DYSREGULATION OF NF-kB AND JAK2/STAT3 SIGNALING IN THE HIPPOCAMPUS OF FEMALE WKY STRAIN, A GENETIC ANIMAL MODEL OF DEPRESSION

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ABSTRACT. Depression is a serious disorder with a large impact on both an individual's quality of life and society as a whole. This study aimed to evaluate the potential involvement of nuclear factor kappa-B (NF-kB) and the Janus activated kinase (JAK) and Signal transducer and activator of transcription proteins (STAT) signaling pathway in the pathogenesis of genetically predisposed depression in female rats. The obtained results showed increased phosphorylation in JAK2 and STAT3, and increased protein levels of NF-kB in the hippocampus of Wistar Kyoto rats compared to Wistar rats. These results suggest that disturbance in these pathways could have a significant role in the pathophysiology of genetically predisposed depression in females.

Keywords: Wistar Kyoto, female rats, hippocampus, depression, JAK2/STAT3 signaling pathway

INTRODUCTION

Depression is a serious disorder with a large impact on both an individual's quality of life and society as a whole. The incidence of depression in women is nearly double that in men and depressed females typically experience prolonged or recurrent depression more than depressed males (KORNSTEIN *et al*., 2000). The use of animal models of depression has contributed to understanding the pathophysiology of depression. Although complex psychiatric disorders can never be truly recapitulated in animal models, there is a conservation of certain phenotypes throughout species allowing us to measure behavior and neurobiological factors that have relevance from animals to humans. Various kinds of animal models of depression have been established for the mechanism research on depression susceptibility (CZEH *et al*., 2016). Among these models, the Wistar Kyoto (WKY) rat strain has been proposed as a valid animal model with endogenous depression and may be suitable for investigations of the genetic factors in depression (ALEKSANDROVA *et al*., 2019). This strain of rats is first used as a normotensive control for the spontaneously hypertensive rats

(OKAMOTO and AOKI, 1963). The WKY rat exhibits neurobiological and behavioral characteristics that are comparable to those seen in clinical cases of depression and is resistant to conventional antidepressants (LÓPEZ-RUBALCAVA and LUCKI, 2000). Additionally, the WKY strain demonstrates elevated anxiety and depressive-like behaviors (RITTENHOUSE *et al*., 2002). Sex differences in the WKY model are rarely examined (D'SOUZA and SADANANDA, 2017; MILLARD *et al*., 2019). Most studies are focused on males, even though sexual dimorphism is prevalent in the pathophysiology and etiology of depression. Considering that depression is more prevalent in women than in men, in the present study, female rats were used. Although the exact genetic or molecular mechanisms underlying the depressive-like phenotype of WKY rats are still unresolved and published reports are not always consistent, various abnormalities in different neurotransmitter and endocrine systems have been demonstrated in WKY compared to control outbred rats (SCHOLL *et al*., 2010; BRUZOS-CIDON *et al*., 2014).

