NK-1 and NK-3 Type Tachykinin Receptor mRNA Expression in the Rat Spinal Cord Dorsal Horn Is Increased during Adjuvant or Formalin-induced Nociception

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Substance P (SP) and other related tachykinins such as neurokinin B (NKB) have been studied widely as mediators of sensory information. The release of SP into the dorsal horn of the spinal cord is increased during nociception, and SP activates nociception-specific dorsal horn neurons. The tachykinin NKB has antinociceptive effects in the spinal cord and is contained in intrinsic spinal neurons; thus, NKB may also contribute to the processing of sensory information. Both neurokinin-1 (NK-1) and neurokinin-3 (NK-3) receptors have been localized in the superficial laminae of the dorsal horn. This study investigated changes in NK-1 and NK-3 receptor mRNA expression during nociception. Following injection of either formalin or complete Freund's adjuvant (CFA) into one hindpaw, the levels of expression of NK-1 and NK-3 mRNAs in the spinal cord dorsal horn and preprotachykinin (PPT) mRNA expression in the lumbar dorsal root ganglia (DRG) were quantitated using solution hybridization–nuclease protection assays. Peptide and receptor mRNA expression levels were normalized to β -actin mRNA levels, which did not change during the treatments. Formalin (2 or 6 hr) or CFA (4 d) injection produced approximately a twofold increase in SP-encoding PPT mRNA expression in the ipsilateral lumbar DRG. Increased activity in primary afferent neurons containing SP may stimulate the production of SP precursors, providing substrate for increased SP production, release, and turnover in the dorsal horn and periphery. Formalin or CFA increased NK-1 receptor mRNA expression by about twofold in the ipsilateral dorsal horn. Activated SP-containing primary afferent neurons may provide the neuronal drive for this increase. Formalin or CFA also increased NK-3 receptor mRNA expression by about twofold in the dorsal horn. Nociception may trigger increased activity of intrinsic NKB antinociceptive systems that could act to limit or modify transmission of nociceptive information to higher brain centers. These results provide evidence for activity-induced plasticity of tachykinin receptor expression in nociceptive sensory systems during subchronic nociceptive activation.

[Key words: substance P, neurokinin B, NK-1 receptor, NK-3 receptor, nociception, formalin test, adjuvant]

Received April 13, 1993; revised July 16, 1993; accepted July 22, 1993.

This work was supported by National Institutes of Health Grant NS21937. K.E.M. was supported by NRSA 54782 and NRSA 5-T32-NS0707.

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The undecapeptide substance P (SP) and other tachykinin peptides such as neurokinin B (NKB) have been widely studied as mediators of sensory information in the nervous system. The tachykinins are a family of peptide neurotransmitters and/or neuromodulators that share a highly conserved carboxy-terminal amino acid sequence. The actions of SP in the spinal cord are widely accepted as being involved in nociception (Pernow, 1983; Otsuka and Yanagisawa, 1987). The release of SP into the spinal cord can be evoked by noxious stimulation of the periphery (Yaksh et al., 1980; Kuraishi et al., 1983; Oku et al., 1987; McCarson and Goldstein, 1991), and intrathecal injection of SP produces various pain-related behaviors (Doi and Jurna, 1981; Piercey et al., 1981; Hylden and Wilcox, 1983a,b; Matsuura et al., 1985). Afferent terminals containing SP have been shown to appose primarily nociception-specific dorsal horn neurons (DeKoninck et al., 1992), and SP selectively activates dorsal horn cells that normally receive nociceptive afferent input (Henry, 1976).

Three different tachykinin receptors have been cloned and characterized. Each exhibits binding selectivity for one of the various tachykinins, and all show a similarity to other G-protein-coupled receptors (Nakanishi, 1991; Krause et al., 1993). In the spinal cord, two of these receptor sites have been implicated in sensory transmission. The neurokinin-1 receptor (NK-1 or SP receptor; Yokota et al., 1989; Hershey and Krause, 1990) has the highest affinity for SP of the tachykinin receptors. In the spinal cord, NK-1 sites are found primarily in the superficial dorsal horn (laminae I and II) in the region of termination of SP-containing primary afferent neurons (Yashpal et al., 1990; Moussaoui et al., 1992). The mechanism of action of SP at the NK-1 receptor involves phospholipase C-mediated phosphoinositol hydrolysis (Hanley et al., 1980; Hunter et al., 1985), which subsequently mobilizes internal Ca^{2+} stores (Streb et al., 1983) and activates protein kinase C (Kishimoto et al., 1980). The rate of phospholipid hydrolysis has been shown to be directly proportional to the number of SP binding sites in the rat CNS (Mantyh et al., 1984). The activation of nociception-specific dorsal horn neurons (Salter and Henry, 1991) and certain aspects of thermal and chemical nociception (Yamamoto and Yaksh, 1991; Yashpal et al., 1993) can be blocked by the non-peptide NK-1 receptor antagonist CP-96,345. Thus, the NK-1 receptor appears to have an important role in the processing of nociceptive information in the dorsal horn, and regulation of NK-1 receptor expression may play an important role in the regulation of pain transmission. Less is known about the actions of NKB in the spinal cord. The majority of NKB in the dorsal horn is found in intrinsic spinal neurons (Ogawa et al., 1985),

and NKB has been reported to have antinociceptive effects in the spinal cord (Laneville et al., 1988). The neurokinin-3 (NK-3) receptor (NKB receptor or neuromedin K receptor; Shigemoto et al., 1990) is found in the superficial dorsal horn (laminae I and II) of the spinal cord (Yashpal et al., 1990), and NKB has the highest affinity of the tachykinins for the NK-3 receptor (Helke et al., 1990). Thus, the primary role of NKB in the dorsal horn of the spinal cord could be related to the processing of afferent nociceptive information.

