Aconitum-Derived Bulleyaconitine A Exhibits Antihypersensitivity Through Direct Stimulating Dynorphin A Expression in Spinal Microglia

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Abstract: Aconitine and its structurally-related diterpenoid alkaloids have been shown to interact differentially with neuronal voltage-dependent sodium channels, which was suggested to be responsible for their analgesia and toxicity. Bulleyaconitine A (BAA) is an aconitine analogue and has been prescribed for the management of pain. The present study aimed to evaluate the inhibitory effects of BAA on pain hypersensitivity and morphine antinociceptive tolerance, and explore whether the expression of dynorphin A in spinal microglia was responsible for its actions. Single intrathecal or subcutaneous (but not intraventricular or local) injection of BAA blocked spinal nerve ligation-induced painful neuropathy, bone cancer-induced pain, and formalin-induced tonic pain by 60 to 100% with the median effective dose values of 94 to 126 ng per rat (intrathecal) and 42 to 59 µg/kg (subcutaneous), respectively. After chronic treatment, BAA did not induce either self-tolerance to antinociception or cross-tolerance to morphine antinociception, and completely inhibited morphine tolerance. The microglial inhibitor minocycline entirely blocked spinal BAA (but not exogenous dynorphin A) antinociception, but failed to attenuate spinal BAA neurotoxicity. In a minocycline-sensitive and lidocaine- or ropivacaine-insensitive manner, BAA stimulated the expression of dynorphin A in the spinal cord, and primary cultures of microglia but not of neurons or astrocytes. The blockade effects of BAA on nociception and morphine tolerance were totally eliminated by the specific dynorphin A antiserum and/or κ-opioid receptor antagonist. Our results suggest that BAA eliminates pain hypersensitivity and morphine tolerance through directly stimulating dynorphin A expression in spinal microglia, which is not dependent on the interactions with sodium channels. Perspective: The newly illustrated mechanisms underlying BAA antinociception help us to better understand and develop novel dynorphin A expression-based painkillers to treat chronic pain.

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Key words: Bulleyaconitine A, anti-hypersensitivity, morphine tolerance to antinociception, spinal microglia, dynorphin A, sodium channel.

For centuries, preparations of the Aconitum genus have been used for analgesic, antirheumatic and neurological indications in China and other Asian countries. As a principal group of compounds present in Aconitum, 170 alkaloids have been identified and classified into 4 categories: C20-, C19-, C18-, and bis-diterpenoid alkaloids. The C19-diterpenoid group represented by aconitine comprises the most toxic alkaloids. Bulleyaconitine A (BAA), isolated from Aconitum bulleyanum, belongs to the “aconitine-like” alkaloids but differs from aconitine with 2 hydroxyl groups at C3 and C15 and a p-methoxy-benzoylester group at C14. The chemical structures of aconitine and BAA are presented in Fig 1. Because it has lower toxicity and a wider therapeutic window than aconitine, BAA was introduced into clinic in China for the treatment of chronic pain for 3 decades. Administration of BAA blocked acetic acid- and formalin-induced pain, with a...
affinity to the open state of sodium channels, causing Aconitum contrast, aconitine, the main diterpenoid alkaloid in their analgesia and toxicity,2,14 although there is no with neuronal sodium channels and be responsible for its alkaloids have generally been believed to interact and herbal catalpol produced antinociception and neuroprotection, leading to antinociception and neuroprotection, which was separate from the blockage of sodium channels that presumably mediated neurotoxicity.

Methods

Drugs and Reagents

BAA was purchased from Zelang Bio-Pharmaceutical (Nanjing, China) and ropivacaine mesylate was obtained from Xi’an Libang Pharmaceutical Co (X’ian, China). Morphine hydrochloride, lidocaine, and minocycline were purchased from Northeast Pharmaceuticals Group (Shenyang, China), Chengdu First Pharmaceuticals Group (Chengdu, China), and Yuanye Biotech (Shanghai, China), respectively. CTAP and nor-binaltorphimine dihydrochloride (nor-BNI) were obtained from Abcam (Cambridge, United Kingdom) and naltrindole from Tocris Bioscience (Bristol, United Kingdom). The rabbit polyclonal antibodies neutralizing dynorphin A and β-endorphin were purchased from Phoenix Pharmaceuticals (Burlingame, California) and Abcam, respectively. On the basis of the manufacturers’ information, the dynorphin A antiserum was specific to dynorphin A (100%), but not to dynorphin B (0%), β-endorphin (0%), α-neo-endorphin (0%), or leu-enkephalin (0%); the β-endorphin antiserum was specific to β-endorphin and did not cross-react with methionine-enkephalin, leucine-enkephalin, dynorphin A or B, γ-endorphin, α-endorphin, adrenocorticotropic hormone, or α-melanocyte-stimulating hormone. The specificities of the dynorphin A antiserum18,79 and β-endorphin antiserum62 were also validated by using the antigen absorption tests from other laboratories. All of the drugs and reagents were dissolved or diluted in 0.9% normal saline.

Experimental Animals

Male and female adult (200 ± 20 g body weight) and 1-day-old neonatal Wistar rats were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in plastic cages with 4 per cage and thick sawdust bedding at standard room temperature (22 ± 2°C), under conditions of a 12/12-hour reversed light-dark cycle (7:00 AM–7:00 PM), and received food and water ad libitum. They were accustomed to the laboratory environment for 3 to 5 days before the experiments. Experimental study groups (n = 6 per group except for the BAA neurotoxicity study in which there were 12 per group) were randomly assigned, and the researchers (T.F., H.F., Y.X.W.) were blinded for the behavior tests. The research protocols were approved and performed in accordance with the Animal Care and Welfare Committee of Shanghai Jiao Tong University and carried out in accordance with the Animal Care Guidelines of the National Institutes of Health.

