model, after subtraction of the vasomotion signals (*12*). We also analyzed several data sets from different species at the full wavelength range using a more rigorous algorithm similar to that proposed by Mayhew. We found that the initial dip persisted or disappeared depending on the parameters used in the model, and the residuals of the curve fitting could not be used as reliable criteria for the validity of the model parameters [U. Lindauer *et al.*, *Neurosci. Abstr.* **25**, 1639 (1999)].

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18. All surgical and experimental procedures were in accordance with NIH guidelines. Details are described elsewhere (*7, 8*). Cats were anesthetized during the recording phase with continuous infusion of sodium pentothal. A craniotomy overlying area 18 of the visual cortex was performed and the dura removed. Stimulation protocol: (i) blank screen (prestimulus time, 1.5 to 5 s); (ii) moving gratings for 4 s (high contrast, 0.2 cycles per degree, duty cycle = 0.3, 6 cycles per second), and (iii) a blank screen (poststimulus time, at least 30 s). Up to 96 such trials were averaged.
19. E. H. Ratzlaff and A. Grinvald, *J. Neurosci. Methods* **36**, 127 (1991). Instead of a tungsten halogen lamp, we used a flash lamp (flash lamp model LABPAC 1200 power supply, with trigger unit LITE-PAC FYD-1150 and flashtube FX-1150, providing a flash of 3 μs exponential decay time; EG&G Electro Optics, Salem, MA).
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21. The other factors affecting the phosphorescence decay time are temperature, salinity, and pH. By their nature, salinity and temperature changes are much slower than the phenomenon under examination. The upper limit for a pH change should be smaller than the arterovenous pH difference of $\Delta pH = 0.03$. Using pH calibration data (*17*), the effect of ΔpH ($pH = 7.4$ was taken as the resting pH) on the decay time is negligible, about 7% of the difference we observed during the deoxygenation phase. The initial dip detected with phosphorescence decay time measurements cannot be attributed to blood volume rearrangements among microvascular compartments, because the latter's onset is delayed by ~500 ms (Fig. 2B, inset). In addition, in those high-resolution volume measurements (4 μm), the volume increase observed in venules and veins was neither faster nor larger than in the other compartments. Previous (*7, 19*) and recent (I. Vanzetta, R. Hildesheim, A. Grinvald, unpublished data) fluorescent tracer imaging measurements confirmed these results. The visualization of a stimulus-dependent increase in red blood cell velocity in individual capillaries [D. Kleinfeld, P. P. Mitra, F. Helmchen, W. Denk, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 15741 (1998)], also showed that the flow increase starts late (> ~0.5 s).
22. A weighted nonlinear least squares analysis was used to obtain the decay parameters (*30*). At least three exponential components are expected for the three vascular compartments. However, the true decay function is even more complex; in each compartment a wide distribution was found (*16*). However, a single exponential-fit approximation is sufficient to show the initial decrease in oxygen tension claimed here. The graphs in Fig. 2 are based on such fit. To rule out the possibility that the observed increase in activity-dependent decay time is not an artifact of the single component fit to a complex decay function, we also performed multicomponent analyses. For a two-components fit we obtained $\tau_1 = 65.9 \pm 0.3$ μs, $\tau_2 = 274 \pm 1$ μs, with amplitudes of $A_1 = 67.1 \pm 0.5\%$ and $A_2 = 32.9 \pm 0.5\%$. The initial dip was clearly present and statistically significant (95% confidence) in the time course of the shortest decay time. For three-components analysis, the initial dip was observed in the second component. However, as expected, the same goodness of fit was provided by very different decay parameters. For example set 1: $\tau_1 = 24$ μs, $\tau_2 = 70$ μs, $\tau_3 = 281$ μs, $A_1 = 5\%$, $A_2 = 63\%$, $A_3 = 32\%$; set 2: $\tau_1 = 59$ μs, $\tau_2 = 182$ μs, $\tau_3 = 620$ μs, $A_1 = 57\%$, $A_2 = 36\%$, $A_3 = 7\%$. These large differences underscore the inherent ambiguities of multiexponential analysis [(*30*) and references therein]. Adding constraints to some of the parameters that can be obtained from independent measurements should allow more quantitative analysis.
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28. It has been argued on theoretical (*24*) as well as experimental grounds (comparing the amplitude of the dip at 1.5 and 4 T) that the amplitude of the initial dip seen with fMRI depends on the second power of the field strength (K. Ugurbil, personal communication).
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Transmission of Chronic Nociception by Spinal Neurons Expressing the Substance P Receptor

