- FINAL RESEARCH REPORT -

The role of endocannabinoid signaling in the modulation of challenge responding and trauma resilience (NKFIH PD112787)

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1. Overall outcome

We have successfully accomplished the aims of the research plan. During the project period we carried out all major planned experiments and revealed important, novel aspects of endocannabinoid involvement in the regulation of responses to traumatic events and their contribution to the regulation of long–term trauma-induced effects. Our studies resulted in 1 peer-reviewed international article and a manuscript currently under preparation as well as numerous oral and poster presentations of our results at international and domestic scientific meetings.

2. Aims of the study

The endocannabinoid (eCB) system –cannabinoid receptors type-1 (CB_1R) and -2, their ligands anandamide (AEA) and 2-arachindonoilglycerol (2-AG) and the enzymes required for their synthesis and degradation– plays an important role in the neural regulation of cognitive and emotional processes (Zanettini et al. *Front Behav Neurosci*. 2011; 5: 57). Previous work from our group showed that eCBs are particularly involved in the interpretation of and coping with stressful environmental challenges (Haller et al. *Psychopharmacology*. 2013; 230: 353-62), especially in the case of traumatic events when AEA signaling contributes to the regulation of acute responses to the trauma promoting resilience against its adverse effects (Haller et al. *Psychopharmacology*. 2014; 231: 593-601). While recent reports suggested that 2-AG exerts different effects compared to AEA in several behavioral paradigms (Busquets-Garcia et al. *Biol Psychiatry*. 2011; 70: 479-86), ligand-specific eCB effects are still unknown regarding the regulation of responses to and resilience against trauma. In our project we aimed to i.) characterize such specific eCB effects and ii.) their anatomical and pharmacological basis.

Identification of such details of eCB involvement in the neurobiological background of trauma-related behavioral aspects might significantly contribute to our understanding of the pathomechanism of trauma-induced mental disorders, most importantly posttraumatic stress disorder (PTSD), a frequent and highly debilitating condition developing following exposure to traumatic events and of which treatment is still only partially resolved to this day.

3. Results

3.1. Modeling trauma exposure in laboratory rats

In a set of pilot experiments we validated the rat model of trauma exposure in which we further assessed the effects of eCB signaling on the behavioral outcome of traumatic experiences. After submitting rats to a series of electric footshocks representing a traumatic event, we studied acute and conditioned fear responses, social behavior and impulsivity as all were reported to be altered following traumatic events. Employing a protocol in which three 2 sec long, 2.4 mA electric footshocks were delivered, each 30 sec apart (for detailed protocol see Figure 1A), led to marked acute and conditioned fear responses measured by the duration of freezing behavior, a typical fearful behavioral response in rodents. Interestingly, trauma-induced social deficits (measured in

the social avoidance test) and increased impulsivity (measured in the delay discounting test) could not be shown in our model, therefore instead of studying several different aspects of trauma-induced behavioral changes we shifted the focus of our work towards the more detailed description of eCB contribution to the regulation of acute fear responses during traumatic exposure and the formation and extinction of traumatic memories. Furthermore, as during the early phases of our work new findings emerged describing direct functional interactions between AEA and 2-AG signaling on the cellular level (Lee et al. *J Neurosci*. 2015; 35: 10039-57.) in addition to clarifying ligand-specific eCB effects we also aimed to assess functional eCB interactions on the behavioral level as well. The novel focus of our project is highly relevant as a number of recent reports suggest that eCB signaling is fundamental in the regulation of traumatic memory dynamics but no specific eCB effects or interactions were directly studied so far regarding this issue.

