Effects of RORγt overexpression on the murine central nervous system

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Research note

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Abstract

Objective T-helper 17 (Th17) cells are a subset of CD4+ T cells that produce interleukin (IL)-17A. Recent studies showed that an increase in circulating IL-17A causes cognitive dysfunction, although it is unknown how increased systemic IL-17A affects brain function. Using transgenic mice overexpressing RORγt, a transcription factor essential for differentiation of Th17 cells (RORγt Tg mice), we examined changes in the brain caused by chronically increased IL-17A resulting from excessive activation of Th17 cells.

Results RORγt Tg mice exhibited elevated Rorc and IL-17A mRNA expression in the colon, as well as a chronic increase in circulating IL-17A. We found that the immunoreactivity of Iba1 and density of microglia were lower in the dentate gyrus of RORγt Tg mice compared with wild-type mice. However, GFAP+ astrocytes were unchanged in the hippocampi of RORγt Tg mice. Levels of synaptic proteins were not significantly different between RORγt Tg and wild-type mouse brains. In addition, novel object location test results indicated no difference in preference between these mice. Our findings indicate that a continuous increase of IL-17A in response to RORγt overexpression resulted in decreased microglia activity in the dentate gyrus, but had only a subtle effect on murine hippocampal functions.

Introduction

CD4+ helper T (Th) cells play an important role in immune responses by helping B cells and orchestrating CD8+ T cells and macrophages against pathogens. Functionally distinct subsets of Th cells produce different cytokines. One such subset is Th17 cells, whose signature cytokine is interleukin (IL)-17. IL-17 promotes inflammation by stimulating recruitment of neutrophils and monocytes [1], which are involved in autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and psoriasis [1–3]. Tumor growth factor β (TGF-β) and IL-6 are reportedly required for Th17 cell differentiation from naive T cells [4]. These mediators act in concert to activate the orphan nuclear receptor RORγt, a transcription factor indispensable for Th17 cell differentiation [5–7]. Th17 cells and IL-17A play a key role in neurological disorders such as multiple sclerosis, Alzheimer’s disease, and schizophrenia through auto-inflammatory mechanisms [2]. In addition, IL-17 enhances microglial function [8] and activates astrocytes following spinal cord injury [9]. Recent research in mice indicates that maternal IL-17A promotes autism-related phenotypes in offspring by binding to IL-17 receptors on fetal cortical neurons [10, 11], and an increase in circulating IL-17 leads to cognitive dysfunction by reducing nitric oxide production [12]. To investigate the effect of IL-17 on neuronal and glial cells in the brain, we made use of mutant mice overexpressing RORγt (RORγt Tg mice) [13, 14]. As expected, these mice exhibited increased serum IL-17A levels compared with wild-type mice. Immunoreactivity of ionized calcium binding adaptor molecule 1 (Iba1) and the density of microglia were unexpectedly decreased in the hippocampal dentate gyrus (DG) of RORγt Tg mice. However, despite the chronic increase in serum IL-17A in RORγt Tg mice, we found no changes in astrocytes, neurogenesis, hippocampal expression of synaptic molecules, or defects in spatial memory.

Materials And Methods
Detailed methods are described in the supplementary information.

**Animals**

RORγt Tg mice were generated on a C57BL/6 background in which transgene expression was driven by the CD2 promoter [13, 14]. Throughout the study, we used male mice at 16 weeks of age (16W). All animals were housed under standard laboratory conditions (12/12 h light/dark cycle, free access to food and water) and maintained under pathogen-free conditions in the Laboratory Animal Resource Center at the University of Tsukuba (Tsukuba, Japan).

**Real-time PCR**

Real-time PCR was performed as previously described [14], using primer pairs for Rorc and IL17A (Supplementary Table 1, Fig. 1A). Relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method using HPRT as an internal control.

**Enzyme-linked immunosorbent assay (ELISA)**

Blood samples (> 0.5 mL) were obtained from the hearts of male mice. Serum concentrations of IL-6 and IL-17A were measured using an ELISA system (PM1700; R&D Systems, Minneapolis, MN, USA) (Fig. 1B).

