Published in final edited form as:

Brain Behav Immun. 2014 August ; 40: 226–234. doi:10.1016/j.bbi.2014.03.019.

Emotional regulatory function of Receptor Interacting Protein 140 revealed in the ventromedial hypothalamus

S Flaisher-Grinberg¹, HC Tsai^{1,2}, X Feng¹, and LN Wei¹

¹Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

²Department of Neurosurgery, Chang-Gung Memorial Hospital and University, Tao-Yuan, Taiwan, R.O.C

Abstract

Receptor-interacting protein (RIP140) is a transcription co-regulator highly expressed in macrophages to regulate inflammatory and metabolic processes. However, its implication in neurological, cognitive and emotional conditions, and the cellular systems relevant to its biological activity within the central nervous system are currently less clear. A transgenic mouse line with macrophage-specific knockdown of RIP140 was generated (MΦRIPKD mice) and brain-region specific RIP140 knockdown efficiency evaluated. Mice were subjected to a battery of tests, designed to evaluate multiple behavioral domains at naïve or following site-specific RIP140 re-expression. Gene expression analysis assessed TNF- α , IL-1 β , TGF-1 β , IL1-RA and Neuropeptide Y (NPY) expression, and in-vitro studies examined the effects of macrophage's RIP140 on astrocytes' NPY production. We found RIP140 expression was dramatically reduced in macrophages within the ventromedial hypothalamus (VMH) and the cingulate cortex of MΦRIPKD mice. These animals exhibited increased anxiety- and depressive-like behaviors. VMH-targeted RIP140 re-expression in MΦRIPKD mice reversed its depressive- but not its anxiety-like phenotype. Analysis of specific neurochemical changes revealed reduced astrocytic-NPY expression within the hypothalamus of MΦRIPKD mice, and in-vitro analysis confirmed that conditioned medium of RIP140-silenced macrophage culture could no longer stimulate NPY production from astrocytes. The current study revealed an emotional regulatory function of macrophage-derived RIP140 in the VMH, and secondary dysregulation of NPY within hypothalamic astrocyte population, which might be associated with the observed behavioral phenotype of MΦRIPKD mice. This study highlights RIP140 as a novel target for the development of potential therapeutic and intervention strategies for emotional regulation disorders.

© 2014 Elsevier Inc. All rights reserved.

Correspondent Author: Li-Na Wei, Ph.D., Professor, Department of Pharmacology, University of Minnesota, Minneapolis, USA. Phone: 612-625-9402, Fax: 612-625-8408, weix009@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

RIP140; immune-system; ventromedial-hypothalamus; anxiety; depression; NPY

Introduction

Receptor-interacting protein 140 (RIP140), also known as nuclear receptor-interacting protein 1 (NRIP1), is recognized for its functional role as a wide-spectrum transcriptional co-regulator (1). A significant body of evidence demonstrates that translocation and accumulation of RIP140 in the cytoplasm promotes adipocyte dysfunctions, thus contributing to the development and progression of metabolic disease via altered regulation of glucose uptake, adiponectin secretion and lipolysis (1). In addition, RIP140 has been found to affect inflammatory potential in macrophages through its function as a co-activator for NF- κ B to promote pro-inflammatory cytokine production following the administration of Toll like-Receptor ligands (2).

In difference from the established roles of RIP140 in modulating macrophage's activity to regulate metabolic and inflammatory processes, its involvement in human neurological, cognitive and emotional disorders and/or in animal models for such conditions has only recently been implicated. For instance, altered expression of RIP140 has been identified in Down Syndrome patients (3) and in a rodent model of aging (4). In addition, it was suggested as a candidate gene in autism (5) and in experience-dependent cortical plasticity (6). Recently, direct evidence for its effects on cognitive and emotional processes was provided by Duclot et al (7), who demonstrated long-term memory and stress-response deficits in adult mice with constitutive, whole body inactivation of RIP140 gene expression.

In the central nervous system (CNS) of human and rodents, RIP140 has been detected in various regions [e.g., cerebral cortex, hippocampus and pituitary gland (3, 7, 8)] and cell types [neurons and immune cells (7, 9)]. Delineating the contributions of RIP140 expression in specific CNS targets to the rigorously regulated cognitive and emotional responses may advance our understanding of the mechanisms by which the brain translates external and internal stimuli into integrated biological responses and point the way toward selective next-generation intervention strategies aimed to promote human mental and emotional well-being. One of these targets may be the immune system. CNS-induced immune activation has been associated with various neurophysiological processes, entailing both functional neurodestructive and neuroprotective properties (10–13). While several studies indicate that microglia and monocyte-derived macrophages stimulation may yield protective effects via the production of neurotrophic factors including nerve growth factor, brain-derived neurotrophic factor (BDNF), and neurotrophin-4/5 (12, 13), dysregulation of macrophage/microglia-induced cytokine signaling has also been linked with the occurrence of a number of pathological conditions including schizophrenia, anxiety/depression, cognitive dysfunction and neurological diseases (14–16). In line with the pivotal role of RIP140 in modulating the inflammatory potential of macrophages (2, 17) we chose to explore the behavioral effects of RIP140-silencing within macrophage-lineage.

A mouse line with macrophage-specific knockdown of RIP140 (MΦRIPKD), was generated on a C57BL/6 background (2), and mice were subjected to a test battery which evaluated multiple behavioral domains at naïve or following site-specific RIP140 re-expression. Gene expression analysis evaluated brain-region specific RIP140 knockdown and re-expression efficiency as well as possible neurochemical correlates of RIP140 expression alternation. These included the pro-inflammatory cytokines TNF- α and IL-1 β and the anti-inflammatory cytokines TGF-1 β and IL1-RA, genes implicated in CNS-derived neuroinflammatory processes, depression, stress and anxiety in human and animal models [e.g., (15, 18)]. Because of the involvement of neuropeptide Y (NPY) in the etiology of mood, stress and resilience processes (19–21) and its known action in adaptive and innate immunity (22, 23), we also examined the expression level of this neuromodulator agent. Finally, in-vitro studies were performed to examine if RIP140 expression in macrophages could modulate NPY production from astrocytes.