Based on our pilot experiments, in our further studies we employed a protocol in which adult male Wistar rats received electric footshocks then were replaced to the footshock context daily (with no further footshocks) during the next consecutive 7 days. 28 days after the footshock session another reminder was conducted (for detailed protocol see Figure 1B). Measurement of freezing behavior allowed to determine acute fear responses (footshock session) and the formation and extinction of the traumatic memory (contextual reminders). To assess the involvement of eCBs in the studied behavioral phenomena we specifically enhanced eCB signaling by pharmacological blockade of the respective degrading enzymes of AEA, fatty acid amid hydrolase by URB597 and 2-AG, monoacylglycerol lipase by JZL184. Treatments were delivered either systemically or locally to brain sites highly relevant in the regulation of responses to traumatic events and the dynamics of traumatic memory.

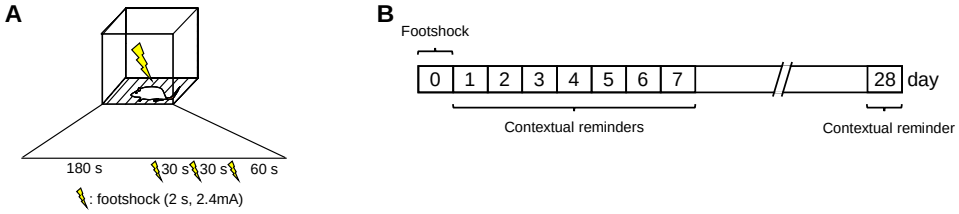

Figure 1. Protocol of the electric footshock session (**A**) and the behavioral paradigm (**B**) employed in our studies.

3.2. The effects of systemically enhanced endocannabinoid signaling on acute fear responses during a traumatic event and the formation of traumatic memories

In our first experiment we studied the effects of systemically enhanced AEA and 2-AG signaling *via* acute URB597 and JZL184 treatments on acute fear responses and formation of traumatic memories. In order to temporally limit treatment effects to the period when acute fear responses and formation of traumatic memories occur treatments were delivered before traumatization. Enhancement of 2-AG but not AEA signaling decreased fear responses during the traumatic event. Interestingly, the effect of 2-AG enhancement was dampened by simultaneous enhancement of AEA signaling, suggesting a functional interaction between the two eCBs in the regulation of acute fear responses to trauma (Figure 2A). Regarding traumatic memory formation, enhanced AEA signaling led to the formation of robust and lasting fear memories which effect was dampened by simultaneously enhanced 2-AG signaling (Figure 2B). Simultaneous pharmacological blockade of CB_1Rs by systemic treatment with antagonist AM251 revealed that this modulating effect of 2-AG was mediated *via* CB_1Rs.

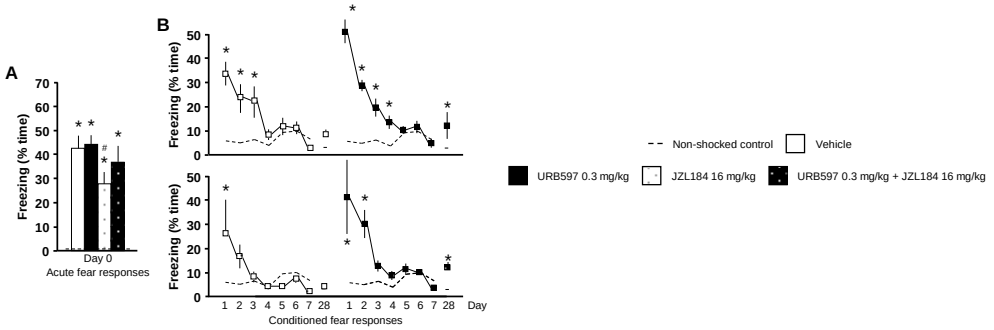

Figure 2. The effects of systemically administered URB597-induced enhanced AEA and/or JZL184-induced enhanced 2-AG signaling on acute fear responses during footshocks (**A**) and conditioned fear responses throughout the daily contextual reminders on day 1-28 (**B**). Systemic JZL184-treatment dampened footshock-induced increases in freezing duration, which effect was blocked by simultaneous URB597-treatment (**A**). Freezing levels in URB597-treated rats returned to non-shocked levels later compared to vehicle controls, and showed marked spontaneous recovery 28 days after traumatization, suggesting the formation of more robust and lasting traumatic memories. This effects was dampened by simultaneous administration of JZL184 (**B**). *: significant difference from non-shocked control on the same day; #: significant difference from vehicle control on the same day ($p<0.05$, one-way and repeated measures ANOVA, post-hoc Duncan test).