Michael L. Nichols,[1,2] Brian J. Allen,[1,2] Scott D. Rogers,[1,2] Joseph R. Ghilardi,[1,2] Prisca Honore,[1,2] Nancy M. Luger,[1,2] Matthew P. Finke,[1,2] Jun Li,[1] Douglas A. Lappi,[3] Donald A. Simone,[1] Patrick W. Mantyh[1,2]*

Substance P receptor (SPR)–expressing spinal neurons were ablated with the selective cytotoxin substance P–saporin. Loss of these neurons resulted in a reduction of thermal hyperalgesia and mechanical allodynia associated with persistent neuropathic and inflammatory pain states. This loss appeared to be permanent. Responses to mildly painful stimuli and morphine analgesia were unaffected by this treatment. These results identify a target for treating persistent pain and suggest that the small population of SPR-expressing neurons in the dorsal horn of the spinal cord plays a pivotal role in the generation and maintenance of chronic neuropathic and inflammatory pain.

Chronic pain conditions are caused by ongoing disease states or tissue damage that result in sensitization of primary afferent and spinal cord neurons. This sensitization results in an increased sensitivity to both noxious (hyperalgesia) and non-noxious (allodynia) stimuli that is frequently difficult to treat with current pharmacological or surgical approaches (*1*).

Spinothalamic (STT) and spinoparabrachial (SPB) neurons are involved in the ascending conduction of acute noxious stimuli. Sensitization of these neurons results in hyperalgesia (*2*). Although SPR-expressing neurons represent less than 5% of the total neurons in the dorsal horn of the spinal cord

[1]Departments of Preventive Sciences, Psychiatry, and Neuroscience and Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA. [2]Molecular Neurobiology Lab (151), Veterans Affairs Medical Center, Minneapolis, MN 55417, USA. [3]Advanced Targeting Systems, 11175-A Flintkote Avenue, San Diego, CA 92121, USA.

*To whom correspondence should be addressed. E-mail: manty001@maroon.tc.umn.edu

(*3*), the majority of STT and SPB neurons in lamina I of the dorsal horn of the spinal cord express SPR. The majority of lamina I SPR neurons are STT and SPB neurons (*4*). SPR activation appears to be involved in the excitation and sensitization of STT neurons as well as the development of hyperalgesia (*5*).

When a conjugate of substance P (SP) and the ribosome-inactivating protein saporin (SAP) was intrathecally (i.th.) infused into the spinal cord, the SP-SAP conjugate was specifically concentrated in SPR-expressing neurons. Thirty days after infusion, there was a loss of lamina I spinal cord neurons that express the SPR (*5*). This treatment attenuated thermal and mechanical hyperalgesia as well as nocifensive behavior produced by capsaicin injection (*5*).

To determine the concentration-response relation for SP-SAP in ablating SPR-expressing spinal neurons and in blocking capsaicin-induced pain behaviors, we infused 10 μl of 10^{-7} M, 10^{-6} M, or 5×10^{-6} M SP-SAP 30 days before plantar administration of 10 μg of capsaicin (*5*). In normal animals, capsaicin results in a profound nocifensive behavior, thermal hyperalgesia, and mechanical allodynia (*5*). Thirty days after infusion of SP-SAP, there was a distinct concentration-related reduction in these behaviors. The reductions in these pain-related behaviors were all significantly correlated with the concentration-related loss of SPR-expressing lamina I neurons (all $r > 0.95$; Fig. 1A). On the basis of these results and the lack of any observable side effects at the concentration of 10^{-6} M, i.th. infusion of 10 μl of 10^{-6} M SP-SAP was used in the remaining experiments.

