

The Involvement of μ -Opioid Receptors in the Central Nervous System in the Worsening of Allergic Airway Inflammation by Psychological Stress in Mice

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Key Words

Bronchial asthma • Psychological stress • μ -Opioid receptors • Morphine

Abstract

Background: Psychological stress has a recognized association with asthma symptoms. However, the mechanisms linking stress to the exacerbation of asthma are not well defined. μ -Opioid receptors (MOR) have been shown to be involved in the shift of the immune system toward a Th2-predominant response caused by psychological stress. **Objective:** To test the hypothesis that MOR play a role in the worsening of allergic airway inflammation evoked by psychological stress. **Methods:** Sensitized mice were exposed to restraint stress followed by antigen challenge. The levels of corticosterone and ovalbumin (OVA)-specific IgE in the blood and the levels of inflammatory cells and cytokine contents in bronchoalveolar lavage fluid were compared between stressed and nonstressed mice. The effects of MOR gene deletion and MOR antagonists/agonists were also investigated. **Results:** Stress exposure was confirmed by an increase in corticosterone levels. Although OVA-specific IgE levels were not sig-

nificantly different, the numbers of inflammatory cells and Th2 cytokine levels after antigen challenge in stressed mice were significantly higher than in nonstressed mice. MOR gene deletion ameliorated the stress-induced worsening of antigen-induced airway inflammation, and the administration of morphine, a MOR agonist, reproduced the stress-induced antigen-induced airway inflammation. Selective blocking of MOR in the central nervous system (CNS) significantly reduced stress-induced inflammatory exacerbation, but the blocking of peripheral MOR did not. **Conclusions:** MOR in the CNS are involved in psychological stress-induced aggravation of allergic airway inflammation.

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Introduction

Bronchial asthma is a chronic airway inflammatory disease characterized by the accumulation and activation of inflammatory cells, such as eosinophils and mast cells, in the bronchial wall and airway lumen. The behavior of inflammatory cells in asthmatic airways is tightly regulated by a network of Th2 cytokines such as IL-4, IL-5 and

IL-13. Inflammatory mediators released from these cells upon stimulation, such as arachidonic acid metabolites and reactive oxygen species, evoke the airway narrowing and airway hyper-responsiveness observed in asthmatics [1–4].

Various clinical studies have demonstrated an association between psychological stress and aggravation of asthma symptoms [5, 6]. For example, one study found that 20–35% of asthmatics experience exacerbation of symptoms during periods of stress [7]. In childhood asthma, high levels of ongoing chronic stress combined with an acutely negative life event increase the risk of asthma attacks [8]. Furthermore, psychological stress has effects on the inflammatory and immune responses implicated in asthma, such as Th2 cytokines. For example, chronic stress caused by academic examinations was shown to increase IL-5 production and eosinophil accumulation after antigen inhalation in college students with mild asthma [9, 10]. However, the precise mechanisms linking psychological stress to exacerbation of asthma are not well understood.

Psychological stress upregulates the production of endogenous opioids, including endorphins, enkephalins and dynorphins. These endogenous opioids exert their effects, such as analgesia, neuroendocrine functions and immunoregulation, through binding to opioid receptors consisting of 3 subtypes, μ , δ and κ [11, 12]. The hypothalamic-pituitary-adrenal (HPA) axis and sympathetic and adrenomedullary (SAM) system are activated by opioids in stressful conditions through the binding of ligands to μ -opioid receptors (MOR). Activation of the HPA axis and SAM system results in enhanced secretion of cortisol and catecholamines, respectively [13–15]. These stress hormones have been reported to shift the immune response from Th1 to Th2 by downregulating Th1 cytokine expression [16]. MOR are also expressed on inflammatory cells, including T cells, monocytes/macrophages and dendritic cells [17, 18]. The activation of MOR on T cells was reported to induce the differentiation of Th2 cells and a shift from Th1 to Th2 responses [19–21]. Furthermore, we recently reported that psychological stress, in this case ‘restraint stress’, exacerbated antigen-induced airway inflammation in wild-type mice but not in MOR gene knockout (MORKO) mice [22].

These findings led us to hypothesize that MOR play an important role in the worsening of allergic airway inflammation caused by psychological stress. To address this hypothesis, we examined the effects of opioid receptor antagonists, as well as MORKO, on stress-induced asthma exacerbation and the potential of MOR agonists

to exacerbate allergic airway inflammation using a murine model of asthma. In addition, to clarify which MOR in the central nervous system (CNS) or on inflammatory cells are involved in such exacerbation, the effects of intracerebroventricular and intraperitoneal injection of MOR antagonists were investigated.

Materials and Methods

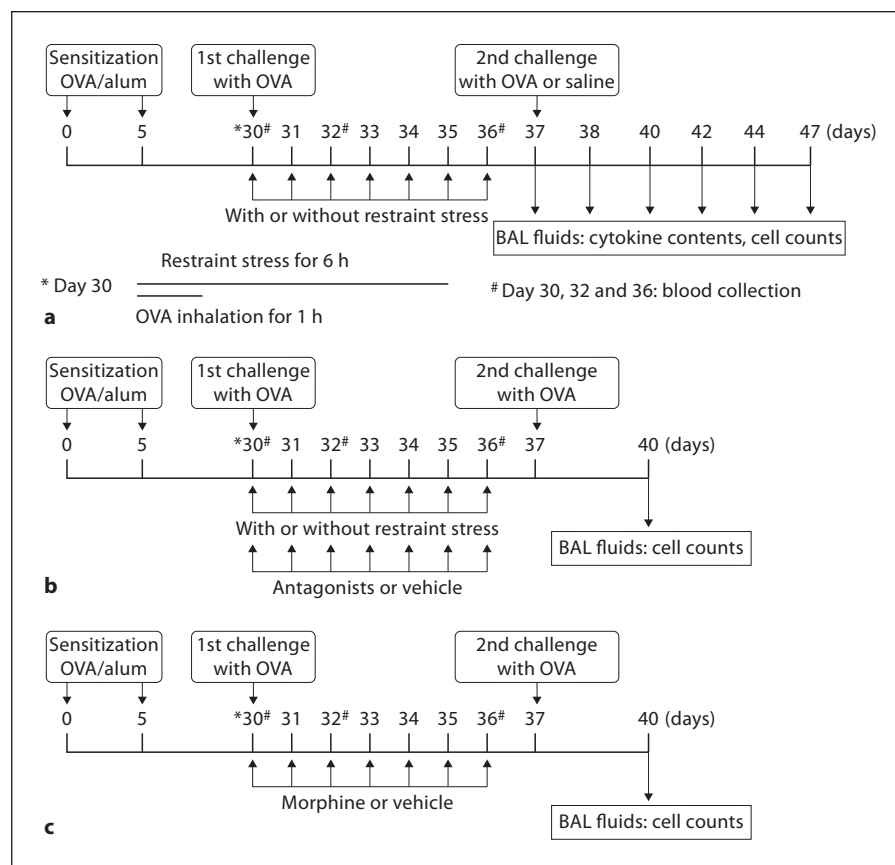
Animals

Specific pathogen-free female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). MORKO mice were generated as described previously [23]. Congenic MORKO mice backcrossed with C57BL/6 mice [24], and wild-type C57BL/6 mice (Japan SLC) were used as controls in this study. Animals were housed under a 12-hour light/dark cycle at a constant temperature ($22 \pm 2^\circ\text{C}$). Sterilized food and water were available *ad libitum*. All experiments described below were approved by the Committee of Animal Experiments at Tohoku Pharmaceutical University and conformed to its guidelines.

