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Glial TLR4 signaling does not contribute to opioid-induced depression of respiration

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Abstract

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Opioids activate glia in the central nervous system in part by activating the toll-like receptor 4 (TLR4)/myeloid differentiation 2 (MD2) complex. TLR4/MD2-mediated activation of glia by opioids compromises their analgesic actions. Glial activation is also hypothesized as pivotal in opioid-mediated reward and tolerance and as a contributor to opioid-mediated respiratory depression. We tested the contribution of TLR4 to opioid-induced respiratory depression using rhythmically active medullary slices that contain the pre-Bötzinger Complex (preBötC, an important site of respiratory rhythm generation) and adult rats in vivo. Injection with DAMGO (μ-opioid receptor agonist; 50 μM) or bath application of DAMGO (500 nM) or fentanyl (1 μM) slowed frequency recorded from XII nerves to 40%, 40%, or 50% of control, respectively. This DAMGO-mediated frequency inhibition was unaffected by preapplication of lipopolysaccharides from *Rhodobacter sphaeroides* (a TLR4 antagonist, 2,000 ng/ml) or (+)naloxone (1–10 μM, a TLR4-antagonist). Bath application of (−)naloxone (500 nM; a TLR4 and μ-opioid antagonist), however, rapidly reversed the opioid-mediated frequency decrease. We also compared the opioid-induced respiratory depression in slices in vitro in the absence and presence of bath-applied minocycline (an inhibitor of microglial activation) and in slices prepared from mice injected (ip) 18 h earlier with minocycline or saline. Minocycline had no effect on respiratory depression in vitro. Finally, the respiratory depression evoked in anesthetized rats by tail vein infusion of fentanyl was unaffected by subsequent injection of (+)naloxone, but completely reversed by (−)naloxone. These data indicate that neither activation of microglia in preBötC nor TLR4/MD2-activation contribute to opioid-induced respiratory depression.

Keywords: opioid, pre-Bötzinger complex, rat, respiratory depression, toll-like receptor

THE ANALGESIC EFFECTS OF OPIOIDS, which result from their activation of classical opioid receptors in the

central nervous system (CNS), underlie their widespread clinical use. Unwanted side effects include opioid tolerance, dependence, reward, hyperalgesia, and perhaps most serious in acute terms, opioid-induced respiratory depression, which can be life-threatening. This is particularly problematic in premature infants with immature respiratory networks and unstable breathing (18); it contributes significantly to the challenge of providing adequate pain control in the pediatric population (22, 78). Opioid-induced respiratory depression is counteracted clinically with opioid receptor antagonists [e.g. (−)naloxone], but this also counteracts analgesia. Thus there is a need for pharmacological approaches to alleviate opioid-induced respiratory depression without loss of analgesia (18, 58, 62, 63). Greater understanding of the mechanisms by which opioids depress breathing would greatly facilitate the development of such approaches. Respiratory depression is primarily dependent on activation of μ-opioid receptors (64), however, the location, cell type, and cellular mechanism(s) are incompletely defined. The direct actions of opioids on μ-opioid receptors expressed by respiratory neurons in a brainstem region critical for generating inspiratory rhythm, the pre-Bötzinger Complex (preBötC) (3, 16, 51), are considered a major contributor to this respiratory depression. However, recent data indicate that in addition to their action on classical opioid receptors, opioids also act by binding to and activating the toll-like receptor 4 (TLR4)/myeloid differentiation 2 (MD2) complex (25), which is expressed almost exclusively on glia (microglia and astrocytes) (82).

TLR4 is an innate, immune-pattern-recognition receptor that is activated by endogenous danger signals and opioids, leading to activation of microglia that then induce activation of astrocytes (82). Activated glia release a variety of compounds including proinflammatory cytokines [i.e., tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-18] and other immune mediators [i.e., brain-derived neurotrophic factor (BDNF), prostaglandin E_2, and nitric oxide] that in turn influence neuronal excitability and plasticity in a variety of neural networks including hippocampus (12), spinal cord (11, 33, 39, 42, 87, 88), dorsal horn (8), and ventromedial medulla (83), by regulating gene transcription and translation. In addition, recent studies demonstrating TLR involvement in neurogenesis, and learning and memory in the absence of underlying infectious etiology suggest that TLR signaling can rapidly influence network activity independent of major inflammatory processes (21).

The net effect of opioids on CNS function therefore reflects the activation of classical neuronal opioid receptors and TLR4 (and perhaps other TLRs) on glia. Accumulating evidence suggests that opioid-induced TLR4-mediated glial activation actually opposes opioid analgesia and contributes to many of the negative side effects of opioids such as tolerance, dependence, and reward (82); involvement in opioid hyperalgesia controversial (13). Most relevant to this study is the demonstration in rats in vivo that the microglia attenuator, minocycline, reduces the opioid-induced depression of breathing (23). If glia and TLR4 signaling contribute to the respiratory depressant actions of opioids, stereoselective antagonism of opioid activation of TLR4 with (+)naloxone (which blocks TLR4 but not opioid receptors) could provide a pharmacological means of reducing the respiratory depressant actions of opioids without altering analgesia.

The objectives of this study were therefore to determine the contribution of glia or TLR4 signaling to the opioid-mediated depression of central respiratory networks and whether acute TLR4 activation can modulate respiratory network activity. We examined the effects on the opioid-induced respiratory depression of LPS-RS (lipopolysaccharides from *Rhodobacter sphaeroides*) (a TLR4 antagonist), (−)naloxone (an opioid receptor antagonist that blocks opioid receptors and TLR4), (+)naloxone (which blocks TLR4 but not opioid receptors), and minocycline (an agent that inhibits proinflammatory microglial activation). This was performed both in vitro using rhythmically active medullary slices in which opioid actions were limited to the slice or just the preBötC, and in vivo to reproduce the clinically relevant setting in which opioids can act throughout the CNS to depress breathing.

METHODS

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All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Alberta Animal Ethics Committee.

Rhythmic Medullary Slices

Medullary slices that generate rhythmic inspiratory-related activity were prepared with methods similar to those described previously (44, 66, 69). Briefly, neonatal Sprague-Dawley rats of either sex [postnatal day 0–4 (P0–P4)] were anesthetized through inhalation of isoflurane and decerebrated. The brainstem–spinal cord was then isolated in cold artificial cerebral spinal fluid (aCSF) containing the following (in mM): 120 NaCl, 3 KCl, 1.0 $CaCl_2$, 2.0 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 20 D-glucose, equilibrated with 95% O_2/5% CO_2. The brainstem–spinal cord was pinned to a wax chuck, and serial sections (100–200 μm) were cut in the rostral-to-caudal direction using a vibratome (VT1000S; Leica, Nussloch, Germany) and transilluminated to identify anatomical landmarks as described elsewhere. Structure of the subnuclei of the inferior olive was particularly useful in defining this boundary (66). One rhythmic, transverse, 700-μm-thick medullary slice was cut with the preBötC at the rostral surface of the slice, ~0.35 mm caudal to the caudal aspect of the facial nucleus (44, 66, 69). Slices were pinned with the rostral surface up on Sylgard resin in a recording chamber (volume 5 ml) and perfused with aCSF that was recirculated at a flow rate of 15 ml/min. The concentration of K^+ in the aCSF ($[K^+]_e$) was raised from 3 to 9 mM at least 30 min before the start of data collection. Slices generate rhythmic inspiratory-related activity at 3 mM $[K^+]_e$ that lasts 1–2 h (66). The majority of protocols in this study involved multiple interventions, and therefore required slices that produced stable inspiratory-related rhythm for extended periods (i.e., >5 h). Therefore, the $[K^+]_e$ was raised from 3 to 9 mM to produce prolonged, stable rhythm (66).

Electrophysiological Recordings

Inspiratory activity was recorded using glass suction electrodes (A-M Systems, Carlsborg, WA) from cut ends of XII (hypoglossal) nerve rootlets and directly from the ventrolateral surface of the slice. Surface recordings were made to guide drug injections into the preBötC (75). Signals were amplified, bandpass-filtered (100 Hz to 3 kHz), full-wave rectified, integrated using a leaky integrator (τ = 25 or 50 ms), and displayed using Axoscope 9.2 (Molecular Devices, Union City, CA). Data were saved to a computer using a Digidata 1322 A/D board and AxoScope 9.2 software (Molecular Devices) for off-line analysis. All recordings were conducted at room temperature (22–24°C).

Drugs and Their Application

Lipopolysaccharides (LPS) from *Escherichia coli* (LPS, serotype 0111:B4; a TLR4 agonist, 200 ng/ml microinjected in vitro) and minocycline (an inhibitor of microglia activation, 0.5 μM bath application or 45 mg/kg ip) were obtained from Sigma-Aldrich (Oakville, ON, Canada). LPS from *Rhodobacter sphaeroides* (LPS-RS; a TLR4 antagonist, 2,000 ng/ml microinjected in vitro) was obtained from InvivoGen (San Diego, CA). Fentanyl citrate (an opioid agonist, 1 μM bath-applied or 40 μg/kg iv) was obtained from Sandoz (Boucherville, QC, Canada). (+)Naloxone (a TLR4 antagonist, 10 μM bath, 3–60 mg/kg iv) was a kind gift of K.C. Rice (National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, Rockville, MD). Under in vivo conditions, (+)naloxone was used first at a concentration similar (5-fold higher) to that at which (−)naloxone is effective in antagonizing opioid actions. When this concentration was ineffective, the concentration was increased 10-fold to 30 mg/kg iv (n = 4), and finally to 60 mg/kg iv (n = 1) to ensure the lack of antagonism was not due to insufficient concentration. Drugs obtained from Tocris Bioscience included [Sar^9-$Met(O_2)^{11}$]-substance P (SP), an NK1R agonist, 1 μM microinjected in vitro; DAMGO [(D-Ala^2, NMe-Phe^4, Gly-ol^5)-enkephalin], a μ-opioid receptor agonist,

50 μM microinjected in vitro, 0.5 μM bath-applied; and (−)naloxone [(5α)-4,5-epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride], an opioid antagonist, 0.5 μM bath-applied, 0.6 mg/kg iv.

