G-Protein Activation by Neurokinin-1 Receptors Is Dynamically Regulated during Persistent Nociception

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ABSTRACT

Previous work has demonstrated that persistent nociception evokes increased neurokinin-1 receptor (NK-1) gene expression in the spinal cord dorsal horn of the rat within 2 h but has failed to elucidate the relationship between increased NK-1 gene expression at later time points and functional regulation of NK-1 receptor signaling. This study was undertaken to assess changes in NK-1 receptor mRNA levels in models of persistent inflammatory hyperalgesia and to relate them to changes in the functional coupling of NK-1 receptors to G-protein activity in the dorsal horn of the rat. Thus, unilateral intraplantar formalin or complete Freund’s adjuvant was used to alter mechanical and thermal withdrawal thresholds in the inflamed paw. One to 96 h later, NK-1 receptor mRNA levels were quantified using solution hybridization-nuclease protection assays. Formalin-evoked inflammation produced a 2-fold unilateral increase in NK-1 receptor mRNA levels apparent from 2 to 96 h postinjection. Histological sections of the lumbar cord from similarly treated rats were used to generate concentration-response curves using GTPγS35 functional binding assays stimulated by an NK-1 selective agonist. Results showed that formalin evoked a transient, bilateral decrease in the maximal functional response to 35% of control in the treated side at 24 h postinjection and as much as a 10-fold leftward shift in the EC50 of the agonist at 12 to 96 h postinjection. These results provide novel evidence that peripheral nociceptive activation promotes a central mechanism of hyperalgesia through increased functional sensitivity of NK-1 receptors in the spinal cord dorsal horn.

The neurokinin-1 receptor (NK-1) is a G-protein coupled receptor located on both central and peripheral targets (Deguchi et al., 1989; Jacques et al., 1989; Tsuchida et al., 1990; Hershey et al., 1991; Ansel et al., 1996; McCarson, 1999) and is concentrated in the superficial lamina of the spinal cord dorsal horn (Tsuchida et al., 1990; Hershey et al., 1991). Our previous results demonstrated elevated levels of mRNA for both the NK-1 receptor and its endogenous peptide ligand substance P (SP) during peripheral inflammation induced by formalin or complete Freund’s adjuvant (CFA) (McCarson and Krause, 1994, 1995, 1996; McCarson, 1999). Although an activity-dependent increase in NK-1 mRNA expression may contribute to sensory sensitization (McCarson and Krause, 1994), the net contribution of increased NK-1 mRNA levels to receptor protein production and, more importantly, receptor functional coupling, has not yet been established.

The NK-1 receptor and SP have been widely implicated in nociceptive mechanisms (Radhakrishnan and Henry, 1991; Sakurada et al., 1993; De Felipe et al., 1998). During activation, SP binds to NK-1 and activates G-protein complexes. Upon activation, the Gαq/11 subunit undergoes an obligatory exchange of GDP for GTP, dissociates from the βγ duplex, and subsequently activates phospholipase C (Krause et al., 1992). Hydrolysis of the GTP allows reassociation with the βγ duplex and formation of a fresh G-protein complex (Collins et al., 1992). Sufficiently intense activation of NK-1 receptors results in clustering of NK-1 receptors on the cell surface (Grady et al., 1995; Mantyh et al., 1995) and subsequent receptor-mediated endocytosis. It has been debated whether the resulting intracellular vesicles contain bound agonist ligand; the internalization of NK-1 receptors occurs within the first 2 min after injection, and receptors are not fully recycled or replaced in the membrane until 90 min postinjection (Wang and Marvizon, 2002). Importantly, the coupling state of the internalized receptors is unclear. Current interpretations suggest that internalization and deactivation of available receptors result in desensitization to further NK-1 agonist application (Ferguson, 2001). However, if the internalized vesicle contains both SP and coupled receptors, superactivation of the second messenger cascade could...
occur and contribute mechanistically to overactivity and/or sensitization of the cell. Thus, the functional capacity of NK-1 receptors is likely to be markedly different during persistent activation and may be altered for very long periods after peripheral injury. These alterations of the function of the NK-1 receptor are measurable by its coupling state and may contribute greatly to the plasticity of nociceptive pathways.

