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Prior chronic stress induces persistent polyI:C-induced allodynia and depressive-like behavior in rats: Possible involvement of glucocorticoids and microglia

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HIGHLIGHTS

- We investigated polyI:C-induced sickness responses in socially defeated rats.
- Both single and repeated social defeat stresses blunted polyI:C-induced fever.
- Only repeated stress group showed prolonged allodynia and depressive-like behavior.
- Pretreatment with RU486 or minocycline abolished these phenomena.
- Corticosterone and microglial activation may play a role in these phenomena.

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ABSTRACT

When animals suffer from viral infections, they develop a set of symptoms known as the "sickness response." Recent studies suggest that psychological stress can modulate the sickness response. However, it remains uncertain whether acute and chronic psychosocial stresses have the same effect on viral infection-induced sickness responses. To address this question, we compared changes in polyI:C-induced sickness responses, such as fever, change of body weight and food intake, mechanical allodynia, and depressive-like behavior, in rats that had been pre-exposed to single and repeated social defeat stresses. Intraperitoneal injection of polyI:C induced a maximal fever of 38.0 °C 3 h after injection. Rats exposed to prior social defeat stress exhibited blunted febrile responses, which were more pronounced in the repeated stress group. Furthermore, only the repeated stress group showed late-onset and prolonged mechanical allodynia lasting until 8 days after injection in the von Frey test and prolonged immobility time in the forced swim test 9 days post-injection. To assess the role of glucocorticoids and microglia in the delayed and persistent development of these sickness responses in rats exposed to repeated stress, we investigated the effect of pretreatment with RU486, a glucocorticoid receptor antagonist, and minocycline, an inhibitor of microglial activation, on polyI:C-induced allodynia and depressive-like behavior. Pretreatment with either drug inhibited both the delayed allodynia and depressive-like behavior. The present study demonstrates that repeated, but not single, social defeat stress followed by systemic polyI:C administration induced prolonged allodynia and depressive-like behavior in rats. Our results show that even though a single-event psychosocial stress does not have any effect by itself, animals may develop persistent allodynia and depressive-like behavior when they suffer from an infectious disease if they are pre-exposed to repeated or chronic psychosocial stress. Furthermore, this study suggests that stress-induced corticosterone and microglial activation play a pivotal role in this phenomenon.

1. Introduction

When animals suffer from viral infections, they develop a set of symptoms, i.e. sickness response, such as fever, allodynia, general fatigue, appetite loss, and depressive-like behavior [1]. Accumulating evidence has suggested that the sickness response is a highly organized strategy that allows animals to eliminate infectious pathogens and facilitate recovery [1,2]. When animals are infected, proinflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferons (IFNs) are released from macrophages and monocytes and act on the brain to induce the sickness response [1]. These locally produced cytokines act on the brain through several pathways, including prostaglandin E_2 synthesis by brain

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microvessel endothelial cells [3], afferent vagal nerves, acting on macrophage-like cells residing in the circumventricular organs, or cytokine-specific transporters at the blood–brain barrier [4]. These immune-to-brain communication pathways ultimately lead to the production of proinflammatory molecules by microglia and induce the sickness response [4].

Recent studies have demonstrated that stress increases proinflammatory cytokine levels in the periphery as well as the central nervous system [5–8]. Furthermore, several studies have shown that psychological stress exaggerates the sickness response in addition to the proinflammatory cytokine response following systemic immune challenge. For example, rats that were exposed to an acute inescapable tail shock exhibited significantly greater fever, higher levels of corticosterone, and greater proinflammatory cytokine responses to lipopolysaccharide (LPS) injection than those without stress exposure [9]. Prior exposure to social disruption stress also augmented peripheral cytokine levels and the scores of sickness behaviors, such as the presence of lethargy, ptosis, curled body posture, and ragged fur, in response to systemic challenge with LPS [10], IFN-α [11], and polyI:C in mice [12].

These findings suggest that prior psychological exposure exaggerates subsequent infection-induced sickness responses. However, the mechanism responsible for this phenomenon is not fully understood yet. We hypothesized that microglia may play a role because it has been demonstrated that microglia are extremely sensitive not only to damage in the central nervous system (CNS), but also to environmental challenges, such as psychological stress [13]. Furthermore, it also is known that psychological stressors can promote structural remodeling of microglia and enhance the release of proinflammatory cytokines from microglia in response to subsequent peripheral immune challenges [13–16]. Interestingly, several studies have reported that stress-induced secretion of glucocorticoids (GCs) plays a pivotal role in microglial priming as an alarm signal of danger [17–19]. Therefore, it is possible that prior psychosocial stress exaggerates the subsequent infection-induced sickness response via GCs and activated microglia.

In clinical settings, it is well known that viral infection triggers the development and exacerbation of physical and psychiatric symptoms in patients with some stress-related, functional syndromes, such as chronic fatigue syndrome (CFS), irritable bowel syndrome (IBS), and depression. They are known as post-infectious CFS [20], post-infectious IBS [21], and post-viral depression [22], respectively. But if psychosocial stress can modulate the development of post-infectious physical symptoms, why do only some, but not all, individuals develop symptoms after viral infection? We hypothesized that the duration of stress exposure is one of the factors that determines the severity and duration of post-infectious symptoms. That is, even though some psychosocial stress may not have any effect by itself, animals may develop an evident or persistent sickness response when suffering from an infectious disease if they are pre-exposed to the stress repeatedly or chronically.

To test this hypothesis, we applied single or repeated (5 times) social defeat stress to rats to emulate psychosocial stressors in humans [23]. Thereafter, we injected polyriboinosinic:polyribocytidylic acid (polyI:C) to mimic viral infection. PolyI:C is a synthetic double-stranded RNA Toll-like receptor 3 (TLR3) agonist that activates the innate immune response [24]. Systemic administration of polyI:C induces not only neuroinflammatory responses, characterized by increased expression of IL-1β, IL-6, TNF-α, IFNs, and cyclooxygenase-2 in the brain [25–27], but also various sickness responses, such as fever [27–30], mechanical hypersensitivity [31], chronic fatigue [30], and depressive-like behavior [25]. Next, we observed the magnitude and time course of polyI:C-induced sickness responses, focusing on fever, allodynia, body weight gain, food intake, and depressive-like behavior in rats. Furthermore, we also assessed the role of GCs and microglial activation in the development of the polyI:C-induced sickness response using pharmacological blockade with RU486, a GC receptor antagonist, and minocycline, a potent inhibitor of microglial activation.

2. Methods

2.1. Animals

Male Wistar rats (SLC, Shizuoka, Japan), 7 weeks old weighing 130–180 g at the start of the experiment (day −8), were used as intruders. After arrival, they were individually caged and allowed to acclimate to the laboratory for 1 week. Male Long Evans rats (SLC, Shizuoka, Japan) weighing 400–600 g were used as residents, and were pair-caged with age-matched females. Both strains were housed in separate rooms maintained at 23 ± 1 °C with a standard 12 h/12 h light–dark cycle (lights on 0700–1900 h), and food and water were available ad libitum. All procedures conformed to the guidelines of animal care by Kyushu University and by the Institute of Laboratory Animals, and were approved by the Ethics Committees of Kyushu University (A26-144-0).