Drugs were prepared as stock solutions in aCSF and frozen in aliquots. Exceptions were (+)naloxone and (−)naloxone, which were made fresh the day of the experiment. The final concentration of K^+ in the drug solutions was matched to that of the aCSF. In slice preparations, drugs were unilaterally applied locally to the preBötC via triple-barreled pipettes (5–6 μm outer diameter per barrel) pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL). Care was taken to ensure that the outer tip diameter was within this range because fluorescent imaging (40× objective) of Lucifer yellow-filled triple-barreled pipettes established that pipettes in this range do not leak, but they do leak if the tip diameter exceeds 6.5 μm. Drug microinjections were controlled by a programmable stimulator (Master-8; A.M.P.I. Instruments, Jerusalem, Israel) connected to a picospritzer (~18 psi, Spritzer4 Pressure Micro-Injector; Bioscience Tools, San Diego, CA). Consecutive agonist applications were separated by a minimum of 15 min. We did not systematically assess whether this was the minimum time interval required for consistent responses, but it was sufficient for reproducible responses. The concentrations of drugs used in the present study should not be directly compared with those in experiments in which similar agents are applied in the bath or directly to isolated cells. The concentration of drug decreases exponentially with distance from the pipette tip, and previous experiments with this preparation have established that drug concentration in the pipette must be approximately 10-fold greater than the bath-applied concentration to produce similar effects (40). The drug injection site was established as described previously (28, 29, 44). Briefly, we first used the location of the ventral respiratory column surface suction electrode as an approximate reference in the transverse plane to the region of most intense respiratory-related activity. The response to SP (1 μM, 10 s) at this site was recorded. The drug pipette was then systematically moved in the dorsoventral and mediolateral directions until SP evoked a frequency increase that occurred within the first breath following drug onset and was at least 2-fold greater than baseline. On the basis of these criteria and the established sensitivity of preBötC networks to SP (16, 28–30, 44) we are confident that positive injections sites were in the preBötC. Once the preBötC was located, LPS, LPS-RS, or DAMGO was microinjected into the same site. All drug injection protocols included control injections of aCSF. These were always without effect.

Minocycline treatment. In one experimental series, DAMGO (50 μM, 10 s) was locally applied into the preBötC. After recovery, minocycline was bath-applied at 0.5 μM for 40 min when a second identical DAMGO application was made (57, 79). Due to the potential for time-dependent changes in DAMGO responses evoked at a 40-min interval, minocycline data were compared with time control experiments in which slices were bathed only in aCSF and DAMGO was locally applied into the preBötC twice at a 40-min interval. In a second series, neonates were injected with minocycline (45 mg/kg ip) or saline 18 h, and again 1 h prior to the isolation of the rhythmic medullary slice (59, 76, 86).

Plethysmographic recording methods. Measurements were made from adult male Sprague-Dawley rats (300–370 g). Rats were anesthetized with isoflurane (3%) in an induction chamber and maintained under isoflurane (2%) anesthesia during tail vein cannulation (P10 size tubing) for iv infusion of drugs. Anesthesia was then removed and animals were placed within a whole-body, Plexiglas plethysmograph (2,000 ml volume) modified to allow exteriorization of the tail. This allowed iv infusion of drugs via the tail vein (KD Scientific infusion pump; Hollistan MA) without disturbing the animal. The plethysmograph had inflow and outflow ports for continuous delivery of a steady flow of fresh air and removal of expired carbon dioxide. Pressure changes were recorded with a pressure transducer (DP 103; Validyne, Northridge, CA), signal conditioner (CD-15; Validyne), analog-digital board (Digidata 1322), and Axoscope software (Molecular Devices, Sunnyvale, CA). The chamber was then flushed with room air to remove any residual isoflurane, 3 min of baseline ventilation data were recorded, and fentanyl infusion was initiated. Fentanyl infusion started at least 5 min after removal of isoflurane, so all measurements were on unanesthetized

animals. It should be noted that our plethysmograph is effective for studying respiratory frequency (f_R). It is not designed for precise quantification of tidal volume (V_T, ml/g). The physical principle underlying whole-body plethysmography with this system is the detection of pressure changes in the chamber resulting from the heating and humidification of inspired gas. However, V_T measurements may also be influenced by gas compression effects related to the airway resistance. Because of these limitations, our whole-body plethysmographic system provides only semiquantitative measurements of V_T, which we report as changes relative to the baseline.

Data Analysis

For in vitro experiments, the effects of a drug on frequency of integrated inspiratory bursts recorded via suction electrodes from XII nerve roots were assessed off line using pClamp 9.2 (Clampfit) and Microsoft Excel software. Values of frequency during the drug were compared with the average value during the 2-min control period that immediately preceded drug application. The maximum effect of a drug on frequency was determined as the maximum (or minimum) value measured in the moving average of inspiratory frequency (calculation based on the average of three consecutive events) during the first minute after injection. The time course of the response was obtained by averaging data points in 30-s bins for the control period immediately before drug application, in 30-s bins for the first 2 min after the onset of drug application (5 min for minocycline treatment), and in 1-min bins for the remainder of the trial. Parameters are reported relative to control (predrug) levels, as means ± SE. Maximum effects of a drug correspond to greatest (or lowest) values of frequency obtained from the moving average of instantaneous frequency during the first 3 min following the start of opioid administration.

For in vivo experiments, inspiratory frequency was calculated from the airflow trace. Relative inspiratory V_T, reported relative to baseline levels, was calculated from the area under the airflow curve. Relative changes in minute ventilation were calculated from the product of the relative frequency and V_T data.

Unless otherwise stated, statistical comparison of means for in vitro and in vivo data was performed on raw data using GraphPad Prism version 4 (GraphPad Software, La Jolla, CA). Statistical comparison of relative data was performed only after verification of normality. Values are reported as means ± SE. Differences between means were identified using a one-way or two-way ANOVA with Bonferroni correction for multiple comparisons or a Student's *t*-test (for unpaired data). Values of $P < 0.05$ were assumed significant.

RESULTS

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TLR Antagonism Does Not Affect DAMGO-Mediated Respiratory Inhibition In Vitro

Sites that underlie the depressant actions of opioids on respiratory rhythm are not fully known, but recent data implicate the preBötC as a key site of action (50). To test whether opioid activation of TLR4-mediated signaling, which primarily occurs in microglia (25, 31, 70), contributes to the opioid-induced respiratory depression in the preBötC, we compared the effects on frequency of locally applying the μ-opioid agonist DAMGO (50 μM, 10 s) into the preBötC before and immediately after local preapplication of the TLR4 antagonist LPS-RS (2,000 ng/ml; 90 s). The injection site for DAMGO within the preBötC was established using the previously developed SP mapping protocol (28, 29, 44). It is based primarily on the functional criterion that in the rhythmic slice, SP evokes its maximum effect on frequency when it activates NK1R in the preBötC (16, 30). When SP (1 μM, 10 s) was injected into the site of maximum sensitivity (i.e., the preBötC), it produced a rapid-onset, 2.80 ± 0.58-fold increase in burst frequency that peaked within 10 s of SP application and remained significantly greater than baseline frequency until 90 s after drug onset ($n = 5$, data not shown). As observed previously (3, 16, 44, 45, 65, 73), when applied alone, DAMGO produced a significant reduction in frequency that reached a nadir at 0.32 ± 0.12 of control baseline frequency ~1 min

after drug onset (Fig. 1*A*; $n = 5$; two-way ANOVA, time factor, $P < 0.0001$). Preapplication of LPS-RS had no significant effect on the DAMGO-induced decrease in frequency (Fig. 1, *B* and *C*; $n = 5$; two-way ANOVA, LPS-RS factor, $P = 0.109$; also no significant interaction between factors time and LPS-RS, $P = 0.578$). After LPS-RS, DAMGO depressed frequency to a nadir that was 0.65 ± 0.09 of the control value. The DAMGO-evoked inhibition of frequency was repeatable after a 15-min washout period when frequency fell to 0.49 ± 0.17 of control.

We next tested the efficacy of two naloxone isomers in antagonizing the opioid-induced respiratory depression. (−)Naloxone is used commonly in clinical situations to counteract the actions of opioids. It is a nonselective antagonist that acts at both μ-opioid receptors and TLR4. Thus antagonism by (−)naloxone could reflect actions of the opioid agonist at either opioid receptors or TLR4. (+)Naloxone, on the other hand binds to TLR4 rather than μ-opioid receptors, and is useful in discriminating between the actions of opioids at the two types of receptors. We applied the μ-opioid receptor agonists DAMGO (0.5 μM; $n = 7$; Fig. 2, *A* and *C*) or fentanyl (1 μM; $n = 5$; Fig. 2, *B* and *D*) to the aCSF perfusing rhythmically active medullary slice preparations and tested the effects of bath-applying first (+)naloxone and then (−)naloxone on the respiratory depression. DAMGO ($P < 0.0001$) and fentanyl ($P = 0.025$) significantly reduced frequency to 0.32 ± 0.04 (Fig. 2*C*) or 0.68 ± 0.06 (Fig. 2*D*) of control, respectively (one-way ANOVA, multiple comparison test with Bonferroni correction). Following bath application of the TLR-selective antagonist, (+)naloxone (10 μM), the frequency in DAMGO was 0.55 ± 0.04 of control (Fig. 2*C*), whereas that in fentanyl was 0.48 ± 0.10 of control [Fig. 2*D*; i.e., (+)naloxone had no significant effect on the respiratory depression evoked by DAMGO ($P = 0.17$) or fentanyl ($P = 0.26$, one-way ANOVA, multiple comparison test with Bonferroni correction)]. In contrast, subsequent addition of (−)naloxone (0.5 μM) to the bath rapidly (within 5 min) and completely reversed the respiratory depression in vitro. Inspiratory frequency increased significantly from opioid levels, returning to 1.11 ± 0.16 or 1.33 ± 0.07 of control in slices with DAMGO ($P < 0.0001$; Fig. 2, *A* and *C*) and fentanyl (Fig. 2, *B* and *D*), respectively ($P < 0.0001$, one-way ANOVA, multiple comparison test with Bonferroni correction).

Effects of LPS on preBötC Rhythm

Although TLR4 activation does not appear to contribute to the opioid-induced depression of inspiratory rhythm in the rhythmic slice, this does not exclude the possibility that acute activation of TLR is capable of influencing inspiratory rhythm-generating networks. The hypothesis that glia and TLR4 signaling contribute to the respiratory depressant actions of opioids (23) requires a mechanism of action that can occur within minutes. To test whether TLR activation can directly modulate the activity of preBötC rhythm-generating networks on this type of time scale, we locally applied LPS to the preBötC of rhythmically active medullary slices. The injection site for LPS within the preBötC was established using the SP mapping protocol described above (28, 29, 44). In these experiments, when SP (1 μM, 10 s) was injected into the preBötC, it produced a rapid-onset, 2.07 ± 0.14-fold increase in burst frequency that peaked within 10 s of SP application and remained significantly greater than baseline frequency until 90 s after drug onset (Fig. 3*A*; $n = 4$; $P < 0.0001$, one-way ANOVA, multiple comparison test with Bonferroni correction). Local injection of LPS (200 ng/ml, 10 s) into the same site had no significant effect on inspiratory-related frequency, as shown for a representative slice (Fig. 3*B*) and group data (Fig. 3*C*; $n = 4$; $P = 0.29$, one-way ANOVA, multiple comparison test with Bonferroni correction).

