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Research report

IL-1 receptor-antagonist (IL-1Ra) knockout mice show anxiety-like behavior by aging

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Abbreviations

ANOVA, analysis of variance; **DA**, dopamine; **DOPAC**, 3,4-dihydroxyphenylacetic acid; **EDTA**, ethylenediaminetetraacetic acid; **EPM**, elevated plus-maze; **GABA**, γ -amino-butyric acid; **GHB**, γ -hydroxybutyrate; **GR**, glucocorticoid receptor; **5-HIAA**, 5-hydroxyindole acetic acid; **HPA**, hypothalamic-pituitary-adrenal; **HPLC**, high-performance liquid chromatography; **5-HT**, 5-hydroxytryptamine; **HVA**, homovanillic acid; **IL-1**, interleukin-1; **IL-1Ra**, interleukin-1 receptor antagonist; **KO**, knockout; **MR**, mineralocorticoid receptor; **SDS**, sodium dodecylsulfate; **WT**, wild-type.

ABSTRACT

Interleukin 1 (IL-1) plays a critical role in stress responses, and its mRNA is induced in the brain by restraint stress. Previously, we reported that IL-1 receptor antagonist (IL-1Ra) knockout (KO) mice, which lacked IL-1Ra molecules that antagonize the IL-1 receptor, showed anti-depression-like behavior via adrenergic modulation at the age of 8 weeks. Here, we report that IL-1Ra KO mice display an anxiety-like phenotype that is induced spontaneously by aging in the elevated plus-maze (EPM) test. This anxiety-like phenotype was improved by the administration of diazepam. The expression of the anxiety-related molecule glucocorticoid receptor (GR) was significantly reduced in 20-week-old but not in 11-week-old IL-1Ra KO mice compared to wild-type (WT) littermates. The expression of the mineralocorticoid receptor (MR) was not altered between IL-1Ra KO mice and WT littermates at either 11 or 20 weeks old. Analysis of monoamine concentration in the hippocampus revealed that tryptophan, the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA), and the dopamine metabolite homovanillic acid (HVA) were significantly increased in 20-week-old IL-1Ra KO mice compared to littermate WT mice. These findings strongly suggest that the anxiety-like behavior observed in older mice was caused by the complicated alteration of monoamine metabolism and/or GR expression in the hippocampus.

Key words: anxiety-like phenotype, elevated plus-maze test, glucocorticoid receptor, hippocampus, IL-1 receptor antagonist

1. Introduction

Chronic severe stress causes psychological illnesses, such as anxiety and depression, but the mechanisms underlying their induction remain obscure. Interleukin (IL)-1, which is one of the most studied pro-inflammatory cytokines, is induced by stress and activates the hypothalamic-pituitary-adrenal (HPA) axis [1-4]. It has also been shown that IL-1 administration induces anxiety- [5, 6] and depression-like behaviors [7]. In addition, IL-1 receptor knockout (KO) mice show decreased anxiety-like behavior in the elevated plus-maze (EPM) test [8], suggesting that depression and anxiety are closely associated with IL-1 signaling.

The stress response is terminated by a negative feedback mechanism through the corticosteroid receptors in the brain [9]. Two subtypes of corticosteroid receptors exist in the brain: mineralocorticoid receptor (MR, type I) and glucocorticoid receptor (GR, type II) subtypes [10]. Both subtypes are expressed in the hippocampus [10, 11], and corticosterone action in the brain is mediated by either GRs or MRs [12]. Several lines of evidence suggest a potential role of GRs in anxiety modulation. For example, the stress induces a decrease of GR mRNA and protein expression in the hippocampus [13-15] and the anxiety-like phenotype was observed with the decreased GR expression [16]. These results suggest that changes in GR expressions affect anxiety-related behavior.