2.2. Surgery and monitoring of core body temperature (Tc)

Tc was measured using a telemetry system (Data Sciences International, St. Paul, MN, USA) as described previously [32,33]. Each Wistar rat was anesthetized intraperitoneally (i.p.) with a combination of 0.15 mg/kg of medetomidine hydrochloride (Orion Corp, Espoo, Finland) and 2.0 mg/kg of midazolam (Sando, Tokyo, Japan) and 2.5 mg/kg of butorphanol (Meiji Seika, Tokyo, Japan). TA10TA-F40 radiotelemeters were implanted into the peritoneal cavity via a small abdominal incision. After closure of the cavity with suture, the wounds were dressed with 0.1% gentamicin ointment (MSD, Tokyo, Japan), and the animals were housed individually for 7 days to recover from the surgery. During this postsurgical period, rats were habituated to handling. Tc signals were received by an antenna located below the animal's cage and relayed to a signal processor (Dataquest A.R.T.™ System, Data Sciences International, St. Paul, MN, USA) connected to a server computer. At least 72 h before the experiment, the telemetric transmitters were activated using a magnet to start recording Tc each minute. Tc values were then averaged into 15 min time periods during the experiment. Only rats that showed stable diurnal changes in Tc were used for the following experiments. In order to match the animal's conditions, Wistar rats used in Experiments 2, 3, 4 and 5 also underwent sham surgery even though telemetry systems were not used in these experiments. In brief, a small incision was made, the incision was closed, and the wound was treated as described above.

2.3. Social defeat stress

Wistar rats were exposed to social defeat stress by placing them in the home cage of dominant Long-Evans rats following a modified resident–intruder confrontation procedure [32–35]. Briefly, from the home cage of paired Long-Evans rats, the female was removed and in exchange, a Wistar rat (the intruder) was placed into the cage of the male Long-Evans rat (the resident) for 60 min. In most cases, the intruder was attacked and defeated by the resident within 1 min as was evident from intruder freezing behavior or submissive posture. As soon as the intruder was found to be defeated, the animals were separated by inserting a wire-mesh partition to avoid further physical injury. Thus, the intruder was protected from direct physical contact while remaining in olfactory, visual and auditory contact with the resident for the rest of the stress period. After this stress procedure, no wounds were found on the intruders. Following the stress period, the intruder was returned to its home cage. To avoid individual differences in defeat intensity, each day the intruders were confronted with a different resident. This stress procedure was performed in the morning, between 1000 and 1130 h, when the circadian changes in Tc were minimal. The sham stress rats were housed in their home cages for 60 min instead of being placed into residents' cages, and were gently lifted by their tails at the start and end of the period. Following the social defeat or

sham stress, the animals in both groups were left undisturbed until the time of injection 2 days later (day 0).

2.4. Drugs

PolyI:C (Sigma, St. Louis, MO, USA), a synthetic double-stranded RNA, was dissolved in sterile physiological saline at a concentration of 1 mg/ml and injected i.p. at a dose of 3 mg/kg. RU486 (mifepristone; Sigma, St. Louis, MO, USA), a GC receptor antagonist, was dissolved in dimethyl sulfoxide (DMSO; Wako, Osaka, Japan), and injected i.p. at a dose of 50 mg/kg. Minocycline hydrochloride (Sigma, St. Louis, MO, USA) was diluted in sterile physiological saline, and injected i.p. at a dose of 50 mg/kg.

2.5. Von Frey test

A modified von Frey behavioral test was performed to assess mechanical allodynia, following Honda's methods [36]. Rats were placed individually into a cubical Plexiglas chamber with a wire mesh bottom and allowed to be habituated at least 15 min in the chambers before testing was initiated. After rats had adapted to the environment, a von Frey filament (Semmes-Weinstein monofilaments, Stoelting, IL USA) with a strength of 10 g was exerted vertically into the mid-plantar surface of the left hind paw through the mesh floor and held for 3 s with the filament slightly buckled. The degree of mechanical allodynia was measured as the frequency of paw withdrawal response when the filament was applied 10 times to the plantar surface with intervals of 5 s between measurements. Results were expressed as the percent response frequency of paw withdrawals (100 × number of withdrawal/10).The frequency of paw withdrawal responses with a filament of 10 g was approximately 20% or less in naive rats. Therefore, we considered it to be an appropriate value of the baseline for the assessment of mechanical allodynia.

2.6. Forced swim test

To assess depressive-like behavior, we conducted the modified single forced swim test (FST), following Porsolt's methods [37,38]. Rats were individually forced to swim for a 15-min session in glass cylinders (height: 60 cm; diameter: 18 cm) containing 22 cm of 23 °C water so that rats could not touch the bottom. Experiments were recorded with a video camera. The cylinder was cleaned and filled with fresh water for each test session. An experimenter blinded to the treatments the animals had received observed the recorded video and measured the immobility time for 15 min. The rat was judged to be immobile whenever it remained floating passively in the water in a little hunched but upright posture, its nostrils just above the water surface.

2.7. Experimental protocols

2.7.1. Experiment 1

The purpose of Experiment 1 was to test the hypothesis that prior social defeat stress would affect the fever response and time course of Tc after administration of polyI:C (Fig. 1). After implant surgery, rats were divided into 5 groups as follows: 5 days of repeated social defeat stress followed by polyI:C injection (Repeated/PolyI:C $n = 8$); 4 days of sham stress plus 1 day of social defeat stress followed by polyI:C injection (Single/PolyI:C $n = 5$); 5 days of repeated social defeat stress followed by physiological saline injection (Repeated/Saline $n = 6$); 5 days of sham stress followed by polyI:C injection (Sham/PolyI:C $n = 6$); 5 days of sham stress followed by saline injection (Sham/Saline $n = 6$). In this experiment, we did not include a Single/Saline group, i.e., rats that received 4 days of sham stress plus 1 day of social defeat stress followed by physiological saline injection, because our previous studies repeatedly demonstrated that the Tc of a Single/Saline group was not different from the Sham/Saline group between day 0 and day 7, and to keep the number of experimental animals to a minimum. PolyI:C or physiological saline was injected at 1000 h. To assess if anticipatory hyperthermic responses would affect the Tc of the subsequent day of the final social defeat stress, 3 mg/kg of polyI:C or equivalent volume of saline was administered to rats 48 h after the beginning of the last defeat session (day 0). At least 5 rats were used in each group, and their Tc was monitored for 9 days (day −1 to day 7). Over this time period, we measured the body weight just prior to intraperitoneal drug injection and the amount of food pellets at day −8, day 0 and day 8, and calculated the cumulative body weight gain and food intake over the individual eight day periods (day −8 to day 0 and day 0 to day 8).