Primary Neuronal and Glial Cell Culture

The 1-day-old neonatal rats and adult rats (180 ± 20 g body weight) were decapitated under anesthesia
The fold change was calculated using the ΔΔCT method after normalization to glyceraldehyde-3-phosphate dehydrogenase (gadph). The primers were: 5′-CCA AGG TCA TCC ATG ACA AC-3′ (gadph forward), 5′-TCC ACA GTC TTC TGA GTG GC-3′ (gadph reverse), 5′-ACT GCC TGT CCT TGT GTT CC-3′ (prodynorphin forward), and 5′-CCA AAG CAA CCT CAT TCT CC-3′ (prodynorphin reverse).15

**Measurement of Dynorphin A Using Fluorescent Immunoassay**

The dynorphin A concentrations were measured in the ipsilateral spinal dorsal lumbar enlargements from sham and neuropathic rats and primary cell cultures. For the spinal cords, rats were deeply anesthetized with ether and decapitated, and the spinal lumbar enlargements were obtained and dissected after dorsal laminectomy. The samples were immediately frozen in liquid nitrogen and stored at −80°C until further measurement. The rat ipsilateral spinal dorsal lumbar enlargements (1 g per 5 mL) were homogenized (4,000 rpm) for 15 seconds with a homogenizer (Fluko Equipment Co, Shanghai, China) in 10 mM Tris-hydrochloric acid (pH 7.4) and centrifuged (1,500 rpm) at 4°C for 15 minutes. Protein concentrations were determined using the bicinchoninic acid method using a commercially available kit (Beyotime Institute of Biotechnology, Shanghai, China).

For primary cultures, neurons, astrocytes, and microglia originating from the spinal dorsal horn of neonatal rats were placed in 24-well plates (5 × 10⁵ cells per well) and washed twice with 1 mL of warm DMEM containing 2 mg/mL of bovine serum albumin and 15 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid. Cells were exposed to 100 nM BAA for 2 hours and supernatant samples were processed. The dynorphin A contents were assayed using an enzyme-linked fluorescent immunoassay kit (Phoenix Pharmaceuticals) that was validated by running linear standards curves with testing samples simultaneously to determine the peptide concentrations. According to the manufacturer, the cross-reactivity of the assay included dynorphin A (100%), but not dynorphin B (0%), β-endorphin (0%), α-neo-endorphin (0%), or leu-enkephalin (0%). The relative fluorescence units were measured using a Fluorescence Microplate Reader (Thermo Labsystems, Vantaa, Finland), and the concentrations of the samples were determined by comparisons with a calibration curve running at the same time.

**Immunofluorescence Staining**

Single and double immunofluorescence labeling of dynorphin A and/or the biomarkers of primary cultures of microglia, astrocytes, and neurons were observed using a TCS SP8 confocal microscope (Leica Microsystems, Wetzel, Germany). Microglia, astrocytes, and neurons originating from the spinal dorsal horn of neonatal rats were placed in 6-well plates (5 × 10⁵ cells per well) with poly-L-lysine coated coverslips at the bottom, and were fixed in 4% paraformaldehyde then incubated in 10% goat serum (vol/vol) and 0.5% Triton-X 100 (vol/vol) in phosphate buffer solution for 1 hour. The cells were

RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction

RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction (PCR) were performed as described previously.13 The primary cultures of neurons, astrocytes, and microglia were collected and isolated using TRizol reagent (Invitrogen) on ice and total RNA was isolated. A sample of 1 μg of total RNA was reverse-transcribed using a ReverTra Ace qPCR RT-Kit (Toyobo Co, Osaka, Japan). Real-time quantitative PCR was carried out with a Mastercycler ep realplex (Eppendorf, Germany) using RealmasterMix (SYBR Green I; Toyobo Co). The fold change was calculated using the...
incubated with OX42 (mouse polyclonal; Abcam; 1:100 for tissue sections, 1:200 for cell flasks) for microglia, GFAP (mouse polyclonal; Millipore, Boston, MA; 1:100 for tissue sections, 1:200 for cell flasks) for astrocytes, NeuN (mouse polyclonal; Millipore; 1:60 for tissue sections, 1:100 for cell flasks) for neurons, and dynorphin A antibody (rabbit polyclonal, Phoenix Pharmaceuticals; 1:100 for tissue sections, 1:200 for cell flasks) for 24 hours at 4°C. All antibodies were tested for optimal dilution, the absence of cross-reactivity, and nonspecific staining. Dynorphin A was visualized with Alexa 555-conjugated goat anti-rabbit secondary antibody (1:200; Invitrogen), and other marker antibodies were detected with Alexa 488-conjugated goat anti-mouse secondary antibody (1:200; Invitrogen) for 1 hour at room temperature. Staining with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was also used to determine the cell nuclei.

**Intrathecal Catheterization and Injection in Rats**

An 18-cm polyethylene catheter (PE-10: 0.28 mm inner diameter and 0.61 mm outer diameter; Clay Adams, Parsippany, NJ) with a volume of 13 μL was inserted into the rat lumbar level of the spinal cord as described previously under inhaled isoflurane anesthesia (4% for induction and 1% for maintenance) run by an anesthesimeter (Ugo Basile Gas Anesthesia System, Comerio, Italy). Two days after recovery from anesthesia, the installation of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μL followed by 15 μL of saline for flushing) with a 50-μL microinjector (Shanghai Anting Micro-Injector Factory, Shanghai, China). Only rats that had no motor impairment after insertion of the intrathecal catheter were considered for the study, and only rats that developed immediate bilateral paralyzation of the hind limbs after intrathecal administration of lidocaine were selected for the study.

**Intracerebroventricular Catheterization and Injection in Rats**

The methods for intracerebroventricular catheterization and injection in rats have been described previously. Briefly, the animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and placed on a stereotaxic apparatus (Stoelting, Wood Dale, IL). After exposing the skull of the rat, a 24-gauge guide cannula (.55 mm) was stereotactically implanted 3 mm above the right lateral ventricle (anterior–posterior: −1.1 mm; height: +1.5 mm relative to the bregma; dorsal ventral: +2.8 mm below the surface of the skull). The guide cannula was anchored to 2 stainless steel screws using dental acrylic. The viability of the guide cannula was maintained using a dummy cannula, which was removed daily and cleaned during the handling procedure. Animals were allowed to recover for at least 7 days before undergoing behavioral tests. Control and test materials were delivered slowly over 1 minute through the injection needle (diameter: .3 mm), which was connected to a 10-μL microsyringe via a polyethylene tube held in the same place for 5 minutes. Each rat was intraventricularly injected with 5 μL of Indian ink dye after the experiment to ensure the accuracy and completeness of the surgical procedure.

**Rat Formalin Test**

Rats were individually acclimatized to the observation cage for 30 minutes before testing. The formalin test was performed as described previously by injecting 50 μL of 5% formalin in .9% saline subcutaneously on the dorsal side of the left hind paw of the rats that were immediately placed in a 23 × 35 × 19-cm transparent polycarbonate box. Nociceptive behavior was quantified manually by counting the number of the formalin-injected paw flinches over 1-minute time periods. Measurements were taken at 10-minute intervals, beginning immediately after the formalin injection and ending 90 minutes later.