Protocols for Sensitization, Challenge and Stress Exposure

Mice 6–8 weeks of age were sensitized and made to inhale antigen as described previously [22, 25], but with a minor modification. Briefly, mice were sensitized by intraperitoneal injections of chicken ovalbumin (OVA; grade V, Sigma, St. Louis, Mo., USA; $8 \mu\text{g}/\text{mouse}$) adsorbed with aluminum hydroxide (Wako Pure Chemical Industries, Osaka, Japan; $4 \text{ mg}/\text{mouse}$) on days 0 and 5. For restraint stress, each mouse was placed in a 50-ml conical centrifuge tube with multiple ventilation holes for 6 h a day. Mice were not physically squeezed and felt no pain. This restraint allowed the mice to rotate from a supine to prone position, but not to turn their heads toward their tails or to consume food and water [22]. Restraint stress is generally regarded as inducing psychological stress in animals [26]. On day 30, stressed mice in 50-ml conical centrifuge tubes were left in the plastic chambers for 6 h. For the first hour of the period, aerosolized OVA flowed through the chambers so that the mice in the tubes were exposed to OVA. Stressed mice were further exposed to the restraint stress on 6 consecutive days, days 31–36, at the same time each day. Nonstressed mice were only deprived of food and water for the same period of time as the stressed mice were exposed to stress. Deprivation of food and water has been used as a nonstress condition in other rodent experiments that investigated the effect of restraint stress [27, 28]. Before and immediately after the restraint stress on day 30, and immediately after the restraint stress on days 32 and 36, a retro-orbital blood sample was taken from the mice after anesthetization with diethyl-ether (Wako) and stored on ice until further processing. Blood was collected from nonstressed mice in the same way. Plasma samples separated from blood samples were stored at -80°C for corticosterone concentration measurements by enzyme immunoassay (EIA). On day 37, stressed and nonstressed mice were challenged with aerosolized OVA or saline (second challenge). Before and after the second challenge, as shown in figure 1a, bronchoalveolar lavage (BAL) was performed as described previously [22, 25]. Briefly, the lungs were dissected and the trachea

Fig. 1. Diagrammatic representation of the protocols for sensitization, challenge and stress exposure. **a** Stress exposure protocol. **b** Stress exposure plus opioid receptor antagonists protocol. **c** μ -Opioid receptor agonist protocol.



was cannulated with a polyethylene tube (Becton Dickinson, Sparks, Md., USA). The lungs were lavaged twice by the injection of 0.25 ml of cold saline through the tube, and the recovered saline was pooled. Approximately 0.4 ml of instilled fluid was consistently recovered from each mouse. BAL fluid was processed for the measurement of cytokine contents and for counting total cells and cell differentials. After centrifugation of BAL fluid, supernatants were stored at -80°C for cytokine measurements by ELISA (fig. 1a). In experiments with opioid receptor antagonists (fig. 1b), β -funaltrexamine (β -FNA; a MOR antagonist; Tocris, Bristol, UK), naltrindole (a δ -opioid receptor antagonist; Sigma), nor-binaltorphimine (nor-BNI; a κ -opioid receptor antagonist; Sigma) and naloxone methiodide (a nonselective opioid receptor antagonist; Sigma) were intraperitoneally or intracerebroventricularly administered 1 h before each period of stress at the dose indicated [29–31] to the stressed and nonstressed mice. These mice were then exposed to OVA on day 37 (second challenge). In experiments with the MOR agonist (fig. 1c), morphine hydrochloride (Sankyo, Tokyo, Japan; 0.3, 1, 3 and 10 mg/kg body weight) was administered subcutaneously to the nonstressed mice instead of stress exposure. These mice were then exposed to OVA on day 37 (second challenge). The antagonists and agonist were dissolved in sterilized calcium- and magnesium-free phosphate-buffered saline (PBS; Wako), which was used as a control for the agents.

Corticosterone Assays

The corticosterone concentration in plasma was measured using an EIA kit (Assay Designs Inc., Ann Arbor, Mich., USA) according to the manufacturer's protocol. The sensitivity was 32 pg/ml.

OVA-Specific IgE Measurement

Serum levels of OVA-specific IgE on day 37 before the second challenge were measured with ELISA, as described previously [32].

Cytokine Assays

IL-4, IL-5, IL-13 and IFN- γ concentrations in the BAL fluid were measured using an ELISA kit (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's protocol. The sensitivity of detection was 2 pg/ml for IL-4 and IFN- γ , 7 pg/ml for IL-5 and 1.5 pg/ml for IL-13.

Statistical Analysis

Data are expressed as means $\pm$ SD. The number values are from multiple independent experiments with 2–4 mice. Significant differences between two groups were determined using the nonparametric Mann-Whitney U test. Significant differences in corticosterone concentrations in plasma after stress exposure, in the cell counts in BAL fluid during the time period after the second challenge and in BAL fluid from mice treated with various

doses of morphine were determined using one-way analysis of variance followed by Bonferroni's test. These analyses were performed using Prism 4 (GraphPad Software, San Diego, Calif., USA). A p value of less than 0.05 was considered significant.

Results

The Effect of Restraint Stress on Plasma Corticosterone Concentrations

While there were no significant differences in corticosterone concentrations between stressed and nonstressed mice before stress exposure, corticosterone concentrations in stressed mice were significantly higher than in nonstressed mice after the 1st, 3rd and 7th stress exposure [1st exposure: 368.7 ± 135.9 (n = 7) vs. 223.7 ± 53.2 ng/ml (n = 7), $p < 0.05$; 3rd exposure: 228.0 ± 53.3 (n = 5) vs. 144.2 ± 17.9 ng/ml (n = 5), $p < 0.05$; 7th exposure: 197.7 ± 52.0 (n = 4) vs. 89.5 ± 10.2 ng/ml (n = 4), $p < 0.05$] (fig. 2). Corticosterone concentrations after the 1st stress exposure were significantly increased in both stressed (restraint stress with food and water deprivation) and nonstressed (only food and water deprivation) mice compared to concentrations before the 1st stress exposure. The increase in nonstressed mice could have been due to food and water deprivation. Therefore, the differences in concentrations between stressed and nonstressed mice would only reflect the effect of the restraint stress. Although corticosterone concentrations tended to decrease with repeated exposure to stress in the stressed group and over time in the nonstressed group, this decrease did not reach significance (fig. 2).

