Studies on age-dependent acute and chronic neuronal activity in limbic and midbrain stress-regulated areas in the rat

Doctoral PhD. thesis

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Introduction

Stress response regulation

Stress is a physiological and/or behavioral response to stimuli which may potentially threat the individual's physiological and/or psychological integrity. Stress response initiates and supports the adaptation processes. The primary aim of the process is to maintain the internal integrity (homeostasis) of the body.

During stress, usually environmental stimuli activate the hypothalamus-pituitary-adrenocortical (HPA) axis resulting in corticotropin-releasing factor (CRF) secretion of neurons in the parvocellular part of hypothalamic paraventricular nucleus (PVNp). CRF is secreted to the median eminence via axonal transport and it is finally released into capillary loops. Then, through the hypophyseal portal veins, CRF is carried to the adenohypophysis. CRF binding to the CRF1 receptor induces the synthesis of proopiomelanocortin (POMC). The cleavage of POMC creates the adrenocorticotropic hormone (ACTH) in the adenohypophysis. Finally, the ACTH is secreted into the bloodstream to reach the *zona fasciculata* of the adrenal cortex where it induces the synthesis and secretion of glucocorticoids.

The adrenal cortex-derived glucocorticoids (cortisol in humans, and corticosterone (CORT) in rats) are required for the individual's adaptation response. Glucocorticoids increase the metabolism, decrease appetite and inflammatory processes and promote the proper cardiovascular functioning. Glucocorticoids affect the attention also and their increased blood titer is frequently associated with mood disorders, especially with major depressive disorder. Finally, both gluco- and mineralocorticoids control their own synthesis and secretion by a negative feedback via the limbic system and CRF neurons of the PVNp.

Higher-order control of stress response

Numerous cortical and subcortical brain areas control the HPA axis. The gamma-amino-butyrate (GABA)-secreting neurons in the hippocampus and prefrontal cortex (PFC) inhibit the stress axis activity by decreasing the CRF expression in the PVNp neurons. At the end of stress response, the hippocampus and PFC help the recovery of the HPA axis and restore its basal (i.e. pre-stress) steady state.

The extended amygdala exerts complex effects on the stress response. The classic amygdala nuclei such as the central (CeA), medial (MeA) and basolateral nuclei (BLA) stimulate the

HPA axis. The nuclei of the extended amygdala, such as the anterior part of bed nucleus of the stria terminalis (BNST) increases the stress response also, meanwhile the posterior nuclei of the BNST have opposite effect.

It has to be stated that some nuclei of the extended amygdala contain CRF-producing neurons also, which control the CRF neurons in the PVN, thus, they have ability to modify the stress response.

Besides diencephalic and telencephalic structures, brainstem centers have also great importance in stress regulation. The serotoninergic dorsal raphe nucleus (DR) neurons promote the stress response, meanwhile the urocortin1 (Ucn1)-producing neurons in the centrally projecting Edinger-Westphal nucleus (cpEW) downregulate the HPA axis activity.

CRF peptide family and mood disorders

CRF is a 41 amino-acid neuropeptide, one of the four members of the mammalian CRF peptide family (CRF, Ucn1, Ucn2, Ucn3). The existence of CRF has been proven in numerous tissues of the body. Importantly, within the peptide family, CRF exerts the greatest central nervous system levels. CRF is expressed in several cortical areas and can be found in numerous subcortical territories such as in the PVNp, in the CeA, and the subdivisions of BNST [such as in the fusiform (BNSTfu) ventral (BNSTv) and oval (BNSTov) nuclei]. Brainstem CRF cells and their fibers contribute to the control of cerebellum as well. CRF in the PVNp plays critical role in stress adaptation response. The lack of CRF decreases the HPA axis activity and it is associated with decreased anxiety level in mice. Local lentivirus-driven CRF overexpression in the CeA decreases anxiety-like behavior. Conversely, selective CRF overexpression in the BNSTov was found to increase depression-like behavior in the same experiment. Increased central CRF level is associated with major depressive disorder in humans.

Studies on neural activity markers in stress response

Examination of immediately-early gene (IEG) expression is a frequently used technique to assess the rise of neural activity. Members of *Jun/Fos* proto-oncogene family are the most extensively examined IEGs. FOS proteins form heterodimers with other *Jun* gene products to create the transcription factor activator protein 1 (AP-1). AP-1 controls numerous cellular processes including *Crf* gene expression also.