**Immunohistochemistry**

Mice at 16W were deeply anesthetized with sodium pentobarbital (60 mg/kg body weight, intraperitoneal injection; Somnopentyl; BCM International, Hillsborough, NJ). Brain tissue sections (40 µm) were prepared with a sliding microtome and stained using the following primary antibodies: Iba1 (1:2000, 019-19741; Fujifilm-Wako Pure Chemical, Tokyo, Japan), glial fibrillary acidic protein (GFAP; 1:1000, Rb-Af800; Frontier Institute, Hokkaido, Japan), and doublecortin (DCX; 1:2000, AB2253; Millipore, Burlington, MA, USA). Fluorescence intensities of Iba1$^+$ and GFAP$^+$ cells (Fig. 2B and 2E) were quantified with a BZX-710 microscope and its integrated software (BZX-710; Keyence, Osaka, Japan). Densities of Iba1$^+$, GFAP$^+$, and DCX$^+$ cells (Figs. 2C, 2F, and 2H) were counted using Photoshop CS5 (Adobe, San Jose, CA, USA).

**Western blotting**

Anti-NR2B (1:500, A-6474; Thermo Fisher Scientific, Waltham, MA, USA), NR2A (1:500, A-6476, Thermo Fisher Scientific), postsynaptic density protein 95 (PSD-95; 1:2000, ab13552, Abcam, Cambridge, UK), and PSD-93 (1:1000, AB_2571834, Frontier Institute) were used as primary antibodies. For normalization of proteins in each sample, α-tubulin (1:1000, T9026; Sigma-Aldrich, St. Louis, MO, USA) was used as a control (Fig. 2I). Protein bands were visualized using a LI-COR C-Digit Blot Scanner with Image Studio software (LI-COR, Lincoln, NE, USA).

**Behavioral analysis**
Novel object location testing was performed as previously described with slight modifications [15]. Two cylinder blocks were used as objects (Fig. 3A). Quantitative analysis of the retrieved data was performed using EthoVision XT (Noldus, Wageningen, Netherlands).

**Statistical analysis**

Differences between the two groups were analyzed with Student’s t-test. Probability values < 0.05 were considered significant. All data are expressed as mean ± standard error of the mean.

**Results**

**Elevated levels of Rorc and IL17A mRNA and serum IL-17A in RORγt Tg mice**

Th17 cells are constitutively present in lamina propria of the gut [16, 17], and differentiate from naive T cells upon stimulation with IL-6 and TGF-β [18]. mRNA levels of Rorc (the gene encoding RORγt) and IL17A were determined by qPCR and compared between RORγt Tg and wild-type (WT) mice (Fig. 1A). In the colon, levels of both Rorc and IL17A mRNA were significantly higher in RORγt Tg mice compared with WT mice (Fig. 1A, n = 4, p < 0.05). Consistently, IL-17A concentrations were significantly higher in RORγt Tg mice compared with WT mice (Fig. 1B, 2.72 ± 1.11 vs. 9.54 ± 1.77 pg/mL, respectively; p < 0.05); however, levels of IL-6 were unaltered (Fig. 1B, 3.91 ± 0.11 vs. 4.63 ± 0.49 pg/mL, respectively). These results indicate significantly elevated serum levels of IL-17A in RORγt Tg mice, which occurred concomitantly with upregulation of Rorc and IL-17A mRNA in the bowel.

**Microglia and astrocytes in the hippocampus of RORγt Tg mice**

IL-17A binds to a receptor complex of IL-17 receptor subunit A (IL-17RA) and IL-17RC [19]. In the mouse brain, IL-17RA is expressed in microglia [20, 21]. To investigate potential cellular alterations induced by overexpression of RORγt in the mouse brain, we visualized microglia and astrocytes using immunohistochemistry in RORγt Tg mice (Fig. 2A and 2D).

