The role of endocannabinoid signaling in the modulation of responses to environmental stimuli

Doctoral Theses

Manó Aliczki

Semmelweis University
János Szentágothai School of Neurosciences

Supervisor: Dr. József Haller, D.Sc., scientific advisor

Official referees: Dr. György Bárdos, D.Sc., university docent
Dr. Júlia Timár, Ph.D., university docent
Chairman of committee of comps: Dr. Béla Halász, member of the Hungarian Academy of Sciences, professor emeritus
Members of committee of comps: Dr. Tibor Bartha, D.Sc., professor
Dr. Gergely Zachar, Ph.D., research assistant

Budapest
2013

INTRODUCTION

The discovery of the components of the endocannabinoid signaling system (the cannabinoid receptors, the endogenous ligands and their metabolizing enzymes) and of the retrograde endocannabinoid signaling pathway as whole, revealed an important new mechanism of the function of the central nervous system at the end of the 20th century. This discovery opened new perspectives in the understanding of the fine functioning of the central nervous system and –since the endocannabinoid system is a crucial component of the neural regulation of emotional behavior– it may also contribute to the therapy of psychiatric disorders which constitute a serious burden to the society. Therefore, the detailed exploration of this system attracted great attention.

In the recent years, the main direction of endocannabinoid research shifted from the manipulation of cannabinoid receptors with receptor agonists or antagonists towards more specific indirect manipulations e.g. the blockade of endocannabinoid reuptake and metabolism. Although the findings obtained so far underscore the importance of endocannabinoid signaling in behavioral control, the characteristics of cannabinoid effects on behavior still remain unclear, most probably because of the complexity of the mechanisms affected by this signaling pathway, which appear to depend largely on environmental conditions.

As an attempt to clarify the reasons of the discrepant findings obtained earlier, we studied here the behavioral effects of cannabinoids from a new perspective. We indirectly enhanced endocannabinoid signaling by the blockade of endocannabinoid metabolism, and then we studied how subjects responded to different testing conditions; particularly we studied how increased endocannabinoid signaling altered the strategy adopted by subjects when coping with environmental challenges. The impact of endocannabinoid signaling on coping responses was studied in conjunction with the hypothalamus-pituitary-adrenal gland-axis (HPA-axis), an endocrine cascade known to be affected by endocannabinoids also known for its role in coping.

In the first part of our work, we enhanced anandamide (AEA) signaling by the blockade of its degrading enzyme fatty acid amide hydrolase (FAAH) and studied the impact of this treatment in behavioral tests performed under varying levels of aversiveness. In addition, we also investigated the effects of AEA on coping in test paradigms directly assessing coping styles.

During our studies, a specific inhibitor of monoacilglycerol lipase (MAGL), the hydrolyzing enzyme of the other endocannabinoid, 2-arachidonoylglycerol (2-AG) became available, which allowed us to study the behavioral roles of 2-AG signaling. As first steps, we investigated (i) the effects of MAGL blockade in behavioral tests performed under varying levels of aversiveness, and (ii) the relationship of these effects with stress responses. The latter approach was justified by both our own observations on the interactions between MAGL blockade and behavioral effects and by the known interactions between endocannabinoid signaling and HPA-axis. It is also worth noting that stress reactivity plays a crucial role in the modulation of coping styles. Besides behavioral testing we also measured different physiological parameters, e.g. changes in body temperature and plasma corticosterone levels.

AIMS

The questions addressed by our studies were the followings:

I. The effects of FAAH blockade on responses to environmental stimuli and coping styles

1. Does the blockade of FAAH affect an important characteristic of coping styles, e.g. the responsiveness to environmental stimuli in the rat elevated plus-maze?
2. Does the blockade of FAAH activity alter behavioral patterns in a paradigm specifically used to study coping styles e.g. the tail pinch test performed in rats?
3. Are the effects of FAAH inhibition on coping styles mediated by type-1 cannabinoid receptors (CB_1R)?
4. Is it possible that the effects of FAAH blockade on coping styles are secondary to the anxiolytic properties of the treatment?
5. Do coping styles or FAAH blockade affect pain thresholds and can these effects alter behavior shown in the tail-pinch test of coping?
6. Does FAAH inhibition alter the impact of water temperature on behavior shown by mice in the forced swimming tests i.e. in a different species and a test involving a different sensory modality?