Just minutes after acute neural activation, the *Fos* mRNA expression becomes detectable. FOS peaks at protein level 2 hours after the stimulus and it returns to the basal level 4-6 hours after an acute challenge.

The detection of FOSB protein is a frequently used tool to measure the chronic neuronal activation. In contrast to FOS, FOSB exerts slower dynamics and longer half-life time (9,5 hours). A shorter splice variant of FOSB, designated as ΔFOSB has an even more chronic dynamics than full-length FOSB. Additionally, as ΔFOSB may accumulate in the cell, it stays detectable even days after the last stimulus of a repetitive stress exposure. For instance, chronic variable mild stress (CVMS) is a commonly used model to evoke ΔFOSB response.

Aging and stress

It is well-known that in the course of ageing the functions of several organ systems are affected. The stress response of the HPA axis is also a function of age. In newborn rats, the stress hyporesponsive period is characterized by low HPA axis activity and low CORT response to stress. Prepubertal rats display a prolonged CORT response to acute stress stimuli compared to adults. In aged rats the increased stress sensitivity is characteristic, however, the results of CRF and CORT measurements gave contradictory results in earlier studies.

Although some age-related changes were found in the HPA axis and in stress centers of the brain, we did not find any studies in the literature providing a systematic description on the age-related dynamics of acute and chronic stress response.

Aims and hypotheses

Examination of acute stress-induced FOS protein response in the course of aging

We hypothesized that the FOS protein production upon acute restraint stress (ARS) exposure is a function of age. We put forward to examine eight age groups (i.e. 1; 1,5; 2; 3; 6; 12; 18 and 24 months old) of rats and measured their FOS immunoreactivity in the following stress-associated brain areas upon ARS exposure: PVNp, PVN magnocellular part (PVNm), MeA, CeA, BLA, BNSTov, dorsolateral BNST (BNSTdl), dorsomedial BNST (BNSTdm), BNSTv, BNSTfu, EWcp, DR. We also assessed the FOS immunoreactivity in the somatosensory barrel cortex (S1). The activity of the HPA axis was also examined.

Examination of age-dependent dynamics of IEG expression in CRF neurons

The examination of our first hypothesis revealed that age affects FOS activation, moreover important CRF-containing centers (PVN, BNSTov and CeA) were found to show age-dependent FOS expression profile. Based on this, we hypothesized that the CRF-producing cells show age-dependent FOS dynamics in these nuclei. Using the same acute model in the eight age groups defined above, we assessed FOS and FOSB immunoreactivities. As CRF neurons contribute both to acute and chronic stress adaptation, we expanded the experimental setup with chronic variable mild stress (CVMS) exposed groups in six age groups (i.e. 2; 3; 6; 12; 18 and 24 months old). Here we hypothesized that the chronic stress response of CRF neurons (i.e. ΔFosB content) is a function of age in the PVN, BNSTov and CeA.

Methods

Experimental setup

To test the first hypothesis, we used 73 animals divided into 8 age groups (1; 1,5; 2; 3; 6; 12; 18 and 24 months old animals). In each age group we created control and ARS subgroups. ARS animals were exposed to 60 min acute restraint stress. Two hours after onset of stress exposure we perfused our animals.

In order to examine our second hypothesis, 127 animals of the same age groups were used. Besides the subgroups described above, we founded a third CVMS subgroup of 2; 3; 6; 12; 18 and 24 months old rats. ARS animals were treated as described above. CVMS animals were exposed to two weeks chronic variable mild stress. In this, we exposed our animals randomly to a short day-time stress (shaking on laboratory shaker, acute restraint stress, tilted cage, dark room) and a longer night-time stress (social isolation, wet bedding) daily.

The animals' plasma CORT level was determined by radioimmunoassay (RIA). In the second experiment we registered the animals' bodyweight, and finally we measured their thymus- and adrenal weights also.

Immunolabeling

In the first experiment the neural activity was determined by FOS immunohistochemistry using diamino-benzidine (DAB) chromogen in 13 brain areas. In the second study we conducted a triple label immunofluorescence for CRF, FOS and FOSB.

Microscopy and digital analysis

DAB labelled slides were photographed using a Nikon light microscope equipped with a digital camera. The triple-labelled fluorescent preparations were digitalized by an Olympus confocal microscope. For each brain area, we examined 5 representative photos per animal. In DAB labelled preparations, we counted the number of FOS positive nuclei. In the fluorescent images, we counted CRF, FOS and FOSB immunoreactive cells. Additionally, we quantified the co-localizations and the specific signal density (SSD) of CRF.