Two widely used animal models of subchronic nociception consist of the injection of formalin or complete Freund's adjuvant (CFA) into the plantar aspect of one hindpaw of a rat. The formalin test (Dubuisson and Dennis, 1977) produces a biphasic pain-related behavioral response within the first hour after injection into the hindpaw and an inflammation of the paw that is long lasting (Tjolsen et al., 1992). Formalin injection into the hindpaw has been shown to increase SP immunoreactivity in the dorsal horn (Kantner et al., 1985; McCarron and Goldstein, 1989, 1990) and increase the expression of SP-encoding preprotachykinin (PPT) mRNAs in the lumbar dorsal root ganglia (DRG) (Noguchi et al., 1988). Injection of CFA into one hindpaw produces a longer-lasting monoarthritic inflammation that peaks after about 4 d (Iadarola et al., 1988a,b). The use of CFA as a nociceptive activator has been shown to potentiate SP release into the dorsal horn of the spinal cord (Oku et al., 1987). CFA-induced nociception also increases the levels of SP-encoding PPT mRNAs in the lumbar DRG and spinal cord dorsal horn (Minami et al., 1989; Donaldson et al., 1992).

Although much is known about the regulation of expression of the tachykinin peptides and their role in nociception, little is known about how nociceptive activation may alter tachykinin receptor expression or sensitivity, or the role that these receptors play in nociceptive processing. Further, the altered expression and/or release of tachykinin peptides may directly or indirectly affect tachykinin receptor function and expression during nociception. Therefore, this study was undertaken to determine the levels of NK-1 receptor (NK-1R) and NK-3 receptor (NK-3R) mRNA expression in spinal cord regions and SP precursor (PPT) mRNA expression in the lumbar DRG following peripheral nociceptive activations.

Materials and Methods

Animals and nociceptive treatments. Twenty male Sprague-Dawley rats (250–300 gm; Harlan Sprague-Dawley Inc., Indianapolis, IN) were used for this study. Ten rats received a subcutaneous injection of 100 μ l of 5% formalin (Sigma Chemical Co., St. Louis, MO) into the plantar aspect of the right hindpaw. Tissues were removed from five subjects 2 hr after formalin injection; tissues were removed from the remaining five subjects 6 hr after formalin injection. Five additional subjects received a subcutaneous injection of Freund's complete adjuvant (50 μ g *Mycobacterium butyricum* in an oil-saline emulsion, 100 μ l; Sigma) in the plantar aspect of the right hindpaw. Tissues were removed from these monoarthritic animals 4 d after CFA injection. In all cases, animals were visually monitored after injection to confirm inflammation of the hindpaw and for appearance of the appropriate pain-related behaviors. Formalin-injected animals were observed during the 30 min immediately after treatment to confirm the appearance of both early and late phase bouts of pain-related behaviors (e.g., biting, licking, and lifting) directed toward the treated paw (Dubuisson and Dennis, 1977); CFA-injected animals were observed for appearance of pain-related behavior (guarding of the treated paw) within the first 60 min after treatment and periodically during the 4 d after treatment to confirm continued, profound inflammation of the injected paw (Iadarola et al., 1988b). Animals were housed individually in containers with excess bedding material with standard chow provided in the bedding to minimize discomfort

during the treatment period. All animal procedures related to nociceptive treatments followed NIH guidelines and were reviewed and approved by the Washington University Institutional Animal Care and Use Committee. Five uninjected animals served as naive controls, resulting in four experimental groups with five subjects in each group. At the time of tissue removal, subjects were anesthetized with sodium pentobarbital (Vet Labs Ltd., Lenexa, KS; approximately 40 mg/kg, i.p.). A laminectomy was then performed to expose the lumbar spinal cord and six lumbar DRGs (three DRGs, L4–L6, from each side). Tissues were quickly removed and frozen on dry ice. The spinal cord lumbar enlargements were dissected into longitudinal quarters along the sagittal midline and just above the central canal. The left and right side tissues were assayed separately, with the three DRGs from each side combined as one tissue sample. Animals were then killed with an overdose of sodium pentobarbital (approximately 150 mg/kg, i.p.).

Isolation of total RNA. Total cellular RNAs were extracted from spinal cord and DRG tissue samples using a rapid guanidinium isothiocyanate-phenol/chloroform extraction and subsequent precipitation with 0.3 M sodium acetate and 75% ethanol (a modification of the method described by Chomczynski and Sacchi, 1987; unless otherwise stated, all reagents were of the highest purity available). The resulting RNA samples were subjected to spectrophotometric analysis and 5 μ g aliquots were electrophoresed on 1.5% agarose gels containing 6% formaldehyde to ensure integrity of each RNA preparation (Krause et al., 1987). One dorsal quarter of lumbar spinal cord yielded approximately 45 μ g total RNA; three lumbar DRGs together yielded approximately 30 μ g total RNA. These quantities were sufficient for multiple assays to be performed on one tissue sample. It should be noted that this method extracts a much larger amount of tRNA and very small mRNAs than traditional cesium chloride ultracentrifugation methods. This may result in specific mRNA expression levels per microgram of total RNA that appear lower than those reported using other extraction methods (see Results).

