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Anxiety-like behavior in the elevated-plus maze tests and enhanced IL-1β, IL-6, NADPH oxidase-1, and iNOS mRNAs in the hippocampus during early stage of adjuvant arthritis in rats

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ABSTRACT

We studied anxiety-like behavior in the elevated plus-maze (EPM) tests in male Lewis rats on days 2 and 4 of adjuvant arthritis (AA). In plasma we analyzed C-reactive protein (CRP), albumin, ACTH, corticosterone, in the hippocampus the mRNA expression of interleukin-1β (IL-1β), interleukin-6 (IL-6), corticotrophin releasing factor (CRH), NADPH oxidases NOX1 and NOX2, and inducible NO-synthase (iNOS). EPM tests showed a higher anxiety index in AA rats on days 2 and 4 and reduction of total entries. On days 2 and 4 we found reduced plasma albumin, enhanced CRP, ACTH and corticosterone, and in the hippocampus enhanced mRNA for NOX1 and IL-1β in AA rats, on day 4 we found enhanced mRNAs for iNOS and IL-6, and reduced mRNA for CRH. The mRNA for NOX2 did not change on any experimental day. These results suggest enhanced anxiety, as well as locomotor impairment during the early phase of AA that correlate with enhanced mRNA expressions of parameters of oxidative stress NOX1, iNOS, and inflammatory cytokines IL-1β and IL-6 in the hippocampus.

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation, pain and enhanced levels of circulating proinflammatory cytokines. Patients with RA manifest symptoms of pain, depression, anxiety, fatigue, reduced socialization and loss of appetite. The prevalence of depression in RA patients is approximately 20%. A complex relationship has been shown between the exacerbation of these symptoms and cytokine levels produced by activated immune cells [16]. Social dysfunction with a less adaptive defensive profile, involving anxiety related conflicts, has been observed during the early stages of RA [12]. Moreover, it has recently been described that up-regulation of cytokines and chemokines predates the onset of RA symptoms [13].

Experimental studies investigating the potential mechanisms relevant to understanding the behavioral problems of RA patients are scarce. RA patients, upon diagnosis, usually experience pain and physical discomfort that may accentuate the effect of inflammation. One of the experimental models of human polyarthritis is adjuvant arthritis (AA) in rats. AA displays various neuroendocrine and immune changes in the very early stage of the disease, before joint damage and pain manifestation: peak levels of pro-inflammatory cytokines and enhanced C-reactive protein (CRP) in plasma, have been described on day 1 of the disease [20], and enhanced levels of nitrate on day 3 [15]. The hypothalamo-pituitary-adrenocortical (HPA) axis hormones are activated from day 7 of AA [21].

Immune challenges induced by the bacterial endotoxin lipopolysaccharide (LPS) or by interleukin-1β (IL-1β) in rodents are known to trigger loss of appetite, reduced locomotor and exploratory activities, reduced social interaction and anhedonia. This syndrome has been defined as sickness behavior, and reflects the complex communication between the immune cells and the brain as a part of host' defense mechanisms [7]. Administration of LPS to rats has been shown to enhance the anxiety index and reduced exploration activity as measured by the time spent in the open and closed arms in the elevated plus-maze (EPM) test [18]. Subsequently, similar behavioral effects have been demonstrated along with depressed locomotion in mice treated with LPS or IL-1β [26]. The mechanisms associated with the expression of sickness behavior are complex and involve multiple mechanisms, possibly other cytokines, although IL-1β is presumed to play a pivotal role [8]. The involvement of IL-6 in sickness behavior has not been conclusively established: in a series of anxiety and depression-related tests, using in IL-6 knock-out mice, no behavioral differences between IL-6 deficient and wild type mice have been observed [25]. On the other hand, reduced helplessness and/or

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anhedonia in IL-6 deficient mice compared to wild type mice have been observed [5].