The Effect of Restraint Stress on OVA-Specific IgE in Plasma

The levels of OVA-specific IgE in stressed mice were not significantly different from those in nonstressed mice [5.1 ± 2.0 (n = 8) vs. 6.4 ± 2.0 EU/ml (n = 7)] on day 37 before the second challenge.

Worsening of Antigen-Induced Airway Inflammation due to Restraint Stress

In stressed mice, the numbers of total cells, eosinophils and lymphocytes in BAL fluid 3 days after the second OVA challenge (day 40) were significantly increased compared to those before the second OVA challenge (day 37) [day 40 (n = 11) vs. day 37 (n = 6) in the stress/OVA group: total cells, 178.8 ± 88.9 vs. $35.9 \pm 19.0 \times 10^4$ cells/ml, $p < 0.01$; eosinophils, 131.9 ± 77.6 vs. $22.5 \pm 18.5 \times 10^4$ cells/ml, $p < 0.01$; lymphocytes, 22.8 ± 10.9 vs. $3.8 \pm 2.2 \times 10^4$ cells/ml, $p < 0.05$] (fig. 3b). However,

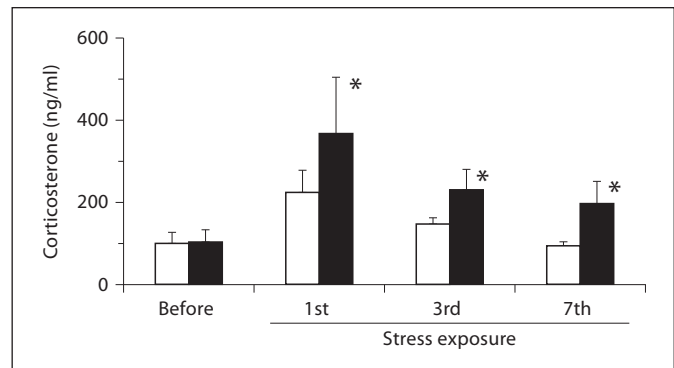
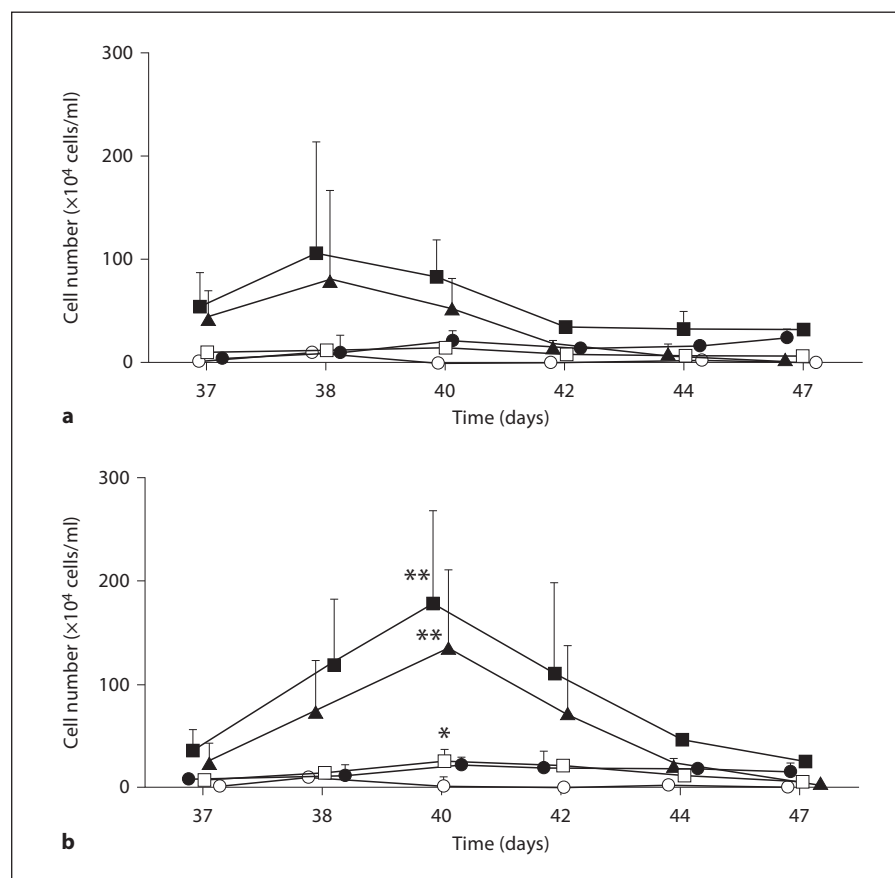


Fig. 2. The effect of restraint stress on blood corticosterone concentrations. Blood was collected from stressed (black bars) and nonstressed (white bars) mice before the 1st stress and after the 1st, 3rd and 7th stress exposure, and the corticosterone levels in blood were measured by EIA. Data are expressed as means $\pm$ SD (n = 4–7). * $p < 0.05$ compared to nonstressed mice.

the second challenge with saline in stressed mice did not increase the numbers of those cells [day 40 (n = 6) vs. day 37 (n = 9) in the stress/saline group: total cells, 16.1 ± 9.4 vs. $36.6 \pm 20.7 \times 10^4$ cells/ml; eosinophils, 0.7 ± 1.4 vs. $16.8 \pm 14.2 \times 10^4$ cells/ml; lymphocytes, 2.1 ± 2.5 vs. $5.0 \pm 4.1 \times 10^4$ cells/ml] nor of macrophages and neutrophils. In stressed mice, there were significant differences in the numbers of total cells ($p < 0.01$), eosinophils ($p < 0.01$) and lymphocytes ($p < 0.01$) between the OVA and saline challenge on day 40. On the other hand, in nonstressed mice, there was no statistically significant increase in the numbers of total cells, eosinophils and lymphocytes, nor of macrophages and neutrophils, in BAL fluid at any time point after the second OVA challenge compared to the numbers of cells before the second OVA challenge (day 37) [day 40 (n = 10) vs. day 37 (n = 6) in the nonstress/OVA group: total cells, 85.4 ± 33.4 vs. $54.3 \pm 21.4 \times 10^4$ cells/ml; eosinophils, 52.0 ± 29.2 vs. $41.2 \pm 27.8 \times 10^4$ cells/ml; lymphocytes, 12.3 ± 5.6 vs. $7.8 \pm 3.9 \times 10^4$ cells/ml] (fig. 3a). There was also no significant increase in the numbers of inflammatory cells after the second challenge with saline [day 40 (n = 6) vs. day 37 (n = 8) in the nonstress/saline group: total cells, 21.6 ± 4.2 vs. $51.4 \pm 33.1 \times 10^4$ cells/ml; eosinophils, 2.8 ± 2.7 vs. $29.7 \pm 28.0 \times 10^4$ cells/ml; lymphocytes, 4.0 ± 1.8 vs. $6.4 \pm 3.5 \times 10^4$ cells/ml]. However, in nonstressed mice, there were significant differences in the numbers of total cells ($p < 0.01$), eosinophils ($p < 0.01$) and lymphocytes ($p < 0.01$) between the OVA and saline challenge on day 40. Comparing the numbers of inflammatory cells between stressed and nonstressed mice on

Fig. 3. Time course of changes in cell numbers in the BAL fluid from stressed and nonstressed mice after the second OVA inhalation. In the BAL fluid from nonstressed (a) and stressed (b) mice challenged with OVA, the numbers of total cells (■), macrophages (●), eosinophils (▲), neutrophils (○) and lymphocytes (□) were counted at the time points indicated. Data are expressed as means $\pm$ SD (n = 6–11). * $p < 0.05$, ** $p < 0.01$ compared to prior to the challenge (day 37).



day 40 (3 days after the second OVA challenge), significant differences were found in numbers of total cells ($p < 0.01$), eosinophils ($p < 0.01$) and lymphocytes ($p < 0.05$), while there were no significant differences in the numbers of any type of cell between the groups on day 37 (before the second challenge). Therefore, in the following experiments in this study, the numbers of inflammatory cells in BAL fluid were evaluated on day 40.

