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Gender-specific effects of fluoxetine on hippocampal glucocorticoid receptor phosphorylation and behavior in chronically stressed rats

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ABSTRACT

Chronic psychosocial isolation stress (CPSI) modulates glucocorticoid receptor (GR) functioning in Wistar male rat hippocampus (HIPPO) through alteration of nuclear GR phosphorylation and its upstream kinases signaling, which parallels animal depressive-like behavior. The current study investigated potential gender specificities regarding the effect of chronic therapy by an antidepressant fluoxetine (FLU) on GR signaling in HIPPO and depressive-like behavior in CPSI animals.

FLU was administrated to female and male naïve or CPSI rats for 21 days and GR protein, its phosphorylation status and upstream kinases, as well as GR and BDNF mRNA were followed in HIPPO together with animal serum corticosterone (CORT) and depressive-like behavior.

The results showed that CPSI increased immobility in males *versus* hyperactivity in females and disrupted nuclear pGR232-Cdk5 pathway and JNK signaling in a gender-specific way. In contrast, in both genders CPSI increased the nuclear levels of GR and pGR246 but decreased CORT and mRNA levels of GR and BDNF. Concomitant FLU normalized the depressive-like behavior and altered the nuclear pGR232-Cdk5 signaling in a gender-specific manner. In both females and males, FLU reversed the nuclear levels of GR and pGR246 without affecting CORT and GR mRNA levels. In contrast, FLU exhibited gender-specific effect on BDNF mRNA in CPSI animals, by increasing it in females, but not in males. In spite of normalization the total nuclear GR level upon FLU treatment in both gender, down-regulation of GR mRNA is possibly maintained through prevalence of pGR232 isoform only in males.

The gender-specific alterations of pGR232-Cdk5 signaling and BDNF gene expression in HIPPO and normalization of depressive-like behavior upon FLU treatment distinguishes this signaling pathway as potential future antidepressant target for gender-specific therapy of stress related mood disorders.

1. Introduction

Exposure to chronic stress has been associated with the pathophysiology of many psychiatric disorders including depression (Kendler and Karkowski-Shuman, 1997). Although, the stress response is essential for maintenance of homeostasis, maladaptive response to stress can elevate risk factors that lead to the disease (Bale, 2006; Jentsch et al., 2002). Several theories have been proposed to explain stress-related pathogenesis of depression, including glucocorticoid hormones (GCs) and glucocorticoid receptor (GR) (Holsboer, 2000), as possible depressogenic mediators and potential targets for antidepressant action (Budziszewska et al., 2000; Calfa et al., 2003; Pariante and Miller, 2001). Epidemiological studies demonstrate that women are more vulnerable to stress-related psychopathologies than men, since depression occurs twice as frequently in women (Carter-Snell and Hegadoren, 2003; Kessler, 2003; Lin et al., 2009; Sun and Alkon, 2006). Furthermore, women tend to show poorer response rates and slower clinical improvement with tricyclic antidepressants (TCAs) and appear to respond better to selective serotonin reuptake inhibitors (SSRIs) (Davidson and Pelton, 1986; Hamilton et al., 1996; Kornstein et al., 2000; Martenyi et al., 2001). However the mechanisms underlying these clinical facts, as well as molecular specificities that underlie possible sex differences, including the GCs and GR signaling are not well defined.

Notably, studies carried out in depressed patients as well as studies on animal models of depression showed consistently dysfunctional limbic hypothalamus–pituitary–adrenal (LHPA) axis activity, leading to altered levels of circulating GCs (Gass et al., 2001; Holsboer, 2000; Juruena et al., 2004; Pariante and Miller, 2001; Ridder et al., 2005; Zunszain et al., 2011). The activity of LHPA axis is controlled through inhibitory feedback mechanisms

