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Essential role of interleukin-15 receptor in normal anxiety behavior

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Abstract

The interactions between the cytokine interleukin (IL)-15 and the classical neurotransmitter GABA have been shown in IL15R α receptor knockout mice by observations of memory deficits and reduction of GABA. To test the hypothesis that IL15 affects anxiety-like behavior, knockout mice without IL15, IL15R α , or the co-receptor IL2R γ were subjected to open-field and elevated plus maze tests. All three strains showed reduction of anxiety, with greater changes in the IL15R α knockout mice than in the IL15 or IL2R γ knockout mice. This unexpected observation is opposite to the reported increase of anxiety in mice lacking other proinflammatory cytokines or their receptors. The reduced anxiety was not associated with changes in associated serum cytokines. However, western blotting, qPCR, and immunohistochemistry all showed that IL15R α knockout mice had mild microgliosis and astrogliosis in the hippocampus. To determine whether this gliosis plays a role in decreasing anxiety, IL15R α knockout mice were treated with minocycline, but this did not cause a change in open field performance. To determine whether IL15 plays a direct role in anxiety, wildtype mice were treated with IL15 by intraperitoneal injection. This also failed to cause a change in open field behavior under the experimental conditions tested. Thus, IL15R α is essential for normal anxiety-like behavior, but inhibition of gliosis in the fearless IL15R α knockout mice or IL15 treatment of normal mice did not acutely modulate behavioral performance as tested.

Keywords

Anxiety; Open-field; IL15; Cytokine receptor; Astrogliosis; Microgliosis; Neuroinflammation; Hippocampus

Introduction

Interleukin (IL)-15 is a 14 kD cytokine belonging to the four-alpha-helix bundle family of cytokines that includes IL2. Binding of IL15 to its specific receptor IL15R α results in recruitment of the co-receptors IL2R β and IL2R γ and formation of a heterotrimeric receptor complex (Waldmann *et al.*, 1998; Waldmann and Tagaya, 1999). IL15R α activation results in phosphorylation of Janus kinases (JAK)-1 and 2 and Signal Transducer and Activator of Transcription (STAT)-3 and 5 (Johnston *et al.*, 1995; Giron-Michel *et al.*, 2003). The mammalian brain expresses both IL15 and its receptors (Hanisch *et al.*, 1997; Kurowska *et*

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et al., 2002). In the human fetus, the hippocampus and cerebellum express high levels of mRNA for IL15 and IL15R α , in comparison with the cortex and thalamus (Kurowska *et al.*, 2002). An essential neuroprotective role of IL15 signaling is shown by increased motor neuron death in IL15R α knockout (KO) mice after facial nerve axotomy (Huang *et al.*, 2007), by increased deficits in IL15 KO mice after induction of experimental autoimmune encephalomyelitis (EAE), and by the effect of IL15 treatment in ameliorating the symptoms of EAE (Wu *et al.*, 2010a). Produced in the periphery as well as the central nervous system, IL15 has limited permeation across the blood-brain barrier, but this passage can be modulated by lipopolysaccharide (Pan *et al.*, 2008c) and EAE (Hsueh *et al.*, 2009).

IL15R α KO mice also show baseline behavioral deficits in the absence of inflammatory or immune challenge. There is decreased retention of both spatial memory and contextual fear. Despite a compensatory increase of the synthesizing enzymes for γ -aminobutyric acid (glutamic acid decarboxylase, GAD65 and GAD67) in selective hippocampal regions, there is a concurrent reduction of GABA concentration in hippocampal homogenates and increase of GABA uptake in synaptosomes (He *et al.*, 2010a). Oxygraph metabolic chamber studies have shown that these mice are hyperactive, with increased oxygen consumption, carbon dioxide production, and heat dissipation. They also have higher body temperature and reduced adiposity despite hyperphagia (He *et al.*, 2010b) as well as depressive-like behavior and deficits in serotonin transmission (Wu X *et al.*, manuscript in revision in PNE).