Method

For procedural details of behavioral assays, Reverse-Transcriptase PCR, immunofluorescence, western blotting and astrocytes viability assay, see SI Appendix.

Animals and housing

MΦRIPKD male mice and WT littermates were bred in the animal facility of the University of Minnesota and used at 9–12 weeks of age. Mice were housed in a temperature controlled room (22±1 °C) on a 14/10 light dark cycle (lights on/off at 0600/2000) with ad-lib food and water. Experimental procedures were conducted according to NIH guidelines and approved by the University of Minnesota Institutional Animal Care and Use Committee. For the derivation of transgenic mice that overexpress shRNA to target RIP140 in macrophage lineage, the shRNA for RIP140 was mimicked as endogenous microRNA following reported method (24). The expression of this shRNA was driven by hCD68 promoter (25) and the expression DNA fragment was cloned into pWhere vector. Transgenic DNA fragment was excised by PacI and then injected into C57BL/6 mouse oocytes (Mouse genetics laboratory, University of Minnesota). Transgenic founder mice were genotyped by PCR using the following primer set: Forward: 5'-GAGTTCTCAGACGCTGGAAAGCC-3' and Reverse" 5'-GTCCAATTATGTCACACCACAGAAG-3' (for additional information, see Fig. 1A–B). F1–F3 progeny were used for this study.

Experimental design

The effect of RIP140 knockdown on the behavioral phenotype of naïve mice was evaluated using 4–11 MΦRIPKD mice and 4–7 WT littermates, subjected to a test battery designed to assess general activity, balance/motor coordination, muscle strength, sensory functioning, pain sensitivity, anxiety and depressive-like behaviors. The behavioral effect of RIP140 re-expression in MΦRIPKD and WT mice was evaluated using 9 RIP140-OE-lentivirus-injected MΦRIPKD mice, 9 GFP-control-injected MΦRIPKD mice and 8 GFP-control-injected WT mice (following the exclusion of 3 GFP-control-MΦRIPKD and one WT mice from analysis based on lentivirus-localization/expression analysis and the inability of one MΦRIPKD-RIP140-OE and one WT mice to recover from the surgical procedure). Gene

expression analysis included 8–13 MΦRIPKD and 6–9 WT littermates. Experiments were performed at 2–3 partially overlapping replication and a separate cohort of mice was used for each replication.

Behavioral assessments

All behavioral assessments were performed during the light phase following habituation to the test room for 1 hour on each day. General activity was assessed using the automated open field environments, motor coordination and balance using the grid walk test and the Rotarod apparatus, neuromuscular strength using the wire hang test, simple sensory functioning using the adhesive removal test and sensitivity to a painful stimulus using the hot plate test. These behavioral modalities were tested as major physical abnormalities (e.g., motor, muscular or sensory) may indicate that mice are sick or physically unable to perform the procedural aspects of more complex behavioral tasks (26). The elevated plus maze (EPM), emergence test and light-dark box (LDB) were used for the assessment of anxiety-like behaviors. The forced swim test (FST) and tail suspension test (TST), common antidepressant- and depression-related behavior-screening paradigms (27) were used for the assessment of depressive-like behaviors. These behavioral domains were tested based on (7), which pointed to the possible involvement of RIP140 in stress-response and associated depressive-like behavioral phenotype. Tests were conducted either at minimal intrusion condition (*baseline phenotype*) or at a condition of behavioral stimulation, following stress-inducing assessments (*provoked phenotype*). For *baseline phenotype*, order of tests was arranged from the less to the more intrusive, a method commonly implemented in studies involving genetically altered mice (26, 28) and suggested to partially overcome the effect of consecutive behavioral testing (26). In this conditions, tests were also separated by 24 hours (preliminary open field environments and EPM) or 2 hours (grid walk, adhesive removal test, wire hang test, FST, repeated open field environments) interval. For *provoked phenotype*, tests were conducted 5 minutes following the termination of another, stress-inducing procedure (emergence test after repeated FST exposure, TST after hotplate exposure and LDB at the termination of the complete battery, for details, See Table S1). The behavioral battery used for the assessment of RIP140 knockdown and re-expression was similar with the exception of body temperature evaluation, Rotarod and repeated open field exposure, which yielded no difference between naïve MΦRIPKD mice and WT littermates and was thus not included in the re-expression experiments. All mice were assessed daily for fur and whiskers condition, home cage activity (running, jumping, circling, freezing, general activity, defecation, urination and social behaviors; body contacts and attacks). Weight was taken every 2 days.

Lentiviral vector design, production and validation

Lentiviral vector (pCDH-CMV-Flag RIP140-EF1-GFP) was constructed to produce GFP-fused, CMV promoter-driven expression of wild type mouse RIP140 (RIP140-OE)/GFP-control (Fig. 3A) as described earlier (29). Lentivirus was produced in HEK293T cells, using lentivirus packing system (pCDH-CMV-MCS-EF1-copGFP, System Biosciences), harvested at 48, 72, 96 and 120 hours post-transfection, filtered through 0.45-mm-pore cellulose acetate filters and concentrated by ultracentrifugation. Aliquots were kept at –80°C. Viral titers were determined by infection of HEK293T cells and green fluorescent

protein (GFP) visualization. The ability of the RIP140-OE vector to express RIP140 was assessed in-vitro, using RT-PCR analysis and in-vivo, using immunofluorescence detection. For in-vitro validation, HEK293T cells were infected with RIP140-OE-lentivirus or with GFP-control with polybrene (10 µg/ml), harvested 72 hours post-infection, total RNA extracted with TRIzol and processed as described (see SI Appendix). For in-vivo validation, WT mice were anesthetized as described below and RIP140-OElentivirus/GFP-control microinjected into the primary motor cortex, selected as the expression of RIP140 within this area is detectable but not excessive. The coordinates relative to bregma were as follows: RIP140-OE: ML +0.75, AP -1.46, DV -1. GFP-control: ML -0.75, AP -1.46, DV -1; according to the Franklin and Paxinos mouse brain atlas.