One of the mechanisms involved in host defenses is oxidative stress in the brain; Microglia, in response to over-activation, produce excessive amounts of reactive oxygen-species (ROS) mediated by the NADPH oxidase system [3]. Co-activation of NADPH oxidase and iNOS promotes formation of highly toxic pro-oxidant, peroxynitrite [27]. Excessive production of NO-radicals after LPS treatment has been shown to cause neuronal cell death in slices from the rat hippocampus [14]. Moreover, in vivo oxidative stress in the hippocampus induced anxiety-like behavior and decreased locomotor activity in mice [19].

In the present experiments we used a model of early phase of AA (up to day 4). During this time confounding factors such as reduced mobility, due to edema, and/or pain are not expected. Nagakura et al. [17] observed no changes in pain threshold of uninjected paws for 8 postinoculation days. In our laboratory we did not find any c-fos activation in neurons from the lumbar spinal cord through day 9. We studied behavioral performance in the EPM, as well as the inflammatory and oxidative changes in the hippocampus. We measured anxiety and locomotion in the EPM test, plasma levels of CRP, albumin, ACTH, corticosterone, and in hippocampus the expression of mRNA for IL-1β, IL-6, CRH, NOX1, NOX2, and iNOS.

6-Week old male Lewis rats were obtained from Charles River (Charles River, Germany). Rats were housed four rats per cage in an animal room in the Department of Normal, Pathological and Clinical Physiology, Third Medical Faculty of Charles University, Prague. Animals were kept under a 12 h light/dark cycle (light on from 6:00 a.m. to 6:00 p.m.) with controlled humidity and temperature. Animals had free access to water and a standard pellet diet. Animals were allowed to adjust to laboratory conditions for one week prior to the testing. During this time they were handled daily by the experimenter to minimize handling stress during experiments. The animals were treated in accordance with the national law of the Czech Republic regarding the use of laboratory animals no. 167/1993 (fully compatible with European Community Council directives 86/609/EEC).

AA was induced in 7 week old rats by a single intradermal (2 cm from the base of the tail) injection of 100 μl of complete Freund's adjuvant (cFA). Control rats received saline. The study was comprised of 2 experiments.

In the first experiment the tests were performed in 14 control and 13 AA rats on days 2 and 4 after cFA or saline administration. The EPM apparatus consisted of two open arms (10 cm × 30 cm) and two closed arms (10 cm × 30 cm), which extended from a central platform (10 cm × 10 cm). The arms were elevated by a central support to a height of 40 cm above the floor. The open arms had Plexiglas railings 0.5 cm high along the edges of the maze. The closed arms had opaque side and end walls extending 40 cm above the arms and were open at the top. On each test day the rats were moved, in their home cages, to the experimental room 1 h before testing, for acclimatization. EPM tests were performed during the hours of highest activity, between 3 p.m. and 5 p.m. Each animal was tested once. The animal was placed on the central platform facing an open arm; its activity was then videotaped for 5 min. Between tests the maze was cleaned with ethanol. An arm entry was defined as all four paws of the rat in the arm. The number of entries into the open (OAE) and closed (CAE) arms and time spent in the open (OAT) and closed (CAT) arms were evaluated using ODLog Software (RegSoft.com). The total spent in the arms time (TT) was calculated as the sum of OAT and CAT.

In the second experiment control and AA rats (8 rats per group) were decapitated between 8:00 and 9:00 a.m. Trunk blood was collected into tubes containing EDTA, centrifuged, and stored at −70 °C. The hippocampus was removed from the rat brain, snap-frozen in liquid nitrogen, and stored at −70 °C until extracted for RNA. Assays: ACTH was determined by a double antibody technique using a commercial kit (Johnson & Johnson, Amersham, UK). The lower limit of the assay was 25 pg/ml and the upper limit was 1000 pg/ml. The assay sensitivity was 25 pg/ml. Corticosterone was extracted with methylene chloride (Merck, Germany) and analyzed by radioimmunoassay using specific antibodies (Sigma–Aldrich, Germany) and [1,2,6,7-3H]-corticosterone (Amersham, UK). CRP was estimated using a commercial specific rat CRP – ELISA kit (Immunology Consultants Laboratory, Inc., Oregon, USA). Albumin was measured spectrophotometrically using an Albumin (BCG)SYS 1 BMI Hitachi kit (Boehringer, Germany) based on the formation of albumin bromocresol green complex.