As enhanced eCB activity is reported to exert hypomotor and analgesic effects, we assessed whether the changes described above were secondary to such eCB-induced alterations. Neither motor activity during the 3 min long habituation period before the first footshock (Figure 3A) nor pain threshold measured in the hot plate test (Figure 3B) showed treatment-induced changes, suggesting that the above described effects on fear responses and traumatic memory were specific and not secondary to altered motor activity of perception of footshock-induced pain.

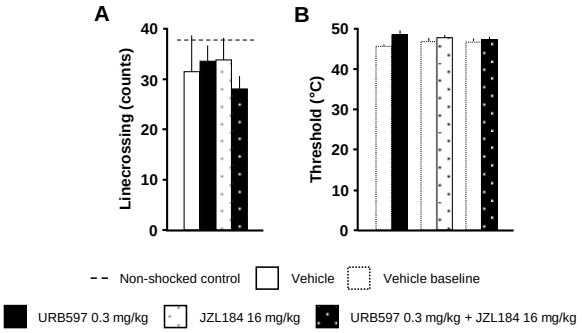

Figure 3. The effects of systemically administered URB597-induced enhanced AEA and/or JZL184-induced enhanced 2-AG signaling on motor activity before the first footshock (measured by the number of lines crossed on a virtual grid over the platform) (**A**); and on pain sensitivity in the hot plate test (measured by a temperature threshold when animals first lick their paws on a continuously warming plate) (**B**).

Taken together, our study employing systemic enhancements of eCB signaling revealed that eCBs differentially and interactively regulate acute fear responses and the formation of traumatic memory: 2-AG dampens acute fear responses during a traumatic event which process is modulated by AEA, while formation of traumatic memory is promoted by AEA which is mediated by a CB_1R-dependent 2-AG mechanism. These effects are not secondary to motor or pain effects.

3.3. The effects of locally enhanced endocannabinoid signaling at relevant brain sites on acute fear responses during a traumatic event and the formation of traumatic memories

To further dissect the eCB mechanisms through which acute fear responses during traumatic events and traumatic memory formation are modulated we employed local pharmacological enhancement of eCB signaling at brain areas highly relevant in the regulation of fear responses and

formation of traumatic memories: the prelimbic subregion of the prefrontal cortex (PrL), the ventral part of the hippocampus (vHC) and the basolateral subregion of the amygdala (BLA). Cannulae were bilaterally implanted targeting these regions then URB597 and/or JZL184 treatments were delivered *via* the cannulae allowing to anatomically limit treatment effects to the targeted brain sites (for anatomical localization of cannulae tips see Figure 4). After validation of the surgical processes and technical aspects of local treatment administration to reach the needed anatomical specificity, we studied the effects of local enhancement of eCB signaling in our model of trauma exposure.

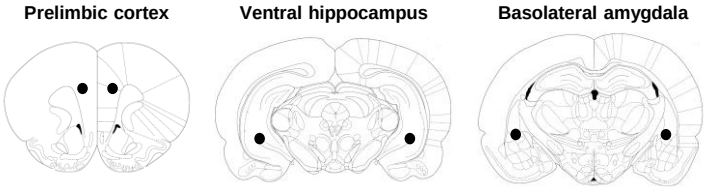

Figure 4. Anatomical localization of bilateral cannulae tips in the prelimbic cortex, ventral hippocampus and basolateral amygdala indicated by black dots. Based on the rat brain atlas of Paxinos and Watson (Academic Press, 1998).