The effect of SP-SAP on three models of inflammatory pain was tested 30 days after i.th. administration of 10 μl of 10^{-6} M SP-SAP (*5*). Subcutaneous (s.c.) injection of formalin into the hind paw produces a distinct biphasic, nocifensive behavior consisting of an early phase followed by a prolonged second phase lasting nearly 1 hour (*6*). SP-SAP did not significantly affect the first phase (0 to 10 min); however, in the second phase (20 to 50 min), the paw-flinching behavior was significantly reduced in SP-SAP–treated animals (Fig. 1B). Injection of λ-carrageenan or complete Freund's adjuvant (CFA) into the hind paw produced local inflammation, thermal hyperalgesia, and mechanical allodynia (*5*), peaking at about 3 hours and 3 days after injection, respectively (*7*). SP-SAP significantly reduced the thermal hyperalgesia and the mechanical allodynia produced by carrageenan (Fig. 1C) or CFA (Fig. 1D).

The effects of SP-SAP were also assessed in a model of neuropathic pain (*8*). Tight ligation of the L5 and L6 spinal nerves resulted in long-lasting mechanical allodynia. Thirty-day pretreatment with SP-SAP reduced the mechanical allodynia that is present 7 days after nerve ligation surgery (Fig. 1E). When SP-SAP was administered 7 days after nerve ligation, there was also significant reduction in the mechanical allodynia (Fig. 1F). These findings demonstrate that SP-SAP treatment will inhibit the allodynia associated with nerve injury when administered before or after the development of the neuropathic pain.

To assess long-term effects, we examined measurements of capsaicin-induced pain behaviors and several neuronal and glial cell markers in both spinal cord and dorsal root ganglia at 30, 100, and 200 days after infusion of 10 μl of 10^{-6} M SP-SAP (Fig. 2 and Table 1) (*5*). In all cases, saline or 10^{-6} M

