Title
Lactoferrin enhances opioid-mediated analgesia via nitric oxide in the rat spinal cord

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Keywords
formalin test; tail-flick test; N⁶-nitro-L-arginine methyl ester; D-Phe-Cys-Tyr-D-Trp-Orn-Thr-NH₂; tolerance

Abstract
Lactoferrin (LF) is a multifunctional protein that is found in milk, neutrophils, and other biological fluids, and its receptors have also been identified in the central nervous system. Recently, we found that bovine milk-derived LF (BLF) produced analgesia via µ-opioid receptor-mediated response in the spinal cord. However, the precise mechanism of this analgesic effect remains unclear. In this study, spinally applied BLF produced analgesia that was reversed by co-administration with a nitric oxide (NO) synthase inhibitor, N⁶-nitro-L-arginine methyl ester (L-NAME), during phase 1 and phase 2 in the formalin test. Spinal co-administration of a µ-opioid receptor agonist, morphine, with
sub-effective dose of BLF produced a much more highly potentiated analgesia compared to that of morphine alone during both phases in the formalin test. This potentiated analgesia by morphine with BLF was reversed by a µ-opioid receptor antagonist, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-NH2 (CTOP), or by L-NAME. In the tail-flick test, continuous spinal infusion of morphine via a mini-osmotic pump over 6 days resulted in development of tolerance by day 4, but no tolerance of BLF was observed throughout the experiment. These results suggest that BLF acts as an enhancer of the spinal opioidergic system via a NO-mediated mechanism.

Introduction

Lactoferrin (LF) is a single-chain glycoprotein with a molecular weight of about 80 kD that belongs to the family of transferrins (1). Human colostrum milk contains 5-7 mg/ml LF and mature breast milk contains 1-3 mg/ml LF (19). Bovids and other species also have LF in their milk (25). That is, the infants of many mammalian species constantly take exogenous LF from their mother’s milk. LF can also be detected in blood, saliva, nasal secretions, tears, bronchial mucus, hepatic bile, pancreatic juice, seminal fluid, female cervical mucus, urine and cerebrospinal fluid (19, 22, 24, 35). Under inflammatory conditions, LF production is increased in the periphery by neutrophils (1, 19) and in the central nervous system (CNS) by the microglia (9). Thus, endogenous LF is also available for the adult animals.

LF has many peripheral functions, inducing primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation (1). Although LF and its receptor have also been identified in the central nervous system (CNS) (6, 20, 22, 35), its physiological function is still unclear. LF enters into the cerebrospinal fluid through the blood-brain barrier via receptor-mediated transcytosis (8). Previously, we reported that orally administered bovine milk-derived lactoferrin (BLF) entered into the cerebrospinal fluid in piglets (10) and calves (35). We also confirmed the presence of BLF in the rat cerebrospinal fluid following oral or intraperitoneal administration in the preliminary study. This macromolecular transcytosis may be enhanced in inflammatory conditions inducing pain, as suggested by a report that the proinflammatory
cytokine, TNF-α, increased the rate of transendothelial transport of BLF in a blood-brain barrier model (7).

Recently, we found that oral, intraperitoneal, or intrathecal administration of BLF produces a μ-opioid receptor-mediated antinociceptive activity in the rat CNS, especially in the spinal cord (11). It is now clear that many classes of dorsal horn neurons, fibres descending from the brain, and primary afferent fibres themselves exert a powerful modulatory influence upon the onward transfer of nociceptive information from the spinal cord to the brain (27). In these modulatory processes, the activation of spinal opioid receptors exerts a pivotal role that causes analgesia in animals (12, 21, 28, 29, 38, 43) and humans (2). Thus, the pain-modulatory function of LF via opioid receptor-mediated response would be of substantial physiological importance. Although various types of opioid ligands have been found in milk or milk digests (37), BLF neither bound to the μ-opioid receptor nor changed its binding affinity to the opioid ligands in our preliminary study. Thus, we hypothesize that BLF does not act as an opioid agonist, but rather as an enhancer of endogenous opioid signaling in the spinal cord.