According to our findings, URB597-induced enhancement of AEA signaling in the vHC decreased acute fear responses during traumatization, which effect was blocked by simultaneous JZL184-induced enhancement of 2-AG signaling at the same brain site. In contrast, enhancement of AEA or 2-AG signaling at the PrL and BLA did not affect these responses (Fig 5A). Interestingly these effects of local enhancement of eCB signaling were not present when eCB signaling was systemically enhanced in our previous experiments, suggesting that eCB activity at several brain sites collectively and interactively modulate acute fear responses during traumatic events. Formation of traumatic memories was promoted by AEA signaling at the PrL and vHC: enhancement of AEA signaling in these regions resulted in robust, lasting traumatic memories, resistant to extinction. These effects of enhanced AEA signaling were blocked by 2-AG (Fig 5B). Similar effects were shown at systemic enhancement of eCB signaling (Fig 2B), suggesting a predominant role of PrL and vHC eCB signaling mechanisms in formation of traumatic memories. Interestingly, eCBs showed anatomically dependent effects in our studies as enhanced AEA signaling in the BLA completely abolished formation of traumatic memories in a 2-AG independent manner (Fig 5B).

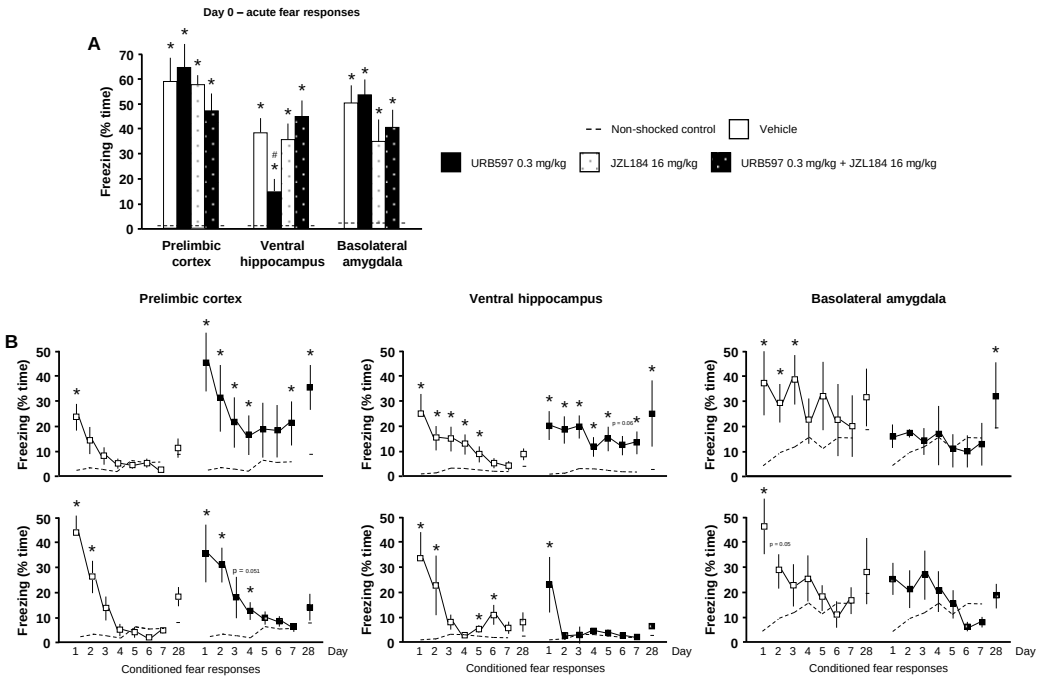

Figure 5. The effects of locally administered URB597-induced enhanced AEA and/or JZL184-induced enhanced 2-AG signaling on acute fear responses during footshocks (**A**); conditioned fear responses throughout the daily contextual reminders on day 1- 28 (**B**). Ventral hippocampal URB597-treatment dampened footshock-induced increases in freezing duration, which effect was blocked by simultaneous JZL184-treatment (**A**). Freezing levels in intra prelimbic cortex and ventral hippocampus URB597-treated rats did not return to non-shocked levels and showed high levels 28 days after traumatization, suggesting the formation of more robust and lasting traumatic memories. This effects was dampened by simultaneous local administration of JZL184. In contrast, intra basolateral amygdala URB597 treatment freezing levels did not differ from non-shocked levels at any time point (**B**). *: significant difference from non-shocked control on the same day; #: significant difference from vehicle control on the same day ($p<0.05$, one-way and repeated measures ANOVA, post-hoc Duncan test).