It is well known that defective GABA transmission is implicated in anxiety. Benzodiazepines that bind to the GABA $_A$ receptor have been widely used clinically to treat anxiety, and mice deficient in GAD65 show increased anxiety-like behavior (Aihara *et al.*, 1994). Several cytokines and chemokines, including interferon α , C-C motif ligand 2 (CCL2, or MCP-1), and tumor necrosis factor α (TNF), are known to increase anxiety behavior (Fahey *et al.*, 2007; Silverman *et al.*, 2007; Breese *et al.*, 2008). IL2 also appears to be an inducer of anxiety. This is not only reflected by reduced fearfulness to a novel environment in the IL2R β KO mice (Petitto *et al.*, 2002), but also by behavioral responses to intrastriatal injection of IL2 (Pawlak *et al.*, 2003). Rats with a high level of anxiety in the elevated plus maze test have higher expression of IL2 in the striatum than those with a low level of anxiety. Microinjection of IL2 into the striatum produces time- and dose-dependent effects. A higher dose of IL2 (25 ng) is anxiolytic whereas a lower dose (1 ng) is anxiogenic (Pawlak and Schwarting, 2006; Karrenbauer *et al.*, 2009). Lastly, a paradoxical effect of IL1 β has also been shown. After intracerebroventricular injection, IL1 β can be anxiolytic (Montkowski *et al.*, 1997) or anxiogenic (Cragolini *et al.*, 2006), and IL1 β receptor null mice show decreased anxiety-like behavior (Koo and Duman, 2009). These reports provide a strong basis for determining the link between IL15R α and anxiety.

Materials and Methods

Mice and reagents

All studies were conducted following a protocol approved by the Institutional Animal Care and Use Committee. IL15R α KO (IL15RKO; B6.129X1-IL15ra^{tm1Ama}/J) and IL2R γ KO (B6.129S4-IL2rg^{tm1Wjl}/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) with their matching control mice. IL15 KO mice (C57BL/6NTac-IL15^{tm1Imx}) and their controls were obtained from Taconic (Hudson, NY). For IL15 KO mice, the control was C57BL/6NTac (B6 mice from Taconic). For IL15R γ KO mice, the control was B6.129SF2 from Jackson Laboratory (F1 littermate controls). For IL2R γ KO mice, the control was C57BL/6J (B6 mice from Jackson Laboratory). All mice were housed in the animal care facility for at least 2 weeks before study. To estimate the impact of minocycline on behavior, the IL15R α KO mice received minocycline (20 mg/kg/mouse, ip) or PBS vehicle for 3 consecutive days followed by the open field test. Minocycline was purchased from Sigma (St. Louis, MO). To

assess the effect of IL15 on the behavior of normal mice, we treated C57BL/6J mice with IL15 (Peprotech, Rocky Hill, NJ) ip at a dose of 100 ng/mouse/day, or PBS vehicle, for three consecutive days. Afterwards, the mice were subjected to the open field test. Behavioral studies were performed when the mice were 2.5 – 3.5 months old. The same KO and control mice were used for open field and elevated plus maze tests, with one week rest in between the two tests. Separate mice were used for the IL15 treatment study. A third batch of mice was used for minocycline treatment.

Open Field Test

The open field test was performed in a square arena (50×50 cm) that was surrounded by black walls (30 cm height) and well illuminated. The arena was divided into 16 equally sized squares. The four squares in the center were defined as the central region while the other squares were considered peripheral regions. The animal was placed in the center at the beginning of the experiment. The frequency of crossing the central region, rearing, and grooming were recorded for 5 min. The arena was cleaned with 70% ethanol after each trial.

Elevated Plus Maze Test

The plus-maze consisted of two open (30×5 cm) and two wall-enclosed arms (30×5×15 cm). The arms were connected by a central platform (5×5 cm). The maze was elevated 75 cm above the floor (Pellow *et al.*, 1985). The test was conducted under 40 lux lighting. Time spent completely in the open arm, partially (both forepaws) in the open arm, and looking over the edge of the open arms were recorded for 5 min. The arena was wiped with 70 % ethanol after each trial.

