Analysis of genes linked to depressive-like behaviors in interleukin-18-deficient mice: Gene expression profiles in the brain

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Abstract. Interleukin (IL)-18 is an interferon γ-inducing inflammatory cytokine associated with function of the immune system and other physiological functions. IL-18-deficient (Il18−/−) mice exhibit obesity, dyslipidemia, non-alcoholic steatohepatitis and depressive-like behavioral changes. Therefore, IL-18 has a number of important roles associated with immunity, energy homeostasis and psychiatric conditions. In the present study, gene expression in the brains of Il18−/− mice was analyzed to identify genes associated with the depressive-like behaviors and other impairments displayed by Il18−/− mice. Using whole genome microarray analysis, gene expression patterns in the brains of Il18−/− and Il18+/+ mice at 6 and 12 weeks of age were examined and compared. Subsequently, genes were categorized using Ingenuity® Pathway Analysis (IPA). At 12 weeks of age, 2,805 genes were identified using microarray analysis. Genes related to ‘Major depression’ and ‘Depressive disorders’ were identified by IPA core analysis, and 13 genes associated with depression were isolated. Among these genes, fibroblast growth factor receptor 1 (Fgfr1); protein tyrosine phosphatase, non-receptor type 1 (Ptpn1); and urocortin 3 (Ucn3) were classed as depression-inducing and the other genes were considered depression-suppressing genes. Subsequently, the interactions between the microarray results at 6 weeks of age and the above three depression-inducing genes were analyzed to search for effector genes of depression at 12 weeks of age. This analysis identified cyclin D1 (Ccnd1) and NADPH oxidase 4 (Nox4). The microarray analysis results were correlated with the results of reverse transcription-quantitative PCR (RT-qPCR).

Overall, the results suggest that Fgfr1, Ptpn1 and Ucn3 may be involved in depression-like changes and Ccnd1 and Nox4 regulate these three genes in IL-18-deficient mice.

Introduction

Major depressive disorder (MDD) has a high worldwide prevalence. Several hypotheses regarding the mechanism of development of MDD have been proposed, including an immunological mechanism involving cytokines (1,2). Increased levels of proinflammatory cytokines, such as interleukin (IL)-6 and tumor necrosis factor-α (TNF-α), have been identified in patients with MDD by meta-analyses (3). Clinical trials of a cyclooxygenase-2 inhibitor to suppress inflammation in patients with MDD produced symptomatic improvements; however, the immunological mechanisms underlying MDD are not understood in detail.

IL-18 was initially identified as an interferon-γ inducing factor (4). An inactive 24-kDa precursor form of IL-18 is cleaved by activated caspase-1 to a mature 18-kDa active form (4-7). In addition to its role as an inflammatory cytokine, IL-18 also serves a role in energy metabolism, and in the central nervous system it is involved in psychiatric disorders such as depression (8-12). Regarding energy homeostasis, IL-18 deficiency induces bulimia, corpulency and insulin resistance which resembles type 2 diabetes mellitus (8). IL-18-deficient mice also exhibit dyslipidemia leading to nonalcoholic fatty liver disease and steatohepatitis (9), and hippocampal impairments that may cause depression-like behavioral changes (12). Therefore, IL-18 is closely related to energy homeostasis, central nervous system function, and the occurrence of psychiatric disorders such as depression. However, the biological mechanism by which IL-18 contributes to these processes remains unclear.

In our previous study (12), it was demonstrated that the aforementioned behavioral changes were the result of hippocampal impairments; however, other parts of the brain, including the prefrontal cortex and amygdala, are also responsible for depression (13,14). Therefore, to determine the biological and molecular mechanisms responsible for depression caused by IL-18 deficiency, additional analyses of other brain regions are required.