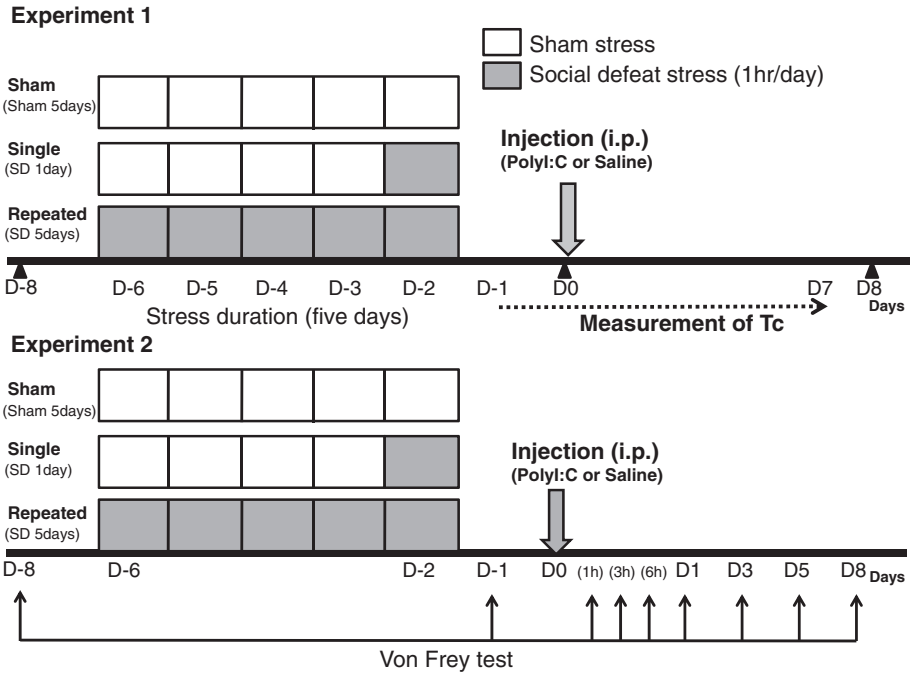

Fig. 1. Experimental design for Experiments 1 and 2. SD: social defeat; Tc: core body temperature; D: day; ▲: measurement of body weight and amount of food intake.

2.7.2. Experiment 2

Experiment 2 tested the hypothesis that exposure of single and repeated social defeat stress would affect the time course of mechanical allodynia induced by polyI:C (Fig. 1). After the sham surgery, rats were divided into the 5 groups as in Experiment 1. Von Frey tests were performed 8 days before (baseline) and 1 day before (post-stress) the injection and 1, 3, and 6 h and 1, 3, 5, and 8 days after the injection.

2.7.3. Experiment 3

We tested the hypothesis that the combination of social defeat stress and polyI:C administration would affect the time course of depressive-like behavior in FST. After the sham surgery, rats were divided into the 5 groups described in Experiment 1. Then, 15 min of single FST was performed on day 1 and day 9. To measure immobility time on day 1 and day 9, separate groups of rats were videotaped during the entire swim session for later analysis.

2.7.4. Experiment 4

To test the hypothesis that prolonged allodynia and the depressive-like behavior found in the Repeated/PolyI:C group is mediated by stress-induced corticosterone, 12 rats (Repeated/PolyI:C group) were injected i.p. with either RU486 (50 mg/kg) or DMSO (vehicle), daily, 20 min before exposure to social defeat stress for 5 consecutive days. Furthermore, to assess whether administration of RU486 would affect the withdrawal response or immobility time by itself, we included 10 rats (Repeated/Saline group) that were pre-treated with RU486 or DMSO (vehicle) for 5 consecutive days. Thus, rats were divided into 4 groups as follows: Repeated(Vehicle)/PolyI:C ($n = 6$), Repeated(RU486)/PolyI:C ($n = 6$), Repeated(RU486)/Saline ($n = 5$) and Repeated(Vehicle)/Saline ($n = 5$). Von Frey tests were conducted at the same time point as in Experiment 2. FST was performed on day 9 at the conclusion of the von Frey test.

2.7.5. Experiment 5

To test the hypothesis that prolonged allodynia and depressive-like behavior in the Repeated/PolyI:C group is mediated by activated microglial cells, 12 rats were injected i.p. with minocycline (50 mg/kg) diluted in physiological saline, or saline 20 min before exposure to social defeat stress for 5 consecutive days. In addition, to assess whether administration of minocycline would affect the withdrawal response or immobility time by itself, we included 11 rats (Repeated/Saline group) those were pre-treated with minocycline (Mino) or saline (vehicle) for 5 consecutive days. Thus, rats were divided into 4 groups as follows: Repeated(Vehicle)/PolyI:C ($n = 6$), Repeated(Mino)/PolyI:C ($n = 6$), Repeated(Mino)/Saline ($n = 6$) and Repeated(Vehicle)/Saline ($n = 5$). Von Frey tests and FST were conducted at the same time point as in Experiment 4.

2.8. Statistical analysis

All data are reported as means ± standard error of the mean (SEM). In Experiment 1, measurements of Tc were averaged over 15 min and then analyzed using two-way repeated-measures analysis of variance (ANOVA), with treatment as the between subjects factor and time as a within subjects repeated measure for the light period (0700–1900 h) and the dark period (1900–0700 h) from days 1 to 7. On day 0 (the day of polyI:C or saline administration), the data for the first 2 h after the drug injections were excluded in these analyses to avoid interference of stress-induced hyperthermia (SIH) [39] occurring after the injections. Then, two-way repeated measures of ANOVA were used to evaluate the differences among the measured points (1200–1800 h), and the values at each time point were compared by one-way ANOVA followed by Dunnett's post-hoc test. Furthermore, fever data were also calculated as a cumulative fever index (area under the curve above 36.5 °C) between 120 and 480 min (1200–1800 h) following either polyI:C or saline injection and then analyzed using one-way ANOVA followed by Dunnett's post-hoc test.

Statistical analysis of the obtained values, for example, change in food intake and body weight gain and immobility time in FST were analyzed using one-way ANOVA followed by Dunnett's test or Tukey–Kramer test, if Sham/Saline group is not included. In Experiment 2, the time course of withdrawal response frequency was also analyzed using two-way repeated measures of ANOVA with time and treatment as the main effects and the values at each time point were compared by one-way ANOVA followed by Dunnett's test. In Experiments 4 and 5, statistical analysis of withdrawal response frequency was also analyzed using two-way repeated measures of ANOVA and the values at each time point were compared by one-way ANOVA followed by Dunnett's post hoc test. All p values of less than 0.05 were considered statistically significant. All statistical analyses were performed with JMP® 9.0.2 for Windows (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Inhibitory effects of social defeat stress on polyI:C-induced fever