7. Does the blockade of FAAH activity alter the behaviors shown by mice in the back test of coping?

II. The effects of MAGL blockade on behavioral and endocrine stress response

8. Does MAGL blockade alter locomotor activity and anxiety in more aversive unfamiliar environments and what is the temporal dynamics of these effects?
9. Do the above effects depend on the animal strain employed?
10. Does MAGL blockade change locomotion and body temperature in less aversive, familiar environments and what is the temporal dynamics of these changes?
11. Does the inhibition of MAGL activity change basal or stress-induced corticosterone levels and what is the temporal dynamics of these effects?
12. Do the behavioral effects of MAGL blockade occur in an HPA-axis-dependent manner?

MATERIAL AND METHODS

Subjects

Subjects were 2-3 months old male Wistar rats weighting 300-350 g and 2-3 months old CD1 and C57BL/6J mice, weighting 30-35 g, respectively (Charles River Laboratories, Budapest). Animals were kept in a 12:12 hours light-dark cycle, tap water and rodent food was available *ad libitum*. Rats were housed in groups of 4, while mice were housed individually, as mice form strong hierarchy when housed in groups, which can alter the results of behavioral experiments. Socially housed rats were isolated for 3 days before experimentation. Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Behavioral test and analysis

Behavioral experiments were conducted in the early hours of the light phase in a dedicated experimental room. The behavior of subjects was recorded with a digital camcorder during testing. Video recordings were later analyzed with the H77 event recorder software (Haller József, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest).

Elevated plus-maze test (EPM)

The elevated plus-maze test is one of the most frequently used testing procedures to study the anxiety of rodents in biomedical research. The test apparatus is elevated 80 cm from the ground and consists of 2 closed and 2 open arms, connected by a central platform. Subjects are placed on the central platform and their behavior is recorded for 5 minutes. Time spent in the open arms indicates anxiety, while entries in the closed arms represent locomotor activity. We also analyzed "ethological" parameters, particularly risk assessment behavior as an indicator of anxiety.

Open-field test (OF)

The open-field test is a widely used procedure to study the locomotor activity of laboratory rodents. The open-field apparatus is a white plastic box, covered with transparent Plexiglas lids. Subjects are placed in one of the corners of the box and their behavior is recorded for 5 minutes. The distance traveled in the apparatus represents locomotor activity, while time spent in the central area of the open-field indicates anxiety.

Forced swimming test (FST)

The forced swimming test is a widely used paradigm in the studies of depression-related behavior in laboratory rodents. The apparatus is a glass cylinder filled with tap water. In our studies we did not use the procedure for its original purpose, i.e. investigation of treatment effects on depression, but we measured the responses of the subjects to different environmental contexts. Subjects were placed in the water and their behavior was recorded for 6 minutes. We measured three behavioral indicators: floating, struggling and swimming, respectively. In some of our studies we employed the forced swimming procedure as a stressor instead of a behavioral testing procedure.

Tail pinch test

We used the tail pinch test for studying coping styles in rats. We placed a binder clip on the tail of our subjects and recorded their behavior

for 5 minutes. We conducted a detailed ethological analysis on the behavior of the subjects. We studied two of the observed parameters, clamp gnawing and clamp-independent exploration, respectively, as these two parameters occurred in 85% of the testing time. We considered the former a „problem-oriented", active response, while the latter a „problem ignoring", passive response. Animals were categorized to primarily active or passive coper groups, based on the ratio of these two behavioral parameters.

