THE ROLE OF NEURON-MICROGLIA FRACTALKINE SIGNALING IN ORGANIZATION OF RESPONSES TO ACUTE AND CHRONIC STRESSORS

PhD thesis

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Budapest

2017

Introduction

Stressors trigger physiological and behavioral responses that are aimed at reinstating homeostasis, help us cope with the challenges posed by exogenous and endogenous environment. Stress results in activation of the sympathetic nervous system and hypothalamo-pituitary-adrenocortical (HPA) axis. The coping is effective, if the stress response activated rapidly when necessary, and terminated when adjustments are efficient. The processes that underlie the stress response have been collectively termed "allostasis". If the stress response is inadequate or excessive and prolonged, the cost of restoring homeostasis might become too high, and can lead to maladaptation (metabolic, immunological diseases and stress-related brain disorders).

Stressors can be divided into four main categories: physical; psychological; social; physiological. In terms of duration, stressors can be classified into two main categories: acute or chronic exposure. In the stress response, fast-acting agents (such as catecholamines, neuropeptides) and slower, gene-mediated corticosteroid effects contribute to an adequate response to the stressor, which leads to enhanced vigilance, focused attention, and in general, increase gluconeogenesis, glycogenolysis, proteolysis or lipolysis providing energy supply to "crucial" organs. Corticotropin-releasing hormone (CRH), which is produced in the parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) is essential for initiating the neuroendocrine stress response and regulating the behavioral and metabolic responses to stress. Several afferent inputs trigger the CRH neurons in the PVN, including ascending brainstem pathways that convey visceral and sensory stimuli, and a complex limbic pathway which is activated by psychological stressors. Metabolic- and nutrient-related information is mediated by local hypothalamic inputs originating in the arcuate nucleus.

The arcuate nucleus of the hypothalamus contains two major population of neurons that control energy balance; the orexigenic neurons releasing neuropeptide Y and agouti-related protein (NPY/Agrp) and anorexigenic neurons producing pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART). These neurons express receptors for detecting energy-related nutrients (glucose and FFA), hormones (leptin, insulin and ghrelin) and equipped with projections ascending to second-order neurons in the hypothalamic sub-regions such as PVN.

Stress, as a danger signal, results in an immune arousal. Microglia, the resident immune cells of the central nervous system (CNS) are sensitive to various perturbations of the environment,

such as infection, brain damage, and stress exposure and play a role in regulating of various homeostatic functions.

Information processing at the synapses is not only defined by neurons, but may be modulated by glial cells, such as astrocytes and microglia, which together form a functional unit referred to as quartide synapse. Bidirectional microglia-neuron communication occurs in at these sites. The motile microglial processes make physical contact with the pre- and postsynaptic neuronal elements and able to sense neuronal signals via neurotransmitter, neurohormone, cytokine and chemokine receptors. The activation of microglia from the default "surveying state" implies robust migratory, and morphological changes (retraction of long processes, changing number of branches and, in extreme case, transformation into an amoeboid phagocytosing form. Furthermore, activated microglia are able to express different spectrum of pro- or anti-inflammatory cytokines depending on the circumstances.

Among the pathways mediating stress-related neuronal cues to microglia, the fractalkine-fractalkine receptor (CX_3CR1) signaling has a crucial role. Fractalkine is the only member of CX_3C chemokine family, which is expressed by neurons (and astrocytes) in the central nervous system (CNS). It is found either in membrane bound or secreted forms, and has distinct functions, such as cell-cell adhesion and chemotaxis, repectively. Fractalkine interacts with its unique Gαi-coupled seven-transmembrane receptor (CX_3CR1), which is expressed by microglial cells solely in the CNS.

In my dissertation I aimed to reveal the role of hypothalamic microglia - via fractalkine signaling - in modulation of stress response, induced by acute or chronic exposure of psychogenic and physiological stressors. By using transgenic mice, in which the fractalkine receptor gene was disrupted ($CX_3CR1^{-/-}$), we explored hormonal and behavioral responses to acute and chronic stressful stimuli along with changes in hypothalamic microglia.

Objectives

The aim of the present studies was to determine if acute/chronic exposure of psychogenic or physiological stressful stimuli activate microglia in the hypothalamus -the main integrator of stressful afferents of the brain.