Anxiety is one of the psychiatric disorders observed in the general human population, which is caused by the chronic stressful event [17, 18]. But the molecular and cellular mechanisms that cause anxiety disorders are still unclear. Our previous study showed no difference between IL-1Ra KO mice and their wild-type (WT) littermates in the light/dark-box test [19]. In our present study, we examined anxiety-like behavior in IL-1Ra KO mice, especially in the EPM test at young (8 and 11 weeks) and old (20 weeks) ages. To clarify the relationship between IL-1 and anxiety, we analyzed monoamine concentrations, especially those of dopamine (DA), 5-hydroxytryptamine (5-HT, serotonin) and their metabolites in the hippocampus of mice at 4, 8, and 20 weeks old and compared them between the genotypes, because the involvement of the alteration of monoamine contents in the hippocampus in anxiety is not fully understood. Further, we analyzed GR and MR protein expressions in the hippocampus of young (11 weeks old) and old (20 weeks old) mice. Finally, we

discussed possible mechanisms by which aging affected brain functions in IL-1Ra KO mice.

2. Materials and methods

The methods of EPM test, drug administration, western blot, and HPLC analysis were described in Supplementary Materials and methods.

2.1. Animals

IL-1Ra KO mice were generated as described previously [20] and were backcrossed to C57BL/6J mice for more than 10 generations. The littermate WT mice were used as control. Mice were housed under specific pathogen-free (SPF) conditions at the Center for Experimental Medicine and Systems Biology, Institute of Medical Science at University of Tokyo (IMSUT) or National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP) in standard cages with wood chips under the condition between 24-26°C temperature and 45-55% humidity with a 12/12-h modified dark/light cycle (light on at 8:00-20:00). Food and water were available *ad libitum*. All of the experiments were carried out according to the IMSUT's and NCNP's Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Statistical analyses

One-way factorial analysis of variance (ANOVA) was used to analyze the diazepam effects on behaviors of IL-1Ra KO mice in the EPM test. Tukey's corrected pairwise comparisons were used as the *post-hoc* tests. In the comparison between genotypes, the data of the EPM test and of hippocampal GR and MR expression were analyzed by Student's *t*-tests. HPLC data were analyzed by Mann-Whitney's *U*-test. A *p* value < 0.05 was regarded as statistically significant. All the data are presented as the mean $\pm$ S.E.M. Analyses were conducted with SPSS ver. 21.0 (IBM Japan, Tokyo, Japan).

3. Results

3.1. *IL-1Ra KO mice showed anxiety-like behaviors at 20 weeks old*

The EPM test was performed to determine whether KO mice showed an anxiety-like phenotype. The anxiolytic phenotype was not observed at either 8 or 11 weeks old (Fig. 1A-D and Fig. 1E-H, respectively), although the number of total entry was tended to have decreased in 8 weeks old [$t = 1.89$, $df = 14$, $p = 0.079$; Fig.1D] and 11 weeks old [$t = 1.38$, $df = 14$, $p = 0.188$; Fig.1H], respectively. We found that all the four parameters in the EPM test, including the number of open-arm entries [$t = 3.36$, $df = 30$, $p = 0.002$; Fig.1I], the open-arm stay [$t = 3.21$, $df = 30$, $p = 0.004$; Fig. 1J], the total distance traveled [$t = 4.26$, $df = 30$, $p < 0.001$; Fig. 1K], and the number of total entries [$t = 3.13$, $df = 30$, $p = 0.004$; Fig. 1L], were significantly reduced in 20-week-old IL-1Ra KO mice compared with littermate WT mice.