Total RNA was isolated from the right and left hippocampus separately using an RNAqueous® – 4PCR kit (Applied Biosystems, Czech Republic). DNase1 treatment was included to prevent DNA contamination. RNA was reverse transcribed to cDNA using the High-Capacity® cDNA Reverse Transcription kit (Applied Biosystems, Czech Republic). PCRs were performed using TaqMan® gene expression products (Applied Biosystems, Czech Republic). The multiplex PCR mix contained cDNA, universal PCR master mix and TaqMan probes. TaqMan eucaryotic 18S mRNA was used as endogenous control, which was labeled with VIC reporter dye. Target genes (IL-1β, IL-6, CRH, NOX1, NOX2 – gp91^{phox} and iNOS) were labeled with FAM reporter dye. Samples were analyzed in triplicate. Thermal cycling was done according to the manufacturer's instructions with 2 initial setup steps: 2 min at 50 °C and 10 min at 95 °C, and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Expression of target gene mRNA was quantified using an ABI Prism® 7000 Sequence Detector and software (Applied Biosystems, Czech Republic).

For evaluation of the EPM tests, a two-way ANOVA with repeated measures on factor day was used. Humoral parameters were calculated using one-way ANOVA followed by the Bonferroni test. The differences in mRNA expressions between control and AA groups were evaluated using the unpaired Student's *t*-test. In all tests, a difference was considered significant if $p < 0.05$. Results are expressed as means ± S.E.M.

Both control and AA rats showed a significant reduction of closed arm entries ($F_{(1,25)} = 10.11$; $p < 0.005$) and open arm entries ($F_{(1,25)} = 9.14$; $p < 0.01$) over time (interaction treatment × day, N.S.), presumably due to the habituation. However, AA rats showed a lower number of open arm entries compared to control animals ($F_{(1,25)} = 9.14$; $p < 0.01$), while the number of CAE did not differ between the groups. Consequently the total ambulatory activity of AA rats was significantly reduced ($F_{(1,25)} = 6.40$; $p < 0.02$). The percentage of open arm entries was lower in AA rats ($F_{(1,25)} = 7.14$; $p < 0.02$), as was the ratio of open arm entries/closed arm entries ($F_{(1,25)} = 4.53$; $p < 0.05$). As for the time spent in individual arms, AA rats spent less time in the open arms of the maze ($F_{(1,25)} = 4.63$; $p < 0.05$), and more time in closed arms ($F_{(1,25)} = 8.14$; $p < 0.01$), and consequently total time spent in the arms was not different. Furthermore, the percentage of the open arm time for AA rats was significantly reduced. ($F_{(1,25)} = 5.21$; $p < 0.05$) (Fig. 1).

The specific inflammatory indicator, CRP, was enhanced to the same extent on both examination days (AA day 2 vs. controls $p < 0.001$; AA day 4 vs. controls, $p < 0.001$). The inflammatory process was also reflected by a gradual reduction of the negative indicator of the hepatic acute phase response, albumin (AA day 2 vs. controls, $p < 0.001$; AA day 4 vs. AA day 2, $p < 0.001$) (Fig. 3). Plasma levels of ACTH and corticosterone were equally elevated on days 2 and 4. Elevated levels of ACTH in AA rats (AA day 2 vs. controls, $p < 0.001$; AA day 4 vs. controls, $p < 0.01$) were followed by enhanced corticosterone levels (AA day 2 or 4 vs. controls, $p < 0.01$) (Fig. 2).

Hippocampal mRNA expression is shown in Fig. 3. The columns represent average values from the right and left hippocampus, which were assayed separately. The mRNA for IL-1β showed a mild