Surgical procedure

Mice were anesthetized with ketamine mixed with Xylazine hydrochloride injection (100/10 mg/kg, respectively) and placed on a stereotaxic instrument (Stoelting, Wood Dale, IL). One µl of lentiviral vectors were delivered bilaterally using a 10ml Hamilton syringe connected to a motorized injector, at a rate of 0.2 µl min⁻¹. To allow the solution to diffuse into the brain tissue, the needle was left in the brains for an additional 5 minutes after injection. The coordinates relative to bregma were as follows: VMH ML ±0.5, AP -1.46, DV -5.6; according to the Franklin and Paxinos mouse brain atlas. Seven days after surgery mice were subjected to behavioral analysis followed by sample collection and processing.

RNA interference-mediated silencing of RIP140 in RAW 264.7 cells, preparation of conditioned medium and treatment of primary astrocytes

Raw 264.7 macrophage cell line, used for conditioned medium preparation was purchased from American Type Culture Collection and maintained in high glucose DMEM medium with 10% FBS and 1% antibiotics. Transfection was performed by using Hiperfect (Qiagen) according to the manufacturer's instructions with RIP140-specific siRNA plasmids (target 1, cat. Number SI00242984, target 2, cat. Number SI02698759) and scrambled siRNA control plasmid (Qiagen). Seventy two hours following transfection, medium was collected, centrifuged at 1000 g for 5 minutes at 4°C and filtered through 0.22µm PVDF membrane. Medium was applied to primary astrocytes at 1:1 concentration with DMEM/F-12, GlutaMAX medium for 4 hours, and astrocytes were collected for RT-PCR analysis/ immunofluorescence. RAW 264.7 cells were collected and processed by western blotting for verification of silencing efficiency. Astrocytes were dissected and cultured as described previously (30). Briefly, brains were quickly removed from neonatal mice, and whole cerebral cortex isolated. Tissues were incubated for 15 minutes in 0.25% trypsin at 37°C. The dissociated cells were cultured in poly-D-lysine (5µg/mL)-coated wells in DMEM/F12 medium (10% FBS, 100U/mL penicillin) at 37°C under 5% CO₂. The medium was changed twice weekly. After 2 weeks, neurons were removed by shaking the flasks in the culture box for 15 h at 250 rpm, astrocytes were maintained in DMEM/F12 medium and the 3–5 week old cells were used for the experiments. Cultures consisting of more than 95% astrocytes were determined by GPAP staining.

Data Analysis

Statistical differences between groups were analyzed by repeated measures one- or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Independent-sample t tests were used to compare two independent groups. All analysis was carried out using GraphPad Prism 5 for windows (GraphPad Software, La Jolla, CA). All tests were performed at a significance level of $p < 0.05$ and data are presented as the mean $\pm$ SEM.

Results

Complete statistical summaries of behavior and gene expression are provided in the SI Appendix, Tables S3–S4, respectively.

RIP140 Knockdown mice demonstrate increased anxiety and depressive-like phenotype

In comparison to WT littermates, MΦRIPKD mice demonstrated increased baseline and provoked anxiety-like behavior, as indicated by its reduced entries into- and time spent in the open arms of the EPM (Fig. 1C–D), less time spent out of 'shelter' in the emergence test (Fig. 1E), reduced entries and trend towards less time spent in the lit area of the LDB (Fig. 1F–G). In addition, MΦRIPKD mice demonstrated higher immobility time and shorter latency to immobility in the FST (Fig. 1H–I) and higher immobility time in the TST (Fig. 1J), indicative of higher depressive-like behavior. Importantly, this behavioral phenotype was highly specific, as both groups demonstrated no differences in measures of general activity, balance/motor coordination, muscle strength, sensory functioning or pain sensitivity, also attesting against possible action of these measures to confound the anxiety/depressive-like phenotype. Specifically, there were no significant differences between the groups in general activity level in the open field environments on either day 1 (Fig. 1K–L) or when tested across the battery (Table S3). In this test, the significant effect of repeated exposure (Day) but the lack of effect of strain or interaction between both factors suggest that normal habituation to the apparatus occurred in both groups. No significant differences were found between the groups in measures of motor balance and coordination, measured by the grid walk (Table S3) and the Rotarod test (Fig. 1M). In the Rotarod, the significant effect of exposure progression (Session) but the lack of effect for strain or interaction between both factors suggest that normal enhancement of motor performance occurred in both groups. No significant group differences were found in sensory functioning measured using the adhesive removal test, neuromuscular strength measured using the wire hang test or sensitivity to painful stimulus measured using the hot plate apparatus. When tested for body temperature, at basal and following 6 minutes swim at 30°C (the temperature used for FST), the exposure to swim affected both group similarly, revealing no significant group difference in response to this condition (see Table S3 for data). Finally, no difference in fur, whiskers, home-cage activity, social behavior or weight was found on any day of the battery (data not shown).

Significant reduction of RIP140 expression in the ventromedial hypothalamus of MΦRIPKD mice

Immunofluorescence performed on WT and MΦRIPKD mice brain samples showed that RIP140 expression could be easily detected within the prefrontal cortex (PFC),

hippocampus, hypothalamus, amygdala and additional areas of the WT mouse brain. However, in MΦRIPKD mice, a noticeable reduction in RIP140 expression was identified within the macrophage/microglia population of the ventromedial hypothalamus (VMH), and, to a lesser extent, the dorsomedial (DMH) hypothalamus (Fig. 2A,C). Other distinguished areas included the cingulate cortex, area 1 (Cg1), and, to a lesser extent, the prelimbic (PrL) cortex (Fig. 2B, C), as defined by the Franklin and Paxinos mouse brain atlas. Comparison of RIP140 mRNA expression in the hypothalamus, PFC, hippocampus and amygdala of WT and MΦRIPKD mice provided significant support for the preferential reduction of RIP140 expression within hypothalamic and PFC tissues of MΦRIPKD mice (Fig. 2D–E).

