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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 the 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.

Keywords

Five-lipoxygenase activating protein (FLAP); animal models; behavior; anxiety

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Contributors
Y.B.J. and D.P. designed the study.
Y.B.J. performed the experiments, collected the results, and analyzed the data.
Y.B.J. and D.P. discuss and interpret the data and results.
Y.B.J. wrote the first draft of the manuscript.
Y.B.J. and D.P. approved the final version.

Conflict of interest
The authors declare that they have no relevant conflicts of interest that might influence the study design, data acquisition, interpretation, or other parts of this work.

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.

Materials and Method

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 hours 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 24h after behavioral data was collected.

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.

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.

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.

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 hr and then incubated with primary antibodies overnight at 4°C. After three washing cy cles with T-TBS, membranes were incubated with IRDye 800CW or IRDye 680CW-labeled secondary antibodies (LI-COR Bioscience) at 22°C for 1 hr. 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).

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 Praticò, 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.

Immunohistochemistry

Mouse brains were prepared for immunohistochemistry as previously described (Joshi *et al.,* 2012). Briefly, 6 μm brain sections were incubated over night 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.

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.

Results

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 towards 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 shown in Fig. 1B, FLAPKO animals did not differ from WT counterparts in total entries made in the elevated plus maze at 3 months of age ($P<0.90$), but had lower entries at both 6 months ($P<0.002$) and 12 months ($P<0.002$).

Absence of FLAP does not influence working-memory

To investigate whether knockout of FLAP modulated behavior in a context that does not evoke an anxiety response, we challenged all animals in the Y-maze paradigm. As shown in Fig. 2A, no differences were observed in total arm entries at 3 months ($P<0.09$), 6 months ($P<0.49$), or 12 months ($P<0.14$). Similarly, there was no change in alternating behavior as shown in Fig. 2B at 3 months ($P<0.55$), 6 months ($P<0.66$) or 12 months ($P<0.69$).

cFOS is reduced in the brains of 12 month-old FLAPKO mice

Because recent studies have indicated that cFOS protein may be aberrantly regulated *in vivo* in several models of anxiety, and others have noted that that inhibition of FLAP prevents induction of cFOS, we investigated whether cFOS protein level was altered in the brains of FLAPKO animals (McQuade, *et al.,* 2006; Muigg *et al.*, 2009; Haliday *et al.,* 1991). As shown in figure 3, we did not find any changes between WT and FLAPKO animals in any of the assayed proteins at 3 and 6 months. However, at 12 months of age, compared to control, we found significantly lower cFOS expression in FLAPKO animals ($P<0.02$). Additionally, the same tissues were assayed for CREB and phosphorylated CREB, postsynaptic density protein 95 (PSD95), and synaptophysin (SYP), but no significant changes were detected for any of these proteins (Figure 3A,B). To see whether the change in cFOS protein levels was accompanied by a similar change in its mRNA levels, we performed quantitative real-time PCR. As shown in Fig 3C we found that cFOS mRNA was significantly reduced in the brains of FLAPKO animals compared to control ($P<.0.001$).

To further support our findings, we assayed for cFOS using immunohistochemistry in the brains of animals, and as shown in Fig. 3D, we found reduced cFOS staining in cortical neurons of FLAPKO animals compared to WT.

Discussion

In this study we found that FLAPKO animals, indistinguishable from WT mice at 3 months of age in the elevated plus maze, which is used to measure anxiety-like responses in rodents, showed an anxiety phenotype by 12 months. Associated with the development of this anxiety phenotype we found a decline in brain levels of the transcription factor cFOS at the protein as well as message level. By contrast, we did not observe any significant differences between the two groups of animals when CREB, another transcription factor, or PSD95 and synaptophysin, two synaptic proteins, were assayed. Finally, FLAPKO were not different from their controls when spontaneous alternating behavior in the Y-maze, which is considered a measure for working memory, was tested.

Taken together these findings suggest that cognition is not globally altered in these animals and that the effect of FLAPKO on anxiety is specifically associated with modulation of cFOS levels, a marker of general neuronal activity previously implicated in numerous neuronal processes (Williams *et al.* 1990; Abraham *et al.* 1991). FLAP is an indispensable player in the 5LO-dependent formation of important lipid mediators (Dixon *et al*, 1990). While FLAP and 5LO have been well studied in contexts such as inflammation, new roles for these proteins are being discovered in neurological and psychiatric contexts (Stewart *et al.,* 2001, Whitney *et al.,* 2001; Uz *et al.,* 2008). FLAPKO animals are healthy, breed normally and do not have any obviously apparent motor deficits, but in the current study we found that at 6 and 12 months of age they demonstrated significantly lower total arm entries in the elevated plus maze compare to WT animals. While this observation could in part suggest that the difference in the anxiety behavior of the FLAPKO animals might have been due to a locomotor difference from WT animals, it is important to stress that we found similar behavior in total entries also in the Y-maze between genotypes. Since locomotor activity is known to diminish as a function of age, and both WT and FLAPKO animals exhibited fewer arm entries in both behavioral paradigms at 6 and 12 months compared to 3 months, we interpret our findings to mean that WT and FLAPKO animals follow different trajectories of locomotor decline (Fahlstrom *et al.,* 2011).