This study quantified dynamic changes in NK-1 receptor gene expression for 4 days after peripheral inflammation using solution-hybridization nuclease protection assays combined with concurrent measurement of the function of NK-1 receptors using a ligand-stimulated GTP$\gamma$S$^{35}$S binding assay that measures function of the receptor by incorporating a radiolabeled nonhydrolyzable GTP$\gamma$S$^{35}$S onto activated Gα subunits at the point of dissociation from the βγ duplex. After activation, the G-protein becomes locked and measurable by autoradiography and densitometry (Sovago et al., 2001). The selective NK-1 receptor agonist Sar9Met11(O)2 substance P (smSP; Lew et al., 1999) was used to activate NK-1 receptors in the ex vivo tissue sections. This technique quantifies the number of functional receptors by analyzing $E_{\text{max}}$ values and receptor G-protein coupling affinity through analysis of $E_{C50}$. The results of this study directly quantify pain-evoked dynamic alterations in NK-1 receptor coupling in the context of ongoing up-regulation of spinal NK-1 receptor gene expression during inflammation-evoked behavioral hyperalgesia.

Materials and Methods

Animals and Experimental Design. Seventy-six male Sprague-Dawley rats (Harlan, Indianapolis, IN) were assigned to one of nine treatment groups. Control animals received no injection ($n = 22$). Formalin animals received a subcutaneous injection of 100 µl of 5% formalin (Fisher Scientific Co., Pittsburgh, PA) into the plantar aspect of the right hind paw and were sacrificed from 1 to 96 h after injection ($n = 3–13$). The CFA animals received a 50-µl injection of complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO) into the plantar aspect of the right hind paw and were sacrificed 1 or 4 days after injection ($n = 3–5$). All animals were sacrificed by decapitation, and the spinal cord and hind paws were removed as described previously (McCarson, 1999). All procedures were performed as outlined in the Association for Assessment and Accreditation of Laboratory Animal Care Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

Behavioral Measurements. Thermal withdrawal latencies were measured with a Thermal Analgesimeter (University of California, San Diego, CA). Freely moving rats were placed in a Plexiglas chamber, and a high-intensity light beam was focused on the plantar surface of a hind paw or tail as a noxious thermal stimulus (Dirig et al., 1997). Baseline measurements of both hind paws and tail were measured with a Thermal Analgesimeter (University of California, San Diego, CA). Freely moving rats were placed in a Plexiglas chamber with a wire mesh grid bottom to quantify mechanical stimulation was repeated three times at 5- to 10-min intervals, with randomization of order of testing for each paw (adapted from Brennan et al., 1996).

Assays of NK-1 Receptor Gene Expression. Lumbar spinal cord samples were dissected into dorsal quarters, and left and right
total RNA samples were isolated and assayed separately for NK-1 receptor and β-actin mRNAs using solution hybridization-nuclease protection assays as described previously (Krause et al., 1989; McCarson, 1999). Samples of 15 to 50 μg of total RNAs were assayed for NK-1 receptor, or 5 μg of total RNA was assayed for β-actin mRNA levels. Aliquots of 20 to 100 pg of cRNA quantitation standards were used to generate a standard curve, and *Escherichia coli* tRNA was used as a negative control. Densitometric images of the resulting denaturing gels were generated using a GE Healthcare PhosphorImager SP and analyzed using IP Lab Gel (Signal Analytics Corporation, Vienna, VA). Data values are reported as picograms of NK-1 mRNA nanograms of β-actin mRNA (mean ± S.E.M.). Data were analyzed using ANOVA with Fisher’s tests used for post hoc comparisons, with significance considered to be \( p < 0.05 \). Correlation analyses across time points and biochemical and behavioral endpoints were conducted using parametric models to assess correlation coefficients (\( r^2 \)) between measures for mRNA levels, behaviors, and receptor function. The degree of interaction between measures was determined by two-factor ANOVA, with interactions of \( p \leq 0.05 \) considered significant. Pearson correlation coefficients were determined and linear regression analyses were performed using Sigma Plot.

**Results**

Initially, the behavioral impact of the inflammatory stimuli used was quantified. Rats injected with formalin, but not CFA, exhibited licking and biting of the injected paw that subsided by 1 h postinjection. Either formalin or CFA injection caused significant edema in the injected paw apparent from 24 to 96 h after injection (Table 1). Formalin-injected paws became hypoalgesic, whereas CFA-injected paws developed hyperalgesia to thermal stimuli as soon as 1 h postinjection, and this behavioral sensitization persisted for the duration of the measurements (Fig. 1). Neither stimulus elicited overt changes in thermal withdrawal thresholds in either the contralateral uninjected paw or the tail (data not shown). Mechanical hyperalgesia was seen in both the plantar and dorsal surfaces of the CFA-injected paw evident at 1 and 6 h, respectively, after injection and persisting for the duration of the experiment (Fig. 2). Formalin also produced mechanical hyperalgesia on the dorsal surface of the injected paw that was apparent earlier than CFA (2 h) and similarly lasting to 96 h but created significant hypoalgesia on the plantar (injected) surface.