In the pathogenesis of depression, the hippocampus is closely related to the physiological and behavioral responses to stress. A large body of evidence demonstrates that chronic stress may affect both the structure and function of the hippocampus (BARCH *et al*., 2019; PRICE and DUMAN, 2020; PREVIOUS *et al*., 2022). The previous studies have demonstrated reduced volume in the hippocampus of WKY rats (TIZABI *et al*., 2010). Although emerging evidence has suggested depression as a multi-gene syndrome resulting from a complex interaction of biological, psychological, and social factors (BAGOT *et al*., 2016), the exact molecular mechanisms underlying the susceptibility to depression are still largely unknown and thus require further studies. In addition to neurotransmission theory of depression, inflammatory processes and disrupted signaling pathways also play key roles in the pathophysiology of depression. Many cytokines and growth hormones use the Janus activated kinase (JAK)-Signal transducer and activator of transcription proteins (STAT) signaling pathway, an intracellular protein network, to activate the expression of particular genes (GAŁECKA *et al*., 2021). When a ligand binds to a membrane receptor, the associated tyrosine kinase (JAK) is activated, which phosphorylates the receptor and causes it to become active. The STAT proteins then bind to active domains, which, during the dimerization process, separate from them and travel to the cell nucleus, where they link to the proper promoter and start the transcription process of the corresponding gene (IMADA and LEONARD 2000; SCHINDLER and PLUMLEE, 2008; HARRISON, 2012). JAK-STAT system is important in all cell types, including neurons (NICOLAS *et al*., 2013). The importance and participation of signaling pathways in the pathogenesis of depression is confirmed by the fact that JAK can regulate the expression or function of several neurotransmitter receptors, including gamma-aminobutyric acid (GABA) (LUND *et al*., 2008), cholinergic muscarinic (CHIBA *et al*., 2009), N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors, which are strongly associated with depressive symptoms (ORELLANA *et al*., 2005; MAHMOUD and GROVER 2006; XU *et al*., 2008). STAT expression is weaker in the CNS, although these proteins may be important in various brain areas, including the cerebral cortex, hippocampus, hypothalamus, and cerebellum. The JAK-STAT signaling system is activated by the factors produced during inflammation. Concerning the JAK isoforms, it seems that JAK1 is more involved in astrocytic differentiation while JAK2, seems essential for neural stem cells proliferation (BONNI *et al*., 1997; KIM *et al*., 2010). Through a JAK2-dependent mechanism, oxidative stress and certain cytokines (e.g., IL-6) activate both STAT1 and STAT3 (PLANAS *et al*., 2006). We chose JAK2/STAT3 signaling because NICOLAS et al. (2012) reported the involvement of both JAK2 and STAT3 in hippocampal synaptic plasticity independently of their ability to regulate gene expression. Since NF-kB could interact with a bunch of genes involved in inflammation, researchers observed the activation of nuclear factor kappa-B (NF-kB) in many inflammatory diseases (MONACO *et al*., 2004). WANG *et al*. (2018) demonstrated that CUMS induced depressive-like behavior and spatial memory

damage, and overexpression of cytokines and NF-κB in the frontal cortex and hippocampus in C57BL/6 strain.

Therefore, the current study was designed to investigate the potential involvement of NF-kB and the JAK/STAT signaling pathway in the pathogenesis of genetically predisposed depression in female rats.

MATERIAL AND METHODS

Subjects

Adult Wistar (WIS) and WKY female rats (9 weeks old, 150-250g) were kept in transparent plastic cages size 40×25×15 cm. The cages were placed in room with stable environmental conditions (e.g. light/dark cycle, temperature (22 ± 2°C), and humidity (45 ± 5%)). Food and water were available *ad libitum*. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee for the use of laboratory animals of the "Vinca" Institute based on Directive 2010/63/EU. All procedures with animals were approved by Ethical Committee for the use of laboratory animals of the "Vinca" Institute and Ministry of Agriculture and Environmental Protection, Authority for Veterinary permission No. 323-07-01498/2022-05.

The animals were divided into two matched groups: WIS and WKY rats. All female rats were housed in groups of three. For further analyses we used only samples from 6 female rats per group that were in the diestrus phase at the end of the experiment to avoid the influence of sex hormones on the results. At the end of the study, all animals were decapitated with a guillotine (Harvard - Apparatus, USA), the hippocampus was quickly removed on ice, frozen in liquid nitrogen and stored at -80°C until biochemical analyses.