Intra-VMH administration of lentivirally-introduced RIP140 restores the depressive-like behavioral phenotype of MΦRIPKD mice

Given the significant reduction of hypothalamic (VMH)-RIP140 expression in MΦRIPKD mice, the modulatory role of RIP140 within this nuclei on the animals' behavioral profile was tested. Specifically, MΦRIPKD mice received either intra-VMH microinjection of RIP140-OE-lentiviral vector or a GFP-expressing negative control (GFP-control), WT mice received intra-VMH microinjection of GFP-control, and all animals were subjected to the test battery. Preliminary estimation of RIP140-OE-lentiviral vector validated its efficiency in-vitro and in-vivo. Specifically, RT-PCR analysis revealed high expression of mouse RIP140 following the infection of human HEK293T cells with RIP140-OE-lentivirus (Fig. 3B). In addition, while both viruses, when microinjected into the primary motor cortex of WT mice, yielded detectable GFP signal, only the administration of RIP140-OE-lentivirus produced significant increase in RIP140 production (Fig. 3C). Additionally, GFP visualization and microinjection localization following Intra-VMH administration of RIP140-OE-lentivirus/GFP-control enabled the recognition and elimination of mice with non-specific or diffused signal, thus allowing the strict inclusion of mice with distinct and specific VMH cue in behavioral analysis (for representative immunofluorescence data see Fig. 3D–E).

As expected, in comparison to WT mice receiving GFP-control, MΦRIPKD mice receiving GFP-control maintained the behavioral phenotype identified in naïve mice, demonstrating both increased anxiety and depressive-like behaviors, assessed using the EPM and emergence test as well as the FST and TST, respectively. Surprisingly, while intra-VMH microinjection of RIP140-OE-lentivirus was able to fully and significantly reverse the depressive-like behavior of MΦRIPKD mice, it had minor effect on its anxiety-like phenotype. Specifically, in comparison to MΦRIPKD mice receiving GFP-control, MΦRIPKD mice receiving RIP140-OE-lentivirus exhibited significantly reduced immobility in both the FST (Fig. 3F) and TST (Fig. 3G) procedures. In contrast, MΦRIPKD mice receiving GFP-control or RIP140-OE-lentivirus did not differ in the percentage of entries into- and time spent- in the open arms of the EPM (Fig. 3H,I) or in time spent out of 'shelter' in the emergence test (Fig. 3J). No significant difference was found between the groups in general activity level, motor balance/coordination, sensory functioning or neuromuscular strength (Table S3).

Reduced NPY expression in the hypothalamus of MΦRIPKD mice

Next, hypothalamic tissues of MΦRIPKD and WT mice were analyzed to assess the expression profiles of TNF- α , IL-1 β , TGF-1 β , IL1-RA and NPY, genes implicated in CNS-derived neuroinflammatory and neuropsychopathological processes in human and animal models. Results revealed significant attenuated expression of NPY gene (Fig. 4A) as well as high correlation between RIP140 and NPY gene expression within hypothalamic tissues of WT and MΦRIPKD mice (Pearson $R = 0.647$, $R^2 = 0.4$, Fig. 4B). Immunofluorescence analysis revealed reduced NPY protein expression in hypothalamic tissues of MΦRIPKD mice, attributed mainly to its astrocyte population (Fig. 4C). To determine the functional role for macrophage/microglia-expressed RIP140 in modulating astrocytic NPY expression, in-vitro RNA interference-mediated silencing of RIP140 was performed in the macrophage RAW 264.7 cell line, and the conditioned medium was applied to mouse primary astrocyte cultures. Results demonstrate that the application of conditioned medium from RIP140-silenced RAW 264.7 cells (silencing, 80%, Fig. 4D) led to a significantly reduced expression of NPY in these astrocytes (Fig. 4E–F), an effect not attributable to reduced cell viability [MTT assay (data not shown), Two way ANOVA, Treatment, $F(2,11)=0.151$, NS, Time of measurement, $F(1,11)=0.074$, NS, Interaction, $F(2,11)=2.002$, NS). These findings support a causal relationship between RIP140 level in CNS-macrophage lineage and NPY expression in proximate astrocyte population.

Discussion

Investigating the impact of targeted cellular systems within specific brain region/pathways might shed light into the integrated biological responses underlying complex behaviors. The current study explored the effects of RIP140 silencing in the macrophage lineage on mouse behavioral phenotype, and identified increased anxiety- and depressive-like behaviors, associated with region-specific, system-selective alternation of intercellular crosstalk that modulates a specific neuropeptide production from astrocytes. The results suggest the RIP140 in microglia within the ventromedial hypothalamus contributes to disorders of emotional regulation.

The current study demonstrates that RIP140, mostly known for its metabolic and inflammatory regulatory roles (1, 2), is involved in CNS-mediated neurophysiological processes. These results are in line with its identification as a candidate gene for autism and Down Syndrome etiology in human patients [(3, 5) respectively] and cortical plasticity and aging processes in animal models [(4, 6) respectively]. The study also provides a specific cellular context for the effect of RIP140 in the brain, as recently reported in a study (7) that demonstrated despair-like and stress-sensitivity deficits in whole body RIP140 knockout mice. The current research utilized a targeted silencing approach to selectively knockdown RIP140 within the macrophage lineage (2), and thus was able to dissect the behavioral effects exerted specifically by RIP140 expressed in the innate immune cells, sparing the survival-imperiling alternations of lipid and glucose metabolism in whole-body deletion of RIP140 (2).