In Experiment 1, rats were injected with 3 mg/kg of polyI:C or an equivalent volume of physiological saline 3 h after lights were turned on (1000 h) on day 0 (Figs. 1 and 2). All groups exhibited a transient hyperthermia due to SIH peaking 0.5 h after the injection. There was a significant effect of treatment ($F_{4,26} = 6.07$, $p = 0.0014$) and a time effect ($F_{24,624} = 4.06$, $p < 0.0001$) but not a significant treatment × time interaction ($F_{96,624} = 1.14$, $p = 0.1771$) in Tc across the measured points. The Sham/PolyI:C group showed a monophasic fever peaking 180–225 min (1300–1345 h) after polyI:C injection. This group exhibited a maximum fever of 38.0 °C and resulted in a significant elevation of Tc 180–435 min (1300–1715 h) after injection, except for 2 time points (1400, 1415 h), compared with the control (Sham/Saline) group. The Single/PolyI:C group reached a peak Tc at 1300 h (37.8 °C) and returned to baseline gradually. There were no time points significantly higher than the control group. With prior repeated stress (Repeated/PolyI:C), the Tc at 1300 h was the lowest among the 3 polyI:C-treated groups, and only 2 time points were significantly higher than the control group (1515, 1530 h). This group showed a delayed Tc peak 390 min after the injection (1630 h). For the Repeated/Saline group, there were no time points significantly higher than the control group. Next, we compared the fever index among the five groups (Fig. 3), one-way ANOVA yielded a significant difference ($F_{4,26} = 6.21$, $p = 0.0012$), and post hoc analysis revealed that only Sham/PolyI:C group showed significant increased fever index compared to the Sham/Saline (control) group ($p = 0.0006$). Tc during the dark and light periods did not differ among the 5 groups from day 1 to day 8 (data not shown). Neither did Tc at day −1 differ among the 5 groups, indicating that single or multiple exposures to social defeat did not induce anticipatory hyperthermia [39].

3.2. Effects of single or repeated exposures to social defeat followed by polyI:C injection on body weight gain and food intake

In parallel with the Tc measurement, we assessed changes in cumulative body weight and food intake before (day −8 to day 0) and after (day 0 to day 8) the injection (Fig. 4). The Sham/Saline (control) group gained 50.6 ± 1.5 g (Fig. 4A upper panel) and one-way ANOVA yielded a significant difference ($F_{4,26} = 9.81$, $p < 0.0001$) among the 5 groups during days −8 to 0. In this time period, Dunnett's post-hoc analysis showed significant reduced body weight gain in the Repeated/PolyI:C and Repeated/Saline groups ($p = 0.0094$, 0.0012, respectively) compared to the Sham/Saline (control) group. Regarding food intake, the control group consumed 128.9 ± 4.6 g from day −8 to day 0 (Fig. 4A lower panel). Though one-way ANOVA revealed a significant difference among the 5 groups ($F_{4,26} = 3.23$, $p = 0.0279$), post-hoc analysis did not reveal any significant differences among the control

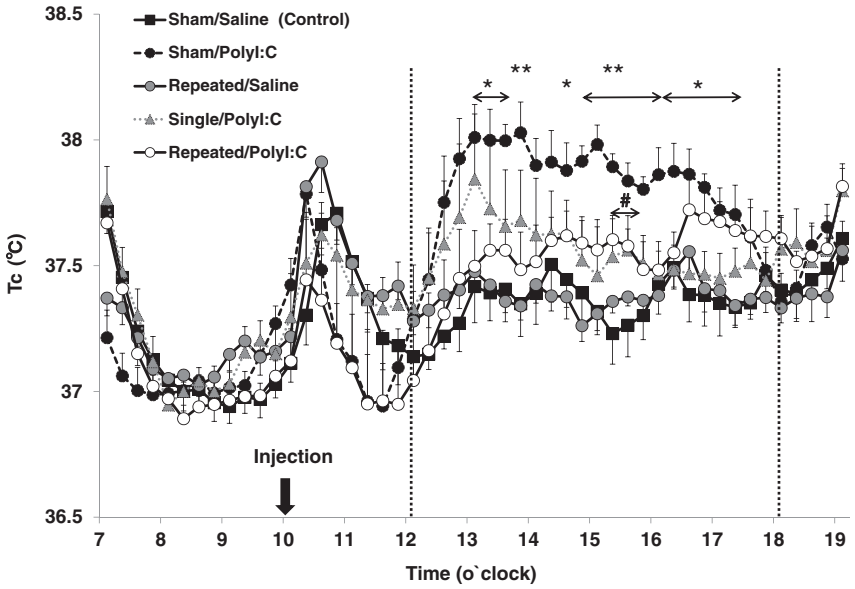

Fig. 2. Effects of single or repeated exposure to social defeat on polyI:C-induced fever. PolyI:C (3 mg/kg) or physiological saline was injected at 1000 h, 48 h following the last stress session on day 0 (D0). Data represent the mean ± SEM at 15 min intervals. The number of rats for each group were as follows: Repeated/PolyI:C (○, $n = 8$), Single/PolyI:C (▲, $n = 5$), Repeated/Saline (●, $n = 6$), Sham/PolyI:C (●, $n = 6$), or Sham/Saline (■, $n = 6$). $^{**}P < 0.01$, $^{*}P < 0.05$ Sham/PolyI:C versus Sham/Saline. $^{\#}P < 0.05$ Repeated/PolyI:C versus Sham/Saline. The dotted lines indicate the duration analyzed (1200–1800 h).

and experimental groups. After the drug injection, the Sham/Saline (control) group gained 43.6 ± 1.5 g during days 0 to 8 (Fig. 4B upper panel). There was a significant difference among the 5 groups ($F_{4,26} = 6.39$, $p = 0.0010$), and only the repeated stress groups (Repeated/PolyI:C and Repeated/Saline) showed significantly reduced body weight gain compared to the control group ($p = 0.0145$, 0.0009, respectively). Over this time period, one-way ANOVA revealed a significant difference among the 5 groups ($F_{4,26} = 6.14$, $p = 0.0013$), and post-hoc Dunnett's test revealed significantly reduced food intake in the Repeated/PolyI:C and Repeated/Saline groups ($p = 0.0053$, 0.0052, respectively) compared to the control group (Fig. 4B lower panel).

3.3. Effects of single or repeated exposure to social defeat on the time course of polyI:C-induced allodynia

We also observed changes in paw-withdrawal frequency in the 5 groups (Fig. 5). Two-way repeated ANOVA revealed a significant effect of treatment ($F_{4,25} = 9.95$, $p < 0.0001$), a significant time effect ($F_{8,200} = 14.13$, $p < 0.0001$), and also a significant treatment × time interaction effect ($F_{32,200} = 14.67$, $p < 0.0001$). With pre-exposure to sham or single social defeat stress, peak paw-withdrawal responses reached more than 70% between 1 h and 3 h after polyI:C injection, after which the response returned to baseline within a day. In contrast, the Repeated/PolyI:C group did not exhibit a significant increase in paw-withdrawal response frequency compared to the control group at day 0. In contrast, the paw-withdrawal response frequency of the Repeated/PolyI:C group began to increase significantly ($p = 0.0128$) compared to the Sham/Saline group from day 1 (46.7 ± 8.4%) and gradually increased until day 8 (70.0 ± 7.3%). In order to investigate how long this phenomenon sustains, we assessed mechanical allodynia until 8 weeks after polyI:C injection using a separate group of rats (Fig. 6) Two-way repeated ANOVA revealed a significant effect of treatment ($F_{13,6} = 144.99$, $p < 0.0001$), a significant time effect ($F_{13,78} = 11.54$, $p < 0.0001$), and also a significant treatment × time interaction effect ($F_{13,78} = 10.62$, $p < 0.0001$). As was seen in Fig. 5, significant polyI:C-induced allodynia appeared from day 1 (42.5 ± 4.8%, $p = 0.0027$), peaked from day 3 to day 28, began to decrease from day 35 (65.0 ± 11.9%, $p = 0.0102$), and returned to baseline level from day 42 to day 56.