Statistical analysis

After testing the normal distribution and homogeneity of data, datasets were evaluated with two-way analysis of variance (ANOVA). Pairs of groups were compared with Tukey's post-hoc test. Our data were subjected to Spearman's rank correlation analyses to test the age-dependency of changes.

Results

Model validation

In order to test if the HPA axis response to ARS and CVMS is a function of age, the CORT titer was determined. ARS increased the CORT titer in all ARS groups compared to their respective controls. One month old animals showed a smaller but significant CORT increase upon ARS than all older rats, except the three months animals. Although the CORT response did not decline with age, a stress and age interaction was detectable.
In our CVMS model, only 3 month-old stressed animals showed significantly higher CORT titer than their respective controls. CVMS reduced the animals' bodyweight and increased their relative adrenal gland weights although this was significant in the 2 month-old CVMS group only. The relative thymus weight upon CVMS significantly decreased, except for the oldest group.

Examination of neuronal activity patterns in the ARS model

We confirmed that stress and age affect FOS cell count in all examined nuclei of the extended amygdala (i.e. MeA, CeA, BLA, BNSTov, BNSTdl, BNSTdm, BNSTv, BNSTfu). However, the interaction of stress and age was proven only in the CeA, BLA and BNSTfu. In four additional nuclei (i.e. PVNp, PVNm, DR, cpEW) the FOS cell count also showed age- and stress-dependence with a second order effect of age and ARS. Although the magnitude of FOS reactivity upon stress differed in the examined nuclei, the highest FOS cell count was detected in two months old rats in all areas. The greatest, 29-fold activity increase was observed in the PVNp. Spearman's correlation analyses confirmed the age-dependent decrease of FOS cell count in all nuclei both in control and ARS animals, except for EWcp and DR.

Neuronal activity of CRF cells in acute and chronic stress models

In ARS animals, both FOS and FOSB increased in the PVN-CRF cells. The magnitude of this rise decreased with age. The Spearman's test proved that the IEG product content of CRF cells decreased linearly with age in the ARS groups. The CRF SSD in the PVN increased with the number of triple labelled cells (CRF-FOS-FOSB).
Both stress and age affected FOS content of CRF cells in the CeA and BNSTov nuclei. However, contrary to the PVNp, the interaction of both factors could not be confirmed. The

magnitude of FOSB immunoreactivity in CeA- and BNSTov-CRF cells decreased also linearly with age in ARS animals.

In CVMS animals, PVN-CRF cells showed increased FOSB immunoreactivity which did not change with age. Significantly increased FOSB content in the PVN-CRF cells was observed in 3- and 18-month-old animals, compared to their controls. The magnitude of PVN-CRF SSD correlated the number of cells co-localizing CRF and FOSB. In contrast to the PVN, the FOSB content of CeA and BNSTov CRF neurons did not change upon CVMS, but decreased with age. The age-dependent decrease of CRF-FOSB cell count in both the CeA and BNSTov was also confirmed by correlation analyses.

Discussion

The validity of our animal models

ARS was effective in both experiments as supported by CORT titers reflecting the activity of HPA axis. The increased FOS contents in stress-sensitive brain areas also supported the efficacy of ARS.

The CVMS protocol's effectivity was also supported by our CORT RIA results. Although the thymus- and adrenal gland weight data support the validity of CVMS, we have to state that the changes were not significant in all age groups. The greatest differences were observed in young animals (1,5; 2; 3; 6 months old groups), while in aged rats (12; 18; 24 months old groups) we did not detect statistically significant changes in organ-, body weights or CORT levels. Earlier studies described that some rat strains (Sprague-Dawley, Brattleboro, Wistar) show reduced response to stressful conditions in old age (e.g.: decreased CORT response). Other studies reported also, that stress does not have always remarkable impact on thymus weight.

In summary, the data on organ- and bodyweights changes, the CORT response and the FOS and FOSB content of PVN-CRF neurons support our models' validity.

Age-dependent effects of ARS on FOS expression

Most of stress-associated nuclei of young (1 and 1.5 month old) animals did not show significantly increased FOS content upon ARS. Meanwhile in line with earlier studies in 2 months old animals in all examined stress-associated areas showed significant rise in FOS content. The highest FOS immunoreactivity was detected in the 2 months old animals that may be explained by the increased stress sensitivity of these brain areas in the late adolescence and post-puberty life period. The age-dependent sensitivity of a particular brain area may refer to its characteristic stress-vulnerable life periods.

