Persistent Pain Produces Stress-like Alterations in Hippocampal Neurogenesis and Gene Expression

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Abstract: Clinical observations have shown that patients with chronic pain are often depressed, suggesting the importance of the affective or emotional component of pain and its impact on cognition. In this study we investigated pain-induced activation of the hippocampus to address possible molecular and cellular events that may underlie the comorbidity of chronic pain and depression. Rats received either an acute (formalin) or chronic (complete Freund’s adjuvant) inflammatory stimulus to the hind paw or an acute or chronic immobilization. Results demonstrated that pain can alter hippocampal morphology and gene expression. Bromodeoxyuridine (BrdU) staining indicated that neurogenesis in the hippocampal dentate gyrus was significantly reduced after long-term inflammatory nociception, similar to previous observations after various stress models. Important activators of nociception-induced spinal central sensitization, the neurokinin-1 (NK-1) receptor and brain-derived neurotrophic factor (BDNF), have also been intimately associated with depressive processes in the limbic system. In situ hybridization assay results demonstrated that either pain or stress (acute or chronic treatments) reduced the levels of both NK-1 receptor and BDNF mRNAs in the cornu ammonis 1-3 sublayers of the hippocampus, suggesting a possible role of these neuromediators in processing of pain in higher brain centers.

Perspective: The findings in this study demonstrate that persistent pain induces stress-like damaging modulatory effects in the hippocampus, which is one of the limbic regions involved in the pathophysiology of depression. Targeting these mechanisms (which are potential contributors to the emotional impact of pain) may provide novel therapeutic approaches for relieving depression-like aspects of chronic pain.

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Key words: NK-1 receptor, BDNF, immobilization stress, formalin, CFA, BrdU.

Chronic pain is a debilitating disease state characterized by complex alterations in both peripheral and central nociceptive pathways. In addition to the sensory-discriminative facet, pain also possesses a robust stress-like component, reflecting a significant affective-cognitive aspect. This concept is supported by reports suggesting that the majority of patients suffering from chronic pain also express various symptoms of clinical depression. Although some clinical evidence implicates dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, the main physiologic and biochemical factors underlying the comorbidity of chronic pain and depression are yet to be determined.

The hippocampus, a central component of the limbic system involved in regulation of mood or affect, is one of several brain regions capable of continuous cell proliferation and neurogenesis throughout adulthood in hu-
mians and other animals. However, previous reports have revealed that the rate of neurogenesis can be influenced by various environmental, endocrine, and pharmacologic stimuli. Furthermore, it has been demonstrated that stressful experiences have an inhibitory effect on formation of new granule neurons in the hippocampal dentate gyrus in various mammalian species. In addition to decreased neurogenesis, other stress-induced morphologic changes in the hippocampus, primarily the atrophy and loss of CA3 pyramidal neurons, as well as diminished expression of brain-derived neurotrophic factor (BDNF), are considered to be some of the cellular and molecular processes that contribute to the development and long-term pathophysiology of depression. In contrast, long-term antidepressant administration can elevate BDNF levels, up-regulate neurogenesis, and consequently reverse or block many of the detrimental effects of stress on the hippocampus.

The tachykinin neuropeptide substance P (SP) and neurotrophin BDNF, as well as their preferred receptors, the neurokinin-1 (NK-1) receptor and tyrosine kinase B (TrkB), respectively, have been previously implicated in several important aspects of nociceptive sensory processing in the nervous system. Clinical observations that NK-1 receptor antagonists have antidepressant effects have suggested that the effects of pain and stress may converge in higher brain centers sensitive to both types of stimuli. However, nociception-induced modulation of NK-1 receptor and BDNF gene expression as well as their function in the context of processing chronic pain in higher brain centers associated with regulation of affect/mood (eg, the amygdala, hippocampus, and hypothalamus) remain poorly understood.

