Early life adversity in the rodent neurobehavioral development and three hit concept model of major depressive disorder

Doctoral (PhD) thesis

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Pécs
2017

Introduction

Major depressive disorder (MDD)

The Diagnostic and Statistical Manual of Mental Disorders (DSM 5) defines MDD as a debilitating disease. It has a substantial impairing effect on various fields of the patient's life resulting sometimes in suicide. Besides the psychiatric aspects, depression can be blamed for the increased likelihood of developing certain somatic diseases. MDD has a high impact on human life globally. According to the Global Burden of Disease Study, MDD was within the first three contributors of years lived in disability in 2013. Depression has serious economic effects: approximately 1% of European GDP is spent on the treatment annually. MDD affects 350 million people globally. MDD is similarly widespread in high-, middle- and low-income countries, disproving the idea that it might be a disease of developed countries.

Significant early and late life events in the background of MDD

The body is continuously challenged by noxious factors, which endanger the equilibrium. Selye János in 1936 defined the nonspecific response of the body to such threatening demand as stress. In humans, the link between stressful life events and the occurrence of depressive episodes is well documented. Such factors may be for instance exposure to environmental traumas, terrorism and violence, subjugation to domestic violence, sexual abuse, chronic or life threatening health problems, loss of employment, neglect, financial instability, separation or grief.

The perinatal and childhood events have strong correlation with psychosocial development. Significant amount of evidence suggests that childhood maltreatment potentially increases the risk of developing depression and other mental and somatic diseases in later life.

More recently, the three hit concept of depression has been developed postulating that inherited genetic factors (hit 1), epigenetic alterations caused by significant (early) life events (hit 2) and additional environmental stress in later life (hit 3) may precipitate the symptoms in humans.

Effect of PACAP on stress response and early development of nervous system

In 1989 Arimura and his coworkers isolated a new member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon family, the pituitary adenylate cyclase-activating polypeptide (PACAP). PACAP's main effect is the stimulation of cAMP formation in cells expressing PAC1, VPAC1 and VPAC2 receptors. Since the discovery, PACAP and its receptors were found in the HPA axis too. In addition to its various roles, PACAP has important functions in the embryonic development of various tissues, and it is also considered as a trophic factor during neuronal development and in case of neuronal injuries. Due to the roles played by PACAP in neuronal development, it is not surprising that severe behavioral abnormalities have been described in PACAP knockout mice. Hashimoto has found altered phenotypic signs of depression and anxiety in these animals.

Aims of the studies

1. Maternal deprivation study

The aim of this project was to describe the neurobehavioral development, early motor coordination and early open-field activity in Wistar after 2 weeks of maternal deprivation. Several effects of maternal deprivation are known to be gender-dependent, therefore, we evaluated neurodevelopment of male and female rats separately.

2. PACAP mutant mouse development study

The aim of the PACAP mutant mouse development study was to describe the postnatal development of physical signs and neurological reflexes in mice with the partial (heterozygous, HZ) or complete lack of PACAP (homozygous PACAP-deficient, KO animals). We examined developmental hallmarks during the first 3 weeks of the postnatal period and described the neurobehavioral development using a complex battery of tests.

3. Depression model study

The third part of this PhD project aimed to develop and validate a new mouse model for the three hit theory of human depression. Offspring of PACAP HZ pairs (hit 1) were subjected to maternal deprivation (hit 2), and later, adult mice were subjected to chronic variable mild stress (CVMS, hit 3) *vs.* controls. The model was validated by studying the effect of stress using physical, endocrinological, behavioral and functional-morphological tools.

Materials and methods

1. Laboratory animals

Animals were housed in temperature and humidity controlled 12 h light-dark cycle environment (lights on at 6 am) in standard cages in three to four rats per cage and four to six mice per cage groups at the animal facility of the Department of Anatomy, University of Pécs Medical School. Animals were provided *ad libitum* with standard rodent chaw and drinking water (BA02/2000-25/2011, BA02000-39/2016).

