the hind paw formalin test, including effects on formalin-induced FOS expression in the superficial vs. the deep laminae of the dorsal horn. The specific thermal and chemical algesia tests in the present study were chosen to permit comparison between the SP-sap and SSP-sap across a range of conditions to see if the lesion produced by the more potent and selective SSP-sap results in similar, or different, alterations in responses to noxious thermal or chemical stimuli.

## EXPERIMENTAL PROCEDURES

### Subjects

Adult female Long Evans hooded (250–350 g) and male Sprague–Dawley albino (300–400 g) rats (Harlan Industries, Inc., Indianapolis, IN, USA) were housed two to a shoebox cage in HEPA-filtered controlled temperature racks with 12-h light/dark cycles and free access to food and water. All procedures were approved by the Vanderbilt University IACUC and conformed to the NIH guide for care and use of laboratory animals. Standard power analysis techniques (minimum effect size = 25–35%, $\alpha$ = 0.05, $\beta$ = 0.8) were used to ensure that group sizes were the minimum necessary. All procedures also were designed and conducted to minimize animal discomfort consistent with obtaining necessary data. Two groups of Long Evans female rats were used: 1, 33 rats total which included 8 control rats that received intrathecal injections of saline vehicle, 13 rats received various doses of intrathecal SP-sap and 12 rats that received various doses of SSP-sap, and 2, 11 rats (6 vehicle and 5 SSP-sap) initially were used for the hotplate time course followed by operant escape testing. The NK-1R staining of the control and SP-sap rats (group 1) was previously reported (Vierck et al., 2003). All of the rats in both Long Evans female groups were used for pre-terminal formalin testing and subsequent FOS and NK-1R staining comparisons. In addition, 12 male Sprague–Dawley rats (6 vehicle controls, 6 SSP-sap) were used for formalin testing and subsequent FOS and NK-1R staining. Data for the male Sprague–Dawley rats are always reported separately and not combined with the Long Evans female data.

### Lesioning NK-1R-expressing dorsal horn neurons

Substance P-saporin and SSP-sap were supplied by Advanced Targeting Systems (San Diego, CA, USA). The toxin conjugates were dissolved in sterile, preservative-free normal saline containing 1% bovine serum albumin and 0.1% Fast Green dye to permit visualization of injection volumes. Concentrated stock solutions were stored at −40 °C. Working dilutions were made fresh and stored on water ice until used the same day and then discarded. Rats were anesthetized by i.p. injections of a ketamine–xylazine–acepromazine mixture supplemented with additional ketamine as needed to maintain insensibility. Stretched PE10 catheters were inserted into the spinal subarachnoid space through a small incision in the atlanto-occipital membrane as per the technique of Yaksh and Rudy (LoPachin et al., 1981). The catheters were inserted to a depth of 8–8.5 cm from the dural incision. Toxin or saline vehicle was injected in a volume of 10 $\mu$l followed by a 10 $\mu$l flush of vehicle and the catheters removed 15 min later. The wounds were closed with Michel clips and rats warmed until awake then returned to home cages. Animals were carefully evaluated for presence of hindlimb motor deficits daily beginning the day after surgery. Any rat with a deficit that persisted more than 2–3 days after intrathecal injection or developed at any later time was removed from the study and killed. Termination for motor deficits was a very unusual occurrence and always associated with the immediate postoperative period. Delayed onset motor deficits typical of toxin effects were not seen in any of the SSP-sap treated rats.

### Anatomy procedures

*Tissue preparation.* Two weeks (male Sprague–Dawley rats only) or 4 months (Long Evans female rats) after toxin or vehicle injections, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with 200–300 ml of cold normal saline containing 5 mM sodium phosphate, pH 7.5, 1 g/l sodium nitrite (vasodilator) and 1000 units/l sodium heparin (anticoagulant) followed by 4% formaldehyde prepared from paraformaldehyde in 100 mM sodium phosphate, ph 7.5. Spinal cords were postfixed for at least 1 h and stored in fixative at 4 °C. The day prior to sectioning, lumbar enlargement spinal cord blocks were equilibrated overnight in 30% sucrose in 5 mM sodium phosphate, pH 7.5. Transverse sections of the entire lumbosacral spinal cord were cut at 40 $\mu$m thickness on a freezing sliding