This study addressed whether persistent pain initiates changes in hippocampal cellular architecture and gene expression similar to those evoked by immobilization stress. Results show decreased neurogenesis in the dentate gyrus and reduced NK-1 receptor and BDNF mRNA levels in the hippocampal CA1-CA3 subregions. These findings also represent novel pain-induced biologic end points that may reveal additional information about affective aspects of chronic pain and their relationship to depression.

Materials and Methods

Animal Housing and Handling

Young adult male Sprague Dawley rats (Harlan Farms, Indianapolis, IN), used for all experiments, were age matched (7-8 wks old) at the beginning of the treatment. Rats were allowed at least 1 wk of habituation before any treatments were applied. The maintenance of the rat colony and all the animal treatments were in accordance with National Institutes of Health laboratory care standards and approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Efforts were made to minimize animal suffering and to reduce the number of animals used in this study. Animals were housed (12-h light/dark cycle) in groups of 3 per cage with ad libitum access to food and water; they were mixed together so there was one member of each treatment group in every cage. All rats, including the control group, were handled in the same way to reduce the effects of stress associated with handling on the results.

Pain and Stress Treatments

Rats (200-300 g) were either injected with an inflammatory stimulus or subjected to immobilization (Fig 1). Acute inflammatory pain (n = 15) rats received a single subcutaneous (SC) injection of 100 µL of 5% formalin (Fisher Scientific Co, St Louis, MO) into the plantar aspect of the right hind paw; animals were decapitated either 45 min (corticosterone assay) or 24 h later. Chronic inflammatory pain (n = 20) rats received either a single (1x; injection on day 0) or 3 (3x; injections on days 0, 7, and 14) SC injections of 50 µL complete Freund’s adjuvant (CFA) (Sigma Chemical Co, St Louis, MO) into the plantar aspect of the right hind paw over the 21 days. Additional animals (n = 5/group) were injected once with CFA and killed either 45 min, 2h 45 min, or 21 days later to provide supplemental time points for the corticosterone (CORT) measurement assays and behavior studies. Acute stress (n = 15) rats were immobilized in restraint bags (Rodent Restraint Cones; Harvard Apparatus, Holliston, MA) once for 45 min, and chronic stress (n = 20) rats were immobilized for 45 min once daily for 10 consecutive days. Sham (n = 20) animals received no hind paw treatment but were momentarily restrained and their right hind paw manipulated. Otherwise, they were handled identically to the treatment animals. The animal handling consisted of identical housing regimes, daily transport from the animal facilities to the laboratory, and interactions with the handler.

Corticosterone Study

Animals used in this experiment were subjected to the pain or stress models as described above. To minimize diurnal rhythm variations, trunk blood from all subjects was collected between 2:00 and 3:00 pm, centrifuged and the serum stored at −20°C until analysis. Serum CORT levels were determined with a commercially available radioimmunoassay (RIA) kit (Coat-A-Count Rat Corticosterone; DPC, Los Angeles, CA), using 50 µL of sample and 50 µL of 125I tracer per tube coated with low-cross-reactivity antibodies to rat CORT. The working range for the assay was 20-2,000 ng/mL, with a minimal detectable concentration of approximately 5.7 ng/mL.

Behavioral Measurements

A Thermal Paw Analgesiometer (Department of Anesthesiology, University of California, San Diego, CA) was used to measure thermal withdrawal sensitivity as previously described. For quantification of mechanical sensitivity thresholds, von Frey monofilaments (Stoelting, Wood Dale, IL) of graded bending forces (2.6-522 mN) were used as previously described.
Thermal and mechanical baseline measurements of both hind paws were taken for each animal before pain or stress stimuli and at 2, 4, 7, 9, 11, 14, 18, and 21 (where applicable) days following the treatments to measure hyper- or hypoalgesia. When the animals were killed, hind paws were removed just above the tibiotarsal joint and weighed to measure edema. All behavioral measurements were conducted by an experimenter blind to animal treatments.