Western blot analysis

Hippocampal tissue was homogenized in RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, sc-24948). After centrifugation (12 000 rpm, 20 mins at 4 ^{0}C), the supernatant was taken and protein concentration was determined by the method of LOWRY et al. (1951). 30μg of hippocampal proteins extracts separated by 12% SDS-poly-acrylamide gel electrophoresis were transferred to a supported PVDF membrane (Immobilon-P Transfer membrane, Catalog No. UPVH 00010, pore size: 0.45 μm, Merck Millipore Ltd, Ireland). The membranes were blocked in 5% non-fat dry milk in Tris-Buffered Saline-Tween 20 (TBST) for 1h at room temperature. All following washes (three times for 15 mins) and antibody incubation (overnight at 4$^°$C for primary antibody and 1h at room temperature for secondary antibody) were also performed in TBST at ambient temperature on a shaker. For measuring JAK2, pJAK2, STAT, pSTAT3 and NF-kB protein levels anti-JAK2 mouse monoclonal primary antibody (C-10): sc-390539, Santa Cruz Biotechnology (dilution 1:1000); anti-phospho-JAK2 (Tyr1007/1008) rabbit polyclonal primary antibody, Sigma Aldrich (dilution 1:1000); anti- STAT3 mouse monoclonal primary antibody (F-2): sc-8019, Santa Cruz Biotechnology (dilution 1:1000); anti-p-STAT3 mouse monoclonal primary antibody (23G5): sc-56747, Santa Cruz Biotechnology (dilution 1:1000); anti-NF-kB mouse monoclonal primary antibody (F-6): sc-8008, Santa Cruz Biotechnology (dilution 1:200), were used respectively. Washed membrane was further incubated in the horseradish peroxidase conjugated secondary anti-mouse (Goat Anti Mouse IgG (HRP), Catalog No. sc2005, dilution 1:5000, Santa Cruz Biotechnology, USA) and anti-rabbit antibody (Goat Anti-Rabbit IgG (HRP), Catalog No. ab6721, Abcam, dilution 1:1000, United Kingdom) for luminol based detection. The secondary antibody was visualized by Immobilion Western

Chemiluminescent HPR Substrate (Catalog No. WBKLS 0100, Millipore Corporation, USA) and exposed to Hyperfilm™ ECL™ (GE28-9068-36 hyperfilm ECL, 18x24 cm, 50 sheets, Sigma Aldrich) for Western Blot Detection. The relative density of the protein immunoblot images was analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Amounts of all analyzed proteins were normalized to β-actin levels (dilution 1:5000, Catalog No. sc-47778, Santa Cruz Biotechnology, USA).

Statistical analysis

The results in this paper were presented as a mean ± standard error (S.E.M). The significance of the differences in protein content of JAK2, STAT3 and NF-kB in the hippocampus of the WIS and WKY group were estimated using the Student's t-test in Origin software, version 9 (Jandel Corporation, USA) and Statistica, version 7 (StatSoft, Inc., Tulsa, USA). The level of statistical significance was set at $p < 0.05$.