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In the present study, candidate genes which may be involved in the induction of depressive-like behavioral changes were identified by comparing gene expression in the brains of \( \text{Il18}^{-+} \) and \( \text{Il18}^{-} \) mice. At 12 weeks of age, a time at which \( \text{Il18}^{-} \) mice start to exhibit depression-like behavioral changes, the genes with a \( >1.5 \) fold change were identified and analyzed using Ingenuity® Pathway Analysis (IPA) as described previously (10,11). Subsequently, to explore candidate genes associated with depression at 12 weeks of age, microarray analysis was performed to compare expression at 6 and 12 weeks of age to screen for effector genes that induce depression. Finally, to confirm the microarray results, reverse transcription-quantitative PCR (RT-qPCR) was performed.

Materials and methods

Details regarding RNA purification, microarray analysis, RT-qPCR, and IPA (Ingenuity® Systems; ingenuity.com) have been described in our previous studies (15-17).

Animals. \( \text{Il18}^{-+} \) C57BL/6 male mice were used in the present study (18). Littermate \( \text{Il18}^{-+} \) male mice were used as the controls. Mice were housed in groups of 3-5 in polycarbonate cages in a controlled environment (temperature, 22±1°C; humidity, 50-60%; and a 12-h light/dark cycle) with free access to water and food (MF; Oriental Yeast Co., Ltd.). A total of three \( \text{Il18}^{-+} \) and three \( \text{Il18}^{-} \) mice were used for microarray and RT-qPCR analysis.

Animal experiments performed in the present study were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Animal Care Committee of Hyogo College of Medicine (Hyogo, Japan; approval nos. #28041, #13-062, #14-020 and #16-013) prior to performing the animal experiments.

Sample collection. Mice were euthanized by deep anesthesia using 5% isoflurane inhalation at 10:00 a.m., and lack of ventilation was used to confirm euthanasia. The brains were removed and immediately placed in liquid nitrogen and subsequently kept at -80°C until required.

RNA purification. Total RNA was extracted and purified from mice brains using a Sepasol-RNA I Super kit (Nacalai Tesque, Inc.) according to the manufacturer's protocol. Genomic DNA contamination was removed using 5 units RNase free DNase I at 37°C for 30 min. After phenol/chloroform extraction and ethanol precipitation, total RNA was dissolved in de-ionized water. RNA concentrations were measured using NanoDrop 1000 spectrophotometry (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Microarray analysis. Expression profiling was performed as described previously (9). Raw data were imported into Subio platform version 1.18 (Subio, Inc.) and normalized. Differentially expressed genes between \( \text{Il18}^{-+} \) and \( \text{Il18}^{-} \) mouse brains were defined by signal ratios of \( >1.5 \) -fold. Data can be accessed in Gene Expression Omnibus (accession no. GSE64307).

Heatmap design. Heatmaps were created using the R package (heatmaps with row and/or column covariates and colored clusters; github.com/alexploener/Heatplus) in R version 2.30.0.

IPA. IPA (version spring 2019) was used to determine the functionality of microarray results and for the interpreting the gene profiling data. To investigate molecular mechanisms affected by IL-18 expression, core analysis was performed with the following settings: Tissue, Brain; all other settings, default. The network explorer of IPA was used to detect relevant interactions among genes extracted at 6 and 12 weeks of age and to reveal the shortest direct pathways between genes without protein-protein interactions.

RT-qPCR. Brain samples from mice at 6 or 12 weeks of age were used for analysis of expression. A total of 5 ng/reaction total RNA was used, extracted as described above and was analyzed using RNA-direct SYBR-Green Real-Time PCR Master mix and a One-step qPCR kit (Toyobo Life Science). Samples were run in duplicate reactions in 384-well plates. Median threshold cycle values were used to calculate the fold changes between group samples. The fold change values were normalized to \( \text{Gapdh} \) levels using the relative standard curve method. qPCR was performed using QuantStudio™ 12K Flex (Thermo Fisher Scientific, Inc.). The reverse transcription and thermocycling conditions were: 90°C for 30 sec and 20 min at 61°C for reverse transcription; followed by 45 cycles of 98°C for 1 sec, 67°C for 15 sec and 74°C for 35 sec. The primer sequences for RT-qPCR are shown in Table I.