**Bromodeoxyuridine Labeling and Immunohistochemistry**

For quantification of Bromodeoxyuridine (BrdU)-positive cells, rats were administered a single dose of BrdU (75 mg/kg; Sigma) just before the last treatment. Twenty-four hours after the BrdU injection, rats were anesthetized and transcardially perfused: 0.01 mol/L cold phosphate-buffered solution (PBS) for 5 min followed by 4% cold paraformaldehyde for 15 min. The brain was subsequently removed and incubated in 4% paraformaldehyde overnight at 4°C and then stored in 30% sucrose solution for several days at the same temperature. After gradual freezing at −70°C, brains were cut transversely into 40-μm-thick sections using a cryostat (Jung Frigocut 2800; Leica). Sections through the entire hippocampus were made and stored in 30% sucrose solution at −20°C. The BrdU staining process was performed on free-floating brain sections, starting with DNA denaturation by 2 h incubation in 50% formamide/2× standard sodium citrate buffer (SSC) at 65°C, followed by 2 TBS rinses. After 30 min incubations at 37°C in 2 N HCl and 10 min in 0.1 mol/L boric acid, sections were exposed to 2% H2O2 for 20 min to quench endogenous peroxidases. After several washes in TBS and blocking with 3% normal horse serum in 0.01% Triton X-100, an overnight incubation at 4°C with primary mouse monoclonal anti-BrdU antibody (1:100; Becton Dickinson, San Jose, CA) followed. The next day, sections were incubated in biotinylated secondary horse antimouse antibody for 1 h (1:200; Vector Laboratories, Burlingame, CA), followed by amplification with an avidin-biotin complex and exposure with DAB (Vector Laboratories).

**Figure 1.** Diagram showing the experimental design and time course for paradigms modeling acute and persistent pain or stress. (A) Acute stress: Rats were exposed to a single 45-min immobilization in a restraint bag. (B) Acute pain: Rats were injected with 100 μL of 5% formalin (an inflammatory agent), into the plantar aspect of the right hind paw. (C) Chronic stress: Rats were immobilized in restraint bags for 45 min daily for 10 consecutive days. (D) Chronic pain: Rats received an inflammatory agent into the plantar aspect of the right hind paw. A single 50-μL injection of CFA (1x, day 0) or 3 injections of CFA (3x, days 0, 7, and 14) were given over a 21-day period. Experiments measuring neurogenesis or gene expression only used the 3-injection paradigm, which kept the animals in a state of persistent nociception. In the neurogenesis experiments, BrdU was given 24 h before animals being perfused (S).
Quantification of BrdU Labeling

To avoid counting the same neuron in multiple sections, every sixth section throughout the hippocampus was processed for BrdU staining. During quantification of BrdU labeling, a modified unbiased stereology protocol was used, specifically confirming that each BrdU-labeled cell was counted only once, and that the area counted was consistent across all sections. BrdU-labeled cells in the dentate gyrus (granule cell layer) and hilus regions of the dentate gyrus were counted by an experimenter blinded to the treatment group using X-100 for 10 min, rinsed briefly in depc-H2O, and placed into 0.1 mol/L triethanolamine, pH 8.0, with 0.25% acetic acid and probes were precipitated by addition of 3% 3 acid and probes were fragmented by alkaline hydrolase.3 Briefly, antisense probes were diluted with an or probes were additionally fragmented by alkaline hydrolase.3 Briefly, antisense probes were diluted with an equal part carbonate buffer (80 mmol/L NaHCO3, 120 mmol/L Na2CO3), pH 10.2, and incubated at 60°C for 9 min to yield fragments 100 to 150 bp in length. The reaction was terminated by addition of 5% (v/v) 10% acetic acid and probes were precipitated by addition of 3% 3 mol/L NaOAc, pH 6.0, and 2.5 volumes of ethanol.