The reactivity of stress-associated nuclei decreases gradually with age, but the magnitude of this decrease is brain area dependent. Compared to the two months old ARS animals, a significant age-related decrease of FOS immunoreactivity was found over the third month in the DR and CeA, over sixth months of age in the MeA, BNSTov, BNSTdm, BNSTfu and PVN, and in the BNSTdl just over the twelfth month. In seven nuclei (MeA, CeA, BNSTov, BNSTdl, BNSTdm, BNSTfu, PVNp) the correlation analyses supported the age-dependent decrease of FOS positive cell counts in ARS animals. In four other nuclei (EWcp, PVNm,

BNSTv, BLA) the age-related decrease of FOS immunoreactivity was confirmed in control rats as well.

We need to state that the FOS activity did not correlate with the plasm CORT levels, hence assessment of FOS immunoreacitivity *per se* is not sufficient to determine the HPA axis activity. Indeed, the FOS labelling has a number of limitations. The technique does not differentiate between types of functional changes in the neurons (i.e. excitatory *vs.* inhibitory), since it just indicates the changes at gene-expression level. FOS is not produced in all kinds of neurons, but other IEGs may be expressed, such as Arc. Finally, there are neurons with tonic inhibitory character which do not show IEG and FOS immunoreactivity at all, when they are active. Additionally, besides these limitations one has to consider also, that there are several brain regions which were not studied here (e.g. PFC, hippocampus), but they could have modified the HPA axis activity, for instance via the glucocorticoid feedback.

The two-way ANOVA revealed significant interactions between age and stress in seven nuclei, but correlation analyses verified the age-dependent FOS cell count decrease unequivocally only in three nuclei (CeA, BNSTfu, PVNp). The age-related decline of PVNp activity might be related to the decline of CeA and BNSTfu FOS reactivity, as these nuclei play crucial role in the control of glucocorticoid response. The other examined nuclei (MeA, BNSTov, BNSTdl, BNSTdm, BNSTv) did not show univocal age-associated decline of the FOS response. As ARS-CORT levels remained stable in old age, we assume that these latter five nuclei may have contributed to the maintenance of constant CORT response in senescence.

Finally, in order to exclude that the reduction of FOS content would be caused by the aging-related natural reduction of the sensitivity of sensory systems in our aged rats, we evaluated the FOS content of the somatosensory barrel cortex (S1). However, we saw some-age related change in the FOS content of this cortical area, this showed U-shaped dynamics. Therefore, we did not find linear correlation between age and FOS content of the S1 in contrast to the regions related to stress adaptation response.

In summary, we propose that the observed age-dependent dynamics of FOS response is characteristic for the examined stress-associated nuclei, but not for the whole brain. The decreased reactivity of the nuclei cannot be explained by the decreased sensitivity of the sensory systems.

An unexpected observation of our studies was that some brain areas showed relatively high FOS content in young control animals. Among the studied nuclei, the CeA, BNSTov and EWcp showed considerable FOS signal in one month old controls which later started to

decrease over 1.5 month of age. The importance of this finding is currently unknown, but it is interesting that stress-related neuropeptides are produced here (i.e. CRF in the CeA and BNSTov; Ucn1 in the EWcp). To explain this, one may anticipate that during the post-weaning period the FOS activation is necessary for adequate stress adaptation. To test this hypothesis further co-localization studies would be necessary.

Age-dependent effects of ARS on CRF-FOS-FOSB immunoreactivity

It has been repeatedly reported that ARS increases the number of FOS containing CRF cells. It is also known that the magnitude of this increase is lower in young juvenile animals than in adult rats. These findings were also supported by our study. Besides this, we proved first time that the number of CRF-FOS positive cells continues to decrease till 24 month of age. In our second experiment we saw decreased FOS content in CRF cells in old rats, without any significant changes of CORT response in ageing. This suggests that the hypophyseotrop neurons do not express necessarily FOS when they stimulate the HPA axis or their stress-induced CRF content does not co-exist with detectable FOS immunosignal in the nucleus. Our experiments support that two hours after the start of stress stimulus, the FOSB immunoreactivity has already increased significantly, which strongly correlates with the FOS data, despite that the peak of the FOSB signal occurs later.

CRF cells of the CeA and BNSTov did not show remarkable FOS signal. This indicates that the ARS-induced FOS signal occurs in not CRF-producing cells (e.g. metenkephalinergic neurons). In contrast, we detected considerable FOSB immunoreactivity in CRF neurons. The magnitude of the FOSB signal was not affected by ARS, but it decreased dramatically with age both in control and ARS animals. In the CeA and BNSTov, the age-related decrease of CRF-FOSB cell number was greater than in the PVN neurons. As both the CeA and BNSTov modulate the PVN's activity through indirect connections only, the dynamics of neuronal activity in the amygdala and PVN may differ.