It is reported that BLF induces NO secretion from macrophages in rats (33). Several reports suggest that nitric oxide (NO) is involved in the central opioidergic system (4, 13, 14, 15, 41). NO is a free radical that is synthesized from L-arginine by NO synthase (NOS). It produces an increase in intracellular cyclic GMP through activation of soluble guanylate cyclase. In the CNS, NO is produced by a constitutive (neuronal) form of NO synthase (nNOS), an enzyme localized to the neurons, or by an inducible form of NOS (iNOS) localized to glia (5, 26, 31). It has been reported that intrathecal administration of a NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME), enhances opioid-induced analgesia in the rat tail-flick and formalin tests (21, 43). Another NOS inhibitor, 7-nitroindazole, also enhances opioid-induced analgesia in the rat tail-flick test following intrathecal administration (21). However, a NO precursor, L-arginine, has been shown to produce analgesia via a central opioidergic mechanism in rats and mice with carrageenin-induced hyperalgesia (13, 14), or in the mouse tail-flick test (15). It has also been reported that a NO-donor, nitroglycerin, acts as an adjuvant to morphine (μ-opioid agonist) in cancer patients (17). Together, then, the available data
on the involvement of NO in the central opioidergic system are not consistent and are still a matter of debate.

The aim of the present study was to assess three points. First, would NO be involved in BLF-induced analgesia? Second, could spinally applied BLF potentiate a spinal µ-opioid receptor-mediated antinociceptive response? If so, would NO also be involved in this potentiation by BLF? Last, would BLF cause tolerance? In this study, we used the formalin test and the tail-flick test in rats. The formalin test is widely used as a peripheral inflammatory nociceptive test (23, 40) involving peripheral and central sensitization (18, 23). The tail-flick test is the most used thermal-evoked nociceptive test to assess the development of opioid tolerance in the rat spinal cord (28, 29).

Materials and Methods

Animals
Male Wistar-Imamichi rats (8-9 weeks old; body weight, 250-330 g) were used in all experiments. All animals were maintained at a controlled temperature (22±2°C) under a regular light/dark cycle (light period: 7:00 to 19:00 h) with free access to food and water. Behavior tests were conducted during the light period. All experiments were conducted in accordance with the guidelines of the Physiological Society of Japan regarding the care of experimental animals.

Intrathecal catheterization
Chronic lumbar intrathecal catheters were implanted in rats under pentobarbital anesthesia (50 mg/kg i.p.) according to a previous report with modifications (42). The rat was mounted in a conventional stereotaxic instrument. After shaving and sterilizing the overlying skin, the posterior superficial neck muscles were separated to give a clear visual field of the atlanto-occipital membrane. The catheter (SP-10; Natsume, Tokyo, Japan) was inserted through a small incision at the center of the membrane and gently advanced caudally 8.5 cm down to the lumber subarachnoid space. The external end of the i.t. catheter was capped with a closed tube (JB-30; Eicom, Kyoto, Japan), and covered by the head skin until the day of the experiment. Only animals showing normal motor function 5-10 days after
the surgery were used.

**Drugs and administrations**

Bovine lactoferrin (BLF; MW=approximately 78000; Tatura, Morrinsville, New Zealand), naloxone hydrochloride (naloxone; Sigma, Tokyo, Japan), D-Phe-Cys-Tyr-D-Trp-Orn-Thr-NH2 (CTOP; Sigma), morphine hydrochloride (morphine; MW=375.8; Sankyo, Tokyo, Japan), NG-nitro-L-arginine methyl ester (L-NAME; Sigma), and NG-nitro-D-arginine methyl ester (D-NAME; Sigma) were dissolved in saline solution for administration. All control groups were treated with saline. For the spinal application, under light ether anesthesia, a small incision was made to inject drugs into the catheter covered by the head skin. Drugs were then injected at a volume of 5 µl (single injections) or 10 µl (coadministration), followed by 10 µl of saline for flushing 15 min prior to the test. For the continuous spinal infusion, we used osmotic mini-pumps (model 2001: 1 µl/h; Alzet®, Cupertino, CA) filled with BLF or morphine dissolved in sterilized saline. The mini-osmotic pumps were implanted subcutaneously on the back of the rat under pentobarbital anesthesia (50 mg/kg i.p.) and connected to the intrathecal catheters.