NK-1 Receptor and BDNF Probes

The NK-1 receptor and BDNF sense and antisense cRNA probes were generated by an in vitro transcription reaction.15,40,43 Probes were double-labeled by the addition of 35S-rCTP and 35S-rUTP and purified through a NucAway spin column (Ambion, Austin, TX). The NK-1 receptor probes were additionally fragmented by alkaline hydrolysis.3 Briefly, antisense probes were diluted with an equal part carbonate buffer (80 mmol/L NaHCO3, 120 mmol/L Na2CO3), pH 10.2, and incubated at 60°C for 9 min to yield fragments 100 to 150 bp in length. The reaction was terminated by addition of 5% (v/v) 10% acetic acid and probes were precipitated by addition of 3% 3 mol/L NaOAc, pH 6.0, and 2.5 volumes of ethanol.

NK-1 Receptor and BDNF mRNA In Situ Hybridization

Immediately after decapitation, rat brains were removed and washed twice with 50 mmol/L PBS for 10 min and dehydrated briefly with 50% and 70% isopropanol incubations. Two to five million cpm/slide of NK-1 receptor or BDNF probes in hybridization buffer (50% formamide (v/v), 3× SSC, 50 mmol/L NaPO4, 10 mmol/L DTT, 1× Denhardt’s solution, 0.25 g/L tRNA, 10% dextran SO4) were applied to the sections and allowed to hybridize at 60°C overnight in a humid chamber. The following day, slides were washed in 2× SSC for 20 min and 1× SSC for 20 min and treated with RNase solution (0.5 mol/L NaCl, 10 mmol/L Tris-Cl pH 8.0, 1 mmol/L EDTA pH 8.0, 20 μg/mL RNase A, 1 U/mL RNase T1) for 30 min at 37°C. Slides were then transferred to 1× SSC for 20 min, 0.5× SSC for 20 min, 0.2× SSC for 20 min, and 0.2× SSC for 1 h at 60°C. Wash with 0.2× SSC for 10 min followed and then a brief dehydration in 50% and 70% isopropanol. After air drying for a few hours, slides were exposed to Kodak MR autoradiographic film for 16 to 48 h. Images were captured using a computer-controlled digital camera (Cohu, Poway, CA) and imported into Image J (Scion Corp, Frederick, MD) for densitometric analysis. Using the manufacturer’s calibration scale, raw densitometry data were converted to nCi of C14/g of tissue, which is linearly related to the tissue levels of the specific mRNA.

Statistical Analysis

Data from all the experiments were analyzed using analysis of variance (ANOVA) with Student-Newman-Keuls’ statistical tests used for post-hoc comparisons, except for the mechanical sensitivity experiment, where nonparametric Kruskal-Wallis analysis with Dunn’s post-test was used (InStat; GraphPad Software, San Diego, CA). Significance was considered to be P ≤ .05; all comparisons were made to naive controls.

Results

Formalin Injection or Immobilization Stress Activates the HPA Axis

In order to compare the effects of pain and stress on cellular morphology and molecular events occurring in the hippocampus, we initially addressed the effects of inflammatory pain and immobilization stress paradigms on the activation of the HPA axis (Fig 2). Acute or chronic stress produced approximately 130% increases (845 ± 51 mg/mL, 72 ng/mL, respectively) in CORT when compared with nontreated (356 ± 72 ng/mL) animals. The HPA axis was stimulated in a similar manner 45 min after an acute nociceptive stimulus (single formalin injection into the hind paw); serum CORT levels were elevated to 862 ± 68 mg/mL, consistent with previous reports.56 Interestingly, animals that received a chronic pain treatment (single or multiple injections of CFA) showed no such effect; CORT levels, measured without additional manipulations of the inflamed paws, were not significantly different from controls at 45 min, 2 h 45 min, or 21 days after CFA injections.
Thermal and Mechanical Sensitivity After Chronic Pain or Stress

Injections of either formalin or CFA produced significant edema in the treated paws within the first 24 h after injections (data not shown). The behavioral impact of acute inflammatory nociceptive stimuli on thermal and mechanical sensitivity were recently published63; therefore, the outcomes of acute treatments were not addressed in this study. The effects of chronic immobilization (10 days) and CFA-induced nociception over 21 days on the thermal withdrawal latencies and mechanical withdrawal thresholds are shown in Fig 3. Animals receiving 45 min immobilization for 10 consecutive days did not display alterations in paw sensitivity during the entire time course of immobilization treatment. A single CFA injection at the beginning of the treatment paradigm produced significant thermal (Fig 3A) and mechanical (Fig 3B) hyperalgesia within the first 48 h after the injection. However, rats that did not receive any additional CFA treatments (1x) exhibited reduced hypersensitivity by day 14 and were returning to baseline by the end of the 21-day time course. However, in rats that subsequently received 2 additional weekly CFA injections (3x), continuous thermal and mechanical hyperalgesia was evident throughout the duration of the experiment.