Expression of the NK-1 receptor gene was quantified at several late time points following peripheral inflammatory stimuli. Figure 3 shows that, in the ipsilateral dorsal horn of the spinal cord, unilateral peripheral formalin injection resulted in a significant (approximately 2-fold) increase in NK-1 receptor mRNA levels that was apparent at 2 h and lasted at least 96 h. Expression levels of the NK-1 receptor gene were also slightly, although not significantly, increased in the contralateral spinal cord dorsal horn.

**Table 1**

<table>
<thead>
<tr>
<th>Hind paw edema evoked by chemogenic inflammatory stimuli</th>
<th>$g$</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-0.05 \pm 0.06$</td>
</tr>
<tr>
<td>24-h CFA</td>
<td>$1.50 \pm 0.06^*$</td>
</tr>
<tr>
<td>96-h CFA</td>
<td>$0.50 \pm 0.04^*$</td>
</tr>
<tr>
<td>24-h Formalin</td>
<td>$0.94 \pm 0.12^*$</td>
</tr>
<tr>
<td>48-h Formalin</td>
<td>$0.50 \pm 0.15^*$</td>
</tr>
<tr>
<td>96-h Formalin</td>
<td>$0.49 \pm 0.08^*$</td>
</tr>
</tbody>
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* $p < 0.01$ compared with control (ANOVA, Fisher’s PLSD; \( n = 3–7 \)).

The concentration-response curve for CFA-treated animals was similar to the curve for control animals (data not shown). Inflammation induced by CFA...
evoked shifts in the EC50 that were bilateral and not significant at this time point. However, the decrease in the Emax on the treated side was significantly different from control (Fig. 5B).

To test the specificity of the agonist, three naive control animals were used to generate a competition curve of 4.5 μM smSP in the presence of the potent selective nonpeptide NK-1 receptor antagonist L-733,060 (Seabrook et al., 1996). As seen in Fig. 6, the highest concentration of antagonist used (10 μM) was able to completely block the stimulation by 4.5 μM smSP. Stimulation of NK-1 receptors with 4.5 μM smSP

Fig. 2. Mechanical hind paw withdrawal thresholds to monofilament stimulation of the dorsal or plantar surface of the contralateral and ipsilateral hind paws after injection of CFA or formalin. Data are reported as force applied by the monofilament (in grams). Note that either chemogenic stimulus reduced withdrawal thresholds to mechanical stimulation of the dorsal surface of the ipsilateral paw (A). In contrast, only CFA evoked mechanical hypersensitivity to stimulation of the plantar surface of the injected paw (C). Formalin-injected plantar surfaces were significantly hypoalgesic. Neither stimulus elicited overt changes in mechanical withdrawal thresholds of either surface of the contralateral paw (B and D). *p < 0.05 as compared with preinjection baseline (ANOVA, Wilcoxon test for treatment comparisons, Dunnett’s for individual time point comparisons; n = 3–8).

Fig. 3. Time course of spinal NK-1 receptor gene expression after injection of formalin into the right hind paw. Data values are reported as picograms of NK-1 receptor mRNA/nanograms of β-actin mRNA. Note that NK-1 receptor mRNA levels are significantly increased from 2 to 96 h after formalin injection (for clarity, previously published data are included for 1–6-h time points; McCarson and Krause, 1994, 1995). *p < 0.05 compared with control (ANOVA, Fisher’s PLSD; n = 4–14).

Fig. 4. Autoradiographic images of representative lumbar spinal cord sections assayed for NK-1 receptor-activated GTPγS35 binding. Nonspecific binding was conducted in the presence of 10 μM unlabeled GTPγS; basal activity in the presence of drug vehicle. Agonist stimulation for control and formalin are shown at 36 μM [Sar9Met11(O)2] substance P.
evoked a maximal response of 233.22 ± 54.53% stimulation over basal, and the addition of 10 μM L-733,060 decreased the maximal response to 4.15 ± 3.69% stimulation over basal. The NK-1 antagonist produced an identical blockade of smSP-evoked stimulation in ex vivo spinal cord sections of animals tested either 24 h after formalin injection or 4 days following CFA.

Once changes in NK-1 receptor function were characterized at these time points, a time course after formalin injection was performed to determine the series of events and duration of changes initially seen at 24 h. In this phase of the study, four control animals were used and three to five animals each were used for time points of 1, 6, 12, 24, and 96 h postformalin. At 1 h, the $E_{\text{max}}$ of both contralateral and ipsilateral sides was significantly reduced (Fig. 7). Values for $E_{\text{max}}$ rebounded toward baseline before declining again at 12 to 24 h. Measurement at 96 h shows that $E_{\text{max}}$ values again return toward preinjection levels, although treated-side values remain significantly lower than control. Similar but less intense changes in the number of functionally coupled NK-1 receptors are evident on the contralateral side as well. Treatment with formalin decreased the EC$_{50}$ of smSP on the treated side but had no significant effect on the untreated side (Fig. 8). This decrease was significant at 12 h and was maintained throughout the duration of the time course out to 96 h postformalin.