The finding associating an anxiety- and depressive-like phenotype with altered gene expression in macrophage population of the VMH and DMH are in line with the body of

evidence implicating these brain targets in modulating anxiety and depression in animal models. Both nuclei are depicted as part of a neurobiological substrate controlling rodents' avoidance and defensive behaviors, related to fear, anxiety and panic-like responses (31, 32). Specifically, the manipulation of GABAA receptor in both nuclei was shown to modulate rodents' defensive behaviour, utilized as an experimental model of panic attack (33, 34). Also, CNS-specific, knockout of the nuclear receptor steroidogenic factor 1 (SF-1), mostly affecting the VMH, was found to result in increased anxiety-like behavior in rodents (35, 36), possibly through altered distribution of stress and anxiety-like behavior related genes, including BDNF and the type-2 receptor for CRH (Crrh2) [for review, see (35)]. In this context, increased Crrh2 binding was also detected in the VMH of wistar rats bred to demonstrate hyper-anxiety/depressive-like phenotype (37). Importantly, the current study also highlights the Cg1 and PrL cortex as implicated in the detected behavioral phenomena, in line with the association of these brain targets with depressive and anxiety disorders in human and animal models (33, 34, 38–40). Given the evidence for PrL-modulation of VMH/DMH-induced panic-like behavior in mice, probably via the pathways connecting these structures (33, 34), it is possible that RIP140 might have a systemic, rather than discrete modulatory role within this pathway.

The finding that reducing RIP140 levels in macrophages yielded substantial anxiogenic and depressogenic effects in mice expands some of the current knowledge concerning immune-behavior interactions. A significant body of evidence points to various pathways and mechanisms via which the immune system influences brain and behavior. In human and rodents, immune activation and increased pro-inflammatory cytokine-signaling have been shown to induce and exacerbate depression, anxiety and cognitive dysfunction or have been suggested as biomarkers for these conditions [(41), for review see (15, 18)]. Given that RIP140 functions in macrophages to promote Toll-like receptor-induced production of pro-inflammatory cytokines such as TNF, IL-1 β and IL-6 (2), it could have been expected that the attenuation of macrophage-specific RIP140 expression would improve mice behavioral phenotype rather than be associated with behavioral deficits. Surprisingly, we found that silencing RIP140 in macrophages led to increased anxiety and depressive-like behavior, revealing a subtle and complicated immune-cytokine behavioral interaction. This is in line with the idea that pro-inflammatory cytokines-elicited immune responses in the CNS may not only yield deleterious effect but may also prove beneficial, depending the affected modulatory factors, their concentration, site, and duration of action (11–13, 15). In fact, within hypothalamic tissues of M Φ RIPKD and WT mice, the pro-inflammatory cytokines TNF- α and IL-1 β , and the anti-inflammatory cytokines TGF-1 β and IL1-RA appeared to be expressed at comparable levels. This may indicate that other factors modulate the behavioral phenotype demonstrated by M Φ RIPKD mice.

One of these factors seems to be the NPY system. The potential role of NPY in the etiology and pathophysiology of mood and anxiety disorders has been extensively studied (21, 42). In addition, NPY have been found to antagonize the behavioral consequences of stress in human and animal models and has been suggested as a biological marker of stress resilience, acting in the brain to help “shut off the alarm” (19, 20). Notably, NPY is also considered as a neuroimmune messenger, and known to modulate multiple functions of the immune system. These include immune cell trafficking, T helper cell differentiation, cytokine

secretion, natural killer cell activity, phagocytosis and the production of reactive oxygen species (22, 23). The finding that MΦRIPKD mice had significantly reduced hypothalamic NPY expression in comparison to WT mice indicates that this alternation could possibly be related to their behavioral profile. Evidence of pronounced anxiogenic-like phenotype in NPY-knockout mice (43) and of NPY downregulation in rats whose behavior was disrupted by the application of extreme stress (42) support this hypothesis. However, this requires future studies to directly manipulate NPY levels in these brain areas in MΦRIPKD mice, evaluate the regulatory processes possibly involved in RIP140-NPY correspondence and assess NPY levels following vector administration.

The finding that the reduction of NPY expression in MΦRIPKD mice can be at least partially located to the astrocytic population of the hypothalamus was supported using studies of astrocyte cultures. We found that production of NPY in astrocyte cultures supplemented with macrophage-conditioned medium was abolished by supplementing astrocyte cultures with RIP140-silenced macrophage conditioned-medium. This finding is in line with the notion of macrophages/microglia-astrocytes crosstalk in the brain, which is implicated in the balance of protective and destructive actions by these cells [e.g., (13, 44)]. As CNS astrocytes can synthesize neuropeptides and secrete them in a stimulation-dependent manner (45), it is likely that the reduction of NPY expression within astrocyte population is secondary to the silencing of RIP140 within macrophage-derived cells of the CNS.

RIP140 can act as coactivator or corepressor of various transcriptional factors and nuclear receptors (2), including those involved in stress and depression, such as glucocorticoid receptors, estrogen receptors or retinoic acid (7). Experimental and clinical evidences show that a rise in glucocorticoids concentration contribute to the behavioral changes associated with depression [for review see (18)]. In addition, estrogen receptor polymorphisms is associated with the risk of depression (46) and reduced anxiety and depression have been shown in cystic-acne patients after successful treatment with oral isotretinoin, a synthetic retinoid (47). Of specific interest are findings in mice with genetically engineered CNS/VMH localized-deletion of SF-1 which show increased anxiety and depressive-like behaviors (35, 36), as RIP140 was found to regulate SF-1-dependent transcription (48). However, despite all these findings attributing the effects of transcription programs to behavioral outcomes, the cellular cross-regulation and the relevance of the physiological context is still unclear. In the current study, our finding reveals a potential cellular crosstalk, such as between macrophages and astrocytes in the brain, which is possibly involved in augmenting very specific behavioral responses in selected brain regions. This finding also alerts future data interpretation when results are obtained using global gene manipulation strategies. This is particularly important in understanding complicated behavioral phenotypes of many genetically-manipulated animal models in which the defects could be highly dependent on the physiological context and inter-cellular crosstalk (7).