Fluorescence intensity of Iba1, a marker of microglia, was significantly decreased in the DG of RORγt Tg mice compared with WT mice (Fig. 2B, 16571.93 ± 1150.53 vs. 25387.14 ± 1706.29, respectively; p < 0.05). However, no significant difference in fluorescence intensities was observed in the cornu ammonis 1 (CA1) region of the hippocampus between RORγt Tg and WT mice (Fig. 2B, 15245.00 ± 1302.7 vs. 17636.00 ± 817.87, respectively; p = 0.18). Accordingly, the density of Iba1+ cells was significantly decreased in the DG of RORγt Tg mice compared with WT mice (Fig. 2C, 4.71 ± 0.27 vs. 7.71 ± 0.36 /10^4 µm^2, respectively; p < 0.05), but not in the CA1 (6.11 ± 0.51 vs. 5.47 ± 0.52 /10^4 µm^2, respectively; p = 0.17), without any marked morphological changes. Thus, the activity and density of Iba1+ microglia was specifically reduced in the DG of RORγt Tg mouse brains.
Immunofluorescence intensity of GFAP, a marker of astrocyte activation, was unaltered in the hippocampal DG and CA1 of RORγt Tg mice compared with WT mice (Fig. 2E, DG: 49249.33 ± 202.19 vs. 48719.25 ± 4324.98, respectively; \( p = 0.93 \); CA1: 25387.14 ± 1706.29 vs. 16571.93 ± 1150.53, respectively; \( p = 0.17 \)). There was also no difference in the density of GFAP+ astrocytes between WT and RORγt Tg mice (Fig. 2F, DG: 11.56 ± 0.63 vs. 10.67 ± 0.57 /10^4 \mu m^2, respectively; \( p = 0.93 \); CA1: 6.11 ± 0.51 vs. 6.67 ± 0.29 /10^4/\mu m^2, respectively; \( p = 0.31 \)). No substantial changes were observed in the activity or density of GFAP+ astrocytes in these hippocampal regions of RORγ Tg mice despite constitutively high levels of serum IL-17A.

Activation of microglia causes a decrease in the number and activity of stem cells, thereby reducing neurogenesis in the DG [22, 23]. To evaluate the potential effects of altered glial-cell activity on neurogenesis in the brains of RORγ Tg mice, we performed immunohistochemistry with an antibody against DCX, a marker of immature neurons (Fig. 2G). The density of DCX+ neurons was almost the same in WT and RORγt Tg mice (Fig. 2H, 23.88 ± 2.34 vs. 31.63 ± 4.79 /10^4 \mu m^2, respectively; \( p = 0.157 \)), indicating that reduced microglial activity does not affect neurogenesis in the DG of adult RORγ Tg mice.

**Hippocampal levels of synaptic molecules were maintained in RORγt Tg mice**

To examine potential changes in synaptic molecules in response to a constitutive increase of IL-17A in RORγt Tg mice, western blot analysis was performed using hippocampal tissues (Fig. 2I). Protein levels of N-methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B, which are important for synaptic plasticity [24], and postsynaptic density proteins PSD93 and PSD95 were compared between RORγt Tg and WT mice. There was no significant difference in protein expression of these four synaptic molecules between RORγt Tg and WT mice (NR2A: \( p = 0.130 \); NR2B: \( p = 0.211 \); PSD93: \( p = 0.101 \); and PSD95: \( p = 0.964 \)).

**Object location recognition testing revealed no difference in preference between WT and RORγt Tg mice**

To examine whether increased IL-17A and microglial alterations in RORγt Tg mice influenced hippocampus-dependent brain function, an object location recognition test was performed [15]. RORγt Tg mice exhibited preference to the displaced object to the same extent as WT mice (Fig. 3, \( p = 0.0006 \) and \( p = 0.020 \), respectively). These results suggest that RORγt Tg mice have normal hippocampus-dependent spatial memory function.

**Discussion**
In this study, to evaluate the effects of long-term upregulation of IL-17A on the CNS in vivo, we used transgenic mice overexpressing RORγt [13, 14]. Serum levels of IL-17A in RORγt Tg mice were found to be elevated to three or more times the expected level, indicating that differentiation of Th17 cells is accelerated in response to overexpression of Rorc (RORγt), and IL-17A was excessively produced. Importantly, Th17 cell-mediated inflammatory responses contribute to disruption of blood-brain barrier integrity, and can cause cognitive impairment ([25, 26]). Thus, we concentrated our analysis on the hippocampus of RORγt Tg mice.