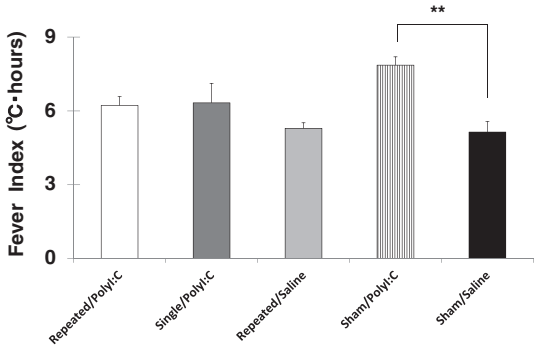

Fig. 3. Fever data calculated as a cumulative fever index (area under the curve above 36.5 °C) between 120 and 480 min (1200–1800 h) following either polyI:C or saline injection. Data represent mean ± SEM. $^{**}P < 0.01$ Sham/PolyI:C versus Sham/Saline.

3.4. Repeated exposure to social defeat increased depressive-like behavior

The immobility times in 15 min FST were compared among the 5 groups at days 1 and 9 using separate groups (Fig. 7). Although there was no significant difference at day 1 ($F_{4,25} = 1.10$, $p = 0.3771$), there was a significant difference at day 9 ($F_{4,26} = 4.37$, $p = 0.0078$) among the 5 groups. Post-hoc tests indicated that only the immobility time of the Repeated/PolyI:C group was significantly longer than that of the Sham/Saline (control) group ($p = 0.0145$).

3.5. RU486 treatment improved the time course of mechanical allodynia and decreased depressive-like behavior in rats exposed to repeated social defeat

Fig. 8A shows the time course of mechanical allodynia in rats that were pre-treated with RU486 or vehicle before stress exposure. There was a significant effect of treatment ($F_{3,18} = 20.18$, $p < 0.0001$), and was also a significant time effect ($F_{8,144} = 13.72$, $p < 0.0001$) and a significant treatment × time interaction ($F_{24,144} = 20.53$, $p < 0.0001$). The time course of RU486-treated Repeated/Saline group was not different from Vehicle-treated Repeated/Saline group, and withdrawal responses were within the range of 10–20%. The vehicle-treated Repeated/PolyI:C group showed delayed onset and persistent mechanical allodynia peaking at day 8, which was consistent with the results of Experiment

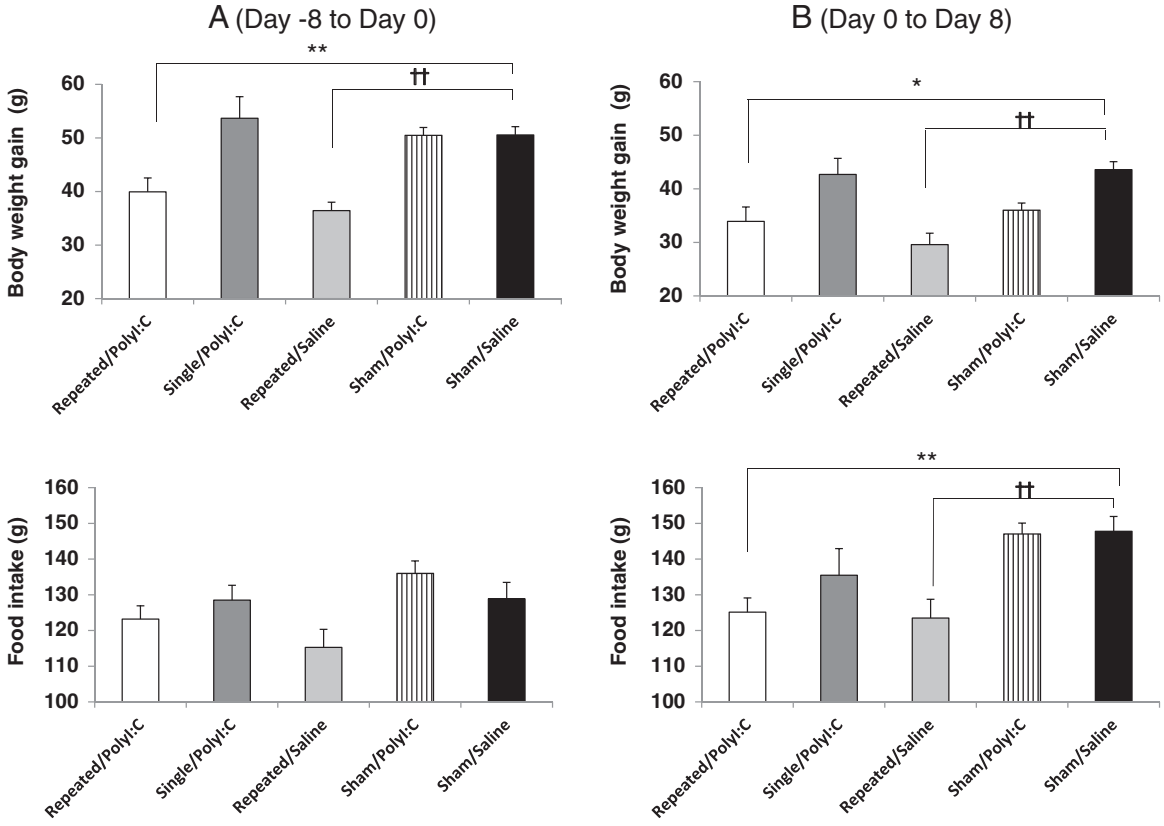

Fig. 4. Change in cumulative body weight gain and food intake of rats from day −8 to day 0 (A) and day 0 to day 8 (B). Data represent mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ Repeated/PolyI:C versus Sham/Saline. $^{\dagger\dagger}P < 0.01$ Repeated/Saline versus Sham/Saline.

2 (Fig. 5). However, the RU486-treated group showed maximal withdrawal responses after 3 h following polyI:C injection, and the emergence of mechanical allodynia from days 1 to 8 was blocked completely. FST immobility time was significantly different among the 4 groups ($F_{3,18} = 5.57$, $p = 0.0070$), and the Tukey–Kramer test indicated the significant difference between vehicle-treated Repeated/PolyI:C and RU486-treated Repeated/PolyI:C group ($p = 0.0115$) demonstrating a difference in immobility time about 140 s (Fig. 8B).