Correlation analyses were conducted between behavioral and biochemical endpoints, revealing significant relationships among three key measurements evaluated, namely mechanical sensitivity, NK-1 receptor gene expression, and NK-1 receptor affinity (Fig. 9). Both receptor affinity (EC$_{50}$) and mechanical sensitivity (von Frey threshold) were significantly correlated with NK-1 receptor gene expression (Fig. 9, A and B).

**Discussion**

Several lines of evidence have established the importance of substance P/neurokinin-1 receptor mechanisms in the...
eral reduction in maximal NK-1 receptor responsiveness at 1 h postformalin injection, but the specific relationships among NK-1 receptor activation, gene expression, receptor-mediated endocytosis, and receptor protein trafficking remain unclear from the perspective of their eventual impact on central sensitization and maintenance of inflammation-evoked hyperalgesia, but the specific relationships among NK-1 receptor activation, gene expression, receptor-mediated endocytosis, and receptor protein trafficking remain unclear from the perspective of their eventual impact on central sensitization and maintenance of inflammation-evoked hyperalgesia. Previous studies have attempted to characterize changes in NK-1 receptor density or affinity during afferent neuronal activation (Stucky et al., 1993; Kar et al., 1994; Henry et al., 1999). The variable nature of the results of such studies is especially understandable in light of subsequent studies describing the rapid internalization events associated with agonist-induced activation of the NK-1 receptor (Collins et al., 1992; Grady et al., 1995). This study quantified dynamic changes in NK-1 receptor gene expression combined with concurrent measurement of the function of NK-1 receptors utilizing a ligand-stimulated GTP-$\gamma$S binding assay performed on sections of lumbar spinal cord, providing valuable new insights into changes in the function and expression of the NK-1 receptor after peripheral inflammation.

**Behavioral Impact of Inflammatory Stimuli.** Extensive quantification of the hyperalgesia resulting from the inflammatory pain models used in this study was performed; the formalin-evoked hypersensitivity of peripheral tissues (Fu et al., 2000, 2001) is sparsely studied compared with its ability to evoke spontaneous pain-related behaviors (Dubisson and Dennis, 1977; Hunskaar et al., 1985). As in previous reports, injection of either CFA or formalin produced mechanical or thermal hyperalgesia in the rat (Ma and Woolf, 1996). Plantar thermal and mechanical measurements in formalin-injected animals were confounded because of the overlap of the testing surface and the tissue area injured by the injection. Formalin, although a potent nociceptive activator, also causes irreversible damage to the site of injection due to protein cross-linking. The thermal analgesimeter used in this study allowed testing only of the plantar surface of the paw or tail. Mechanical thresholds measured on the dorsal surface, however, showed formalin-injected rats to be hyperalgesic, but these measurements could not be confirmed through plantar thermal or mechanical testing.

Formalin injection evoked mechanical hyperalgesia on the dorsal surface of the injected hind paw at times when de novo synthesis and receptor affinity were increasing and functional receptor number was decreasing. It is surprising that the most robust hyperalgesia was not associated with increased functional density of NK-1 receptors but rather with their coupling affinity, as described in further detail below. Nonetheless, the temporal pattern of similarities between behavioral sensitization and dynamic changes in NK-1 function suggests that these phenomena are mechanistically linked.

**Alterations in Maximal NK-1 Receptor Responsiveness ($E_{\text{max}}$).** At 1 h postformalin, a significant decrease in the $E_{\text{max}}$ of NK-1 stimulated GTP-$\gamma$S binding was apparent (Fig. 7). This decrease could be attributed to a depletion of available NK-1 receptor binding sites at the surface membrane due to internalization events or to modification of the receptor protein via phosphorylation or interactions with accessory proteins (Collins et al., 1992). It has been previously reported that NK-1 receptors are rapidly internalized after peripheral injection of formalin but are recycled or replaced in the membrane within 90 min postinjection (Wang and Marvizon, 2002). The current results robustly support this finding and may functionally confirm that receptors within the internalized vesicles are either not coupled to G-proteins or are unavailable for ligand binding. However, time course data (Fig. 7) show that, by 6 h, the number of receptors in the membrane functionally coupled to G-proteins is increasing.