Taken together, experiments employing local pharmacological enhancement of eCB signaling revealed, that i.) while AEA signaling in the vHC dampens acute fear responses during traumatic events the overall behavioral response is rather shaped by eCB signaling at several interacting brain sites; ii.) in contrast, AEA signaling in the vHC and PrL was shown to have a predominant role in promoting the formation of lasting traumatic memories, which effects of AEA are under 2-AG control. iii.) Interestingly, AEA had anatomically specific effects regarding its role in the regulation of traumatic memory formation as AEA signaling in the BLA was shown to abolish the formation of such memories. Our findings on the interactions of AEA and 2-AG signaling in the regulation of acute fear responses and formation of traumatic memories are currently under preparation for publishing in a peer reviewed, international journal.

3.4. The effects of enhanced endocannabinoid signaling on extinction of traumatic memories

After the description of specific eCB effects and interactions regarding acute responses to trauma and formation of traumatic memories we assessed whether AEA and 2-AG interact in the extinction of traumatic memory. Following the above described protocol but temporally limiting treatment effects to the contextual reminders by systemically administering pharmacological agents URB597 and JZL184 before the first contextual reminder we were able to determine the direct effects on traumatic memory extinction. Interestingly, regarding extinction AEA and 2-AG synergistically accelerated the extinction of traumatic memories with no interactions (Figure 5).

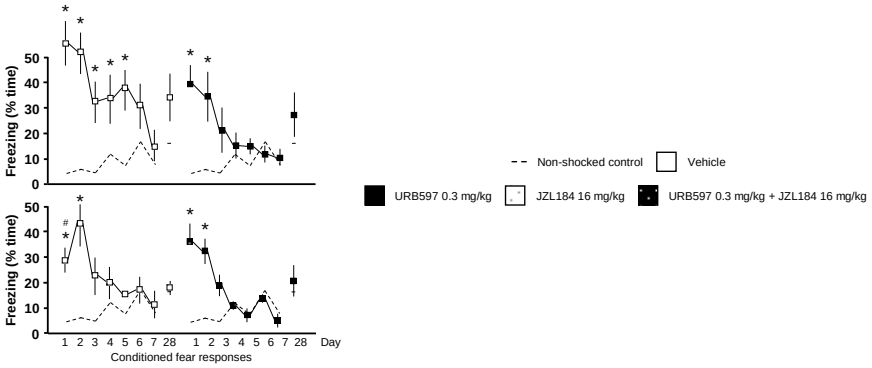

Figure 5. The effects of systemically administered URB597-induced enhanced AEA and/or JZL184-induced enhanced 2-AG signaling on the extinction of traumatic memories. Freezing levels in systemic URB597- and/or JZL184-treated rats returned to non-shocked levels more rapidly than in the case of vehicle control rats, suggesting accelerated extinction of traumatic memories. *: significant difference from non-shocked control on the same day; #: significant difference from vehicle control on the same day ($p<0.05$, one-way and repeated measures ANOVA, post-hoc Duncan test).

Taken together, eCBs appear to interact only at the regulation of specific phases at traumatic memory dynamics, as they showed no interactions and synergistically accelerated traumatic memory extinction in our model.