3.2. *Anxiety-like behaviors in 20-week-old IL-1Ra KO mice were improved by diazepam*

Next, we examined whether the anxiety-like phenotype observed in 20-week-old IL-1Ra KO mice was improved by diazepam, a GABA_A-receptor agonist. In control experiments, vehicle treatment did not improve the impaired performance in the EPM test in IL-1Ra KO mice: % open-arm entry [$t = 3.34$, $df = 15$, $p = 0.004$; Fig. 2A], % open-arm stay [$t = 3.95$, $df = 15$, $p = 0.001$; Fig. 2B], total distance travelled [$t = 3.69$, $df = 15$, $p = 0.002$; Fig. 2C], and the number of total entries [$t = 4.41$, $df = 15$, $p = 0.001$; Fig. 2D]. One-way ANOVA analysis revealed that there was a significant diazepam effect in % open-arm entry [$F(2,22) = 6.37$, $p = 0.007$; Fig. 2A], % open-arm stay [$F(2,22) = 3.95$, $p = 0.034$; Fig. 2B], and the number of total entries [$F(2,22) = 5.71$, $p = 0.010$; Fig. 2D]. The *post-hoc* analysis revealed that diazepam was effective against the impairment in % open-arm entry [$p < 0.05$ (0.5 mg/kg), $p < 0.01$ (2 mg/kg); Fig. 2A], % open-arm stay [$p < 0.05$ (2 mg/kg); Fig. 2B], and the number of total entries [$p < 0.01$ (2 mg/kg); Fig. 2D]. Total distance, however, did not show significant improvement by diazepam [$F(2,22) = 3.40$, $p = 0.052$, $p = 0.85$ (0.5 mg/kg), $p = 0.055$ (2 mg/kg); Fig. 2C].

3.3. *GR but not MR protein expression was decreased in IL-1Ra KO mice at 20 weeks old*

Because corticosteroid receptors are closely related to anxiety, we measured

the GR and MR protein levels in the hippocampus. The western blotting image of GR and MR expression in the hippocampus of littermate WT and IL-1Ra KO mice at 11 or 20 weeks old was shown in Fig. 3A and 3D, respectively. We found that both GR and MR expression in the hippocampus was not different between young IL-1Ra KO mice and WT littermates at 11 weeks old [$t = 1.35$, $df = 4$, $p = 0.247$; Fig. 3B and $t = -0.927$, $df = 4$, $p = 0.406$; Fig. 3C, respectively]. On the other hand, significant reduction of GR protein expression was observed in the hippocampus of IL-1Ra KO mice at 20 weeks old [$t = 2.7$, $df = 5$, $p = 0.042$; Fig. 3E]. The MR expression level was not different between IL-1Ra KO and WT littermate mice [$t = -0.231$, $df = 5$, $p = 0.826$; Fig. 3F].

3.4. Tryptophan, the 5-HT metabolite 5-HIAA, and the dopamine metabolite HVA were increased in IL-1Ra KO mice by aging

We also analyzed monoamine and their metabolite levels in the hippocampus in 4-, 8- and 20-week-old KO and littermate WT mice. In young mice, there was no significant difference in monoamine and their metabolites between IL-1Ra KO and littermate WT mice at the age of 4 and 8 weeks. At 20 weeks old, we found that tryptophan ($p < 0.01$), the 5-HT metabolite 5-HIAA ($p < 0.01$), and the dopamine metabolite HVA ($p < 0.05$) were significantly increased in IL-1Ra KO mice compared to WT littermates (Table 1).

4. Discussion

In this report, we examined the behavior in the EPM test that is one of the test batteries for estimating anxiety state. Anxiety-like phenotypes were observed in IL-1Ra KO mice compared to littermate WT mice at 20 weeks old but not at either 8 or 11 weeks old and were improved by the administration of diazepam. GR expression but not MR expression in the hippocampus was significantly decreased in 20-week-old but not in 11-week-old IL-1Ra KO mice compared to littermate WT mice. By aging, tryptophan, 5-HIAA and HVA were significantly increased in the hippocampus of IL-1Ra KO mice compared to WT littermates.