**Formalin test**

The formalin test was performed according to a previous report with modifications (40). To reduce additional stress to the animal, we chose a minimum concentration (2.0%) and injection volume (50 µL) of formalin that produced a stable flinching behavior in Wistar-Imamichi rats in the preliminary experiment. Rats were given a single subplantar injection of formalin (2.0%, 50 µL) into the right hindpaw using a 27 gauge needle before immediate transfer to a clear perspex observation chamber (base 20 × 28 cm, height 15 cm). A video camera and a display monitor placed behind the observation chamber enabled the observer to view the injected hindpaw at all times. Nociceptive behavior was quantified by counting the incidents of spontaneous flinching/shaking of the injected paw. The flinches were counted over 5 min periods for 60 min following the injection. Two phases of spontaneous flinching behavior were observed: Phase 1 started immediately after formalin injection and declined during the second period (5-10 min). Phase 2 began after 10 min with a maximum
response typically observed at approximately 20-30 min after the injection. Immediately after the test, rats were euthanised with an excess dose of pentobarbital (150 mg/kg i.p.).

The data for the phase 1 (1-10 min), and phase 2 (10-60 min) observations were considered separately. In each case, the mean value of saline-treated rats was considered as the control value. We calculated the percent of analgesia in the respective rats using the following equation:

\[
\text{percent analgesia} = \frac{\text{control value} - \text{test value}}{\text{control value}} \times 100.
\]

**Tolerance test (Tail-flick test)**

The tail-immersion version of the tail-flick test was performed according to a previous report with modifications (32). The rat was held in a cloth restrainer during testing. This method of restraint is a less stressful means of containing rats during tail-flick testing and has been shown to reduce variability in response latencies when compared to commercial restrainers (30). To perform the test, the end of the tail (5 cm) was placed in a 50°C water bath (49.5-50.5°C). This water-bath temperature was shown to produce a stable noxious stimulus-evoked response in this rat strain in the preliminary experiment. The tail-flick latency was defined as the time required to elicit a flick of the tail. The cut-off time was 30 sec. Immediately after control testing, the mini-osmotic pumps were implanted as described above. Since BLF cannot be solved in saline more than 1.25 µmol/ml, we used this concentration of BLF (1.25 µmol/ml) in this test.

**Statistics**

For the dose-response analysis, the dose response lines for phase 1 and phase 2 of the formalin test were fitted using least squares linear regression analysis, and ED50 values (the dose which produced 50% analgesia) and their 95% confidence intervals (CI) were calculated. Data are expressed as the mean ± S.E.. Differences between treatment groups were assessed by Student’s *t*-test or, when appropriate, ANOVA followed by Dunnett’s *post-hoc* test for multiple comparisons. In all cases, a probability (P) value of <0.05 was considered to indicate statistical significance.
Results

Dose response of BLF on the formalin-evoked nociception

As shown in Fig. 1A, rats were given a formalin (2.0%, 50 µl) injection into the right hindpaw, and showed spontaneous flinching/shaking of the injected paw. This pain-related behavior can be divided into two phases: an intense initial response beginning immediately after formalin injection and decreasing over the subsequent 5-10 min (phase 1), and a phase beginning at 10 min after injection, with a maximum response typically occurring at 20-30 min after the injection (phase 2). Spinally applied BLF (1.25-1250 pmol/rat) produced a dose-dependent inhibition of formalin-evoked nociceptive behavior during both phase 1 and 2. Spinal administration of a µ-opioid receptor agonist, morphine (27 nmol/rat), also produced a marked analgesia in this test. These data on BLF are summarized in Fig. 1B as a percent of analgesia. Spinally applied BLF induced significant analgesia at doses from 12.5 pmol to 1250 pmol during both phases (p<0.01 vs. control). ED50 (95% confidence interval: CI) values for BLF were 318.0 (19.4-5224) pmol/rat in phase 1 and 43.3 (3.1-596) pmol/rat in phase 2, respectively. Based on this result, we chose a BLF dose of 125 pmol/rat for the subsequent experiments.