3.5. The effects of enhanced anandamide signaling in the basolateral amygdala and prelimbic cortex on coping with stressful challenges

As URB597-induced enhanced AEA signaling altered responsivity to traumatic experiences in our previous reports (Haller et al. *Psychopharmacology*. 2013; 230: 353-62; Haller et al. *Psychopharmacology*. 2014; 231: 593-601) as well as in the current project, we hypothesized that this eCB ligand plays an important role in the regulation of coping with environmental challenges. In a set of studies we assessed the neurobiological localization of such effects of AEA. We implanted URB597 into the PrL and BLA thus chronically enhancing AEA signaling in these regions and studied the effects on behavioral responses to changes in environmental aversiveness during a stressful challenge. According to our findings, chronic enhancement of AEA signaling in the PrL led to a more problem oriented, less distracted behavioral phenotype when the individual faced environmental challenges as these animals showed dampened reaction to changes in environmental aversiveness. Interestingly, these effects of AEA were predominantly mediated in the PrL as similar treatments in the BLA did not lead to such changes (Figure 7).

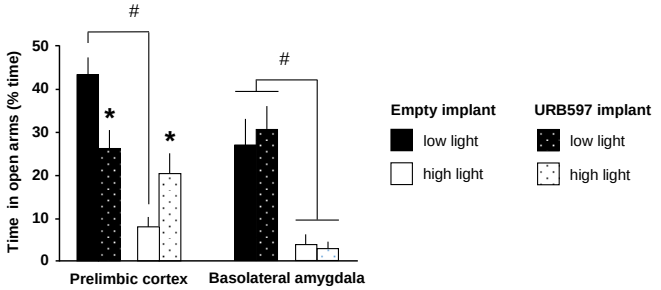

Figure 7. Times spent in the open arms of the elevated plus-maze in low and high light in animals with empty or URB597 containing implants in their prelimbic cortex or basolateral amygdala. While animals with empty implants reacted to high light conditions (considered to be aversive for rats) with a marked decrease in open arm exploration on the elevated plus-maze, rats with URB597 implants in their prelimbic cortices but not in their basolateral amygdalae did not react to changes in light intensity. *: significant effect of URB597 compared to controls tested under similar light conditions; #: significant effect of light ($p < 0.05$, factorial ANOVA, post-hoc Duncan test).

Taken together, our findings suggest, that AEA signaling in the PrL has a predominant role in shaping acute responses during stressful events, particularly *via* fine tuning the responses according to environmental factors. Our report on these findings was accepted for publishing at Psychopharmacology (IF: 3.875) in 2016 (Aliczki et al. *Psychopharmacology*. 2016 May; 233 (10): 1889–1899).

4. Summary

PTSD is a relatively prevalent mental disorder that represents a large burden to the individual and the society as well. The lack of understanding the neurobiological mechanisms contributing to the development of PTSD hampers its therapy. In our current project, we have successfully characterized novel and highly relevant neurobiological aspects of the behavioral outcome of trauma exposure employing a laboratory rat model. Our work was the first to demonstrate that the two eCBs, AEA and 2-AG functionally interact in the regulation of acute fear responses during a traumatic event and the dynamics of traumatic memories. The main findings of our project showed that i.) acute fear responses during traumatic events are predominantly regulated by 2-AG signaling under AEA control; ii.) traumatic memory formation is promoted by AEA signaling in the PrL and vHC, where 2-AG tuned these effects *via* CB_1R activation, while AEA signaling in the BLA blocks traumatic memory formation; iii.) extinction of traumatic memories are facilitated by both eCBs. Revealing novel details of eCB involvement in the behavioral aspects of trauma exposure contributes to our knowledge on the pathomechanism of trauma-induced mental disorders, most importantly PTSD and may lead to the identification of effective treatment strategies. We thank NKFIH for funding the research project.