PACAP knock out mice on CD1 background

PACAP KO mice were used for the depression model study and for the PACAP KO mouse development study.

Wistar rats

Six litters of in-house bred Wistar rats (n=10±1 pups per litter) were used. Both male and female offspring were included into the study.

2. Experimental design

Maternal deprivation study in the rat

- Removal of the dam from the nest in a randomized manner, on PND 1–14 between 8 and 11 am for 180 minutes.
- Maternally deprived (MD) rats, n = 16 males, 19 females.
- Control rats: shortly handled, for the duration of the neurobehavioral testing (n = 12 males, 13 females).

PACAP mutant mouse development study

- 46 offsprings of PACAP HZ mice (6 litters) were used
- 11 WT, 22 HZ and 13 KO animals.
- Mice reaching weaning age were included (both genders were observed)

Depression model study

- 37 litters of PACAP HZ parents were used.
- Three main groups: animal facility rearing conditions (AFR): 12 litters, 15 minutes maternal separation (MS15) 12 litters; 180 minutes maternal deprivation (MD180): 13 litters
- Separation and deprivation on PND 1-14.
- Only male offspring (AFR: n=56; MS15: n=58; MD180: n=44) were further used.
- Two experimental subgroups: control (CTRL) group *vs.* mice exposed to CVMS.
- Based on quality of maternal care, stress factor and genotype, 18 groups of mice were created, with four to nineteen subjects.

3. Tests for neurobehavioral development (PACAP mutant mouse development, maternal separation study)

- Physical signs: Body weight, physical characteristics (eye opening, incisor eruption ear unfolding).
- Neurological reflexes: surface righting reflex, negative geotaxis, crossed extensor reflex, sensory reflexes, limb placing, limb grasp, gait, auditory startle, air righting.

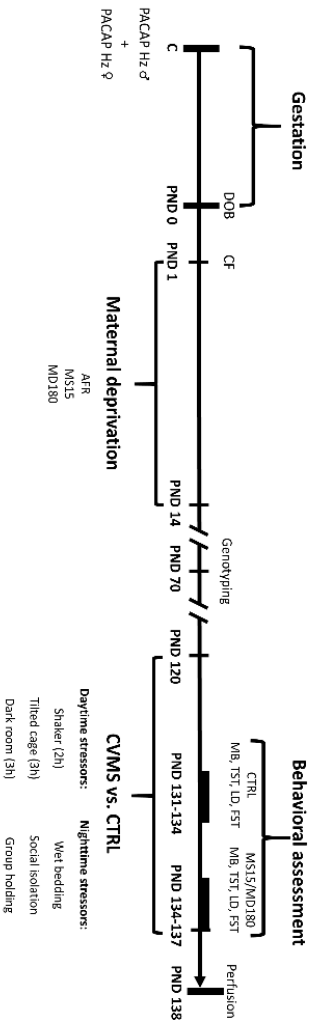
- Motor coordination tests: grid-walking and footfault test, walking initiation, rope suspension test, inclined board test, rotarod test.
- Open-field activity

4. Chronic variable mild stress (Depression model study)

Adult animals were subjected to CVMS composed by various mid-day and overnight stressors in a completely random fashion to avoid habituation. The mid-day stress paradigm consisted of the following stressors: tilted cage, shaker, dark room. The overnight stress paradigm consisted of social isolation and wet bedding, group holding

Behavioral tests

- Marble burying (MBT)
- Tail suspension test (TST)
- Light-dark test (LDT)
- Forced swim test (FST)



Experimental setup of our three hit model (Conception – C, Post-natal day – PND, Date of Birth – DOB, Marble Burying Test – MB, Tail Suspension Test – TST, Forced Swim Test – FST, Chronic Variable Mild Stress – CVMS, cross fostering - CF)

5. Functional-morphological assessment

Free floating double-label immunofluorescence

In order to assess the activity of a) corticotropin-relaeasing facor (CRF) positive neuronal activity of the BSTov, b) the urocortin1 (Ucn1) expressing cells of the centrally projecting Edinger-Westphal nucleus (EWcp), and c) serotonin (5-HT) containing perikarya of the dorsal raphe nucleus (DR) we carried out double immunolabelings for CRF, Ucn1, 5-HT and FosB, respectively.