Furthermore, my specific aims were to determine if fractalkine/ fractalkine receptor signaling between neurons and microglia:

- implicated in stress-induced activation of microglia,
- influence the coping strategy or anxiety- and depression-related behavior evoked by acute or chronic psychological stressors,
- contribute to hormonal responses induced by acute/chronic psychogenic or physiological challenges,
- have an effect on metabolic phenotype, and on the counter-regulatory responses induced by insulin-induced hypoglycemia.

Methods

Animals

Experiments were performed in male fractalkine receptor deficient ($CX_3CR1^{-/-}$) and the background strain, C57BL/6 mice. In these mice, the *cx3cr1* gene was replaced by a *gfp* reporter gene such that $CX_3CR1^{-/-}$ mice express green fluorescent protein, GFP in cells of the myeloid linage and lack functional CX_3CR1. To assess the role of IL-1 in hypoglycemia, IL-1 a/b KO mice were used (also on C57BL6 background).

In vivo **experiments:**

- *Acute psychogenic stress - Restraint:*

Restraint stress was performed using transparent ventilated Falcon tubes fitted to the size of the animals. This procedure minimized the space around the animal, prevented them from turning and provided stressful stimulus, without being harmful.

- *Chronic psychogenic stress - Two-hit stress protocol*

Two-hit stress protocol is a combination of early life adversity (maternal separation, MS) followed by chronic variable stress (CVS) paradigm in the adulthood. During maternal separation pups were removed from their dams for 3 h daily, in postnatal days 1–14. CVS is a commonly used paradigm designed to introduce recurrent physical, psychological and social stress that is unpredictable and unavoidable. In the CVS paradigm used here, adult mice were randomly exposed to two psychogenic stressors daily for 3 weeks. Unseparated control (Control) litters were left undisturbed, except for change of bedding once a week.

- *Acute physiological stress – Insulin-induced hypoglycemia:*

Following overnight fast, insulin (1.0 IU/kg, Actrapid) or saline was injected intraperitoneally. 60 min after insulin administration mice were either transcardially perfused or decapitated for collection of brain and plasma samples. Metabolic parameters were analysed in another set of animals.

- *Chronic physiological challenge – High-fat diet:*

22-25 days old mice were fed with normal diet (ND) or high-fat diet (HFD) for 10 weeks, body weight was regularly measured, and mice were transcardially perfused. A separate set of obese and lean mice underwent cold tolerance test.

- *Intracerebroventricular (icv.) injections:*

C57BL/6 mice were anesthetized with ketamine/xylazine cocktail and a single dose of minocycline (Sigma-Aldrich, 20 μg/total volume of 2 μl), or IL-1RA (anakinra;Kineret, 100mg/0,67ml; SOBI) was injected into the right lateral ventricle of the brain using stereotaxic apparatus. After icv. injection mice were exposed to overnight fasting and intraperitoneal insulin injection.

***In vitro* experiment**

Mouse microglial BV2 cell line was obtained from the Cell Culture Core Facility at the Institute of Experimental Medicine and cultured in humidified atmosphere of 5% CO_2 in DMEM/F12 (1:1) media with 10% FCS containing Glutamax and penicillin/streptomycin. Cells were treated with 2-deoxy-glucose (Sigma) 1 mM for 3h or with insulin 1nM overnight and harvested.

- *Behavior tests:*

Open field test, elevated plus maze test, forced swim test, tail suspension test, sucrose consumption test were performed to compare the anxiety and depression-related behaviour of wild-type and $CX_3CR1^{-/-}$ mice during basal conditions and after chronic variable stress in the early light phase of the day. Behavioral tests were video recorded and analyzed later with the H77 computer based event recorder software or with EthoVision XT video tracking software.

- *Analysis of metabolic parameters:*

Mice were singly housed in TSE Phenomaster cages (TSE Systems GmbH Bad Homburg, Germany) and acclimatized for 1 day followed by 72 hours data collection of basal food consumption, X-Y-Z locomotor activity, oxygen consumption (ml/h/kg) (VO_2) and CO_2 production (ml/h/kg) (VCO_2). Energy expenditure (EE (kcal/h)) and the respiratory exchange ratio (RER) and fatty acid oxidation were calculated. Data collection was performed during overnight fasting, and after ip. insulin injection.

