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Final report

Two distinct serotonergic systems in the brain: implications for the control of social behavior

The major aim of our study was to reveal the distict role of two raphe nuclei, the median (MR) and dorsal (DR) brainstem nuclei in regulation of the social behaviour.

At the very beginning of the project the leader moved to another institute, thus, let another senior researcher lead the research. This shifted the focus a bit toward MR raphe and the role of different cell-types in diverse behaviours. Originally we intended to focus on the serotonin. To do so we purchased a serotonin transporter (SERT) Cre miceline. However, breeding teh line took more time than was expected, and in the meantime we moved towards the exaineation of other cell-types.

During the course of the study one PhD student defended her thesis (Diána Balázsfi) and moved to another lab, while several new PhD students (Bibiána Török, Csilla Fazekas, Pedro Correia and Tiago Chaves) were involved in the project. Our previous assistant (Katalin Pelczer) retired and a new assistant (Krisztina Bánrévi) was employed. Toward the end of the project the new leader also had a parttime job at an other instiute, which delayed the work a bit. Therefore the project was prolonged with one year.

All in all, we published 16 articles, 6 in a journal with higher than 5 impact factor, two other papers were also submitted (Journal of Integrative Neuroscience, International Journal of Molecular Sciences) and three others is still in progress.

Our major findings were the followings:

(I) In relation to social behaviour

our data (D. Balazsfi et al. 2018) confirmed that **optogenetic stimulation** of the **MR,** which is considered to be a serotonergic nucleus, in male mice phasically reduced aggression. On the other hand, an increase in friendly social relationships was observed after dorsal raphe (**DR**) stimulation containing more serotonin. These may be due to different neurotransmitter releases in the prefrontal cortex, a a major site of aggression control. MR stimulation rapidly but transiently increased serotonin release, and induced a lasting increase in glutamate levels. DR stimulation had no effect on glutamate, but elicited a lasting increase of serotonin release. Prefrontal serotonin levels remained elevated for at least 2 h subsequent to DR stimulations. The stimulation of both nuclei increased GABA release rapidly and transiently. Our findings revealed a surprisingly strong behavioral task division between the two raphe nuclei, which was associated with a nucleus-specific neurotransmitter release in the prefrontal cortex.

Further **chemogenetic** studies (Fazekas et al. 2021) confirmed our findings for the role of MR stimulation and social behaviour. However, we ruled out the role of MR as a thermoregulatory centrum in the process, but found that chemogenetic activation of the MR decreased locomotion and depressive-like behaviour in the forced swim test (FST). Most interestingly, only depressive-like behavior was accompanied by changed body temperature regulation, which was also observed in human depressive disorders previously.
Despite low penetration, the ligand of the arteficial receptor, used in chemogenetics, clozapine-N-oxide (CNO) acts centrally, but does not influence the examined basic parameters, being suitable for repeated behavioral testing. Our data on CNO penetrability and repeated usage might have broader interest for researcher.

Our recent, still unpublished data in SERT-Cre mice using chemogenetics confirmed that social behaviour was not affected after stimulation of **MR-serotoninergic cells** in any of the tests (sociability, social interaction, resident-intruder). However, the excitation of MR-SERT+ neurons marginally increased floating and significantly decreased struggling during the FST. Furthermore, the decrease in core body temperature during and after the FST was reproduced in the SERT-Cre excitatory group (manuscript is in preparation).

The glutamatergic cells of the MR first was characterized by the presence of the vesicular glutamate transporter 3 (VGluT3), however, subsequent studies found, that approx. 20% of the cells contain **VGluT2** (Szonyi et al. 2019). We confirmed, that chemogenetic stimulation of the MR-VGluT2 cells made the mice more aggressive, which can be part of their depressive-like behavioural profile. Indeed, as a sign of anhedonia, the very same animals preferred the sweet, pleasurable sucrose solution less.

Vast majority of the MR cells are **GABAergic**. and social interactions, with minor role of their GABAergic cells. Chemogenetic stimulation of the MR-GABAergic cells (Chaves et al. 2021) had no effect on locomotion or working and social memory (which might confound the results of other tests); however, it increased social interest during sociability and social interaction but not in resident-intruder tests. Accordingly, c-Fos (a neuronal activation marker) elevation in MR-GABAergic cells was detected after sociability, but not resident-intruder tests. All in all, we confirmed the role of MR-GABAergic cells in promoting social interest. However, different subpopulations (e.g. long vs short projecting, various neuropeptide containing) might have divergent roles, which might remain hidden and requires further studies.