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involving different brain structures with main research focus on hippocampus (HIPPO). At the molecular level, GCs exert their effects by activating the GR, a key regulator of the negative feedback of the LHPA axis activity (Holsboer, 2000; Pariante et al., 2001b; Tronche et al., 1999). Besides GCs, the GR activity could be also controlled by specific modification *via* phosphorylation of GR which is accomplished through activation of several cellular kinases (Chrousos and Kino, 2005; Kino et al., 2007). Such kinases among others include cyclin-dependent kinases (CDKs) targeting the GR at 232 serine residue (pGR232) and c-Jun N-terminal kinases (JNKs) that target GR at serine residue 246 (pGR246) (Ismaili and Garabedian, 2004; Itoh et al., 2002; Krstic et al., 1997; Miller et al., 2005; Rogatsky et al., 1998; Webster et al., 1997). The phosphorylation of GR at these specific epitopes may influence its stability and nucleo-cytoplasmic shuttling (Blind and Garabedian, 2008; Chen et al., 2008; Davies et al., 2008) resulting in enhanced or inhibited GR transcriptional activity (Ismaili and Garabedian, 2004) and thereby modifying the cellular processes.

In our previous studies we observed that male Wistar rats exposed to chronic psychosocial isolation stress (CPSI) exhibited depressive-like behavior in forced swimming test (FST) (Djordjevic et al., 2012a,b). We found that male Wistar rats exposed to CPSI exhibit decreased serum corticosterone levels (CORT), increased GR phosphorylation at serine232 *vs* 246 in HIPPO and prefrontal bran cortex (PFC). These changes were accompanied with increase in Cdk5 *vs* decrease in JNKs activities, and with altered GR and BDNF gene expression (Adzic et al., 2009a). In the current study we investigated whether the parameters that were altered by CPSI could be normalized by concomitant antidepressant treatment and whether they are changed in gender-specific manner. Namely, the complexity of GR regulation may suggest multiple points at which antidepressant treatment could affect the GR activity (Anacker et al., 2011; Funato et al., 2006b). In particular, we investigated the ability of chronic SSRI antidepressant fluoxetine (FLU) to modulate serum corticosterone levels (CORT), hippocampal cytoplasm–nuclear distribution of GR and its phosphorylation status, pGR232 and pGR246, as well as its upstream signaling kinases, Cdk5 and JNKs, in female and male Wistar rats exposed to CPSI. Also, the transcriptional activity of the GR was followed by measuring the hippocampal GR and BDNF mRNA levels. Finally, we applied FST to analyze depressive-like behavior in rats exposed to CPSI and FLU treatment.

2. Materials and methods

2.1. Preparation of fluoxetine–hydrochloride solution

The capsules of Flunirin were emptied and dissolved in distilled, sterile water with the aid of ultra-sonication, and the solution was filtered through Whatman No. 42 filter paper. The fluoxetine–hydrochloride (FLU) concentration in the solution was determined using Ultra Performance Liquid Chromatography (UPLC) (Djordjevic et al., 2005).

2.2. Animals and treatment

The experiments were performed on adult 3 months old, female (body mass 250–300 g) and male (body mass 330–400 g) Wistar rats. All animals were housed at 20 ± 2 °C, with a 12 h light/dark cycle (lights on at 07:00 h), with food (commercial rat pellets) and drinking water available *ad libitum*.

All experiments on female and male rats were conducted in parallel. As depicted in Fig. 1, both female and male animals were divided into four experimental groups, each containing 12 animals housed 4 animals per cage: Control + Vehicle, Control + Fluoxetine, Stress + Vehicle and Stress + Fluoxetine group. The experiment consisted of two phases and lasted for 6 weeks (42 days). The first experimental phase (Stress) lasted 21 days, during which animals of Stress + Vehicle and the Stress + Fluoxetine were submitted to chronic psychosocial isolation (CPSI). The CPSI procedure was carried out according to our standard protocol (for details see Adzic et al., 2009a,b). The second experimental phase consisted of the vehicle (VEH) or fluoxetine (FLU) treatment for 21 days, while animals remained in the CPSI. The 21 day of CPSI was used as a model of chronic stress during which animals had

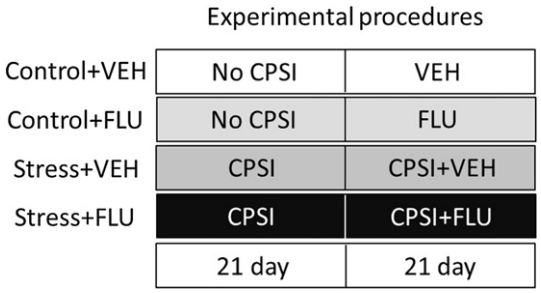

Fig. 1. Experimental groups and design. For details, see the Materials and methods.

normal auditory and olfactory experiences, but were deprived of any visual or tactile contact with other rats.

