

Social defeat stress induces phosphorylation of extracellular signal-regulated kinase in the leptomeninges in mice

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Aims: Animal studies using various stress models have shown that excessive environmental stress induces depression- and anxiety-like behaviors through inflammatory responses in the brain and periphery. Although the leptomeningeal cells have multiple functions related to inflammatory responses in the brain, whether environmental stress influences the leptomeninges remains unknown. In this study, we aimed to examine phosphorylation of the extracellular signal-regulated kinase (ERK) in the leptomeninges.

Methods: We subjected C57BL/6 male mice to a single episode of social defeat stress and analyzed the expression of phosphorylated ERK in the leptomeninges by immunohistochemistry.

Results: Social defeat stress in mice induced phosphorylation of ERK in the leptomeninges, adjacent to vascular endothelial cells and the glia limitans. This ERK phosphorylation was maintained for at least one hour after the stress.

Conclusions: This study shows the effect of environmental stress on the leptomeninges for the first time and paves the way for elucidating its functional role in stress-induced changes in neural functions.

KEYWORDS

depression, inflammation, MAP Kinase, meninges, psychological stress

1 | INTRODUCTION

Excessive or prolonged environmental stress predisposes to mental illnesses including mood and anxiety disorders in humans.¹ Animal studies using a variety of stress models such as social defeat stress, chronic mild stress, and restraint stress in rodents have revealed that exposure to environmental stress induces inflammatory responses in the brain and systemic circulation, thereby contributing to emotional alternations.^{2–6} For example, social defeat stress activates microglia in several brain areas including the medial prefrontal cortex, and this activation is indispensable for dendritic atrophy in the medial prefrontal cortex and

the induction of social avoidance.² Social defeat stress also induces the production of interleukin-6 in bone marrow-derived leukocytes.⁷ The circulating IL-6 has been shown to enter the brain parenchyma due to concomitant disruption of the blood-brain barrier for the induction of social avoidance.³ In addition, social defeat stress recruits bone marrow-derived monocytes to the brain, and this monocyte infiltration is thought to induce anxiety-like behavior through eliciting inflammatory responses in the brain parenchyma.^{8,9} Together, these evidences suggest that social defeat stress induces microglial activation in the brain and infiltration of monocytes and cytokines from the periphery, and consequently alters emotional behaviors.

The leptomeninges consists of the arachnoid and the pia mater, which are interconnected by arachnoid trabeculae, and the surface

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of the leptomeninges is covered by leptomeningeal cells. The leptomeninges have multiple functions related to inflammatory responses in the brain. For example, it has been reported that peripheral inflammation induces the leptomeningeal expression of TNF α , which is responsible for astrocytic and microglial responses to peripheral inflammation.¹⁰ Brain antigen-specific T cells enter the brain parenchyma through the leptomeninges to damage myelinating axons in experimental autoimmune encephalomyelitis in mice.¹¹ For clinical relevance, it has been reported that lipocalin-type prostaglandin D synthase (L-PGDS) and its product prostaglandin (PG) D₂ in the CSF, which are thought to be derived from the leptomeninges, are reduced in major depressive patients.¹² Notably, L-PGDS-dependent PGD₂ synthesis is involved in sleep/wake regulation.^{13,14} Given a close link between stress, depression, and sleep dysregulation, it is plausible that the leptomeninges play a role in stress-related pathophysiology in the brain.

We previously reported that single exposure to social defeat stress induces the phosphorylation of extracellular signal-regulated kinase (ERK) at Thr202 and Tyr204 residues coupled to dopamine D1 receptor activation in the medial prefrontal cortex in mice.¹⁵ In this study, we examined the ERK phosphorylation in other brain areas or cell types and happened to find that single exposure to social defeat stress induces the ERK phosphorylation in the leptomeninges.

2 | METHODS

2.1 | Animals

Eight-week-old male C57BL/6N mice and male ICR mice retired from breeding were purchased from Japan SLC (Shizuoka, Japan) and used for the behavioral and histological analyses. All the mice were maintained on a 12 hours light/12 hours dark cycle with food and water available ad libitum.