RESULTS

To clarify the possible involvement of JAK2/STAT3 and NF-kB signaling pathways in the higher vulnerable WKY to stress we examined the ratio of pJAK2/JAK2 and pSTAT3/STAT3 and protein levels of NF-kB in the hippocampus. The results of JAK2 protein levels in WKY and WIS rats have presented in Fig. 1. Our results indicate increased phosphorylation of JAK2 (by 24%, $p < 0.05$) and ratio pJAK2/tJAK2 (by 15%, $p < 0.05$) in the hippocampus of WKY in relation of WIS rats.
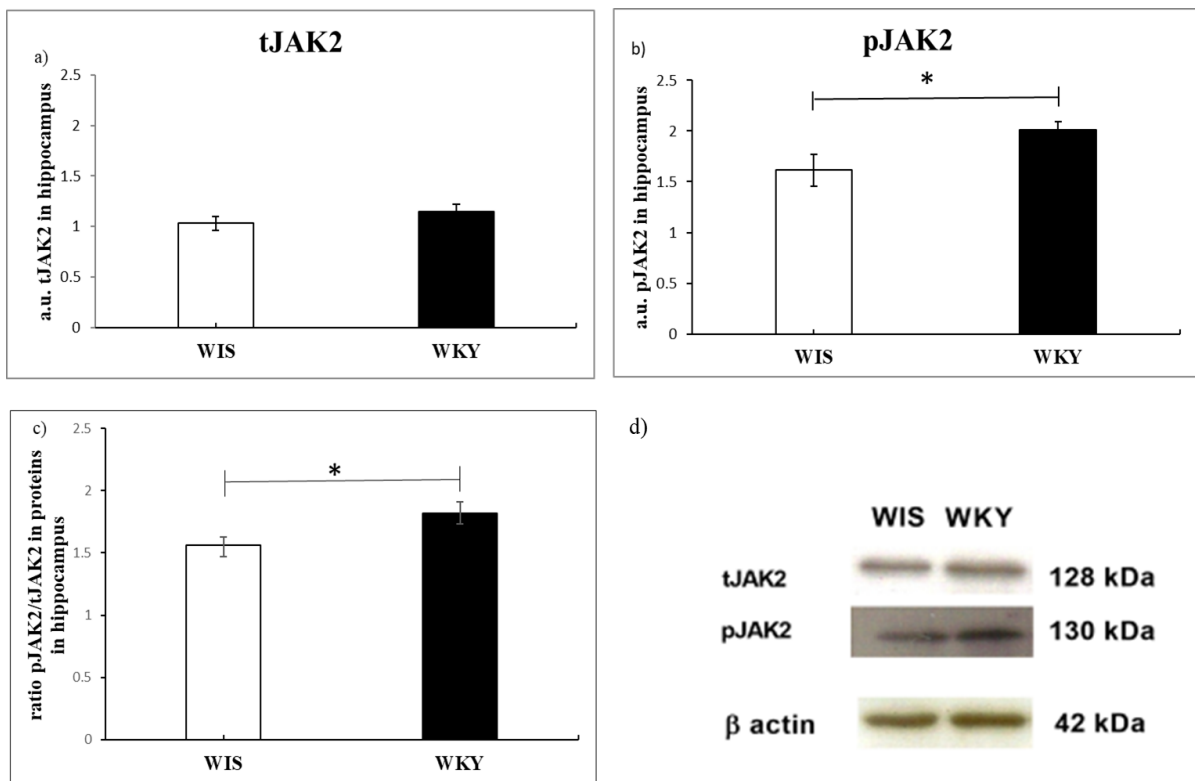

Figure 1. Western blot quantification analysis of a) total, b) phosphorylated JAK2 and c) ratio pJAK2/tJAK2 levels in hippocampal whole-cell protein extracts obtained from WIS and WKY rats. Each column represents the mean ± SEM. The number of animals per experimental group: n=6. β actin was used as a loading control. Statistical significance * $p < 0.05$ WIS vs. WKY. d) Representative Western blots for phosphorylated and total JAK2 proteins.

