Postnatal changes of interleukin-18 receptor immunoreactivity in neurons of the retrosplenial cortex in wild-type and interleukin-18 knock out mice

By

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Summary: Interleukin-18 (IL-18), which is involved in the inflammatory response, is also found in the cerebral cortex. IL-18 receptor-immunoreactive (IL-18R-ir) neurons are present in layer V of the retrosplenial cortex (RSC). In the adult IL-18 knock out (KO) mice, no IL-18R-ir neurons but many degenerated neurons are present in layer V of the RSC, suggesting that any changes in the neurons of layer V have occurred during postnatal development. We examined changes of IL-18R expression during postnatal development. In the wild-type mice, many IL-18R-ir neurons were present in layers II, III and VI of the RSC in 2-week-old mice, whereas they were sparsely observed in only layer III in 3-week-old mice. No IL-18R-ir neurons were present in 4- and 5-week-old mice. In older than 6-week-old mice, many IL-18R-ir neurons were present in layers V and VI. The IL-18KO mice showed IL-18R-ir neurons in layers II, III and VI at 2-weeks-old, and a few in layer III at 3-week-old mice, similar to that in the wild-type mice. No IL-18R-ir neurons were found in mice older than 4 weeks of age. Thus, IL-18 or IL-18R seem to be involved in the construction of neural circuits corresponding to events after 3-weeks of age.

Introduction

Interleukin 18 (IL-18) was originally isolated as an interferon-gamma inducing factor from Kupffer cells of mice (Okamura et al., 1995). IL-18 is thought to be a proinflammatory cytokine in T lymphocytes and play proapoptotic, proatherogenic roles in several diseases (Dinarello and Fantuzzi, 2003; Reddy, 2004; Dinarello, 2006). IL-18 has also been found in nonimmune tissues, such as, the adrenal gland, the mammary gland, the pituitary gland, the renal tubule, the ependymal cells, the neurons in the medial habenula, Purkinje neurons in the cerebellum, and the glial cells of the dorsal root ganglion and the trigeminal spinal nucleus caudalis. (Conti et al., 2000; Takahata et al., 2001; Sugama et al., 2002; Nagai et al., 2005; Wang et al., 2006; Dinarello, 2007; Miyoshi et al., 2008; Daigo et al., 2012). IL-18 synthesis in the microglia is elevated by neuropathic pain (Miyoshi et al., 2008; Daigo et al., 2012). There are also neurons contain-

ing the IL-18 receptor (R) in the central nervous system, such as in the retrosplenial cortex (RSC), hippocampus, hypothalamus, septum, supraoptic nucleus and Purkinje neurons in the cerebellum (Sugama et al., 2002; Andoh et al., 2008; Alboni et al., 2009; Hayakawa et al., 2016; Kuwahara-Otani et al., 2017).

Behavioral studies of the IL-18 knock out (KO) mice have shown that rearing activity is significantly suppressed, retention latency is much shorter, and acquisition latency is significantly prolonged in the water maze test for IL-18KO mice as compared with those for the wild-type mice (Yaguchi et al., 2010; Too et al., 2014). Lesion studies of different parts of the RSC in rats have shown that differential degrees of an impairment for alternations in spatial memory and motor activity (Van Hoesen et al., 1991; Wyss and Van Groen, 1992; Vann et al., 2003; Vann and Aggleton, 2004; Sipos et al., 2007; Pothuizen et al., 2008). These results suggested that the degeneration of the IL18R-ir neurons in the RSC may cause behavioral

abnormalities for the IL-18KO mouse.

Our previous study (Hayakawa *et al.*, 2016) has revealed that the adult IL-18KO mice showed no IL-18R-immunoreactive (IL-18R-ir) neurons in the RSC, whereas many IL-18R-ir neurons are found specifically in layers V and VI of the adult wild-type mice. The IL-18R-ir neurons were not recognized in the RSC even in 5-week-old IL-18KO mice. Electron microscopic observation has shown that many small to medium-sized electron-dense neurons are present in layer V of the RSC in IL-18KO mice (Hayakawa *et al.*, 2016). Thus, the IL-18R-ir neurons are thought to degenerate in IL-18KO mice that are older than 5 weeks. These results suggested that IL-18R-ir neurons may change their immunoreactivity or degenerate in IL-18KO mice younger than 5 weeks old. However, it is not clear when the IL-18R-ir neurons stop expressing immunoreactivity and when the IL-18R-ir neurons degenerate or why the IL-18R-ir neurons disappear in the RSC. Furthermore, there have not been any studies investigating the change of immunoreactivity during postnatal development of IL-18R-ir neurons in the RSC of the wild-type and the IL-18KO mice.