The role of **GABAergic** cells was also examined in **maternal behavior** (Dimen et al. 2021). Chemogenetic stimulation of preoptic GABAergic cells resulted in elevated maternal motivation and caring behavior in females and mothers but aggression toward pups in males. Behavioral effects were the opposite following inhibition of preoptic GABAergic neurons suggesting their physiological relevance. In addition, increased anxiety-like and depression-like behaviors were found following chemogenetic stimulation of the same neurons in females, whereas previous pup exposure increased only anxiety-like behavior suggesting that not the pups, but overstimulation of the cells can lead to depression-like behavior. A sexually dimorphic projection pattern of preoptic GABAergic neurons was also identified, which could mediate sex-dependent arenting and associated emotional behaviors.

Furthermore, chemogenetic inhibition of **VGluT3-containing MR cells** has increased social interest (manuscript is in preparation).

In a review article we summarized practical knowledge on the usage of **optogenetics** in behavioural studies (Zelena et al. 2017). Optogenetics was the method of the year in 2010 according to Nature Neuroscience. Since then this method has become widespread, the use of virally delivered genetic tools has extended to other fields such as pharmacogenetics, and optogenetic techniques became frequently applied with genetically manipulated animals for in vivo circuit analysis and behavioural studies. However, several issues should be taken into consideration when planning such experiments.

(II) Conditioned fear, as a putative model of posttraumatic stress disorder (PTSD) as well as learning and memory from teh viewpoint of the brainstem

In our experiments, we also observed that **optogenetic stimulation of MR** elicited a long-term fear response (D. G. Balazsfi et al. 2017) characteristic of post-traumatic stress disorder. The optogenetic stimulation patterns (50Hz theta burst and 20Hz) used in our tests elicited serotonin release *in vitro* and lead to activation primarily in the periaqueductal gray examined by c-Fos immunohistochemistry. Earlier studies demonstrated that fear can be induced acutely by stimulation of several subcortical centers, which, however, do not generate persistent fear memories. In our experiments we showed that the MR also elicits fear, but this

develops slowly over time, likely by plastic changes induced by the area and its connections. These findings assign a specific role to the MR in fear learning. Particularly, we suggested that this area is responsible for the durable sensitization of fear circuits towards aversive contexts, and by this, it contributes to the persistence of fear memories. This suggests the existence a bottom-up control of fear circuits by the MR, which complements the top-down control exerted by the medial prefrontal cortex.

At the time of our experiments glutamatergic cells, characterized by the presence of VGluT3 was thought to be a special, important subpopulation of MR neurons. Therefore, we studied the role of **VGluT3 in fear memory formation** in connection with its role in stress (D. Balazsfi et al. 2018). VGluT3 KO mice spent more time with freezing during the contextual fear test, less time in the open arm of the elevated plus maze (a conventional behavioural test of anxiety) and travelled a smaller distance in the open field, with less entries into the central area (another measure of anxiety). However, there was no trauma and genotype interaction suggesting that VGluT3 does not influence the fear conditioning, rather determines anxiety-like characteristic of the mice. The resting hypothalamic CRH mRNA was higher in KO mice with reduced stressor-induced corticosterone elevations. Immunohistochemistry revealed the presence of VGluT positive fibers in the paraventricular nucleus of hypothalamus, but not on the hypophysis. As a summary, we confirmed the involvement of VGluT3 in innate fear, but not in the development of fear memory and generalization, with a significant contribution to HPA alterations.

Take into consideration the role of hippocampus in learning and memory and the strong VGluT3ergic innervation of the hippocampus by MR we next conducted a deep examination on the role of **VGluT3 in learning and memory** formation using an extended behavioural test battery (Fazekas et al. 2019). Reversal learning was examined to test the cognitive flexibility. The VGluT3 KO mice clearly exhibited the ability to learn. The social recognition memory of KO mice was intact. The y-maze test revealed weaker working memory of VGluT3 KO mice. No significant learning impairments were noticed in operant conditioning or holeboard discrimination paradigm. In avoidance-based learning tests (Morris water maze and active avoidance), KO mice exhibited slightly slower learning process compared to wild-type mice, but not a complete learning impairment. In tests based on simple associations (operant conditioning, avoidance learning) an attenuation of cognitive flexibility was observed in KO mice. In conclusion, knocking out VGluT3 results in mild disturbances in working memory and learning flexibility. Apparently, this glutamate transporter is not a major player in learning and memory formation in general. Based on previous characteristics of VGluT3 KO mice we would have expected a stronger deficit. The observed hypolocomotion did not contribute to the mild cognitive disturbances herein reported, either.

In a review article (Horvath et al. 2018) we summarized the available data on VGluT3 and concluded that **VGluT3 participates in stress adaptation** regulation. The neuroendocrine changes observed in VGluT3 knockout mice may contribute to their anxious, fearful phenotype.