2.2 | Social defeat stress

Social defeat stress was performed as previously described with minor modifications.^{6,15} In the present study, we subjected 9-week-old male C57BL/6N mice to single social defeat stress. Prior to social defeat stress, male ICR mice were screened for their aggressiveness to a novel C57BL/6N mouse for 3 minutes daily for 3 days. We assessed the aggression of the ICR mice by the latency and the frequency of attacks during the observation period, and only used those showing stable aggression for further experiments. Prior to social defeat stress, male C57BL/6N mice were housed singly with foods and water available ad libitum. These mice were then transferred to the home cage of a male ICR mouse for 10 minutes. Control mice were instead transferred to a novel cage and were allowed to freely explore for 10 minutes. We included all the data for the analyses without any exclusion. The mice were sacrificed for immunohistochemistry immediately or 1 hour after the social defeat stress or the cage transfer (control).

2.3 | Immunohistochemistry

Immunohistochemistry was performed as previously described.¹⁵ Briefly, immediately or 1 hour after a single exposure to the social defeat stress or the cage transfer (control), mice were deeply anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and were transcardially perfused with ice-cold 4% paraformaldehyde fixative following Dulbecco's phosphate-buffered saline (D-PBS). Brains were carefully removed and postfixed in the same fixative overnight. Following cryoprotection in 30% sucrose in D-PBS and rapid freeze in OCT compound, the brains were coronally cut at 30 μ m thickness by cryostat (Leica Microsystems, Nussloch, Germany). The sections were collected in cryoprotectant and kept at -30°C until use. In immunohistochemistry, the brain sections were incubated in blocking buffer (0.3% Triton X-100 and 3% donkey serum in D-PBS) for 60 minutes at room temperature, reacted with primary antibodies (see below for detail) diluted in the blocking buffer for two overnights at 4°C , reacted with secondary antibody conjugated with either Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (Thermo Fisher Scientific, Rockford IL, USA or Abcam, Cambridge, UK) at 1:1000 dilution in the blocking buffer overnight at 4°C , and then incubated with Hoechst 33342 at 2 $\mu\text{g}/\text{mL}$ in D-PBS for 15 minutes at room temperature. The primary antibodies used in the present study were as follows: mouse anti-glia fibrillary acidic protein (GFAP) antibody (MAB360; Millipore, Burlington, MA, USA) at 1:1000 dilution, rabbit anti-phospho-ERK antibody (#4370; Cell Signaling Technology, Beverly, MA, USA) at 1:1000 dilution and rat anti-CD31 antibody (Cat No. 550274; BD Pharmingen, San Diego, CA, USA). In between each step, the sections were washed in D-PBS containing 0.3% Triton X-100 for 10 minutes three times. After the staining, the sections were mounted in ProLong Gold Antifade Mountant (Life Technologies, Carlsbad, CA, USA) on an APS-coated glass slide (Matsunami Glass, Osaka, Japan). Fluorescent images were acquired with LSM700 confocal microscope (Carl Zeiss Micro Imaging, Göttingen, Germany), except with BZ-X microscope (Keyence, Osaka, Japan) for Figure 1A-C to visualize broader distribution of ERK phosphorylation. We manually set regions of interest including the cells lining on the brain parenchyma based on the nuclear staining (Hoechst 33342) and measured the signal intensities of the phosphorylated ERK by ImageJ software in the cells adjacent to and excluding the glia limitans positive for the GFAP staining. For the analyses, we selected three brain slices per animal in the range of bregma +1.4 mm to +2.1 mm and measured the signal intensities in the primary motor cortex (M1), the secondary motor cortex (M2), the primary somatosensory cortex (S1), and the piriform cortex of each brain slice. Since the ERK phosphorylation in leptomeningeal cells was similar across the analyzed brain regions and slices, we compared the signal intensities among immediately and 1 hour after the stress and the control for these cells adjacent to the primary motor cortex in a single representative slice from each mouse (Figure 1D). As to the line profile plot, we set a line vertical to the surface of the brain parenchyma and measured the signal intensity of each pixel along the line in each channel (Figure 2).