Luminex assay of serum cytokine concentrations

A Milliplex™ Map Kit (Millipore, Billerica, MA) was used to measure the concentrations of a series of cytokines (IL2, IL4, IL6, IL10, and IL15) in sera of the KO and their control mice. These assays were performed on a Luminex Labmap 200 (Luminex Corp., Austin, TX). The effective range of the assay was between 6.4 and 2000 pg/ml. Many cytokine levels examined were below the detection limit under normal conditions. To facilitate the illustration of the figures, the data of those cytokines are shown by extrapolation of the standard curves.

Real-time RT-PCR (qPCR)

To determine whether IL15Rα KO results in hippocampal astrogliosis and microgliosis, the KO and control mice were anesthetized by ip injection of ketamine/xylazine and decapitated (n = 5 /group). Total RNA was extracted from the hippocampus and reversely transcribed as described previously (Wu *et al.*, 2010a). Real-time PCR was conducted with SYBR Green PCR master mix on an ABI 7900 HT system (Applied Biosystems, Carlsbad, CA) as described previously (Pan *et al.*, 2008b). The primers for GFAP were FP: CATCGAGATCGCCACCTACA and RP: CAGGAATGGTGATGCGGTTT. The primers for CD11b were FP:CTTCTCCCAGAACCTCTCAAGTG and RP: GATCCCGGAAATTGGAGTGA. The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously. Standard curves for quantification were generated with template plasmids containing fragments of the respective target genes. The levels of GFAP and CD11b expression were normalized to that of GAPDH.

Western blotting (WB)

To determine whether the regulatory changes of GFAP and CD11b also occur at the protein level, hippocampi from wildtype and IL15Rα KO (n = 6 /group) were homogenized in protein lysis buffer containing protease inhibitor cocktail, and the supernatant after centrifugation was used for WB. After quantification by BCA assay, 30 µg of protein from each sample was loaded

into the well of 10 % SDS-PAGE gel for electrophoresis followed by electro-transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then probed with antibodies against CD11b (1:300, ab8878, AbCam, Cambridge, MA), glial fibrillary acidic protein (GFAP, marker for astrocytes, 1:1000, G3893, Sigma, St Louis, MO), and β -actin (1:20000, A5441, Sigma) overnight at 4°C. After thorough wash, the membrane was incubated with fluorescently conjugated secondary antibodies. Images were obtained on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The relative integrated intensity for CD11b and GFAP was normalized to that of β -actin in the same sample.

Immunohistochemistry (IHC)

IL15R α KO or control mice were anesthetized by ip urethane and perfused intracardially with saline followed by 4 % paraformaldehyde ($n = 3$ /group). The brain was post-fixed, cryoprotected, and processed for 20 μ m coronal sections on a cryostat as described previously (Pan *et al.*, 2006;2008a). The sections were stored at -20 °C in cryoprotectant (30 % sucrose and 30 % ethylene glycol in 0.1 M phosphate buffer) until use. After permeabilization and blocking, the sections were incubated with a primary antibody against GFAP (1:600, Sigma) overnight at 4 °C, and then Alexa488-labeled secondary antibody for 1 h at room temperature, with thorough washes in between. The sections incubated only with secondary antibody were used as negative controls. Fluorescent images in the hippocampal region were acquired with an Olympus FV1000 confocal microscope with the same setting for the control and IL15R α KO mice.

Statistical analyses

Group design and sample size are indicated in the Results section. Group size was determined from preliminary data to assure sufficient statistical power (0.75). Means are presented with standard errors. Student's t-test was performed to determine the difference between each control and experimental groups.