Astrocytes are associated with the blood-brain barrier, causing them to confront infiltrating molecules into the CNS; thus, they may detect and be more sensitive to changes in IL-17 levels. Activation of astrocytes affects microglial function during neuroinflammation [27]. Contrary to our expectation, we did not observe any differences in the fluorescence intensity or density of GFAP+ astrocytes in RORγt Tg mice. However, the activity of Iba1+ microglia, which express IL-17RA [20, 21], was significantly downregulated in the DG of RORγt Tg mice. In this regard, our previous study demonstrated that continuous elevation of IL-17A weakens poly(I:C)-mediated inflammatory responsiveness during pregnancy [14]. Thus, it is possible that persistently high levels of IL-17A activate inhibitory immune system responses and suppress microglial activity. Multiple regulatory pathways inhibit IL-17, including Th1, Th2, and regulatory T (Treg) cells, and interleukins such as IL-4 and interferon γ [14]. Among these regulatory systems, the balance between Th17 and Treg cells is important for the regulation of Th17-related immune responses. We considered that a continuous excess of Th17 cells caused by RORγt overexpression might potentiate these inhibitory systems and cytokines other than IL-17, such as IL-10, could be responsible for the observed hypoactivity of microglia.

We found no alterations in neurogenesis or levels of synaptic proteins in RORγt Tg mice. As inflammation may cause cognitive dysfunction, we expected to observe changes in expression of synaptic molecules between RORγt Tg and WT mice. These results suggest that the level of inflammation induced by elevation of IL-17A was not enough to cause robust changes in synaptic structure or function in the hippocampus of RORγt Tg mice.

In conclusion, our data indicate that chronically increased levels of IL-17A elicited by overexpression of RORγt resulted in reduced microglial activity in the DG, with subtle effects on murine hippocampal functions. Considering that changing genetic background of the RORγt Tg mice from C57BL/6 to mixed C57BL/6 and BALB/c resulted in polyclonal plasmacytosis due to over production of IL-17 and IL-6 [13], it is possible that phenotypes in central nervous system are also dependent upon genetic background. Future work is necessary to elucidate the details of regulatory immune systems responsible for the brain phenotypes of microglia in RORγt Tg mice.

Limitations
In the present study, we used early aged mice of 16W. Therefore, the factor of aging may influence the state of chronic inflammation. By using additional age groups, it may be able to more clearly delineate the effect of chronic inflammation due to excess IL-17A on the nervous system. In addition, a more complicated test set, such as the Morris water maze, could be used to further examine behavior. Because this test takes several days to perform, differences between groups can be visualized in more detail.

**Abbreviations**

CA: cornu ammonis; CD: cluster of differentiation; cDNA: complementary deoxyribonucleic acid; DCX: doublecortin, DG: dentate gyrus; GFAP: glial fibrillary acidic protein; HRP: horseradish peroxidase; IL: interleukin; NMDA: N-methyl-D-aspartate; NR: N-methyl-D-aspartate receptor; PBS: phosphate buffered saline; PCR: polymerase chain reaction; PSD: postsynaptic density; RNA: ribonucleic acid; ROR: retinoid acid-related orphan receptors; TBST: Tris-buffered saline containing Tween-20; TGF-β: transforming growth factor-β; Th: T helper cells; Treg: regulatory T cells; W: weeks of age.

**Declarations**

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**Availability of data and materials**
The datasets, which were used and/or analyzed in the current study, are available from the corresponding author on reasonable request.

**Author contributions**

RN and TS performed the experiments. ST provided the mutant mice. TS and YT designed the study and wrote the initial draft of the manuscript. All authors approved the final version of the manuscript.

**Ethics approval and consent to participate**

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba, and National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978). The study was approved by the Institutional Review Board. All efforts were made to minimize animal suffering and the number of animals used in experiments.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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Figures
Fig. 1

A

\[
\begin{align*}
\text{Rorc} & \quad * \\
\text{IL17A} & \quad *
\end{align*}
\]

mRNA Expression Level (relative to WT)

|       | WT | Tg |
|-------|----|----|
| Rorc  | 1  | 2  |
| IL17A | 0  | 20 |

B

\[
\begin{align*}
\text{IL-6} & \quad \text{IL-17A} \\
\text{Serum Concentration (pg/ml)} & \quad (pg/ml)
\end{align*}
\]

|       | WT | Tg |
|-------|----|----|
| IL-6  | 4  | 4  |
| IL-17A| 0  | 20 |
(A) mRNA expression levels of Rorc (left) and IL17A (right) in colon (n = 4 per group, Student’s t-test, *p < 0.05). The Rorc gene encodes RORγt. Rorc and IL17A mRNA expression levels were significantly higher in the colon of RORγt Tg mice compared with WT mice. (B) Concentrations of IL-6 (left) and IL-17A (right) in serum (n = 4 per group, *p < 0.05). Serum IL-17A concentrations were significantly higher in RORγt Tg compared with WT mice.
Fig. 2