Microglia and Opioid-Induced Respiratory Depression

The previous experiments exclude involvement of TLR-mediated signaling in the opioid-induced respiratory depression and demonstrate that preBötC networks are insensitive to acute TLR activation. Microglia, however, may still play a role, as suggested by the demonstration that ip injection of

minocycline attenuates a weak opioid-mediated respiratory depression in adult rats in vivo (23). To test the contribution of microglia in the opioid-mediated respiratory depression, we compared the effects on inspiratory frequency of locally applying DAMGO into the preBötC in the absence and presence of bath-applied minocycline in rhythmically active medullary slices of Sprague-Dawley rats. The injection site for DAMGO within the preBötC was established using the SP mapping protocol in which SP (1 μM, 10 s) produced a rapid-onset, 2.65 ± 0.29-fold increase in burst frequency ($n = 6$). Local injection of DAMGO (50 μM, 10 s; $n = 6$) into the same site depressed inspiratory burst frequency to 0.52 ± 0.06 of control. Following 40 min of incubation in minocycline, DAMGO was reapplied into the same site where it similarly reduced frequency to 0.52 ± 0.06 of control. Time control data using different slices ($n = 6$) showed that in naïve slices treated exactly the same way, application of DAMGO into the SP-identified preBötC (2.30 ± 0.22-fold increase) reduced inspiratory burst frequency to 0.47 ± 0.06 of control on the first application and 0.43 ± 0.06 of control on the second application 40 min later. Statistically, to take into consideration any time-dependent changes in consecutive DAMGO responses, we compared the DAMGO response in minocycline (which was the second DAMGO application in the minocycline group) with the second DAMGO response in the time control group. Data indicate that although DAMGO caused a significant depression in both groups (Fig. 4*A*; two-way ANOVA; $n = 6$; time factor $P < 0.0001$), minocycline had no significant effect on the DAMGO-induced frequency depression (Fig. 4*A*; $n = 6$; two-way ANOVA, minocycline factor, $P = 0.69$; also, no significant interaction between factors, minocycline, and time, $P = 0.071$, indicating that the time course of the DAMGO response was the same in untreated time-control slices and minocycline-treated slices).

Due to uncertainties about the time required for minocycline to inhibit microglial activation under in vitro conditions and its efficacy in arresting an activation process that may be initiated during the slicing process (82), we performed a second series of experiments in which minocycline was applied via ip injection (45 mg/kg; $n = 9$) to neonatal rats 18 h and again 1 h prior to the generation of rhythmic medullary slices. We compared the frequency response to locally applied DAMGO (50 μM, 10 s) into the preBötC of slices from control animals (different group from that depicted in Fig. 4*A*) and minocycline-pretreated animals (different control and minocycline groups). In these experiments, SP evoked a 2.23 ± 0.05 ($n = 10$) and 2.51 ± 0.16 ($n = 9$) increase in control and minocycline-treated slices, respectively (Fig. 4*C*). The time course (Fig. 4*B*) of the effects of DAMGO on inspiratory frequency is shown for the two groups. DAMGO reduced frequency significantly in both groups to 0.49 ± 0.05 and 0.47 ± 0.03 in control ($n = 10$) and minocycline-treated slices ($n = 9$), respectively (two-way ANOVA, time factor, $P < 0.0001$). However, the time course of the DAMGO inhibition was not affected by minocycline (two-way ANOVA, minocycline factor, $P = 0.2554$; there was no significant interaction between factors, minocycline ip and time, $P = 0.187$, indicating that the time course of the DAMGO response was the same in untreated control slices and slices pretreated with minocycline).

Peak and minimum frequencies evoked by SP and DAMGO, respectively, in control conditions, in bath-applied minocycline and ip-injected minocycline are shown relative to baseline (of 1.0) in Fig. 4*C*. Peak and nadir were calculated from the maximum or minimum values recorded from a moving average of three consecutive bursts during the first 3 min after DAMGO application. SP and DAMGO produced significant increases and decreases in frequency, respectively. Minocycline, however, had no effect on the DAMGO response whether bath-applied ($P = 0.2124$, *t*-test) or injected ip ($P = 0.754$, *t*-test).

(+)Naloxone Has No Effect On the Opioid-Evoked Depression of Frequency In Vivo

The previous data suggest that TLR4 signaling does not play a role in the opioid-mediated depression of breathing. However, because all data are based on responses to local injection of opioid agonists into the preBötC or bath application to the medullary slice, it remains possible that glia or TLR4 signaling

contributes to the opioid-induced respiratory depression through a region that is either outside the preBötC or not contained in the rhythmic slice. Certainly, under clinical conditions opioids are administered iv or orally where they can act throughout the CNS. In addition, because the in vitro experiments are performed on neonatal networks, it is possible that TLR4 glial signaling has not matured to the point at which it can influence respiratory activity. Therefore, as a final test of the hypothesis that TLR4 signaling contributes to the opioid-mediated depression of respiration, we compared the ability of (+)naloxone and (−)naloxone to reverse the respiratory depression produced by tail vein administration of fentanyl (40 μg/kg delivered over a 20-min period) to adult rats in vivo. The effects of fentanyl reached a stable level after ∼8 min of infusion and remained stable throughout the infusion. Fentanyl markedly suppressed respiratory frequency (to 0.53 ± 0.09 of control) and V_T (to 0.42 ± 0.08 of control). Intravenous administration of (+)naloxone (3 mg/kg, $n = 1$; 30 mg/kg, $n = 4$; 60 mg/kg, $n = 1$) began 10 min after onset of fentanyl administration. Ventilation was monitored for 8 min following the onset of (+)naloxone; it had no effect on the fentanyl-induced respiratory depression (Fig. 5, *A* and B). Respiratory frequency and V_T remained at 0.55 ± 0.12 ($P = 0.99$) and 0.45 ± 0.10 ($P = 0.97$, one-way ANOVA, Tukey's multiple comparison test) of control, respectively. In contrast, ventilation began to recover within 30 s of beginning iv administration of (−)naloxone (0.6 mg/kg; $n = 6$). Respiratory frequency and V_T increased significantly to 1.05 ± 0.07 ($P < 0.001$) and 0.94 ± 0.06 ($P < 0.001$, one-way ANOVA, Tukey's multiple comparison test) of baseline (prefentanyl) levels within minutes.

DISCUSSION

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A growing body of evidence suggests that glial activation via TLR4 opposes opioid analgesia and may contribute to many of the negative side effects of opioids (23, 25, 26, 31, 82). Respiratory depression, arguably the most serious of side effects due to its associated mortality, may also have a TLR4-mediated glial component because the microglial inhibitor, minocycline, attenuates the opioid-induced depression of breathing (23). Understanding the mechanisms underlying opioid-induced respiratory depression is essential for developing approaches to minimize the respiratory depression while maintaining opioid-mediated analgesia. We therefore tested whether a nonneuronal, nonopioid receptor-based mechanism contributes to opioid-induced respiratory depression by asking whether inhibition of TLR4 or microglial activation can reduce the respiratory depression and whether activation of TLR4 can change breathing on a time scale consistent with the opioid-mediated depression of breathing. Data from analysis of in vitro respiratory networks revealed that the inspiratory inhibition evoked by application of opioids (DAMGO and fentanyl) directly into the critical site of inspiratory rhythm generation, the preBötC, was unaffected by block of TLR4 [with LPS-RS or (+)naloxone], specifically within the preBötC or throughout the slice. In contrast, the opioid inhibition was completely reversed by (−)naloxone (which antagonizes both μ-opioid receptors and TLR4). Inhibition of microglial activation with minocycline also had no effect on the opioid-induced respiratory depression in vitro. Moreover, when fentanyl was administered iv to adult rats in vivo, which is more clinically relevant because it can work throughout the CNS, the respiratory depression was unaffected by (+)naloxone, but completely reversed by (−)naloxone. Finally, exogenous activation of TLR4 within the preBötC via local application of LPS had no effect on baseline respiratory rhythm, indicating that TLR4 activation had no acute effect on preBötC inspiratory activity in neonates in vitro. These data strongly suggest that glial TLR4 signaling does not contribute to the opioid-induced respiratory depression nor is it a candidate system for altered respiratory drive during infection.

Neither TLR4 Signaling Nor preBötC Microglia Contribute to Opioid Respiratory Depression

Opioids are used extensively for their analgesic properties but they have a variety of unwanted side effects, including tolerance, dependence, reward, and most significantly, they depress respiration (82). Like

analgesia, these side effects have historically been attributed to the actions of opioids on opioid receptors. However, analysis of opioid receptor–knockout mice revealed that opioids activate glia through mechanisms that are distinct from classical opioid receptors (14), including TLR4. What is more, growing evidence suggests that opioid activation of glia via TLR4 opposes opioid analgesia and contributes to many opioid side effects (82), including opioid-induced reward (54, 77), dependence (6, 9, 41, 81), and withdrawal (23, 25, 26, 41).

Although the majority of these negative side effects develop with prolonged exposure (days), respiratory depression occurs rapidly (minutes-hours) with the first exposure. Mechanisms underlying respiratory depression and other side effects may therefore differ. Even side effects that develop over time, such as tolerance and hyperalgesia, are produced via different signaling pathways (13). Here we specifically examined the hypothesis that opioids act through TLR4 on microglia and contribute to respiratory depression (23). This hypothesis is based on the observation that the respiratory depression evoked in rats by subcutaneous administration of morphine (10 mg/kg) is attenuated by preadministration of minocycline, an inhibitor of microglial activation (23). The efficacy of minocycline may derive from its ability to block the TLR4-mediated activation of microglia by morphine. We examined this question because although the opioid-induced respiratory depression in the study by Hutchinson et al. (23) resulted from reductions in V_T and frequency, the attenuation of the respiratory depression by minocycline was achieved via blockade of the opioid-induced reduction in V_T. The morphine-induced respiratory frequency decrease was not affected by minocycline. In vivo, the major depressive effect on breathing of opioids in the brain is to reduce respiratory frequency (16, 48, 50). Effects on V_T can occur through central actions on motoneurons (20, 43) or premotoneurons (38, 50, 71). Opioid-induced reductions in V_T are also attributed to reductions in lung, chest-wall, or airway compliance (5, 67) via unknown mechanisms. Thus it is possible that the actions of minocycline were through indirect action on chest wall mechanics or pre/motoneuron excitability. Regardless of the mechanism underlying the changes in V_T, the observation that minocycline did not counteract the effects of opioids on frequency (23) raises the possibility that minocycline did not reverse the central depressant effects of opioids on respiratory rhythm-generating networks.

Our data convincingly demonstrate that the TLR4-preBötC microglial signaling cascade is not involved in opioid-induced respiratory depression. First, minocycline had no effect on the ability of opioids to depress inspiratory rhythm in vitro, whether applied in a bath for 40 min or injected in vivo 18 and 1 h prior to slice preparation. There are two potential caveats. Minocycline measurements were limited to rhythmically active in vitro preparations to avoid potentially confounding effects of drug-induced changes in chest wall mechanics. Thus a potential effect of minocycline on microglia outside of the preBötC cannot be excluded. A role for microglia in mature networks also cannot be excluded because the morphology, number, and expression of cytokines and chemokines by microglia change significantly during development (68). The opioid-induced respiratory depression also changes postnatally. Neonatal respiratory networks are more susceptible to depression (10, 37), which may reflect developmental changes in the expression of opioid receptor subtypes in respiratory networks (36) and associated signaling pathways. Microglia may even influence the development of this response. Recurrent hypoxia during development, which can activate microglia (72), increases the sensitivity of respiratory networks to opioid-mediated depression (52). The specificity of minocycline as an inhibitor of microglial activation is also a potential concern. Since its initial use an inhibitor of microglial activation (86), the mechanisms underlying its inhibition of microglia remain unknown, but numerous additional effects including potent anti-inflammatory, immunomodulatory, and neuroprotective actions have been documented (19, 32, 34, 46, 47, 56, 76, 84). Although the high concentration of minocycline used here increases the potential for nonspecific actions, these are of minimal consequence in the context of this study because minocycline was without effect on the preBötC opioid-induced respiratory depression. The high concentration is in fact a strength of this study because it

minimizes the possibility of a false negative result (i.e., that we missed an effect of minocycline by using a subthreshold concentration).