The levels of IL-4, IL-5 and IL-13 in BAL fluid from stressed and OVA-challenged mice were significantly higher than those in nonstressed and OVA-challenged mice on day 38 (1 day after the second OVA challenge; table 1). In stressed mice, the levels of these cytokines were decreased on day 40 (3 days after the second challenge) and were below the limit of detection on day 42 (5 days after the second challenge), while these cytokines were not detected on either day 40 or 42 in nonstressed mice. Similar levels of IL-13 were detected even after the saline challenge in stressed and nonstressed mice, although the levels were significantly lower than those in the OVA-challenged mice. The presence of IL-13 in sa-

Table 1. Cytokine contents in BAL fluids from the stressed and nonstressed B6 mice on day 38

2nd challenge	IL-4 pg/ml	IL-5 pg/ml	IL-13 pg/ml	IFN- γ pg/ml
Nonstressed				
OVA	3.7 ± 4.0	10.9 ± 8.4	50.6 ± 49.3	3.5 ± 0.5
Saline	0.0 ± 0.0	0.0 ± 0.0	23.3 ± 7.5	0.0 ± 0.0
Stressed				
OVA	$26.5 \pm 7.2^{**}$	$23.1 \pm 10.2^*$	$195.3 \pm 133.7^*$	4.3 ± 1.4
Saline	0.0 ± 0.0	0.0 ± 0.0	26.8 ± 15.8	0.0 ± 0.0

Data are expressed as means $\pm$ SD (n = 4–7). * $p < 0.05$, ** $p < 0.01$ compared to the nonstressed mice.

line-challenged mice could be due to the effect of the first OVA challenge on day 30. IFN- γ was detected in stressed and nonstressed mice only on day 38, after the second OVA challenge, when the levels were not significantly different between the groups (table 1).

Fig. 4. Comparison of cell numbers in BAL fluids between stressed and nonstressed MORKO mice after the second OVA challenge. The numbers of total cells, macrophages, eosinophils, neutrophils and lymphocytes in BAL fluids from stressed (black bars) and nonstressed (white bars) MORKO mice 3 days after OVA challenge were counted. Data are expressed as means $\pm$ SD (n = 7).

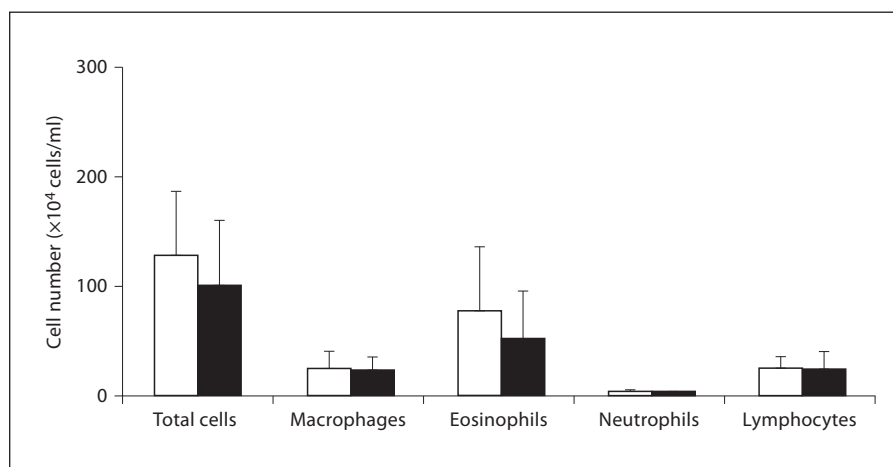


Table 2. Cytokine contents in BAL fluids from the stressed and nonstressed MORKO mice on day 38

	IL-4 pg/ml	IL-5 pg/ml	IL-13 pg/ml	IFN- γ pg/ml
Nonstressed	29.6 $\pm$ 19.7	36.8 $\pm$ 14.1	41.8 $\pm$ 41.8	12.8 $\pm$ 4.1
Stressed	21.4 $\pm$ 13.8	36.8 $\pm$ 2.5	92.1 $\pm$ 71.4	10.5 $\pm$ 0.5

Data are expressed as means $\pm$ SD (n = 4–7).

Abolishment of Restraint Stress-Induced Aggravation in MORKO Mice

In both stressed and nonstressed MORKO mice, there were no significant increases in the numbers of any type of cell in BAL fluids on day 40 (3 days after the second OVA challenge) compared to those on day 37 (before the second challenge), and there was no significant difference in the numbers of any type of cell in BAL fluid between stressed and nonstressed MORKO mice on either day 37 (data not shown) or day 40 (fig. 4). The levels of IL-4, IL-5, IL-13 and IFN- γ in BAL fluid of stressed MORKO mice were not significantly different from those of nonstressed MORKO mice on day 38 (1 day after the second OVA challenge; table 2).

Worsening of Antigen-Induced Airway Inflammation by Morphine, a MOR Agonist

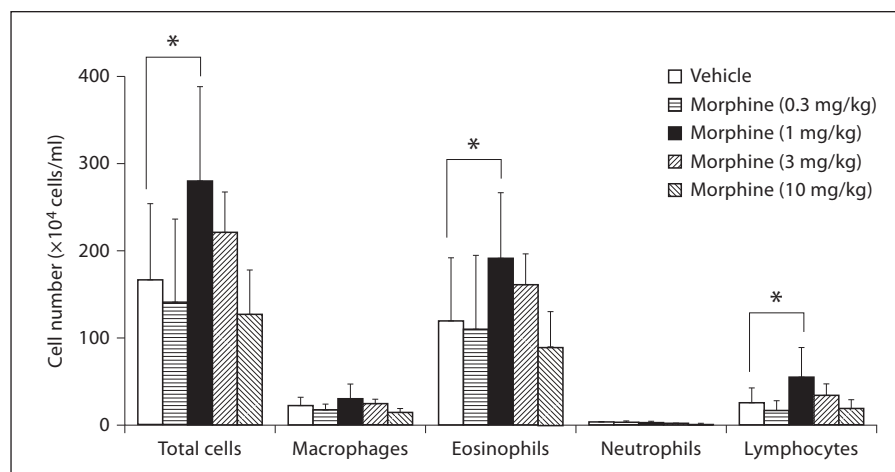
To investigate whether MOR activation exacerbates antigen-induced airway inflammation, we examined the effect of administration of the MOR agonist morphine as a replacement for restraint stress in wild-type mice with-