Chronic Pain or Stress Treatments Decrease the Number of BrdU-Positive Cells in the Hippocampus

Neurogenesis can be detected by immunohistochemical staining of BrdU, a thymidine analog, when incorporated into the newly formed DNA of actively dividing cells. In the hippocampus, the production of new neuronal cells in adult rats is limited to the dentate gyrus, particularly the SGZ and hilus subregions (Fig 4). Previous reports have shown that progenitor cells located in the SGZ are able to divide and migrate to the granule cell layer where a majority mature into neurons and astrocytes.7,37 Using a modified stereology protocol, hippocampal BrdU-labeled cells were counted 24 h after BrdU injection. The 24-h survival time was adopted from Malberg et al37 and allows for the completion of at least 1 cell cycle by cells in S phase at the time of BrdU admin-

Figure 2. Histogram showing serum corticosterone levels following immobilization stress or inflammatory nociception. In the stress models, blood was collected immediately after the animals were taken out of the restraint bags. Note that acute nociception (formalin) produced an increase in corticosterone levels similar to that produced by immobilization, whereas long-term nociception (single (1x) or triple (3x) CFA injections over 21 days) had no effect. All values are given in ng/mL (mean ± SEM; n = 5/group). *P < .05 compared with the control group (ANOVA and Student-Newman-Keuls’ post-hoc test).

Figure 3. Histogram showing (A) thermal nociceptive withdrawal latencies and (B) mechanical withdrawal thresholds to stimulation of the plantar surface of the ipsilateral (right) paw following either persistent pain or stress. Baseline measurements were taken before the application of any treatments. Chronic immobilization (45 min daily for 10 days) did not alter paw sensitivity at any time point. CFA injections, however, evoked both thermal and mechanical hypersensitivity within the first 2 days after the initial injection. Although initially hyperalgesic, animals that received only 1 CFA injection (1x) displayed less hypersensitivity by day 14 and were approaching baseline values by day 21. Rats that received 3 weekly CFA treatments (3x) exhibited thermal and mechanical hyperalgesia over the entire time course of 21 days. Thermal withdrawal latencies are expressed in seconds, and mechanical withdrawal thresholds are reported as grams of force (mean ± SEM; n = 5). *P < .05 compared with baseline (thermal data: ANOVA and Student-Newman-Keuls’ post-hoc test; mechanical data: Kruskal-Wallis and Dunn’s post-hoc test).

Figure 4. Neurogenesis can be detected by immunohistochemical staining of BrdU, a thymidine analog, when incorporated into the newly formed DNA of actively dividing cells. In the hippocampus, the production of new neuronal cells in adult rats is limited to the dentate gyrus, particularly the SGZ and hilus subregions (Fig 4). Previous reports have shown that progenitor cells located in the SGZ are able to divide and migrate to the granule cell layer where a majority mature into neurons and astrocytes.7,37 Using a modified stereology protocol, hippocampal BrdU-labeled cells were counted 24 h after BrdU injection. The 24-h survival time was adopted from Malberg et al37 and allows for the completion of at least 1 cell cycle by cells in S phase at the time of BrdU admin-
Results demonstrated that acute pain (24 h post-formalin) or stress (single 45-min immobilization) treatments did not produce a significant effect on the number of BrdU-labeled cells in the dentate gyrus when compared with controls (Fig 5A; control: 3,947 ± 609; formalin: 3,471 ± 299; stress: 3,611 ± 314 BrdU-labeled cells; mean ± SEM; n = 5/group). In contrast, when persistent inflammatory pain (3 CFA injections over 21 days) or chronic immobilization stress (45 min/day × 10 days) treatments were applied, a significant damaging impact on hippocampal cellular architecture was observed. In comparison with the nontreated animals, chronic pain or stress each reduced the number of BrdU-labeled cells in the total dentate gyrus by 50% and 43%, respectively (Fig 5B; control: 3,468 ± 195; CFA: 1,737 ± 60; stress: 1,975 ± 124 BrdU-labeled cells; mean ± SEM; n = 5/group). The unilateral administration of the nociceptive stimulus did not produce sided differences in the hippocampus, so the BrdU-labeling data are shown as bilateral averages. The most robust changes were observed in the the SGZ (pain 60% vs stress 53% decrease) and to a lesser extent in the hilus (pain 28% vs stress 21% decrease).

