Cellular/Molecular

Windup in Dorsal Horn Neurons Is Modulated by Endogenous Spinal μ-Opioid Mechanisms

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The μ-opioid receptor (MOR) plays a critical role in morphine analgesia and nociceptive transmission. However, the physiological roles for endogenous MOR mechanisms in modulating spinal nociceptive transmission, and particularly in the enhanced excitability of spinal nociceptive neurons after repeated noxious inputs, are less well understood. Using a MOR gene knock-out (−/−) approach and an MOR-preferring antagonist, we investigated the roles of endogenous MOR mechanisms in processing of acute noxious input and in neuronal sensitization during windup-inducing stimuli in wide dynamic range (WDR) neurons. Extracellular single-unit activity of WDR neurons was recorded in isoflurane-anesthetized $MOR^{-/-}$ and wild-type C57BL/6 mice. There were no significant differences between the genotypes in the responses of deep WDR cells to acute mechanical stimuli, graded electrical stimuli, and noxious chemical stimuli applied to the receptive field. Intracutaneous electrical stimulation at 1.0 Hz produced similar levels of windup in both genotypes. In contrast, 0.2 Hz stimulation induced significantly higher levels of windup in $MOR^{-/-}$ mice compared with the wild-type group. In wild-type mice, spinal superfusion with naloxone hydrochloride (10 mM, 30 μl) significantly enhanced windup to 0.2 Hz stimulation in both deep and superficial WDR cells. A trend toward facilitation of windup was also observed during 1.0 Hz stimulation after naloxone treatment. These results suggest that endogenous MOR mechanisms are not essential in the processing of acute noxious mechanical and electrical stimuli by WDR neurons. However, MORs may play an important role in endogenous inhibitory mechanisms that regulate the development of spinal neuronal sensitization.

Key words: **μ-opioid receptor; spinal cord; wide dynamic range neuron; pain; neuronal plasticity; transgenic mice**

Introduction

The μ-opioid receptor (MOR) modulates nociceptive processing along the pain signaling pathway and plays a critical role in morphine analgesia (Hurley and Hammond, 2000, 2001; Trafton et al., 2000; Kieffer and Gaveriaux-Ruff, 2002). Noxious stimuli activate dorsal horn neurons primarily through release of glutamate and peptide neurotransmitters from presynaptic terminals (Smullin et al., 1990; Riedel and Neeck, 2001). Intense noxious inputs also trigger release of opioid peptides into the dorsal horn from multiple sources (Yaksh and Elde, 1981; Basbaum and Fields, 1984; Iadarola et al., 1986; Song and Marvizon, 2003). Among them, endomorphins and enkephalins are important for modulating pain transmission, mainly through activating an MOR-mediated antinociceptive system (Cesselin et al., 1989; Zadina et al., 1997; Martin-Schild et al., 1998; Snyder, 2004; Trafton and Basbaum, 2004). Therefore, a dynamic balance between excitatory and MOR-mediated inhibitory mechanisms may determine the level of neuron excitability and fine-tune the efficiency of spinal pain transmission (Basbaum, 1999).

A state of dorsal horn neuronal hyperexcitability (central sensitization) may underlie persistent pain and hyperalgesia (Woolf and Thompson, 1991; Woolf, 1994; Amantea et al., 2000; Ji and Woolf, 2001), yet its cellular and molecular mechanisms remain to be established (Woolf and Salter, 2000; Melzack et al., 2001). Wide dynamic range (WDR) neurons in the dorsal horn represent an important component in spinal pain transmission and a target for opioid actions (Homma et al., 1983; Collins et al., 1984; Willcockson et al., 1986; Wang et al., 1996; You et al., 2003). Importantly, WDR neurons are readily sensitized by intense noxious inputs, and they exhibit a frequency-dependent, progressive increase in the neuronal excitability in response to repeated electrical activation of afferent C-fibers (windup) (Mendell and Wall, 1965; Herrero et al., 2000). Although windup is different from central sensitization (Woolf, 1996; Magerl et al., 1998), it mimics some characteristic features of central sensitization and may share some fundamental mechanisms (Li et al., 1999; Herrero et al., 2000). Importantly, windup also occurs during natural stimulation of C-fibers (Schouenborg and Sjolund, 1983) and hence represents an important experimental tool for exploring mechanisms of activity-dependent spinal neuronal plasticity. Although excitatory and amplification mechanisms underlying spinal pain processing and neuronal plasticity have been studied extensively (Dickenson and Sullivan, 1987; Woolf and Thompson, 1991;

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Randic et al., 1993; Liu and Sandkühler, 1998), the role of endogenous MOR mechanisms in these processes is unclear.

Endogenous opioids may act as neurotransmitters in the CNS, and endogenous MOR mechanisms may regulate CNS neuronal excitability (Bramham, 1992; Beregovoi et al., 2003; Meilandt et al., 2004). Using an MOR gene knock-out approach and an MOR-preferring antagonist, we examined the involvement of endogenous MOR mechanisms in the responses of WDR neurons to acute noxious inputs and in regulating the dynamic responsiveness of WDR neurons during windup stimulation. Our study suggests that elimination of MORs or spinal blockade of MOR activation had no significant impact on processing of acute noxious inputs in WDR neurons but significantly facilitated windup.

Materials and Methods

Animals. Age-matched (16–36 weeks old) wild-type and $MOR^{-/-}$ male mice (C57BL/6, 25–35 g) were used in these experiments. The investigator who performed the neurophysiologic recordings was blinded to the genotype conditions. Mice deficient in the MOR gene were generated by gene targeting as described previously (Sora et al., 1997). Embryonic stem cells were used to obtain germ-line transmission, and all tissues in the resulting mice were deficient in the MOR gene. The genomic status was monitored by PCR to confirm the presence of the knock-out gene marker. Mice received food and water *ad libitum* and were kept in a 12 h light/dark cycle in microisolator cages. The $MOR^{-/-}$ mice were fertile and were not distinguishable by their appearance and behavior from wild-type mice. All procedures were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee as consistent with the National Institutes of Health *Guide for the Use of Experimental Animals* that ensured minimal animal use and discomfort.