out stress. The systemic administration of morphine (1 mg/kg) produced significant increases in the numbers of total cells, eosinophils and lymphocytes in BAL fluid compared to the administration of PBS [morphine (1 mg/kg) (n = 9) vs. PBS (n = 11) in the nonstress/OVA group: total cells, 277.8 $\pm$ 107.0 vs. 164.9 $\pm$ 86.5 $\times$ 10⁴ cells/ml, p < 0.05; eosinophils, 192.0 $\pm$ 73.2 vs. 118.4 $\pm$ 71.3 $\times$ 10⁴ cells/ml, p < 0.05; lymphocytes, 55.0 $\pm$ 32.9 vs. 25.1 $\pm$ 18.1 $\times$ 10⁴ cells/ml, p < 0.05). However, doses of 0.3, 3 and 10 mg/kg morphine did not produce significant effects on the numbers of any types of cells in BAL fluid compared with PBS (fig. 5). In contrast to the results for wild-type mice, in MORKO mice, administration of morphine (1 mg/kg) did not significantly affect the numbers of inflammatory cells in BAL fluid compared with PBS administration (data not shown).

Inhibitory Effects of Antagonists for μ -, but Not κ - and δ -Opioid Receptors on Stress-Induced Aggravation

To investigate whether other opioid receptors, such as κ - and δ -receptors, rather than MOR were involved in stress-induced exacerbation of antigen-induced airway inflammation, we examined the effect of systemic administration of antagonists for opioid receptors in wild-type mice. The increase in total cells, but not eosinophils and lymphocytes, in BAL fluid from stressed mice treated with PBS was abolished in stressed mice treated with β -FNA (10 mg/kg, i.p.), a MOR antagonist [PBS (n = 7) vs. β -FNA (n = 8) in the stress/OVA group: total cells 291.1 $\pm$ 106.7 vs. 172.7 $\pm$ 83.5 $\times$ 10⁴ cells/ml, p < 0.05]. Treatment with nor-BNI (10 mg/kg, i.p.), a κ -opioid receptor antagonist, and naltrindole (10 mg/kg, i.p.), a δ -

Fig. 5. The effect of morphine on cell numbers in the BAL fluid after the second OVA challenge. The numbers of total cells, macrophages, eosinophils, neutrophils and lymphocytes in the BAL fluid from vehicle- or morphine-treated mice 3 days after OVA challenge were counted. Data are expressed as means $\pm$ SD ($n = 9-11$). * $p < 0.05$.



opioid receptor antagonist, had no significant effect on the increase in total cells, eosinophils and lymphocytes in BAL fluid from stressed mice (data not shown).

Abolishment of Stress-Induced Aggravation by a MOR-Selective Antagonist in the CNS

The above results revealed that MOR were involved in stress-induced aggravation of antigen-induced airway inflammation. Since MOR are expressed not only in the CNS [11, 12], but also on immune cells [17, 18], as a next step, we investigated which MOR present in the CNS or on immune cells are involved in aggravation, by intracerebroventricular injection of β -FNA to selectively antagonize MOR in the CNS, and by intraperitoneal injection of naloxone methiodide for opioid receptors, including MOR, in the periphery. The increase in the numbers of total cells, eosinophils and lymphocytes in the BAL fluid caused by restraint stress was abolished by intracerebroventricular administration of β -FNA (5 nmol/mouse) [PBS ($n = 7$) vs. β -FNA ($n = 7$) in the stress/OVA group: total cells, 297.5 ± 119.1 vs. $124.6 \pm 42.5 \times 10^4$ cells/ml, $p < 0.01$; eosinophils, 217.3 ± 84.0 vs. $91.2 \pm 39.9 \times 10^4$ cells/ml, $p < 0.01$; lymphocytes, 45.6 ± 21.8 vs. $18.3 \pm 8.4 \times 10^4$ cells/ml, $p < 0.05$). Stressed mice that were injected intracerebroventricularly with β -FNA showed similar levels of cells to nonstressed mice that were injected intracerebroventricularly with vehicle (fig. 6a). By contrast, treatment with a peripherally restricted opioid antagonist, naloxone methiodide (10 mg/kg), did not reduce the increase in the inflammatory cells in BAL fluid of stressed mice that were treated intraperitoneally with vehicle, and stressed mice that were injected intraperitoneally with naloxone methiodide still

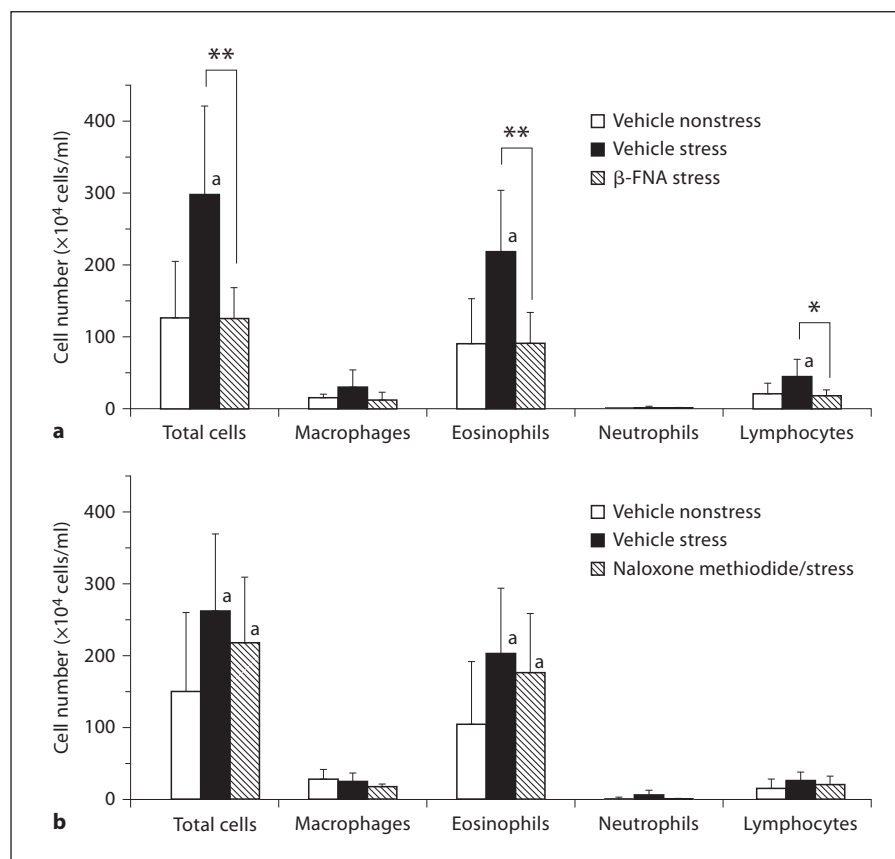
showed a significant increase in the numbers of total cells and eosinophils compared to nonstressed mice that were injected intraperitoneally with vehicle (fig. 6b).