Statistical analysis. All results are expressed as the mean ± standard deviation. Sigmagplot™ (version 11.0; Systat Software, Inc.) was used for all statistical analyses. The microarray results were analyzed using a Student's t-test and correlations between the microarray and RT-qPCR results were analyzed using Spearman's rank correlation tests. All analyses were performed >2 times to confirm the results. \( P<0.05 \) was considered to indicate a statistically significant difference.

Results

Identification and categorization of differentially expressed genes in \( \text{Il18}^{-} \) mice. The heatmaps of the microarray results at 6 and 12 weeks of age are shown in Fig. 1A and B, respectively. A total of 699 and 2,805 genes showed a \( >1.5 \) fold-change \((\text{Il18}^{-/+}\text{Il18}^{-+})\) in expression at 6 and 12 weeks of age, respectively, and were extracted for further analysis. The results of IPA core analysis are shown in Table II. Based on the results of IPA, 13 genes associated with ‘Major depression’ and ‘Depressive disorder’ were identified: Aquaporin 4 (Aqp4), BTG anti-proliferation factor (Btg1), Btg2, CD46, chromogranin A, complexin 1, corticotropin releasing hormone, eukaryotic translation initiation factor 6, fibroblast growth factor receptor 1 (Fgfr1), hepatocyte growth factor-regulated tyrosine kinase substrate (Hgs), kinesin family member 5B (Kif5b), protein tyrosine phosphatase, non-receptor type 1 (Ptpn1) and urocortin 3 (Ucn3). The heatmap of extracted genes is shown in Fig. 1C.
Table I. Primer sequences.

| Gene (GenBank Accession no.) | Sequence, 5’-3’ |
|-----------------------------|----------------|
| Aqp4 (NM_009700)            |                |
| Forward                     | CTCCGCCCATCGAATGCTC |
| Reverse                     | CGACATTGGCAGCACATTTGCTC |
| Bdnf (NM_007540)            |                |
| Forward                     | CGGCCCATGAAAGAAGTAAACGTCCA |
| Reverse                     | GGCCCATTCACGCTCTCC |
| Btg1 (NM_007569)            |                |
| Forward                     | GGCTTTGTCCGCATTTCCATAGCAG |
| Reverse                     | CTTTGGCCCATGAGGTACACGTTT |
| Btg2 (NM_007570)            |                |
| Forward                     | CTGCCCTCTGGTCTCATCATCAGTCTT |
| Reverse                     | ACAGTCCAGCTCTAGGGT |
| Cd46 (NM_010778)            |                |
| Forward                     | GCCTACTCATCCTACCAAGCCT |
| Reverse                     | TTGGCTAAATAATCTCCTTCACGGGAC |
| Chga (NM_007693)            |                |
| Forward                     | CCAATACCAATATCAACACCAGC |
| Reverse                     | TCTCAGTCTCTCCAGGTC |
| Cplx1 (NM_007756)           |                |
| Forward                     | TCTGCAAGGTTGTCCTTAAGAATTCCA |
| Reverse                     | ACCCGCTCATAATCTATTGC |
| Crh (NM_205769)             |                |
| Forward                     | AGAGAGCCCTATATACCCCTATTAGCAT |
| Reverse                     | AGCATGGGCAATAACAAATAACG |
| Drp2 (NM_010078)            |                |
| Forward                     | CCACAACAGGAGTCAGT |
| Reverse                     | GAGCCATTGCACTGACTAC |
| Eif6 (NM_010579)            |                |
| Forward                     | CACCCTAAACTTCTATAGGAGGACCA |
| Reverse                     | CTCGGTTCACAGTGCC |
| Fgfr1 (NM_010206)           |                |
| Forward                     | ATCATAATGAGTTCTCTGAGT |
| Reverse                     | CTCAATTCACAGT |
| Gapdh (NM_008084)           |                |
| Forward                     | CTTTCCGTTTTTCTACCCCATC |
| Reverse                     | TGATGTCATCATACTTGGCAGGTTT |
| Hgs (NM_001159328)          |                |
| Forward                     | GACTCTCAGCCATAACTCCCC |
| Reverse                     | CTCAATGGCCTCCTCCGACT |
| Htr2c (NM_008312)           |                |
| Forward                     | GCATAAACATGAACGTCAGT |
| Reverse                     | GGCAGCTCTAATAATCTCATCG |
| Kif5b (NM_008448)           |                |
| Forward                     | CTCACGTTTATGCAAGACAGACGA |
| Reverse                     | CACGGCTCCTCCAAAACC |
| Ptpn1 (NM_011201)           |                |
| Forward                     | ACACTGGAGGCTCACAACGG |
| Reverse                     | CCAGGCTGCTTTCATCCCC |
Interactions between depression-associated genes and \( \text{II18}^{-/-} \)-specific genes at 6 weeks of age. Interaction pathway analysis was used to identify genes responsible for regulating depression-associated genes. As shown in Fig. 2, the interactions deemed to be significant in the pathway analysis are shown for 10 genes: Adenosine A2a receptor (Adora2a), cyclin D1 (Ccnid1), CCAAT enhancer binding protein delta, D-box binding PAR bZIP transcription factor, endothelin 1, Fos proto-oncogene, AP-1 transcription factor subunit, FosB proto-oncogene, AP-1 transcription factor subunit, Htr2c, 5-hydroxytryptamine receptor 2C.
HRas proto-oncogene, GTPase, Notch1 and NADPH oxidase 4 (Nox4).