Microscopy, digital imaging and morphometry

Olympus FluoView 1000 confocal microscope was used for imaging. Sequential scanning in photon count mode was used for the respective fluorophores to detect semi-quantitable fluorescent signal. For densitometry, ImageJ software (version 1.42., NIH, Bethesda, MD) was used.

6. Corticosterone radioimmunoassay

The corticosterone (CORT) titer was determined by radioimmuessay in plasma samples to assess the hypothalamus-pituitary-adrenal (HPA) axis activity.

7. **Statistical analysis**

Data were expressed as mean ± standard error of the mean in all cases. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Dunn's *post hoc* test in the maternal deprivation study Fisher's multiple comparison test in the PACAP transgenic development study. Results in the depression model study were evaluated by

multifactorial analysis of variance (MANOVA) followed by Fisher's *post hoc* test. Homogeneity of variance (Bartlett's Chi-square test) and normality (Shapiro–Wilk test) were tested for all data groups. When data were not normally distributed, a square root mathematical transformation was applied. Results were considered significant when $p < 0.05$.

Results

1. Maternal deprivation study

Male MD rats showed a tendency for a faster weight gain than control males, which reached a significant level toward the end of the 3rd week. They weighed significantly more than MD females from the 4th day throughout the whole observation period. Further signs of somatic development did not show significant differences. No gender difference was revealed within the control and MD groups and no marked differences were found in the appearance of neurological reflexes between control pups and those subjected to maternal separation. Male rats, however, showed a tendency of enhanced development. In the motor coordination test only the percentage of foot faults was significantly lower in MD male rats on the 3rd week. In the open field tests, we found no statistical difference.

2. PACAP mutant mouse development study

The weight gain of PACAP KO mice, both in males and females, was slower than in WT mice. The slowest weight gain was observed in PACAP HZ mice. Results show that weight gain was fastest in the WT group, except for the first week. Homozygous KO mice had a more variable weight gain pattern. Signs of

somatic development did not show any significant difference between groups. Regarding reflex development we observed a significant delay in appearance of forelimb grasp in female HZ mice compared to female WT and KO. Air righting reflex appearance showed a similar pattern. Gait initiation reflex appearance was also delayed in female HZ mice compared to their WT mates. In males, only a tendency was observed. In the foot fault test female HZ mice made significantly more mistakes than WT or KO animals on week 3, while males showed only a similar tendency with no statistical significance. On week 4 male HZ and KO mice made more mistakes than WT animals. Females only showed a similar tendency.

3. Depression model study

Efficacy of the paradigm

CVMS exposure in WT and HZ AFR animals caused a statistically significant decrease in their *body weight* compared to the AFR controls. In MS15 mice, CVMS decreased the body weight in all genotypes. CVMS exposure on MD180 HZ mice *vs.* controls had a significant decreasing effect on body weight. *Adrenal gland weight* measurements proved the effectivity of our CVMS paradigm. Although the CVMS-related rise of adrenal gland weights did not reach the significant value in all pairs of groups, CVMS exposure of HZ AFR mice resulted in a 28% elevation of the adrenal weight.

WT and HZ animals with AFR history reacted to CVMS with a *CORT* increase by 78% and 76.7%, respectively, while KO mice showed negligible change. CVMS exposure of MS15

mice caused a 99.13% rise of CORT level. MD180 KO animals reacted with a significant CORT increase.

In the FST, upon MD180 history, immobility time was significantly increased when AFR CTRL WT animals were compared to MD180 CTRL WT mice. AFR WT animals reacted with a significantly increased immobility time to CVMS. Upon CVMS exposure, HZ mice with MD180 history showed a robust, 130% increase in their immobility time. In MBT, after CVMS exposure in AFR group all genotypes, while in MS15 group WT and HZ mice showed a robust and statistically significant increase in the number of buried marbles. In MD180 group CVMS caused a significant increase only in HZ animals.