Discussion

The present study demonstrated for the first time that MOR in the CNS are involved in psychological stress-induced aggravation of airway inflammation in a murine model of allergic asthma using MORKO mice and MOR antagonists/agonists.

The effect of restraint stress as a model of psychological stress was confirmed by the findings shown in figure 2, in which the levels of corticosterone, one of the stress hormones [33], were significantly higher in stressed mice than in nonstressed mice. Corticosterone levels in both stressed (restraint stress + deprivation of food and water) and nonstressed (only food and water deprivation) groups declined with repeated stress, although the decline was not statistically significant. It has been suggested that repeated stress induces a state of hyporesponsiveness of the HPA axis, whereby corticosterone secretion is attenuated [34]. In other rodent models, repeated stress ranging from 4 to 14 days was required to induce worsening of allergic airway inflammation [22, 35–37]. By contrast, short-term restraint stress reduced allergic airway inflammation [22, 35]; therefore, prolonged period of repeated stress was used in this study, as in our previous study [22]. Allergen-induced airway inflammation in stressed mice was more severe than in nonstressed mice, in association with greater amounts of Th2 cytokines. These findings are consistent with previous reports using

Fig. 6. The effects of antagonists for central MOR and peripheral opioid receptors on cell numbers in the BAL fluid after OVA challenge. The numbers of total cells, macrophages, eosinophils, neutrophils and lymphocytes in the BAL fluid from stressed and nonstressed mice 3 days after OVA challenge were counted. β -FNA (5 nmol/mouse, intracerebroventricular) (a) and naloxone methiodide (10 mg/kg, i.p.) (b) were administered before each stress exposure. Data are expressed as means $\pm$ SD ($n = 7$). * $p < 0.05$, ** $p < 0.01$. ^a $p < 0.05$ compared to vehicle/nonstressed mice.



mouse models with different types of psychological stress, such as sound stress, restraint stress and forced swim stress [22, 35]. All experiments described here were conducted in B6 mice. There are several pieces of evidence that support the idea that allergic inflammatory responses in airways vary widely in different strains of mice [38]. BALB/c mice have been commonly used for mouse models of allergic asthma. To ensure that the effect of restraint stress was not specific to B6 mice, we previously analyzed the same protocol in BALB/c mice [22]. While the results of the experiments using BALB/c mice were similar to those obtained using B6 mice, stress was relatively more effective in B6 mice. Furthermore, the genetic background of the MORKO mice used in the current study was B6.

In MORKO mice, the numbers of inflammatory cells in BAL fluid were not significantly different from those in wild-type mice under nonstressed conditions, although the levels of IL-4 and IL-5 were significantly higher. The reason for the differences in cytokine levels between MORKO and wild-type mice was not determined.

However, in contrast to the results for wild-type mice, no significant worsening of inflammatory cell numbers and cytokine levels in BAL fluid was observed in MORKO mice under stress conditions. These results suggest that MOR are involved not in the development but the exacerbation of allergen-induced airway inflammation. The involvement of MOR in stress-induced aggravation of allergic airway inflammation was further investigated by the replacement of stress with a MOR agonist, morphine. As shown in figure 5, the administration of 1 mg/kg morphine, but not of 0.3, 3 or 10 mg/kg, induced an increase in inflammatory cells in BAL fluid after the second allergen challenge. The analgesic effect exerted by 1 mg/kg morphine was similar to that evoked by the restraint stress used in this study [39]. Administration of 0.3 mg/kg might not be sufficient to stimulate MOR. Administration of higher doses of morphine could have desensitized MOR, an idea that is supported by findings that repeated administration of morphine at doses of 3 mg/kg or 10 mg/kg for 5–7 days resulted in tolerance to morphine-induced analgesia [40, 41]. There are several pieces

of evidence showing that desensitization of MOR via the activation of protein kinase C and internalization of MOR via activation of arrestin are major mechanisms that reveal antinociceptive tolerance due to chronic treatment with morphine [42, 43]. The MOR-dependent morphine effect was confirmed by the loss of the effect in MORKO mice. Severe asthma exacerbations related to the use of opioids such as heroin and morphine (a heroin metabolite) have received attention as a public health issue. The possible mechanisms have been thought to be sensitization against opioid antigen and pharmacological degranulation of mast cells independent of opioid receptors [44, 45]. The results of the current study suggest the possibility that opioid use exacerbates allergen-induced asthma attacks through MOR activation.

Endogenous opioids released upon exposure to psychological stress are able to bind to 3 subtypes of opioid receptors, i.e. μ , δ and κ [12, 13]. MOR involvement was pharmacologically investigated with opioid receptor antagonists specific for each subtype. The simultaneous administration of β -FNA, a MOR antagonist, with restraint stress significantly reduced the number of total cells in BAL fluid compared to the simultaneous administration of vehicle. However, the reduction in the numbers of eosinophils and lymphocytes was not statistically significant, the reason for which will be discussed below. On the other hand, naltrindole, a δ -opioid receptor antagonist, and nor-BNI, a κ -opioid receptor antagonist, showed no significant effects on the increase in total cells, nor on eosinophils and lymphocytes, under the stress conditions. These observations suggest that subtypes of opioid receptors other than MOR are unlikely to be involved in stress-induced aggravation of airway inflammation.

Opioid-induced immunomodulation is thought to be mediated centrally by MOR localized in the CNS and peripherally by MOR expressed on immune cells [46]. To clarify which MOR contribute to stress-induced aggravation, MOR in the CNS were selectively antagonized by intracerebroventricular injection of β -FNA, and MOR in the periphery were selectively antagonized by intraperitoneal injection of naloxone methiodide, a nonselective opioid receptor antagonist that is unable to pass through the blood-brain barrier. Stress-induced aggravation was significantly reduced by intracerebroventricular injection of β -FNA, but not by intraperitoneal injection of naloxone methiodide. These results indicate that the MOR involved in stress-induced aggravation could be localized in the CNS. The participation of the CNS in stress-induced aggravation of airway inflammation and obstruction

has been suggested in previous reports. Rosenkranz et al. [47] showed that activation in the anterior cingulate cortex and insula in response to asthma-relevant emotional stimuli, demonstrated by functional magnetic resonance imaging, was associated with airway inflammation and obstruction in asthmatics exposed to antigen. This study suggests a functional link between emotion-processing circuits in the brain and peripheral physiological processes relevant to airway inflammation and narrowing subsequent to antigen exposure in the lungs [47]. Joachim et al. [48] reported that, in asthmatics, stress perception was correlated with serum levels of brain-derived neurotrophic factor involved in central stress processing and, furthermore, that forced expiratory volume in 1 s was negatively correlated with brain-derived neurotrophic factor. In contrast to intracerebroventricular administration (fig. 6a), intraperitoneal administration of β -FNA showed no significant effects on the numbers of eosinophils and lymphocytes, as mentioned above. This lack of effect might be due to the fact that the intraperitoneal dose of β -FNA in the CNS was not sufficient to antagonize MOR in the CNS.