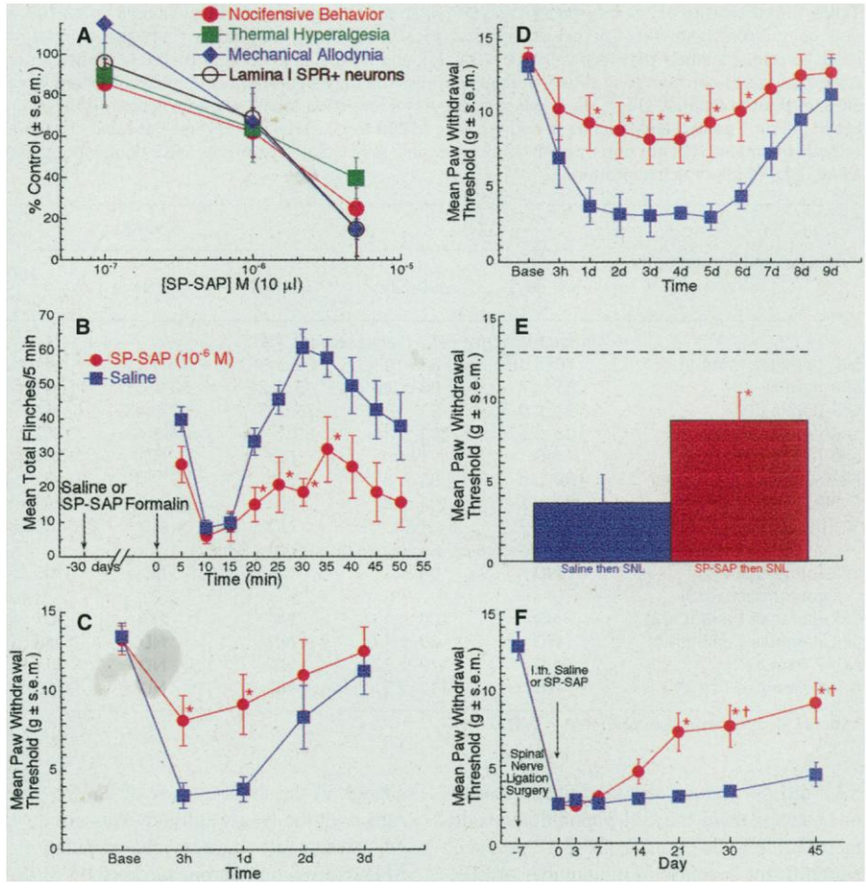

Fig. 1. (**A**) SP-SAP produces a concentration-related reduction in capsaicin-induced pain behaviors and ablation of SPR-expressing lamina I neurons. Concentration-response relations of SP-SAP (10 μl, i.th.) on capsaicin-induced nocifensive behavior (red), thermal hyperalgesia (green), mechanical allodynia (blue), and loss of lamina I SPR-positive neurons (open circles) 30 days after SP-SAP treatment. $n = 5$ per group. (**B** to **D**) SP-SAP attenuates pain behaviors in three models of inflammatory pain. In all cases, SP-SAP (10^{-6} M, 10 μl) was administered 30 days before injection of the inflammatory agent. (**B**) The behavioral effects of administration of formalin in the rat hind paw. Formalin results in about 90 min of robust paw flinching (*6*) in saline-treated animals (blue, $n = 7$). There is a significant reduction in both the number of flinches and the overall length of the flinching behavior in the second phase (20 to 50 min) produced by formalin in SP-SAP–treated animals (red, $n = 9$). (**C**) SP-SAP (red, $n = 5$) attenuates the mechanical allodynia that is present after injection of carrageenan (*7*) when compared with saline-treated controls (blue, $n = 4$); h, hours; d, days. (**D**) SP-SAP (red, $n = 6$) pretreatment attenuates the mechanical allodynia present after injection of CFA (*7*) when compared with saline-treated controls (blue, $n = 4$). There is no significant allodynia in saline-treated animals by 3 days after carrageenan injection or at 7 days after CFA injection. (**E** and **F**) SP-SAP attenuates nerve injury (spinal nerve ligation model)–induced allodynia when administered either 30 days before or 7 days after nerve ligation (*8*). The dashed lines indicate the paw withdrawal threshold (g) in the normal animal. (**E**) Thirty-day pretreatment with SP-SAP (red, $n = 6$) reduces the tactile allodynia in nerve-injured animals when compared with saline-treated controls (blue, $n = 6$). (**F**) The antiallodynic 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 antiallodynic effect of SP-SAP becomes significant 21 days after infusion. Error bars represent standard error of the mean. Asterisks represent statistical significance from control ($P < 0.05$). Crosses represent statistical significance from baseline ($P < 0.05$).