We also summarized the **role of brainstem nuclei in stress adaptation** (Chaves et al. 2021). Two major regulatory pathways exist: the hypothalamic–pituitary–adrenocortical axis (HPA) and the sympathetic adrenomedullary axis. They act in unison, ensured by the enormous bidirectional connection between their centers, the paraventricular nucleus of the hypothalamus (PVN), and the brainstem monoaminergic cell groups, respectively. PVN and especially their corticotropin-releasing hormone (CRH) producing neurons are considered to be the centrum of stress regulation. However, the brainstem seems to be equally important. Therefore, we summarized the present knowledge on the role of classical neurotransmitters of the brainstem (GABA, glutamate as well as serotonin, noradrenaline, adrenaline, and dopamine) in stress adaptation. Neuropeptides, including CRH, might be co-localized in the brainstem nuclei. Here we focused on CRH as its role in stress regulation is well-known and widely accepted and other

CRH neurons scattered along the brain may also complement the function of the PVN. Although CRH positive cells are present on some parts of the brainstem, sometimes even in comparable amounts as in the PVN, not much is known about their contribution to stress adaptation. Based on the role of the Barrington's nucleus in micturition and the inferior olivary complex in the regulation of fine motoric—as the main CRH-containing brainstem areas—we might assume that these areas regulate stress-induced urination and locomotion, respectively.
(III) In a broader sense, stress and memory are both participate in adverse memory formation during PTSD.

Therefore, we summarized the available **animal models** (Torok et al. 2019), which are useful tools to reveal the neurobiological basis of the vulnerability to traumatic events, and to develop new treatment strategies, as well as predicting treatment response contributing to personalized medicine approach. Different models have different construct, face and predictive validity and they model different symptoms of the disease. The most prevalent models are the single prolonged stress, electric foot-shock and predator odor. Freezing as 're-experiencing' in cluster B and startle as 'arousal' in cluster E according to DSM-5 are the most
frequently studied parameters; however, several other symptoms related to mood, cognitive and social skills are part of the examinations. Beside behavioral characteristics, symptoms of exaggerated sympathetic activity and HPA axis as well as signs of sleep disturbances are also warranted. Test battery rather than a single test is required to describe a model properly and the results should be interpreted in a comprehensive way, e.g. creating a z-score. Research is shifting to study larger populations and identifying the features of the resilient and vulnerable individuals, which cannot be easily done in humans. Incorporation of the "three hit theory" in animal models may lead to a better animal model of vulnerability and resilience. As women are twice as vulnerable as men, more emphasize should be taken to include female animals. Moreover, hypothesis free testing and big data analysis may help to identify an array of biomarkers instead of a single variable for identification of vulnerability and for the purpose of personalized medicine.

We, indeed, utilized hypothesis free testing concentrating on a specific biomarker group, the n-glycans (Fazekas et al. 2020). **N-linked glycosylation**, by modifying protein functions, may provide an important environmental link predicting vulnerability. First, we confirmed that our rat model was indeed working, and identified resilient and vulnerable individuals among the traumatized ones. Innate anxiety, measured on the elevated plus maze, did not predict vulnerability, but pretrauma levels of PGP10(FA1G1Ga1), PGP11(FA2G2) and PGP15(FA3G2) glycans correlated positively with it, the last one being the most sensitive. Traumatic stress induced a shift from large, elaborate N-glycans towards simpler neutral structures in the plasma of all traumatized animals and specifically in the prefrontal cortex of vulnerable rats. In plasma trauma increased PGP17(A2G2S) level in vulnerable animals. In all three brain regions BGP11(F(6)A2B) was more abundant in vulnerable rats, while most behavioral correlations occurred in the prefrontal cortex. In conclusion, we found N-glycans (especially PGP15(FA3G2)) in plasma as possible biomarkers of vulnerability to trauma that warrants further investigation. Posttrauma PGP17(A2G2S1) increase showed overlap with human results highlighting the utility and relevance of this animal model. Prefrontal cortex is a key site of trauma-induced glycosylation changes that could modulate the behavioral outcome.

(IV) In continuation of our previous results we were publishing some ideas on the role of vasopressin in stress adaptation as well.

Vasopressin is a ubiquitous molecule playing an important role in a wide range of physiological processes thereby implicated in the pathomechanism of many disorders. **Epigenetic** changes may also play a role in the development of these disorders. In a review

article we were focusing on the possible epigenetic mechanisms and how they were influenced by vasopressin or their receptors (Torok et al. 2021).

Chronic hypernatremia activates the central osmoregulatory mechanisms and inhibits the function of the HPA axis (Matuska et al. 2020). Noradrenaline (NE) release into the periventricular anteroventral third ventricle region (AV3V), the supraoptic (SON) and PVN from efferents of the caudal ventrolateral (cVLM)and dorsomedial (cDMM) medulla has been shown to be essential for the hypernatremia-evoked responses and for the HPA response to acute restraint. Notably, the medullary NE cell groups highly coexpress prolactin-releasing peptide (PrRP) and nesfatin-1/NUCB2 (nesfatin). To test whether they contributed to the reactions to chronic hypernatremia we compared two models: homozygous Brattleboro rats with hereditary diabetes insipidus (DI) and Wistar rats subjected to chronic high salt solution (HS) intake. Our data suggested that HPA axis responsiveness to restraint depends on the type of hypernatremia, and on NE capacity in the cVLM. Additionally, NE and PrRP signalization primarily of medullary origin is increased in the SON, PVN and AV3V in HS rats. This suggests a cooperative action in the adaptation responses and designates the AV3V as a new site for PrRP's action in hypernatremia.