In the present study, we investigated when IL-18R-ir neurons or presumed IL-18-containing neurons in the RSC, disappear in the IL-18KO mice. We also attempt to clarify if IL-18R immunoreactivity changes in neurons in the RSC during postnatal development from 2 weeks to 8 weeks old in wild type and IL-18KO mice. Because IL-18 and IL-18R are thought to be co-localize in the neurons of the brain (Andoh *et al.*, 2008; Alboni *et al.*, 2009; Kuwahara-Otani *et al.*, 2017), we used the IL-18R immunoreactivity as a marker for IL-18-containing neurons.

Materials and methods

A total of 45 wild-type mice (C57BL/6, male) obtained from Japan SLC and IL-18 KO (IL-18^{-/-}, male) mice from 2 to 8 weeks of ages were used. IL-18^{-/-} mice gifted by Prof. S. Akira (Osaka Univ.) were backcrossed with C57BL/6 mice; F8 mice were used (Takeda *et al.*, 1998). The number of mice used at each week of age is shown in Table 1. All procedures were approved by the Animal Care and Use Committee of Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To determine the distribution of IL-18R-ir neurons in the RSC, wild-type mice or IL-18KO mice at each week old of ages were anesthetized first with 2% isoflurane in

30% oxygen and 70% nitrous oxide in a chamber, which they breathed spontaneously. A tube was then attached to the nose to administer isoflurane, and the anesthetized mouse was placed in a supine position. The mouse was perfused first with 5 ml of saline and then with 30 ml of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The brain was immediately removed and placed in the same fixative for 1 day. Serial frontal sections were made at a thickness of 70 μ m using Microslicer™ (DosakaEM, Kyoto). The sections were incubated with a polyclonal goat anti-IL-18R alpha serum (R&D Systems, Minneapolis, MN; 1:200, AF856) in 0.1 M phosphate buffer at pH 7.4 containing 0.3% Triton X-100 for 1 day. The treated sections were incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA; 1:200) for 5 h, then incubated with Vectastain® ABC reagents (Vector Laboratories) for 1 day, and finally reacted with a solution of 0.1% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.01% H₂O₂ in 0.05 M phosphate buffer at pH 7.4 for 5 min to produce brown reaction products. Control sections of both wild type and IL-18KO mice were proceeded the same with the exception of the primary antibody incubation.

Results

In 2-week-old wild type mice, many IL-18R-ir neurons were distributed in the RSC (Fig. 1). The IL-18R-ir neurons were present throughout the rostrocaudal extent of the RSC. At the caudal end of the RSC, the IL-18R-ir neurons extended to the subiculum. Many IL-18R-ir neurons were found specifically in layers II and III, and fewer IL-18R-ir neurons were found in layer VI. The IL-18R-ir neurons in layer II and III were small and round in shape, having small granular DAB reaction products (Fig. 1D), and were distributed only in the granular RSC (Figs. 1A–C). The IL-18R-ir neurons in layer VI were oval or triangular in shape (Fig. 1E), and were distributed mainly in the granular RSC, though some labeled neurons extended to the agranular RSC (Fig. 1A–C). No IL-18R-ir fibers and terminals were found. In 3-week-old mice, the IL-18R-ir neurons had mostly disappeared, but a few IL-18R-ir neurons were found in layer III throughout the rostrocaudal extent of the RSC. At this age, the number of IL-18R-ir neurons was small and their immunoreactivity was low (Fig. 2A).