It has become increasingly apparent that psychological stress increases the frequency, duration and severity of asthmatics' symptoms by enhancing airway inflammatory responses to environmental triggers such as inhaled allergen, characterized by further skewing of the balance toward a Th2-dominant cytokine profile [49]. Students with asthma showed increases in eosinophils and IL-5 in sputum and enhanced and reduced production of Th2 and Th1 cytokines, respectively, by mononuclear cells in peripheral blood during exam periods compared to non-exam periods [9, 10, 50]. It has been reported that lower socioeconomic status and more stressful life events are associated with higher levels of Th2 cytokine production and eosinophil counts in peripheral blood [51–53]. In animal models of asthma, various types of psychological stress such as electrical footshock stress, sound stress, restraint stress and forced swim stress have been shown to exacerbate allergen-induced airway inflammation [22, 35–37]. The HPA axis and SAM system have been proposed as relevant pathways through which stress modifies inflammatory/immune responses to induce asthma exacerbation. Stimulation of the HPA axis and SAM system results in the release of stress hormones corticosterone and catecholamine, which have the potential to shift immune responses to Th2 predominance [33, 34, 54]. However, the psycho-neuro-endocrino-immunological cascade in the worsening of asthma symptoms due to psychological stress is not fully understood. There

are only a few reports suggesting interactions between psychological stress and peripheral neuronal responses. Joachim et al. [55, 56] used a murine model to show that increased expression of substance P and its interaction with NK-1 receptors in diseased airways were involved in sound stress-aggravated airway inflammation.

In summary, the present study identified for the first time that MOR are one group of candidates in the CNS linking psychological stress to the worsening of allergen-induced airway inflammation. The mechanism by which the activation of MOR in the CNS was triggered by psychological stress to induce immune alterations favoring

asthmatic exacerbations should be further investigated, paying particular attention to activation of the HPA axis and SAM system.

Acknowledgments

This article was checked for correct English usage by American Journal Experts (Certification Number: 0805-16C9-6EE3-7CA3-5EB1). This work was supported by Grants-in-Aid for Scientific Research (No. 16616007 and 19590909 to I.O.) and by the High Technology Research Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM: Asthma: from bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; 161:1720–1745.
- Holgate ST: Airway inflammation and remodeling in asthma: current concepts. *Mol Biotechnol* 2002;22:179–189.
- Kay AB: The role of T lymphocytes in asthma. *Chem Immunol Allergy* 2006;91:59–75.
- Barnes PJ: Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;8:183–192.
- Rietveld S, Everaerd W, Creer TL: Stress-induced asthma: a review of research and potential mechanisms. *Clin Exp Allergy* 2000; 30:1058–1066.
- Chetta A, Foresi A, Marangio E, Olivieri D: Psychological implications of respiratory health and disease. *Respiration* 2005;72: 210–215.
- Isenberg SA, Lehrer PM, Hochron S: The effects of suggestion and emotional arousal on pulmonary function in asthma: a review and a hypothesis regarding vagal mediation. *Psychosom Med* 1992;54:192–216.
- Sandberg S, Paton JY, Ahola S, McCann DC, McGuinness D, Hillary CR, Oja H: The role of acute and chronic stress in asthma attacks in children. *Lancet* 2000;356:982–987.
- Kang DH, Coe CL, McCarthy DO, Jarjour NN, Kelly EA, Rodriguez RR, Busse WW: Cytokine profiles of stimulated blood lymphocytes in asthmatic and healthy adolescents across the school year. *J Interferon Cytokine Res* 1997;17:481–487.
- Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, Busse WW: School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 2002;165:1062–1067.
- Lopez-Caderon A, Ariznavarreta C, Chen CL: Influence of chronic restraint stress on pro-opiomelanocortin mRNA and β -endorphin in the rat hypothalamus. *J Mol Endocrinol* 1991;7:197–204.
- Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier L-F: Role of endogenous opioid system in the regulation of the stress response. *Prog Neuropsychopharmacol Biol Psychiatry* 2001;25:729–741.
- Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, Walker JM: Endogenous opioids: biology and function. *Annu Rev Neurosci* 1984;7:223–255.
- Buckingham JC: Stimulation and inhibition of corticotropin releasing factor secretion by β endorphin. *Neuroendocrinology* 1986;42: 148–152.
- Kiritsey-Roy JA, Appel NM, Bobbitt FG, Van Loon GR: Effects of μ -opioid receptor stimulation in the hypothalamic paraventricular nucleus on basal and stress-induced catecholamine secretion and cardiovascular responses. *J Pharmacol Exp Ther* 1986;239: 814–822.
- Elenkov IJ, Chrousos GP: Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends Endocrinol Metab* 1999;10:359–368.
- Chuang TK, Killam KF, Chuang LF, Kung HF, Sheng WS, Chao CC, Yu L, Chuang RY: μ opioid receptor gene expression in immune cells. *Biochem Biophys Res Commun* 1995;216:922–930.
- Makarenkova VP, Esche C, Kost NV, Shurin GV, Rabin BS, Zozulya AA, Shurin MR: Identification of δ - and μ -type receptors on human and murine dendritic cells. *J Neuroimmunol* 2001;117:68–77.
- Sacerdote P, Manfredi B, Gaspani L, Panerai AE: The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in BALB/c mice. *Blood* 2000;95:2031–2036.
- Wang J, Charboneau R, Barke RA, Loh HH, Roy S: μ -opioid receptor mediates chronic restraint stress-induced lymphocyte apoptosis. *J Immunol* 2002;169:3630–3636.
- Roy S, Wang J, Gupta S, Charboneau R, Loh HH, Barke RA: Chronic morphine treatment differentiates T helper cells to Th2 effector cells by modulating transcription factors GATA 3 and T-bet. *J Neuroimmunol* 2004; 147:78–81.
- Okuyama K, Ohwada K, Sakurada S, Sato N, Sora I, Tamura G, Takayanagi M, Ohno I: The distinctive effects of acute and chronic psychological stress on airway inflammation in a murine model of allergic asthma. *Allergol Int* 2007;56:29–35.
- Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL, Uhl GR: Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci USA* 1997;94:1544–1549.
- Hall FS, Li XF, Goeb M, Roff S, Hoggatt H, Sora I, Uhl GR: Congenic C57BL/6 μ -opioid receptor (MOR) knockout mice: baseline and opiate effects. *Genes Brain Behav* 2003; 2:114–121.
- Kumagai K, Ohno I, Okada S, Ohkawara Y, Suzuki K, Shinya T, Nagase H, Iwata K, Shirato K: Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J Immunol* 1999;162:4212–4219.
- Posevitz V, Vizler C, Benyhe S, Duda E, Borsodi A: Restraint stress and anti-tumor immune response in mice. *Acta Biol Hung* 2003;54:167–176.
- Mercado AM, Padgett DA, Sheridan JF, Marucha PT: Altered kinetics of IL-1 α , IL-1 β , and KGF-1 gene expression in early wounds of restrained mice. *Brain Behav Immun* 2002;16:150–162.