3.6. Minocycline treatment improved the time course of mechanical allodynia and decreased depressive-like behavior in rats exposed to repeated social defeat

Fig. 9A shows the time course of mechanical allodynia in rats that were pre-treated with minocycline or vehicle before stress exposure. There was a significant effect of treatment ($F_{3,19} = 16.83$, $p < 0.0001$), and was also a significant time effect ($F_{8,152} = 10.68$, $p < 0.0001$) and a significant treatment × time interaction ($F_{24,152} = 29.71$, $p < 0.0001$). The withdrawal responses of minocycline-treated and vehicle-treated Repeated/Saline groups were around 20% throughout the observation

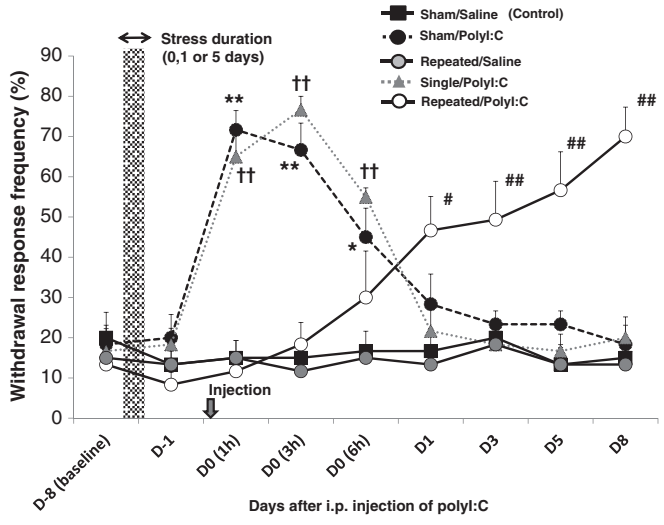

Fig. 5. Effects of single or repeated exposure to social defeat on the time course of PolyI:C-induced allodynia. Mechanical allodynia was measured as the paw withdrawal response frequency (%) to stimulation with a 10 g von Frey filament. The number of rats for each group were as follows: Repeated/PolyI:C (○, $n = 6$), Single/PolyI:C (▲, $n = 6$), Repeated/Saline (●, $n = 6$), Sham/PolyI:C (●, $n = 6$), or Sham/Saline (■, $n = 6$). Data represent mean ± SEM $^{**}P < 0.01$, $^{*}P < 0.05$ Sham/PolyI:C versus Sham/Saline, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ Repeated/PolyI:C versus Sham/Saline. $^{\dagger\dagger}P < 0.01$ Single/PolyI:C versus Sham/Saline.

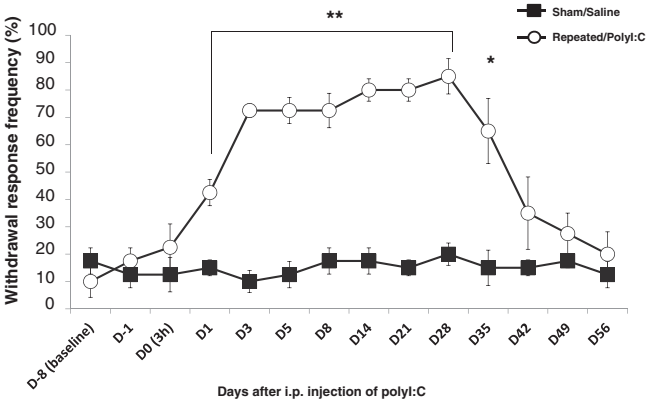

Fig. 6. The time course of PolyI:C-induced allodynia in Repeated/PolyI:C group. The number of rats for each group were as follows: Repeated/PolyI:C (○, $n = 4$), Sham/Saline (■, $n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$ Repeated/PolyI:C versus Sham/Saline.

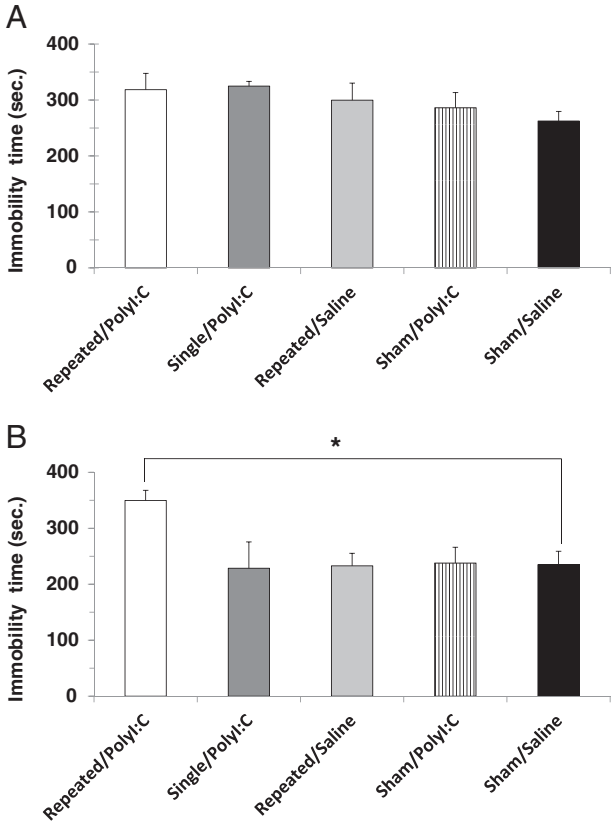

Fig. 7. Effects of single or repeated exposure to social defeat followed by polyI:C injection on immobility time in a forced swim test on day 1 (A) and day 9 (B) in rats. Data represent mean ± SEM. (A) For each group, $n = 6$. (B) Repeated/PolyI:C ($n = 8$), Single/PolyI:C ($n = 5$), Repeated/Saline ($n = 6$), Sham/PolyI:C ($n = 6$), or Sham/Saline ($n = 6$). $^{*}P < 0.05$ Repeated/PolyI:C versus Sham/Saline.