In our previous study, the anxiety-like behavior was not detected in either 8- or 20-week-old IL-1Ra KO mice in the light/dark-box test [19]. Here, we found that 20-week-old IL-1Ra KO mice showed an anxiety-like phenotype (Fig. 1I-L) although either 8- or 11-week-old KO mice did not (Fig. 1A-D and Fig. 1E-H, respectively) in the EPM test, suggested that the EPM test might be more sensitive than the light/dark-box test. Further, this phenotype was improved by diazepam that is clinically used for patients with anxiety (Fig. 2), although the decrease of total distance in IL-1Ra KO mice was not improved by diazepam (Fig. 2C). Lines of evidence suggested that diazepam did not affect to the total distance traveled in the EPM test, although the distance traveled in the open arms was increased by diazepam [21]. In addition, the administration of diazepam decreased locomotor activity in the activity cage [22]. Considered to these observations, it might be difficult to show the significant improvement by diazepam in total distance in the EPM test. We think that the IL-1Ra KO mouse at 20 weeks old would be a good animal model for anxiety.

We also examined biochemical changes in the hippocampus to elucidate the molecular mechanism for the induction of anxiety. The hippocampus is closely related to anxiety and depression [23] and is easily affected by stress. In our study, the expression of GRs (Fig. 3C), but not MRs (Fig. 3D), in the hippocampus of IL-1Ra KO mice was decreased at 20 weeks old, although there was no alteration in the expression of either GRs (Fig. 3A) or MRs (Fig. 3B) in young mice (11 weeks old). Toritos et al. have reported that stress-induced decrease of GR mRNA expression was inhibited by diazepam [24]. It would be interesting to examine whether GR protein expression in the hippocampus of IL-1Ra KO mice is also recovered by diazepam.

Further studies would be needed to solve these problems in the future.

The involvement of monoamine in anxiety has not yet become clear. In our study, a piece of evidence was obtained that tryptophan concentration was spontaneously increased by aging in the hippocampus of IL-1Ra KO mice as well as the 5-HT metabolite 5-HIAA and the dopamine metabolite HVA (Table 1). Gobaille et al. have reported that the increase of tryptophan availability and facilitation of 5-HT turnover are induced by administration of a high dose of γ -hydroxybutyrate (GHB) in mice [25]. GHB is synthesized [26] and released in the brain [27] and the receptor for GHB exists particularly in the hippocampus, cortex and striatum [28]. Although a low dose of GHB exhibits anxiolytic action in the EPM test [29], there is no evidence whether a high dose of GHB affects the state of anxiety and how the variation of the concentration of endogenous GHB in the brain regulates anxiety behavior. It is interesting to test whether GHB affects the behavior of IL-1Ra KO mice.

There is an interesting report that when the lymphocytes from chronically social defeat-stressed mice that confer anti-depression-like phenotype were transferred to non-stressed Rag2 KO mice, which lack mature lymphocytes, the recipient Rag2 KO mice showed anti-depression-like phenotype [30]. We guess that the activation of peripheral immune system might induce the anti-depression-like behavior in 8-week-old IL-1Ra KO mice [19]. In addition, Wohleb et al. reported that an anxiety-like phenotype was induced by the repeated social defeat stress-induced recruitment of the CD11b⁺CD45^{high}Ly6C^{high} macrophages to the brain or the activation of macrophages/microglia in the brain [31] and β -adrenergic receptor blockade prevented an anxiety-like phenotype [32]. In consideration of all the above information, we hypothesize that the anxiety-like phenotype begins with the activation of adrenergic signaling, followed by the accumulation of peripheral macrophages in the brain or the activation of microglia/macrophages in the brain during aging. Further studies are required to test this hypothesis.

5. Conclusion

In conclusion, the IL-1Ra KO mice show anxiety-like phenotypes spontaneously by aging that resemble anxiety in humans in that diazepam is effective in both cases. The abnormal tryptophan accumulation and the increase of HVA and

5-HIAA in the hippocampus may become a novel biomarker of the state of anxiety.