No IL-18R-ir neurons were present in 4- and 5-week-old mice in any layers of the RSC (Fig. 2B). In 6-week-

Table 1. The number of mice used at each week of age

	2 weeks	3 weeks	4 weeks	5 weeks	6 weeks	7 weeks	8 weeks
Wild type	3	3	3	3	3	2	6
IL-18 KO	3	3	3	3	2	2	3

Fig. 1. Photomicrographs of immunohistochemistry for interleukin-18 receptor (IL-18R) in the RSC of 2-week-old wild type mice. Many IL-18R immunoreactive neurons are present in layer II, III and VI. (A) Rostral portion of the RSC. Double-arrow indicates the border between the granular and agranular cortex of the RSC. (B) Mid-portion of the RSC at the level of the habenula. (C) Caudal portion of the RSC. (D) High power photomicrograph of the IL-18R-immunoreactive neurons in layers II and III of the RSC. (E) High power photomicrograph of the IL-18R-immunoreactive neurons in layer VI of the RSC. CC, corpus callosum. Scale bars = 100 µm in A, B, C and 20 µm in D and E.

old mice, many IL-18R-ir neurons were present in layers V and VI. The IL-18R-ir neurons in layer V were distributed mostly in the outer part of layer V and the granular RSC (Fig. 2C). The IL-18R-ir neurons in layer VI were distributed mainly in the granular RSC, but some extended to the agranular RSC. In 7- and 8-week-old mice, many IL-18R-ir neurons were found in layers V and VI of the RSC (Fig. 2D). The IL-18R-ir neurons were round or oval in shape, and were medium-sized neurons containing granular DAB reaction products. They were distributed specifically in the outer part of layer V and the granular RSC. The number of IL-18R-ir neurons was larger and their immunoreactivity was higher than those of 6-week-old mice (Fig. 2C and D). Many IL-18R-ir neurons were also found in layer VI. They were distributed mostly in the granular RSC but some IL-18R-ir neurons extended to the agranular RSC, similar to those of 6-week-old mice. No IL-18R-ir fibers and terminals were found. IL-18R-ir neurons were also found in the subiculum, the medial septum and the diagonal band of Broca in all ages of the wild-type mice.

In 2-week-old IL-18KO mice, many IL-18R-ir neurons were found in the RSC (Fig. 3A), specifically in layers II and III, and were distributed in the granular RSC throughout the rostrocaudal extent. Many IL-18R-ir neurons were also found in layer VI, and were distributed mostly in the granular RSC, though some extended to the agranular RSC similar to the distribution in the wild type mice. At the caudal end, the labeled neurons extended to the subiculum (Fig. 3B). In 3-week-old mice, a few IL-18R-ir neurons were found in only layer III (Fig. 3C), and they were distributed throughout the rostrocaudal extent of the RSC. The number of IL-18R-ir neurons was small, and their immunoreactivity was low. IL-18R-ir neurons were found in the subiculum, the medial septum and the diagonal

Fig. 2. Immunohistochemistry for IL-18R in the RSC of wild-type mice at 3-week-old (A), 4-week-old (B), 6-week-old (C) and 7-week-old (D) wild type mice. (A) Some IL-18R immunoreactive neurons (arrows) were found in layer III. (B) No IL-18R immunoreactive neurons were found in the RSC. (C) Several IL-18R immunoreactive neurons (arrows) were found in layers V and VI. (D) Many IL-18R immunoreactive neurons (arrows) were found in the outer part of layers V and VI. Scale bars = 100 µm.

Fig. 3. Immunohistochemistry for IL-18R in the RSC of 2-week-old (A and B), 3-week-old (C), and 7-week-old (D) of the IL-18 knock out mice. (A) Many IL-18R immunoreactive neurons were present in layers II, III and VI at the rostral part of the RSC. (B) IL-18R-immunoreactive neurons in layer VI extended to the subiculum (SUB) at the caudal end of the RSC. (C) Some IL-18R immunoreactive neurons (arrows) were present in layer III. (D) No IL-18R-immunoreactive neurons were present in the RSC. CC, corpus callosum. Scale bars = 100 μ m in A, B and D, and 50 μ m in C.