Age-dependent effects of CVMS on CRF-FOS-FOSB immunoreactivity

Our present study further supports that CVMS increases the FOSB immunoreactivity in CRF cells of the PVN. In contrast to the observation in the ARS model, the CVMS-induced FOSB rise in PVN-CRF cells is independent from age. Another interesting finding was that while

PVN CRF cells did not show detectable basal FOSB, the CeA and BNSTov exerted considerable FOSB expression in controls animals also.

CVMS did not change the FOSB content of CRF cells in the CeA and BNSTov which is in line with earlier findings. The FOSB labelling in the CeA and BNSTov revealed that CRF neurons had considerable FOS content in both control and CVMS groups. In contrast to PVN, the CeA and BNSTov CRF cells showed decreased FOSB immunoreactivity in old age in both control and CVMS animals. The difference in the FOSB dynamics in these centers may be explained by the neurochemical character of the neurons, since the amygdala nuclei contain mostly GABAergic CRF neurons, meanwhile the PVN harbors mostly glutamate-producing CRF cells. Moreover, it is known for some GABAergic neurons that they do not show *Fos* gene expression upon external stimuli. The significance of age-related activity decline of CeA and BNSTov neurons is to date unknown. Further studies are required to test if the main projection areas (e.g. DR, locus coeruleus) of these CRF cells also show age-related functional changes. The age-dependent activity of these nuclei is known, but there is no information available if these may be explained by the ageing-related decline of CRF neuronal activity.

The age-dependent expression of FOS proteins in CRF neurons

Our studies have confirmed the findings of earlier works that FOS is an excellent cellular activity marker of acute changes. On the other hand, FOSB (ΔFOSB) labeling is a useful technique to assess the changes of chronic neural activity. One major outcome of our experiment was that the magnitude of ARS-induced FOS immunoreactivity in PVN CRF neurons declined remarkably with age. This FOS signal strongly correlated with FOSB two hours after the beginning of ARS in PVN CRF neurons.

Chronic stress increased the FOSB content of the PVN CRF cells, which however did not change with age. In contrast to PVN, we observed a considerable FOSB immunoreactivity in the CeA and BNSTov cells that was not affected significantly in CVMS.

However FOS and FOSB have different expression dynamics, our experiment proved that the FOSB labelling may be an alternative technique of the FOS labelling in the PVN two hours after the start of acute stimulus.

The triple labelling does not provide information on the identity of the genes which were affected by the AP1. Therefore, this technique does not allow direct functional insights into the functional significance of gene expression changes at neuronal level. Nevertheless, the

technique was useful to assess the CRF neurons' activity in the CeA, BNSTov and PVN in acute and chronic stress exposure. Moreover, this is the first study that provided a throughout-lifespan systematic assessment of the CRF neurons' IEG response. In contrast to the PVN, CRF neurons in the CeA and BSNTov showed remarkably reduced FOSB content in old age.

Conclusions

To our knowledge, this study is the first to provide a systematic comparison of both basal and ARS exposure-induced FOS content of the 13 stress-related brain areas of Wistar rats in eight age groups from young age till senescence. FOS immunoreactivity was found to be a function of brain area and age both in control and stressed groups. Therefore, the major methodical result of this study is that in similar setups the age of the animals has to be selected with care. If young animals are used, one has to predict considerable basal FOS immunoreactivity in some nuclei (e.g. CeA, BNSTov, EWcp).

According to our results, the stress-induced FOS reactivity correlated negatively with age in seven brain areas (i.e. MeA, CeA, BNSTov, BNSTdl, BNSTdm, BNSTfu, PVNp). Therefore, their contribution to the control of the HPA axis and that of other stress-related systems may also be a function of age. Further extensive systematic research is required to test the age-dependent contribution of these areas to the age dependency of stress adaptation response.

The characterization studies in CRF-producing brain areas confirmed that FOS co-localized mainly with CRF cells of the PVN. In line with the FOS mapping results in the first study, both the FOS and FOSB decreased with aging in CRF cells. In contrast, the CVMS-induced CRF FOSB/ΔFOSB immunoreactivity was not affected by age.