Plasmid construction. Six different plasmid constructs were used for the analysis of RNA in this study. Four previously constructed plasmids were used. For the analysis of SP-encoding PPT mRNAs, the pG1- β PPT and pG2-31-1 (γ -PPT) plasmids were used (Krause et al., 1989). For the analysis of NK-1R mRNA, an NK-1R 3' coding region plasmid [pSPR(+1213 $\rightarrow$ +1800)] and a full coding region plasmid [pSPR(+577 $\rightarrow$ +1800)] were used (Hershey et al., 1991).

In order to quantitate NK-3R mRNA, a plasmid containing 345 bases of the 3' end of the coding region of the NK-3R cDNA including three bases of the 3' untranslated region (Shigemoto et al., 1990) was constructed. An NK-3R insert was generated using the polymerase chain reaction (PCR) with buffer conditions, taq polymerase, and a thermal cycler from Perkin-Elmer Cetus Corp. (Norwalk, CT). The oligonucleotides used corresponded to the 3' end of the seventh transmembrane domain (5' +1014 CAACCCCATCATCTACTGCT +1033 3') and to the opposite strand of the 3' end of the coding region (5' +1359 TTAGGAATATTCATCCA +1343 3'; Shigemoto et al., 1990). These oligonucleotides were synthesized by the Washington University Protein Chemistry Facility. PCR was carried out for 30 cycles, and 20% of the reaction product was analyzed by electrophoresis on a 1% agarose gel prior to its isolation and molecular cloning. The resulting PCR fragment was cloned into the HincII restriction site of pBLUESCRIPT (Stratagene, La Jolla, CA). The sequence of the receptor insert was confirmed by chain termination methods (Sanger et al., 1987) with T7 and T3 primers using the Sequenase nucleotide sequencing system as recommended by the manufacturer (U.S. Biochemical, Cleveland, OH). The resulting plasmid was called pBS-rNKBR (+1014 $\rightarrow$ +1359).

Another plasmid was constructed for β -actin mRNA analysis. A 210 base Sau 3A restriction fragment of the rat β -actin cDNA clone pR- β A1 (a gift of Dr. K. L. O'Malley, Washington University School of Medicine) was isolated and subcloned into the BamHI restriction site of pBLUESCRIPT. The identity of the insert as rat β -actin cDNA (Nudel et al., 1983) was confirmed by restriction digest mapping and by specific hybridization to a BglII restriction fragment of pR- β A1. This plasmid was called pBS-r β A₂₁₀.

cRNA transcription reactions and quantitation of cRNA standards. Antisense ³²P-labeled cRNA probes were synthesized using α -³²P-UTP (3000 Ci/mmol) from Amersham (Arlington Heights, IL). The plasmid pG1- β PPT was linearized with HindIII and a cRNA probe generated using T7 RNA polymerase (Promega Biotec, Madison, WI). This resulted in an antisense cRNA probe of 567 bases (508 bases coding sequence, 59 bases vector) with a specific activity of 4.5×10^8 dpm/ μ g. pSPR(+1213 $\rightarrow$ +1800) or pBS-r β A₂₁₀ was linearized with XbaI and

antisense probes generated using T7 RNA polymerase. For the NK-1R plasmid an antisense cRNA probe of 696 bases (588 bases coding sequence, 108 bases vector) was generated at a specific activity of 3.5×10^8 dpm/ μ g. For the β -actin plasmid the antisense cRNA probe was 293 bases (210 bases coding sequence, 83 bases vector) with a specific activity of 3.9×10^8 dpm/ μ g. The NK-3R plasmid pBS-rNKBR(+1014 $\rightarrow$ +1359) was linearized with XhoI and an antisense RNA probe generated using T3 polymerase (Boehringer-Mannheim, Indianapolis, IN). This resulted in an antisense cRNA probe of 447 bases (345 bases coding sequence, 102 bases vector) with a specific activity of 5.3×10^8 dpm/ μ g.

Unlabeled message-sense cRNAs were also generated for use as quantitation standards in nuclease protection assays. pG2-31-1 (γ -PPT) was linearized with EcoRI; pSPR(+577 $\rightarrow$ +1800) and pBS-rNKBR(+1014 $\rightarrow$ +1359) were linearized using XbaI, and synthetic mRNAs were generated from all three using T7 RNA polymerase. pBS-r β A₂₁₀ was linearized with XhoI and sense RNA generated using T3 RNA polymerase. All transcription reactions were carried out using procedures suggested by Promega. Template DNA was subsequently digested using RQ1 DNase (Promega). Message-sense cRNAs were quantitated using spectrophotometric analysis and confirmed by electrophoresis and subsequent visualization of similar amounts of each cRNA (0.1–1.0 μ g) with a quantitative RNA size ladder (Promega) on denaturing polyacrylamide gel using the Mighty Small II minigel apparatus (Hofer, San Francisco, CA). These gels were stained and fixed for 10 min with 0.1% bromophenol blue in 10% acetic acid and destained in distilled water for 1 hr, allowing visualization of the cRNA species and comparative quantitation with the RNA size standards.