**Rat Neuropathic Pain Model**

To induce neuropathic pain, the rats were subjected to spinal nerve ligation essentially as described previously. Spinal nerve ligation was used to induce peripheral neuropathy and neuropathic hypersensitivity. The unilateral ligation of 2 spinal nerves (L5 and L6) was performed under inhaled isoflurane anesthesia run by an anesthesimeter (Ugo Basile Gas Anesthesia System). The left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread. After ligation, the wound was sutured and the rats were allowed to recover. Of the spinal nerve-ligated animals, only those with marked unilateral allodynia to mechanical stimulation (hind limb withdrawal thresholds on the side of the procedure <8g) and no major impairment were included in the study. A sham procedure was performed on rats using the same procedures but without isolation and ligation of spinal nerves. Drug testing started on 2 to 4 weeks after the surgery of spinal nerve ligation.

**Rat Bone Cancer Pain Model**

The rat bone cancer pain model involved female rats anesthetized with pentobarbital (50 mg/kg intraperitoneally) as described previously. Bilateral superficial incisions were made in the skin overlying the patella. Further incisions were then made along the patellar ligament to expose the head of the tibia with minimal damage. A 23-gauge needle was inserted at the site of the intercondylar eminence and pierced 7 mm below the knee joint into the medullary cavity of the tibia. The needle was then removed and attached to a 10-μL microinjection syringe. Walker 256 carcinoma cells (4 × 105) in 10 μL phosphate buffer solution were slowly injected into the left tibia cavity. The syringe was left in place for an additional 1 minute to prevent the carcinoma cells from leaking out. The injection site was closed with aseptic bone wax while the syringe was removed. The wound was then closed and dusted with penicillin powder. After recovery from the inoculation surgery, rats were returned to their home cages for...
14 days to allow the development of allodynia in the ipsilateral paws. The withdrawal thresholds of contralateral and ipsilateral hind limbs were measured by the application of monofilaments.

**Behavioral Assessments of Mechanical Allodynia and Heat Hyperalgesia in Rats**

To evaluate mechanical allodynia, the animals were acclimatized for at least 30 minutes to the test environment, namely a plexiglass box on a metal grid (0.5 x 0.5 cm). The hind paw withdrawal threshold was measured using a 2450 CE Electronic Von Frey hair (IITC Life Science Inc, Woodland Hill, CA). An electronic hand-held transducer with a number 15 monofilament was applied perpendicularly to the medial surface of the hind paws with increasing force (ranging from .1 to 90g) until the rat suddenly withdrew or licked the hind paw. The lowest force that produced a withdrawal response was considered to be the threshold; this was on the basis of 3 repeated measurements with a 5-minute intervals, and the mean of the 3 threshold values for each hind paw at each time point was used.

To assess heat hyperalgesia, rats were put in a plexiglass box on an elevated glass surface. After an adaption period of at least 30 minutes, radiant heat was applied to the plantar medial surface of each hind paw. The hind paw withdrawal latency was measured by a 390G Plantar Test Analgesia Meter (IITC Life Science Inc). To prevent tissue damage, the latency cutoff was set at 30 seconds. The paw withdrawal latency was defined as the time from the onset of radiant heat application to the withdrawal of the hind paws. Both hind paws were tested independently 3 times with a 5-minute interval between the trials. The result for each test was calculated as the mean of the 3 repeated measurements.

**Data Evaluation and Statistical Analysis**

The maximal possible effect (% MPE) was calculated using the formula: (post-drug threshold in ipsilateral hind limb – baseline threshold in ipsilateral hind limb)/(baseline threshold in contralateral hind limb – baseline threshold in ipsilateral hind limb) x 100.5 The % MPE values near 100 indicate normal mechanical thresholds (ie, near contralateral thresholds) and values near 0 indicate allodynia. For the dose–response curve analysis, the parameters (ie, minimum effect [Emax], maximum effect [Emax], median effective concentration [EC50] or dose, or median lethal dose [LD50] and Hill coefficient [n]), were calculated from individual dose–response curves (GraphPad Prism, version 5.01, GraphPad Software, La Jolla, CA). To determine the parameters of the dose–response curves, values of response (Y) were fitted by nonlinear least squares curves to the relation Y = a + bx, where x = [D]/(ED50 + [D]), [C]/(EC50 + [C]), or [D]/(LD50 + [D]), to give the value of ED50, EC50 or LD50 and b (Emax) yielding a minimum residual sum of squares of deviations from the theoretical curve.73

The data are presented as means ± standard error of the mean or 95% confidence limits, and no data were missing. Statistical significance was evaluated using a χ2-test (for the rate data), an unpaired Student t-test or a 1-way or 2-way repeated-measures analysis of variance (ANOVA), which was followed by a post hoc Student–Newman–Keuls test when a statistically significant drug (dose) effect was observed (for the 1-way ANOVA, the factor was drug [dose]; for the 2-way ANOVA, the factors were drug [dose], time, and their interaction). Probability values were 2-tailed, and the statistical significance criterion P value was .05.

**Results**

**Systemic BAA Produced Antihypersensitivity Effects in Neuropathic Pain**

The antihypersensitivity effects of systemic BAA were examined in 5 groups of LS/L6 spinal nerve–ligated neuropathic rats, which received a single subcutaneous injection of normal saline (1 mL/kg) or BAA (10, 30, 100, or 300 µg/kg). The paw withdrawal thresholds to von Frey monofilaments and withdrawal latencies to radiant heat (10 minutes later) were subsequently measured before, and .5, 1, 2, and 4 hours after drug injection. As shown in Figs 2A and 2B, spinal nerve ligation induced marked mechanical allodynia and heat hypersensitivity compared with the contralateral paws. Paw withdrawal responses remained unchanged in saline-treated rats during the 4-hour observation period. Subcutaneous injection of BAA significantly increased both paw withdrawal thresholds to mechanical stimulus and withdrawal latency to heat stimulus in ipsilateral paws in a time- and dose-dependent manner. BAA did not affect paw withdrawal thresholds in contralateral paws. The % MPE values of BAA were calculated and the dose–response analyses for allodynia and hyperalgesia were projected at 1 hour after the injection. For the blockade effect on mechanical allodynia, the Emax value was 70.1% MPE and the ED50 value was 59.4 µg/kg (95% confidence limits: 23.5–149.8 µg/kg; Fig 2C). For the blockade effect on thermal hyperalgesia, the Emax value was 99.4% MPE and the ED50 value was 42.4 µg/kg (95% confidence limits: 14.3–125.7 µg/kg; Fig 2D).