Pain or Stress Decreases Hippocampal BNDF and NK-1 Receptor Gene Expression

In situ hybridization analyses were used to assess which hippocampal subregions and neuronal populations exhibited changes in BDNF (Fig 6A) and NK-1 receptor (Fig 6B) gene expression in response to pain or stress. Initially, the results from the assays were analyzed separately on the ipsilateral vs contralateral sides of the hippocampus, because the inflammatory stimuli were only injected into the right hind paws of the rats. However, neither formalin nor CFA treatments produced any significant sided differences in the expression level of either the NK-1 receptor or BDNF genes. Therefore, the mRNA levels are
reported as hippocampal bilateral averages. Significant decreases in the intensity of BDNF mRNA in situ hybridization signals were observed in the CA1-CA3 pyramidal cells of the hippocampus after either formalin or acute immobilization (Fig 7A). Furthermore, BDNF gene expression was also down-regulated in the dentate gyrus granule cell layers after acute immobilization, whereas formalin had no significant effect on BDNF gene expression within this subregion. Following the same acute pain or stress stimuli, NK-1 receptor gene expression was decreased in similar fashion across the hippocampal subregions except for the CA2 layer, where no changes were observed (Fig 7B). Long-term pain or stress treatments (CFA-induced persistent inflammatory noiception over 21 days or 10 days of immobilization) also evoked significant decreases in both BDNF (Fig 7C) and NK-1 (Fig 7D) mRNA in situ hybridization signals in the CA1-CA3 subregions. Nevertheless, no changes in gene expression in response to either of the chronic stimuli were observed in the granule cells of the dentate gyrus layer (the same cell layer where alterations in neurogenesis took place).

Discussion

Clinical reports estimate that over half of chronic pain patients also display symptoms of clinical depression, yet little emphasis has been placed by pain neurobiologists on studying the mechanisms contributing to the emotional component of pain or its impact on perception and cognitive function. The physiologic basis for the comorbidity of chronic pain and depressive illnesses is not well understood, although various studies have indicated that stress can have profound effects on the hippocampus, one of the main modulators of affect. Stress-induced alterations in gene expression, inhibited neurogenesis, increased atrophy, and loss of hippocampal neurons are considered to be some of the main neurologic events underlying the pathophysiology of depression. The results of this study demonstrate that peripheral nociception also affects the hippocampus and induces biomolecular changes that could help further characterize the affective-cognitive aspects of pain, including the relationship between pain and mood, individual coping strategies, and formation of memories about painful stimuli.