Solution hybridization–nuclease protection assays and slot blot RNA analysis. The RNA samples were assayed for PPT, NK-1R, or NK-3R mRNAs using solution hybridization–nuclease protection assays. DRG samples were assayed for SP-encoding PPT mRNAs and spinal cord samples were assayed for both NK-1R and NK-3R mRNAs. The use of protection assays to quantitate SP-encoding and NK-1 encoding mRNAs has been described previously (Krause et al., 1987, 1989; Hershey and Krause, 1990; Hershey et al., 1991). NK-3R mRNAs were quantitated similarly. Briefly, 2×10^5 dpm of the specific 32 P-labeled antisense cRNA probe was coprecipitated with 5–10 μ g of total cellular RNA, cRNA quantitation standards (0.5–7.0 pg), or *Escherichia coli* tRNA negative controls (each sample was made up to 25 μ g with *E. coli* tRNA), and the precipitate was resuspended in 10 μ l of hybridization buffer [40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% (v/v) deionized formamide]. The RNAs were allowed to anneal with cRNA probes for 16–20 hr at 45°C. Annealed portions of the mRNAs were protected from nuclease digestion, which was performed for 30 min at 37°C. Nuclease S₁ (1000 U/ml; Sigma) was used for PPT assays; S₁ digestion was terminated by addition of 75 μ l of stop buffer (2.5 M NH₄O-acetate, 50 mM EDTA). Nucleases A (40 μ g/ml; Sigma) and T₁ (2 μ g/ml; Sigma) were used for NK-1R and NK-3R assays; nuclease digestion was followed by a 15 min digestion with proteinase K (100 μ g/sample; Boehringer-Mannheim). Digestion reactions were precipitated with an equal volume of 2-propanol prior to resuspension and electrophoresis at 25 V/cm on 6% acrylamide gels containing 7 M urea. Gels were fixed, dried, and exposed to Hyperfilm autoradiographic film (Amersham) at –80°C with an overlying intensifying screen. Densitometric analysis of the resulting autoradiograms was performed using a Bio-Rad model 620 video densitometer (Richmond, CA). Lanes were scanned individually to allow removal of small lane-to-lane variations in background intensity. Autoradiographic signals for the total RNA samples were compared to those for the message-sense cRNA quantitation standards in order to calculate the relative amount of specific mRNA in each total RNA sample.

The use of denaturing acrylamide gels to separate protected RNA species by electrophoretic mobility allows the precise determination of specific mRNA species. The nuclease protection assay is a more sensitive detection method than Northern blot analysis since hybridization with the antisense cRNA probe is driven essentially to completion. However, this increased sensitivity can easily be lost or masked by the overexposure of the autoradiograms. Overexposure can result in minimization or obliteration of subtle variations in signal intensities. It was imperative that exposure times resulted in signal intensities within the linear response range of the film used (Krause et al., 1989). In this study, exposures varying from 18 hr to 5 d were made from gels of each assay type to determine the appropriate (nonsaturating) exposure time for RNA samples in each assay. This resulted in autoradiograms on which

the signals for cRNA standards were linearly related to the mass amount of cRNA run on the gel.

β -Actin mRNA was quantitated by hybridizing slot blots of individual RNAs to the 32 P-labeled antisense cRNA probe generated using the pBS-r β A₂₁₀ plasmid. Aliquots of total RNA samples (0.1 μ g and 1.0 μ g) were applied through a Schleicher & Schuell (Keene, NH) Minifold II slot blot apparatus in 400 μ l of elution buffer [4.5 M formaldehyde, 10 $\times$ standard sodium citrate (SSC)] onto Nytran nylon membranes (Schleicher & Schuell). Aliquots of message-sense cRNA (20–200 pg) were included as quantitation standards, and *E. coli* tRNA was used as a negative control RNA. The manifold wells were then washed with 400 μ l of 10 $\times$ SSC. The resulting slot blots were dried at room temperature for 20 min and then fixed in a vacuum oven at 80°C for 2 hr. Before hybridization, blots were preincubated at 55°C for 1 hr in hybridization buffer (Krause et al., 1987). Hybridization was performed for 24 hr at 55°C in hybridization buffer containing 1×10^6 cpm/ml 32 P-cRNA probe, and the blots subsequently washed four times for 15 min at 65°C in 500 ml of wash buffer (1 $\times$ SSC, 0.1% SSPE). Northern blots of spinal cord RNA were included with the slot blots during hybridization in order to ensure that the resulting β -actin signal:noise ratio was acceptable. The hybridized slot blots were then subjected to autoradiographic and densitometric analysis similar to that used for protection assays.

Statistical analysis. Data were analyzed using analysis of covariance, and Dunnett's test was used for post hoc comparisons. Analysis of covariance was used because the error between assays significantly contributed to the error between treatment groups. Significance was considered to be $p \leq 0.05$. All data are expressed as picograms of specific mRNA/ng β -actin mRNA (mean $\pm$ SEM), and all comparisons were made to naive controls of the same side.

Results

Schematic illustrations of the cRNA probes used in this study along with results from representative nuclease protection experiments are shown in Figures 1–3. In all assays discussed below, the autoradiographic signals from message-sense cRNAs and tissue RNA samples were linear with respect to mass of cRNA, and expression levels of the control β -actin RNA were not altered by any nociceptive treatment (the variability of β -actin expression between individuals was greater than the variability of β -actin levels between treatment groups). Thus, β -actin was considered an appropriate control species for the normalization of peptide and receptor mRNA expression. An assay for SP-encoding PPT mRNAs is shown in Figure 1A. Three protected bands are visible for the RNA species protected by β -PPT (508 bases) and γ -PPT (295 and 168 bases) mRNAs; the bands protected by the less abundant α -PPT mRNA (364 and 90 bases) are not visible in this exposure. There was no evidence of any alteration of the normal ratio of PPT mRNAs (<1%:20%:80%, α : β : γ ; Krause et al., 1987) following either formalin or CFA treatments. Some residual undigested cRNA probe (567 bases) was still visible after nuclease digestion but did not interfere with quantitation of any specific signals. Quantitation of PPT mRNAs was obtained by analysis of the 295 base band protected by γ -PPT mRNA (see Fig. 1B). This band accounted for 64% of the mass of γ -PPT mRNA present, and thus represents about 50% of the total PPT mRNAs present in lumbar DRG. Each DRG sample represents the signal protected by 5 μ g of total RNA isolated from three lumbar DRGs obtained from a single rat. Average γ -PPT mRNA expression levels for the left and right lumbar DRGs in naive subjects were 10.5 ± 1.4 and 11.4 ± 1.7 pg/ng β -actin mRNA, respectively (approximately 3.2 pg of γ -PPT mRNA/ μ g DRG total RNA).