Back test

The back test was originally developed to piglets then later adapted to laboratory rodents as well. Subjects were manually restrained on their backs for 1 minute and their behavior was recorded. Coping styles were determined by the ratio of time spent with "escape attempts" and "resting", respectively.

Hot plate test

The hot plate test is a widely used behavioral test for studying pain threshold. Subjects were placed on a hot plate, covered by a transparent Plexiglas box. Animals could habituate to the box for 3 minutes then the temperature of the plate started to increase gradually. When the animal licked one of its paws for the first time, the increase in temperature stopped and the current temperature of the plate was recorded.

In vivo biotelemetry measurements

For the measurements of body temperature and home-cage locomotor activity, subjects were implanted with *in vivo* biotelemetry e-mitters (HR E-Mitter, PDT-4000; Mini Mitter Company, Bend, OR, USA). Signals transmitted by the e-mitter were received with a receiver unit under the cage of the subjects. The receiver transmitted the signals to the VitalView Data Acquisition System software (Mini Mitter Company, Bend, OR, USA) running on a computer. Prior to pharmacological treatments, 2 hours of baseline levels were recorded for both parameters. After treatments we studied the changes to these baseline levels.

Blood sampling and hormone measurements

Blood samples for hormone measurements were collected from tail incisions or trunk blood, respectively. Samples were centrifuged then blood

plasma was stored at -20°C until hormone measurements. Corticosterone levels were measured with radioimmunoassay. ^{125}I-labelled corticosterone-carboximethyloxime-tyrosine-methyl esther was used as tracer. The interference with plasma transcortin was under 0.05%, except deoxicorticosterone (1.5%) and progesterone (2.3%). The sensitivity of the assay was 1 pmol/ml. Intra- and inter-coefficient of variation was 10 and 15%, respectively.

Statistical analysis

Data were analyzed with one-, two-way or repeated measures analysis of variance (ANOVA). ANOVA assumptions were evaluated by the Levene's test; where assumptions were not fulfilled, data were square root transformed. For pairwise comparisons, we conducted *post-hoc* Duncan test. Correlations between variables were assessed with the Spearman correlation test, while distributions were compared with χ^2–test. For statistical analyses we used the Satistica 11 software (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

I. The effects of FAAH blockade on responses to environmental stimuli and coping styles

Increases in environmental aversiveness decreased open arm exploration in the rat EPM. FAAH blockade-induced enhanced AEA signaling dampened these responses to aversive conditions. Dampened reactivity might be reminiscent of an active coping style, as active copers are characterized by autonomous behavior that is weakly influenced by environmental conditions. This assumption was strengthened by our findings obtained in the tail pinch test of coping, where FAAH inhibition increased the share of active copers. We also showed that the effects of FAAH blockade on coping styles were mediated via CB_1R in rats and were not the secondary to decreases in anxiety induced by the treatment. The effects were not attributable to changes in pain perception as this was neither altered by FAAH blockade nor by coping styles. Similar to rats, mice adapted their FST behavior to environmental conditions. Inhibition of FAAH activity dampened these responses. As in the case of rats, we assumed that these changes were due to a switch towards a more active way

of coping. This assumption was supported by the findings of the back test study, where FAAH blockade promoted an active coping style.

Thus, FAAH blockade dampened the impact of environmental conditions on behavior and promoted active responses to challenges. We interpret these findings in terms of coping styles, as active coping is characterized by problem-focused and routine-driven behaviors that are weakly affected by environmental conditions. We hypothesize that enhanced AEA signaling promotes an active coping style, which assumption is in line with previously reported indirect evidence.

In addition to their theoretical implications, these findings suggest therapeutic options of the blockade of AEA hydrolysis as well. Individual coping styles might have a number of therapeutic possibilities, particularly in psychiatry. Promoting active coping can be an important component in the therapy of particular disorders, such as disorders characterized by passive coping, e.g. depression or disorders characterized by exaggerated, inadequate responses to environmental stimuli, e.g. various forms of anxiety disorders such as phobias or posttraumatic stress disorder (PTSD). Blockade of AEA metabolism might suggest an important next step in the therapy of PTSD, a disorder constituting a serious burden to the society, as this disorder remains to be difficult to treat, and identifying alternative treatment options is imperative.