Morphological findings

CRF-FosB in BSTov

In the AFR group, the CVMS exposure caused a significant increase in the CRF-FosB co-localization both in the WT and HZ animals. The CRF positive cell count in the MD180 CTRL WT animals was significantly higher than in AFR CTRL WT and in MS15 WT mice. Similarly, MD180 CTRL HZ and KO animals had more CRF cells in the BSTov, than their AFR CTRL counterparts. The MD180 CTRL WT animals showed significantly higher CRF signal density compared to AFR CTRL and MS15 CTRL animals.

Ucn1 in the cpEW

In the AFR group CVMS caused a significant growth in the Ucn1-FosB co-localization in WT and HZ animals compared

to CTRL counterparts. KO animals reacted to CVMS by a 48% rise in Ucn1-FosB expressing neuron count, compared to CTRL counterparts, although this remained under the significant value. MD180 increased the basal FosB expression.

The comparison of CVMS-exposed MD180 mice revealed that KO mice showed 2.8 times, while HZ mice 2.1 times higher Ucn1-FosB cell count when compared to WT animals.

Serotonin in DR

In the AFR group CVMS exposure increased the number of 5-HT-FosB co-localizing cells in all genotypes but the increase was statistically significant only in WT animals. 5-HT-FosB cell count of the MD180 CTRL group in comparison to their AFR counterparts both in WT and HZ animals showed significantly elevated basal values.

Discussion

1. Maternal deprivation study

The results revealed that a 3h-long daily maternal separation did not lead to marked delay or enhancement in reflex development and motor coordination. A subtle enhancement was observed in the appearance of hind limb grasp and gait reflexes, and a better performance in foot fault test in male rats exposed to maternal deprivation. In contrast, female MD rats displayed a slight delay in forelimb grasp and air righting reflex appearance, and surface righting performance.No significant effect of maternal separation was observed in the open-field test at such a young age.

2. PACAP mutant mouse development study

Our present study revealed even greater alterations in heterozygous PACAP knockout mice than in homozygous animals in the disturbance of some early neurobehavioral development during the first 3 weeks. Considering physical signs, we found that PACAP-deficient mice had slower weight gain throughout the observation period. PACAP HZ mice weighed significantly less than did homozygous mice. There was no difference between male and female mice during the first 3 weeks. Incisor teeth erupted earlier in mice lacking PACAP. On the elevated grid, homozygous KO mice took significantly more steps but also made more mistakes. This can be related to the previously described hypermotility and explosive behavior of PACAP knockout mice. The explanation in these cases may be that the complete lack of PACAP may induce the upregulation of other trophic factors compensating for the genetic defect of PACAP, but the partial lack may not have such an effect.

3. Depression model study

Here we aimed to validate an animal model for the three hit theory of resilience and vulnerability. Our hypothesis was that the combination of these paradigms may help to induce a depression-like status in mice. Genetic predisposition was modeled by the mutation(s) of PACAP gene(s). Early life adversity, such as maternal deprivation applied in this study shapes the epigenome of mouse pups resulting in long-lasting changes in stress adaptation. Finally, CVMS, as a third hit, superimposed to the genetic predisposition and history of MD may precipitate the symptoms.