Surgical preparation for neurophysiological recordings. Mice were initially anesthetized with sodium pentobarbital (70–80 mg/kg, i.p.; Sigma, St. Louis, MO) to maintain areflexia to sensory stimuli (e.g., no withdrawal reflexes, no corneal reflex) during surgery. The trachea was cannulated (1.0 mm outer diameter, 0.6 mm inner diameter; Harvard Apparatus, South Natick, MA) to enable controlled ventilation. A laminectomy was performed at vertebral levels T12–L1 corresponding to lumbar enlargement at spinal segments L3–S1. Mice were placed in a stereotaxic frame, and the vertebral segments were clamped to stabilize the spinal cord. The dura mater was incised and retracted longitudinally. All exposed tissue was covered with warm agar (1.5%), except the recording segments of the spinal cord column that were continually bathed in a pool of warm saline (37°C). Core body temperature was maintained in the normal range (36.0–37.0°C) with a circulating hot-water pad. During neurophysiological recording, mice were paralyzed with pancuronium bromide (0.15 mg/kg, i.p.; Elkins-Sinn, Cherry Hill, NJ) with intermittent injection given as needed (0.05 $mg \cdot {}^{kg-1} \cdot h^{-1}$, i.p.) to facilitate controlled ventilation and to eliminate muscular contractions during electrical stimulation. Mechanical ventilation was delivered by a small animal ventilator (model 683; Harvard Apparatus) and was set at a rate of 130 cycles/min with a stroke volume of 0.2–0.3 ml. Inhalation anesthesia was maintained with a constant level of isoflurane (1.5%; Abbott Laboratories, North Chicago, IL) in a mixture of room air and 100% O_2 (1:1) throughout the experiment, which was deemed sufficient to retain a state of complete areflexia in rodent (Zuurbier et al., 2002; Cuellar et al., 2005a,b). The volatile anesthetic allows us to maintain a long period of anesthesia at a stable depth, without breathing complications or notable changes in systemic circulatory parameters (Szczesny et al., 2004). The concentration of isoflurane (<1.5%) has been shown to have minimal influence on spinal nociceptive synaptic transmission and dorsal horn neuronal plasticity (Antognini and Carstens, 1999; Rygh et al., 2000; Benrath et al., 2004; Cuellar et al., 2005a,b). Importantly, isoflurane was always kept at constant concentration for the duration of the experiment, and the same concentration (1.5%) was used in all groups. Electrocardiogram was monitored throughout the experiment. A sufficient depth of anesthesia was monitored during the experiment and judged from the areflexia to sensory stimuli (e.g., no withdrawal reflexes, no corneal reflex) in the unparalyzed state and the absence of gross fluctuations of heart rate under isoflurane anesthesia, which was maintained at a normal range of 450–500 beats/min during muscular paralysis (Szczesny et al., 2004). Room temperature was kept at 22°C. Mice were killed at the conclusion of each experiment by an overdose of sodium pentobarbital (300 mg/kg, i.p.).

Dorsal horn neuron recording. Extracellular recordings of single dorsal horn neuronal activity with defined receptive fields (RFs) in the plantar region of the hindpaw were obtained by using fine-tip (<1.0 μm) paralyn-coated tungsten microelectrodes (8 mΩ at 1 kHz; Frederick Haer Company, Brunswick, ME). The microelectrode was advanced using a hydraulic micropositioner (model 650 D; David Kopf Instruments, Tujunga, CA). Neural activity was amplified, filtered (high pass, 300 Hz; low pass, 30 kHz) (model DAM80; World Precision Instruments, Sarasota, FL), audio monitored (Grass AM8 audio monitor; Grass Instruments, West Warwick, RI), and displayed on an oscilloscope. A real-time computer-based data acquisition and processing system (DAPSYS 4; Brian Turnquist, Johns Hopkins University, Baltimore, MD) provided window discriminators for real-time sorting of different action potential (AP) waveforms (for details, see http://www.dapsys.net). Once a neuron was isolated, its amplitude was optimized by moving the electrode in the dorsoventral plane. Recordings were made from single neurons whose amplitude could be easily discriminated from background and other units, if present. Waveforms passing a selected threshold level were saved for off-line analysis. Depth of recording site was estimated from the microdrive coordinates reading, which has been shown to be comparable with that confirmed histologically (Martin et al., 2001; Weng et al., 2001).

Experimental design. Using an MOR gene knock-out approach, we first examined the neurophysiologic responses of deep WDR neurons to acute mechanical stimuli in $MOR^{-/-}$ and wild-type mice. In the initial electrophysiological studies, we focused on phenotyping responses of WDR neurons located in the deep laminas of the lumbar dorsal horn (segment L4–L5) to allow comparisons with other studies and, importantly, to avoid potential pitfalls in interpretation attributable to possible differences in the neurophysiological properties of superficial and deep WDR cells (Schouenborg and Sjolund, 1983; Mokha 1992; Herrero et al., 2000; Eckert et al., 2003). Deep WDR cells were identified according to depth (350–700 μm) ranging from spinal lamina III to V in mice, as well as their characteristic responses (Martin et al., 2001; Weng et al., 2001). Mechanical search stimuli ranged from mild to noxious and consisted of stroking the plantar skin with a cotton swab and mild pinching with the experimenter's fingers. The cutaneous RFs of mechanosensitive units were mapped with a suprathreshold von Frey monofilament (4.0 g; Stoelting, Wood Dale, IL). A single site (most sensitive site) near the center of the RF was then chosen for application of a series of mechanical test stimuli applied in the following order: brushing with a small camel-hair brush, indentation of the plantar skin with von Frey monofilaments (0.09, 0.17, 0.42, 0.90, 1.25, 1.65, 2.5, 4.0, 6.0, 9.0, and 12.0 g) applied in an ascending order with an interstimulus interval of 15 s, and pinching with calibrated serrated forceps (6 N/mm^2). The intensities of the mechanical stimuli applied ranged from non-noxious to noxious and are based on previous behavioral studies conducted on the same strain of mice (Mansikka et al., 2004). Each mechanical stimulus was applied for 3 s. Noxious mechanical stimulus with serrated forceps was not used repeatedly to avoid sensitizing nociceptors. Audible tones generated by the DAPSYS software provided cues for accurate manual delivery of the mechanical stimuli. Neurons were classified functionally according to their responses evoked by graded intensities of mechanical stimuli applied to the receptive field (Menetrey et al., 1977; Li et al., 1999; Weng et al., 2001). Specifically, neurons that responded in a graded manner with increasing firing rates to the stimulus range from non-noxious to noxious intensity were classified as WDR cells. On average, two to three WDR neurons per animal were examined for the mechanical responses. Before the application of test stimuli, the spontaneous activity of WDR neurons was counted for 3 s and subtracted from the response obtained after each stimulus. Neurons that displayed high levels of spontaneous activity (>5 Hz) were not investigated in this study because of the difficulty in obtaining a stable neuronal response to peripherally applied mechanical stimuli.

We then characterized responses of deep WDR cells to graded intracutaneous electrical stimuli and to windup-inducing stimuli in a subgroup of animals including both genotypes. Electrical search stimulus (3.0 mA, 2 ms, constant current; model DS3; Digitimer, Hertfordshire, UK) was applied through a pair of fine needles inserted subcutaneously at the central area of the hindpaw. In our study, the mean distance between the stimulation site and the recording electrode was 7.3 ± 0.2 cm. Based on the axon conduction velocities, WDR neuronal responses to single intracutaneous electrical stimulus (3.0 mA, 2 ms) were separated as a short latency A-fiber-mediated responses (0–40 ms, excluding stimulus artifact, A-component) and a long-latency C-fiber-mediated responses (40–250 ms, C-component) (see Fig. 3*A*). With some neurons, a prolonged period of firing occurs after the C-fiber latency band, which was considered as afterdischarge (250–1000 ms). This A- and C-fiber latency range is comparable with that used by others (Suzuki et al., 2003). The stimulus intensity–response functions (S–R function) of A- and C-fiber-mediated responses were determined by application of intracutaneous electrical stimuli in steps (0.02, 0.05, 0.1, 0.2, 0.3, 0. 4, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mA, 2 ms). The intensity that evoked at least one spike within the C-fiber latency range was considered the C-fiber threshold. The responses evoked by A- and C-fibers were quantified by separating the action potential firing on a latency basis, as described above. WDR neurons were further assessed for their ability to show action potential windup phenomenon to repeated computer-controlled intracutaneous electrical stimuli that are suprathreshold for C-fiber activation. Three stimulus trains (16 pulses, 3.0 mA, 2ms, constant current) of increasing frequency (0.1, 0.2, and 1.0 Hz) were applied to the receptive field with a 10 min interval between each trial.