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Figure legends

Fig. 1. Age-dependent anxiety-like phenotypes in IL-1 receptor antagonist (IL-1Ra) KO mice. Results in the EPM test in 8- (A-D), 11- (E-H), and 20-week-old (I-L) IL-1Ra KO and littermate WT mice. (A, E, I) The % ratio of the number of the open-arm entries to that of the total entries. (B, F, J) The % ratio of the time of the open-arm stay to the total observation time. (C, G, K) The total distance traveled. (D, H, L) The number of total entries. 8 and 11 weeks old: WT, $n = 9$; KO, $n = 7$. 20 weeks old: WT, $n = 13$; KO, $n = 19$. $*p < 0.01$, $**p < 0.001$ (Student's t -test). Data represent mean $\pm$ S.E.M.

Fig. 2. Effects of diazepam on anxiety-like behavior in 20-week-old IL-1 receptor antagonist (IL-1Ra) KO mice and WT littermates in the elevated plus-maze test. (A) The % ratio of the number of the open-arm entries to that of the total entries. (B) The % ratio of the time of the open-arm stay to the total observation time. (C) The total distance traveled. (D) The number of total entries. $*p < 0.01$ vs. WT vehicle group (Student's t -test). $\#p < 0.05$, $\##p < 0.01$ vs. IL-1Ra KO vehicle group (one-way ANOVA followed by *post hoc* Tukey's test). v: vehicle (WT, $n = 9$; KO, $n = 8$); 0.5 mg/kg diazepam (WT, $n = 9$; KO, $n = 8$); 2 mg/kg diazepam (WT, $n = 10$; KO, $n = 9$). Data represent mean $\pm$ S.E.M.

Fig. 3. The expression level of glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) in young (11 weeks old) and old (20 weeks old) IL-1 receptor antagonist (IL-1Ra) KO mice. The hippocampus of 11- and 20-week old KO mice and their WT littermates was removed and equivalent amounts of protein were applied for Western-blot analysis. (A,D) Representative Western blot images of GR and MR expression 11-week (A) and 20-week (D) old mice. Protein expression level of GR (B,E) and (C,F) MR expression level in 11-week (B,C) and 20-week (E,F) old mice were shown, respectively. The levels of MRs and GRs were quantified by densitometry. The data were normalized to the level of the WT controls. The blot of β -actin was shown as an internal control. 11 weeks old: $n = 4$ for each genotype; 20 weeks old: $n = 6$ for each genotype. $*p < 0.05$ (Student's t -test). Data represent mean $\pm$ S.E.M.

Fig. 1

Fig. 2

Fig. 3

Table. 1. HPLC analysis of monoamine and its metabolites in the hippocampus.

	4-week-old		8-week-old		20-week-old	
	WT (N = 9)	IL-1Ra KO (N = 8)	WT (N = 16)	IL-1Ra KO (N = 18)	WT (N = 14)	IL-1Ra KO (N = 19)
DA	0.92 ± 0.25	0.66 ± 0.23	1.99 ± 0.42	1.97 ± 0.99	2.03 ± 0.39	1.98 ± 0.44
DOPAC	0.47 ± 0.04	0.54 ± 0.04	1.07 ± 0.19	0.81 ± 0.12	1.22 ± 0.16	2.43 ± 0.60
HVA	0.30 ± 0.05	0.36 ± 0.03	0.42 ± 0.06	0.40 ± 0.06	0.45 ± 0.04	0.68 ± 0.08*
TRY	30.4 ± 2.23	33.3 ± 1.95	43.0 ± 4.23	47.4 ± 5.91	44.8 ± 2.03	60.7 ± 4.15**
5-HT	4.21 ± 0.55	5.39 ± 0.50	4.60 ± 0.65	4.21 ± 0.49	5.61 ± 0.82	7.93 ± 1.28
5-HIAA	3.17 ± 0.21	3.24 ± 0.20	5.00 ± 0.46	5.45 ± 0.65	4.42 ± 0.46	7.05 ± 0.50**

DA: dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, TRY: tryptophan, 5-HT: 5-hydroxytryptamine, 5-HIAA: 5-hydroxyindole acetic acid.

p* < 0.05, *p* < 0.01; Mann-Whitney’s *U*-test.