Our results bring to light a relationship between cFOS and anxiety, which has been implicated in other studies as well. However, depending on different experimental treatments, measurements of the anxiety phenotype and stimuli employed, cFOS has been reported to be up- or down-regulated in the context of anxiety, which suggests that cFOS is responsible for a complex phenomenology (Meyza *et al.* 2011; Shaw *et al.*, 2011; Beiderbeck *et al.,* 2012). This may also be partly due to the fact that cFOS is highly sensitive to environmental stimuli, and so even difference in length of time after which animals are subjected to behavioral assessment and then are sacrificed can alter its levels. In our experimental setting, which is characterized by global brain knockout of FLAP, cFOS reduction is associated with an age-dependent development of an anxiety phenotype. Earlier work in cultured adipogenic TA1 cells as well as in rat spinal cord has also shown cFOS is downregulated by the addition of FLAP/5LO pathway inhibitors (Haliday *et al.* 1991; Yoo *et al* 2009. It is important to note, however, that while earlier work supports the idea that FLAPKO directly downregulates cFOS, we are unable to rule out the possibility that changes in cFOS are compensatory rather than causally related. This is especially important to note given that we observed tendencies in anxiety behavior at 6 months of age without observing similar trends in cFOS protein at the same age. Future work dissecting the pathway between FLAP and cFOS would be critical to better answer this question.

It is curious that knockout of FLAP only resulted in anxiety-like behavior at 12 months and not at 3 or 6 months. One possible explanation of our findings could be because the FLAP/5LO enzymatic pathway is more active in the aging brain (Chinnici *et al.*, 2007). To this

end, we speculate that the FLAP/5LO system is privileged towards later in life, and that we do not observe FLAPKO-mediated anxiety behavior in younger rodents because other signaling pathways may compensate. Interestingly, the neurobiological substrates of anxiety disorders are thought to be modulated in aged individuals, and clinical data from the United States National Institute of Mental Health on suicidality, which is associated with anxiety disorders, show that adults older than 65 have a dramatically increased risk compared to the general population (Sareen *et al.*, 2005; Blay and Marinho, 2012). Regardless, it probably unlikely that the FLAP/5LO system directly modulates anxiety-behavior, but rather is involved in multiple pathways that are altered as a function of aging which are relevant to anxiety. Intriguingly, FLAP inhibition alters GluR1 receptor dynamics, and we speculate that this could also be at play in FLAP-mediated behavioral alterations (Imbesi *et al.* 2007; Uz *et al.* 2008b).

Our present work informs the current discussion surrounding classes of drugs that target the FLAP/5LO/leukotriene pathway which include the leukotriene receptor-blocker montelukast (trade name Singulair) and the 5LO inhibitor zileuton (trade name Zyflo), which have been linked to reports of anxiousness, mood changes and suicidal ideation (Schumock *et al.* 2011a; Schumock *et al.* 2011b). Since allergic and asthmatic conditions for which such drugs are prescribed independently elevate risk for adverse neuropsychiatric events, some commentators have expressed the need for large observational cohort or case-control studies investigating the relationship between 5LO/leukotriene receptor drugs and behavioral changes. Our results offer support to the idea that targeting the FLAP/5LO pathway may result in anxiety. Because gene polymorphisms in 5LO and FLAP have been described, it is also possible that potential neuropsychiatric risk is increased only in certain subpopulations —interestingly, single nucleotide polymorphisms in leukotriene A4 hydrolase is associated with depression, but only in women (Zhao *et al* 2009). Future clinical studies assessing behavioral changes that employ pharmacogenetic strategies to fully understand how both drug exposure and genetic vulnerability interact would be particularly powerful.

In conclusion, our work is the first to describe a relationship between FLAP and anxiety which seems to be associated with a reduction in cFOS. Exploration of FLAP-mediated changes in brain levels of cFOS could be fruitful for development of anxiolytics as well as a better understanding of the molecular neurobiology of anxiety.

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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	phorphosylated CREB
SYP	synaptophysin
PSD95	post-synaptic density protein 95

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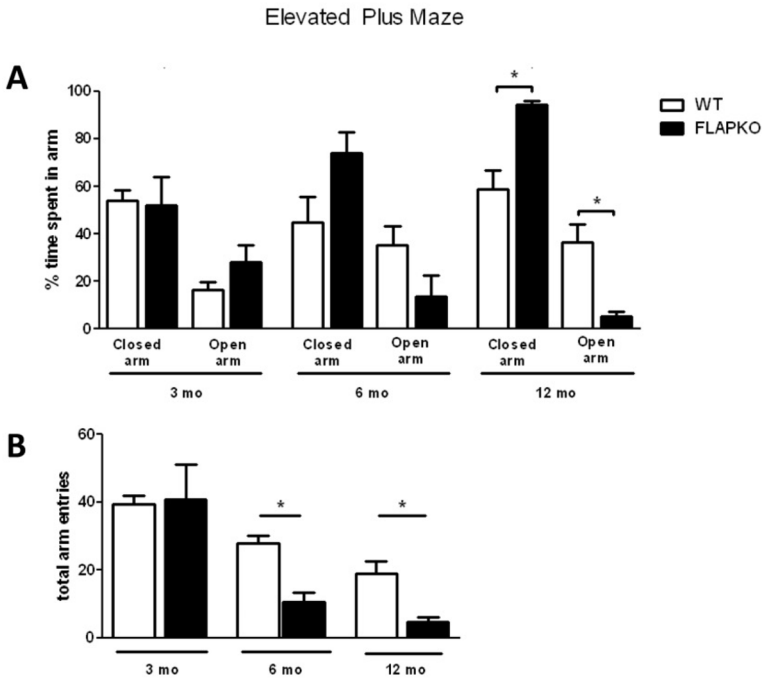