We also determined acute responses of deep WDR neurons to mustard oil-induced noxious chemical stimulation in a subgroup of randomly assigned animals including both genotypes. WDR neurons with RFs on plantar skin of the hindpaw were identified. Before application of mustard oil, warm-heat stimuli (42 and 51°C) were delivered in an ascending order by ipsilateral hindpaw immersion (5 s) into a preheated water bath (interstimulus interval of 90 s). Room temperature was set at 22°C. Therefore, WDR neurons that were recruited in the mustard-oil test were also confirmed to be thermal-responsive units that responded with increasing firing rates to stimuli ranging from warm (42°C) to noxious (51°C) heat. To avoid sensitization of neurons induced by repetitive noxious thermal stimuli, investigation of endogenous MOR mechanisms in thermal nociception was not pursued in this study. Once a thermal-responsive WDR cell was isolated, a small piece of cotton compress (2 × 2 mm) soaked with mustard oil (3-isothiocynato-prop-1-ene; Sigma) diluted to 50% with mineral oil was then applied for 5 s over the receptive field located at plantar area of the hindpaw. The baseline spontaneous activity and evoked responses of WDR cells to mustard oil were then counted as the number of action potentials for 1 min before application and for a total of 10 min after application, respectively. No additional cell was studied, and no additional test was conducted after mustard-oil application.

In a second study, we conducted pharmacological experiments in a separate group of wild-type mice to examine the effects of spinal superfusion with naloxone hydrochloride on the neurophysiologic responses of both deep (depth of 350–700 μm) and superficial (depth of <350 μm) WDR neurons to electrical stimulation. The optimal dose and pharmacokinetics for spinal application of naloxone to block endogenous opioid actions on MOR was not known in mice. In unblinded pilot experiments, we observed that the effect of naloxone (1, 10, and 20 mM, 30 μl) on windup was detected as early as 10 min after application and lasted for at least 45 min. The neuronal response returned to the pre-naloxone baseline level 45 min after washing out with saline. The specificity of drug effects and actions of drug on deep WDR cells were also verified in the pilot study. Spinal application of morphine (0.5 mM, 30 μl) was found to attenuate windup in a subgroup of deep WDR cells encountered ($n = 3$). This inhibitory effect of morphine on windup substantially diminished at 45–60 min after washing out with saline. Posttreatment with spinal naloxone (1 mM, 30 μl) before the recovery reversed the inhibition of morphine. The drug dose (10 mM, 30 μl) used in the present study was based on previous observations using intrathecal injection of naloxone to block endogenous and/or exogenous activation of MOR in the spinal cord in rat (Wongchanapai et al., 1998; Hu et al., 1999; Palea and Pietra, 1999; Sluka et al., 1999; Rygh et al., 2000; Yu et al., 2002; Hayashida et al., 2003; Watanabe et al., 2003) and was confirmed by our multiple-dosing pilot study to be effective and suitable for the current study. The S–R function of A- and C-fiber-mediated responses and windup phenomena to repetitive stimuli of 0.2 and 1.0 Hz were assessed before drug application. Naloxone or vehicle was applied directly onto the exposed surface of the spinal cord at the recording section 15 min after the predrug control tests. The tests were then repeated in the same sequence from 15 to 45 min after drug application. Because the drug was applied topically on the dorsal aspect of the spinal cord and left in place, this approach creates a constant concentration bath at the recording segment and provides a desirable period of controlled drug condition for various testing. Stock solutions were freshly prepared by dissolving naloxone in 0.9% sterile saline, and the pH was neutralized. Saline was used as a drug control. The postdrug responses were compared with the predrug treatment conditions. Because the drug redistribution and rate of metabolism/elimination was not known under the current experimental conditions, only one neuron was studied in each animal.

Data analysis. The stored digital record of unit activity was analyzed off-line. The number of action potentials evoked by acute mechanical, chemical, or graded electrical stimuli was compared between the genotypes, using a two-way mixed-model ANOVA with Fisher's protected least significant difference (LSD) *post hoc* test. For analysis of windup, the primary parameter studied was the number of APs in the C-component responses evoked by each stimulus in a train of repetitive electrical stimulation. Because the number of APs in the C-component varies between WDR neurons, the raw data for each cell were normalized with 100% representing the response to the first stimulation in each trial (input). The normalized responses among cells were then averaged. Windup graphs were then created by plotting the normalized values against the stimulation number in a train of 16 stimuli. A two-way mixed-model ANOVA (Fisher's protected LSD *post hoc* test) was used to compare windup at each frequency tested and to compare the average number of C-component responses for the last 10 stimuli for the three different frequencies of stimulation in the two genotypes. After naloxone application, the first stimulus in each train (input) produced a variable number of APs in the C-component response of WDR neurons. In some WDR neurons, the C-component response was completely blocked at the beginning of the stimulation. Therefore, the number of APs was used to analyze windup phenomenon in the pharmacological study. Windup graphs were created by plotting the number of APs against the stimulation number in a train of 16 stimuli. A two-way repeated-measures ANOVA with Fisher's protected LSD *post hoc* test was used to compare group difference between predrug and postdrug conditions in wild-type mice. In addition, a two-way mixed-model ANOVA (Fisher's protected LSD *post hoc* test) was used to compare input and average number of C-component for the last 10 stimuli between superficial and deep WDR cells. In instances in which *t* tests were used for specific analysis, all comparisons were made using Bonferroni's correction. Data are presented as mean ± SEM. $p < 0.05$ was considered significant.

Results

There were no significant differences in mean recording depths of deep WDR neurons between $MOR^{-/-}$ and wild-type mice in each test category. These recording depths are comparable with that of deep WDR cells studied by others (Martin et al., 2001; Weng et al., 2001).

Responses of deep WDR neurons to mechanical stimulation in $MOR^{-/-}$ and wild-type mice

Quantitative mechanical testing was performed on 21 WDR neurons in $MOR^{-/-}$ mice (depth, 567 ± 26 μm) and 17 WDR neurons (depth, 577 ± 23 μm) in wild-type mice with receptive fields on the glabrous skin of the hindpaw. Spontaneous activity was not common, and WDR neurons showed typical increased re-

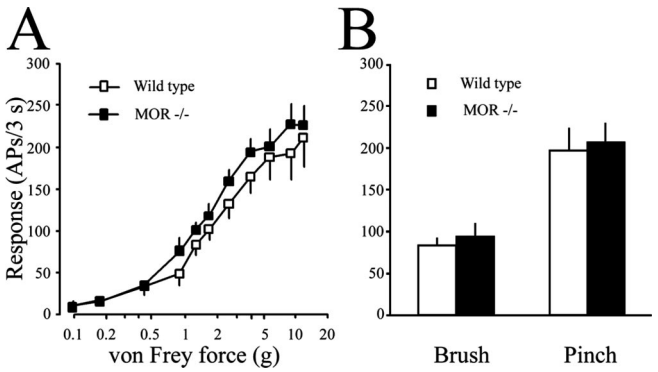

Figure 1. Responses of deep WDR neurons in the spinal cord to mechanical stimuli were not altered in $MOR^{-/-}$ mice. ***A***, Deep WDR neurons in $MOR^{-/-}$ mice ($n = 21$) showed stimulus intensity–response functions similar to those of wild-type mice ($n = 17$) to brief punctuate mechanical stimuli (0.09–12.0 g von Frey probe, 3 s) applied to the cutaneous receptive field. ***B***, The mean evoked responses of deep WDR neurons to brush and pinch stimuli (3 s) in $MOR^{-/-}$ mice were not significantly different from that in wild-type mice. Data are expressed as mean ± SEM.

sponses to increasing intensities of acute mechanical stimuli in both groups. The S–R functions to graded punctate mechanical stimuli (0.09–12.0 g, von Frey probe, 3 s) in $MOR^{-/-}$ mice were similar to that in wild-type mice (Fig. 1*A*). There were no significant differences in the mean number of action potentials evoked by graded punctate mechanical stimuli, brushing, and noxious pinching between the two genotypes (Fig. 1*A*,*B*).