Results

1. IL15R α KO mice show reduced anxiety in the open field test

The difference between the IL15R α KO ($n = 9$) and its control ($n = 10$) in the open field mainly occurred in the time moving in the inner grids. The IL15R α KO mice showed a significant elevation of the number of inner grids crossed ($t_{17} = 4.124$, $p < 0.005$), but there was no change in the outer grids crossed during the 5 min test interval (Fig.1A). The IL15R α KO mice also showed a decrease of defecation ($t_{17} = 4.811$, $p < 0.005$), tended to rear more frequently ($t_{17} = 2.084$, $p = 0.053$), and showed “wet dog shakes”. By contrast, the IL15 KO mice ($n = 10$ /group) only showed a trend toward an increase ($t_{18} = 2.056$, $p = 0.055$) in the number of inner grids crossed (Fig.1B), and the IL2R γ KO mice ($n = 14$ /group) did not show a change in comparison with their respective controls (Fig.1C).

2. IL15R α KO mice show reduced anxiety in the elevated plus maze test

Consistent with results from the open field test, a different batch of IL15R α KO mice ($n = 11$) showed more time spent in the open arm ($t_{21} = 4.945$, $p < 0.005$). While moving in the open arm, they more frequently looked out over the edge of the arm ($t_{21} = 4.834$, $p < 0.001$). They also spent more time stretching toward and only partially in the open arm from their start position in the center of the maze at the junction between the open and closed arms ($t_{21} = 2.925$, $p < 0.005$) (Fig.2A). The IL15 KO mice ($n = 9$) showed a tendency to stay in the open arm ($t_{16} = 1.992$, $p = 0.06$) and spent more time looking over the edges of the open arms ($t_{16} = 2.154$, $p < 0.05$) (Fig.2B). The IL2R γ KO mice ($n = 14$ /group) the same mice used for the open field test) also showed a significant increase of time staying in the open arm ($t_{28} =$

2.101, $p < 0.05$) (Fig.2C). The different scales of the y-axes (time spent during the 5 min test duration) in the three strains of mice indicate the basal difference in the open arm /close arm ratio that may reflect strain and slight age differences, as well as the influence of circadian rhythms. There was no strong gender difference, and most studies included both male and female mice. Although the closed arm entries give an index of activity and whether a locomotor bias exists, we did not record them; nonetheless, the total number of grids crossed in the open field (Fig.1) and the performance in the rotarod test (not shown) did not differ between the KO mice and their respective controls. Overall, these results support reduced fear and anxiety in the IL15R α KO mice, and to a lesser extent in the IL15 KO and IL2R γ KO mice.

3. Serum interleukin concentrations

The elevated serum IL15 concentration in the IL15R α KO mice ($n = 4$ /group) was expected for end-organ resistance in the absence of a functional IL15R α . There was no difference among other cytokines tested. The concentrations of IL2, IL4, and IL6 were below detection sensitivity (Fig.3A). In the IL15 KO mice ($n = 4$ /group), the concentrations of IL10 did not differ from those of controls, and those of IL2, IL4, IL6, and IL15 were below detection sensitivity (Fig. 3B). In the IL2R γ KO mice ($n = 4$ /group), there was a significant increase of IL2 and IL10. The concentrations of IL4, IL6, and IL15 remained low (Fig.3C).

4. Gliosis in the hippocampus of the IL15R α KO mice in the resting state

The hippocampus of IL15R α KO mice had higher levels of expression of CD11b, a marker for microglia. The difference was statistically significant ($t_{10} = 4.115$, $p < 0.01$). Similarly, the IL15R α KO mice had a higher level of expression of GFAP, a marker for astrocytes in the adult brain ($t_{10} = 2.333$, $p < 0.05$). Not only was there an increase at the protein level (Fig.4A-B), but the mRNA for both CD11b ($t_8 = 3.529$, $p < 0.01$) and GFAP ($t_8 = 3.176$, $p < 0.05$) also showed a significant increase (Fig.4C).