A

|   | DG | CA1 |
|---|----|-----|
| WT | ![image] | ![image] |
| Tg | ![image] | ![image] |

B

- DG
- CA1

|   | Immuno| fluorescence | area (µm²) |
|---|-------|-------------|------------|
| WT | ![image] | ![image] |
| Tg | ![image] | ![image] |

C

|   | Microglia density (1×10^6/µm²) |
|---|-------------------------------|
| WT | ![image] | ![image] |
| Tg | ![image] | ![image] |

E

- DG
- CA1

|   | Immuno| fluorescence | area (µm²) |
|---|-------|-------------|------------|
| WT | ![image] | ![image] |
| Tg | ![image] | ![image] |

F

|   | Astrocite density (1×10^6/µm²) |
|---|-------------------------------|
| WT | ![image] | ![image] |
| Tg | ![image] | ![image] |

G

|   | DCX | DAPI | Merge |
|---|-----|------|-------|
| WT | ![image] | ![image] | ![image] |
| Tg | ![image] | ![image] | ![image] |

I

- NR2A
- NR2B
- PSD93
- PSD95
- α-tubulin

|   | WT | Tg |
|---|----|----|
| NR2A | ![image] | ![image] |
| NR2B | ![image] | ![image] |
| PSD93 | ![image] | ![image] |
| PSD95 | ![image] | ![image] |
| α-tubulin | ![image] | ![image] |

J

|   | WT | Tg |
|---|----|----|
| Expression levels | ![image] | ![image] |
Figure 2

(A) Representative images of immunohistochemistry of Iba1 in the DG (left) and CA1 (right) regions of WT (upper panel) and RORγt Tg (lower panel) mice. (B) Semi-quantitative results of Iba1 immunofluorescence in DG (left) and CA1 (right) of WT and RORγt Tg mice (n = 3 per group, *p < 0.05). (C) Density of Iba1+ microglia in the DG (left) and CA1 (right) of WT and RORγt Tg mice (n = 3 per group, *p < 0.05). Microglial activity and density were significantly lower in the DG of RORγt Tg compared with WT mice. (D) Representative images of immunohistochemistry for GFAP in DG (left) and CA1 (right) regions of WT (upper panel) and RORγt Tg (lower panel) mice. (E) Semi-quantitative results of GFAP immunofluorescence in the DG (left) and CA1 (right) of WT and RORγt Tg mice (n = 3 per group). (F) Density of GFAP+ astrocytes in the DG (left) and CA1 (right) of WT and RORγt Tg mice (n = 3 per group). There were no significant differences in astrocyte activity or density between RORγt Tg and WT mice. (G) Representative images of immunohistochemistry of DCX (green) and DAPI (blue) in DG regions of WT (upper panel) and RORγt Tg (lower panel) mice. (B) Density of DCX+ immature neurons in the DG of WT and RORγt Tg mice (n = 4 per group). (I) Western blot of synaptic molecules (NR2A, NR2B, PSD-93, and PSD-95) in the hippocampi of WT (left) and RORγt Tg (right) mice. For normalization of proteins in each sample, α-tubulin was used as a control (the lowest band). Each lane corresponds to a sample derived from one mouse (n = 3 per group). (B) Comparison of relative expression levels of NR2A, NR2B, PSD-93, and PSD-95 between WT and RORγt Tg mice (n = 3 per group). Scale bars = 50 µm.
Fig. 3

A

Training

Displaced object

Interval

Test

B

Novel location recognition test

WT

\[ p = 0.020 \]

Tg

\[ p = 0.006 \]
Figure 3

(A) Schematic of novel location recognition test. On Day 1, a single mouse was placed in the arena. Left and right blocks (green) were placed collinear to each other. On Day 2, the task was repeated with the right block placed at a different location (dotted arrow). (B) Percentages of preference for the displaced object. Both WT and RORγt Tg mice showed a preference for the object moved to a novel location. The level of preference was not different between WT and RORγt Tg mice.

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