Responses of deep WDR neurons to noxious chemical stimulation in $MOR^{-/-}$ and wild-type mice

The baseline spontaneous activity level during the 1 min period before mustard-oil application was not significantly different between deep WDR cells in $MOR^{-/-}$ (depth, 468 ± 40 μm; $n = 6$) and wild-type (depth, 469 ± 20 μm; $n = 5$) mice. The overall pattern of response to mustard oil (50%, 5 s) was similar between the two genotypes (Fig. 2*B*). Mustard oil induced an initial barrage of activity in WDR neurons in both groups, usually occurring at 1–3 min after application to the receptive field, followed by a gradual decay in ongoing firing rate toward baseline (Fig. 2*A*). In both groups, a 10 min observation period covered the initial peak response and the early ongoing discharge evoked by mustard-oil application. Peak response frequencies were calculated by constructing peristimulus time histograms (bin width, 1 s) for each cell and selecting the maximum value. The peak frequencies (wild type, 28.6 ± 9.6 APs/s, $n = 5$; $MOR^{-/-}$, 32 ± 2.5 APs/s, $n = 6$) and the total response (after subtracting 10 × baseline spontaneous activity) during the first 10 min period after application (wild type, 2280 ± 245 APs; $MOR^{-/-}$, 2638 ± 289 APs) were not significantly different between the two genotypes. Furthermore, the two groups did not differ after application in the mean responses at each minute from 0 to 10 min (Fig. 2*B*). The ongoing responses of most WDR neurons remained at an elevated level (>200% of baseline spontaneous activity) at 10 min after application in both $MOR^{-/-}$ (six of six) and wild-type (four of five) mice.

Responses of deep WDR neurons to graded electrical stimulation in $MOR^{-/-}$ and wild-type mice

Deep WDR neurons in wild-type (depth, 538 ± 34 μm; $n = 13$) and $MOR^{-/-}$ (depth, 554 ± 34 μm; $n = 14$) mice showed similar S–R functions of A-fiber-mediated and C-fiber-mediated responses to graded intracutaneous electrical stimuli (Fig. 3*B*).

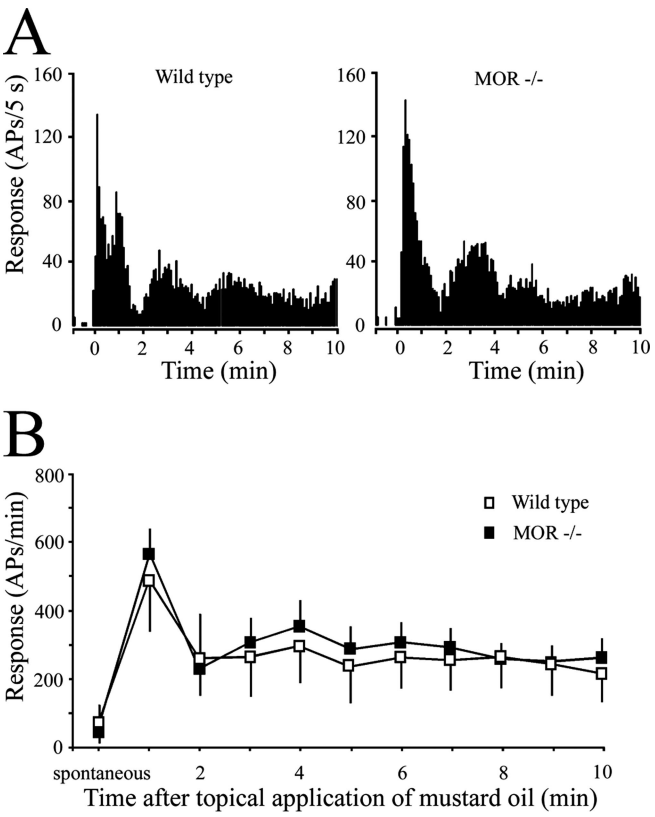

Figure 2. Acute response of deep WDR neurons to noxious chemical stimuli was not altered in $MOR^{-/-}$ mice. ***A***, Peristimulus time histograms showed examples of representative responses of single WDR neurons in a wild-type mouse (left) and in a $MOR^{-/-}$ mouse (right) during the first 10 min after application of mustard oil (50%, 5 s) to the receptive field (bin size, 5 s). ***B***, The mean response evoked by mustard oil during each minute from 0 to 10 min after topical application were not significant different between $MOR^{-/-}$ ($n = 6$) and wild-type ($n = 5$; $p > 0.05$) groups. Data are expressed as mean ± SEM.

There were no significant differences between the two genotypes in the mean numbers of APs in the A-component and C-component, respectively, at each intensity tested (0.02–5.0 mA) (Fig. 3*B*). In addition, there were no significant differences in threshold for activation of a C-fiber-mediated response (wild type, 0.89 ± 0.16 mA; $MOR^{-/-}$, 0.84 ± 0.21 mA) and in latency of the first C-fiber-mediated AP (at C-fiber activation threshold) between the two groups (wild type, 98.9 ± 7.7 ms; $MOR^{-/-}$, 88.6 ± 9.1 ms).

Windup of C-fiber-mediated responses of deep WDR neurons in $MOR^{-/-}$ and wild-type mice

In wild-type mice, WDR neurons showed a progressive increase in the number of C-fiber-mediated responses (windup) (Fig. 4*A*) to a train of electrical stimuli applied at 1.0 Hz (Fig. 4*D*) but not to stimulation applied at 0.1 or 0.2 Hz (Fig. 4*B*,*C*). Repetitive stimulation applied at 1.0 Hz induced similar patterns of windup (slope and peak level) in the two genotypes (Fig. 4*D*). Compared with the C-component response to the first stimulus (input), the average C-component response for the last 10 stimuli were significantly increased during both 0.2 Hz ($p < 0.05$) and 1.0 Hz ($p < 0.01$) stimulation in $MOR^{-/-}$ mice ($n = 14$), but it was increased significantly only during 1.0 Hz stimulation in wild-type mice ($n = 13$; $p < 0.01$) (Fig. 4*E*). There was no significant difference in the average C-component response for the last 10 stimuli during 1.0 Hz stimulation between the two genotypes (wild type, 173 ± 23%; $MOR^{-/-}$, 179 ± 29%) (Fig. 4*E*). In