Pain and stress are known activators of the HPA axis, and stimulation of this system may contribute to the hippocampal plasticity addressed in this study. Direct comparison between inflammatory nociception and stress models used in these experiments indicated that injection of formalin or 45 min of immobilization stimulated comparable increases of HPA axis activity, as demonstrated by blood CORT levels (Fig 2). Chronic immobilization stress also enhanced CORT levels similarly to those following acute treatments. Corticosterone measurements were only made immediately after the last immobilization on the 10th day; it is not known whether the HPA axis was continuously stimulated over the entire time course. Complete Freund’s adjuvant induces a delayed immune system–modulated inflammation that develops several hours after the injection but persists for days. However, CORT levels were not significantly altered at any time point measured, including after the 21 days of repeated (3 weekly CFA treatments) nociceptive stimulation. These findings could be interpreted in two ways. First, the time points when CORT was measured may have been temporally inadequate to detect a transient change in serum CORT, and meaningful conclusions about the effects of elevated serum CORT on the measured end points cannot be drawn. Second, these data imply that a continuously hyperactivated HPA axis is not a major contributor to the long-term neurochemical changes in the hippocampus during persistent pain. Importantly, clinical evidence suggests that HPA axis dysfunction and disrupted negative feedback mech-
Anisms, rather than hyperactivity, may be common factors underlying the comorbidity of chronic pain and depression. In the context of activation of higher brain centers, the models of pain and stress used in this study significantly differ in terms of the application and physiologic location of the stimulus; CFA-induced nociceptive processes originate in the periphery, whereas immobilization has a more direct effect on the limbic system. Therefore, it was important to assess the effects of these two fundamentally different stimuli on pain-related alterations of thermal and mechanical behavioral sensitivity. When compared with the baseline levels measured before any treatment, a single CFA treatment produced robust thermal and mechanical hyperalgesia within the first 2 days after the injection (Fig 3A and B). These initially hypersensitive animals started to display attenuated sensitivity by day 14 and were returning to baseline levels by the end of the 21-day treatment time course. In contrast, rats that received 2 additional weekly CFA injections displayed continuous hypersensitivity over the full 3 weeks, so this model of chronic pain (3x CFA within 21 days) was used in subsequent experiments as a model of robust persistent inflammatory pain.

Although stress-induced analgesia is a well-documented phenomenon, the chronic immobilization stress paradigm used in this experiment did not produce any significant changes in either thermal or mechanical sensitivity of the hind paws. Thus, mechanisms of descending inhibition or facilitation are probably not robustly activated by these immobilization stress paradigms. However, it is not known whether the stress-like activation of the central nervous system (CNS) by CFA-induced nociception contributes to a latent activation of descending modulatory systems during CFA-induced hyperalgesic behaviors.

The rate of production of new neurons in the adult mammalian hippocampus is one of the key neurologic processes thought to contribute to the cognitive abnormalities often present in patients with mood disorders. Hippocampal neurogenesis can be increased through voluntary exercise, electroconvulsive seizures, antidepressant drug treatments, or various environment enrichment conditions, including social interactions, learning, and memory formation. Furthermore, hippocampal neurogenesis is sensitive to, and can be downregulated by, other factors, such as stress, glucocorticoids, and normal aging processes. Complex neuronal networks connect this limbic structure to other brain regions, such as the thalamus and the amygdala, suggesting that the hippocampus may indirectly receive nociceptive inputs from the periphery, primarily via spinothalamic and parabrachial ascending pathways. Therefore, in this experiment we investigated whether inflammatory nociception, like immobilization, evoked similar morphologic changes within the hippocampus.

The rate of neurogenesis was quantified by counting BrdU-positive cells in the hippocampal dentate gyrus 24 h after the administration of BrdU. Results demonstrated that exposure to either acute nociception (formalin) or acute stress (a single 45-min immobilization) did not alter the number of BrdU-labeled cells relative to the controls (Fig 5A). Previous reports have shown that acute exposures to certain types of stress can decrease hippocampal neurogenesis. Failure to observe such effects after the acute paradigms used in this study could be attributed to differences in the intensity of the stimuli, as well as to the discrepancies in species and age of