Second, in vitro application of two TLR4 antagonists, LPS-RS (via local injection) and (+)naloxone (via bath application), had no effect on the respiratory depression produced in the preBötC either by local or bath application of DAMGO or fentanyl (bath-applied only). DAMGO and fentanyl both bind to the μ-opioid receptor. Both also bind TLR4 and have immunomodulatory effects (24). Thus the observation that TLR4 inhibition with LPS-RS or (+)naloxone had no effect on respiratory depression in vitro, whereas (−)naloxone completely reversed it, indicates that in this reduced preparation TLR4 does not contribute to the opioid-induced respiratory depression that is produced within the preBötC. Our rationale for using the rhythmic slice in these experiments was that it provides direct access to the preBötC network, which is a major site mediating the opioid-induced respiratory depression. Opioid-sensitive neurons in the preBötC are a significant contributor to the respiratory depression (3, 16, 17, 45, 51). In fact, recent in vivo data suggest, at least in rat, that the preBötC is the primary site underlying respiratory depression (50). Bilateral administration of (−)naloxone into the preBötC, but not other brain regions, via reverse microdialysis reverses the respiratory depression evoked by iv administration of morphine (50).

A limitation of the rhythmic slice is that additional sites not contained in the slice may contribute to opioid-induced respiratory depression. As a result, our in vitro data do not exclude the possibility that TLR4 microglial signaling in other brain regions contributes to opioid-induced respiratory depression. Our in vivo data, however, address this limitation for TLR4. Under clinically relevant conditions in which drugs were applied iv to affect the entire CNS, the respiratory depression evoked by fentanyl was unaffected by (+)naloxone, but rapidly and completely reversed by (−)naloxone. These data provide compelling evidence that TLR4 signaling does not contribute to the opioid-induced respiratory depression; the possibility remains that microglia outside of the preBötC slice are involved.

The site in the brain at which opioids act to inhibit breathing is a critical question. Several sites in the brain that have a direct or indirect influence on the respiratory network, and are implicated in dyspnea in humans, express μ-opioid receptors, including higher brain centers such as the insula, thalamus, anterior cingulate cortex, cerebellum, periaqueductal gray, and prefrontal cortex (4, 60). Mu-opioid receptors are also abundant throughout brainstem respiratory control centers (80, 85), which are believed to be the main areas through which opioids act to depress breathing. Our in vitro data, in which local application of opioids into the preBötC reduced frequency, add to similar data supporting the view that opioid-sensitive neurons in the preBötC are a significant contributor to respiratory depression (3, 16, 17, 45, 51). Consistent with this, recent in vivo data suggest in rat that the preBötC is a primary site contributing to respiratory depression (50). However, there may be species differences; the preBötC appears much less important in mediating respiratory depression in goat and dog (35, 53). Detailed discussion is beyond the scope of this study because our experiments were not designed to address this question and do not provide additional insight. The significant point is that block of TLR4 signaling throughout the CNS via iv administration of (+)naloxone had no effect on respiratory depression evoked by iv administration of fentanyl, whereas (−)naloxone rapidly and completely reversed it.

Our data are consistent with the majority of literature suggesting that opioid-induced respiratory depression is primarily dependent on activation of μ-opioid receptors (60). Most compelling among this large database are genetic studies in which knockout mice lacking the μ-opioid receptor display no analgesia or respiratory depression with morphine, reinforcing clinical observations that analgesic and respiratory effects of opioids are strongly linked (64). These data also suggest that there is unlikely to be any clinical potential in using TLR4-selective antagonists [such as (+) naloxone] in combination with opioids as a means of directly counteracting the respiratory depressant actions of opioids. However, the fact that (+)naloxone can potentiate acute opioid analgesia (82) suggests that analgesia may be achieved with lower doses of opioid,

indirectly reducing opioid-induced respiratory depression.

Acute TLR4 Activation in the preBötC Does Not Modulate Rhythm

For glial signaling to contribute to opioid-induced respiratory depression (23), the proinflammatory mediators released from the opioid-activated TLR4-glial pathway would need to affect neuronal excitability on a relatively rapid time scale (i.e., in the order of minutes). It is well established that microglia play an important role in the long-term modulation of neural networks. TLR4 activation of microglia also influences neuronal networks in several CNS pathologies including Alzheimer's disease (49, 74), ischemic stroke (1, 7), and chronic pain (15, 55). Whereas the slow time scale required for activation of these effects is not consistent with the rapid onset of the opioid-induced respiratory depression, some microglial and inflammatory mediators operate on faster, more relevant time scales. The TLR4 transcriptional events that occur in the initiation of pathological pain and with acute inflammation occur within 4 h throughout the CNS including the lumbar spinal cord, brain stem, and forebrain (61). TLR4-gated intracellular signaling pathways, such as Akt1, are turned on very rapidly as shown a in mouse macrophage cell line that stably expresses green fluorescent protein (GFP)-tagged Akt1. Under basal conditions, Akt1 is diffusely distributed in the cytosol, but within a minute of LPS application, the GFP fluorescence begins to redistribute to the cell membrane via a TLR4-dependent pathway (26). Consistent with this rapid onset, inflammatory cytokines released in response to TLR4 activation inhibit hippocampal long-term potentiation on the time scale of minutes (12). Similarly, microglial mediators TNF-α, IL-1β, and BDNF in the spinal cord enhance excitatory synaptic transmission, suppress inhibitory synaptic transmission (11, 33, 39), and induce spinal long-term potentiation (42, 87, 88) (via TNF-α and BDNF), all on relatively short time scales. Thus glia-derived proinflammatory cytokines appear to affect information processing on a time scale sufficient to mediate at least part of the opioid-induced respiratory depression.

The activity of respiratory networks is also sensitive to inflammatory mediators, but the time course of these cascades has not been examined in a paradigm that would help assess whether microglia might contribute to the acute effects of opioids. Systemic LPS injection alters breathing frequency and minute ventilation 24 h after injection in unanesthetized Lewis rats (27). Systemic LPS also decreases the hypoxic ventilatory response in a variety of animals (27). These LPS-induced respiratory changes could reflect direct actions of inflammatory mediators on brainstem centers controlling frequency or indirect actions via sensory pathways. Central nervous system involvement is supported by the observation in neonatal rat that intratracheal endotoxin increases expression of proinflammatory cytokines in the caudal nucleus tractus solitarius and area postrema of the central respiratory network, and that these changes contribute to the blunting of the ventilatory response to hypoxia (2). However, time scale is uncertain because inflammatory mediators were applied peripherally in these studies rather than directly into the CNS. Our data, however, suggest that TLR4 activation in the preBötC or brainstem does not have an acute effect on breathing. Microinjection of the TLR4 agonist LPS directly into the preBötC in vitro had no immediate effect on respiratory rhythm. We are confident in this negative conclusion because injection of the NK1R agonist SP into the same site produced a robust frequency increase, confirming injection into the preBötC. Thus whereas over long time scales it appears that LPS-induced inflammatory processes can modulate central respiratory network activity, possibly playing a role in the adaptive/plastic changes that occur in respiratory control during chronic disease, LPS-sensitive pathways do not acutely influence preBötC network activity.

In summary, our in vitro and in vivo data strongly suggest that the respiratory depression evoked by acute opioid administration does not involve TLR4 or preBötC microglia. Whether chronic opioid activation of TLR4 signaling and/or microglia beyond the preBötC have longer-term effects on respiratory control is not known.

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DISCLOSURES

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Go to:

Author contributions: J.D.Z., Y.Z., M.R.H., L.R.W., J.J.G., and G.D.F. conception and design of research; J.D.Z., Y.Z., and J.R. performed experiments; J.D.Z., Y.Z., and J.R. analyzed data; J.D.Z., Y.Z., J.R., and G.D.F. interpreted results of experiments; J.D.Z., Y.Z., and J.R. prepared figures; J.D.Z. and G.D.F. drafted manuscript; J.D.Z., Y.Z., J.R., M.R.H., K.C.R., L.R.W., J.J.G., and G.D.F. edited and revised manuscript; J.D.Z., Y.Z., J.R., M.R.H., K.C.R., L.R.W., J.J.G., and G.D.F. approved final version of manuscript.

REFERENCES

Go to:

1. Abate W, Alghaithy AA, Parton J, Jones KP, Jackson SK. Surfactant lipids regulate LPS-induced interleukin-8 production in A549 lung epithelial cells by inhibiting translocation of TLR4 into lipid raft domains. J Lipid Res 51: 334–344, 2010 [PMCID: PMC2803235] [PubMed: 19648651]

2. Balan KV, Kc P, Hoxha Z, Mayer CA, Wilson CG, Martin RJ. Vagal afferents modulate cytokine-mediated respiratory control at the neonatal medulla oblongata. Respir Physiol Neurobiol 178: 458–464, 2011 [PMCID: PMC3150618] [PubMed: 21397055]

3. Ballanyi K, Lalley PM, Hoch B, Richter DW. cAMP-dependent reversal of opioid- and prostaglandin-mediated depression of the isolated respiratory network in newborn rats. J Physiol 504: 127–134, 1997 [PMCID: PMC1159942] [PubMed: 9350624]

4. Baumgartner U, Buchholz HG, Bellosevich A, Magerl W, Siessmeier T, Rolke R, Hohnemann S, Piel M, Rosch F, Wester HJ, Henriksen G, Stoeter P, Bartenstein P, Treede RD, Schreckenberger M. High opiate receptor binding potential in the human lateral pain system. Neuroimage 30: 692–699, 2006 [PubMed: 16337817]

5. Benvenga MJ, Del Vecchio RA, Capacchione JF, Jerussi TP. An in vivo alpha-2 assay reversal of opioid-induced muscular rigidity and neuroleptic-induced ptosis. J Pharmacol Toxicol Methods 27: 45–50, 1992 [PubMed: 1349839]

6. Burns LH, Wang HY. PTI-609: a novel analgesic that binds filamin A to control opioid signaling. Recent Pat CNS Drug Discov 5: 210–220, 2010 [PubMed: 20726836]

7. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115: 1599–1608, 2007 [PubMed: 17372179]

8. Choi JI, Svensson CI, Koehrn FJ, Bhuskute A, Sorkin LS. Peripheral inflammation induces tumor necrosis factor dependent AMPA receptor trafficking and Akt phosphorylation in spinal cord in addition to pain behavior. Pain 149: 243–253, 2010 [PMCID: PMC2860679] [PubMed: 20202754]