FLU was injected intraperitoneally to Control + FLU group and to CPSI + FLU group at a daily dose of 5 mg/kg of body mass at 09:00 h during a 21-day period. Both Vehicle groups (Control and CPSI) received distilled water under the same conditions as matching FLU-treated groups. CPSI groups remained isolated during treatment. For testing the behavior and molecular parameters different set of animals were used and experiments were repeated twice.

Vaginal smears were microscopically analyzed for determination of the estrous cycle stage one week before the start of the experiment and immediately after the sacrifice. Only females with normal 4–5 days cycles were included in the experiment. Neither of treatments (CPSI or FLU or injections) altered the estrous cycle phase distribution.

All animal procedures were approved by the Ethical Committee for the Use of Laboratory Animals of the VINCA Institute of Nuclear Sciences, according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA).

2.3. Behavioral analyses – forced swim test

The forced swim test (FST) that is widely used to investigate the response to antidepressant treatment was conducted according to modified Porsolt (Porsolt et al., 1977; Castro et al., 2010). The test was performed between 10.00 and 11.00 a.m. i.e. at the same time when animals that were used for molecular analyses were sacrificed. Rats were placed in a Plexiglas cylinder (40 cm height, 20 cm in diameter) filled to 30 cm with water (24 ± 0.5 °C). The water depth was adjusted to a height that did not allow the animals to touch the tank bottom with their hind pawns or their tails and under the conditions in which escape was not possible. All animals were placed for a single 5-min session in the swim tank because previous studies have reported that this modified protocol involving a single 5-min session was effective in detecting the anti-immobility effects of established antidepressants is stressed rats (Overstreet et al., 1994, 1995). Each test session lasting for 5 min was videotaped in a room dimly illuminated. The duration of immobility, which is defined as the lack of motion of the whole body except only those movement necessary to keep the animal's head above the water, was scored. After each test the cylinder was cleaned.

2.4. Corticosterone assay

Blood from each animal was collected at the time of sacrifice immediately after the termination of the treatments. Serum was prepared by 15 min centrifugation at 1615 × *g*. The corticosterone (CORT) concentration was determined by using the OCTEIA Corticosterone EIA kit according to manufacturer's instructions (American Laboratory Products Co.). The absorbance at 450 nm was determined by microplate reader (Wallac, VICTOR 1420, PerkinElmer). CORT concentration (ng/ml) was determined using standard curve.

2.5. Preparation of brain tissues

All animals were sacrificed between 10:00–11:00 a.m., i.e. immediately after treatments, by rapid decapitation with a guillotine (Harvard-Apparatus, USA). The hippocampus (HIPPO) was removed and frozen in liquid nitrogen until further preparation.

2.5.1. Preparation of cytoplasmic and nuclear extracts

Different subcellular fractions were prepared from the HIPPO. Frozen HIPPO was weighed and homogenized (1:2 w/v) in ice-cold 20 mM Tris–HCl (pH 7.2) buffer containing 10% glycerol, 50 mM NaCl, 1 mM Na_2EDTA, 1 mM Na_2EGTA, 2 mM DTT, and cocktail of protease (Sigma–Aldrich) and phosphatase inhibitors (Sigma–Aldrich) with 20 strokes of Potter-Elvehjem teflon-glass homogenizer. All operations were conducted at 0–4 °C. Samples were centrifuged 10 min at 2000 × *g* to give a supernatant and a pellet (containing nuclear and cell debris which was further processed for nuclear extracts). Supernatants were ultra-centrifuged for 1 h at 105,000 × *g*, and the final supernatants were used as cytosolic fraction. Nuclear