II. The effects of MAGL blockade on behavioral and endocrine stress response

Inhibition of MAGL activity increased locomotor activity and decreased anxiety in unfamiliar environments (OF and EPM, respectively). These effects occurred 80 minutes after treatment, much later than the biochemical effects of the compound (e.g. the enhancement of brain 2-AG levels). The effects were most probably general as they were present in both mice strains studied by us. In contrast, the effects of MAGL inhibition developed rapidly in familiar context (home cage). In the home-cage, the treatment dampened injection-induced increases in body temperature 20 min after injection, while at the same time point it abolished the injection-induced changes in locomotion patterns. Furthermore, MAGL inhibition increased the time spent with immobility approximately 120 minutes after treatment. This was in contrast with our findings obtained in unfamiliar environments where the very same treatment increased locomotor activity at this time point. We also showed that MAGL blockade rapidly and

transiently increased basal corticosterone levels. Inhibition of MAGL activity did not alter corticosterone stress responses. We also studied the interactions between the behavioral and endocrine effects of MAGL blockade and showed that locomotor effects were independent of corticosterone synthesis while anxiolytic actions were partly caused by the MAGL blockade-induced increase in basal corticosterone synthesis as these effects were abolished by corticosterone synthesis blockade.

Based on these findings we suggest that enhanced 2-AG signaling induced by the blockade of MAGL activity rapidly dampens stress-induced changes in locomotion and body temperature. The effects of 2-AG on behavioral and physiological stress responses are rapid and transient in less aversive conditions e.g. in the home-cage. In contrast, MAGL inhibition decreases anxiety and increases locomotor activity in a lasting, tonic manner in aversive –unfamiliar– environments. Furthermore, we showed that enhanced 2-AG signaling increases basal HPA-axis activity, which seems responsible for certain behavioral effects of MAGL blockade. Corticosterone synthesis-independent behavioral effects were also shown, which are likely mediated by 2-AG-dependent neural mechanisms. We hypothesize that the interactions between MAGL blockade and HPA-axis have a role in the context-dependency and context-dependent temporal dynamics of the effects induced by MAGL blockade.

III. Summary

The specific pharmacological blockade of the MAGL enzyme became possible recently only; as such we had no sufficient time to characterize in detail the role of enhanced 2-AG signaling in challenge responding. Nevertheless, we showed that environmental conditions alter the impact of 2-AG on locomotion; moreover, environmental conditions alter the temporal dynamics of behavioral effects. Based on a study published by our group earlier, a recent study showed that MAGL blockade dampens the anxiety-enhancing effects of aversive conditions. As environmental conditions alter the behavioral effects of MAGL blockade in a manner similar to that seen with FAAH blockade, it is possible that 2-AG signaling plays a role in the modulation of coping strategies as well. This assumption is strangthened by our findings showing that MAGL blockade altered HPA-axis activity, which is an important factor of coping. Therefore, studying the behavioral effects of MAGL blockade in conjunction with environmental aversiveness –as we have done it in the case of FAAH inhibition– appears important. Although the information on

the effects of MAGL blockade is scarce, the available information suggests that this endocannabinoid, similar to anandamide, has an important role in challenge responding. Further studies are necessary on this issue in the future.

Summarizing our results, endocannabinoid signaling had no specific effects on behavior but altered the responses to environmental challenges. Enhanced AEA signaling promoted active coping, which seemed to be a general effect as it occured in different species and paradigms, respectively. However, enhanced 2-AG signaling became available recently, our findings on its behavioral and endocrine effects suggest that this endocannabinoid is involved in the modulation of coping similarly as AEA.