Effects of opioid antagonist or a NOS inhibitor on the BLF-induced analgesia

Spinally applied BLF (125 pmol/rat) produced a significant degree of analgesia during both phase 1 and phase 2 (Fig. 2A, C) (p<0.001 vs. controls). Single administration of naloxone (non-selective opioid antagonist) (10 µg/rat) or D-Phe-Cys-Tyr-D-Trp-Orn-Thr-NH2 (CTOP, µ-opioid antagonist) (1 µg/rat), did not affect formalin-evoked nociception during either phase (Fig. 2A). In accordance with our previous observation (11), co-administration of naloxone (10 µg/rat) with BLF (125 pmol/rat) completely reversed BLF-induced analgesia (p<0.001 vs. BLF in both phases). CTOP also completely reversed BLF-induced analgesia (p<0.001 vs. BLF in both phases). Thus, BLF-induced analgesia on the formalin-evoked nociception is mediated by spinal µ-opioid receptor.

Spinally application of a NOS inhibitor, N^G^-nitro-L-arginine methyl ester (L-NAME; 30-300 µg/rat), did not affect phase 1, but produced a
dose-dependent analgesia during phase 2 (30 µg/rat, p<0.05; 300 µg/rat, p<0.01 vs. controls) (Fig. 2B). On the other hand, spinally applied N^6^-nitro-D-arginine methyl ester (D-NAME, the inactive enantiomer of L-NAME; 300 µg/rat) did not affect the formalin-evoked nociception (data not shown). An L-NAME dose of 30 µg/rat induced significant but weak antinociceptive activity only during phase 2; this dose was therefore chosen for the following co-administration experiments. Co-administration of L-NAME (30 µg/rat) with BLF (125 pmol/rat) completely reversed BLF-induced analgesia in phase 1 of the formalin test (p<0.005 vs. BLF) (Fig. 2C). BLF-induced analgesia in phase 2 was also significantly reversed (from 69% to 18% analgesia) by 30 µg/rat L-NAME, which was antinociceptive by itself (p<0.001 vs. BLF). However, coadministration of D-NAME (30 µg/rat) with BLF (125 pmol/rat) did not affect BLF-induced analgesia. These results suggest that NO participates in a large part of the BLF-induced analgesia.

**Potentiation of spinal μ-opioid receptor-mediated analgesia by BLF: the involvement of NO**

Spinal administration of a μ-opioid agonist, morphine (0.27-27 nmol/rat), produced a dose-dependent analgesia during both phases in the formalin test (Fig. 3). The ED50 (CI) values were 5.1 (37.1-0.7) nmol/rat in phase 1 and 2.5 (20.8-0.3) nmol/rat in phase 2. Analgesia induced by spinal administration of morphine was greatly potentiated by co-administration of BLF (1.25 pmol/rat) during both phases, although this dose of BLF did not affect formalin-evoked nociception per se (Fig. 1A, B). In the presence of BLF (1.25 pmol/rat), ED50 (CI) values for morphine decreased markedly, from 5.1 (37.1-0.7) nmol/rat to 0.10 (0.01-1.0) nmol/rat in phase 1, from 2.5 (20.8-0.3) nmol/rat to 0.02 (0.002-0.2) nmol/rat in phase 2. These results suggest the synergy between BLF and morphine.

As shown in Fig. 4, in the presence of spinally applied naloxone (10 µg/rat) or CTOP (1 µg/rat), the potentiated morphine (0.27 nmol/rat) -induced analgesia by BLF (1.25 pmol/rat) was completely abolished during both phases (p<0.001 in both phases vs. BLF+morphine). Furthermore, in the presence of spinally applied L-NAME (30 µg/rat), this potentiated analgesia (56% analgesia in phase 1, 87% analgesia in phase 2) was also significantly reversed (7.4% analgesia in phase 1, p<0.005; 20.1%
analgesia in phase 2, p<0.001 vs. BLF+morphine;). These results suggest that BLF potentiates the spinal µ-opioidergic system, and that NO is involved in this potentiation.