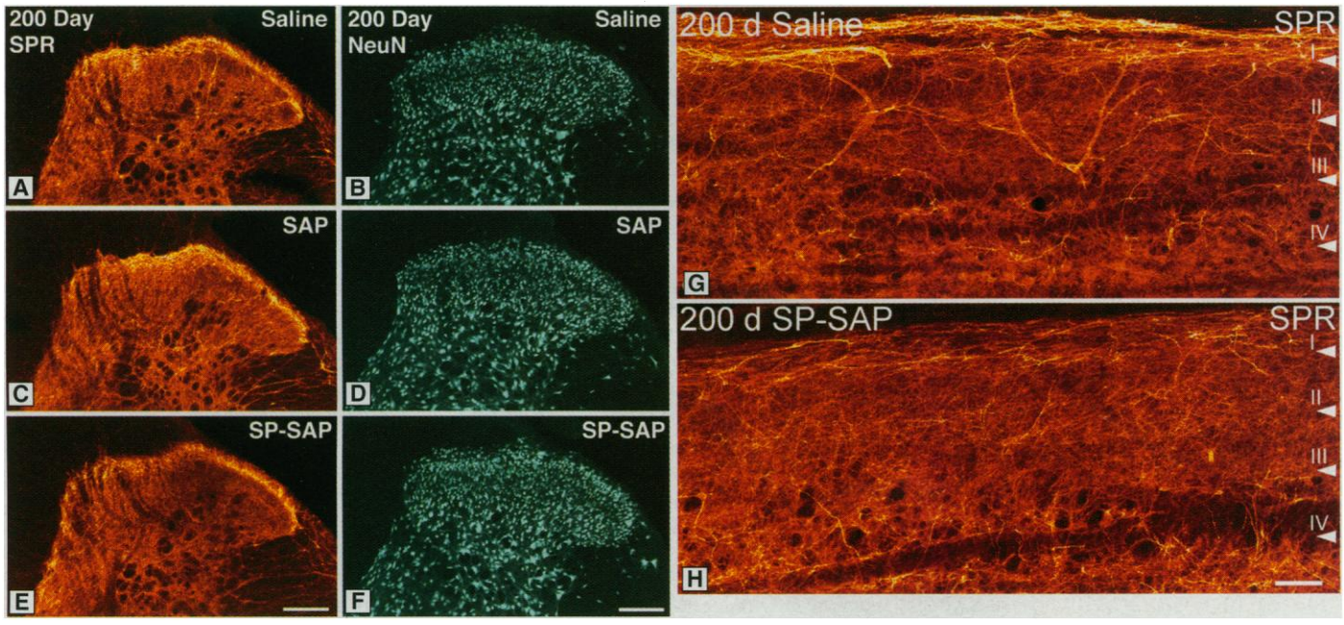

Fig. 2. Fluorescent confocal images of coronal (**A** to **F**) or sagittal (**G** and **H**) sections of the lumbar (L4) rat spinal cord 200 days after saline, SAP, or SP-SAP administration. SPR immunoreactivity (orange) is significantly reduced after SP-SAP treatment (**E**) but not after SAP (C) or saline (A) treatment. In contrast, NeuN labeling shows no significant reduction in the SP-SAP (F), SAP (D), or saline (B) groups. Scale bar (A to F), 200 μm. In the sagittal plane, laminae are indicated by roman numerals, and the loss of SPR-immunoreactive neurons after SP-SAP (H) treatment is apparent in both the superficial and deep laminae when compared with the saline-treated group (G). Scale bar (G and H), 80 μm.

Table 1. Cytotoxicity of i.th. infused SAP and SP-SAP in the L4 segment of the spinal cord at 30, 100, and 200 days after treatment. There were no statistically significant differences between animals that received saline, animals that received SAP (10^{-6} M), and normal animals, and therefore all percentages are expressed as percentage of saline-treated controls. The only significant differences observed were in SP-SAP–treated animals (10^{-6} M), in which there was a reduction in SPR-expressing neurons in laminae I to III (all time points) and laminae IV and V (100 and 200 days). Data are expressed as mean ± standard error of the mean. ND, not determined; CGRP, calcitonin gene-related pepitude; DRG, dorsal root ganglia; GFAP, glial fibrillary acidic protein.

Neuronal cell population	SAP		SP-SAP		
	100 days	200 days	30 days	100 days	200 days
	Immunoreactive cells (versus saline) (%)				
SPR (laminae I and II)	70 ± 18	81 ± 11	69 ± 5*	41 ± 12*	41 ± 3*
SPR (lamina III)	85 ± 7	98 ± 10	45 ± 2*	50 ± 5*	41 ± 3*
SPR (lamina IV)	91 ± 9	81 ± 6	76 ± 3	50 ± 4*	64 ± 2*
SPR (lamina V)	102 ± 5	101 ± 11	89 ± 4	64 ± 6*	63 ± 4*
SPR (lamina X)	ND	ND	ND	ND	104 ± 18
NeuN (lamina I neurons)	99 ± 2	99 ± 8	ND	95 ± 4	97 ± 8
CGRP (motor neurons)	99 ± 5	101 ± 6	ND	96 ± 7	98 ± 4
SP (DRG)	ND	ND	ND	ND	100 ± 4
	Immunofluorescence levels (versus saline) (%)				
SPR (preganglionic sympathetics, T10)	ND	98 ± 1	ND	ND	103 ± 1
SPR (laminae I and II, C2)	ND	100 ± 1	ND	ND	107 ± 2
SPR (laminae I and II, L4)	ND	89 ± 11	ND	ND	49 ± 5*
CGRP (lamina I)	ND	79 ± 22	ND	ND	117 ± 19
GFAP (laminae I to V)	ND	111 ± 5	ND	ND	110 ± 5

* Statistical significance from control ($P < 0.05$).