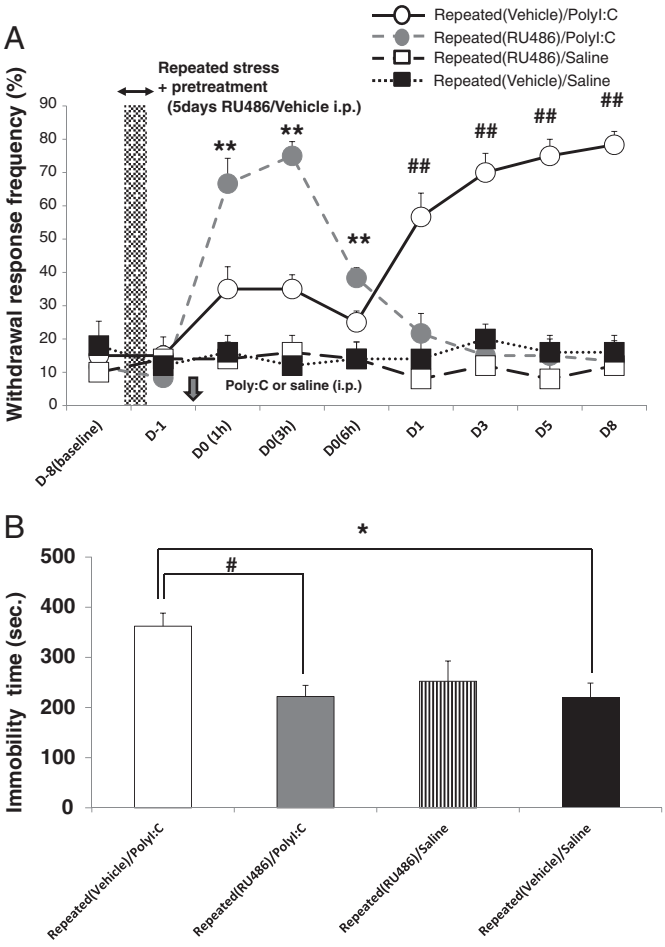

Fig. 8. Effects of RU486 on the time course of mechanical allodynia (A) and on immobility time in a forced swim test at day 9 (B) in Repeated/PolyI:C and Repeated/Saline rats. RU486 (50 mg/kg) or DMSO was injected i.p. 20 min before beginning the social defeat for 5 days. The number of rats for each group were as follows: (A) Repeated(Vehicle)/PolyI:C (○, $n = 6$), Repeated(RU486)/PolyI:C (●, $n = 6$), Repeated(RU486)/Saline (□, $n = 5$), Repeated(Vehicle)/Saline (■, $n = 5$). Data represent mean ± SEM. $^{**}P < 0.01$ Repeated(RU486)/PolyI:C versus Repeated(Vehicle)/Saline. $^{\#\#}P < 0.01$ Repeated(Vehicle)/PolyI:C versus Repeated(Vehicle)/Saline. (B) Data represent mean ± SEM. $^{*}P < 0.05$ Repeated(Vehicle)/PolyI:C versus Repeated(Vehicle)/Saline. $^{\#}P < 0.05$ Repeated(Vehicle)/PolyI:C versus Repeated(RU486)/PolyI:C.

period. The vehicle-treated Repeated/PolyI:C group showed delayed onset and persistent mechanical allodynia peaking at day 8. However, the minocycline-treated Repeated/PolyI:C group showed a maximum withdrawal response of 75% at 3 h after polyI:C injection, and the emergence of allodynia from days 1 to 8 was abolished, as was seen in the RU486-treated group (Fig. 8A). On the other hand, FST immobility time was significantly different among the 4 groups ($F_{3,19} = 16.08$, $p < 0.0001$), and the Tukey–Kramer post hoc test revealed the significant difference between vehicle-treated Repeated/PolyI:C and minocycline-treated Repeated/PolyI:C group ($p = 0.0003$). Minocycline-treated Repeated/PolyI:C group demonstrated a shortened immobility time about 160 s compared to the vehicle-treated Repeated/PolyI:C group (Fig. 9B).

4. Discussion

The present study demonstrates that preceding repeated social defeat stress potentiated polyI:C-induced sickness responses, in particular mechanical allodynia and depressive-like behavior.

We first investigated the effects of social defeat stress on the polyI:C-induced febrile response using a telemetry system. To our knowledge, this is the first study that investigated how psychosocial stress modulates the fever response induced by viral mimetics in rats. Both single and repeated social defeat stress attenuated polyI:C-induced fever. Previous results on the effect of stress on febrile responses are conflicting. For example, acute inescapable tail shock stress increased LPS-induced fever in rats [9], whereas immobilization in Guinea pigs [40] or physical restraint in rats [41] and Pekin ducks [42] attenuated it. It is well known that endogenous GCs attenuate fever induced by pro-inflammatory stimuli such as LPS [43,44] and polyI:C [28]. In addition, prior stress exposure augments corticosterone secretion in response to LPS [45]. Therefore, it is likely that prior stress-induced enhanced secretion of corticosterone attenuated polyI:C-induced fever.

As for metabolic parameters, the repeated stress groups, but not single stress groups, showed significant decreases in body weight gain prior to polyI:C injection (days −8 to 0). The attenuated body weight gain we observed in the repeated stress groups was not due to reduced food intake because food intake was not significantly different from others. Rather, it might be caused by increased energy expenditure as social defeat stress increases Tc via sympathetic nerve-mediated nonshivering thermogenesis in the interscapular brown adipose tissues [33]. As repeated exposure to social defeat does not habituate the magnitude of the SIH [32], 5 days of repeated social defeat stress might lead to considerable energy expenditure and result in suppression of body weight gain. In contrast, after the polyI:C or saline injection (days 0 to 8), irrespective of polyI:C administration, only the repeated stress groups showed significant reductions in both body weight gain and food intake compared to the control group. Neither the Single/PolyI:C nor the Sham/PolyI:C groups exhibited any change in these parameters. Systemic administration of polyI:C (750 μg/kg i.p.) was reported to reduce body weight and food intake for 24 h with a return to baseline levels between 24 h and 48 h [27]. Therefore, it is likely that the Sham/PolyI:C and Single/PolyI:C groups transiently decreased body