Depression-inducing genes in Il18−/− mice at 12 weeks of age. The 13 genes extracted in mice 12 weeks of age were separated into depression-inducing and depression-suppressing genes based on previous studies (19-21). Among these 13 genes, Fgfr1, Ptpn1 and Ucn3 may be associated with the development of MDD (depression-inducing), whereas the other 10 genes may be depression-suppressing genes in MDD.

Interactions between depression-inducing and suppressive genes at 12 weeks of age with the 10 extracted genes at 6 weeks of age. The interactions between the three depression-inducing genes at 12 weeks of age, Fgfr1, Ptpn1 and Ucn3, and 10 genes at 6 weeks of age were examined and it was found that Ccnd1 and Nox4 may affect development of depression. Ccnd1 inhibition increases the expression of Fgfr1 (22), and Nox4 decreases Ptpn1 expression (23).

Correlation between microarray and RT-qPCR results. To confirm the microarray analysis results, RT-qPCR was performed, and a correlation test was performed between the results of the microarray analysis and RT-qPCR (Table III). To determine the correlation between microarray and RT-qPCR analysis, Spearman’s rank correlation coefficient analysis was performed for each group (6-week-old group: P=0.014, ρ=0.60; 12-week-old group: P=0.007, ρ=0.64; Fig. 3).

Discussion

The present study identified potentially novel IL-18 pathways in the brain based on the core analysis, 13 genes were identified to be associated with depression at 12 weeks of age; in particular, Fgfr1, Ptpn1 and Ucn3 which may be responsible for causing depression in Il18−/− mice. Ccnd1 and Nox4 at 6 weeks of age interacted with Fgfr1 and Ptpn1, indicating that

| Gene symbol | FC (RT-qPCR) | FC (Microarray) |
|-------------|--------------|-----------------|
| Aqp4 | 0.875 | 0.808 |
| Bdnf | 1.074 | 1.190 |
| Btg1 | 0.702 | 0.651 |
| Btg2 | 1.255 | 1.470 |
| Cd46 | 1.249 | 1.312 |
| Chga | 0.631 | 0.980 |
| Cplxl | 0.714 | 1.029 |
| Crh | 0.817 | 1.290 |
| Drp2 | 0.931 | 1.311 |
| Eif6 | 0.834 | 1.143 |
| Fgr1 | 0.743 | 0.963 |
| Hgs | 1.019 | 1.145 |
| Htr2c | 0.768 | 0.684 |
| Kif5b | 0.875 | 0.636 |
| Ptpn1 | 1.059 | 1.237 |
| Ucn3 | 0.758 | 1.280 |