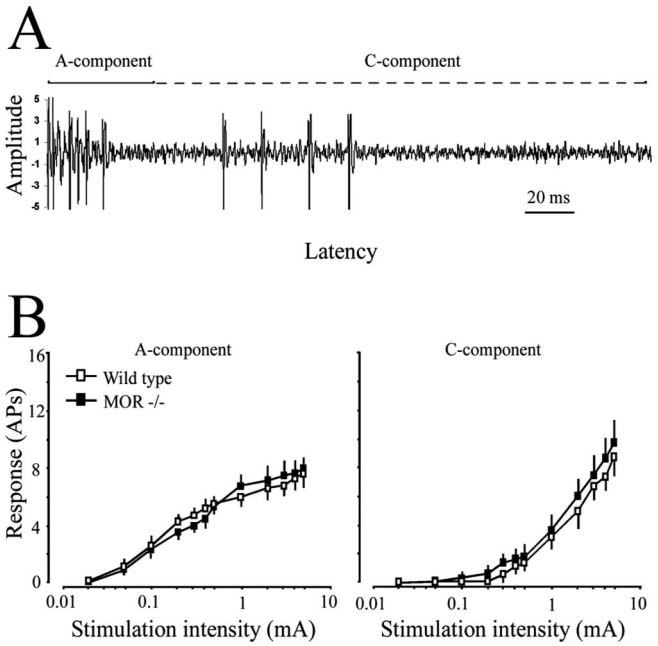

Figure 3. Responses of deep WDR neurons to graded electrical stimuli were not altered in $MOR^{-/-}$ mice. ***A***, An example of an analog recording of a deep WDR neuron displaying A-fiber- and C-fiber-mediated responses to a single intracutaneous electrical stimulus (3.0 mA, 2 ms). A- and C-component of WDR neuronal responses were separated by the early (0–40 ms) and late (40–250 ms) latencies, respectively. ***B***, There were no significant differences in stimulus intensity–response functions of A-fiber-mediated and C-fiber-mediated responses, respectively, to the graded electrical stimuli (0.02–5.0 mA, 2 ms) between $MOR^{-/-}$ ($n = 14$) and wild-type ($n = 13$) mice. Data are expressed as mean ± SEM.

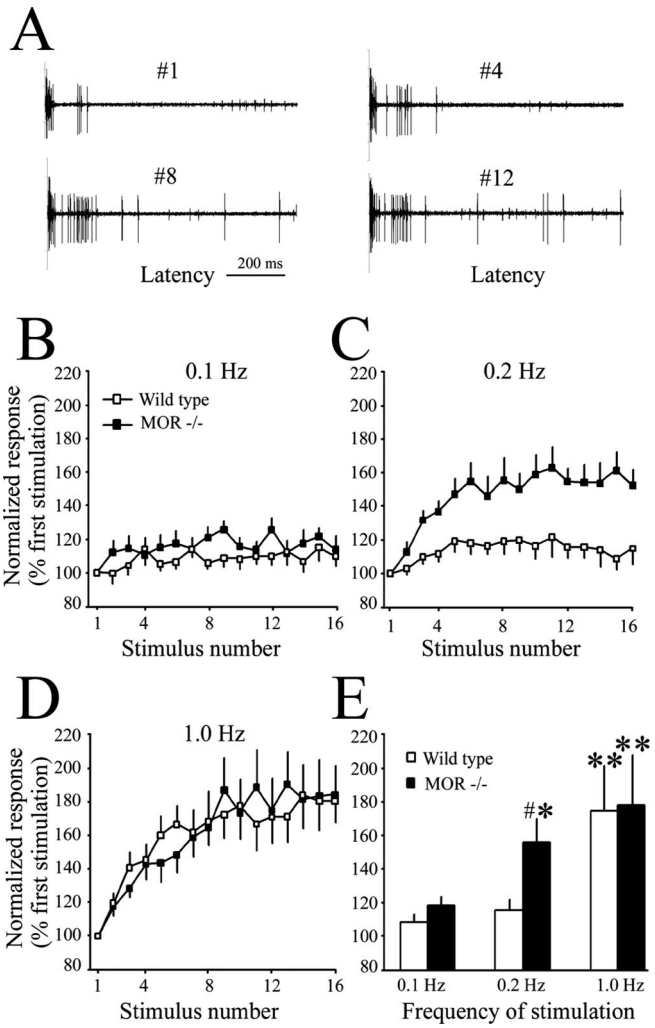

Figure 4. Windup of C-fiber-mediated responses to repeated electrical stimuli was enhanced in $MOR^{-/-}$ mice. ***A***, An example of a deep WDR neuron in wild-type mouse displaying progressive increase in C-fiber-mediated responses (windup) to a train of electrical stimuli (3.0 mA, 2 ms) applied at a frequency of 1.0 Hz. Sequence number of the repetitive stimuli are indicated. ***B–D***, C-component responses of deep WDR neurons to repetitive electrical stimulation applied at a frequency of 0.1 Hz (***B***), 0.2 Hz (***C***), and 1.0 Hz (***D***), respectively, in $MOR^{-/-}$ (filled squares) and wild-type (open squares) mice. ***E***, The average C-fiber-mediated responses for the last 10 stimuli during the repetitive electrical stimulation were plotted for the various frequencies. Stimulation at 1.0 Hz induced a significant level of windup in both genotypes compared with the baseline input (**$p < 0.01$). Notably, stimulation at 0.2 Hz also induced a significant level of windup in $MOR^{-/-}$ mice ($n = 14$; *$p < 0.05$) but not in wild-type mice ($n = 13$). During 0.2 Hz stimulation, the average C-component response for the last 10 stimuli were significantly higher in $MOR^{-/-}$ mice than that in wild-type mice ($^{\#}p < 0.05$). Data are normalized by the response evoked by the first stimulation of each trial.

contrast, it was significantly higher in $MOR^{-/-}$ mice than that in wild-type mice during 0.2 Hz stimulation ($p < 0.05$; wild type, 116 ± 7%; $MOR^{-/-}$, 155 ± 12%) (Fig. 4*E*). Similar results were observed when data were analyzed between electric pulses 5 and 16.

Effects of spinal application of naloxone on responses of WDR neurons to graded electrical stimuli in wild-type mice

In a different group of wild-type mice, we further examined the effects of blocking MOR activation with spinal topical application of naloxone hydrochloride (10 mM, 30 μl). The response to graded intracutaneous electrical stimuli (0.02–5.0 mA, 2 ms) of WDR neurons located at superficial ($n = 14$; depth, 200 ± 15 μm) and deep ($n = 12$; depth, 427 ± 22 μm) layers of the dorsal horn were studied. Population S–R functions of A-fiber- and C-fiber-mediated responses to graded electrical stimuli were similar between pre-naloxone and post-naloxone conditions in superficial (Fig. 5*A*,*B*) and deep (Fig. 5*C*,*D*) WDR cells. There were no significant differences in the mean number of APs in the A-component and C-component, respectively, at each stimulus intensity tested between pre-naloxone and post-naloxone conditions in both populations of WDR cells (Fig. 5). Five deep WDR cells and four superficial WDR cells showed a transient increase in spontaneous firing rate at 1–5 min after naloxone application that lasted ~1–2 min with a gradual decrease to baseline. Saline application, used as a drug control, did not affect the S–R functions of A-fiber- and C-fiber-mediated responses to electrical stimulation ($n = 7$; depth, 463 ± 29 μm) (Fig. 5*C*,*D*, insets) or the spontaneous activity of these WDR neurons. A different picture emerged when effects of naloxone on individual WDR neuronal responses were analyzed. After spinal application of naloxone, we observed variable effects (i.e., enhancement, attenuation, or no change) in the total number of APs in the C-component evoked by graded electrical stimulation from 0.02 to 5 mA. Among 12 deep WDR units studied before and after naloxone treatment, four cells showed an increase and five cells showed a decrease in the C-fiber-mediated response that was >25% of the pre-naloxone value. The remaining three cells showed no remarkable changes (<25% change from the pre-naloxone value). In 14 superficial WDR cells treated with naloxone, five cells showed an increase and six cells showed a decrease in the C-component that was >25% of the pre-naloxone value. The remaining three cells showed no remarkable changes. Application of saline did not affect the response properties of any WDR