9. Coller JK, Hutchinson MR. Implications of central immune signaling caused by drugs of abuse: mechanisms, mediators and new therapeutic approaches for prediction and treatment of drug dependence. Pharmacol Ther 134: 219–245, 2012 [PubMed: 22316499]

10. Colman AS, Miller JH. Modulation of breathing by mu1 and mu2 opioid receptor stimulation in neonatal and adult rats. Respir Physiol 127: 157–172, 2001 [PubMed: 11504587]

11. Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW, De Koninck Y. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 438: 1017–1021, 2005 [PubMed: 16355225]

12. Curran B, O'Connor JJ. The pro-inflammatory cytokine interleukin-18 impairs long-term potentiation and NMDA receptor-mediated transmission in the rat hippocampus in vitro. Neuroscience 108: 83–90, 2001 [PubMed: 11738133]

13. Ferrini F, Trang T, Mattioli TA, Laffray S, Del'Guidice T, Lorenzo LE, Castonguay A, Doyon N, Zhang W, Godin AG, Mohr D, Beggs S, Vandal K, Beaulieu JM, Cahill CM, Salter MW, De Koninck Y. Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl(-) homeostasis. Nat Neurosci 16: 183–192, 2013 [PMCID: PMC4974077] [PubMed: 23292683]

14. Gavériaux-Ruff C, Filliol D, Simonin F, Matthes HW, Kieffer BL. Immunosuppression by delta-opioid antagonist naltrindole: delta- and triple mu/delta/kappa-opioid receptor knockout mice reveal a nonopioid activity. J Pharmacol Exp Ther 298: 1193–1198, 2001 [PubMed: 11504820]

15. Grace PM, Rolan PE, Hutchinson MR. Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. Brain Behav Immun 25: 1322–1332, 2011 [PubMed: 21496480]

16. Gray PA, Rekling JC, Bocchiaro CM, Feldman JL. Modulation of respiratory frequency by peptidergic input to rhythmogenic neurons in the preBötzinger complex. Science 286: 1566–1568, 1999 [PMCID: PMC2811082] [PubMed: 10567264]

17. Greer JJ, Carter JE, Al-Zubaidy Z. Opioid depression of respiration in neonatal rats. J Physiol 485, *Pt* 3: 845–855, 1995 [PMCID: PMC1158049] [PubMed: 7562622]

18. Greer JJ, Funk GD, Ballanyi K. Preparing for the first breath: prenatal maturation of respiratory neural control. J Physiol 570: 437–444, 2006 [PMCID: PMC1479880] [PubMed: 16284077]

19. Gutteridge JM. Iron and oxygen radicals in brain. Ann Neurol 32: S16–S21, 1992 [PubMed: 1510375]

20. Hajiha M, DuBord MA, Liu H, Horner RL. Opioid receptor mechanisms at the hypoglossal motor pool and effects on tongue muscle activity in vivo. J Physiol 587: 2677–2692, 2009 [PMCID: PMC2714030] [PubMed: 19403616]

21. Hanke ML, Kielian T. Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clin Sci 121: 367–387, 2011 [PMCID: PMC4231819] [PubMed: 21745188]

22. Howard RF. Current status of pain management in children. JAMA 12: 2464–2469, 2003 [PubMed: 14612483]

23. Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, Berkelhammer DL, Loram LC,

Rozeske RR, Bland ST, Maier SF, Gleeson TT, Watkins LR. Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. Brain Behav Immun 22: 1248–1256, 2008 [PMCID: PMC2783326] [PubMed: 18706994]

24. Hutchinson MR, Somogyi AA. Characterisation of the in vitro modulation of splenocyte proliferation by non-4,5-epoxymorphinan opioids. Int Immunopharmacol 5: 1713–1722, 2005 [PubMed: 16102521]

25. Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridar M, Sholar PW, Patel SJ, Crysdale NY, Harrison JA, Maier SF, Rice KC, Watkins LR. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci 28: 20–29, 2008 [PMCID: PMC2588470] [PubMed: 18662331]

26. Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR. Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun 24: 83–95, 2010 [PMCID: PMC2788078] [PubMed: 19679181]

27. Huxtable AG, Vinit S, Windelborn JA, Crader SM, Guenther CH, Watters JJ, Mitchell GS. Systemic inflammation impairs respiratory chemoreflexes and plasticity. Respir Physiol Neurobiol 178: 482–489, 2011 [PMCID: PMC3172820] [PubMed: 21729770]

28. Huxtable AG, Zwicker JD, Alvares TS, Ruangkittisakul A, Fang X, Hahn LB, Posse de Chaves E, Baker GB, Ballanyi K, Funk GD. Glia contribute to the purinergic modulation of inspiratory rhythm-generating networks. J Neurosci 30: 3947–3958, 2010 [PubMed: 20237265]

29. Huxtable AG, Zwicker JD, Poon BY, Pagliardini S, Vrouwe SQ, Greer JJ, Funk GD. Tripartite purinergic modulation of central respiratory networks during perinatal development: the influence of ATP, ectonucleotidases, and ATP metabolites. J Neurosci 29: 14713–14725, 2009 [PubMed: 19940166]

30. Johnson SM, Smith JC, Feldman JL. Modulation of respiratory rhythm in vitro: role of Gi/o protein-mediated mechanisms. J Appl Physiol 80: 2120–2133, 1996 [PubMed: 8806921]

31. Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, Langer S, Martin D, Green P, Fleshner M, Leinwand L, Maier SF, Watkins LR. A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J Neurosci 24: 7353–7365, 2004 [PubMed: 15317861]

32. Kalish RS, Koujak S. Minocycline inhibits antigen processing for presentation to human T cells: additive inhibition with chloroquine at therapeutic concentrations. Clin Immunol 113: 270–277, 2004 [PubMed: 15507392]

33. Kawasaki Y, Zhang L, Cheng JK, Ji RR. Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. J Neurosci 28: 5189–5194, 2008 [PMCID: PMC2408767] [PubMed: 18480275]

34. Kloppenburg M, Brinkman BM, de Rooij-Dijk HH, Miltenburg AM, Daha MR, Breedveld FC, Dijkmans BA, Verweij C. The tetracycline derivative minocycline differentially affects cytokine production by monocytes and T lymphocytes. Antimicrob Agents Chemother 40: 934–940, 1996 [PMCID: PMC163234] [PubMed: 8849255]

35. Krause KL, Neumueller SE, Marshall BD, Kiner T, Bonis JM, Pan LG, Qian B, Forster HV. μ-Opioid receptor agonist injections into the presumed pre-Bötzinger complex and the surrounding region of awake goats do not alter eupneic breathing. J Appl Physiol 107: 1591–1599, 2009 [PMCID: PMC2777792]

[PubMed: 19745186]

36. Laferrière A, Liu JK, Moss IR. Mu- and delta-opioid receptor densities in respiratory-related brainstem regions of neonatal swine. Brain Res Dev Brain Res 112: 1–9, 1999

37. Laferrière A, Colin-Durand J, Moss IR. Ontogeny of respiratory sensitivity and tolerance to the mu-opioid agonist fentanyl in rat. Brain Res Dev Brain Res 156: 210–217, 2005

38. Lalley PM. Opiate slowing of feline respiratory rhythm and effects on putative medullary phase-regulating neurons. Am J Physiol Regul Integr Comp Physiol 290: R1387–R1396, 2006 [PubMed: 16284086]

39. Li J, Xie W, Zhang JM, Baccei ML. Peripheral nerve injury sensitizes neonatal dorsal horn neurons to tumor necrosis factor-alpha. Mol Pain 5: 10, 2009 [PMCID: PMC2657778] [PubMed: 19254372]

40. Liu G, Feldman JL, Smith JC. Excitatory amino acid-mediated transmission of inspiratory drive to phrenic motoneurons. J Neurophysiol 64: 423–436, 1990 [PubMed: 1976765]

41. Liu L, Coller JK, Watkins LR, Somogyi AA, Hutchinson MR. Naloxone-precipitated morphine withdrawal behavior and brain IL-1beta expression: comparison of different mouse strains. Brain Behav Immun 25: 1223–1232, 2011 [PMCID: PMC3142610] [PubMed: 21447380]

42. Liu YL, Zhou LJ, Hu NW, Xu JT, Wu CY, Zhang T, Li YY, Liu XG. Tumor necrosis factor-alpha induces long-term potentiation of C-fiber evoked field potentials in spinal dorsal horn in rats with nerve injury: the role of NF-kappa B, JNK and p38 MAPK. Neuropharmacology 52: 708–715, 2007 [PubMed: 17084420]

43. Lorier AR, Funk GD, Greer JJ. Opiate-induced suppression of rat hypoglossal motoneuron activity and its reversal by ampakine therapy. PloS One 5: e8766, 2010 [PMCID: PMC2808240] [PubMed: 20098731]

44. Lorier AR, Huxtable AG, Robinson DM, Lipski J, Housley GD, Funk GD. P2Y1 receptor modulation of the pre-Bötzinger complex inspiratory rhythm generating network in vitro. J Neurosci 27: 993–1005, 2007 [PubMed: 17267553]

45. Lorier AR, Lipski J, Housley GD, Greer JJ, Funk GD. ATP sensitivity of preBötzinger complex neurones in neonatal rat in vitro: mechanism underlying a P2 receptor-mediated increase in inspiratory frequency. J Physiol 586: 1429–1446, 2008 [PMCID: PMC2375674] [PubMed: 18174215]

46. Maier K, Merkler D, Gerber J, Taheri N, Kuhnert AV, Williams SK, Neusch C, Bahr M, Diem R. Multiple neuroprotective mechanisms of minocycline in autoimmune CNS inflammation. Neurobiol Dis 25: 514–525, 2007 [PubMed: 17239606]

47. Mansson R, Hansson MJ, Morota S, Uchino H, Ekdahl CT, Elmer E. Re-evaluation of mitochondrial permeability transition as a primary neuroprotective target of minocycline. Neurobiol Dis 25: 198–205, 2007 [PubMed: 17067803]

48. Manzke T, Guenther U, Ponimaskin EG, Haller M, Dutschmann M, Schwarzacher S, Richter DW. 5-HT4(a) receptors avert opioid-induced breathing depression without loss of analgesia. Science 301: 226–229, 2003 [PubMed: 12855812]

49. Minoretti P, Gazzaruso C, Vito CD, Emanuele E, Bianchi M, Coen E, Reino M, Geroldi D. Effect of the functional toll-like receptor 4 Asp299Gly polymorphism on susceptibility to late-onset Alzheimer's disease. Neurosci Lett 391: 147–149, 2006 [PubMed: 16157451]

50. Montandon G, Qin W, Liu H, Ren J, Greer JJ, Horner RL. PreBotzinger complex neurokinin-1 receptor-

expressing neurons mediate opioid-induced respiratory depression. J Neurosci 31: 1292–1301, 2011 [PubMed: 21273414]

51. Morin-Surun MP, Boudinot E, Dubois C, Matthes HW, Kieffer BL, Denavit-Saubié M, Champagnat J, Foutz AS. Respiratory function in adult mice lacking the mu-opioid receptor: role of delta-receptors. Eur J Neurosci 13: 1703–1710, 2001 [PubMed: 11359522]

