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The involvement of 5-lipoxygenase activating protein in anxiety-like behavior

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ABSTRACT

The 5-lipoxygenase is an enzyme widely expressed in the central nervous system, where its activity is dependent on the presence the 5-lipoxygenase activating protein (FLAP) for the formation of leukotrienes, potent bioactive lipid mediators.

Emerging evidence has shown that the FLAP/leukotriene pathway may play a role in neuropsychiatric disease contexts.

In this study we investigated whether genetic deficiency of FLAP (FLAPKO) modulated some behavioral aspects in mice, and if this effect was age-dependent. While we observed that FLAPKO mice at 3 and 6 months of age did not different from wild type animals in the elevated plus maze, at 12 months of age they manifested a significant increase in anxiety-like behavior. By contrast, we observed no differences between FLAPKO mice and their controls at any of the three ages considered when they were tested for working memory in the Y maze paradigm. Additionally, while we found that cFOS protein and message levels were reduced in the brains of animals lacking FLAP, no changes for other transcription factors were detected.

Taken together our findings suggest a novel role for FLAP in the pathogenesis of anxiety-like behavior. Future studies of FLAP neurobiology may be attractive for development of anxiolytic therapeutics.

1. Introduction

The 5-lipoxygenase (5LO) is an enzyme abundantly present in the central nervous system, where its activity is regulated by the presence and availability of another protein called 5-lipoxygenase activating protein (FLAP). From a biochemical point of view they form a functional complex whose integrity is necessary for the full 5LO enzymatic activity, which catalyzes the oxidation of arachidonic acid to produce lipid molecules with complex signaling properties such as leukotrienes. While FLAP and 5LO function have been extensively interrogated in the context of inflammation, novel roles for these proteins are emerging in the central nervous system. Among them is new evidence that indicates FLAP/5LO may play roles in neurological and psychiatric contexts (Stewart et al., 2001; Whitney et al., 2001; Uz et al., 2008a).

We previously reported that knockout of 5LO results in anxiety-like behavior in female mice (Joshi and Praticò, 2011). However, because 5LO can also use molecules aside from arachidonic acid for substrate, it remains unclear whether the pro-anxiety effects of 5LO knockout we observed were specifically due to the elimination of leukotriene metabolites or due to disruption of other functions of 5LO. In this study, we used animals that possess 5LO but lack FLAP, a protein that is not known to participate in any signaling pathways apart from leukotriene generation to investigate how anxiety behavior is modulated in these animals. We found that knockout of FLAP (FLAPKO) produces an age-dependent increase in anxiety-like behavior in mice.

Associated with this anxiety behavior we found that steady-state protein levels as well as message of the transcription factor cFOS were reduced in the brains of the same animals. Our results are the first to describe the role of FLAP in the context of anxiety, and suggest that FLAP-associated changes of cFOS may be a relevant pathway involved in anxiety behavior.

Abbreviations: 5LO, 5-lipoxygenase; 5LOKO, 5-lipoxygenase knock-out; FLAP, 5-lipoxygenase activating protein; FLAPKO, 5-lipoxgenase activating protein knockout; CRE, cellular response element; CREB, cellular response element binding protein; pCREB, phosphorylated CREB; SYP, synaptophysin; PSD95, post-synaptic density protein 95.

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2. Materials and method

2.1. Animals

Separate groups of naïve female wild-type C57BL/6 (WT; The Jackson Laboratory) and FLAP knockout mice (FLAPKO; The Jackson Laboratory) at 3 months (WT, $n = 4$; FLAPKO, $n = 3$), 6 months (WT, $n = 5$; FLAPKO, $n = 4$), and 12 months (WT, $n = 5$; FLAPKO, $n = 4$) of age were used for this study. We have previously reported the anxiety behavior of the WT mice described in the current study. However, behavioral assays of both WT and FLAPKO animals in this study were conducted at the same time by the same experimenters on the same days (Joshi and Praticò, 2011). All mice were housed on a 12 h light/dark cycle in the Medical Research Building at the Temple University Health Sciences Campus, which is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Standard mouse chow and water were provided *ad libitum*. Animal procedures were conducted in accordance with the National Institute of Health guidelines for the use of experimental animals and approved by the Temple University's Animal Care and Use Committee. All animals were sacrificed 24 h after behavioral data was collected.

2.2. Behavioral paradigms

All animals were pre-handled for 3 days prior to testing. For all behavioral assays, animals were tested in randomized order and all tests were conducted by an experimenter blinded to the genotype. All apparatuses were cleaned with 70% ethanol between animal trials and allowed to dry completely. Elevated plus maze and Y-maze assays were performed in separate rooms, but testing was always performed in the same room and at the same time for each paradigm to ensure environmental consistency.