**BAA Produced Antihypersensitivity at the Spinal Cord**

To determine the site at which BAA produced antihypersensitivity effects, we first tested its intrathecal injection in neuropathic rats. Six groups of neuropathic rats received a single intrathecal injection of normal saline (10 µL) or BAA (10, 30, 100, 300 ng, or 1 µg). The paw withdrawal thresholds and withdrawal latencies were subsequently (with 10-minute intervals) measured before, and .5, 1, 2, and 4 hours after drug injection. BAA increased the thresholds of mechanical allodynia and withdrawal latency of heat hyperalgesia in...
ipsilateral paws in a time- and dose-dependent manner, but did not affect paw withdrawal thresholds in contralateral paws (Figs 3A and 3B). Dose–response analysis 1 hour after the intrathecal injection showed that the Emax and ED50 values for BAA to block mechanical allodynia were 68.1% MPE and 113 ng (95% confidence limits: 57.7–215 ng; Fig 3C). Accordingly, the Emax and ED50 values for BAA to block thermal hyperalgesia were 100% MPE and 126 ng (95% confidence limits: 83.5–190 ng; Fig 3D).

A further determination of antihypersensitivity involved the application of BAA in the spinal cord, brain, and peripheral afferent nerve terminals in the rat formalin test. Two groups of rats received an intrathecal injection of saline (10 μL) or BAA (300 ng) 30 minutes before an intraplantar injection of 5% formalin. Another group of rats received an intrathecal injection of saline (10 μL) 30 minutes before an intraplantar injection of 300 ng BAA dissolved in 5% formalin. As shown in Fig 3E, formalin induced acute and tonic flinching responses in saline-treated rats. Intrathecal BAA did not alter the formalin-induced acute flinching response, but significantly blocked the formalin-induced tonic hyperalgesia by 63.7% after calculation of the area under the flinching-time curve. In contrast, co-intraplantar injection of BAA at the same dose of 300 ng did not significantly inhibit formalin-induced acute or tonic flinching responses. In addition, two groups of rats received an intraventricular injection of 5 μL saline or 300 ng BAA followed by a formalin challenge 30 minutes later. Intraventricular injection of 300 ng BAA did not significantly reduce either the formalin-induced acute or tonic flinching response (Fig 3F).

BAA Produced Antiallodynia in Bone Cancer Pain

Six groups of rats with bone cancer approximately 15 days after a tibia implant of cancer cells received a single intrathecal injection of normal saline (10 μL) or BAA (10, 30, 100, 300 ng, or 1 μg). As shown in Fig 4A, the tibia implant of cancer cells produced mechanical allodynia. Paw withdrawal thresholds and mechanical allodynia in control rats remained unchanged during the 4-hour observation period after intrathecal administration of normal saline. Intrathecal administration of BAA reduced mechanical allodynia in the ipsilateral paws with a peak at 1 hour or earlier and a duration of approximately 4 hours, but did not significantly affect withdrawal thresholds in contralateral paws. The blockage effect of BAA was dose-dependent and the dose–response analysis 1 hour after injection showed that the ED50 value was 93.7 ng (95% confidence limits: 44.2–199 ng) and the Emax value was 59.7% MPE (Fig 4B).

BAA Produced Antiallodynia but Not Neurotoxicity in a Minocycline-Sensitive Manner

To test whether BAA produced antiallodynia and neurotoxicity through spinal microglia, the microglial inhibitor minocycline was applied. Three groups of neuropathic rats received 2 intrathecal injections of
saline (10 μL) + saline (10 μL), saline (10 μL) + BAA (300 ng), or minocycline (100 μg) + BAA (300 ng), respectively. The second treatment was administered 4 hours after the first treatment, and paw withdrawal thresholds were measured at before and 4 hours after the first treatment, and .5, 1, 2, or 4 hours after the second administration. Intrathecal minocycline affected neither contralateral nor ipsilateral paw withdrawal thresholds compared with the saline control. A single intrathecal injection of BAA produced a marked and reversible antiallodynic effect in ipsilateral paws, which was completely inhibited by spinal minocycline (P < .05, 2-way repeated measures ANOVA followed by the post-hoc Student–Newman–Keuls test).

Figure 3. The inhibitory effects of intrathecal injection of BAA on mechanical allodynia (A) and heat hyperalgesia (B) in L5/L6 spinal nerve-ligated neuropathic rats. Dose–response analysis of BAA on mechanical allodynia (C) and heat hyperalgesia (D) 1 hour after intrathecal injection, best projected by the nonlinear least squares method. The effects of intrathecal (E), local (E), and intraventricular (F) injection of BAA on formalin-induced pain in rats. The data are presented as means ± standard error of the mean (n = 6 per group). A Statistical significance compared with the saline group (P < .05, 2-way repeated measures ANOVA followed by the post-hoc Student–Newman–Keuls test).

neuropathic rats received 2 intrathecal treatment regimens of saline (10 μL) + dynorphin A (0.3 μg), or minocycline (100 μg) + dynorphin A (0.3 μg). Intrathecal injection of dynorphin A produced a moderate and reversible antiallodynic effect in the ipsilateral paws, which was not significantly altered by the pretreatment with minocycline (Fig 5B). In addition, 2 groups of neuropathic rats received 2 intrathecal treatment regimens of saline (10 μL) + ropivacaine (200 μg) and minocycline (100 μg) + ropivacaine (200 μg), respectively. Intrathecal ropivacaine produced a moderate and reversible antiallodynia, which was not significantly affected by minocycline (Fig 5C). The spinal neurotoxicity of BAA was also studied in rats. Three groups of neuropathic rats received 2 intrathecal treatments of saline (10 μL) + minocycline (100 μg), saline (10 μL) + BAA (3 μg), or minocycline
the rate or latency. (Figs 6A and 6B) or mortality (Figs 6C and 6D) in neither cline did not significantly alter BAA-induced paralysis 1 hour after injection (Fig 6A) from the primary culture of neurons or astrocytes. Although it is routine to use neonatal animals in cell culture, there are possible changes in BAA-induced dynorphin A expression of glia and neurons in the development. To exclude this possibility, the effects of BAA were also examined in primary cultures of microglia, astrocytes, and neurons from adult rats. Treatment with BAA (1 × 10⁻⁸, 3 × 10⁻⁸, 1 × 10⁻⁷, 3 × 10⁻⁷, and 1 × 10⁻⁶ M) similarly increased prodynorphin gene expression and dynorphin A release from adult microglia, with EC₅₀ values of 55.1 and 47.9 nM, respectively (Figs 8E and 8F). In contrast, BAA at 100 nM did not affect either the prodynorphin gene expression (Fig 8C) or dynorphin A release (Fig 8D) from the primary culture of neurons or astrocytes.