**Figure 6.** Representative autoradiographic images show coronal sections of caudal hippocampus assayed for either (A) BDNF or (B) NK-1 receptor mRNA in situ hybridization signals after nociceptive or immobilization stress treatments. Gene expression was measured in 4 different hippocampal subregions: CA1, CA2, CA3, and dentate gyrus (DG).
test animals and to the timing of the BrdU administration and labeling procedures. When animals were exposed to either prolonged nociception (CFA for 21 days) or stress (10 days of repeated immobilization), the number of BrdU-positive cells in the dentate gyrus was significantly decreased (Fig 5B). The most robust down-regulation of cell proliferation occurred in the subgranular zone, and to a lesser extent in the hilus of the hippocampus. Although CFA injections were unilateral (right hind paw), the neurodegenerative effects on the hippocampus were bilateral, similar to the bilateral responses previously observed at the level of gene expression.15 Most of the BrdU-positive cells were grouped in clusters with irregularly shaped nuclei, both of which are characteristics of immature cells. In this study we did not address the survival and differentiation of newly formed cells; however, previous reports indicate that the majority (75%-85%) of surviving BrdU-positive cells eventually express neuronal markers and have physical characteristics of normal adult neurons.37 Therefore, the pain-induced decreases in hippocampal cell proliferation observed in this study suggest a likely down-regulation of neuronal proliferation (neurogenesis).

The therapeutic effects of antidepressant drugs have been previously attributed to increases in proliferation of neuronal progenitor cells through mechanisms involving up-regulation of hippocampal BDNF levels.33,34,50 Furthermore, studies demonstrating antidepressive properties of NK-1 receptor antagonists38 have provided evidence that these neuromodulators may be important in the context of the processing of emotional aspects of chronic pain within higher brain centers. Our previous studies15 have suggested that painful or stressful stimuli alter the expression of NK-1 receptor and BDNF genes in the hippocampus, but these experiments did not address the anatomic localization of these changes. The complexity of the hippocampal formation network makes possible region-specific responses to painful or stressful stimuli.

Therefore, to directly address the issue of anatomic location of activity-induced changes in hippocampal gene expression in this study, BDNF and NK-1 receptor mRNA levels were determined using in situ hybridization analyses.3,45 The results show that either nociception or immobilization induced significant decreases in both BDNF and NK-1 gene expression similar in magnitude to those produced by the acute treatments. However, neither chronic nociception nor immobilization had any effect on gene expression in the dentate gyrus. *P < .05; significantly different from control group (ANOVA and Student-Newman-Keuls’ post-hoc test; n = 5/group).
neurogenesis occurs, and BDNF has been previously shown to be involved in the regulation of neurogenesis. However, the relationship between BDNF and hippocampal neurogenesis is still not fully characterized, and it is possible that early attenuation of BDNF, as seen in the acute pain and stress models, may have lasting consequences that affect rates of cellular proliferation at later time points.

The differences between the effects of acute vs chronic treatments on NK-1 receptor mRNA levels in the dentate gyrus may result from a discrepancy between high levels of NK-1 receptors and a relatively low density of SP-immunopositive fibers in this subregion of the hippocampus. Nonetheless, the complexity of hippocampal cellular architecture and neuronal connectivity may allow fluctuations in gene expression in one subregion to induce cellular and morphologic alterations in neighboring sublayers. However, current findings provide additional evidence that BDNF and NK-1 receptors are involved in pain- or stress-evoked plastic changes in the CNS, particularly within the limbic system. In addition, the down-regulation of BDNF and NK-1 receptor genes in the hippocampus by persistent noiception is considerably different from the activity-dependent pattern of up-regulated gene expression we have previously described in the spinal cord. These genes may have different roles as modulators of affect in the limbic system following pain compared with their function as important neuromediators in the development of central sensitization in the more “traditional” sensory components of the CNS.

Conclusion
The reduced hippocampal neurogenesis and decreased BDNF and NK-1 receptor gene expression evoked in models of persistent pain or stress suggest that pain and stress converge in higher brain centers and have damaging modulatory effects on “nonsensory” regions of the brain such as the limbic system. Besides providing further insights into the comorbidity of chronic pain and clinical depression, these findings may reflect novel identification of mechanisms contributing to the affective component of pain. Most importantly, the results of this study reveal novel insights into the cellular pathophysiology of the effects of chronic pain in the hippocampus.

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