- In *cold tolerance test,* rectal temperature was measured before and 60,120,180 and 240 min after cold exposure.
- Plasma adrenocorticotrophin hormone (ACTH) and corticosterone concentrations were measured by *radioimmunoassay* (RIA).
- Individual or pooled plasma samples were used for the catecholamine measurements using 2-CAT *ELISA* kits (Labor Diagnostica Nord, Nordhorn, Germany).
- Quantitative and qualitative analysis of gene expression was performed by *quantitative real-time PCR* or by *in situ* hybridization histochemistry.
- Histological analyses were performed on free-floating brain sections. Stress-induced activation of neurons (c-Fos marker) and microglial activation (Iba-1 marker) were visualized by DAB-Ni/DAB/fluorescence *immunohistochemistry*. Images were analysed by Image J software. The nearest neighbour distance was calculated in Matlab program.
- *Statistical analysis* was performed using GraphPad Prism software (ver. 6.01; San Diego, CA, USA) using two-way ANOVA with Bonferroni post hoc test. To determine the significant differences between the two group means, unpaired, two sided t-test was performed.

Results

The general effect of acute or chronic stressors on hypothalamic microglia

I have found that acute and chronic psychogenic stressors stimulate only weak microglial activation in the hypothalamic paraventricular nucleus (PVN). Following restraint stress, quantitative analysis of Iba1-immunoreactivity revealed no significant differences in the density of Iba1+ microglia or in the area of Iba1+ profiles in the PVN area in wild-type mice (C57BL/6). In animals exposed to chronic stress (MS+CVS), the number of Iba1+ cells significantly decreased compared to non-stressed controls.

However, I have provided new evidence that chronic and acute exposure to physiological challenges such as high-fat diet (HFD) and insulin induced-hypoglycemia triggered robust Iba-1 positive microglial activation in the hypothalamic arcuate nucleus (ARC), in the main energy sensor area of the brain. In normal diet fed and fasted control animals, Iba-1 immunostaining revealed predominantly resting form of microglia with moderate perisomatic immunoreactivity and fine branching processes. By contrast, in the hypothalamus of high-fat diet-induced obese wild-type mice and hypoglycemic animals, microglia displayed activated phenotype with thickened branches and increased Iba1-immunoreactivity. Furthermore, I have shown that these "thickened" microglial cells were concentrated around orexigenic NPY+/c-Fos+ activated neuron population in the ARC of the insulin-induced hypoglycemic mice.

The contribution of fractalkine-fraktalkine receptor signaling between neurons and microglia to stress-induced activation of microglia

The acute restraint stress and the chronic psychogenic stressors induced the decrease of the density of Iba1+ microglial cells in the hypothalamic paraventricular nucleus of fractalkine receptor deficient mice ($CX_3CR1^{-/-}$).

Furthermore, the absence of functioning CX_3CR1 attenuated the activation of microglial cells in the arcuate nucleus of the hypothalamus induced by acute hypoglycemia and chronic high-fat diet exposure.

The impact of fractalkine-fraktalkine receptor signaling for the coping strategy or anxiety- and depression-related behavior evoked by acute or chronic psychological stressors

I have detected that mice with non-functioning fractalkine receptor ($CX_3CR1^{-/-}$) displayed more active coping behavior in tests with significant stress component (forced swim test, tail suspension test) than wild-type mice at baseline. The locomotor activity of $CX_3CR1^{-/-}$ mice was not different from C57BL/6 mice neither in the home cage, nor in the novel environment. $CX_3CR1^{-/-}$ mice were resistant to chronic variable stress–induced anhedonia as measured by sucrose consumption test. But $CX_3CR1^{-/-}$ mice did not show less anxious phenotype in elevated plus maze test, and open field test following MS+CVS.

Differences in acute/chronic psychogenic stress-induced hypothalamo-pituitary-adrenocortical axis (HPA) activity in $CX_3CR1^{-/-}$ mice

Acute restraint stress induced c-Fos expression in the PVN and the HPA activity. Furthermore, the number of c-Fos positive cell nuclei within the PVN was significantly higher in $CX_3CR1^{-/-}$ mice after acute restraint, than in wild-type C57BL/6 mice. Plasma corticosterone level was significantly elevated in $CX_3CR1^{-/-}$ animals compared to wild type mice under basal (non-stress) conditions, and after acute exposure of both restraint and forced swim stress.

