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Research paper

Assessment of leukocyte activity in mice devoid of the glucocorticoid receptor in the noradrenergic system (GR^{DBHCre})

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ABSTRACT

Disturbances in brain monoamines, overactivity of the hypothalamo-pituitary adrenal (HPA) axis and pro-inflammatory tendency in the immune system are the key features of depressive disorders. Recently, several murine lines with mutations in glucocorticoid receptors (GRs) have been generated and these animals may be utilized for study depressive-like disorders. In the present study, we have investigated whether selective ablation of GRs in noradrenergic neurons affects functional properties of leukocytes and redirects them towards pro-inflammatory activity.

Transgenic mice selectively devoid of GRs on noradrenergic cells were constructed using the Cre/loxP approach. Peritoneal leukocytes were collected from mutant and wild type (WT) animals of both sexes and were cultured *in vitro* for 24 h both in basal conditions and after application of selected pro- or anti-inflammatory stimuli. Metabolic activity and adherence were measured in basal conditions. Nitric oxide (NO) synthesis and arginase (ARG) activity were assessed as the markers of functional status of the cells. Because adult mutant mice lack adrenal medulla and thereby peripheral adrenaline, we modulated pro- and anti-inflammatory culture conditions by addition of noradrenaline (10–6 M). Finally, effects of *in vivo* pro-inflammatory challenge (with intraperitoneal administration of lipopolysaccharide) on properties of leukocytes were assessed 24 h (in both sexes) and 48 h later (in males only).

The experiments indicated that selective ablation of GR in noradrenergic neurons did not affect fundamental properties of peritoneal leukocytes and exerted effects only under conditions of selected pro- or anti-inflammatory stimuli *in vitro*. Stronger response to pro-inflammatory stimulation in terms of NO synthesis and ARG activity may suggest pro-inflammatory tendency in mutant mice. *In vivo* inflammatory challenge failed to show any effect of GR ablation on selected parameters of leukocyte activity. Both *in vitro* studies and *in vivo* challenge revealed mainly sex-related differences in leukocyte activity.

1. Introduction

Depressive disorders represent one of the most complex human diseases with heterogeneous clinical manifestations comprising emotional, cognitive as well as somatic disturbances. Our understanding of depression has been dominated by monoamine hypothesis (Schildkraut, 1965). However, at present it is widely accepted that apart from the noradrenergic and serotoninergic systems several other mechanisms are also involved, including the glucocorticoid receptor (GR), hypothalamic-pituitary-adrenal (HPA) and immune systems (Zunszain et al., 2011). These three components represent the neuro-immuno-endocrine system maintaining homeostasis of the organism as a whole (Miller and Raison, 2016).

The noradrenergic system is comprised of neurons clustered in several small groups in the brainstem and projecting to almost all regions of the brain, including the prefrontal cortex, hippocampus, hypothalamus and amygdala, structures involved in affective and cognitive processes disrupted in depression (Goddard et al., 2010). Among others, noradrenergic projections directly innervate the paraventricular nucleus (PVN) of the hypothalamus and together with neural inputs from limbic structures regulate activity of the HPA axis, one of the main systems responsible for proper reaction of the organism to environmental and internal stimuli (Goddard et al., 2010). Glucocorticoids released by the adrenal cortex are the main end product of the HPA axis activity and they act by GRs which are widely expressed in many brain regions as well as peripheral tissues, including immune cells. GRs belong to the superfamily of intracellular receptors. After interaction with ligands they exert their actions by binding to specific regions of DNA or interacting with other transcription factors through protein–protein interactions and as a result modulate gene expression (Revollo and

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Cidlowski, 2009). In the periphery, glucocorticoids produce mainly potent anti-inflammatory and immunosuppressive effects, albeit they also promote effective immunity and proper dewelopment of immune system (Taves et al., 2017). In the central nervous system their action is multidirectional and site-specific. Among others, they can modulate activity of the HPA axis by inhibitory feedback to the pituitary, hippocampus and hypothalamus (De Kloet et al., 1998). Noradrenergic neurons of the locus coeruleus express GRs (Czyrak and Chocyk, 2001) and these receptors are involved in the regulation of the expression of tyrosine hydroxylase, a key enzyme in noradrenaline synthesis (Smith et al., 1991; Makino et al., 2002). A proportion of depressed patients display hyperactivity of the HPA axis accompanied by impaired feedback inhibition due to glucocorticoid resistance (reviewed in (Zunszain et al., 2011) and (Anacker et al., 2011)). Under normal conditions, the noradrenergic system can modulate the activity of the HPA axis in response to stress but in depressed patients disruption of this relationship has been demonstrated (Young et al., 2005). Antidepressant drugs ameliorate depressive symptoms as well as abrogate glucocorticoid resistance in depressed patients (Hatzinger et al., 2002). Thus, the noradrenergic and GR/HPA systems are interconnected and play an important role in the pathomechanism of depression.