5. Published articles and conference presentations of the project

5.1. Peer reviewed international scientific articles:

1. Balogh Z, Szente L, Biro L, Varga ZK, Haller J, Aliczki M. Endocannabinoid interactions in the regulation of acute responses to trauma and formation of traumatic memories. ***IN PREPARATION***

2. Aliczki M, Barna I, Till I, Baranyi M, Sperlagh B, Goldberg SR, Haller J. The effects anandamide signaling in the prelimbic cortex and basolateral amygdala on coping with environmental stimuli in rats. *Psychopharmacology*. 2016 May; 233 (10): 1889–1899.

5.2. Oral presentations at international scientific conferences

1. Balogh Z, Szente L, Varga ZK, Biro L, Haller J, Aliczki M. Endocannabinoid interactions in the regulation of behavioral responses to trauma. IACM 9th Conference on Cannabinoids in Medicine, Cologne, Germany, September 29-30, 2017.
2. Aliczki M, Balogh Z, Szente L, Biró L, Varga ZK, Haller J. Interactions of endocannabinoids anandamide and 2-arachidonoylglycerol in the regulation of behavioral responses to traumatic events. The 27th ICRS Symposium on the Cannabinoids. Montreal, Canada, June 25, 2017

5.3. Poster presentations at international scientific conferences

1. Balogh Z, Szente L, Varga ZK, Biró L, Haller J, Aliczki M. Differential involvement of endocannabinoids anandamide and 2-arachidonoylglycerol in the regulation of behavioral responses to trauma. FENS Regional Meeting, Pécs, Hungary, September 20-23, 2017

2. Aliczki M, Balogh Z, Szente L, Varga ZK, Biro L, Haller J. Interactions of endocannabinoids anandamide and 2-arachidonoylglycerol in the regulation of behavioral responses to traumatic

events. 40th Annual Meeting of the Japan Neuroscience Society, Makuhari, Japan, July 20-23, 2017

3. Szente L, Balogh Z, Varga ZK, Biro L, Haller J, Aliczki M. Endocannabinoid interactions in the regulation of behavioral responses to traumatic events. COINS International Conference of Life Sciences, Vilnius, Litvania, 28 February-02 March, 2017
4. Balogh Z, Szente L, Varga ZK, Biro L, Haller J, Aliczki M. Differential involvement of endocannabinoids anandamide and 2-arachidonoylglycerol in the acquisition and extinction of learned fear. 29th ECNP Congress, Vienna, Austria, September 17-20, 2016
5. Balogh Z, Szente L, Varga ZK, Biro L, Haller J, Aliczki M. Differential involvement of endocannabinoids anandamide and 2-arachidonoylglycerol in the acquisition and extinction of learned fear. 26th Annual Symposium of the ICRS, Bukovina Tatrzanska, Poland, June 26 - July 01, 2016
6. Balogh Z, Szente L, Varga ZK, Haller J, Aliczki M. Differential involvement of endocannabinoids anandamide and 2-arachidonoylglycerol in regulating behavioral responses to traumatic experiences. IBRO Workshop, Budapest, Hungary, January 20-21, 2016
7. Szente L, Balogh Z, Varga ZK, Biró L, Haller J, Aliczki M. Endocannabinoid regulation of responses to traumatic experiences – the differential role of anandamide and 2-arachidonoylglycerol. Conference of the Hungarian Etology Society, Dobogoko, Hungary November 27-29, 2015
8. Aliczki M, Barna I, Till I, Baranyi M, Sperlagh B, Haller J. The involvement of the medial prefrontal cortex and basolateral amygdala in the regulation of coping with stressors. 28th ECNP Congress, Amsterdam, Nederland, August 29- September 01, 2015
9. Aliczki M, Barna I, Till I, Baranyi M, Sperlagh B, Haller J. The involvement of the medial prefrontal cortex and basolateral amygdala in the endocannabinoid control of coping with stressors. MNS Meeting, Santa Margherita di Pula, Sardinia, Italy, June 12-15, 2015