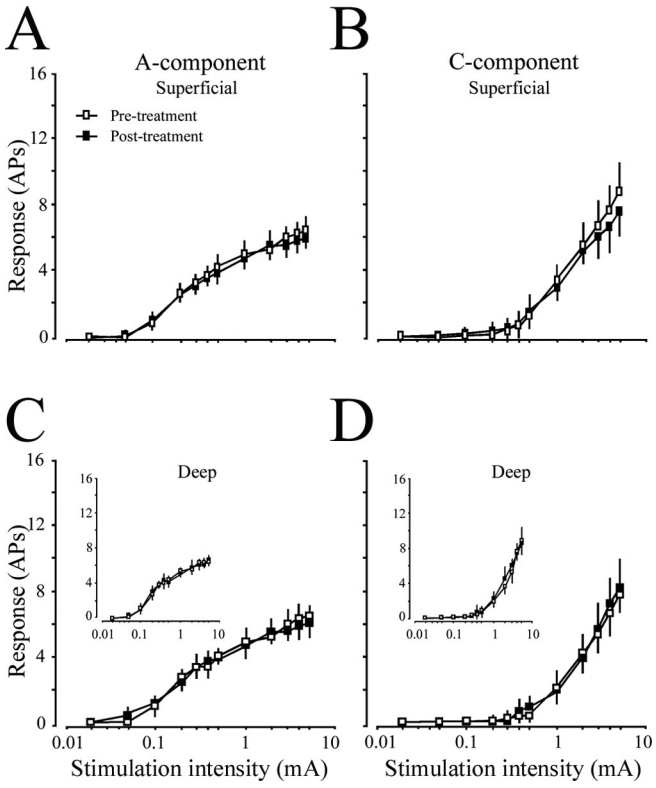

Figure 5. Spinal application of naloxone did not change population responses of superficial and deep WDR neurons to graded intracutaneous electrical stimuli in wild-type mice. There were no significant differences in population stimulus intensity–response functions of A-fiber-mediated (***A***) and C-fiber-mediated (***B***) responses of superficial WDR neurons (depth, <350 μm) to graded electrical stimulation (0.02–5.0 mA, 2 ms) applied in the receptive field between pre-naloxone and post-naloxone conditions (10 mM, 30 μl) in wild-type mice ($n = 14$; $p > 0.05$). In a separate group of wild-type mice ($n = 12$), the population stimulus intensity–response functions of A-fiber-mediated (***C***) and C-fiber-mediated (***D***) responses of deep WDR neurons (depth, 350–700 μm) were not significantly different between pre-naloxone and post-naloxone conditions (10 mM, 30 μl; $p > 0.05$). Saline did not affect stimulus intensity–response functions of A-fiber- and C-fiber-mediated responses in deep WDR neurons (insets in ***C*** and ***D***; $n = 7$; $p > 0.05$). Data are expressed as mean ± SEM.

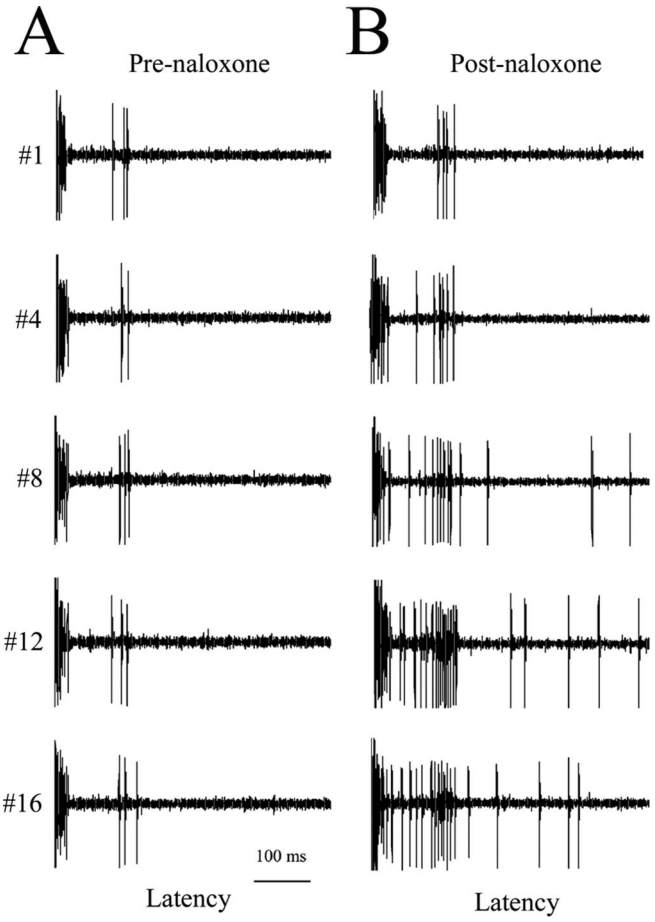

Figure 6. Example of the facilitatory effect of naloxone on the induction of windup during repeated electrical stimuli at 0.2 Hz in wild-type mice. ***A***, An analog recording of responses of a deep WDR neuron in a wild-type mouse to a train of intracutaneous electrical stimuli (16 pulses, 3.0 mA, 2 ms) delivered at a frequency of 0.2 Hz before spinal application of naloxone (10 mm, 30 μl). ***B***, The same WDR unit displays remarkable progressive increase in C-fiber-mediated responses (windup phenomenon) and shows afterdischarges to the same stimulation at 30 min after spinal naloxone administration. Sequential numbers of the repetitive stimuli was also indicated as above.

neuron studied: no unit showed change in total C-component response that was >25% of the pre-saline value.

Effects of spinal application of naloxone on windup of C-fiber-mediated responses of WDR neurons in wild-type mice

Detailed analysis of effects of spinal administration of naloxone (10 mM, 30 μl) on windup profile was performed in the same group of superficial and deep WDR neurons in wild-type mice. There was no significant difference in the C-component induced by the first stimulus of each trial (input) between the pre-naloxone and post-naloxone condition in either superficial or deep WDR neurons (see Fig. 7). In deep WDR cells ($n = 12$), 0.2 Hz stimulation induced a progressive increase in the number of C-fiber-mediated responses (windup) after, but not before, naloxone administration (Fig. 6). Before naloxone application, the average C-component response for the last 10 stimuli was significantly increased only during the 1.0 Hz stimulation ($p < 0.01$) (Fig. 7*F*). In contrast, after naloxone treatment, the average C-component responses for the last 10 stimuli were significantly increased during both 0.2 and 1.0 Hz stimulation ($p < 0.01$) (Fig. 7*E*,*F*). Importantly, there was a significant increase in the average C-component for the last 10 stimuli during the 0.2 Hz stimulation after naloxone treatment compared with that evoked at the pre-naloxone condition ($p < 0.01$) (Fig. 7*E*). Although the average C-component for the last 10 stimuli during 1.0 Hz stimulation was not significantly different between the pre-naloxone and post-naloxone conditions, there was a trend that windup reached the peak level faster after naloxone pretreatment, suggesting a facilitated induction.