The results of STAT3 protein levels in WKY and WIS rats were presented in Fig. 2. Our results also indicate increased phosphorylation of STAT3 (by 111%, p <0.01) and ratio pSTAT3/tSTAT3 (by 77%, p <0.01) in the hippocampus of WKY in relation of WIS rats.
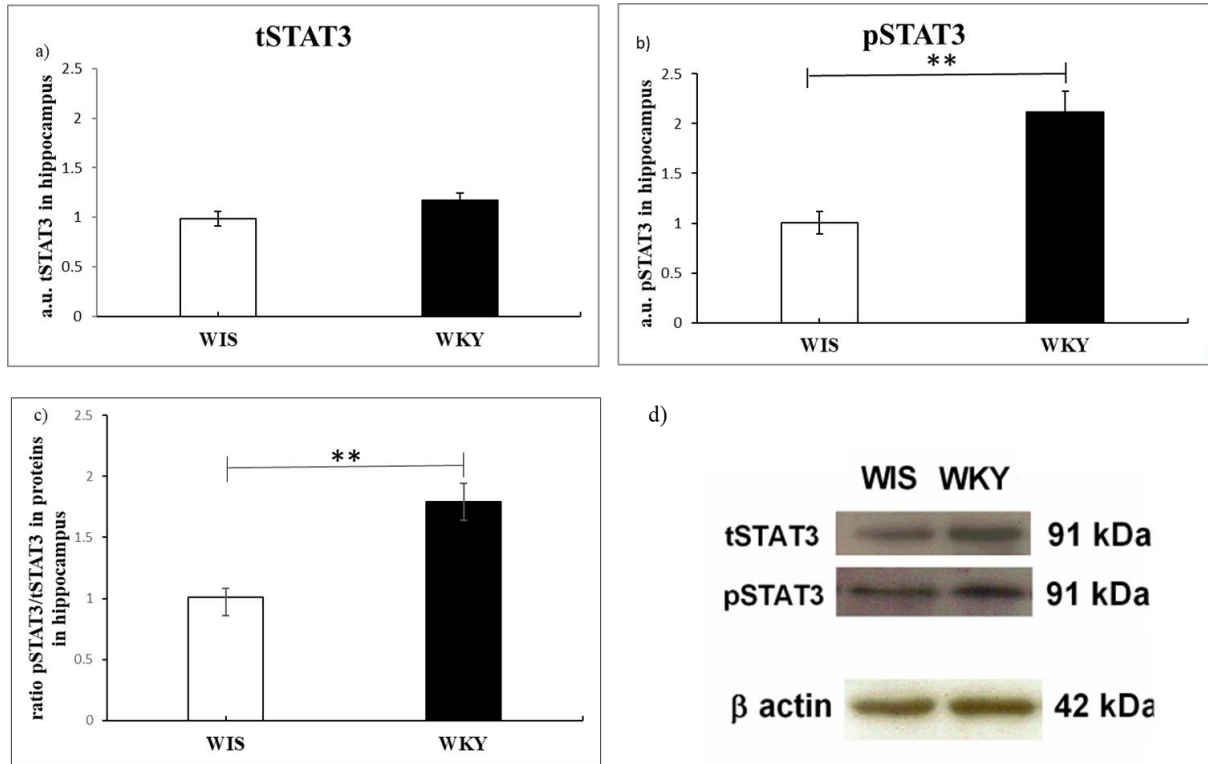

Figure 2. Western blot quantification analysis of a) total, b) phosphorylated STAT3 and c) ratio pSTAT3/tSTAT3 levels in hippocampal whole-cell protein extracts obtained from WIS and WKY rats. Each column represents the mean ± SEM. The number of animals per experimental group: n=6. β actin was used as a loading control. Statistical significance ** p <0.01 WIS *vs.* WKY. d) Representative Western blots for phosphorylated and total STAT3 proteins.

The data presented in Fig. 3 shows the results of NF-kB protein levels in WKY and WIS rats. Nuclear factor NF-kB was significantly increased (by 28%, p <0.05) in WKY compared to WIS rats.
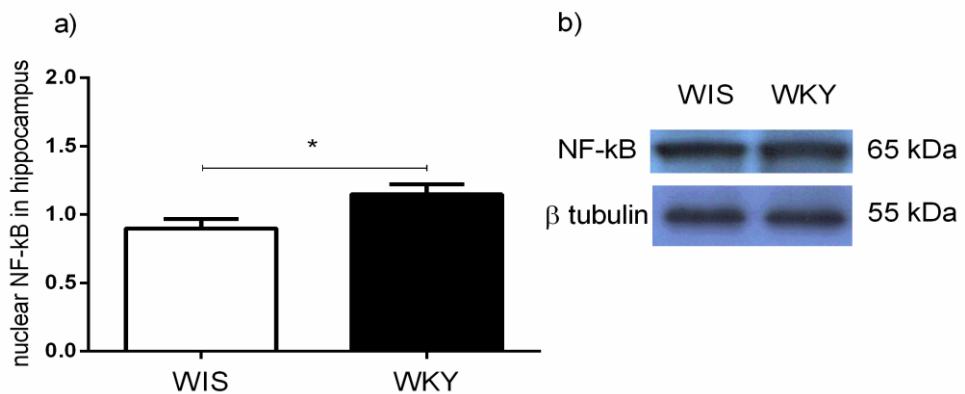

Figure 3. Western blot quantification analysis of NF-κB protein levels in the nuclear fraction of the hippocampal samples obtained from WIS and WKY rats. Results are presented as the means ± S.E.M. Statistical significance * p <0.05 WIS *vs.* WKY. The number of animals per experimental group: n=6. β tubulin was used as a loading control.
b) Representative Western blots for NF-kB protein.