- 28 Ashcraft KA, Hunzeker J, Bonneau RH: Psychological stress impairs the local CD8+ T cells response to mucosal HSV-1 infection and allows for increased pathogenicity via a glucocorticoid receptor-mediated mechanism. *Psychoneuroendocrinology* 2008;33: 951–963.
- 29 Tseng RJ, Padgett DA, Dhabhar FS, Engler H, Sheriden JF: Stress-induced modulation of NK activity during influenza viral infection: role of glucocorticoids and opioids. *Brain Behav Immun* 2005;19:153–164.
- 30 Hiramatsu M, Inoue K, Ambo A, Sasaki Y, Kameyama T: Long-lasting antinociceptive effects of a novel dynorphin analogue, Tyr-D-Ala-Phe-Leu-Arg ψ (CH₂NH) Arg-NH₂, in mice. *Br J Pharmacol* 2001;132:1948–1956.
- 31 Baamonde A, Lasta A, Juárez L, García-Suárez O, Meana A, Hidelgo A, Menéndez L: Endogenous β -endorphin induces thermal analgesia at the initial stage of a murine osteosarcoma. *Peptides* 2006;27:2778–2785.
- 32 Ohwada K, Kanako W, Okuyama K, Ohkawara Y, Sugaya T, Takayanagi M, Ohno I: The involvement of type 1a angiotensin II receptors in the regulation of airway inflammation in a murine model of allergic asthma. *Clin Exp Allergy* 2007;37:1720–1727.
- 33 Wright RJ: Stress and atopic disorders. *J Allergy Clin Immunol* 2005;116:1301–1306.
- 34 Wright RJ, Cohen RT, Cohen S: The impact of stress on the development and expression of atopy. *Curr Opin Allergy Clin Immunol* 2005;5:23–29.
- 35 Forsythe P, Ebeling C, Gordon JR, Befus AD, Vliagoftis H: Opposing effects of short- and long-term stress on airway inflammation. *Am J Respir Crit Care Med* 2004;169:220–226.
- 36 Joachim RA, Quarcio D, Arck PC, Herz U, Renz H, Klapp BF: Stress enhances airway reactivity and airway inflammation in an animal model of allergic bronchial asthma. *Psychosom Med* 2003;65:811–815.
- 37 Datti F, Datti M, Antunes E, Teixeira NA: Influence of chronic unpredictable stress on the allergic responses in rats. *Physiol Behav* 2002;77:79–83.
- 38 Whitehead GS, Walker JKL, Berman KG, Foster WM, Schwartz DA: Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 2003; 285:L32–L42.
- 39 Homayoun H, Dehpour AR: Differential contribution of cholecystokinin receptors to stress-induced modulation of seizure and nociception thresholds in mice. *Pharmacol Biochem Behav* 2004;78:209–215.
- 40 Li S, Lee ML, Brunchas MR, Chan GC, Storm DR, Chavkin C: Calmodulin-stimulated adenylyl cyclase gene deletion affects morphine responses. *Mol Pharmacol* 2006;70:1742–1749.
- 41 Tsuchiya T, Takeuchi T, Hayashida K, Shimizu H, Ando K, Harada E: Milk-derived lactoferrin may block tolerance to morphine analgesia. *Brain Res* 2006;1068:102–108.
- 42 Zeitz KP, Malmberg AB, Gilbert H, Basbaum AI: Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. *Pain* 2001;94: 245–253.
- 43 Bohn LM, Lefkowitz RJ, Caron MG: Differential mechanisms of morphine antinociceptive tolerance revealed in β arrestin-2 knock-out mice. *J Neurosci* 2002;22:10494–10500.
- 44 Cygan J, Trunsky M, Corbridge T: Inhaled heroin-induced status asthmaticus: five cases and a review of the literature. *Chest* 2000; 117:272–275.
- 45 Krantz AJ, Hershow RC, Prachand N, Hayden DM, Franklin C, Hryhorczuk DO: Heroin insufflation as a trigger for patients with life-threatening asthma. *Chest* 2003; 123:510–517.
- 46 Wei G, Moss J, Yuan CS: Opioid-induced immunosuppression: is it centrally mediated or peripherally mediated? *Biochem Pharmacol* 2003;65:1761–1766.
- 47 Rosenkranz MA, Busse WW, Johnstone T, Swenson CA, Crisafi GM, Jackson MM, Bosch JA, Sheridan JF, Davidson RJ: Neural circuitry underlying the interaction between emotion and asthma symptom exacerbation. *Proc Natl Acad Sci USA* 2005;102:13319–13324.
- 48 Joachim RA, Noga O, Sagach V, Hanf G, Fliege H, Kocalevent RD, Peters EM, Klapp BF: Correlation between immune and neuronal parameters and stress perception in allergic asthmatics. *Clin Exp Allergy* 2008;38: 283–290.
- 49 Chen E, Miller GE: Stress and inflammation in exacerbations of asthma. *Brain Behav Immun* 2007;21:993–999.
- 50 Höglund CO, Axén J, Kemi C, Jernelöv S, Grunewald J, Müller-Suur C, Smith Y, Grönneberg R, Eklund A, Stiernä P, Lekander M: Changes in immune regulation in response to examination stress in atopic and healthy individuals. *Clin Exp Allergy* 2006;36:982–992.
- 51 Chen E, Fisher EB, Bacharier LB, Strunk RC: Socioeconomic status, stress, and immune markers in adolescents with asthma. *Psychosom Med* 2003;65:984–992.
- 52 Chen E, Hanson MD, Paterson LQ, Griffin MJ, Walker HA, Miller GE: Socioeconomic status and inflammatory processes in childhood asthma: the role of psychological stress. *J Allergy Clin Immunol* 2006;117:1014–1020.
- 53 Chen E, Chim LS, Strunk RC, Miller GE: The role of the social environment in children and adolescents with asthma. *Am J Respir Crit Care Med* 2007;176:644–649.
- 54 Chrousos GP: Stress, chronic inflammation, and emotional and physical well-being: concurrent effects and chronic sequelae. *J Allergy Clin Immunol* 2000;106:S275–S291.
- 55 Joachim RA, Sagach V, Quarcio D, Dinh QT, Arck PC, Klapp BF: Neurokinin-1 receptor mediates stress-exacerbated allergic airway inflammation and airway hyperresponsiveness in mice. *Psychosom Med* 2004;66:564–571.
- 56 Joachim RA, Cifuentes LB, Sagach V, Quarcio D, Hagen E, Arck PC, Fischer A, Klapp BF, Dinh QT: Stress induces substance P in vagal sensory neurons innervating the mouse airways. *Clin Exp Allergy* 2006;36: 1001–1010.