To further determine whether BAA-induced dynorphin A expression was minocycline-sensitive, minocycline was applied 2 sodium channel blockers, ropivacaine and lidocaine, at a high concentration (1 mM) that was previously shown to block sodium channels completely. In presence of dynorphin A in neurons and astrocytes and further explore whether dynorphin A was also localized in microglia, double immunofluorescence labeling of dynorphin A and the cell-specific markers were examined in primary cultures of spinal neurons, astrocytes, and microglia originated from the spinal dorsal horn of neonatal rats. Dynorphin A immunostaining was found to be colocalized with neurons (NeuN; Figs 7A and 7B) and astrocytes (GFAP; Figs 7C and 7D), as well as microglia (OX42; Figs 7E and 7F). In a separate experiment, microglial cells were cultured with BAA (100 nM) for 2 hours. The density of dynorphin A immunostaining in these cells appeared to be the same as that in microglia without BAA treatment (Figs 7G and 7H), suggesting that BAA did not deplete dynorphin A from microglia.

Furthermore, microglial cells from neonatal rats were treated with different concentrations (3 × 10⁻⁹, 1 × 10⁻⁸, 3 × 10⁻⁸, 1 × 10⁻⁷, 3 × 10⁻⁷, and 1 × 10⁻⁶ M) of BAA, and the cellular prodynorphin gene expression and the level of dynorphin A in culture medium were measured 2 hours later. Treatment with BAA up to 1 μM did not affect microglial viability. In a concentration-dependent manner, BAA increased prodynorphin gene expression, with an EC₅₀ of 44.6 nM (Fig 8A), and stimulated the dynorphin A release into the culture medium, with an EC₅₀ of 46.8 nM (Fig 8B). In contrast, BAA at 100 nM did not affect either the prodynorphin gene expression (Fig 8C) or dynorphin A release (Fig 8D) from the primary culture of neurons or astrocytes. Although it is routine to use neonatal animals in cell culture, there are possible changes in BAA-induced dynorphin A expression of glia and neurons in the development. To exclude this possibility, the effects of BAA were also examined in primary cultures of microglia, astrocytes, and neurons from adult rats. Treatment with BAA (1 × 10⁻⁸, 3 × 10⁻⁸, 1 × 10⁻⁷, 3 × 10⁻⁷, and 1 × 10⁻⁶ M) similarly increased prodynorphin gene expression and dynorphin A release from adult microglia, with EC₅₀ values of 55.1 and 47.9 nM, respectively (Figs 8E and 8F). In contrast, BAA at 100 nM and 1 μM did not alter prodynorphin gene expression (Fig 8G) or dynorphin A release (Fig 8H) from adult neurons or astrocytes.

BAA Stimulated Dynorphin A Expression in Spinal Microglia

Dynorphin A has been reported to be localized and secreted in neurons and astrocytes. To confirm the
contrast to BAA, neither ropivacaine nor lidocaine increased the prodynorphin gene expression in the primary microglial cells from neonatal rats. In addition, ropivacaine and lidocaine did not significantly alter the BAA-induced increase in prodynorphin gene expression (Fig 8K).

Figure 5. The effects of intrathecal injection of the microglial inhibitor minocycline on BAA (A), dynorphin A (B), and the sodium channel blocker ropivacaine (C)-induced antiallodynia in L5/L6 spinal nerve-ligated neuropathic rats. Minocycline (100 μg) was intrathecally injected 4 hours before the spinal administration of BAA, dynorphin A, or ropivacaine. The data are presented as means ± standard error of the mean (n = 6 in each group). a and b Statistical significance compared with the saline and BAA group, respectively (P < .05, 2-way repeated measures ANOVA followed by post hoc Student–Newman–Keuls tests).

Figure 6. The effect of intrathecal injection of the microglial inhibitor minocycline on BAA-induced paralysis (A) and (B) and mortality (C) and (D) in L5/L6 spinal nerve-ligated neuropathic rats. Minocycline (100 μg) was intrathecally injected 4 hours before the spinal administration of BAA. The data are presented as the percentage in (A) and (C), and means ± standard error of the mean in (B) and (D) (n = 12 in each group). a Statistical significance compared with the minocycline group (P < .05, χ²-test or 1-way ANOVA).
**BAA Produced Antiallodynia by Stimulating Spinal Dynorphin A Expression**

To evaluate whether BAA produced antiallodynia via spinal dynorphin A expression, we first tested the minocycline-sensitive stimulating effect of BAA on dynorphin A expression from the spinal cord. Four groups of neuropathic rats 22 days after surgery received 2 intrathecal injections of saline (10 μL) + saline (10 μL), saline (10 μL) + BAA (300 ng), minocycline (100 μg) + saline (10 μL), or minocycline (100 μg) + BAA (300 ng), respectively. The second treatment was administered 4 hours after the first treatment. In addition, 2 groups of sham rats received a single intrathecal injection of saline and BAA (300 ng), respectively. The ipsilateral spinal dorsal lumbar enlargements were obtained 1 hour after intrathecal injection of BAA in sham and neuropathic rats, and the spinal dynorphin A levels were measured. As shown in Fig 9A, intrathecal injection of BAA significantly increased the dynorphin A level by 49.1% (increment in 37 pg/mg protein in spinal homogenates) in the ipsilateral spinal cord. Pretreatment with minocycline did not significantly alter the baseline level of dynorphin A, but completely inhibited the stimulatory effect of BAA. The stimulatory effect of BAA on the spinal dynorphin A level (by 57.7 %, increment in 46 pg/mg protein in spinal homogenates) was also confirmed in sham rats (P < .05, unpaired Student t-test).

Furthermore, 4 groups of neuropathic rats received an intrathecal injection of 10 μL blank rabbit serum (1:10 dilution), dynorphin A antiserum (1:50 or 1:10 dilution), or β-endorphin antiserum (1:10 dilution). A 300-ng dose of BAA was intrathecally injected 30 minutes later. The dose of β-endorphin antiserum was previously shown to completely block the GLP-1 receptor agonist exenatide-induced antinociception in neuropathic rats.24 BAA and the paw withdrawal responses were measured before and .5, 1, 2, and 4 hours after the second administration. Intrathecal pretreatment with antiserum against dynorphin A dose-dependently and completely inhibited BAA-induced antiallodynia although it did not alter the basic mechanical allodynia. In contrast, the antiserum neutralizing β-endorphin was not effective in reducing BAA antiallodynia (Fig 9B).