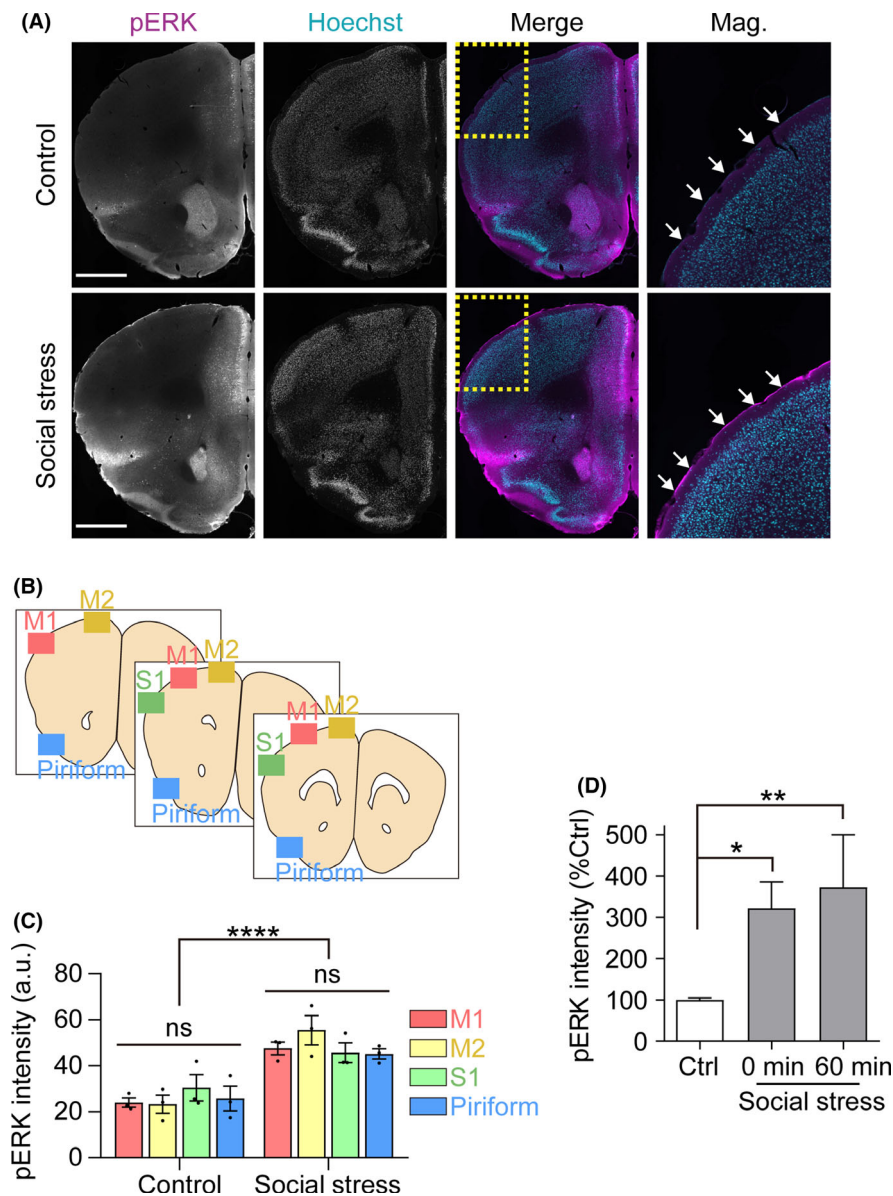


FIGURE 1 Social defeat stress increased expression of phosphorylated ERK in the leptomeninges. A, Immunohistochemistry of phosphorylated ERK (pERK) and nuclei staining (Hoechst 33342) showing the broad expression of pERK in the leptomeninges immediately after the stress, as indicated by arrows. The areas surrounded by yellow dashed boxes are magnified in the right panels. Scale bars = 1000 μ m; B, Schematics of the brain slices and the brain regions used for the semiquantification of the immunohistochemical pERK signal. The primary motor cortex (M1), the secondary motor cortex (M2), the primary somatosensory cortex (S1), and the piriform cortex in each of the three brain slices were examined (see Methods for details); C, The mean signal intensities of pERK for the designated brain region across all the brain slices for each mouse. Mice were perfused immediately after the social defeat stress (n = 3) or the cage transfer (control, n = 3). Each dot represents the data point for each animal; D, Quantification of the pERK intensity in the M1 of the mice that did not receive social defeat stress (Ctrl, n = 8) and that were perfused immediately (0 minute) or 60 minutes after the social defeat (social stress; 0 minute (n = 3) or 60 minutes (n = 3), respectively). Values are expressed as means \pm SEM. ****P < 0.0001 for the main effect of mouse groups and ns, not significant for the main effect of brain regions in two-way analysis of variance (C). *P < 0.05, **P < 0.01 for multiple comparison tests with Tukey-Kramer correction between the indicated groups and control (D)

2.4 | Statistical analyses

Data are expressed as mean values \pm SEM. One-way analysis of variance followed by multiple comparison tests with Tukey-Kramer correction and two-way analysis of variance followed by multiple comparison

tests with Sidak's correction were used for statistical analyses. The analyses were performed with Prism 8.0 software (GraphPad, San Diego, CA, USA). P values < 0.05 were considered to be significant. All the images shown in the figures are representative images from three independent experiments.