**Effect of chronic spinal infusion of BLF on the thermal nociception**

Fig. 5 shows the duration of analgesia and development of tolerance to continuous infusion (1 µl/h) of morphine (27 nmol/h/rat), BLF (1.25 nmol/h/rat) and saline (control) via mini-osmotic pumps over 6 days in the tail-flick test. In the control rats, there was no significant change of tail-flick latency during the experiment. In the morphine-infused rats, the maximum increase of tail-flick latency was observed on day 1 (p<0.001 vs. controls), and the latency declined markedly thereafter. In this experiment, morphine induced a significant degree of analgesia until day 3 (p<0.05 vs. controls). Tolerance to morphine was apparent by day 4, and the tail-flick latency remained at the same level as in the control rats until day 6. The development of tolerance to morphine in this experiment was similar to that of previous reports (12, 28, 29). In the BLF-infused rats, a significant increase of tail-flick latency was observed from day 1 (p<0.001 vs. controls), and the degree of analgesia remained constant until day 6 (p<0.001 vs. controls). No tolerance of BLF was observed during the experiment.

**Discussion**

In the present study, we demonstrated that milk-derived BLF exerts an antinociceptive activity via potentiation of the spinal µ-opioidergic system, and that NO is involved in this potentiation. Although various types of opioid receptor ligands have been found in milk or milk digests (37), BLF does not bind to the opioid receptor (unpublished observations) and does not develop tolerance in the manner of morphine (Fig. 5). It is well known that central endogenous opioid systems are activated during conditions that involve pain. Thus, BLF may exert an antinociceptive effect by potentiating the spinal opioidergic system, rather than by acting as an opioid agonist. In this study, sub-effective dose of BLF (1.25 pmol/rat) markedly potentiated the µ-opioid agonist (morphine)-induced analgesia in the formalin test (Fig. 3). In the presence of BLF, the ED50 values for morphine decreased approximately 50-fold in phase 1 and 100-fold in
phase 2. This result supports our hypothesis that BLF acts as an enhancer of the spinal opioidergic system.

LF and its receptor have been identified in the CNS, especially in the brain (6, 20, 22, 35). However, it has not been reported that whether LF receptor localizes in the dorsal horn of spinal cord, and whether LF receptor co-localizes with opioid receptors. Although present results show that BLF acts in the spinal cord and enhances the spinal opioidergic system, the possibility, BLF may also release opioid peptides, that mediate antinociception can not be excluded from the present data. Further investigation is required to clarify these points.