Dynorphin A produces analgesia by activating κ-opioid receptors.12,33 We further tested the effects of the selective μ-opioid receptor antagonist, CTAP,11 the κ-opioid receptor antagonist, nor-BNI,51 and the δ-opioid receptor antagonist, naltrindole15 on BAA-induced antiallodynia. Four groups of neuropathic rats received the following pairs of intrathecal injections: saline (10 μL) + BAA (300 ng), CTAP (10 μg) + BAA (300 ng), nor-BNI (100 μg) + BAA (300 ng), or naltrindole (5 μg) + BAA (300 ng). The second treatment was administered .5 hours after the first treatment, and the paw withdrawal response was measured before and .5, 1, 2, and 4 hours thereafter. Intrathecal injection of BAA led to time-dependent antiallodynia in the ipsilateral paws. Although the intrathecal injection of nor-BNI did not affect the withdrawal response in either the contralateral or the ipsilateral paws, it almost totally inhibited BAA-induced antiallodynia (P < .05, 2-way ANOVA). In contrast, neither CTAP nor naltrindole significantly reduced BAA antiallodynia (Fig 9C). We later tested whether intrathecal pretreatment with dynorphin A antiserum and nor-BNI totally inhibited systemic BAA-induced antiallodynia. Four groups of neuropathic rats received an intrathecal injection of saline (10 μL), blank rabbit serum (1:10 dilution, 10 μL), dynorphin A antiserum (1:10 dilution), or nor-BNI (100 μg) followed by a subcutaneous injection of BAA (100 μg/kg) 30 minutes later. As shown in Fig 9D, subcutaneous injection of BAA produced marked and reversible antiallodynia in the ipsilateral paws of saline- and blank serum–treated control rats. Treatment with dynorphin A antiserum and nor-BNI completely inhibited systemic BAA antiallodynia.

**BAA Inhibited Morphine Antinociceptive Tolerance via Activating Spinal κ-Opioid Receptors**

We finally tested whether chronic treatment of BAA led to self-tolerance to antinociception or cross-tolerance to morphine antinociception, and also inhibited morphine antinociceptive tolerance. Four groups of neuropathic rats received multiple bidaily injections of saline (10 μL), BAA (300 ng), morphine (20 μg), or BAA (300 ng) + morphine (20 μg) for 7 days. The paw withdrawal thresholds were measured 1 hour after the first injection on each day. As shown in Fig 10A, mechanical allodynia remained unchanged during the 7-day bidaily intrathecal injections of saline. BAA produced antiallodynia and its antinociceptive effect remained unchanged during the 7-day bidaily treatments. In contrast, bidaily injections of morphine for 7 days produced progressive and complete tolerance to its antiallodynic effect. However, coinjections with BAA produced apparently additive antiallodynia with morphine in the acute setting, and completely inhibited morphine antinociceptive tolerance in the chronic setting.

On day 8, all 4 groups of rats subsequently (separated by 6 hours) received a single intrathecal injection of BAA (300 ng) and morphine (20 μg), and paw withdrawal thresholds were measured hourly after injection. Intrathecal injection of BAA and morphine produced marked and reversible antiallodynia in the ipsilateral paws in the 7-day saline-treated neuropathic rats. The antiallodynic effect of BAA in saline-treated rats was the same as that in BAA-, morphine-, and BAA + morphine–treated rats. In contrast, the antiallodynic effect of morphine in saline-treated rats remained the same as that in BAA- and BAA + morphine–treated rats (Fig 10B).

To determine whether the inhibitory effect of BAA on morphine antinociceptive tolerance was associated with the activation of spinal κ-opioid receptors, the κ-opioid receptor antagonist was applied. The 4 groups of rats continuously received bidaily intrathecal injections of saline, BAA, morphine, and BAA + morphine, respectively,
Figure 7. Representative graphs of the expression of dynorphin A in spinal neurons (A) and (B), astrocytes (C) and (D), and microglia (E) and (F). Primary cultures of astrocytes, neurons, and microglia were originated from the spinal dorsal horn of neonatal rats. The
for an additional 7 days. On the 12th day morning, the rats received a single intrathecal injection of nor-BNI (100 μg) 30 minutes before their bidaily injections. As presented in Fig 10C, nor-BNI did not affect baseline mechanical allodynia in saline-treated rats but completely diminished BAA antiallodynia. However, nor-BNI did not alter morphine antinociceptive tolerance, but it completely eliminated the inhibitory effect of BAA on morphine antiallodynic tolerance. The blockade effect of nor-BNI disappeared 2 days after its injection.

Discussion

The Aconitum extracts and their active ingredients, diterpenoid alkaloids, produced effective antinociception in many animal models of pain, such as thermally induced reflex responses, acetic acid-induced writhing responses, formalin-induced flinch responses, postoperative pain, chronic constriction injury induced neuropathic pain, and streptozotocin-induced diabetic pain.2,14,63,78 The present study showed that BAA was efficacious in rat models of pain hypersensitivity and presented several features. 1) BAA was antihypersensitive rather than analgesic. The systemic or intrathecal injection of BAA produced specific, potent, and dose-dependent antinociception in a broad range of pain hypersensitivity states, including formalin-induced tonic pain, spinal nerve ligation–induced neuropathic pain, and cancer cell inoculation–induced mechanical allodynia. In contrast, BAA at the highest doses tested did not significantly affect formalin-evoked acute flinch responses or thermally or mechanically stimulated reflexes in the acute nociceptive pain models. 2) BAA was more sensitive in blocking thermal hyperalgesia (with the E_max of 100% MPE) than mechanical allodynia (with the E_max of 70% MPE) in neuropathic pain. 3) In contrast to morphine, BAA did not induce self-tolerance to antinociception. In addition, it produced apparently additive antiallodynia with morphine in the acute setting and did not induce cross-