band of Broca in the 2- and 3-week-old IL-18KO mice, similar to the distribution in the wild type mice. No IL-18R-ir neurons were found in the RSC in either 4-week-old or 5-week-old IL-18KO mice, similar to the finding in wild type mice. Furthermore, IL-18KO mice older than 6 weeks of age showed no IL-18R-ir neurons in the RSC (Fig. 3D). IL-18R-ir neurons were found in the medial septum and the diagonal band of Broca, but not in the subiculum, in IL-18KO mice older than 6 weeks of age. No IL-18R-ir neurons were found in the control sections for either the wild type or IL-18KO mice. Within the group of each week of age, the distribution patterns of IL-18R-ir neurons were almost same.

Discussion

The present results showed that many IL-18R-ir neurons were present specifically in layers II, III and VI at 2 weeks of age, whereas IL-18R-ir neurons disappeared from 4 to 5 weeks of age in the wild-type mice. Additionally, many IL-18R-ir neurons appeared in layers V and VI of the RSC in wild-type mice older than 6 weeks of age. Interestingly, many IL-18R-ir neurons were also identified specifically in layers II, III and VI in the IL-18KO mice. IL-18R-ir neurons were no longer present in the RSC of IL-18KO mice older than 4 weeks of age (Fig. 4).

Alboni *et al.* (2009) have reported the distribution of total IL-18R alpha mRNAs in adult wild-type mice determined by *in situ* hybridization, which were distributed in

Fig. 4. Schematic drawings of changes in the distribution of IL-18R immunoreactive neurons in the RSC during postnatal development in the wild type (A–D) and the IL-18 knock out (E–H) mice. Black circles indicate IL-18R immunoreactive neurons. CC, corpus callosum; RSA, agranular retrosplenial cortex; RSG, granular retrosplenial cortex.

neurons of the olfactory bulb, cerebral cortex including the RSC, hypothalamus, habenula, hippocampus, and the cerebellar cortex. The mRNAs for IL-18R α were present in all layers of the cortex, where type I IL-18R α was localized primarily in layers V and VI, and type II IL-18R α was mainly in layer V. Immunohistochemistry for IL-18R α revealed a similar distribution to that of the gene expressions determined by *in situ* hybridization. However, the authors did not describe the precise laminar distribution of IL-18R-ir neurons in the RSC. Our present results in adult wild-type mice agree with their reported distribution of IL-18R-ir neurons in the RSC, whereas the distribution in the 2-week old wild-type mice showed IL-18R-ir neurons specifically in layers II, III and VI but not in layer V.

During the early postnatal period, postmitotic precursor cells in the dorsomedial part of the neonatal ventricular zone migrate into the medial limbic cortex and the RSC during the first postnatal week. These neurons migrate and display morphological changes, and enter into layer II of the cortex until around 2 weeks of age. In the RSC, these cells give rise to dendritic bundles in layer I (Zraggen et al., 2012). Parvalbumin and calbindin D28k-immunoreactive neurons are distributed in all layers of the RSC (Luth et al., 1993; Salaj et al., 2015). Changes in parvalbumin and calbindin D28k immunoreactivities have been reported in the RSC of rats during postnatal development (Alcantara et al., 1993). Parvalbumin-immunoreactive neurons first appear in layer V and later in layers VI and IV, and then in II and III at postnatal days 8 or 9. Adult patterns are reached at the end of 3 weeks of age. Calbindin D28K-immunoreactive neurons are present at birth in all cortical layers except the molecular layer. Heavily labeled calbindin D28K-immunoreactive neurons decrease in number from postnatal days 11 to 15, and adult patterns are reached at the end of 3 weeks of age. These findings indicate that parvalbumin and calbindin D28k-immunoreactivities in RSC neurons change during postnatal development. As for IL-18R, the present study showed that immunoreactivity for IL-18R changes in RSC neurons of the wild-type and IL-18KO mice during postnatal development. Because the IL-18R-ir neurons in layers II and V were different in their size and shape, it is likely that they do not migrate together. Adult patterns of IL-18R-ir neurons may reach at the 6 weeks of age different from those of parvalbumin and calbindin D28k-immunoreactive neurons. These differences may cause that parvalbumin and calbindin D28k-immunoreactive neurons play a role for interneurons in the cerebral cortex (De Felipe 1997), but IL-18R-ir neurons may not play important role for neuronal connections or circuits because no axon terminals express IL-18R immunoreactivity in the RSC.