Aqp4, aquaporin 4; Bdnf, brain derived neurotrophic factor; Btg1, BTG anti-proliferation factor; Chga, chromogranin A; Cplxl, complexin 1; Crh, corticotropin releasing hormone; Drp2, Dystrophin Related Protein 2; Eif6, eukaryotic translation initiation factor 6; Fgr1, fibroblast growth factor receptor 1; Hgs, hepatocyte growth factor-regulated tyrosine kinase substrate; Htr2c, 5-hydroxytryptamine receptor 2C; Kif5b, kinesin family member 5B; Ptpn1, protein tyrosine phosphatase, non-receptor type 1; Ucn3, urocortin 3; RT-qPCR, reverse transcription quantitative polymerase chain reaction; FC (RT-qPCR), fold change based on the results obtained from RT-qPCR; FC (microarray), fold change based on the results obtained from microarray analysis.
Ccd1 and Nox4 may be causative genes and the microarray and RT-qPCR results were significantly correlated.

In our previous study, impaired learning and memory, and depressive-like behavioral changes in Il18−/− mice at 12 weeks of age were observed (12). To identify the genes responsible for IL18-associated depression, microarray analysis of gene expression in brains from Il18+/+ and Il18−/− mice and IPA core analysis were performed using previously described procedures (17). In the core analysis results, the function and annotation of ‘Major depression’ and ‘Depressive disorder’ were generated automatically. From the microarray results, 13 genes at 12 weeks of age were associated with “depression”. Among these 13 genes, three, Fgfr1, Ptpn1, and Ucn3, have previously been causally associated with “Major depression” and “Depressive disorder” (19,21). Fgfr1 has been implicated in the development of the brain and self-renewal of neural precursor cells (24,25). In Il18−/− mice, neurogenesis is decreased (12); therefore, the increased expression of Fgfr1 may be a compensatory response to the decrease in neurons during a depressive state. Ptpn1 serves a role in negatively regulating insulin signaling. Ptpn1 deficiency increases insulin sensitivity and obesity resistance (26).

In the present study, in the Il18−/− mice, the expression of Ptpn1 was increased, and it has previously been shown that IL18−/− mice exhibit insulin resistance and obesity (8). Ucn3 is associated with stress-induced anxiety and depression, and with energy homeostasis. Expression of Ucn3 in the rostral perifornical area of the brain regulates not only anxiety-like behaviors but also glucose metabolism of the body (27,28). The expression of Ucn3 in Il18−/− mice at 12 weeks of age was increased; thus, a deficiency in IL-18 might increase the expression of Ptpn1 and Ucn3, resulting in not only depressive-like behavioral changes but also in an energy imbalance. Additionally, in clinical studies on patients with MDD, the mRNA expression levels of Fgfr1 and Ccd1, and RT-qPCR results were significantly correlated. The localization of expression of these genes were higher compared with the Il18+/+ mice based on the microarray results. The localization of expression of these genes were determined using the BrainStairs database (brainstairs.org) (29). Fgfr1 expression is upregulated in the pontine nucleus and hippocampus, Ptpn1 is expressed in the anterior olfactory bulb and hippocampus, and Ucn3 is expressed in the paraventricular hypothalamic nucleus and amygdala. Together, these results suggest that these three genes are expressed in the brain and are involved in depression.

In contrast to these three genes, the other 10 genes identified may have the opposite effect on depression, according to previous findings (19,20,30). For example, upregulated Aqp4 expression in the prefrontal cortex was associated with MDD in patients. However, the expression of Aqp4 in Il18−/− mice was decreased, and the other nine genes also exhibited similar changes. Therefore, these results suggest the existence of a negative feedback process in MDD.