Similar drug effects of naloxone on windup were also observed in superficial WDR cells ($n = 14$). Before naloxone treatment, the average C-component for the last 10 stimuli was significantly higher than the input only during 1.0 Hz stimulation ($p < 0.05$) (Fig. 7*F*). However, after naloxone treatment, the average response of the C-component for the last 10 stimuli was significantly increased during both 0.2 and 1.0 Hz stimulation ($p < 0.01$) (Fig. 7*E*,*F*). Similar to that of deep WDR cells, the average C-component for the last 10 stimuli during 0.2 Hz stimulation in superficial WDR cells was also significantly increased after naloxone treatment compared with the pre-naloxone value ($p < 0.05$) (Fig. 7*E*). Saline had no significant impact on windup of WDR cells at either frequency tested ($n = 7$). These findings suggest that spinal superfusion with naloxone enhanced windup in both deep and superficial WDR cells (Figs. 6, 7).

There was no significant difference in the input between su-

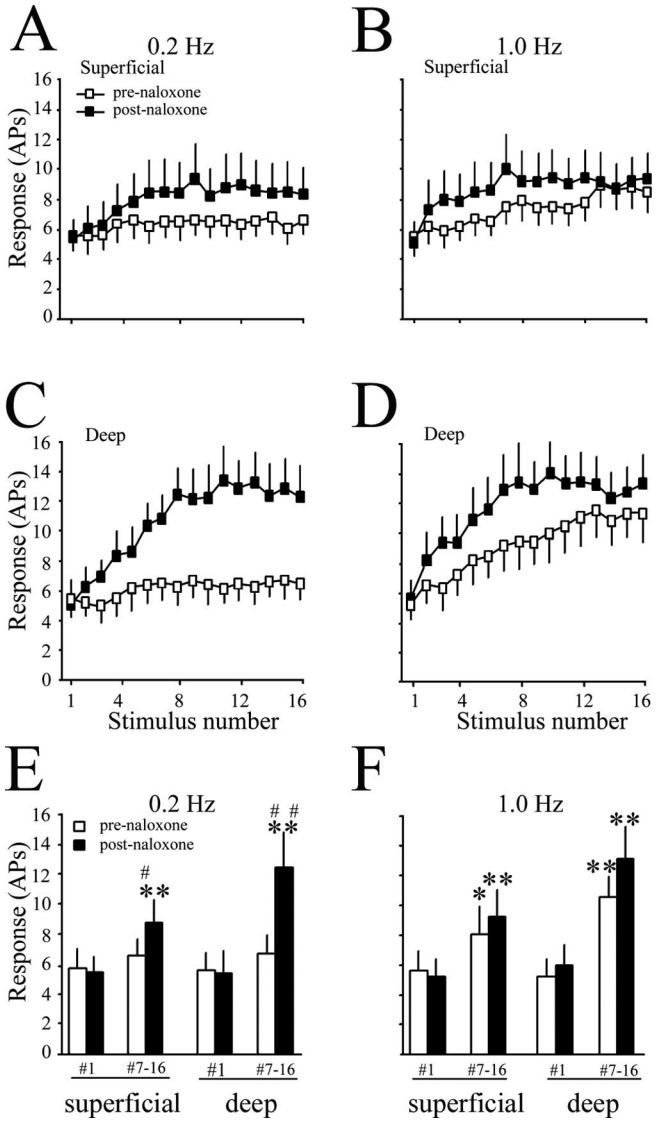

Figure 7. Spinal superfusion with naloxone facilitated the development of windup in superficial and deep WDR neurons in wild-type mice. ***A–D***, The C-component responses to repetitive electrical stimulation (16 pulses, 3.0 mA, 2 ms) are plotted as a function of stimulus number. Average responses from superficial (***A***, ***B***; $n = 14$) and deep (***C***, ***D***; $n = 12$) WDR neurons are plotted for stimulus repetition at frequencies of 0.2 Hz (***A***, ***C***) and 1.0 Hz (***B***, ***D***). Responses before (open squares) and after (filled squares) spinal topical application of naloxone (10 mM, 30 μl) are shown. ***E***, ***F***, Histograms show the mean number of average C-fiber-mediated responses for the last 10 stimuli and for the first stimulus of each trial (input) during 0.2 Hz (***E***) and 1.0 Hz (***F***) stimulation. Repeated stimulation resulted in significant windup phenomenon at both 0.2 and 1.0 Hz in both superficial and deep WDR cells after naloxone treatment ($^{*}p < 0.05$, $^{**}p < 0.01$ compared with the input). Naloxone facilitated the development of windup in both superficial and deep WDR cells. The average C-component responses for the last 10 stimuli during 0.2 Hz but not 1.0 Hz stimulation were significantly increased after naloxone treatment (filled bars) in both superficial and deep WDR cells compared with pre-naloxone responses (open bars) ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the pre-naloxone responses). Data are expressed as mean ± SEM of responses evoked by each stimulus.

perficial and deep WDR neurons at pre-naloxone and post-naloxone conditions. Although not significant, there was a trend that levels of windup to 1.0 Hz stimulation, as indicated by the average C-component for the last 10 stimuli of the trial, were higher in deep WDR neurons than that in superficial ones at both pre-naloxone and post-naloxone conditions (Fig. 7). This phenomenon is consistent with previous findings that a more remarkable windup was observed in deep WDR cells than in superficial ones (Schouenborg and Sjolund, 1983; Mokha, 1992; Herrero et al., 2000).

Discussion

We observed the following: (1) the absence of MOR does not affect responses of deep WDR neurons to acute mechanical stimulation, noxious chemical stimulation, and graded electrical stimulation at A- to C-fiber intensities; (2) spinal naloxone pretreatment in wild-type mice does not change the population S–R functions of A- and C-components of superficial and deep WDR neurons to graded electrical stimulation; and (3) systemic elimination of MORs by targeted gene deletion or spinal naloxone pretreatment in wild-type mice enhanced windup.

The finding that the encoding of acute noxious and non-noxious mechanical stimuli by deep WDR neurons is not altered in $MOR^{-/-}$ mice is in line with previous behavioral observations: $MOR^{-/-}$ mice showed normal pain behavior to acute noxious stimuli (Fuchs et al., 1999; Mansikka et al., 2004). However, psychophysical studies and animal pharmacological experiments yield conflicting results on the effect of naloxone on acute pain perception (El-Sobky et al., 1976; Buchsbaum et al., 1977; Anderson et al., 2002). The group effect of naloxone on the response of WDR neurons to graded electrical stimulation was not significant. However, variable effects were observed in individual cells. The enhancement of C-component could result from the antagonistic effects of naloxone on MORs and/or non-opioid receptors, including relieving a GABA-mediated tonic inhibition (Dingledine et al., 1978). The paradoxical inhibitory effect may result from a partial agonistic effect and/or an interaction with κ-opioid receptors (Vaccarino et al., 1992; Bianchi and Panerai, 1993). Other factors, including location, network properties, and tonic modulations on the specific neuron studied, may ultimately determine the net effect of naloxone (Mokha, 1992). Future study is needed to carefully investigate roles of endogenous MOR mechanism in thermal nociception, because inconsistent observations have been reported (Sora et al., 1997; Matthes et al., 1998; Mansikka et al., 2004).