CONCLUSIONS

Based on our findings we can conclude the following:

1. Enhanced AEA signaling by the blockade of FAAH activity promotes active coping strategy.
    a. Inhibition of FAAH activity induced dampened responses to environmental changes, a characteristic of active coping style.
    b. Enhanced AEA signaling directly promotes active coping.
    c. These effects are not attributed to changes in pain perception or anxiety, enhanced AEA signaling directly affected coping styles *via* CB_1R mediated mechanisms.
    d. The above effects are most likely general, as they occurred in different species and test paradigms as well.

2. Enhanced 2-AG signaling by the inhibition of MAGL caused different effects in differentially aversive environments, partly *via* altered stress reactivity.
    a. Enhanced 2-AG signaling increases locomotor activity, decreases anxiety and dampens changes in locomotion and body temperature induced by aversive stimuli.

b. 120 minutes after treatment blockade of MAGL activity differentially affected locomotor activity in familiar and unfamiliar environment, respectively.

c. The effects occurred in a slow, tonic manner in unfamiliar environment, while in a rapid, phasic manner in familiar context. The effects did not depend on animal strain.

d. MAGL blockade increased basal HPA-axis activity, while did not alter stress reactivity.

e. Anxiolytic effects induced by enhanced 2-AG signaling were partly mediated *via* the increased basal HPA-axis activity, while effects on locomotor activity were independent of altered HPA-axis function.

SUMMARY

Endocannabinoid signaling plays a crucial role in the modulation of emotional behavior. This role is, however, still unclear, as the emotional effects of cannabinoid manipulations depend on the aversivity of the environmental context in a number of reports. The aim of this study was to clarify the context-dependency of cannabinoid effects on emotional behavior. We enhanced endocannabinoid signaling by the inhibition of endocannabinoid catabolism and investigated its behavioral and physiological effects in differentially aversive contexts.

Enhanced anandamide signaling by the blockade of fatty acid amide hydrolase dampened behavioral reactivity to changes in environmental aversiveness and also promoted problem-oriented coping patterns when the individual faced environmental challenges. These changes are reminiscent of an active coping strategy, as the behavioral response to challenges is problem-oriented, based on routines and weakly influenced by environmental stimuli in active copers.

In the second part of our study, we used a newly developed pharmacological compound to enhance 2-arachidonoylglycerol (2-AG) signaling by the inhibition of monoacilglycerol lipase activity, and then we studied the behavioral effects of this treatment in different testing contexts. The treatment decreased anxiety and increased locomotion in aversive environments, but dampened stress-induced changes in locomotion and body temperature in a less aversive contexts. Intriguingly, the temporal-

dynamics of these effects were context-dependent, as in less aversive environments they occurred in a rapid, phasic manner, while they developed in a rather slow, tonic manner in aversive context. In addition, we showed that enhanced 2-AG signaling increased basal HPA-axis activity, which was partly responsible for the anxiolytic actions.

Taken together, endocannabinoid signaling modulates the neural interpretation of environmental stimuli and the behavioral response rather than specifically affecting emotions. This regulatory role is mediated through the promotion of active coping strategy and interactions with the stress response.

PUBLICATIONS OF THE AUTHOR

Publications that form the basis of the Ph.D. dissertation

1. Haller, J., Goldberg, S.R., Gyimesiné Pelczer, K., **Aliczki, M.**, Panlilio, L.V., 2013. The effects of anandamide signaling enhanced by the FAAH inhibitor URB597 on coping styles in rats Psychopharmacology DOI: 10.1007/s00213-013-3161-2 **IF: 4.061**

2. **Aliczki, M.**, Zelena, D., Mikics, E., Varga, Z.K., Pinter, O., Venczkone Bakos, N., Varga, J., Haller, J., 2013. Monoacylglycerol lipase inhibition-induced changes in plasma corticosterone levels, anxiety and locomotor activity in male CD1 mice. Horm Behav DOI: 10.1016/j.yhbeh.2013.03.017 **IF: 3.735**