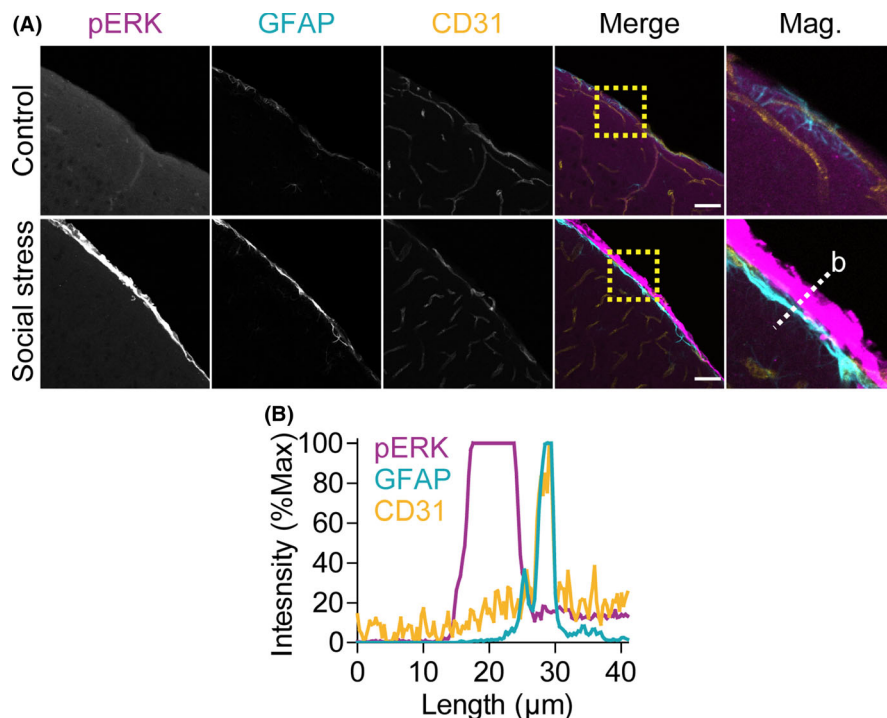


FIGURE 2 ERK phosphorylation (pERK) induced by social defeat stress in the leptomeninges was adjacent to the glia limitans and vascular endothelial cells. A, Immunohistochemistry using anti-pERK, anti-GFAP (astrocyte marker), and anti-CD31 (vascular endothelial marker) antibodies revealed the distinct expression patterns of these molecules. Mice were perfused immediately after the social defeat stress ($n = 3$) or the cage transfer (control, $n = 3$). The areas surrounded by yellow dashed boxes are magnified on the right panels. The signal intensity of each channel along the white dashed line is shown in (B). Scale bars = 50 μm ; B, The line profile plot showing the signal intensity of each channel, normalized by the maximal intensity of each channel. An X axis denotes the distance from a certain point outside the brain parenchyma, where the white dashed line in (A) starts. The expression profile of pERK was distinct from that of GFAP and CD31

3 | RESULTS AND DISCUSSIONS

To examine where in the brain social defeat stress induces the ERK phosphorylation, we subjected C57BL/6N male mice to a single episode of social defeat stress lasting 10 minutes and perfused the mice with paraformaldehyde fixative immediately after the stress. We performed immunohistochemistry using antibodies against the ERK phosphorylation. The ERK phosphorylation was increased broadly in the leptomeninges in the stressed mice relative to the control mice (Figure 1A). We analyzed the leptomeninges from four brain areas, namely the primary motor cortex (M1), the secondary motor cortex (M2), the primary somatosensory cortex (S1), and the piriform cortex (Figure 1B), and found that the ERK phosphorylation was similarly increased across the multiple brain regions in the stressed mice relative to the control mice (Figure 1C). Two-way analysis of variance revealed a significant main effect of mouse groups ($F(1, 16) = 53.41$, $P < 0.0001$), whereas there was no significant main effect of brain regions ($F(3, 16) = 0.3733$, $P = 0.7734$) or the interaction between the two variables ($F(3, 16) = 1.369$, $P = 0.2879$). Post hoc multiple comparison tests with Sidak's correction showed significant differences between the control vs stress conditions in M1 ($P = 0.0061$), M2 ($P = 0.0003$) and the piriform cortex ($P = 0.0252$) and a statistical trend in S1 ($P = 0.098$). This stress-induced ERK phosphorylation was also observed an

hour after the stress, indicating that the effect of the stress on the leptomeninges lasts for at least an hour.