Figure 2A shows representative results from an NK-1R assay. Each sample lane shows one protected band (588 bases) using 10 μ g of total RNA from the right lumbar dorsal horn of a single subject and some residual undigested cRNA probe (696 bases).

Figure 1. *A*, Autoradiogram of a representative solution hybridization-nuclease protection assay for SP-encoding PPT mRNAs. The arrows show the sizes of the undigested cRNA probe (567 bases), and the RNA species protected by γ -PPT mRNAs (295 and 168 bases) and a message-sense γ -PPT cRNA quantitation standard (295 bases) on the autoradiogram of the denaturing urea-polyacrylamide gel. The 295 base band protected by γ -PPT mRNA was analyzed in order to quantitate total PPT mRNA expression levels (see Results). In this example, the γ -PPT mRNA signal from the DRG of the adjuvant-treated subject is less intense than signal representative of the mean for this treatment group. Each lane in these assays represents only one individual; thus, treatment effects may not be fully appreciable by viewing only one assay. *B*, A schematic diagram showing sizes of the PPT cDNA, cRNA probe, and protected mRNA species.

The 588 base protected bands and cRNA standards were used for quantitation of NK-1R mRNA. Average NK-1R mRNA expression levels for the left and right lumbar dorsal horns in naive subjects were 1.36 ± 0.17 and 1.08 ± 0.12 pg/ng β -actin mRNA, respectively (approximately 0.41 pg of NK-1R mRNA/ μ g dorsal horn total RNA). These NK-1R mRNA expression levels are similar to but somewhat less than those reported by Tsuchida et al. (1990) or Hershey et al. (1991). This may be due to differences in total RNA extraction techniques (see Materials and Methods). In the lumbar ventral horns, NK-1R mRNA expression levels for the left and right sides of naive

Figure 2. *A*, Autoradiogram of a representative solution hybridization-nuclease protection assay for NK-1R mRNA. The arrows show the sizes of the undigested cRNA probe (696 bases) and RNA species protected by NK-1R mRNA (588 bases) and a message-sense NK-1R cRNA quantitation standard (588 bases) on the autoradiogram. The 588 base protected band was analyzed for quantitation of NK-1R mRNA. *B*, A schematic diagram showing sizes of the NK-1R cDNA, cRNA probe, and protected mRNA species. The Roman numerals above the NK-1R cDNA schematic represent putative transmembrane domains of the deduced protein.

subjects were 1.70 ± 0.17 and 1.70 ± 0.10 pg/ng β -actin mRNA, respectively (approximately 0.48 pg of NK-1R mRNA/ μ g ventral horn total RNA).

Representative results from an NK-3R assay are shown in Figure 3*A*. Each sample lane shows the single 345 base band protected using 10 μ g of total RNA from the right lumbar dorsal horn of a single subject. This autoradiographic signal was used for quantitation of NK-3R mRNA. Also visible are a quantitation standard (408 bases) and residual undigested cRNA probe (447 bases and the three less than full-length products apparent in the tRNA and sample lanes). Average NK-3R mRNA expression levels for the left and right lumbar dorsal horns in naive subjects were 2.84 ± 0.08 and 2.48 ± 0.40 pg/ng β -actin mRNA, respectively (approximately 0.68 pg of NK-3R mRNA/ μ g dorsal horn total RNA). In the lumbar ventral horns, NK-3R mRNA expression levels for the left and right sides of naive subjects were 4.76 ± 0.91 and 4.34 ± 1.16 pg/ng β -actin mRNA, respectively (approximately 1.02 pg of NK-3R mRNA/ μ g ventral horn total RNA).

Figures 4–6 show mRNA changes in subjects receiving nociceptive activations of the hindpaw. Figure 4 shows the effects of formalin or CFA injection on the expression of an SP-encoding PPT mRNA in the L4–L6 DRGs. Formalin treatment showed a trend toward increased γ -PPT mRNA levels in the ipsilateral L4–L6 DRGs 2 hr after hindpaw treatment (from 11.4 ± 1.7 to 16.0 ± 1.3 pg/ng β -actin mRNA). Steady state

Figure 3. *A*, Autoradiogram of a representative solution hybridization-nuclease protection assay for NK-3R mRNA. The arrows show the sizes of the undigested cRNA probe (447 bases) and the RNA species protected by NK-3R mRNA (345 bases) and a message-sense NK-3R cRNA quantitation standard (408 bases) on the autoradiogram. The one major and three minor bands evident in the tRNA lane correspond to undigested full length and incompletely transcribed cRNA probe, respectively. The 345 base protected band was analyzed for quantitation of NK-3R mRNA. *B*, A schematic diagram showing sizes of the NK-3R cDNA, mRNA probe, and protected mRNA species. The Roman numerals above the NK-3R cDNA schematic represent putative transmembrane domains of the deduced protein.