In the hippocampus, the immunofluorescent intensity of GFAP was higher in the IL15R α KO mice than the controls (Fig.5A). The morphology of the astrocytes demarcated by GFAP staining appeared to be normal, without apparent engorgement either in brain parenchyma or at the astrocytic endfeet surrounding microvessels. However, in several areas of the hippocampus, including the stratum lacunosum-moleculare (LMol), CA3, and dentate gyrus (DG), there was an increase in GFAP (+) cell number and fluorescent intensity (Fig.5B).

5. Lack of effect of minocycline and peripheral IL15 treatment on open-field behavior

Minocycline is an inhibitor of microgliosis and, to a lesser extent, astrogliosis (Tikka *et al.*, 2001; Kerr and Patterson, 2004; Henry *et al.*, 2008; Jiang *et al.*, 2009; Kim and Suh, 2009). In IL15R α KO mice treated with minocycline ($n = 10$ /group), there was no change in the inner and outer grids crossed in the open field test (Fig.6A). In B6 mice treated with IL15 for three consecutive days ($n = 10$ /group), there was no change in comparison with the control group injected with PBS (Fig.6B). The preservation of biological effects of this batch of IL15 has been verified both in mice with the same dose and cultured cells (He *et al.*, 2010; Wu *et al.*, 2010b).

Discussion

Results from the open field and elevated plus maze tests show that IL15R α KO mice had less anxiety than the controls or the other cytokine KO mice examined. The presence of astrogliosis and microgliosis in these mice was unusual and unprecedented. Although we have seen predominantly neuronal expression of IL15 and IL15R α in mouse brain in previous studies (He *et al.*, 2010a; Wu *et al.*, 2010a), IL15 and all of its receptors are also produced by microglial cells (Hanisch *et al.*, 1997) and the cerebral endothelia composing the blood-brain barrier (Pan

et al., 2009). It is not yet clear how the absence of IL15R α results in neuroinflammatory changes. Both direct mechanisms and indirect mediators that alter cell-cell interactions may play a role.

To determine whether elevation of other cytokines in the IL15R α KO mice induces secondary gliosis after they permeate the blood-brain barrier, we measured serum concentrations of several candidate interleukins. In several situations of enhanced innate immunity, such as in lipopolysaccharide-challenged mice, IL6 plays an essential role in alterations of hippocampal biochemistry, structure, and functions (Dantzer *et al.*, 1999; Goshen *et al.*, 2007; Richwine *et al.*, 2008). In the IL15 ligand and receptor KO mice, however, serum IL6 remained low. Preliminary results also showed that IL1 β did not show a significant change, although blood-borne IL1 β crosses the blood-brain barrier by a saturable transport system (Banks *et al.*, 1991). The increase of serum IL10 as well as IL2 in the IL2R γ KO mice is another novel finding, indicating the interactions of the two cytokine systems.

Although it is possible that the higher concentrations of IL15 induced signaling through its co-receptors IL2R β and IL2R γ resulting in the formation of a low affinity ligand-receptor complex, the behavioral defects and presence of gliosis indicate that the role of IL15R α predominates. Other interleukins in the 4-helix bundle family of cytokines, including IL2, did not show a compensatory increase. By contrast, IL15 ligand KO and IL2R γ KO mice showed much milder changes, indicating that deficiency of a cytokine ligand or shared receptor can be partially compensated by pleiotropic cytokine pathways. The reduced anxiety-like behavior in these mice is consistent with the reported findings in IL2R β KO mice that have reduced fearfulness to a novel environment (Petitto *et al.*, 2002).