52. Moss I, Brown KA, Laferrière A. Recurrent hypoxia in rats during development increases subsequent respiratory sensitivity to fentanyl. Anesthesiology 105: 715–718, 2006 [PubMed: 17006070]

53. Mustapic S, Radocaj T, Sanchez A, Dogas Z, Stucke AG, Hopp FA, Stuth EA, Zuperku EJ. Clinically relevant infusion rates of mu-opioid agonist remifentanil cause bradypnea in decerebrate dogs but not via direct effects in the pre-Bötzinger complex region. J Neurophysiol 103: 409–418, 2010 [PMCID: PMC2807229] [PubMed: 19906886]

54. Narita M, Miyatake M, Narita M, Shibasaki M, Shindo K, Nakamura A, Kuzumaki N, Nagumo Y, Suzuki T. Direct evidence of astrocytic modulation in the development of rewarding effects induced by drugs of abuse. Neuropsychopharmacology 31: 2476–2488, 2006 [PubMed: 16407899]

55. Nicotra L, Loram LC, Watkins LR, Hutchinson MR. Toll-like receptors in chronic pain. Exp Neurol 234: 316–329, 2012 [PMCID: PMC3303935] [PubMed: 22001158]

56. Nikodemova M, Watters JJ, Jackson SJ, Yang SK, Duncan ID. Minocycline down-regulates MHC II expression in microglia and macrophages through inhibition of IRF-1 and protein kinase C (PKC)alpha/betaII. J Biol Chem 282: 15208–15216, 2007 [PubMed: 17395590]

57. Nutile-McMenemy N, Elfenbein A, Deleo JA. Minocycline decreases in vitro microglial motility, beta1-integrin, and Kv1.3 channel expression. J Neurochem 103: 2035–2046, 2007 [PubMed: 17868321]

58. Oertel BG, Felden L, Tran PV, Bradshaw M, Angst MS, Schmidt H, Johnson S, Greer JJ, Geisslinger G, Varney MA, Lötsch J. Selective antagonism of opioid-induced respiratory depression by an AMPAKINE molecule in humans without loss of opioid analgesia. Clin Pharmacol Ther 87: 204–211, 2010 [PubMed: 19907420]

59. Osikowicz M, Skup M, Mika J, Makuch W, Czarkowska-Bauch J, Przewlocka B. Glial inhibitors influence the mRNA and protein levels of mGlu2/3, 5 and 7 receptors and potentiate the analgesic effects of their ligands in a mouse model of neuropathic pain. Pain 147: 175–186, 2009 [PubMed: 19782473]

60. Pattinson KT, Governo RJ, MacIntosh BJ, Russell EC, Corfield DR, Tracey I, Wise RG. Opioids depress cortical centers responsible for the volitional control of respiration. J Neurosci 29: 8177–8186, 2009 [PubMed: 19553457]

61. Raghavendra V, Tanga FY, DeLeo JA. Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur J Neurosci 20: 467–473, 2004 [PubMed: 15233755]

62. Ren J, Ding X, Funk GD, Greer JJ. Ampakine CX717 protects against fentanyl-induced respiratory depression and lethal apnea in rats. Anesthesiology 110: 1364–1370, 2009 [PubMed: 19461299]

63. Ren J, Poon BY, Tang Y, Funk GD, Greer JJ. Ampakines alleviate respiratory depression in rats. Am J Respir Crit Care Med 174: 1384–1391, 2006 [PubMed: 16973981]

64. Romberg R, Sarton E, Teppema L, Matthes WD, Dahan A. Comparison of morphine-6-glucuronide and morphine on respiratory depressant and antinociceptive responses in wild type and mu-opioid receptor

deficient mice. Br J Anaesth 91: 862–870, 2003 [PubMed: 14633759]

65. Ruangkittisakul A, Panaitescu B, Kuribayashi J, Ballanyi K. Caffeine reversal of opioid-evoked and endogenous inspiratory depression in perinatal rat en bloc medullas and slices. Adv Exp Med Biol 669: 123–127, 2010 [PubMed: 20217334]

66. Ruangkittisakul A, Schwarzacher SW, Secchia L, Poon BY, Ma Y, Funk GD, Ballanyi K. High sensitivity to neuromodulator-activated signaling pathways at physiological [K+] of confocally imaged respiratory center neurons in on-line-calibrated newborn rat brainstem slices. J Neurosci 26: 11870–11880, 2006 [PubMed: 17108160]

67. Scamman FL. Fentanyl-O2-N2O rigidity and pulmonary compliance. Anesth Analg 62: 332–334, 1983 [PubMed: 6829933]

68. Schwarz JM, Sholar PW, Bilbo SD. Sex differences in microglial colonization of the developing rat brain. J Neurochem 120: 948–963, 2012 [PMCID: PMC3296888] [PubMed: 22182318]

69. Smith JC, Ellenberger HH, Ballanyi K, Richter DW, Feldman JL. Pre-Bötzinger complex: a brainstem region that may generate respiratory rhythm in mammals. Science 254: 726–729, 1991 [PMCID: PMC3209964] [PubMed: 1683005]

70. Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. Neurosci Res 39: 281–286, 2001 [PubMed: 11248367]

71. Stucke AG, Zuperku EJ, Sanchez A, Tonkovic-Capin M, Tonkovic-Capin V, Mustapic S, Stuth EA. Opioid receptors on bulbospinal respiratory neurons are not activated during neuronal depression by clinically relevant opioid concentrations. J Neurophysiol 100: 2878–2888, 2008 [PMCID: PMC2585400] [PubMed: 18815346]

72. Tadmouri A, Champagnat J, Morin-Surun MP. Activation of microglia and astrocytes in the nucleus tractus solitarius during ventilatory acclimatization to 10% hypoxia in unanesthetized mice. J Neurosci Res 92: 627–633, 2014 [PubMed: 24510616]

73. Takeda S, Eriksson LI, Yamamoto Y, Joensen H, Onimaru H, Lindahl SG. Opioid action on respiratory neuron activity of the isolated respiratory network in newborn rats. Anesthesiology 95: 740–749, 2001 [PubMed: 11575549]

74. Tan L, Schedl P, Song HJ, Garza D, Konsolaki M. The Toll–>NFkappaB signaling pathway mediates the neuropathological effects of the human Alzheimer's Abeta42 polypeptide in *Drosophila*. PloS One 3: e3966, 2008 [PMCID: PMC2597734] [PubMed: 19088848]

75. Telgkamp P, Ramirez JM. Differential responses of respiratory nuclei to anoxia in rhythmic brain stem slices of mice. J Neurophysiol 82: 2163–2170, 1999 [PubMed: 10561396]

76. Teng YD, Choi H, Onario RC, Zhu S, Desilets FC, Lan S, Woodard EJ, Snyder EY, Eichler ME, Friedlander RM. Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury. Proc Natl Acad Sci USA 101: 3071–3076, 2004 [PMCID: PMC365746] [PubMed: 14981254]

77. Terashvili M, Wu HE, Schwasinger ET, Hung KC, Hong JS, Tseng LF. (+)-Morphine attenuates the (−)-morphine-produced conditioned place preference and the mu-opioid receptor-mediated dopamine increase in the posterior nucleus accumbens of the rat. Eur J Pharmacol 587: 147–154, 2008 [PMCID: PMC2566855] [PubMed: 18448094]

78. Tibboela D, Anandb KJ, van den Ankerc JN. The pharmacological treatment of neonatal pain. Semin Fetal Neonat Med 10: 195–205, 2005

79. Tikka T, Fiebich BL, Goldsteins G, Keinanen R, Koistinaho J. Minocycline, a tetracycline derivative, is neuroprotective against excitotoxicity by inhibiting activation and proliferation of microglia. J Neurosci 21: 2580–2588, 2001 [PubMed: 11306611]

80. Wamsley JK. Opioid receptors: autoradiography. Pharmacol Rev 35: 69–83, 1983 [PubMed: 6195680]

81. Wang HY, Frankfurt M, Burns LH. High-affinity naloxone binding to filamin a prevents mu opioid receptor-Gs coupling underlying opioid tolerance and dependence. PloS One 3: e1554, 2008 [PMCID: PMC2212716] [PubMed: 18253501]

82. Watkins LR, Hutchinson MR, Rice KC, Maier SF. The “toll” of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. Trends Pharmacol Sci 30: 581–591, 2009 [PMCID: PMC2783351] [PubMed: 19762094]

83. Wei F, Guo W, Zou S, Ren K, Dubner R. Supraspinal glial-neuronal interactions contribute to descending pain facilitation. J Neurosci 28: 10482–10495, 2008 [PMCID: PMC2660868] [PubMed: 18923025]

84. Wilkins A, Nikodemova M, Compston A, Duncan I. Minocycline attenuates nitric oxide-mediated neuronal and axonal destruction in vitro. Neuron Glia Biol 1: 297–305, 2004 [PubMed: 18634603]

85. Xia Y, Haddad GG. Ontogeny and distribution of opioid receptors in the rat brainstem. Brain Res 549: 181–193, 1991 [PubMed: 1653081]

86. Yrjanheikki J, Tikka T, Keinanen R, Goldsteins G, Chan PH, Koistinaho J. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. Proc Natl Acad Sci USA 96: 13496–13500, 1999 [PMCID: PMC23976] [PubMed: 10557349]

87. Zhou LJ, Yang T, Wei X, Liu Y, Xin WJ, Chen Y, Pang RP, Zang Y, Li YY, Liu XG. Brain-derived neurotrophic factor contributes to spinal long-term potentiation and mechanical hypersensitivity by activation of spinal microglia in rat. Brain Behav Immun 25: 322–334, 2011 [PubMed: 20933591]

88. Zhou LJ, Zhong Y, Ren WJ, Li YY, Zhang T, Liu XG. BDNF induces late-phase LTP of C-fiber evoked field potentials in rat spinal dorsal horn. Exp Neurol 212: 507–514, 2008 [PubMed: 18565512]