To this end, several considerations must be addressed when interpreting the results of the current study. First, although, as mentioned above, efficient knockdown of RIP140 have been detected in the VMH of MΦRIPKD mice, lentivirally-introduced RIP140 over-expression in this nucleus reversed MΦRIPKD animals' depressive-like behaviors without

affecting anxiety-like behaviors. This is in line with the suggestion that although various external and internal factors can elicit anxious and depressive behaviors simultaneously (18), the underlying biochemistry, circuits and signaling cascades may not overlap (15). It is possible that the anxiogenic phenotype demonstrated by MΦRIPKD mice is associated with its effects within the macrophage-lineage population of the frontal cortex or with additional gene alternation, possibly of the NPY system. Alternatively, it is possible that both phenotypes are dominated by differential timeline. While in the current study, a 5-days test battery was conducted following one week of post-surgery recovery period, future experiments, utilizing diverse timelines to achieve maximal lentiviral-expression efficiency, and targeting the Cg1 and/or the PrL areas of the cortex would assist in elucidating these considerations. Second, although previously global deletion of RIP140 was found to modulate long-term memory processes (7), the current analysis did not include estimation of memory as well as other cognitive processes in MΦRIPKD mice. Future investigations will be designed to expand the behavioral profile of MΦRIPKD mice to include extended emotional, cognitive and neurophysiological phenomena as well as additional cellular systems, in an effort to provide a comprehensive perspective of the biological significance of RIP140 in the CNS. For instance, the open field test can be used in future experiments to provide additional subtle measures of stress/anxiety-like behavior, including time spent at the center and walls of the apparatus [‘Thigmotaxis’ (49)]. Moreover, the crucial involvement of RIP140 in metabolic processes [e.g., (1)], highlight the possibility that metabolic factors may also be regulated in macrophage-specific RIP140 knockdown mice. Thus, future designs will aim to study additional metabolic and hormonal factors possibly affected by RIP140 and manipulate RIP140 expression within metabolic targets, to assess the effects of metabolic-derived RIP140 on mice’ behavioral profile.

In conclusion, the current study assessed the behavioral effects of macrophage-specific knockdown of RIP140 and demonstrated that re-expressing RIP140 within the VMH could play a role in emotional regulatory processes. The study also reveals a potential crosstalk between macrophages and astrocytes via altering RIP140 expression, which could have a profound behavioral outcome. Finally, this study points to RIP140 as a potential target for the development of therapeutic and intervention strategies for emotional regulation disorders. Future studies should continue to elucidate additional relevant brain regions, cellular systems and the biochemical context of RIP140’s biological activity, and thus, provide comprehensive insight into the molecular mechanisms underpinning the effects of RIP140 on complex behavioral phenomena.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Maily Nguyen, Duong Le, Michael Benneyworth and Anders Asp for their technical assistance, and K. D. Wickman and M. J. Thomas for technical guidance. This study was supported by DK60521, DK54733, the Dean’s Commitment and a Distinguished McKnight Professorship from the University of Minnesota (L.-N.W.).

References

1. Ho PC, Wei LN. Biological activities of receptor-interacting protein 140 in adipocytes and metabolic diseases. *Current diabetes reviews*. 2012; 8(6):452–7. [PubMed: 22934550]
2. Ho PC, Tsui YC, Feng X, Greaves DR, Wei LN. NF-kappaB-mediated degradation of the coactivator RIP140 regulates inflammatory responses and contributes to endotoxin tolerance. *Nature immunology*. 2012; 13(4):379–86. [PubMed: 22388040]
3. Gardiner K. Transcriptional dysregulation in Down syndrome: predictions for altered protein complex stoichiometries and post-translational modifications, and consequences for learning/behavior genes ELK, CREB, and the estrogen and glucocorticoid receptors. *Behavior genetics*. 2006; 36(3):439–53. [PubMed: 16502135]
4. Ghosh S, Thakur MK. Interaction of estrogen receptor-alpha ligand binding domain with nuclear proteins of aging mouse brain. *Journal of neuroscience research*. 2009; 87(11):2591–600. [PubMed: 19326447]
5. Iurov I, Vorsanova SG, Saprina EA, Iurov Iu B. Identification of candidate genes of autism on the basis of molecular cytogenetic and in silico studies of the genome organization of chromosomal regions involved in unbalanced rearrangements. *Genetika*. 2010; 46(10):1348–51. [PubMed: 21254553]
6. Heimel JA, Hermans JM, Sommeijer JP, Levelt CN. Neuro-Bsik Mouse Phenomics c. Genetic control of experience-dependent plasticity in the visual cortex. *Genes, brain, and behavior*. 2008; 7(8):915–23.
7. Duclot F, Lapierre M, Fritsch S, White R, Parker MG, Maurice T, et al. Cognitive impairments in adult mice with constitutive inactivation of RIP140 gene expression. *Genes, brain, and behavior*. 2012; 11(1):69–78.
8. Zhang XR, Zeng CM, Du JB, Liang R, Yang LJ, Yu WD, et al. Expression profile of receptor-interacting protein 140 in the brain: experiment with prenatal and postnatal mice. *Zhonghua yi xue za zhi*. 2007; 87(44):3152–4. [PubMed: 18269878]
9. Xue J, Zhao H, Shang G, Zou R, Dai Z, Zhou D, et al. RIP140 is associated with subclinical inflammation in type 2 diabetic patients. *Experimental and clinical endocrinology & diabetes: official journal, German Society of Endocrinology [and] German Diabetes Association*. 2013; 121(1):37–42.
10. Kerschensteiner M, Meinl E, Hohlfeld R. Neuro-immune crosstalk in CNS diseases. *Neuroscience*. 2009; 158(3):1122–32. [PubMed: 18848864]
11. Hohlfeld R, Kerschensteiner M, Meinl E. Dual role of inflammation in CNS disease. *Neurology*. 2007; 68(22 Suppl 3):S58–63. discussion S91–6. [PubMed: 17548571]
12. Smith JA, Das A, Ray SK, Banik NL. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain research bulletin*. 2012; 87(1):10–20. [PubMed: 22024597]
13. Minagar A, Shapshak P, Fujimura R, Ownby R, Heyes M, Eisdorfer C. The role of macrophage/microglia and astrocytes in the pathogenesis of three neurologic disorders: HIV-associated dementia, Alzheimer disease, and multiple sclerosis. *Journal of the neurological sciences*. 2002; 202(1–2):13–23. [PubMed: 12220687]
14. London A, Benhar I, Mattapallil MJ, Mack M, Caspi RR, Schwartz M. Functional macrophage heterogeneity in a mouse model of autoimmune central nervous system pathology. *Journal of immunology*. 2013; 190(7):3570–8.
15. Salim S, Chugh G, Asghar M. Inflammation in anxiety. *Advances in protein chemistry and structural biology*. 2012; 88:1–25. [PubMed: 22814704]
16. Kohman RA, Rhodes JS. Neurogenesis, inflammation and behavior. *Brain, behavior, and immunity*. 2013; 27(1):22–32.
17. Chen WH. Turning down the heat: the potential role of RIP140 in inflammation. *Cellular & molecular immunology*. 2012; 9(3):195–6. [PubMed: 22504951]
18. Leonard BE, Myint A. The psychoneuroimmunology of depression. *Human psychopharmacology*. 2009; 24(3):165–75. [PubMed: 19212943]
19. Heilig M. The NPY system in stress, anxiety and depression. *Neuropeptides*. 2004; 38(4):213–24. [PubMed: 15337373]