2.3. Elevated plus maze

The elevated plus maze behavioral paradigm was carried out as described in Joshi and Praticò (2011). Briefly, anxiety-like behavior was assessed by using the elevated plus maze (SD Instruments, San Diego, CA) behavioral paradigm. Room lighting was adjusted such that closed arm light levels were maintained at ~160 lux and open arm light levels were maintained at approximately ~200 lux. Each mouse was placed in center square facing a closed arm and was allowed to freely explore for 10 min while being video recorded. An entry was counted when the mouse had all four paws in an arm.

2.4. Y-maze

The Y-maze behavioral paradigm was carried out as described in Chu et al. (2012). Briefly, each mouse was placed in the center of the Y-maze and allowed to explore freely through the maze during a 5-min session for the assessment of spontaneous alternating behavior. The sequence and total number of arms entered were video recorded. An entry into an arm was considered valid if all four paws entered the arm. An alternation was defined as three consecutive entries in three different arms (i.e. 1, 2, 3 or 2, 3, 1, etc). The percentage alternation score was calculated using the following formula: (Total alternation number/total number of entries − 2)*100.

2.5. Immunoblotting

Mouse brain homogenates were extracted in RIPA buffer as previously described (Joshi et al., 2012). Total protein concentration was determined by using BCA Protein Assay Kit (Pierce, Rockford, IL). Samples were electrophoretically separated using 8–10% Bis–Tris gels (Bio-Rad, Richmond, CA), according to the molecular weight of the target molecule, and then transferred onto nitrocellulose membranes (Bio-Rad). They were blocked with Odyssey blocking buffer for 1 h and then incubated with primary antibodies overnight at 4 °C. After three washing cycles with T-TBS, membranes were incubated with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience) at 22 °C for 1 h. Signals were developed with Odyssey Infrared Imaging Systems (LI-COR Bioscience). Actin was always used as an internal loading control. Primary antibodies used were as follows: cFOS (1:200, Santa Cruz) pCREB (1:200, Cell Signaling), CREB (1:200, Cell Signaling), PSD95 (1:200, Cell Signaling), synaptophysin (1:1,000, Sigma–Aldrich), actin (1:1,000, Santa Cruz).

2.6. Quantitative real-time RT-PCR

RNA from cortical tissue of mice was extracted and purified using the RNeasy mini-kit (Qiagen), and used as previously described (Chu and Pratico, 2011). Briefly, 80 ng of total RNA was used to synthesize cDNA in a 20 μl reaction using the RT^2 First Strand Kit for reverse transcriptase-PCR (Super Array Bioscience). cFOS gene was amplified by using the corresponding primers obtained from Super Array Bioscience. GAPDH was used as an internal control gene to normalize for the amount of RNA. 2 μl of cDNA was added to 25 μl of SYBR Green PCR Master Mix (Applied Biosystems, CA). Each sample was run in triplicate and analysis of relative gene expression was done by using the $2^{-\Delta\Delta Ct}$ method.

2.7. Immunohistochemistry

Mouse brains were prepared for immunohistochemistry as previously described (Joshi et al., 2012). Briefly, 6 μm brain sections were incubated overnight with primary antibody against cFOS (1:100, Santa Cruz) after blocking in 2% fetal calf serum with citric acid being used to retrieve antigen. Sections were incubated with secondary antibody and finally developed using the avidin–biotin complex method (Vector Laboratories) with 3,3-diaminobenzidine as chromogen.

2.8. Statistical analysis

Data are presented as the mean ± standard error of the mean. For elevated plus maze, percent of time spent in closed and open arms [(total time in arms/600 s)*100] and percent of closed and open arm entries [(total arm entries/total entries)*100] were calculated for each animal. For Y-maze, the percentage alternation score was calculated using the following formula: (Total alternation number/total number of entries − 2)*100. The two-tailed student *t*-test with an alpha of $P < 0.05$ was used to define significance between groups.

3. Results

3.1. Knockout of FLAP results in the development of anxiety-like behavior

We first assessed anxiety-like behavior in animals that lack FLAP. As shown in Fig. 1A, at 3 months of age, no differences were found between WT and FLAPKO animals in percentage of time spent in closed ($P < 0.87$) or open arms ($P < 0.15$). At 6 months of age, FLAPKO animals started displaying tendencies toward a pro-anxiety phenotype, with more time spent in the closed arms ($P < 0.08$) and less time in the open arms ($P < 0.12$). By 12 months of age, FLAPKO animals spent significantly more time in the closed arms ($P < 0.0007$) and less time in the open arms ($P < 0.001$). As