The lack of effect of minocycline on IL15R α KO mice and the lack of effect of IL15 on normal B6 mice in the open field behavioral test indicate that the modulatory changes may be more chronic and involve neural plasticity. In the hippocampal region, we showed that IL15R α KO mice have a relatively well preserved ultrastructure, but this does not exclude the possibility of changes in other regions and neurochemical substrates involved in anxiety behavior. IL15R α KO mice show deficits in GABA transmission (He *et al.*, 2010a), and there are region-specific changes in other neurotransmitters. In particular, there is a reduction of 5-HT $_{1A}$ and an increase of 2-HT $_{2C}$ and SERT in the hippocampus of IL15R α KO mice that also show depressive-like behavior (Wu X *et al.*, manuscript under revision). The wet-dog shakes observed in these mice are consistent with changes in the serotonergic system (Takao *et al.*, 1997). It should be cautioned that we did not perform a dose- and time-dependent analysis nor test different routes of delivery of minocycline or IL15. It remains possible that modulation of gliosis, as well as different treatment regimen to activate or antagonize IL15 signaling, may modulate anxiety-like behavior in a different experimental setting. Nonetheless, the novel findings of neuroinflammation and behavioral changes in the KO mice are important in identifying the mechanistic link between IL15 signaling and neuropsychiatric-like presentations.

While excess anxiety is pathological and leads to dysfunction of animal and human beings, adequate alertness is necessary for survival from predators, and for concentration, learning, and memory. Thus, the unique presentation of the IL15R α KO mice might provide a novel model to address the role of cytokine signaling in emotional and cognitive behavior.

Research highlights

- First study to show that a cytokine receptor (IL15R α) knockout mouse strain shows reduced anxiety

- Comparison of three strains of knockout mice without a specific receptor, a co-receptor, and ligand deficiency in behavioral performance
- Linking behavioral changes with cytokine levels and hippocampal astrogliosis / microgliosis
- Novel evidence that hippocampal gliosis (shown at morphological, protein, and mRNA levels) might result in reduced anxiety in behavioral tests

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Fig.1. Behavior of knockout (KO) mice lacking IL15R α , IL15, or IL2R γ in the open field test. (A) IL15R α KO mice showed an increase in the inner grids crossed ($p < 0.005$). (B) IL15 ligand KO mice showed a trend ($p = 0.06$) toward an increase of the inner grids crossed. (C) IL2R γ KO mice did not show apparent changes.

Fig.2.

Behavior of IL15 ligand or receptor KO mice in the elevated plus maze test. The ordinate represents increases in the time spent completely in the open arm (first set of bars) and frequency (second and third set of bars) looking over the edges while staying fully inside the open arm, or partially (both forepaws) in or stretching toward the open arm while in the center between the closed arms. (A) IL15R α KO mice; (B) IL15 KO mice; and (C) IL2R γ KO mice. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.005$ in comparison with their respective controls.

Fig.3. Serum interleukin concentrations in (A) IL15R α KO mice, (B) IL15 KO mice, and (C) IL2R γ KO mice. Only in the IL2R γ KO mice was there a significant increase of IL2 ($p < 0.005$) and IL10 ($p < 0.01$).

Fig.4.

Presence of microgliosis (showed by elevated CD11b) and astrogliosis (shown by elevated GFAP) in the hippocampus of the IL15R α KO mice. (A) WB showing higher signal intensity of both CD11b and GFAP in the IL15R α KO mice, in comparison with the lack of change of β -actin. (B) Densitometry analysis showed that the difference was statistically significant. (C) qPCR on independent samples showed that the mRNA for both CD11b and GFAP was also increased in IL15R α KO mice. *: $p < 0.05$; **: $p < 0.01$.

Fig.5.

Morphological evidence of astrogliosis in the hippocampus of the IL15Rα KO mice by IHC. (A) Lower magnification images outlining GFAP immunopositive cells in the entire hippocampus. (B) Higher magnification views showed increased GFAP expression in LMol (left panel), CA3 (middle panel), and DG (right panel). LMol: lacunosum molecular layer; Mol: molecular layer of dentate gyrus. Scale bar = 50 μm.

Fig.6. Lack of acute modulation of open field performance by (A) minocycline treatment (20 mg/kg/mouse ip daily × 3 days) in the IL15Rα KO mice; and (B) IL15 treatment (100 ng/mouse ip daily × 3 days) in normal B6 mice.