Figures and Tables

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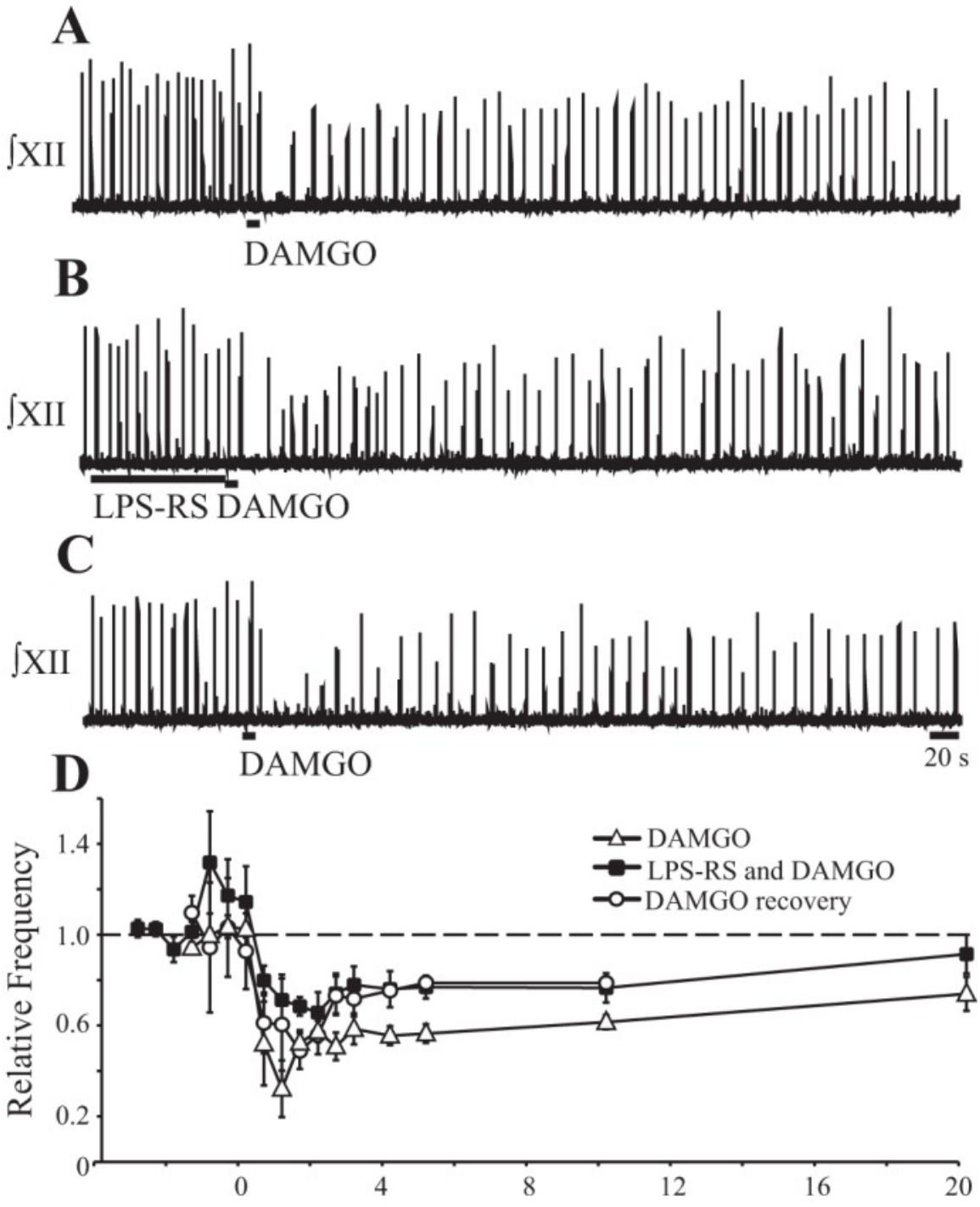
Fig. 1.



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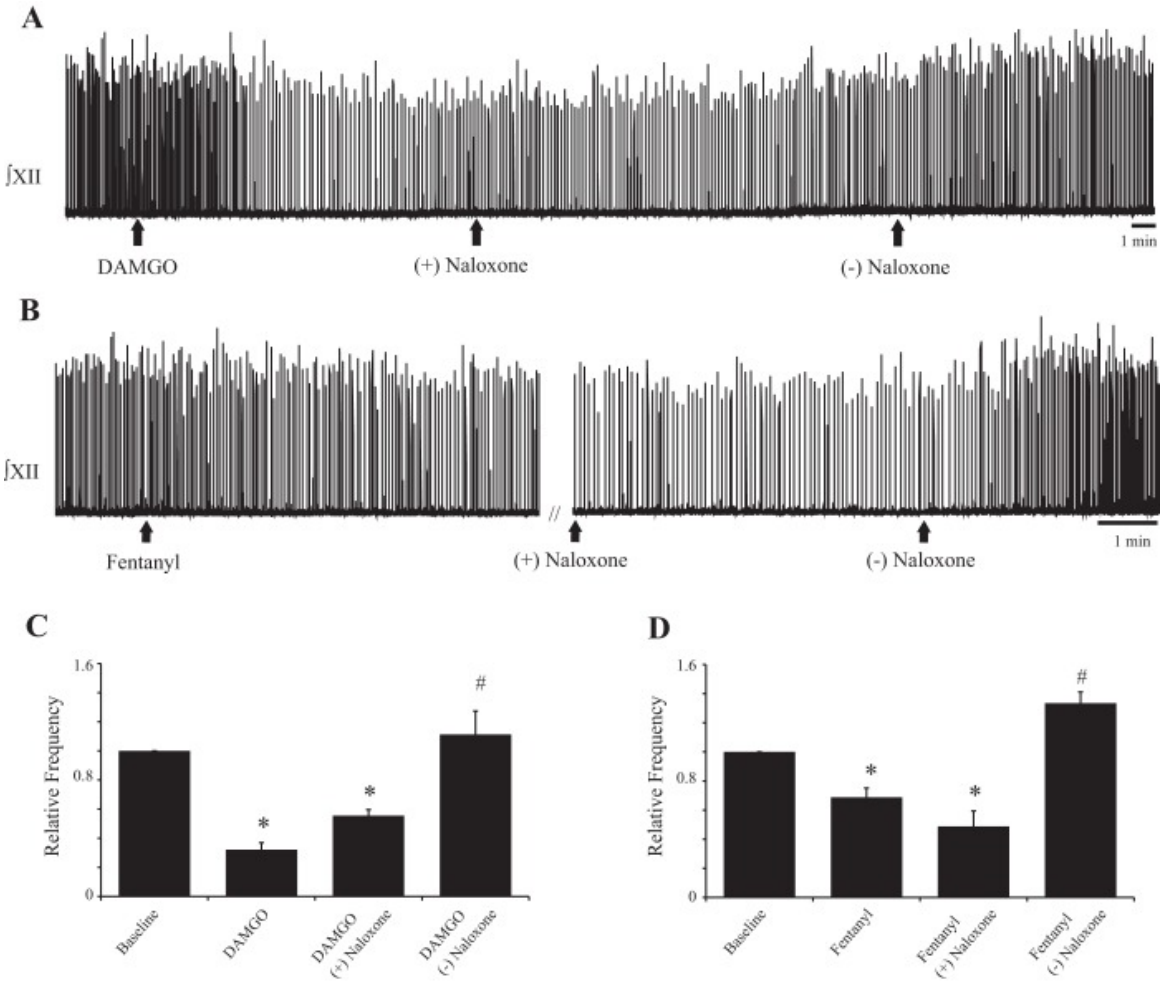
Preapplication of toll-like receptor 4 (TLR4) antagonist LPS-RS (lipopolysaccharide from *Rhodobacter sphaeroides*) has no effect on the DAMGO-mediated inhibition of frequency. Representative recordings of integrated XII nerve activity (∫XII) showing opioid-induced respiratory depression of rat rhythmic slice frequency to locally applied DAMGO (50 μM, 10 s) in control (*A*, open triangle in *D*), after preapplication of TLR antagonist LPS-RS (2,000 ng/ml; 90 s) (*B*, closed square in *D*), and recovery (*C*, open circle in *D*). *D*: group data illustrating the time course of the frequency response (relative to control; $n = 5$). DAMGO caused a significant decrease in frequency in all groups (two-way ANOVA, time

factor). Time course of the DAMGO effect was not significantly different in the presence or absence of LPS-RS (two-way ANOVA, multiple comparison test with Bonferroni correction).

Fig. 2.



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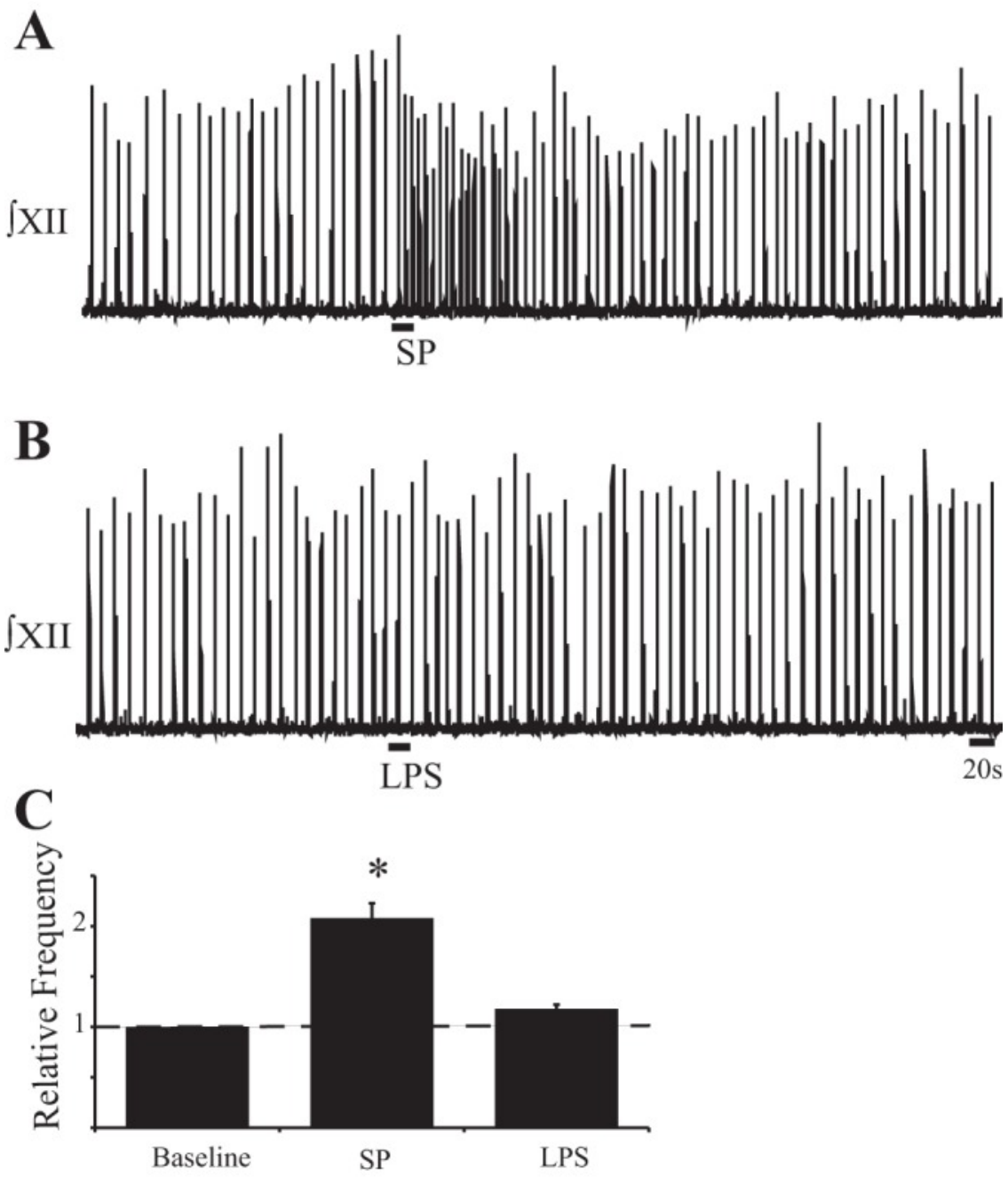
Bath application of the TLR4-selective antagonist, (+)naloxone, has no effect on the opioid-evoked depression of frequency. Representative recordings of integrated XII nerve activity (∫XII) showing opioid-induced respiratory depression of rat rhythmic slice frequency evoked by bath application of DAMGO (0.5 μM, *A*) or fentanyl (1 μM, *B*) under control conditions, and following bath application of the TLR-selective antagonist, (+)naloxone (10 μM), and the classical μ-opioid receptor antagonist, (−)naloxone (0.5 μM). Group data showing effects of (+) and (−)naloxone on the frequency depression (relative to control) evoked by DAMGO (*C*, $n = 7$) and fentanyl (*D*, $n = 5$). *Significant difference from predrug control levels; #significant difference from DAMGO or fentanyl (one-way ANOVA, multiple comparison test with Bonferroni correction).