Figure 1.

FLAPKO mice display an anxiety phenotype. (A) Percentage of time spent in closed and open arms by WT and FLAPKO animals. (B) Total entries made in the elevated plus maze by WT and FLAPKO animals.

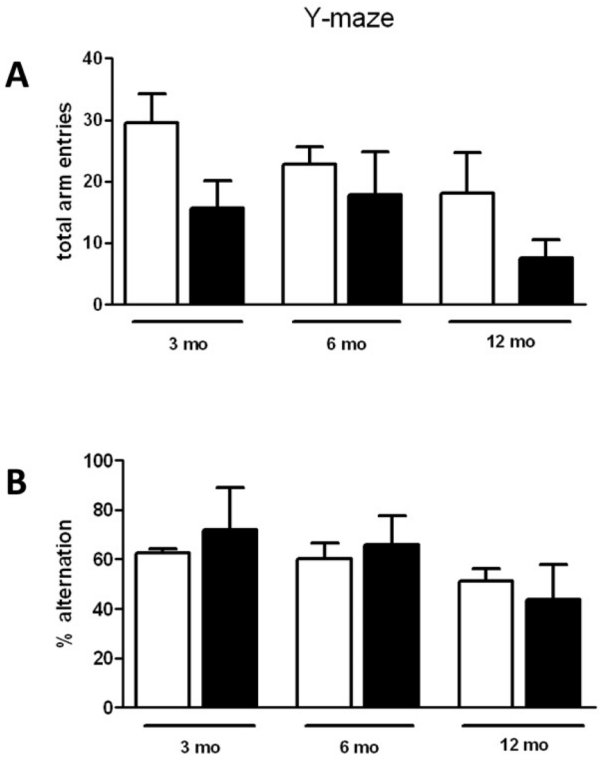

Figure 2.
Y-maze behavior of FLAPKO animals is unremarkable. (A) Total arm entries in the Y-maze by WT and FLAPKO animals. (B) Percentage alternation in the Y-maze by WT and FLAPKO animals.

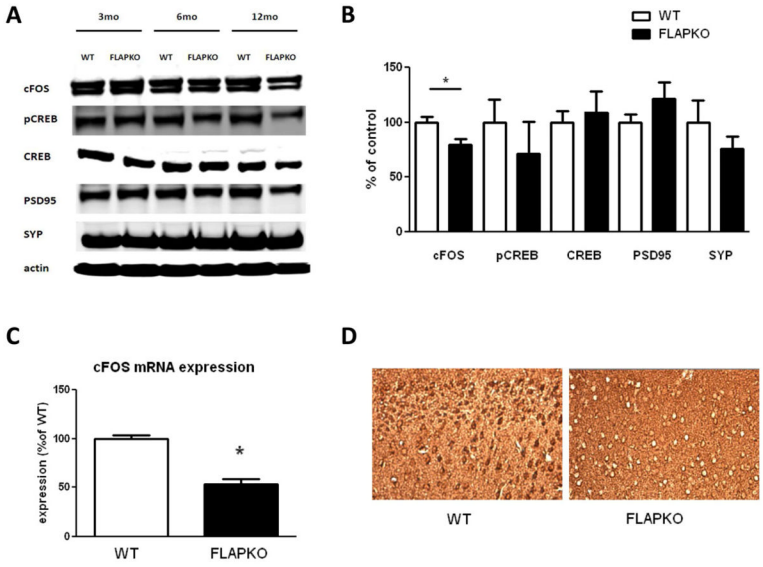

Figure 3.

cFOS is reduced in the brains of FLAPKO animals. (A) Representative western blot analyses of cFOS, CREB, pCREB, PSD95, synaptophysin (SYP) in brain homogenates from wild type (WT) and FLAPKO mice at 3, 6 and 12 months of age. (B). Densitometric analyses of the immunoreactivities to the antibodies shown in the previous panel (*$p < 0.02$). (C) Quantitative real time RT-PCR for cFOS mRNA brain homogenates from 12 month-old wild type (WT) and FLAPKO animals. (D) Representative immunohistochemical staining for cFOS positive areas in brain sections of 12 month-old wild type (WT) and FLAPKO mice. Value represents mean ± standard error of the mean.