SAP did not induce any significant changes in behavior or in the cell populations examined (Table 1). In contrast, SP-SAP produced a significant decrease in the number of SPR-expressing cells in laminae I and III at 30 days and in laminae I, III, IV, and V at 100 and 200 days after infusion (*9*). A significant change in the total number of lamina I neurons with the NeuN antibody was not detected, presumably because the population of SPR-expressing neurons targeted by SP-SAP makes up such a small percentage of these neurons (*3*). SP-SAP treatment results in a long-term inhibition of capsaicin-induced hyperalgesia and allodynia with no evidence of loss of this effect over time (Fig. 3). The effects of SP-SAP did not affect morphine analgesia and appeared to be confined to the spinal segments where it was infused. Thus, after lumbar infusion of SP-SAP, the ability of morphine (15 mg/kg, s.c.) to block mechanical allodynia or thermal hyperalgesia after CFA injection into the hind paw was preserved, and formalin-induced pain behaviors in the forepaw (*6*) were not altered.

These results suggest that SPR-expressing neurons in the dorsal horn of the spinal cord are not the major site of action of morphine and that, after SP-SAP treatment, opiates remain a viable therapy for breakthrough pain. A major reason for the long-term efficacy and apparent lack of side effects after SP-SAP treatment appears to be related to the restricted nature and specificity of SP-SAP action. The actions of i.th. infused SP-SAP are confined to the SPR-expressing neurons in the dorsal horn, many of which are STT and SPB neurons (*3*, *4*).

Previous data have suggested that in the peripheral nervous system, neuropathic and inflammatory pain arise from different mechanisms and are conveyed to the spinal cord by distinct groups of primary afferent neurons. Here, SP-SAP treatment reduced the hyperalgesia and allodynia associated with both neuropathic and inflammatory persistent pain states. Whether it is the same SPR-expressing neurons or different subsets of SPR-expressing neurons that convey neuropathic and inflammatory pain is unknown, but recent evi-

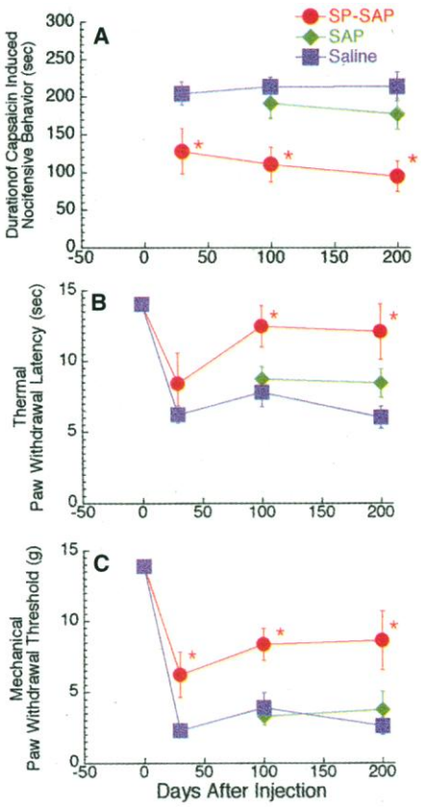

Fig. 3. Time course of antihyperalgesic effect of i.th. infusion of saline (n = 5, 10, and 6 at 30, 100, and 200 days, respectively), 10^{-6} M SAP (n = 6, 6, and 6), or 10^{-6} M SP-SAP (n = 5, 10, and 6). After SP-SAP treatment, there is a reduction in (**A**) capsaicin-induced nocifensive behavior (expressed as duration, in seconds, over 300-s observation), (**B**) thermal hyperalgesia (expressed as paw withdrawal latency, in seconds), and (**C**) mechanical allodynia (expressed as paw withdrawal threshold, in g) that does not diminish with time after injection. Infusion of saline or SAP alone did not produce any changes in these pain behaviors at any of the time points examined. Asterisks represent statistical significance from control ($P < 0.05$).

dence suggests that a uniting feature of many spinal and forebrain neurons that express the SPR is their involvement in the response to tissue injury and stress (*10*). Characterization of the gene and protein changes that SPR-expressing spinal neurons undergo during nociception should provide insight into the spinal mechanisms involved in the generation and maintenance of chronic neuropathic and inflammatory pain.