γ -PPT mRNA levels had significantly increased to 21.7 ± 3.9 pg/ng β -actin mRNA 6 hr after injection of formalin into the hindpaw ($p < 0.05$). Similarly, CFA injection (4 d) significantly increased γ -PPT mRNA expression in the L4-L6 DRGs to 22.8 ± 4.0 pg/ng β -actin mRNA only in the ipsilateral lumbar DRG ($p < 0.05$). There was a slight trend toward increased levels of γ -PPT mRNA in the contralateral DRGs following formalin, but the changes were not significantly different from naive control subjects. It should be noted that although analysis of covariance was used in this study to remove the effects of small variations of mRNA levels in control animals between assays, the use of ANOVA resulted in identical results except that changes in receptor levels marked $p < 0.01$ (see below) were only significant at the $p \leq 0.05$ level. In no case was there a significant difference in mRNA expression levels between the ipsi- and contralateral sides of naive control groups.

The effects of nociceptive activations of the hindpaw on NK-1R mRNA levels in the lumbar enlargement of the spinal cord are shown in Figure 5. Formalin treatment showed a trend toward increased NK-1R mRNA expression in the ipsilateral dorsal horn after 2 hr (from 1.08 ± 0.12 to 1.91 ± 0.31 pg/ng β -actin mRNA; in this case, p is greater than but approximately equal to 0.05; see Fig. 5*A*). Steady state NK-1R mRNA levels

Figure 4. SP-encoding γ -PPT mRNA expression in the L4-L6 DRG. Note that γ -PPT mRNA expression is significantly increased in the right L4-L6 DRGs (ipsilateral to hindpaw treatment) 6 hr after formalin injection and 4 d after adjuvant injection. γ -PPT mRNA expression in the left L4-L6 DRGs (contralateral to hindpaw treatment) remained unchanged. For quantitation purposes, the 295 base protected species (see Fig. 1) was analyzed for comparison purposes (therefore, these amounts reflect only this portion of the total SP-encoding mRNA species; see Results). *, $p < 0.05$ compared to naive controls.

were significantly increased to 2.41 ± 0.23 pg/ng β -actin mRNA 6 hr after injection of formalin into the hindpaw ($p < 0.01$; Fig. 5*A*). NK-1R mRNA expression was significantly increased to 2.15 ± 0.40 pg/ng β -actin mRNA in the ipsilateral dorsal horn 4 d after injection of CFA into the hindpaw ($p < 0.05$; Fig. 5*A*). Neither treatment produced significant alterations in NK-1R mRNA levels in the contralateral dorsal horn (Fig. 5*A*) or in the ventral horn (Fig. 5*B*). However, there was a trend toward increased NK-1R mRNA levels in the ipsilateral ventral horn after formalin injection (Fig. 5*B*).

Figure 6 shows the effects of nociceptive activation of the hindpaw on NK-3R mRNA expression in the lumbar enlargement of the spinal cord. At 2 hr after hindpaw treatment, formalin treatment showed a trend toward increased NK-3R mRNA levels in the ipsilateral dorsal horn (from 2.38 ± 0.40 to 3.83 ± 0.80 pg/ng β -actin mRNA; Fig. 6*A*). Steady state NK-3R mRNA levels were significantly increased to 5.25 ± 0.37 pg/ng β -actin mRNA 6 hr after injection of formalin into the hindpaw ($p < 0.01$; Fig. 6*A*). The expression level of NK-3R mRNA was significantly increased to 4.57 ± 0.72 pg/ng β -actin mRNA in the ipsilateral dorsal horn 4 d after injection of CFA into the hindpaw ($p < 0.05$; Fig. 6*A*). Neither treatment produced significant alterations in NK-3R mRNA expression in the contralateral dorsal horn (Fig. 6*A*) or in the ventral horn (Fig. 6*B*).

Discussion

The results of this study demonstrate that nociceptive activation of the periphery can result in altered tachykinin receptor mRNA expression in the spinal cord. Nociceptive activation of SP-containing primary afferent neurons produces an upregulation in the production of SP-encoding mRNAs in primary afferent neurons, presumably providing substrate for increased SP production, release, and turnover in the dorsal horn and periphery. The twofold increase in γ -PPT mRNA expression in the lumbar DRG evoked by formalin or CFA (Fig. 4) reflects the previous

Figure 5. NK-1R mRNA expression in the spinal cord. *A*, Dorsal horn. Note that NK-1R mRNA expression is significantly increased in the right dorsal horn (ipsilateral to hindpaw treatment) 6 hr after formalin injection and 4 d after adjuvant injection. NK-1R mRNA expression in the left dorsal horn (contralateral to hindpaw treatment) remained unchanged. *B*, Ventral horn. NK-1R mRNA levels in the spinal cord ventral horn are not significantly altered by either nociceptive treatment. *, $p < 0.05$; **, $p < 0.01$; compared to naive controls.

Figure 6. NK-3R mRNA expression in the spinal cord. *A*, Dorsal horn. Note that NK-3R mRNA expression is significantly increased in the right dorsal horn (ipsilateral to hindpaw treatment) 6 hr after formalin injection and 4 d after adjuvant injection. NK-3R mRNA expression in the left dorsal horn (contralateral to hindpaw treatment) remained unchanged. *B*, Ventral horn. NK-3R mRNA expression in the spinal cord ventral horn is not significantly altered by either nociceptive treatment. *, $p < 0.05$; **, $p < 0.01$ compared to naive controls.

findings of other investigators using *in situ* hybridization and dot-blot hybridization techniques (Noguchi et al., 1988; Minami et al., 1989; Donaldson et al., 1992). The results of this study are also consistent with the findings of Noguchi et al. (1988), in that significant upregulation of PPT mRNA expression during nociception takes at least 2 hr (see Fig. 4). This study provides the first evidence for altered NK-1 and NK-3 tachykinin receptor mRNA expression during nociception. Subsequent alterations in the expression or sensitivity of tachykinin receptors at the cell surface may play an important role in determining physiological responses to long-term nociceptive activation.