Mustard oil causes a pure neurogenic inflammation and prolonged activation of C-fiber nociceptors (Reeh et al., 1986; Woolf and Wall, 1986). Unexpectedly, response profiles of WDR neurons to mustard oil were similar between the two genotypes (Fig. 2). $MOR^{-/-}$ mice showed a similar degree of nocifensive behavior as wild-type mice during the first phase of Formalin-induced pain, which primarily results from a direct activation of nociceptive afferents (Zhao et al., 2003). Furthermore, neither naloxone (intraperitoneally) nor CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) (intrathecally) affected the initiation and magnitude of pain behavior in this phase (Wu et al., 2002; Zhao et al., 2003). Intense noxious drive associated with mustard-oil application or intraplantar Formalin injection may trigger release of opioids and activate MOR in the dorsal horn. However, a ceiling effect on neuronal activation by intense noxious chemical stimulation may override MOR-mediated endogenous inhibition and preclude the identification of a potential disinhibition component in $MOR^{-/-}$ mice. In fact, MOR-mediated inhibition of neuronal excitability was revealed after initial extensive noxious drive faded: naloxone (intrathecally or intravenously) and CTOP (intrathecally) significantly rekindle the mustard oil-induced increase in electromyographic activity (Yu et al., 1994; Tambeli et al., 2001).

Stimulation at 0.1 and 0.2 Hz did not induce windup in wild-type mice. This is consistent with previous findings that induction of windup in rodents requires C-fiber activation at frequen-

cies >0.3 Hz under normal conditions (Gozariu et al., 1997; Herrero et al., 2000). An important observation is that the average C-component for the last 10 stimuli during 0.2 Hz stimulation was significantly higher in $MOR^{-/-}$ mice than that in wild-type mice, suggesting an enhanced windup (Fig. 4). The enhancement is unlikely attributable to compensatory changes after deleting the MOR gene, because no significant compensatory changes in the production/release of major neurotransmitters or in the activation mechanisms and functional properties of their receptors, or in major intracellular signaling pathways have been reported in $MOR^{-/-}$ mice under normal conditions (Kieffer and Gaveriaux-Ruff, 2002). Importantly, windup was also enhanced in a similar manner in wild-type mice after spinal naloxone pretreatment (Figs. 6, 7). This pharmacological evidence not only suggests that enhanced windup may result from drug effects at the receptor level but also indicates that an important substrate for activation of endogenous MOR mechanisms involves a spinal site of action.

Windup reflects short-term activity-dependent increase of central neuron excitability to repetitive noxious inputs. In light of an important role of WDR neurons in spinal pain transmission (Willis, 1985; Simone et al., 1991; Herrero et al., 2000), activation of MOR-mediated inhibition may counteract the development and attenuate the level of neuronal hyperexcitability after intense noxious drive. In fact, an increase in spinal nociceptive reflex was more evident in the $MOR^{-/-}$ mice than that in wild-type mice after peripheral nerve injury (Mansikka et al., 2004). The second phase of Formalin-induced pain, which recruits spinal sensitization, was also significantly potentiated in $MOR^{-/-}$ mice (Sugimoto et al., 1986; Zhao et al., 2003) and in wild-type mice after pretreatment with MOR antagonists (Zhao et al., 2003). Naloxone also induced a dose-dependent increase in windup like phenomenon in withdrawal reflexes (Hartell and Headley, 1991; Ramos-Zepeda and Herrero, 2005).

Spatial recruitment of nerve fibers in proximity to the stimulation electrodes is assumed to remain unchanged over time because constant-current stimulation was used. Therefore, it is unlikely that facilitation of windup reflects changes in peripheral terminal excitability or properties of the C-fiber afferents. Windup does not occur in DRG neurons, and the segmental topical application of naloxone unlikely affects the DRG neuron directly. Accordingly, enhanced windup may reflect changes in the central synapses of C-fibers and/or the postsynaptic neurons. Windup is thought to be a centrally mediated phenomenon that reflects temporal summation of slow cumulative depolarizations mediated by NMDA and tachykinin neurokinin-1 receptors on the secondary neurons (Dickenson and Sullivan, 1987; Dubner and Ruda, 1992; Budai and Larson, 1996). Endogenous opioids may limit the duration and/or amount of neurotransmitter released from primary afferent neurons and/or excitatory interneurons through activation of presynaptic μ-opioid autoreceptors (Yaksh et al., 1980; Hori et al., 1992; Trafton et al., 1999). Therefore, facilitated windup could result from an enhanced summation of excitatory synaptic inputs on secondary neurons by lifting the prominent presynaptic inhibition of neurotransmitter release. Because activation of postsynaptic MOR by endogenous opioids leads to membrane hyperpolarization (Yoshimura and North, 1983; Ruda et al., 1984; Glaum et al., 1994), enhanced windup in $MOR^{-/-}$ animals may result from the absence of MORs on the somatodendritic region of WDR neurons. However, it is unclear whether endogenous opioids released during 0.2 Hz stimulation are able to induce postsynaptic MOR signaling.

We observed a trend of more pronounced windup induced in deep WDR cells than in superficial ones, supporting the notion that the capacity of windup is different between deep and superficial WDR cells (Schouenborg and Sjolund, 1983; Mokha, 1992; Herrero et al., 2000). Nevertheless, spinal naloxone pretreatment enhanced windup in both populations of WDR cells, suggesting an endogenous opioidergic modulation on sensory processing at both sites. MORs are concentrated in superficial layers and with a relatively lower concentration found in deeper layers (Besse et al., 1990; Kieffer and Gaveriaux-Ruff, 2002). As discussed above, the effects of naloxone on superficial dorsal horn neurons may be mediated via presynaptic or postsynaptic mechanisms. In addition, deep WDR neurons may be functionally connected with superficial cells. Therefore, drug effects observed in the responses of deep cells may also reflect action at superficial cells.

Like many biological functions, the magnitude of the windup is a sigmoidal function: stimulus frequencies <0.3 Hz cannot induce windup in normal animals. As frequency increases, windup increases until it reaches a plateau level, which normally occurs at ~1–2 Hz (Schouenborg, 1984; Herrero et al., 2000). Removal of MOR-mediated inhibition may move this sigmoidal function to the left. Thus, the response at the high frequencies is not affected (i.e., stays at the plateau level), but the frequency needed to obtain an effect decreases. However, the functions between various stimulation parameters (frequency, intensity, and pulse width) and windup are complicated (Herrero et al., 2000). The relationship between spatial summation and temporal summation is also complex and inadequately characterized. Future studies using other stimulation parameters are needed to confirm the optimal experimental conditions for revealing roles of endogenous opioidergic system in windup. Finally, one should exercise caution when comparing results from studies in $MOR^{-/-}$ animals and pharmacological studies. Although naloxone-induced enhancement in windup may primarily result from its preferential block of MORs, it could also come from its non-MOR antagonistic effects: attenuating inhibition mediated by GABA (Dingledine et al., 1978) and/or δ-opioid receptor (Goldstein and Naidu, 1989; Ossipov et al., 1996). Windup is highly influenced by supraspinal descending modulation (Herrero and Cervero, 1996a,b; Herrero et al., 2000). The differential effects of systemic deletion of MOR versus spinal block of opioid receptors on descending modulation of windup are not clear. These questions need to be addressed in future studies using spinalized animal preparation and highly specific antagonists to different opioid receptor subtypes.

We suggest that endogenous MOR mechanisms are not essential in the processing of noxious mechanical and electrical stimuli by WDR neurons under normal conditions. However, MORs may be functionally activated and mediate an important endogenous inhibitory mechanism modulating dorsal horn neuronal excitability after repetitive noxious inputs. It may represent a compensatory mechanism to counteract the excitatory mechanisms that elicit central hyperexcitability and pathological pain syndromes after tissue or nerve injury (Hao et al., 1998; Mansikka et al., 2004).

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