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6. Rats received an s.c. injection of formalin (2.5%, 50 μl) into the dorsal surface of the hind paw or the forepaw. The number of paw flinches was quantified every 5 min for 50 min after injection [D. Dubuisson and S. G. Dennis, *Pain* **4**, 161 (1977)]. Rats receiving formalin in the forepaw were also observed for time spent licking or guarding the forepaw [B. Calvino, *Physiol. Behav.* **47**, 907 (1990); C. A. Porro, G. Tassinari, F. Facchinetti, A. E. Panerai, G. Carli, *Exp. Brain Res.* **83**, 549 (1991)].
7. Rats received λ-carrageenan (0.2 mg, 0.1 ml) or CFA (50%, 0.1 ml) s.c. into the plantar hind paw 30 days after infusion of SP-SAP (10 μl, 10^{-6} M) and were tested at 3 hours and each day for 3 days (λ-carrageenan) or 9 days (CFA) in the thermal and mechanical assays [L. Kocher, F. Anton, P. W. Reeh, H. O. Handwerker, *Pain* **29**, 363 (1987); M. J. Millan *et al.*, *Pain* **35**, 299 (1988)].
8. Rats were prepared according to the method of S. H. Kim and J. M. Chung [*Pain* **50**, 355 (1992)]. Rats were anesthetized with halothane, and the L5 and L6 spinal nerves were tightly ligated. Animals were allowed to recover for 1 week.
9. The loss of lamina III neurons at 30 days observed in the present study is a result of binning the number of SPR-positive neurons in each individual lamina. Previously, neurons from laminae III to V were analyzed as one group (*5*). If SPR-expressing neurons in laminae III to V are combined and counted as one group, there is no significant loss of laminae III to V neurons at 30 days after infusion of SP-SAP. The delayed loss of SPR-expressing neurons in laminae IV and V compared with neurons in laminae I to III may be due to the rapid diffusion and internalization of SP-SAP in laminae I and III neurons compared with the deeper laminae IV and V neurons. In contrast, the loss of SPR-expressing neurons in laminae I, III, IV, and V all appear to have reached maximal levels at 100 days.
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Specific Lipopolysaccharide Found in Cystic Fibrosis Airway *Pseudomonas aeruginosa*

Robert K. Ernst,[1] Eugene C. Yi,[2] Lin Guo,[1]* Kheng B. Lim,[2]† Jane L. Burns,[3] Murray Hackett,[2] Samuel I. Miller[1]‡

Cystic fibrosis (CF) patients develop chronic airway infections with *Pseudomonas aeruginosa* (PA). *Pseudomonas aeruginosa* synthesized lipopolysaccharide (LPS) with a variety of penta- and hexa-acylated lipid A structures under different environmental conditions. CF patient PA synthesized LPS with specific lipid A structures indicating unique recognition of the CF airway environment. CF-specific lipid A forms containing palmitate and aminoarabinose were associated with resistance to cationic antimicrobial peptides and increased inflammatory responses, indicating that they are likely to be involved in airway disease.

Cystic fibrosis (CF) is the most common inherited disorder of Caucasians (*1*). The respiratory tracts of most patients with CF become infected with the opportunistic gram-negative bacteria *Pseudomonas aeruginosa* (PA) shortly after birth (*2*). Chronic infection results in airwayinflammation, which is the major cause of morbidity and mortality in CF. Despite improved survival when treated with antibiotic therapy, CF patients eventually die of progressive PA pulmonary infection characterized by massive neutrophilic infiltration without bacterial destruction.

Recently, it has been demonstrated that enteric bacteria synthesize different forms of lipid A in response to environmental conditions that include magnesium-limited growth and conditions encountered during mammalian infection (*3*). Salmonellae with these modifications have increased resistance to cationic antimicrobial peptides (CAMPs) and decreased lipopolysaccharide (LPS)–mediated recognition by human cells. Because the PA-CF lung interaction is a remarkable ex-

References and Notes

[5] **Inhibition of Hyperalgesia by Ablation of Lamina I Spinal Neurons Expressing the Substance P Receptor**

Patrick W. Mantyh; Scott D. Rogers; Prisca Honore; Brian J. Allen; Joseph R. Ghilardi; Jun Li; Randy S. Daughters; Douglas A. Lappi; Ronald G. Wiley; Donald A. Simone

Science, New Series, Vol. 278, No. 5336. (Oct. 10, 1997), pp. 275-279.

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