Currently, there are no validated molecular markers for leptomeningeal cells. For example, nestin was reported to be expressed in leptomeningeal cells, but only in about 20% of these cells.¹⁶ Anatomically, beneath the leptomeninges is the subpial space containing blood vessels and the glia limitans, where astrocytes send dense processes.¹⁷ Thus, we visualized vascular endothelial cells and astrocytes using anti-CD31 and anti-GFAP antibodies, respectively. We confirmed that the ERK phosphorylation was induced in leptomeningeal cells, which are adjacent to vascular endothelial cells and the glia limitans (Figure 2A,B). We also found that the GFAP signal in the stressed mice appears to be stronger relative to the control mice (32.2 ± 4.7 and 64.4 ± 12.9 in arbitrary unit in the control mice and the stressed mice, respectively; $t(4) = 2.344$, $P = 0.079$ by Student's t test), suggesting activation of astrocytes adjacent to the leptomeningeal cells with ERK phosphorylation following the social defeat stress.

To the best of our knowledge, this study shows for the first time that social defeat stress induces the ERK phosphorylation in the leptomeninges. Since the leptomeninges mediate the effect of peripheral inflammation in inducing inflammatory responses in the brain parenchyma,¹⁰ inflammation-related molecules from the periphery could be involved in the stress-induced ERK phosphorylation in the leptomeninges.



Leptomeningeal cells express various receptors for cytokines and chemokines, some of which may be responsible for mediating peripherally derived inflammatory signals to the brain.¹⁷ Indeed, it has been reported that single social defeat stress induces IL-6 production from bone marrow-derived leukocytes.⁷ Therefore, the leptomeninges may play a role in mediating inflammation from the periphery to the brain upon stress exposure. Indeed, we reported that single and repeated social defeat stress induces microglial activation in the brain through innate immune signaling.² Since the leptomeningeal ERK phosphorylation occurred immediately after the stress, thus earlier than the microglial activation (ie, 4 hours, but not 90 minutes, after a single exposure to social defeat stress), the leptomeningeal changes appear to precede the microglial activation. Thus, the leptomeningeal changes may be involved in the subsequent microglial activation. However, the stress-induced microglial activation occurred in selective brain regions including the medial prefrontal cortex, whereas ERK phosphorylation was similarly induced in leptomeningeal cells adjacent to multiple brain regions. Thus, it is also possible that these two events are induced separately and play different roles. To address these possibilities, a method to selectively manipulate leptomeningeal cells would be required.

To exert the functions, the leptomeninges produce bioactive substances. L-PGDS, a synthase for PGD₂, is selectively expressed in the leptomeninges in the brain at least under physiological conditions, and L-PGDS and PGD₂ in the cerebrospinal fluids, which are thought to be derived from the leptomeninges, have been shown to be reduced in the cerebrospinal fluids of major depressive patients.¹⁴ Since the phosphorylated ERK increases the production of free arachidonic acid, a precursor for PGD₂ synthesis, by augmenting the activity of cytosolic phospholipase A₂,^{18,19} the leptomeningeal ERK phosphorylation could affect the PGD₂ production. Since PGD₂ from the leptomeninges promotes sleep,¹³ the leptomeningeal ERK phosphorylation could be involved in stress-induced dysregulation of sleep.²⁰ Whether the stress-induced ERK phosphorylation in the leptomeninges affects the production of these substances including PGD₂ and stress-induced changes in neural functions warrants future investigation.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

T.F. designed the study; T.O., H.N., C.N., M.N., and R.S. performed experiments and analyzed the results; H.N. and T.F. wrote the manuscript.

DATA REPOSITORY

We have made our data publicly available in the Supporting Information (Data S1) of our article.

APPROVAL OF THE RESEARCH PROTOCOL BY AN INSTITUTIONAL REVIEWER BOARD

n/a.

INFORMED CONSENT

n/a.

REGISTRY AND THE REGISTRATION NO. OF THE STUDY/TRIAL

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ANIMAL STUDIES

All procedures for animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committees of Kobe University Graduate School of Medicine.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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