Apart from monoamine disturbances and HPA axis overactivity, depression is frequently associated with immunological abnormalities and these observations have led to the enunciation of the immune theory of psychiatric diseases (Beumer et al., 2012). Macrophages are the cells of innate immunity commonly present in all organs of a body. They maintain tissue homeostasis, fight against pathogens as well as participate in tissue healing and reconstruction after eradication of the threat. Macrophages also play an essential role in the regulation of immune response (for review see: (Mills and Ley, 2014)). On the basis of their functional properties, macrophages can be divided into two main subpopulations named M1 and M2 due to the relation with respective subpopulations of T lymphocytes, Th1 and Th2. Polarization of macrophage functionality is reflected by their arginine catabolism (Rath et al., 2014). Pro-inflammatory M1 subpopulation displays high inducible nitric oxide (NO) synthase (iNOS) activity resulting in increased NO synthesis. Anti-inflammatory (or alternatively activated) M2 macrophages are characterized by high arginase I (ARG) activity. The role of macrophages in the pathomechanisms of depression is well established (for review see: (Roman et al., 2013). Their excessive and uncontrolled pro-inflammatory activity is regarded as one of the main mechanisms of depression pathology (Beumer et al., 2012) and successful antidepressive treatment normalizes at least some of the immunological alterations (Dahl et al., 2014).

Progress in pharmacotherapy of depression is related, among others, to development of animal models which can contribute to better understanding the principles of the disease. One of the new transgenic models which may potentially join the immunological context of depression and stress-related response are the mice with selective ablation of GRs in noradrenergic cells (GR^{DBHCre}) constructed by spatiotemporal deletion of GRs in DBH expressing cells using the Cre/loxP approach (Parlato et al., 2009). Inactivation of GRs in noradrenergic system results in degeneration of adrenal medulla in adult GR^{DBHCre} mutant mice (Parlato et al., 2009). Nevertheless, these animals display no visible alterations in basic phenotype (daily cage behavior, weight gain, breeding, spatial memory, spontaneous locomotor activity) and possess similar to controls level of corticosterone both in basal conditions and after stress exposure (Chmielarz et al., 2013). Our previous studies have revealed a gender-dependent depressive-like and anxiety-like behavior observed in female mutants only as revealed in tail suspension test (TST) and light-dark box test (LDB) (Chmielarz et al., 2013). What is more, male GR^{DBHCre} mutants not only did not respond in these tests as female GR^{DBHCre} mice, but were characterized by resistance to behavioral effects of chronic restraint stress procedure, as assessed by the TST and LDB (Chmielarz et al., 2013). We have also found that the mutation affects evening plasma corticosterone levels in female GR^{DBHCre} mice (Chmielarz et al., 2015). These results pointed out that partial disruption of feedback regulation of HPA activity due to ablation of GRs on noradrenergic neurons affects behavior under challenge conditions in sex-dependent manner.

Taking into account aforementioned relationship between the noradrenergic and GRs/HPA systems, these mice can be regarded as a potential model of depressive illness similarly to other murine lines with mutations in the GR system (Müller and Holsboer, 2006; Howell and Muglia, 2006). Keeping in mind the role of macrophages in the pathomechanisms of depression (Beumer et al., 2012; Roman et al., 2013), it seems interesting to assess the activity of the immune system, especially peritoneal leukocytes in these mice. This cell population is heterogenous and comprises of macrophages, lymphocytes, granulocytes and other cells of immune system. Among them macrpophages and B lymphocytes make majority (Nacife et al., 2000; Ghosn et al., 2010; Kolaczkowska et al., 2010; Scotland et al., 2011). Among peritoneal B lymphocytes about 80% makes B-1 cells (Ghosn et al., 2010). They are morphologically and functionally very similar to macrophages (reviewed in (Popi, 2015; Zhu et al., 2016)), and similarly to them are able to NO release (Ghosn et al., 2006), to modulation of immune response (Azevedo et al., 2014; Arcanjo et al., 2017) and wound healing (Oliveira et al., 2010). Thus, the aim of the present study is to investigate whether selective ablation of GRs in noradrenergic neurons affects functional properties of peritoneal leukocytes and redirects them towards pro-inflammatory activity.

2. Materials and methods

2.1. Animals

Experiments were conducted on males and females C57BL/6N mice at the age of 2–3 months selectively depleted of GRs in the noradrenergic system (referred to as GR^{DBHCre}) and their control littermates (referred to as WT). The mutation was obtained using the Cre/loxP approach, as described elsewhere (Parlato et al., 2009). Briefly, transgenic mice bearing Cre recombinase under the dopamine beta-hydroxylase (DBH) promoter were mated with animals carrying the floxed GR gene. Mutant mice were kept with their control littermates of the same sex in self-ventilated cages under standard laboratory conditions (12 h light/dark cycle, food and water *ad libitum*).

All procedures were carried out in accordance with the recommendations set forth in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Ethical Committee at the Institute of Pharmacology, Polish Academy of Sciences (Permit Number: 1053, issued: July 25, 2013).

2.2. Collection of peritoneal leukocytes and culture conditions

Mice were sacrificed by cervical dislocation between 8:00-11:00 a.m. Peritoneal cavities were opened in sterile conditions and resident peritoneal cells were eluted with 2 ml of ice-cold phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, USA). Cell suspensions were centrifuged (250*g*, 3 min), counted and resuspended in culture medium at a concentration of 1×10^6 total white blood cells (WBC) per ml. The culture medium consisted of RPMI 1640 (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine and 8 mM HEPES (all from Sigma-Aldrich, St. Louis, USA). The cells were placed in 96-well, flat-bottomed culture plates (TPP, Trasadingen, Switzerland) in a volume of 100 µl per well and cultured under standard conditions (37 °C, 5% CO2, 95% humidity) for 1 h in order to stabilize culture conditions and cell metabolism. Leukocytes were cultured without further stimulation (basal conditions), or they were stimulated as indicated below. Cell viability exceeded 90%, as assessed by propidium iodide (PI) staining and flow