The nociceptive activations used in this study (formalin or CFA injection into a single hindpaw) have both been widely used as models of subchronic nociception (Dubuisson and Dennis, 1977; Kantner et al., 1985; Oku et al., 1987; Iadarola et al., 1988a,b; Noguchi et al., 1988; McCarron and Goldstein 1989,

1990, 1991; Minami et al., 1989; Noguchi and Ruda, 1992; Tjolsen et al., 1992; Yashpal et al., 1993). The time points after formalin or CFA injection were chosen for this study based on the results of previous studies. Formalin injection into the hindpaw has been shown to increase the amount of immunoreactive SP in the dorsal horn after 1 hr (Kantner et al., 1985; McCarron and Goldstein, 1989, 1990). Similarly, formalin injection increases the number of cells in the lumbar DRG expressing SP-encoding PPT mRNAs as early as 3 hr postinjection (Noguchi et al., 1988). The use of adjuvant as a nociceptive activator increases the expression of SP-encoding PPT mRNAs in the lumbar spinal cord and DRG within 8 hr (Donaldson et al., 1992) to 4 d (Minami et al., 1989) and upregulates expression of both dynorphin and enkephalin mRNAs in the dorsal horn of the spinal cord within 3–4 d (Iadarola et al., 1988a,b). The level of PPT mRNAs and the numbers of cells expressing PPT

mRNA in the dorsal horn of the spinal cord are increased within 4 d after CFA, indicating that SP-containing spinal interneurons may play a role in nociceptive processing (Noguchi and Ruda, 1992).

The role of SP in spinal mechanisms of pain transmission has been widely studied. There is a large body of evidence that associates intrathecal injections of SP with nociceptive behavior (Piercey et al., 1981; Hylden and Wilcox, 1983a) and decreased nociceptive threshold (Doi and Jurna, 1981; Hylden and Wilcox, 1983b; Matsumura et al., 1985). The release of SP from primary afferent neurons can be stimulated by a number of different peripheral manipulations including electrical stimulation of the sciatic nerve (Yaksh et al., 1980), noxious mechanical pinch of the hindpaw (Kuraishi et al., 1983; McCarson and Goldstein, 1991), or adjuvant-induced arthritis of the hindpaw (Oku et al., 1987). In the dorsal horn of the spinal cord, the majority of SP is found in the central terminals of small-diameter finely myelinated (A- δ) and unmyelinated (C) primary afferent fibers (Hokfelt et al., 1975; Barber et al., 1979). Only 20–50% is contained in intrinsic or descending neurons (Jessell et al., 1979; Ogawa et al., 1985). SP-containing afferent neuron terminals have been shown to appose nociception-specific dorsal horn neurons (DeKoninck et al., 1992). Therefore, a likely source of SP for the activation of NK-1 receptors is that released from primary afferent neurons that can be activated by nociception.

If alterations in SP production and release produce direct effects on nociceptive processing in the dorsal horn, then the NK-1 receptors found in the dorsal horn might be expected to play a functional role in nociceptive processing. In the spinal cord, NK-1 receptors are found primarily in the superficial dorsal horn (laminae I and II) in the region of termination of SP-containing primary afferent neurons (Helke et al., 1990; Yashpal et al., 1990). Immunohistochemical studies conducted using recently generated polyclonal antibodies directed toward an amino terminal portion of the NK-1 receptor have confirmed the localization of the NK-1 receptor protein in laminae I and II of the dorsal horn (Moussaoui et al., 1992). There is evidence that NK-1 receptors exist primarily on nociception-specific second-order projection neurons in the superficial dorsal horn. For example, it has been demonstrated that dorsal horn cells, electrophysiologically classified as nociception specific, are activated by exogenously applied SP (Henry, 1976; Salter and Henry, 1991). Importantly, the activation of these nociception-specific dorsal horn cells by SP or noxious peripheral stimuli can be blocked specifically by the nonpeptide NK-1 receptor antagonist CP-96,345 (Radhakrishnan and Henry, 1991) or by peptide NK-1 receptor antagonists (Cridland and Henry, 1988). CP-96,345 does not alter acute responses to noxious thermal stimuli, but has been shown to block the hyperalgesic effects of intrathecal injections of SP or immersion of the tail tip in hot water (a model of endogenous SP release in the spinal cord; Yashpal et al., 1993). Also, CP-96,345 attenuates only the late phase of the formalin response (Yamamoto and Yaksh, 1991; Yashpal et al., 1993), indicating that SP activation of the NK-1 receptor may only play a role in mediating hyperalgesia or long-term states of nociception. Thus, upregulation of SP turnover in primary afferent neurons during nociception may result in increased NK-1 receptor stimulation in the dorsal horn of the spinal cord. The increased tachykinin receptor mRNA expression seen in this study may occur due to increased agonist activation of NK-1 receptors or by increased electrical activity in dorsal horn cells. In either case, second messengers or other downstream medi-

ators could activate transcriptional mechanisms. However, the precise mechanisms by which gene expression is activated during nociception are not understood.