The efficacy of the CVMS paradigm

Bodyweight change is a commonly used physical parameter to assess the efficacy of stress exposure. The decrease of bodyweight upon CVMS in most of our groups support that the CVMS was effective. It has to be underlined that WT and KO mice with MD180 history without CVMS exposure lost some weight, while the bodyweight of HZ remained stable. When CVMS was added, the weight loss of HZ mice was already remarkable, but that of WT and KO mice did not change. This suggests that the strength of the added deleterious factors to cause weight loss in mice with all risk factors was the most ideal in MD180 HZ CVMS mice. Adrenal gland weight is also an effective indicator of stress level. Our statistical analysis proved the significant relationship between adrenal weight and stress exposure underpinning the effectiveness of CVMS in our experiment. However, the heightened basal adrenal weight was refractive to the superimposed CVMS exposure. The increased CORT values resembling the HPA axis activity also proved the effectiveness of our CVMS protocol in WT and HZ AFR mice. In line with earlier studies AFR KO mice did not react. An interesting novel finding of this study is that if PACAP KO mice receive an exposure to short or severe maternal separation, their CORT response to CVMS becomes normal. The effectivity of CVMS exposure was further supported by FST, as stressed AFR WT mice showed longer immobility time. MD180 effectively increased the depression-like behavior in WT. Importantly, to cause increased depression-like phenotype, CVMS exposure was required in MD180 HZ mice, supporting the model value of this group.

The effectivity of CVMS on the anxiety was supported by the MBT test regardless the quality of maternal care. If both PACAP alleles were mutated, anxiety remained uninfluenced by CVMS except for AFR group. The reduced anxiety value of KO animals, presumably due to lower sample size, remained only a tendency in the AFR group of the present study, however in MS15 mice we proved this difference. The important new finding of this study is that MD180 has long lasting effects on the outcome of MBT. Moreover, the CVMS exposure superimposed on MD180 history remains ineffective in increasing anxiety levels assessed by MBT. Interestingly, in MD180 PACAP HZ mice the superimposition of CVMS was required to elevate the anxiety level, therefore, the latter group may be considered as a suitable tool to study mood disorders. Two out of four behavioral tests (i.e. FST for depression-like behavior and MBT for anxiety) support that CVMS of PACAP HZ mice with MD180 history may be used as mouse models of the three hit theory concept.

Morphological findings

CRF in BSTov

Our histological results further support the involvement of BSTov CRF neurons in mood control. First, in line with our recent study, CVMS induced FosB expression in CRF neurons of WT and HZ animals. However, these cells in KO mice did not react at all. Second, the quality of maternal care clearly influenced the CRF peptide content of BSTov neurons as supported both by CRF cell count and specific signal density data. Third, the severe maternal deprivation history set the

expression of FosB in CRF neurons of control mice high, suggesting altered gene expression pattern in these neurons. Presumably this phenomenon is accompanied by altered stress adaptation capacity. Since the CVMS exposure in these mice failed to further increase the FosB expression, we propose that the co-incidence of MD180 history and CVMS expires the adaptation ability of BSTov-CRF neurons in WT and HZ mice. As the magnitude of rise in anxiety level was high in HZ mice only, we propose that the 70% reduced PACAP expression together with the MD180 history and CVMS exposure contributes to the observed phenotype. The relative unresponsiveness of the HPA axis in PACAP HZ mice with HD180 history may be at least in part explained by the CVMS-refractive neuronal activity found in the BSTov.

Ucn1 in cpEW

This study is the first to show that MD180 evokes increased basal FosB expression in cpEW neurons. This phenomenon is especially strong in HZ and KO animals, suggesting altered gene expression, which presumably affects CVMS reactivity in a PACAP expression-dependent manner. The highest number of Ucn1-FosB immunoreactive neurons was found in MD180 KO mice upon CVMS exposure.

Serotonin in DR

Earlier studies indicate that altered DR neuronal 5-HT content, metabolism and/or neuronal activity might be at least in part responsible for the altered stress adaptation ability of PACAP KO mice. In the AFR group all genotypes reacted to CVMS by increasing the cellular activity in the 5-HT positive neurons. Severe maternal deprivation has a robust increasing effect on the

baseline FosB activity of 5-HT system in the DR. At the same time, the 5-HT positive cell number dropped in HZ and KO mice and remained unchanged in the WT group.

Conclusions

The research project aimed to test the effect of early life adversity on the neurobehavioral development of newborn rats. Here we concluded that the effect of this stressor does not cause robust changes in the first three postnatal weeks on the examined variables. The maternal deprivation model per se does not expire the adaptation capacity possibly due to the high neural plasticity which is characteristic for this period of life. The idea arises here, if the superimposition of other detrimental factors may precipitate depression-like effects.