It is reported that BLF induces NO secretion from macrophages in rats (33). Although we did not measure NO level in the spinal cord in this study, we demonstrated the involvement of NO in BLF-induced potentiation of the spinal opioidergic system (Fig. 4). It is now well known that nNOS has different splice variants (nNOS-1 and nNOS-2) that mediate different action. Kolesnikov et al. reported nNOS-1 diminishes the analgesic actions of opioidergic system and causes opioid tolerance, while nNOS-2 enhances opioidergic system and cause analgesia without tolerance (16). BLF enhanced the spinal opioidergic system (Fig.3) but did not develop tolerance (Fig. 5). Thus, BLF may stimulate a nNOS-2 system but not a nNOS-1 system. The limited selectivity of traditional NOS inhibitors can not explore these opposing actions of nNOS-1 and nNOS-2. In the present study, spinally applied L-NAME significantly reversed the BLF-induced analgesia (Fig. 2C). However, L-NAME itself also produced analgesia during the phase 2 in the formalin test (Fig. 2B). BLF may stimulate a nNOS-2 system, which stimulates an opioidergic system. This mechanism leading to antinociception may be blocked at the level of nNOS-2 by L-NAME. On the other hand, upon spinal administration of L-NAME, the activities of both the nNOS-1 and nNOS-2 system were blocked and the antinociceptive potential previously repressed by nNOS-1 appeared and may be the only one that remained as a L-NAME-induced antinociception. Although involvement of NO in the nociception is still a matter of debate, it has been reported that NO exerts an antinociceptive activity via the central opioidergic system in both mice and rats (13, 14, 15), and also that NO potentiates opioid-induced analgesia in the mouse CNS (41). In addition, it has been reported that a transdermal NO-donor, nitroglycerine,
potentiates the opioid-induced analgesia in cancer patients (17). On the other hand, NO inhibits N-type voltage-sensitive Ca channel in human neuroblastoma cells (3). It is well known that the N-type Ca channel is involved in the control of neurotransmitters in both the peripheral nervous system and CNS, and this channel is inhibited by the opioid agonists via a G-protein coupling mechanism. In rats, intrathecal administration of a N-type Ca channel blocker potentiates morphine-induced analgesia, but does not lead to development of tolerance or cross-tolerance to morphine (38). In mice, intrathecal administration of a Ca channel blocker produces an analgesia, which is mediated by µ-opioid receptor (39). In this study, BLF also produced an analgesia that was mediated by µ-opioid receptor (Fig. 2A), and potentiated morphine-induced analgesia (Fig. 3), but did not lead to development of tolerance (Fig. 5). Based on the present data and the reports described above, it seems likely that the NO produced by BLF may induce analgesia via inhibition of the N-type Ca channel in the spinal cord. Nevertheless, the precise mechanism of BLF-induced antinociception seems to be more complicated and needs further investigation (e.g. experiments using nNOS knockout mice or nNOS splicing variants antisense).

In the present study, we demonstrated that NO is involved in BLF-induced analgesia only at the spinal level. However, since LF is an ubiquitous protein in the periphery (1, 19, 24, 25) and the CNS (9, 20, 22, 35), it is possible to imagine that the NO produced by LF may act in both the periphery and CNS. In some pathological conditions, immune-derived opioids cause peripheral antinociception (34). Endogenous LF, which is mainly produced by neutrophils, and exogenous LF may reduce peripheral pain, acting in synergy with immune-derived opioids. Endogenous and exogenous LF that enter the cerebrospinal fluid via receptor-mediated transcytosis through the blood-brain barrier would be enhanced in inflammatory conditions inducing pain, as suggested by a report that the proinflammatory cytokine, TNF-α, increased the rate of transendothelial transport in a blood-brain barrier model (7). Previously, we reported that oral application of bovine milk-derived lactoferrin (BLF) entered the cerebrospinal fluid in piglets (10) and calves (35). We also confirmed the presence of BLF in the rat cerebrospinal fluid following oral or intraperitoneal administration in the preliminary study. The endogenous
and exogenous LF that are transported into the cerebrospinal fluid from the periphery may also act in the CNS to reduce pain under pathological conditions.

The infants of many mammalian species constantly take exogenous LF from their mother’s milk (25). As LF is an ubiquitous protein in the periphery (1, 19, 24, 25) and the CNS (9, 20, 22, 35), endogenous LF is also available for both the infants and the adult animals. We used LF purified from bovine milk in the present study. The LF of humans, bovids, mice, and pigs share 70% overall amino acid sequence and 100% identity in several stretches of 10-15 amino acids at the C terminus (36). We also confirmed that recombinant human LF possesses a level of antinociceptive activity similar to that of BLF in the rat formalin test (11). Thus, not only BLF but also LF from different species will produce analgesia.

In summary, this is the first report to provide evidence of the involvement of NO in potentiation of the rat spinal µ-opioidergic system by milk-derived BLF. This analgesic function of LF involving the NO pathway underscores the importance of the bioactivities of this ubiquitous protein.