Homologous changes in receptor gene expression during pharmacological alteration of surface receptors level have been described in other G-protein-coupled receptor systems (Collins et al., 1992). For example, agonist stimulation of the β_2 adrenergic receptor has been shown to increase homologous receptor gene transcription directly by three- to fourfold via activation of its second messenger, cAMP (Collins et al., 1989). The mechanism of action of SP at the NK-1 receptor involves phospholipase C-mediated phosphoinositol hydrolysis (Hanley et al., 1980). The products of this hydrolysis, inositol trisphosphate and diacylglycerol, have been shown to mobilize internal Ca^{2+} stores (Streb et al., 1983) and activate protein kinase C, respectively (Kishimoto et al., 1980). The 5'-flanking region of the NK-1R gene has been shown to include recognition sequences identical to the Ca^{2+} inducibility sequence of *c-fos* and the cAMP response element, indicating that receptor activation may trigger gene transcription (Hershey et al., 1991). A homologous mechanism for the upregulation of NK-1R gene expression through activation of the NK-1 receptor by SP is consistent with the wealth of evidence implicating SP in the transmission of pain (see above). Alternatively, this mechanism may be heterologous; that is, increased activity of a different neurotransmitter (e.g., excitatory amino acids) may be responsible for alterations in tachykinin receptor mRNA expression. A heterologous activation mechanism has been proposed for the upregulation of preprodynorphin mRNA in the dorsal horn after CFA (Dubner and Ruda, 1992). The inhibition of SP release into the superficial dorsal horn after formalin injection (McCarson and Goldstein, 1991) supports the notion that another transmitter may be responsible for the increased tachykinin receptor mRNA expression demonstrated in this study. Nociception has been shown to evoke the release of many other neuroactive substances such as enkephalin, somatostatin, and excitatory amino acids (Yaksh and Elde, 1981; Kuraishi et al., 1985; Skilling et al., 1988). Furthermore, it is not known whether changes in tachykinin receptor mRNA expression level seen in this study are due to transcriptional or posttranscriptional mechanisms. The NK-1R gene 3'-untranslated region contains pentanucleotide sequence repeats (ATTTA) that may affect mRNA stability and allow rapid turnover of the mature transcript (Ihara and Nakanishi, 1990; Hershey et al., 1991; J. Takeda and J. E. Krause, unpublished observations). It remains to be determined whether the nociceptive paradigms used in this study result in an increased number of functional tachykinin receptors being inserted into receptive areas of the plasma membrane or in increased responsiveness of previously expressed receptors. We hypothesize that changes in steady state receptor mRNA levels could act to reset the sensitivity of dorsal horn cells by altering either receptor number, coupling to G-proteins, or effector activity.

Much less is known about the role that NKB and the NK-3 receptor play in nociception. The majority of NKB mRNA is expressed in cell bodies in lamina III of the dorsal horn (Warden and Young, 1988), primarily in intrinsic spinal neurons (Ogawa et al., 1985). The NK-3 receptor (Shigemoto et al., 1990) is found in the superficial dorsal horn of the spinal cord (Mantyh et al., 1989; Yashpal et al., 1990). NKB has the highest affinity of the tachykinins for the NK-3 receptor (Helke et al., 1990), and the NK-3 site has been shown to mediate the antinociceptive effects of NKB (Couture et al., 1992). Intrathecal injections

of NKB have been shown to have antinociceptive effects (Laneuville et al., 1988). Pretreatment with naloxone blocked these effects, indicating that the antinociceptive effects of NKB are mediated by endogenous opiate substances (Laneuville et al., 1988). This antinociceptive activation by NKB may be similar or identical to that described for endogenous opiate systems (Henry, 1983), and may involve segmental or ascending-descending neuronal reflex systems. Endogenous opiates have been shown to affect dorsal horn SP immunoreactivity during nociception (McCarson and Goldstein, 1989, 1990). Thus, nociception may trigger increased activity of an intrinsic antinociceptive system (mediated via NKB and endogenous opiates) that could act not only to limit or modify transmission of nociceptive information to higher brain centers but also to alter turnover of SP and/or tachykinin receptors locally in the dorsal horn. The increase in steady state NK-3R mRNA levels during nociception demonstrated in this study suggests that cells expressing the NK-3 receptor in the dorsal horn may be activated by nociception. Translation of increased levels of NK-3 receptor mRNA and presumably the elevation of functional NK-3 receptors may contribute to alterations in nociceptive processing. The gene encoding this rat tachykinin receptor has not been characterized to our knowledge, and the molecular mechanisms involved in NK-3 receptor expression are not known.

The results of this study also indicate that expression levels of NK-3 receptor mRNA in the spinal cord dorsal horn are two- to threefold higher than those of NK-1 receptor mRNA (see Figs. 5, 6). However, previous quantitative autoradiographic studies (using subsaturating agonist concentrations) indicate the presence of more NK-1 sites than NK-3 sites in the dorsal horn (Yashpal et al., 1991). This apparent discrepancy could arise if many small cells (e.g., interneurons) express low levels of NK-3 receptor mRNA and few cells (e.g., laminae I and II dorsal horn cells) express high levels of NK-1 receptor mRNA. An individual mRNA may also have different translation efficiencies in various cell types. Additionally, different translation efficiencies may result from intrinsic differences in mRNA sequences that dictate the efficiency of mRNA binding to the 40S ribosomal subunit. For these and other reasons, there may simply be a lack of linearity between amount of steady state receptor mRNAs, amount of mature receptor proteins expressed on the cell surface, and extent of receptor coupling to G-protein(s).

In conclusion, nociception has been shown to alter SP mRNA expression in the DRG and NK-1 and NK-3 type tachykinin receptor mRNA expression in the dorsal horn of the spinal cord. The precise mechanisms are not known, but long-term nociceptive activation of small-diameter afferent neurons may provide a neuronal drive for upregulation of tachykinin receptor mRNA expression in dorsal horn cells and alteration of dorsal horn cell sensitivity. The neuronal changes that occur during long-term nociception may provide the bases for new interpretations of the role that tachykinins and their receptors play in nociception, and perhaps in the future treatment of chronic pain syndromes.

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