Figure 8. Effects of BAA on dynorphin A expression in primary cultures of microglia, neurons, and astrocytes originating from the spinal dorsal horn of neonatal (A), (B), (C), and (D) and adult (E), (F), (G), and (H) rats. Effects of the microglial inhibitor minocycline (I) and (J) and the sodium channel blockers ropivacaine and lidocaine (K) on BAA-stimulated dynorphin A expression in the primary culture of microglia. Minocycline, ropivacaine and lidocaine were applied 1 hour before BAA treatment, and the culture medium and cultured cells were collected 2 hours later. The expression of the prodynorphin gene referred to the gapdh gene in primary cultures of cells and the dynorphin A level in the culture medium were determined using the real-time quantitative PCR and the commercial fluorescent immunoassay kit, respectively. The data are presented as means ± standard error of the mean (n = 3 in each group with 2 repeats). a and b Statistical significance compared with the saline control and BAA groups, respectively (P < .05, 1-way ANOVA followed by post hoc Student–Newman–Keuls test). Abbreviation: mRNA, messenger RNA.
tolerance to morphine antinociception in the chronic setting. The inability of BAA to induce tolerance was supported by the fact that BAA may not deplete dynorphin A, because incubation with BAA did not appear to change the density of dynorphin A immunostaining in the primary culture of microglia, although it stimulated dynorphin A expression. These results support the long-term clinical use of BAA as a painkiller for the management of pain hypersensitivity. Indeed, BAA has been widely used in clinical settings in China for the treatment of chronic pain conditions, including cancer pain, neuopathic pain, and rheumatoid arthritis. 

We further showed that the spinal cord is a principal site where BAA produces antihypersensitivity. This conclusion was supported by the following evidence. The intrathecal injection of BAA produced antinociception in formalin-induced tonic pain, neuropathic pain, and bone cancer pain. Moreover, the ED_{50} values for the intrathecal administration of BAA in spinal nerve-ligated neuropathic rats were 113 and 126 ng for mechanical allodynia and thermal hyperalgesia, respectively, which represented approximately 130- to 80-fold higher values than those of the subcutaneous administration of BAA. In addition, the intraventricular injection and local coinjection of up to 300 ng BAA did not produce antihypersensitivity. More specifically, the antiallodynic effect of BAA given subcutaneously was completely inhibited by the intrathecal pretreatment with the antidynorphin A antiserum and κ-opioid receptor antagonist nor-BNI, both of which interfered with the spinal mechanisms underlying BAA antiallodynia.

One of our striking findings was that BAA antinociception was produced by the stimulation of dynorphin A expression probably in microglia in the spinal cord. Dynorphin A, an endogenous opioid neurotransmitter, is produced in many different parts of the brain, including the hypothalamus, the striatum, hippocampus, and the spinal cord. The localization and secretion of dynorphin A have been found in neurons and astrocytes. We confirmed and extended the previous findings that dynorphin A was also present in microglia by using double immunostaining. In addition, BAA

Figure 9. Stimulatory effects of intrathecal injection of BAA on spinal dynorphin A levels in L5/L6 spinal nerve-ligated neuropathic and sham rats (A). Minocycline was intrathecally injected 4 hours before intrathecal injection of BAA. The ipsilateral spinal dorsal lumbar enlargements in sham and neuropathic rats were obtained 1 hour after BAA injection. The dynorphin A level in the spinal homogenates was measured using a commercial fluorescent immunoassay kit. Blockade effects of the specific dynorphin A antiserum and selective κ-opioid receptor antagonist nor-BNI on intrathecal (B) and (C) and subcutaneous (D) injection of BAA-induced antiallodynia in neuropathic rats. The dynorphin A (and β-endorphin) antisera and opioid receptor antagonists (CTAP, nor-BNI, and naltrindole) were intrathecally injected 0.5 hours before intrathecal or subcutaneous injection of BAA. The data are presented as means ± standard error of the mean (n = 6 per group in neuropathic rats). a and b Statistical significance compared with the saline group and the saline plus BAA groups, respectively (P < .05, unpaired Student t-test, or 1-way or 2-way repeated measures ANOVA followed by post hoc Student–Newman–Keuls tests).
stimulated dynorphin A expression (measured by the levels of dynorphin A and the prodynorphin gene expression) in the primary culture of microglia but not of neurons or astrocytes from neonatal and adult rats, although the latter 2 types of cells also express and secrete dynorphin A. Several groups have shown that peripheral nerve injury evoked dynorphin A expression in the dorsal spinal cord particularly at the early stage.30,39,74 However, our data showed that the spinal level of dynorphin A measured 3 weeks after surgery in spinal nerve–ligated rats were the same as that in sham rats. Our unpublished data in a different study also showed comparable spinal expression of the prodynorphin gene encoding dynorphin A in sham and neuropathic rats. The reason for the conflict results is not clearly known but may be related to the time course of the peripheral nerve injury evoked dynorphin A expression in the dorsal spinal cord particularly at the early stage.30,39,74 Indeed, dynorphin A was reported to be increased by 5 days in neurons in laminae I and II and V to VII in the lumbar spinal cord ipsilateral to the injury. The increase, maximal at 10 days was still present 20 days after the injury but was seen only in neurons in the deep laminae (V–VII).30 Nevertheless, we showed that BAA significantly stimulated dynorphin A expression in the spinal dorsal horn from neuropathic and sham rats compared with the post-surgery baseline values, and its stimulatory effect was completely blocked by the microglial inhibitor minocycline. More directly, the specific antidynorphin A serum (but not β-endorphin antiserum) completely eliminated BAA antiallodynia.

The influential effects of opioid receptor subtypes on Aconitum antinociception were controversial. The antinociceptive effects of crude Aconitum and its processed products were reported to be attenuated by the μ-opioid–preferred antagonist naloxone and the knockout of μ-opioid receptors in an acute pain model.37 In contrast, the antinociceptive effects of the Aconitum extracts were reported to be inhibited by the κ-opioid receptor antagonist nor-BNI but not naloxone in chronic constriction injury neuropathic rats.77 Our data clearly showed that the selective κ-opioid receptor antagonist nor-BNI, but not the μ- or δ-opioid receptor antagonists CTAP or naltrindole, completely inhibited the antiallo- dynic effects of BAA in spinal nerve–ligated neuropathic rats administered intrathecally or subcutaneously. The results indicate that BAA antinociception is governed by κ-opioid receptors and imply that the endogenously expressed dynorphin A acts primarily via the κ-opioid receptors. Finally, our unpublished data also showed that the antinociceptive effects of the main alkaloid aconitine in neuropathic pain was entirely inhibited by minocycline, the dynorphin A antiserum, and nor-BNI. The role of