Chronic stress (MS+CVS) resulted in a significant increase of relative adrenal weight in stressed mice of both genotypes compared to the values of the non-stressed control group. Chronic variable stress significantly decreased the relative thymus weight in $CX_3CR1^{-/-}$ mice, but not in C57BL/6 mice. However, I did not measure significant changes in plasma corticosterone levels and in corticotropin-releasing hormone (CRH) mRNA expression at the end of the 3 week chronic stress procedure.

Effect of fractalkine-fraktalkine receptor signaling on metabolic phenotype, and on the counter-regulatory responses induced by acute/chronic physiological challenges

11 weeks of high-fat diet resulted in significant body weight gain in mice, but the body weights of $CX_3CR1^{-/-}$ mice were significantly lower than HFD fed C57BL/6 mice. The normal diet- and HFD fed $CX_3CR1^{-/-}$ mice displayed improved cold tolerance during cold stress.

Fractalkine receptor deficiency did not affect the baseline feeding behavior, body weight, respiratory exchange ratio, fatty acid oxidation and locomotor activity under standard conditions and overnight starvation, but $CX_3CR1^{-/-}$ mice display increased energy expenditure at baseline. Furthermore, $CX_3CR1^{-/-}$ mice did not develop hypoglycemia and were more active after ip. insulin administration. Real-time quantitative PCR measurement revealed that insulin-induced hypoglycemia stimulated CRH mRNA expression in the hypothalamus, and it was significantly higher in $CX_3CR1^{-/-}$ mice than wild-type mice. Fractalkine receptor deficient mice released significantly higher plasma catecholamines, and overexpressed NPY/AgRP counter-regulatory neuropeptides in the arcuate nucleus.

Danger signals provoke release of proinflammatory cytokines, such as IL-1 from activated glial cells. Significantly elevated IL-1 mRNA level was measured *in vivo* in hypothalamic blocks of C57BL/6 mice in response to insulin-induced hypoglycemia. By contrast, hypoglycemia-induced increase of IL-1 mRNA was not seen in mice with impaired fractalkine signaling. Furthermore, the lack of interleukin-1 in IL1a/b KO mice or central inhibition of IL-1b signaling by IL-1receptor antagonist attenuated hypoglycemic response to insulin. The central blockade of microglial activity by minocycline resulted in improved glycemic control and catecholamine response.

Conclusions

Based on these findings, we can conclude the followings:

1. Acute and chronic-, physiological, metabolic and psychogenic stressors activate hypothalamic microglia in stress- and site-specific manner.
2. The fractalkine-fraktalkine receptor signaling between neurons and microglia contributes to stress-induced activation of microglia.
3. Impaired fractalkine signaling supports active coping with improved activational-hormonal- and counter-regulatory responses.
4. Activated microglia and the microglial interleukin-1 (IL-1) provide a false/ inhibitory signal to metabolic-related neurons which explain why the counterregulatory response to hypoglycemia is not fully triggered.

Altogether, these results highlight differential involvement of microglia in general and fractalkine signaling in particular to control/integrate hormonal-, metabolic- and behavioral responses to acute and chronic stress challenges.

Acknowledgement

First and foremost, I would like to thank my supervisor Krisztina Kovács for guiding and supporting me over the years, and that she promoted my scientific progress from the beginning of my studies.

I am grateful to my all the past and present colleagues of the Laboratory of Molecular Neuroendocrinology: Julianna Benkő, Szilamér Ferenczi, Krisztina Gulyás, Krisztina Horváth, Balázs Juhász, Dóra Kővári, Dániel Kuti, Bernadett Kublerné Pintér, Rókus Kriszt, Ágnes Polyák and Edina Zelei for their help.

I wish to thank to co-workers at Institute of Experimental Medicine, particularly to Ádám Dénes, Nikolett Lénárt, Csaba Fekete, Kornél Demeter, László Barna for their help to extend my technical background.

Last, but not least, I would like to express my thanks to my family and friends for their encouragement during my studies and the preparation of my PhD dissertation.

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