DISCUSSION

Previously studies reported behavioral patterns exhibited by WKY rats which are consistent with the heightened levels of anxiety, including an exaggerated secretion of stress hormones (RITTENHOUSE et al., 2002), reduced time exploring the open field arena and increased defecation rates during a trial (O'MALLEY et al., 2010). The stress-sensitive behavioral phenotype exhibited by WKY rats may have a molecular basis. The data presented in this manuscript illustrates how the genetic make-up of stress-sensitive WKY rats results in alterations in JAK2/STAT3 and NF-kB signaling pathways compared to WIS rats. It is well-known that the JAK/STAT pathway modulates various signals to keep homeostasis in inflammatory conditions. The proinflammatory cytokines promote the recruitment of immune cells and provide an inflammatory microenvironment for the remodeling of hippocampal neurons and the development of mood disorders. Activation of the immune system (with TNF and/or IL-6) can promote the activation of an intrinsic cell survival signaling pathway such as JAK/STAT. Interleukin-6, as a proinflammatory cytokine, forms a complex with IL-6 receptor and coreceptor glycoprotein 130 (gp130), which in turn initiates a cascade reaction including JAK activation, phosphorylation of STAT3, and subsequent dimer formation, nuclear translocation, and gene transcription. There is abundant evidence to show that the sustained activation of the JAK/STAT signaling pathway is closely related to mood disorders (SHARIQ et al., 2018). Our results show increased JAK2 and STAT3 signaling in the hippocampus of WKY female rats. The binding of ligands to its receptors induces the phosphorylation of receptor-associated JAK, which in turn leads to STAT phosphorylation. Phosphorylated STATs are released from the receptor complex and then form homo-or heterodimers and then translocate into the nucleus to regulate the transcription of target genes encoding proinflammatory cytokines and chemokine (O'SHEA et al., 2015). The main consequence of the activation of this pathway is to promote inflammation-associated gene expression. Activated STAT3 accelerated a positive feedback mechanism to produce IL-6 in lipopolysaccharide-induced brain inflammation (BEUREL and JOPE, 2009). This amplification mechanism for IL-6 expression included STAT3 and NF-κB signaling. In the present study, we identified significant upregulation of nuclear NF-kB activation in female WKY rats. There are some reports that NF-κB association with STAT3 increased transcription of IL-6 and, subsequently, accelerated IL-6–JAK-STAT3 signaling, amplifying the production of IL-6 and other chemokines (LAM et al., 2008). MATSUMOTO et al. (2018) reported that an association between STAT3 and NF-κB might be involved in the enhancement of TNF-α-stimulated IL-6 production in pericytes. Sustained STAT3 and NF-κB activation in pericytes could be responsible for CNS diseases, by inducing blood brain barrier dysfunction, glial activation and neuronal damage. The results of KWON et al. (2017) showed that microglia-specific STAT3 knockout mice showed antidepressant-like behavior. There is also evidence that the inhibition of specific JAK/STAT pathways, via JAK inhibitors may be a promising novel treatment for depression (SHARIQ et al., 2018).

Our findings have revealed for the first time the existence of increased levels of NF-kB and JAK2/STAT3 in the hippocampus of WKY female rats compared to WIS female rats. These results suggest that disturbance in these pathways could have a significant role in the pathophysiology of genetically predisposed depression in females. Given that the occurrence of disease is closely linked to the JAK/STAT pathway, therefore, JAK2 and STAT3, are likely to be the most effective targets for the treatment of genetically predisposed depression.

The present data should be interpreted within the limitation. In this study, male WKY and WIS rats were not included considering complex issues regarding the relationship between sex and depression. Future studies should examine whether genetically predisposed depression-like behavior would have a stronger influence on JAK/STAT and NF-kB signaling pathways in male WKY rats. We examined the hippocampus only in the current study.

However, it is unlikely that the observed changes are limited to the hippocampus. Future studies are needed to examine other brain regions such as the prefrontal cortex, amygdala and hypothalamus which are affected also in depression.

Acknowledgments

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