As PACAP has been implicated in several aspects of neurobehavioral development and stress response, we tested the hypothesis if the inheritance of two mutant alleles of PACAP gene would affect the early neurobehavioral development of mice. We found a somewhat slower weight gain and delay in the neurobehavioral development of PACAP KO mice. PACAP KO mice show mild phenotypical anomalies only, but they are known to display markedly reduced adaptation capacity to noxious environmental effects.

Based on these findings, in the third part of this PhD thesis we put forward to combine early life adversity (i.e. maternal deprivation) and genetically reduced adaptation capacity (i.e. PACAP gene mutations) with the chronic variable mild stress exposure to mimic

an aversive environment based on the widely accepted three hit concept of human depressive disorder.

The assessment of our new model based on the classic Willnerian criteria revealed that a) the construct validity was successfully achieved in PACAP HZ mice. b) The face validity criterion was supported by bodyweight data and behavioral tests: PACAP HZ mice with MD180 history show increased depression and anxiety levels. Functional-morphological tools showed that the depression-like phenotype in PACAP HZ mice with MD180 history, may have been related to the elevated basal FosB expression in CRF and Ucn1 neurons which remained unresponsive to CVMS, moreover, to the loss of 5-HT neuronal activity.

Based on these we conclude that PACAP HZ mice upon MD180 and CVMS exposure might be used as a reliable model to study depression. Further tests are in progress to assess the third c) predictive validity criterion by Willner.

The main outcome of this study is the establishment of a new mouse model with promising features to test how risk factors contribute to the disease and how therapy resistant cases develop. Testing of new potential antidepressant compounds in our new model may help to assess their efficacy. Our model might help understanding the neurobiology of monoamine therapy resistant MDD opening new directions of research towards highly effective personalized therapy.

Summary of new results

1. **Severe maternal deprivation does not cause drastic changes in the early postnatal development until PND 21.** The subtle changes in the development of Wistar rats, caused by the maternal separation, were found to be partially gender dependent.

2. **PACAP heterozygous mice show slower neurobehavioral and weight development than the homozygous PACAP knock out and wild type mice.** Our results provide an important piece of information in understanding the significance of endogenous PACAP.

3. **MD180 has long lasting effects on MBT.** The CVMS exposure superimposed on MD180 history remained ineffective in increasing anxiety levels assessed by MBT.

4. **Endogenous PACAP is required for the stress sensitivity of BSTov-CRF neurons.** CVMS exposure increased the FosB activity in CRF neurons of BSTov in HZ and WT mice but did not have an effect in KO mice.

5. **Severe maternal deprivation sets the basal BSTov-CRF neuronal activity high, desensitizes these cells for additional CVMS exposure which phenomenon**

depends on endogenous PACAP. This indicates that if CVMS coincides with MD180 history, the adaptation ability of BSTov–CRF neurons expires.

6. **MD180 evokes increased basal FosB expression in cpEW–Ucn1 neurons.** As this phenomenon is especially remarkable in HZ and KO animals, the CVMS reactivity was influenced in a PACAP expression-dependent manner.

7. **Severe maternal deprivation has a robust increasing effect on the baseline FosB activity of 5-HT expressing DR neurons. Additional CVMS exposure causes a drastic decrease in 5HT-neuronal activity.**

8. **Physical, endocrinological, behavioral and morphological tools support the construct and face validity of our new model for depression based on the three-hit concept. Our PACAP heterozygous mice for the functional gene with a history of maternal deprivation and chronic stress exposure may be used as an animal model for depression.**

Peer-reviewed publications of the author

The thesis is based on the following publications:

- **Farkas J,** Kovacs LA, Gaspar L, Nafz A, Gaszner T, Ujvari B, Kormos V, Csernus V, Hashimoto H, Reglodi D, Gaszner B, *Construct and face validity of a new model for the three-hit theory of depression using PACAP mutant mice on CD1 background.* Neurosci (2017) 354: 11-29 (IF: 3.231)