20. Hughes V. Stress: The roots of resilience. *Nature*. 2012; 490(7419):165–7. [PubMed: 23060171]
21. Wu G, Feder A, Wegener G, Bailey C, Saxena S, Charney D, et al. Central functions of neuropeptide Y in mood and anxiety disorders. Expert opinion on therapeutic targets. 2011; 15(11):1317–31. [PubMed: 21995655]
22. Malva JO, Xapelli S, Baptista S, Valero J, Agasse F, Ferreira R, et al. Multifaces of neuropeptide Y in the brain--neuroprotection, neurogenesis and neuroinflammation. *Neuropeptides*. 2012; 46(6): 299–308. [PubMed: 23116540]
23. Dimitrijevic M, Stanojevic S. The intriguing mission of neuropeptide Y in the immune system. *Amino acids*. 2013; 45(1):41–53. [PubMed: 22143431]
24. Rao MK, Wilkinson MF. Tissue-specific and cell type-specific RNA interference in vivo. *Nature protocols*. 2006; 1(3):1494–501.
25. Vats D, Mukundan L, Odegaard JI, Zhang L, Smith KL, Morel CR, et al. Oxidative metabolism and PGC-1 β attenuate macrophage-mediated inflammation. *Cell metabolism*. 2006; 4(1):13–24. [PubMed: 16814729]
26. Crawley JN. Behavioral phenotyping strategies for mutant mice. *Neuron*. 2008; 57(6):809–18. [PubMed: 18367082]
27. Lockridge A, Newland B, Printen S, Romero GE, Yuan LL. Head movement: a novel serotonin-sensitive behavioral endpoint for tail suspension test analysis. *Behavioural brain research*. 2013; 246:168–78. [PubMed: 23499706]
28. McQuade JA, Xu M, Woods SC, Seeley RJ, Benoit SC. Ethanol consumption in mice with a targeted disruption of the dopamine-3 receptor gene. *Addiction biology*. 2003; 8(3):295–303. [PubMed: 13129831]
29. Ho PC, Wei LN. Negative regulation of adiponectin secretion by receptor interacting protein 140 (RIP140). *Cellular signalling*. 2012; 24(1):71–6. [PubMed: 21872658]
30. Feng XD, Xia Q, Yuan L, Huang HF, Yang XD, Wang K. Gadolinium triggers unfolded protein responses (UPRs) in primary cultured rat cortical astrocytes via promotion of an influx of extracellular Ca²⁺ Cell biology and toxicology. 2011; 27(1):1–12. [PubMed: 20419501]
31. Nascimento JO, Zangrossi H Jr, Viana MB. Effects of reversible inactivation of the dorsomedial hypothalamus on panic- and anxiety-related responses in rats. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas/Sociedade Brasileira de Biofisica* [et al]. 2010; 43(9):869–73.
32. Talalaenko AN, Pankrat'ev DV, Goncharenko NV. Neurochemical characteristics of the ventromedial hypothalamus in mediating the antiaversive effects of anxiolytics in different models of anxiety. *Neuroscience and behavioral physiology*. 2003; 33(3):255–61. [PubMed: 12762592]
33. de Freitas RL, Salgado-Rohner CJ, Biagioni AF, Medeiros P, Hallak JE, Crippa JA, et al. NMDA and AMPA/Kainate Glutamatergic Receptors in the Prelimbic Medial Prefrontal Cortex Modulate the Elaborated Defensive Behavior and Innate Fear-Induced Antinociception Elicited by GABAA Receptor Blockade in the Medial Hypothalamus. *Cerebral cortex*. 2013
34. Freitas RL, Salgado-Rohner CJ, Hallak JE, Crippa JA, Coimbra NC. Involvement of prefrontal medial prefrontal cortex in panic-like elaborated defensive behaviour and innate fear-induced antinociception elicited by GABAA receptor blockade in the dorsomedial and ventromedial hypothalamic nuclei: role of the endocannabinoid CB1 receptor. *The international journal of neuropsychopharmacology/official scientific journal of the Collegium Internationale Neuropsychopharmacologicum*. 2013; 16(8):1781–98.
35. Zhao L, Kim KW, Ikeda Y, Anderson KK, Beck L, Chase S, et al. Central nervous system-specific knockout of steroidogenic factor 1 results in increased anxiety-like behavior. *Molecular endocrinology*. 2008; 22(6):1403–15. [PubMed: 18372344]
36. Kim KW, Zhao L, Parker KL. Central nervous system-specific knockout of steroidogenic factor 1. *Molecular and cellular endocrinology*. 2009; 300(1–2):132–6. [PubMed: 18951950]
37. Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, Liu D, et al. Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*. 2004; 29(1):1–14. [PubMed: 12942143]