Similarly to results of former studies, the FOS and FOSB content of CRF neurons in the CeA and BNSTov did not show remarkable changes upon ARS or CVMS. In controls, without any stress exposure, a considerable FOSB content was detectable in the CRF cells of the CeA and BNSTov that decreased with age.

To the best of our knowledge, this is the first study providing a systematic throughout-lifespan description of neuronal activity in the main hypothalamic and forebrain CRF systems in response to ARS and CVMS in the rat. The neuronal activity of the examined CRF cells was found to be a function of age and brain area. CVMS was found to cause an age-independent FOSB/ΔFOSB neuronal activity in PVN-CRF cells. Therefore, studies applying FOS and FOSB as activity markers should be planned with respect to the age and brain region-specific recruitment of these indicators. Further studies are in progress to describe the ageing-related alteration of neuronal stress responsivity in other stress-recruited circuits (e.g.: EWcp, LC, NTS, DR). Getting an overview on the age-dependency of stress-reactive areas may help to understand why do stress-related brain diseases occur more frequently in adolescence and in old age. This knowledge might ultimately help to find new personalized, eventually age-adjusted strategies for prevention and management of stress-related mood-disorders.

New results

1. This study is the first showing that the FOS content of seven nuclei (i.e. MeA, CeA, BNSTov, BNSTdl, BNSTdm, BNSTfu, PVNp) out of thirteen studied brain regions show negative linear correlation with age.
2. We show that not only stress-exposed, but also young control animals possess considerable FOS immunoreactivity in the CeA, BNSTov and EWcp nuclei.
3. We demonstrate that the ARS-induced FOS and FOSB immunoreactivity in PVN-CRF cells decreases with the course of aging till senescence.
4. The CVMS-related FOSB/ΔFOSB content of PVN-CRF cells does not change with in age.
5. CeA and BNSTov CRF cells contain considerable FOSB immunoreactivity in young naïve animals that does not change upon CVMS exposure.
6. The FOSB content of CeA and BNSTov CRF cells markedly decreases with age in both ARS and CVMS animals.
7. The rise of FOSB immunoreactivity upon CVMS is accompanied with increased CRF content of PVN neurons.

List of publications the thesis is based on

1. Kovács LÁ, Schiessl JA, Nafz AE, Csernus V, Gaszner B. (2018) Both basal and acute restraint stress-induced c-Fos expression is influenced by age in the extended amygdala and brainstem stress centers in male rats. Front Aging Neurosci 10: 248. **IF: 3.633**
2. Kovács LÁ, Berta G, Csernus V, Ujvári B, Füredi N, Gaszner B. (2019) Corticotropin-releasing factor-producing cells in the paraventricular nucleus of the hypothalamus and extended amygdala show age-dependent FOS and FOSB/deltaFOSB immunoreactivity in acute and chronic stress models in the rat. Front Aging Neurosci 11: 274. **IF (2018): 3.633**

   Cumulative impact factor of publications related to the thesis: 7.266

List of publications unrelated to the thesis

1. Kormos V, Gáspár L, Kovács LÁ, Farkas J, Gaszner T, Csernus V, Balogh A, Hashimoto H, Reglődi D, Helyes Z, Gaszner B. (2016) Reduced response to chronic mild stress in PACAP mutant mice is associated with blunted FosB expression in limbic forebrain and brainstem centers. Neuroscience 330: 335-358. **IF: 3.277**
2. Farkas J, Kovács LÁ, Gáspár L, Nafz A, Gaszner T, Ujvári B, Kormos V, Csernus V, Hashimoto H, Reglődi D, Gaszner B. (2017) Construct and face validity of a new model for the three-hit theory of depression using PACAP mutant mice on CD1 background. Neuroscience 354: 11-29. **IF: 3.382**
3. Werling D, Banks WA, Salameh TS, Kvarik T, Kovacs LA, Vaczy A, Szabo E, Mayer F, Varga R, Tamas A, Toth G, Biro Zs, Atlasz A, Reglodi D. (2017) Passage through the ocular barriers and beneficial effects in retinal ischemia of topical application of PACAP1-38 in rodents. Int J Mol Sci 18(3): pii: E675. **IF: 3.687**
4. Werling D, Reglodi D, Banks WA, Salameh TS, Kovacs K, Kvarik T, Vaczy A, Kovacs L, Mayer F, Danyadi B, Lokos E, Tamas A, Toth G, Biro Zs, Atlasz T (2016) Ocular delivery of PACAP1-27 protects the retina from ischemic damage in rodents. Invest Ophthalmol Vis Sci 57 : 6683-6691. **IF: 3.303**

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