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**Fig. 8.** Time course of changes in functional sensitivity ($EC_{50}$) of spinal NK-1 receptor-stimulated GTP-$\gamma$S binding after unilateral injection of formalin into the right hind paw. Data are presented as mmSP concentration (in micromolar). Note that within 12 h, peripheral inflammation resulted in a significant, persistent decrease in the $EC_{50}$ of mmSP that was restricted to the side of the spinal cord ipsilateral to hind paw treatment. *p < 0.05 compared with naive controls (ANOVA, Fisher's PLSD; n = 3–5).

**Fig. 9.** Correlation analyses of changes in NK-1 gene expression, NK-1 receptor affinity in the dorsal horn, and mechanical withdrawal threshold in the paw from 0 to 96 h after unilateral injection of formalin into the right hind paw. Each data point is labeled with the corresponding time point measured, and the overall calculated correlation coefficient is given. Note that in A, there is a significant correlation between increasing amounts of NK-1 mRNA and increasing receptor affinity. Likewise, in B, as levels of NK-1 mRNA increase, mechanical sensitivity increases.
Although the number of coupled receptors on the treated side is still significantly lower than control.

Between 12 and 24 h, the $E_{\text{max}}$ again declined, but this decrease was unlikely due to internalization events, suggesting that a different mechanism is responsible for the decrease in functional receptors seen from 12 to 24 h postformalin. Figure 3 shows a significant increase in mRNA levels for the NK-1 receptor from 2 to 96 h postformalin treatment. Thus, the decrease in the number of functionally coupled NK-1 receptors is not because of down-regulation of NK-1 receptor gene expression. The decrease in $E_{\text{max}}$ could be a result of uncoupling of receptor and G-protein. Inflammation causes an affinity shift to a lower affinity state, but the magnitude of change is still significantly lower than control.

She showed a robust decrease in EC50 to 10 to 15% of the basal value (Fig. 8). NK-1 receptors in the ipsilateral lumbar spinal dorsal horn (Fig. 5), which was further supported by the time course data of mRNA binding assays and Scatchard analyses or evaluation of G-protein number or availability. Ultimately, it appears that the number of functioning NK-1 receptors returns to baseline, an event most likely correlated with elevated levels of NK-1 receptor mRNA and de novo synthesis. The late timing of the return to basal of number of functional receptors may also support the idea that a population of previously non-NK-1-expressing neurons is undergoing a phenotypic switch.

Alters in NK-1 Receptor Sensitivity (EC50). It is interesting that inflammation also evoked a time-dependent leftward shift in the EC50 of the treated side after formalin injection (Fig. 5), which was further supported by the time course data (Fig. 8). NK-1 receptors in the ipsilateral lumbar spinal dorsal horn showed a robust decrease in EC50 to 10 to 15% of control levels, which remained reduced as long as 96 h. This leftward shift, representing an increase in receptor coupling affinity, occurs at times when the animal is profoundly hyperalgesic, and the maintenance of increased receptor affinity could precondition the animal to further nociceptive events. Receptors in the contralateral dorsal horn transiently shift to a lower affinity state, but the magnitude of change was not significant. In this regard, NK-1 receptor function may be controlled by mechanisms similar to those regulating NK-1 gene expression, revealing bilateral effects of a leftward shift, representing an increase in receptor coupling affinity.

Peripheral nociceptive activation promotes a central mechanism of hyperalgesia by which the expression and excitability of NK-1 receptors on the dorsal horn cells are increased. Internalization of NK-1 receptors upon activation results in sequestration of those receptors, mechanistically contributing to functional desensitization as reflected by diminished maximal responsiveness of NK-1 receptors in the spinal cord dorsal horn. Behavioral sensitization after persistent peripheral inflammatory nociception may be produced, in part, by increased functional sensitivity of NK-1 receptors. Understanding dynamic changes in NK-1 receptor functional coupling may ultimately identify novel therapeutic approaches to treatment of chronic inflammatory states.

Conclusions

Peripheral nociceptive activation promotes a central mechanism of hyperalgesia by which the expression and excitability of NK-1 receptors on the dorsal horn cells are increased. Internalization of NK-1 receptors upon activation results in sequestration of those receptors, mechanistically contributing to functional desensitization as reflected by diminished maximal responsiveness of NK-1 receptors in the spinal cord dorsal horn. Behavioral sensitization after persistent peripheral inflammatory nociception may be produced, in part, by increased functional sensitivity of NK-1 receptors. Understanding dynamic changes in NK-1 receptor functional coupling may ultimately identify novel therapeutic approaches to treatment of chronic inflammatory states.

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References


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