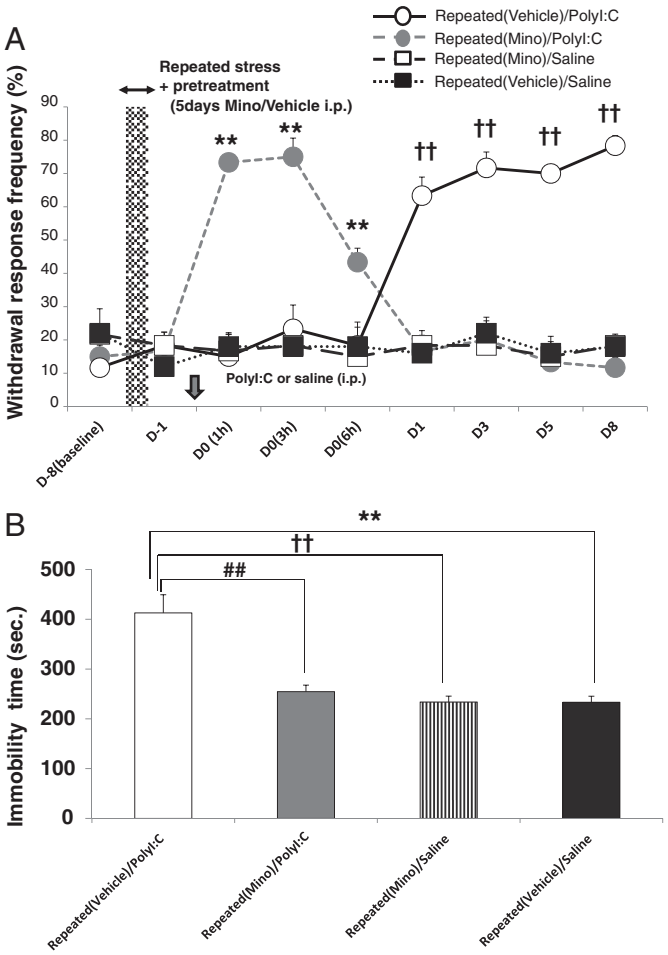

Fig. 9. Effects of minocycline on the time course of mechanical allodynia (A) and on immobility time in a forced swim test at day 9 (B) in Repeated/PolyI:C and Repeated/Saline rats. Minocycline (50 mg/kg) or physiological saline was injected i.p. 20 min before beginning the social defeat for 5 days. The number of rats for each group was as follows: (A) Repeated(Vehicle)/PolyI:C (○, $n = 6$), Repeated(Mino)/PolyI:C (●, $n = 6$), Repeated(Mino)/Saline (□, $n = 6$), Repeated(Vehicle)/Saline (■, $n = 5$). Data represent mean ± SEM. $^{**}P < 0.01$ Repeated(Mino)/PolyI:C versus Repeated(Vehicle)/Saline. $^{††}P < 0.01$ Repeated(Vehicle)/PolyI:C versus Repeated(Vehicle)/Saline. (B) Data represent mean ± SEM. $^{**}P < 0.01$ Repeated(Vehicle)/PolyI:C versus Repeated(Vehicle)/Saline. $^{††}P < 0.01$ Repeated(Vehicle)/PolyI:C versus Repeated(Mino)/Saline. $^{\#\#}P < 0.01$ Repeated(Vehicle)/PolyI:C versus Repeated(Mino)/PolyI:C. Mino: minocycline.

weight and food intake but recovered within the observation period (8 days) in this study. Thus, only repeated social defeat stress had a long-lasting inhibitory effect on both body weight gain and food intake, regardless of polyI:C administration, following cessation of stress exposure.

We next investigated the time course of mechanical allodynia. In this study, neither single nor repeated social defeat stress induced significant allodynia on day −1. In contrast, polyI:C injection induced mechanical allodynia peaking 1–3 h post-injection, with the effect disappearing on the next day. The Single/PolyI:C group displayed allodynia with nearly the same kinetics as the Sham/Poly I:C group. However, it is noteworthy that, in the Repeated/PolyI:C group, significant allodynia was not observed at day 0, but began to develop on day 1 and lasted until day 8. This late-onset and prolonged mechanical allodynia was observed even 4 weeks after PolyI:C injection and started to decline within 6 weeks.

We also assessed immobility time in the FST to evaluate depressive-like behavior at days 1 and 9. Immobility time was not different among the 5 groups at day 1, indicating that rats do not display depressive-like behavior at this time point. However, at day 9, the Repeated/PolyI:C group displayed significantly prolonged immobility time compared to the control group. This suggests that rats develop depressive behavior, not just after, but about 1 week after the polyI:C injection only when they had been preexposed to repeated stress. Interestingly, rats treated with repeated, but not single, social defeat stress followed by polyI:C injection showed both mechanical allodynia and depressive-like behavior about 1 week after polyI:C treatment.

Finally, to verify the role of GC and microglia in the delayed-onset and persistent allodynia and depressive-like behavior, we assessed the effects of RU486, a GC receptor antagonist, and minocycline, an inhibitor of microglial activation. Both pre-treatments dramatically suppressed the late-onset of allodynia and depressive-like behavior in the Repeated/PolyI:C rats. Interestingly, pretreatment with both drugs allowed for the reappearance of allodynia 1–3 h post polyI:C injection, which was observed in rats without stress exposure.

Although the precise mechanism of this phenomenon is unknown, the results suggest that GCs and microglial activation are involved in inhibition of initial (1–3 h post injection) allodynia as well as the development of the late-onset allodynia.

The present study suggests that, even though a single stress event has little effect on the following infection-induced sickness response, exposure to repeated (or chronic) stress can potentiate the severity and time course of post-infectious symptoms. Considering the present findings with the results of previous studies [46,47], microglial priming may play a pivotal role in the exaggerated sickness response. Microglial priming is characterized by a shift in microglial phenotype and exaggerated proinflammatory responses to the subsequent immune trigger [48]. It has been demonstrated that systemic administration of polyI:C induces mechanical pain hypersensitivity by activating microglia in the spinal cord in rats [31,49]. It also induces neuroinflammation in the brain, which is characterized by the expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α [25,50]. These brain-derived cytokines also decrease nociceptive thresholds [51–55] and induce depressive-like behavior [56–58]. It has been shown that acute and chronic psychological stresses can prime the proinflammatory reactivity of microglia to subsequent immune challenge [13–16,19,59]. GCs affect the priming of microglia when the organism is exposed to psychological stress [13,19,60]. Stress and GCs increase the immunoreactivity of microglia [15,19,61] and shift the microenvironment towards a proinflammatory immunophenotype [17]. Furthermore, chronic social defeat stress induced a very large increase in circulating corticosterone compared to acute social defeat stress in mice [62]. Therefore, it is possible that corticosterone induced by repeated stress primed the microglia to a later polyI:C challenge and led to prolonged allodynia and depressive-like behavior.

The sickness response is considered to be an adaptive response that facilitates recovery from acute viral and bacterial infections or inflammatory trauma [2]. However, in some circumstances, such as those described in these experiments, the sickness response becomes more severe and prolonged possibly via sustained neuroinflammation in the CNS. This inappropriately activated or prolonged sickness response could be maladaptive and may lead to the development of post-infectious physical or mental diseases, such as post-infectious CFS and post-viral depression. There is a growing evidence that chronic central neuroinflammation contributes to the pathophysiology of CFS [63–65] and depression [66–68]. It also has been shown that cumulative life stress is involved in the pathophysiology of CFS [69,70] and depression [71]. For example, in a cohort study, it was reported that perceived stress and stressful life events at baseline were associated with post-infectious CFS following infectious mononucleosis at 6 months [20]. Therefore, the present findings shed light on our understanding on the pathophysiological role of psychological stress in CFS or depression, i.e. there is a possibility that stress-induced GCs and microglia play a role in the pathogenesis of post-infectious functional syndromes such as post-infectious CFS and post-viral depression.

This study has several limitations. Firstly, we did not confirm morphological changes of microglia. Secondly, we did not measure the

level of proinflammatory cytokines and corticosterone in the serum or brain. Therefore, further study is needed to decisively demonstrate the involvement of GCs and microglia in prolonged sickness responses. Lastly, we attributed the persistent allodynia and depressive-like behavior to the sustained microglial activation using pharmacological blockade by minocycline in this study. However, this drug has several therapeutic potential, i.e. not only anti-inflammatory effects due to inhibition of microglial activation [72,73], but also has antioxidant properties [74,75], anti-apoptotic properties [73,76], neuroprotective properties [77,78], or decreasing glutamate-induced neurotoxicity [79,80]. Thus, it is possible that these properties other than anti-inflammatory effects might contribute to the inhibition of allodynia and depressive-like behavior.

In conclusion, this study demonstrated that repeated social defeat stress made the development of polyI:C (i.p)-induced mechanical allodynia and depressive-like behavior delayed and persistent. Furthermore, our results suggest that GCs and microglia play a pivotal role in this phenomenon. The present findings may provide insight on our understanding of the pathogenesis of post-infectious diseases such as post-infectious CFS, i.e., chronic psychosocial stress prior to infection plays an important role in the development of post-infectious symptoms [55].

Acknowledgments

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