When the sacral nerve or the trigeminal nerve is injured, a striking increase in IL-18 and IL-18R expression is observed in the glial cells but not in the neurons in the

dorsal horn of the spinal cord or the trigeminal spinal nucleus caudalis (Miyoshi et al., 2008; Daigo et al., 2012). This led to the idea that injury of the trigeminal or sacral nerve induces IL-18 upregulation in glial cells in the sensory nuclei, activating sensory neurons for a possible role of IL-18 in neuropathic pain. These findings would then suggest that pain stimulation does not activate or influence directly to the neurons containing IL-18 or IL-18R. Whereas, IL-18R-ir neurons in layers II and III of RSC disappeared after 3 weeks of ages in both wild type and IL-18 KO mice. These results suggested that the construction of neural network may not relate appearance of IL-18R immunoreactivity in the RSC neurons.

Lesion studies targeting different part of the RSC, such as destruction by mechanical or pharmaceutical lesion, or alternation of projections to the hippocampus from the entorhinal cortex or the RSC in rats have shown a differential degree of an impairment for alterations in spatial memory performance and motor activity (Van Hoesen et al., 1991; Wyss and Van Groen, 1992; Wozniak et al., 1996; Vann et al., 2003; Vann and Aggleton, 2004; Sipos et al., 2007; Pothuizen et al., 2008). Behavioral studies have reported that the rearing activity is significantly suppressed, retention latency is much shorter, and the acquisition latency is significantly prolonged in the water maze test in IL-18KO mice compared with wild-type mice (Yaguchi et al., 2010; Too et al., 2014). These results suggest that behavioral abnormalities of IL-18KO mice may be caused by disappearance of IL-18R or degeneration of presumed IL-18 containing neurons in the RSC.

We have attempted to clarify when immunoreactivity for IL-18R disappears in layer V neurons in IL-18KO mice. Both the wild-type mice and the IL-18KO mice did not show IL-18R-ir neurons in layer V before 6 weeks of age, thus it is difficult to know when immunoreactivity for IL-18R disappeared or when the presumed IL-18R containing neurons degenerate in layer V of the IL-18KO mice. Since IL-18R-ir neurons appeared and disappeared in layers II and III from 2 to 3 weeks of age in both the wild-type and the IL-18KO mice, IL-18R immunoreactivity in layer V may develop around 3 weeks of age. The mammalian visual system is affected by altered visual experiences during a special developmental period known as the critical period from 2 to 3 weeks of ages (Wiesel and Hubel, 1963, 1965; Kawabata et al., 2003). Because mice open their eyes around 2 weeks of age and the RSC has reciprocal connections with the visual cortex (van Groen and Wyss, 1990, 1992, 2003; Shibata et al., 2004), indirect visual inputs may affect the IL-18R-containing neurons with or without IL-18 after 2 weeks of age. IL-18 has been detected in human epithelial cells of the lactating mammary gland by immunohistochemistry, human colostrum contains significantly higher levels of IL-18 compared with early milk and mature milk (Takahata et al., 2001). Further, the study showed a significantly correlation between levels of IL-18 in human milk and

the occurrence of preterm delivery and pregnancy complications of mothers. Since the weaning period of mice is around 2 to 3 weeks of age, these behavioral changes may affect to the RSC neurons, which are involved behavioral activities such as head direction, spatial memory, navigation, or motor activity (Chen *et al.*, 1994a, 1994b; Cooper and Mizumori, 1999; Harker and Whishaw, 2002). Thus, IL-18 or IL-18R seem to be involved in the construction of neural circuits corresponding to events after 3-weeks of ages. Further study will be needed to clarify what kind of effectors influence the immunoreactivity of IL-18R in the RSC neurons.

Conflict of interest

T. Hayakawa, M. Hata and H. Okamura disclose financial interests from Hirakata Ryoikuen related to this research to disclose.

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