Fig. 3.



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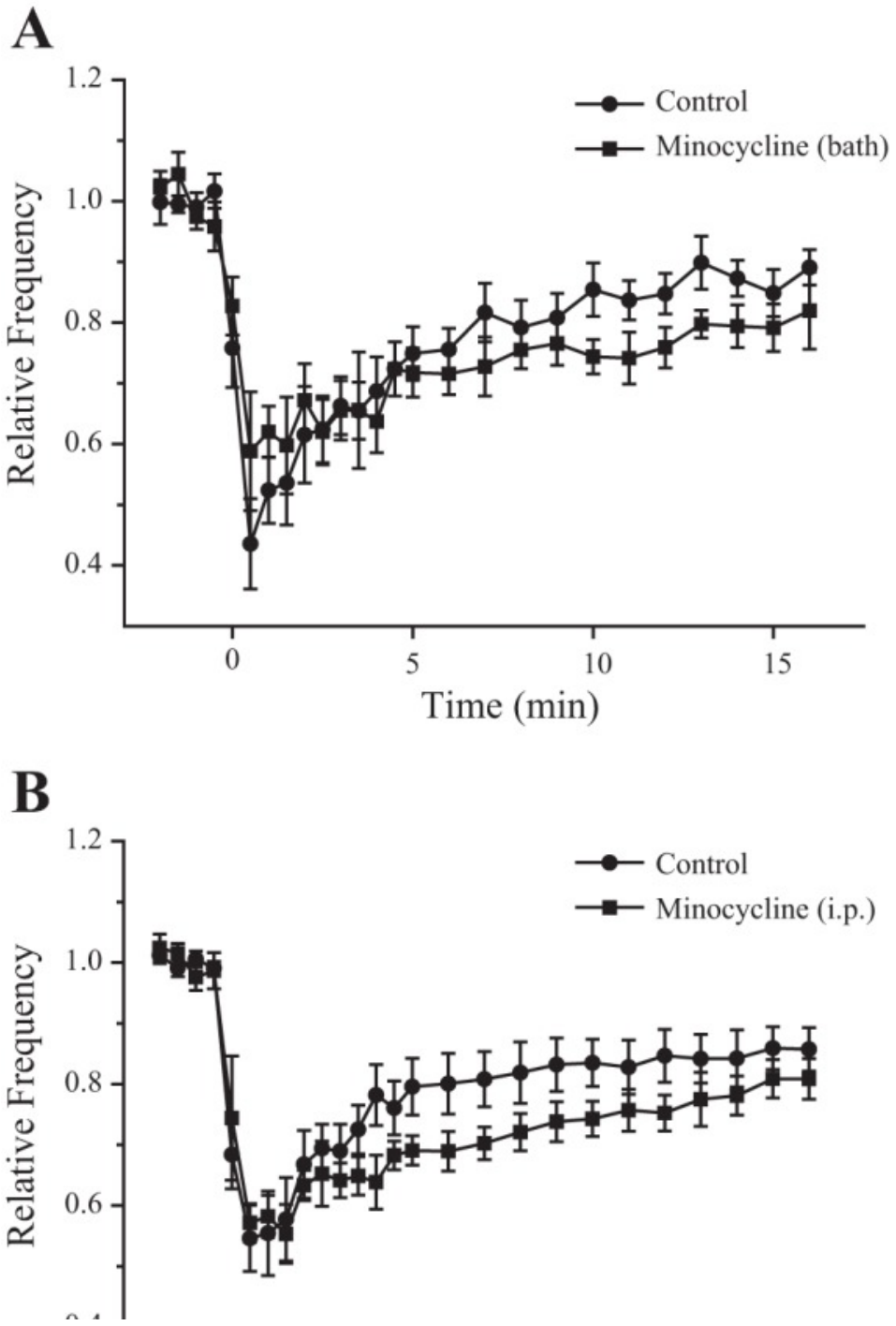
Local application of the TLR4 agonist LPS has no effect on respiratory frequency. Representative recording of integrated XII nerve activity (∫XII) showing the frequency increase evoked in a rhythmic slice by local application of Substance P (SP) (*A*; 1 μM, 10 s) but not LPS (*B*; 200 ng/ml, 10 s). *C*: group data illustrating the peak frequency ($n = 4$). *Significant difference from baseline (one-way ANOVA, multiple comparison test with Bonferroni correction).

Fig. 4.



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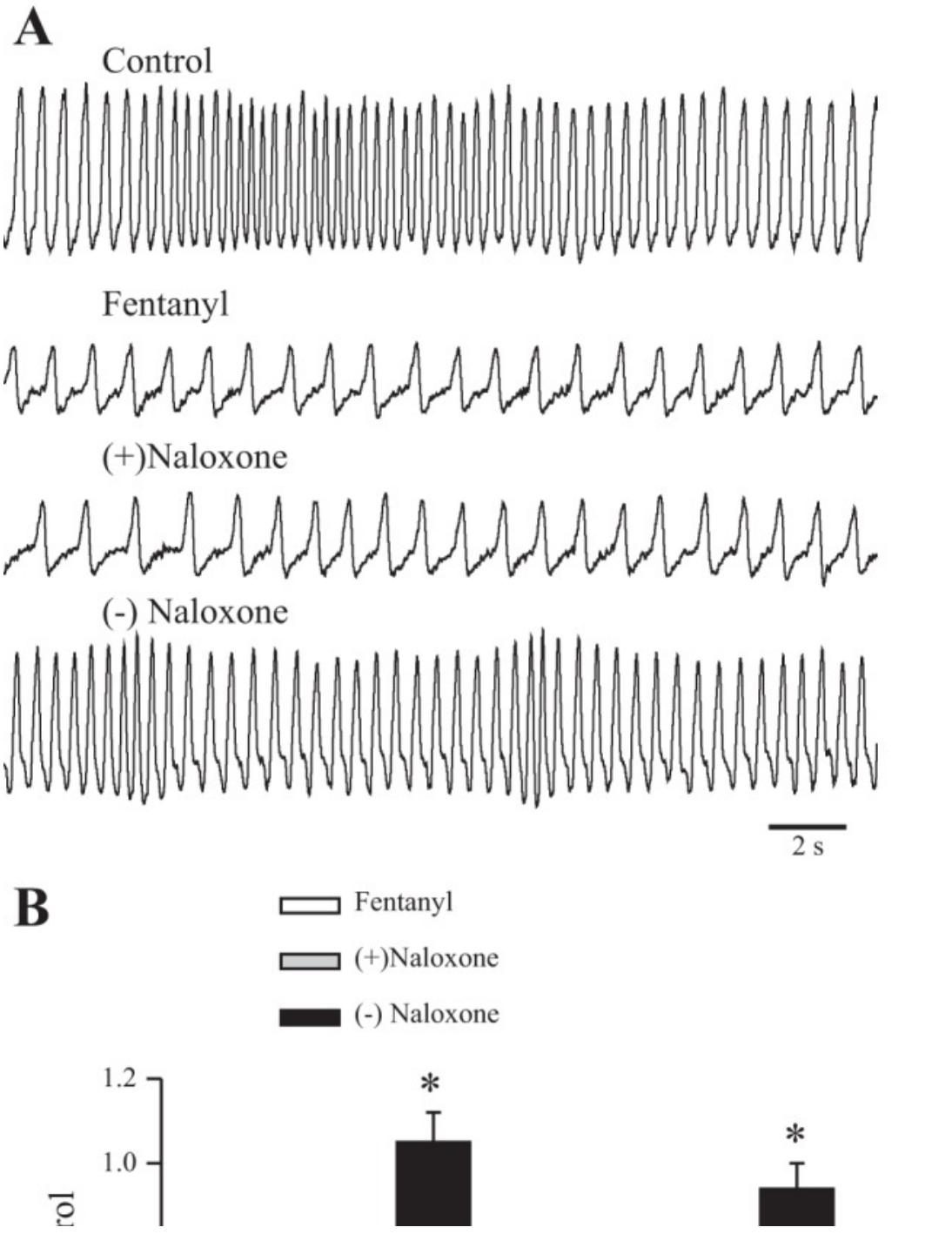
Microglia activation is not required for opioid-induced respiratory depression. *A*: group data illustrating the time course of the frequency response (relative to pre-DAMGO baseline) evoked by the second local application of DAMGO into the pre-Bötzinger Complex (preBötC) of time-control slices (closed circles; 50 μM, 10 s, $n = 6$) and a separate group of slices to which minocycline was bath-applied (closed squares; 500 ng/ml, $n = 6$). *B*: group data illustrating the time course of the frequency response (relative to pre-DAMGO baseline) evoked by the first local application of DAMGO into the preBötC of control slices (closed circles; 50 μM, 10 s, $n = 10$) and slices from minocycline-pretreated neonatal rats (closed squares;

45 mg/kg, *n* = 9). *C*: histogram showing (relative to baseline of 1.0) peak frequency evoked by SP and minimum frequency evoked by first and second applications of DAMGO in time-control slices, in slices before and during bath application of minocycline and in slices from animals injected (ip, first DAMGO application only) with minocycline. N.S., not significant.

Fig. 5.



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(−)Naloxone, but not (+)naloxone counters fentanyl-induced depression of respiratory activity in vivo. *A*: traces are whole-body plethysmographic measurements of the frequency and relative depth of breathing in an adult rat. Control, before fentanyl administration (top trace). Fentanyl (40 μg/kg iv over 20 min) caused depression that stabilized ~8 min after administration at levels demonstrated in the second trace. Injection of (+)naloxone (60 mg/kg iv) 10 min after fentanyl administration had no effect on depression of respiratory frequency and amplitude, as shown in the third trace taken 20 min after administration of (+)naloxone. However, administration of (−)naloxone (0.6 mg/kg iv) rapidly blocked the fentanyl-

induced respiratory depression, as indicated by the bottom trace taken 3 min after (−)naloxone. *B*: population data ($n = 6$) showing the relative effects (compared to prefentanyl baseline levels that are normalized to 1.0) of fentanyl, (+)naloxone (3–60 mg/kg), and (−)naloxone (in the continued presence of fentanyl) on respiratory frequency and tidal volume. *Significant difference compared with other two treatments (using one-way repeated measures ANOVA, Tukey's multiple comparison test).