38. Meyer JH. Neurochemical imaging and depressive behaviours. *Current topics in behavioral neurosciences*. 2013; 14:101–34. [PubMed: 23018753]
39. Dourmes C, Beeske S, Belzung C, Griebel G. Deep brain stimulation in treatment-resistant depression in mice: comparison with the CRF1 antagonist, SSR125543. *Progress in neuropsychopharmacology & biological psychiatry*. 2013; 40:213–20.
40. Kim SS, Wang H, Li XY, Chen T, Mercaldo V, Descalzi G, et al. Neurabin in the anterior cingulate cortex regulates anxiety-like behavior in adult mice. *Molecular brain*. 2011; 4:6. [PubMed: 21247477]
41. Hinwood M, Morandini J, Day TA, Walker FR. Evidence that microglia mediate the neurobiological effects of chronic psychological stress on the medial prefrontal cortex. *Cerebral cortex*. 2012; 22(6):1442–54. [PubMed: 21878486]
42. Cohen H, Liu T, Kozlovsky N, Kaplan Z, Zohar J, Mathe AA. The neuropeptide Y (NPY)-ergic system is associated with behavioral resilience to stress exposure in an animal model of post-traumatic stress disorder. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*. 2012; 37(2):350–63. [PubMed: 21976046]
43. Karl T, Duffy L, Herzog H. Behavioural profile of a new mouse model for NPY deficiency. *The European journal of neuroscience*. 2008; 28(1):173–80. [PubMed: 18616565]
44. Wang T, Gong N, Liu J, Kadiu I, Kraft-Terry SD, Mosley RL, et al. Proteomic modeling for HIV-1 infected microglia-astrocyte crosstalk. *PloS one*. 2008; 3(6):e2507. [PubMed: 18575609]
45. Ramamoorthy P, Whim MD. Trafficking and fusion of neuropeptide Y-containing dense-core granules in astrocytes. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2008; 28(51):13815–27. [PubMed: 19091972]
46. Ryan J, Ancelin ML. Polymorphisms of estrogen receptors and risk of depression: therapeutic implications. *Drugs*. 2012; 72(13):1725–38. [PubMed: 22901010]
47. Rubinow DR, Peck GL, Squillace KM, Gantt GG. Reduced anxiety and depression in cystic acne patients after successful treatment with oral isotretinoin. *Journal of the American Academy of Dermatology*. 1987; 17(1):25–32. [PubMed: 2956296]
48. Mellgren G, Borud B, Hoang T, Yri OE, Fladeby C, Lien EA, et al. Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes. *Molecular and cellular endocrinology*. 2003; 203(1–2):91–103. [PubMed: 12782406]
49. Bambico FR, Cassano T, Dominguez-Lopez S, Katz N, Walker CD, Piomelli D, et al. Genetic deletion of fatty acid amide hydrolase alters emotional behavior and serotonergic transmission in the dorsal raphe, prefrontal cortex, and hippocampus. *Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology*. 2010; 35(10):2083–100. [PubMed: 20571484]

Fig. 1.

The Behavioral phenotype of MΦRIPKD mice. Map describing the generation (A) and representative genotyping data (B) of MΦRIPKD transgenic mice. Mean and SE of percentage of entries into (C) and time spent in (D) the open arms of the EPM, time spent out of 'shelter' in the emergence test (E), time spent in lit area (F) and number of entries into lit area (G) of the LDB, immobility time (H) and latency to immobility (I) in the FST, immobility time in the TST (J), distance traveled (K) and ambulatory count (L) in the open field, and Latency to fall in the Rotarod (M).

Fig. 2.

RIP140 expression within brain samples of MΦRIPKD mice. Representative immunofluorescence data from hypothalamus (A, 20X and 60X magnification) and PFC (B, 4X, 10X and 60X magnification) of MΦRIPKD (empty arrow) and WT (full arrow) mice. Mean and SE of RIP140 fluorescence expression within macrophages of the VMH and Cg1 (C) and of RIP140 mRNA expression within the hypothalamus, PFC, hippocampus and amygdala of MΦRIPKD and WT mice (D) including representative mRNA data (E). Coronal diagrams adapted from the Franklin and Paxinos mouse brain atlas.

Fig. 3.

The Behavioral phenotype of MΦRIPKD mice following intra-VMH administration of RIP140-OE-lentivirus. Schematic representation of the lentiviral construct, designed to over-express mouse RIP140 (RIP140-OE) together with a GFP protein (A), representative RT-PCR image of RIP140-OE in HEK293T cells infected with over-expressing lentivirus or with GFP-expressing negative control (GFP-control) (B), representative immunofluorescence of viruses expression in vivo (motor cortex, 20X and 60X magnification) (C), representative immunofluorescence image of Intra-VMH GFP visualization (10X magnification) and microinjection localization for mice included in the RIP140 re-expression experiment (D–E), mean and SE of immobility time in the FST (F) and the TST (G), percentage of entries into (H) and time spent in (I) the open arms of the EPM and time spent out of 'shelter' in the emergence test (J). CMV=cytomegalovirus. Coronal diagrams adapted from the Franklin and Paxinos mouse brain atlas

Fig. 4. Hypothalamic gene expression in MΦRIPKD mice. Mean and SE of TNF- α , IL-1 β , TGF-1 β , IL1-RA and NPY in hypothalamic samples from MΦRIPKD and WT mice (A), a correlation between hypothalamic RIP140 and NPY mRNA expression (B), NPY immunofluorescence in the hypothalamus of MΦRIPKD and WT mice (C), RIP140 silencing in RAW 264.7 cells (D) and NPY expression in astrocytes treated with RIP140-silenced RAW 264.7-conditioned medium (E-F).