- **Farkas J**, Sandor B, Tamas A, Kiss P, Hashimoto H, Nagy AD, Fulop BD, Juhasz T, Manavalan S, Reglodi D, *Early Neurobehavioral Development of Mice Lacking Endogenous PACAP*. J Mol Neurosci (2017) 61:(4): 468-478 (IF: 2.229)
- **Farkas** J, Reglődi D, Gaszner B, Szőgyi D, Horváth G, Lubics A, Tamás A, Falko F, Besirevic D, Kiss P, *Effects of maternal separation on the neurobehavioral development of newborn Wistar rats*. Brain Res Bull. (2009) 79(3-4):208-214. (IF: 2,184)
- **Farkas J**, Kovacs LA, Gaszner T, Gaszner B In: Dora Reglodi, Andrea Tamas (auth.) *Using PACAP Heterozygous Mice as Models of the Three Hit Theory of Depression Pituitary Adenylate Cyclase Activating Polypeptide* — PACAP. 840 p. Cham (Switzerland): Springer International Publishing, (2016) pp. 731-741. (Current Topics in Neurotoxicity; 11.) (ISBN:978-3-319-35133-9) – Book chapter

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Cumulative IF: 74.209

Acknowledgements

I would like to express my sincere gratitude to my supervisors, *Dóra Reglődi MD, PhD, Dsc* and *Balázs Gaszner MD, PhD* for their compassionate work and unconditional support during my carrier are student research assistant and later as graduate student. They served for me as rolling model both as professionals and as human beings.

I am grateful for the support I received and for the help in the RIA studies from the head of the Doctoral Program, *Valér Csernus MD, PhD, Dsc.*

I would like to express my appreciation towards my fellow graduate and undergraduate colleagues, *Tamás Gaszner MD*, *László Kovács MD*, *Nóra Füredi*, *Balázs Ujvári*, *Anna Nafz* and *László Gáspár* for their professional support during the experiment and for creating a friendly and inspiring atmosphere during my work.

I am grateful for the work of the members of the Anatomy Department at the UPMS. I would like to say special thanks to *Ms. Izabella Orbán* for her professional help in the immunohistological studies and to *Ms. Beatrix Brumán* for her help in the RIAs.

My gratitude goes to my mentors and friends, *Professor Seji Shioda*, *Dr. Tomoya Nakamachi*, *Dr. Hirokazu Ohtaki*, *Professor Sándor Vigh* and *Professor György M. Nagy* who helped to shape the scientist, the educator and the person who I am today.
Last but not least I would like to say thank you to my family, to my wife, *Zsófia* and to my daughter *Luca* who always supported me on my path that lead to the accomplishment of my graduate studies. I am also deeply thankful to my *parents* and to *my sister* who set me on the path and supported me to achieve my goals.

My work was supported by the following grants:
Hungarian Grant OTKA PD 100706 and by the research grant of Pecs University No. KA-2017-01 (to BG), Hungarian Brain Research Program – Grant No. KTIA_13_NAP-A-III/5, MTA TKI 14016 (to BG, DR) and by GINOP-2.3.2-15-2016-00050 – PEPSYS. OTKA K104984, PD109644, National Research, Development and Innovation Fund K119759, GINOP-2.3.2-15-2016- 00050 PEPSYS, PTE AOK Research Grant, Hungarian Brain Research Program—KTIA_13_NAP-A-III/5, Centre for Neuroscience of the University of Pecs. The UNKP-16-4-IV New National Excellence Program of the Ministry of Human Capacities, and Centre for Neuroscience, Idegtudományi Kiválósági Központ, University of Pécs, TAMOP 4.2.4.A/2-11-1-2012- 0001 National Excellence Program. Bolyai János Scholarship , Szodoray Lajos and Magyary Zoltán Funds by the European Union and the State of Hungary, University of Debrecen (RH/751/2015). Hungarian Science Research Fund (OTKA T046589, F67830, 78480), ETT439/2006