Figure 10. Effects of multiple bidaily (Bi-daily; bid) intrathecal injections of BAA, morphine, and the combination of BAA and morphine on mechanical allodynia in L5/L6 spinal nerve–ligated neuropathic rats. Paw withdrawal thresholds were measured 1 hour after each morning injection for 7 days. (A) The inhibitory effect of BAA on morphine antinociceptive tolerance. (B) The cross-effects of BAA and morphine on morphine and BAA antiallodynia. On day 8 after the bid injections, rats received a subsequent single intrathecal injection of BAA and morphine, and paw withdrawal thresholds were measured hourly. (C) The blockade effect of the κ-opioid receptor antagonist nor-BNI on the inhibitory effect of BAA on morphine antinociceptive tolerance. On the 12th day morning, rats received a single intrathecal injection of nor-BNI 30 minutes before the bid injections. The data are presented as means ± standard error of the mean (n = 6 per group).
mitogen-activated protein kinase, but it also has nuclear factor 

minocycline primarily prevents the translocation of both of which were known to respectively interact with neuronal voltage-dependent sodium channels and \(\kappa\)-opioid receptors. Furthermore, ropivacaine and another sodium channel blocker lidocaine neither stimulated dynorphin A expression as did BAA, nor blocked BAA-stimulated dynorphin A expression in the primary culture of microglia. It seems that voltage-dependent sodium channels are not expressed on microglia (H. Kettenmann, personal communication). These data suggest that the activation or inhibition of sodium channels is not required for BAA-induced dynorphin A expression in microglia and its antiallodynia in neuropathic rats.

Intrathecal BAA-induced acute neurotoxicity (including respiratory distress, flaccid paralysis, and death) is characterized by motor blockade, which is shared with dynorphin A neurotoxicity. It is therefore possible for BAA to release a large amount of dynorphin A to produce neurotoxicity especially because dynorphin A expression is associated with BAA antinociception. However, our data ruled out this possibility. First, intrathecal dynorphin A (33 \(\mu\)g)–produced flaccid paralysis was completely blocked by minocycline, the NMDA receptor antagonist, and the matrix metalloproteinase-9 inhibitor, suggesting that dynorphin A induced neurotoxicity by stimulation of microglia, through the NMDA receptor, leading to secretion of proinflammatory cytokines, such as interleukin (IL)-1\(\beta\), IL-6, and tumor necrosis factor (TNF-\(\alpha\)). In our hands, however, minocycline did not affect BAA-induced acute neurotoxicity including paralysis and mortality. Furthermore, BAA may not be able to release a sufficient amount of dynorphin A to produce toxicity. The spinal level of dynorphin A is estimated to be 155 ng/mg protein in the homogenates after its intrathecal injection at the toxic dose of 33 \(\mu\)g, on the basis of our previous study in which the intrathecal injection of 1 \(\mu\)g of \(\beta\)-endorphin led to 4.7 ng/mg protein in the spinal homogenates. On the contrary, intrathecal BAA at the super maximum antinociceptive dose (300 ng) increased the spinal dynorphin A level by only approximately 40 pg/mg protein in the homogenates, which is 3,900-fold less than that caused by intrathecal 33 \(\mu\)g of dynorphin A. All of these results suggest that although the expressed dynorphin A entirely accounts for BAA antiallodynia, it is not primarily responsible for BAA neurotoxicity, which is presumably due to its interactions with neuronal sodium channels. The separation of BAA antinociception from its toxicity may provide a pharmacological base for its analgesic window and for future chemical modifications to remove its sodium channel activity groups.

Morphine is considered a benchmark for analgesics used to relieve severe pain and suffering. However, the clinical usefulness of opiates in the treatment of chronic pain is hampered by morphine analgesic tolerance, which develops rapidly and is a common clinical phenomenon; the amounts of morphine required to elicit pain relief therefore have to be increased to compensate for diminished responsiveness. Preclinical studies have shown that dynorphin A given intrathecally or intravenously was effective in attenuating morphine analgesic tolerance. Our results showed that BAA, in neuropathic rats, inhibited morphine antiallodynic tolerance, which
was completely reversed by the κ-opioid receptor non- 
BNI, consistent with previous findings that the Aconitum 
extracts and their effective ingredient mesaconitine (but 
not aconistine or hyaconitine) inhibited the development 
of morphine antinociceptive tolerance in naive an- 
imals using the tail pressure test.58-60

Accumulated evidence has highlighted that spinal mi-
croglia are activated and upregulated in the spinal dorsal 
horn after inflammatory insult and peripheral nerve 
injury, and play a primary role in neural transmission 
and plasticity, particularly in the induction of central 
sensitization–involved chronic pain.26 After peripheral 
nerve injury or inflammation, microglia are converted 
to an activated state from the resting state through a 
series of cellular and molecular changes.16,26,64 By 
responding to extracellular stimulation, the activated 
microglia can evoke various cellular responses, such as 
migration toward afflicted sites and the secretion of 
proinflammatory cytokines such as IL-1β, IL-6, and 
TNF-α.16,24,26,64 However, recent studies have shown that, in 
addition to their “destructive” inflammatory state, 
microglia have a “protective” state that activates anti-
inflammatory cascades or tissue repair mechanisms.67 
We recently showed that the activation of GLP-1 recep-
tors by peptidic, nonpeptidic, and herbal agonists stimu-
lated spinal and hippocampal microglia to express 
β-endorphin and produce antinociception and neuro-
protection.18,21,24,87 Our current data provide additional 
evidence that microglia express endogenous 
analgesic dynorphins in addition to endorphins, and 
suggest that endogenous morphine-like peptides are 
one of the substance bases for microglia to produce anal-
gesia and anti-inflammation. It is noteworthy that acti-
vation of microglia may not be a prerequisite for BAA 
to induce dynorphin A expression in our conditions. Pe-
ripheral nerve injury is known to induce spinal microglial 
activation and the expression of proinflammatory cyto-
kines such as IL-1β, IL-6, and TNF-α.16,24,26,64 However, 
BAA significantly stimulated dynorphin A expression in 
the spinal cords of neuropathic rats and sham rats to 
the same degree of approximately 50%, although it 
did not exhibit antinociception in the acute nociceptive 
responses. The latter inability of BAA is probably due 
to the lower level of sensitivity to endogenous and 
exogenous opioids in the normal nociceptive condition 
in which central sensitization is not involved.24 In addi-
tion, BAA also induced dynorphin A expression in the 
primary culture of microglia that was not preactivated 
by procedures such as the application of lipopolysaccha-
rides.

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