To analyze the molecular regulation of the three genes at 12 weeks of age (Fgfr1, Ptpn1, and Ucn3), interaction pathway analysis was performed, and based on the results, Ccd1 and Nox4 were extracted. Inhibition of Ccd1 expression increases Fgfr1 mRNA levels in humans (31) and reduced expression of Nox4-activated PTPN1 protein expression in humans (23). These findings suggest that Ccd1 and Nox4 may regulate expression of Fgfr1 and Ptpn1, and thus may be responsible for inducing a depressive state in IL-18 deficiency.

In our previous study, it was shown that decreased expression of Ccd1 in the liver of Il18−/− mice at 6 and 12 weeks of age may be causally associated with energy imbalance through the Wnt signaling pathway (9). In the present study, a similar change was observed in Il18−/− mice at 6 weeks of age. Wnt signaling is indispensable for hippocampal memory function (32) and is also associated with depressive symptoms in an animal model of depression (33). Accordingly, Ccd1 may regulate energy levels and depression in Il18−/− mice.

Another gene extracted at 6 weeks of age, Adora2a, also affected Kif5b expression at 12 weeks of age. The expression of Adora2a was increased and Kif5b was decreased, and Adora2a inhibition may have decreased Kif5b expression in the present study, which differs from the results of a previous study (34). Neuroinflammation is significantly associated with MDD. In particular, inflammatory cytokines, such as TNF-α, have been identified as mediators of MDD by impairing the function of the blood brain barrier (35-37).

### Figure 3. Correlation plots between the results of microarray analysis and RT-qPCR at (A) 6 (P=0.014, p=0.60) and (B) 12 weeks of age (P=0.007, p=0.64). RT-qPCR, reverse transcription-quantitative PCR.
in cerebral endothelial cells may induce blood-brain barrier leakage resulting in the impairment of hippocampal memory function (38). Therefore, these findings indicate that increased expression of Adora2a at 6 weeks of age may be responsible for inducing the behavioral phenotypes of IL18-/- mice through neuroinflammation.

To assess the correlation between the microarray and RT-qPCR results, Spearman's rank correlation coefficient analysis was performed, and a significant correlation was detected between the results.

A limitation of the present study was that the molecular analysis was performed using whole brains; therefore, genes affected by IL-18 only in select areas may not have been detected, and further molecular studies using specific parts of the brain are thus warranted. In addition, a more notable limitation is that the present study did not analyze protein expression levels encoded for by the identified genes. The identified genes may be associated with depression; however the protein expression and protein localization in the brain are required to determine interactions between IL-18 and the proteins encoded for by the extracted genes. Additionally, further studies using in vitro and in vivo models are also required.

In conclusion, the gene expression profile of IL18-/- mice brain was examined and an association between the expression of certain genes and a depressive phenotype in mice was identified. Fgfr1, Ptpn1 and Ucn3 may be associated with depression, and may be regulated by Ccnd1 and Nox4. Furthermore, Adora2a and Ccnd1 may contribute to depression when expression of IL-18 is downregulated.

In our previous study, it was hypothesized that IL-18 may serve as a novel option for treatment of metabolic disorders, such as diabetes mellitus and dyslipidemia, and for neuropsychiatric disorders, such as MDD (9,11,12). The results of such studies indicate that both innate and acquired immunity, and IFN-gamma production. Nature 386: 619-623, 1997.

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Availability of data and materials

The datasets shown and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

KY and HM designed the study. TH, MM, KM, KI, NU, TI, and DO performed experiments. YW, HO, and HY analyzed the data. KY and HM wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Care Committee of Hyogo College of Medicine (Hyogo, Japan; approval nos. #28041, #13-062, #14-020, and #16-013) prior to beginning animal experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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