3. **Aliczki, M.**, Balogh, Z., Tulogdi, A., Haller, J., 2012. The temporal dynamics of the effects of monoacylglycerol lipase blockade on locomotion, anxiety and body temperature. Behav Pharm 23, 348-357 **IF: 2.301**

Other publications of the author

1. Haller, J., **Aliczki, M.**, Gyimesine Pelczer, K., 2013. Classical and novel approaches to the preclinical testing of anxiolytics: A critical evaluation. Neurosci Biobehav Rev DOI: 10.1016/j.neubiorev.2012.09.001 **IF: 9.440**

2. Tulogdi, A., Soros, P., Toth, M., Nagy, R., Biro, L., **Aliczki, M.**, Klausz, B., Mikics, E., Haller, J., 2012 Temporal changes in c-Fos activation patterns induced by conditioned fear. Brain Res Bull 88, 359-370 **IF: 2.935**

3. Panlilio, L.V., Justinova, Z., Mascia, P., Pistis, M., Luchicchi, A., Lecca, S., Barnes, C., Redhi, G.H., Adair, J., Heishman, S.J., Yasar, S., **Aliczki, M.**, Haller, J., Goldberg, S.R., 2012. Novel use of a lipid-lowering fibrate medication to prevent nicotine reward and relapse: Preclinical findings. Neuropsychopharmacology 37, 1838-1847 **IF: 8.678**

4. Haller, J., **Aliczki, M.**, 2012. Current animal models of anxiety, anxiety disorders and anxiolytic drugs. Curr Op Psych 25, 59-64 **IF: 3.422**

5. Zanettini, C., Panlilio, L., **Aliczki, M.**, Goldberg, S., Haller, J., Yasar, S., 2011. Effects of endocannabinoid system modulation on cognitive and emotional behavior. Front Behav Neurosci 5, 57 **IF: 4,758**

6. Toth, M., Mikics, E., Tulogdi, A., **Aliczki, M.**, Haller, J., 2011. Post-weaning social isolation induces abnormal forms of aggression in conjunction with increased glucocorticoid and autonomic stress responses. Horm Behav 60, 28-36 **IF: 3.991**

7. Mikics, E., Vas, J., **Aliczki, M.**, Halasz, J., Haller, J., 2009. Interactions between the anxiogenic effects of CB1 gene disruption and 5-HT3 neurotransmission. Behav Pharm 20, 265-272 **IF: 2.854**

ACKNOWLEDGEMENTS

First of all, I thank my supervisor, Dr. József Haller the opportunity to do my doctoral work at his research group. These findings could never be accomplished without his always available, invaluable professional support and I would not been able to achieve the basics of scientific way of thinking or work.

I am thankful to my colleague, Dr. Éva Mikics for her outstanding theoretical and practical help, her criticism, constant motivation, the good-tempered work together, during which she passed me essential professional knowledge.

I also thank my colleagues and friends, especially Balogh Zoltán, Dr. Barna István, Barsvári Beátá, Biró László, Dr. Fodor Anna, Gyimesiné Pelczer Katalin, Dr. Pintér Ottó, Dr. Tóth Máté, Tulogdi Áron, Varga János, Varga Zoltán, Venczkóné Bakos Nikoletta and Dr. Zelena Dóra for their help in my work, and all my colleagues at the Department of Behavioural Neurobiology at the Institute of Experimental Medicine for their professional support and the friendly atmosphere, which made my work a lot easier and more pleasant. Furthermore, I am grateful for the valuable critics of Dr. Krisztina Kovács about my work and Mária Szűcsné Kazi and Rozália Szafner, employees of the Medical Genetechnological Unit for making my experiments with laboratory animals easier.

I am also grateful to Dr. Steven R. Goldberg and Dr. Leigh V. Panlilio, who collaborated in the present work and who I received a lot of useful experiences from in Baltimore.

And last, I thank the tolerance and trust of my family, with which they were always behind me, I especially thank my sister, Johanna Aliczki for the langue editing of my work. Furthermore, I am thankful for the support of my friends and teammates, all of these could not have been achieved without them.