**Perspective**

In addition to the antinociceptive effects presented here, it is already known that LF has many peripheral functions, including induction of primary defense against bacterial and viral infection, antitumor activity, immunomodulation, and cell growth regulation (1). This wide range of LF activity will potentially be of great benefit to patients. The most common source of exogenous LF is milk, which is optimized for the infants of each mammalian species. The multifunctional milk-derived peptide, LF, *per se* possesses remarkable antinociceptive activity without tolerance, and also dramatically reduces the required dose of morphine, suggesting that LF can be a safely used natural drug for patients with severe pain that requires opioid treatment.

**References**


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**Figure legends**

**Fig. 1 Effect of BLF on formalin-evoked nociception.**
A. Time courses of the formalin-evoked flinching behavior following spinally applied saline (control, n=8), BLF (1.25-1250 pmol/rat, n=8), and morphine (Mor, 27 nmol/rat, n=6).

B. Dose-response curve for spinally applied BLF in the formalin test. The antinociceptive activity produced by BLF for phase 1 (1-10 min, □), and phase 2 (10-60 min, ●) was expressed as the percent of analgesia calculated from experiment A (see Methods). *P<0.01 (vs. control, Dunnett’s test).
Fig. 2 Effects of spinal administration of µ-opioid antagonists or a NOS inhibitor on the BLF-induced analgesia in the formalin test.
All data are presented as the percent of analgesia during phase 1 (□) and phase 2 (■) of the formalin test (see Methods).
A. Effects of spinal co-administration of naloxone (NLX, 10 µg/rat), or CTOP (10 µg/rat) with the BLF (125 pmol/rat) or saline (control) on the BLF-induced analgesia. Six rats were used in each group except the control group (n=8). *P<0.001 (vs. controls, Student’s t-test). #P<0.001 (vs. BLF, Student’s t-test).
B. Analgesia produced by spinally applied L-NAME (30-300 µg/rat). Seven rats were used in each group except the saline-treated group (controls, n=8). *P<0.05, **P<0.01 (vs. controls, Dunnett’s test).
C. Effects of spinal co-administration of L-NAME (30 µg/rat), or D-NAME (30 µg/rat) with the BLF (125 pmol/rat) or saline (control) on the BLF-induced analgesia. Six rats were used in each group except the control group (n=8). *P<0.05, **P<0.001 (vs. controls, Student’s t-test). #P<0.005, ##P<0.001 (vs. BLF, Student’s t-test).

Fig. 3 Potentiation of spinal µ-opioid receptor-mediated antinociceptive response by BLF on the formalin-evoked nociception.
Dose-response curve for spinally applied morphine (2.7-27000 pmol/rat) or saline (control) in the presence (■) or absence (○) of BLF (1.25 pmol/rat), expressed as a percent of analgesia (see Methods), during phase 1 (top) and phase 2 (bottom) in the formalin test. Seven rats were used in each group. *P<0.05, **P<0.01 (vs. control, Dunnett’s test).

Fig. 4 Effects of spinally administration of µ-opioid antagonists or a NOS inhibitor on the potentiated morphine-induced analgesia by BLF in the formalin test.
All data are presented as percent of analgesia during phase 1 (□) and phase 2 (■) of the formalin test (see Methods).
Morphine (270 pmol/rat) with BLF (1.25 pmol/rat) (M/B) was spinally administered in the presence or absence of naloxone (NLX, 10 µg/rat), CTOP (10 µg/rat), or L-NAME (30 µg/rat). Six rats were used in each group except the saline-treated group (control, n=8). *P<0.05, **P<0.001
(vs. controls, Student’s $t$-test). #$P<0.005$, ##$P<0.001$ (vs. BLF, Student’s $t$-test).

**Fig. 5 Tolerance to morphine and BLF in the tail-flick test.**
The time course of changes of the tail-flick latency during continuous (1.0 l/h) spinal infusion of BLF (■, 1.25 nmol/h, n=13), morphine (▲, 27 nmol/h, n=10) and saline (control, ○, n=12) was measured. *$P<0.05$, **$P<0.001$ (vs. controls, Student’s $t$-test).
Fig. 1
Fig. 2
Fig. 4