In perinatal animals we confirmed that the maternal separation-induced ultrasonic vocalization, as a sign of distress, can be influenced by a single treatment with vasopressin receptor antagonist (Torok et al. 2021). However, the modulatory role of the antagonist was independent from their HPA axis influencing effect. Combination of small doses of V1a and V1b receptor antagonist might be even more helpful with presumably less side effects.

All in all, our results added several details to the understanding of the neuronal regulation of social and stress-adaptational processes. A better understanding of fine-tuning processes may lead to the development of more effective treatments for the prevention and treatment of neurological diseases.

Own references

Balazsfi, D., Fodor, A., Torok, B. et al. (2018) Enhanced innate fear and altered stress axis regulation in VGluT3 knockout mice. *Stress,* vol. 21, pp. 151-161. Doi: 10.1080/10253890.2017.1423053

Balazsfi, D., Zelena, D., Demeter, K. et al. (2018) Differential Roles of the Two Raphe Nuclei in Amiable Social Behavior and Aggression - An Optogenetic Study. *Front Behav Neurosci,* vol. 12, pp. 163. Doi: 10.3389/fnbeh.2018.00163

Balazsfi, D.G., Zelena, D., Farkas, L. et al. (2017) Median raphe region stimulation alone generates remote, but not recent fear memory traces. *PLoS One,* vol. 12, pp. e0181264. Doi: 10.1371/journal.pone.0181264

Chaves, T., Fazekas, C.L., Horvath, K. et al. (2021) Stress Adaptation and the Brainstem with Focus on Corticotropin-Releasing Hormone. *Int J Mol Sci,* vol. 22, pp. Doi: 10.3390/ijms22169090

Chaves, T., Torok, B., Fazekas, C.L. et al. (2021) Median raphe region GABAergic neurons contribute to social interest in mouse. *Life Sci,* vol. 289, pp. 120223. Doi: 10.1016/j.lfs.2021.120223

Dimen, D., Puska, G., Szendi, V. et al. (2021) Sex-specific parenting and depression evoked by preoptic inhibitory neurons. *iScience,* vol. 24, pp. 103090. Doi: 10.1016/j.isci.2021.103090

Fazekas, C.L., Balazsfi, D., Horvath, H.R. et al. (2019) Consequences of VGluT3 deficiency on learning and memory in mice. *Physiol Behav,* vol. 212, pp. 112688. Doi: 10.1016/j.physbeh.2019.112688

Fazekas, C.L., Bellardie, M., Torok, B. et al. (2021) Pharmacogenetic excitation of the median raphe region affects social and depressive-like behavior and core body temperature in male mice. *Life Sci,* vol. 286, pp. 120037. Doi: 10.1016/j.lfs.2021.120037
Fazekas, C.L., Sipos, E., Klaric, T. et al. (2020) Searching for glycomic biomarkers for predicting resilience and vulnerability in a rat model of posttraumatic stress disorder. *Stress,* vol. 23, pp. 715-731. Doi: 10.1080/10253890.2020.1795121
Horvath, H.R., Fazekas, C.L., Balazsfi, D. et al. (2018) Contribution of Vesicular Glutamate Transporters to Stress Response and Related Psychopathologies: Studies in VGluT3 Knockout Mice. *Cell Mol Neurobiol,* vol. 38, pp. 37-52. Doi: 10.1007/s10571-017-0528-7
Matuska, R., Zelena, D., Konczol, K. et al. (2020) Colocalized neurotransmitters in the hindbrain cooperate in adaptation to chronic hypernatremia. *Brain Struct Funct,* vol. 225, pp. 969-984. Doi: 10.1007/s00429-020-02049-y
Szonyi, A., Zicho, K., Barth, A.M. et al. (2019) Median raphe controls acquisition of negative experience in the mouse. *Science,* vol. 366, pp. Doi: 10.1126/science.aay8746
Torok, B., Fazekas, C.L., Szabo, A. et al. (2021) Epigenetic Modulation of Vasopressin Expression in Health and Disease. *Int J Mol Sci,* vol. 22, pp. Doi: 10.3390/ijms22179415
Torok, B., Fodor, A., Zsebok, S. et al. (2021) The Effect of Vasopressin Antagonists on Maternal-Separation-Induced Ultrasonic Vocalization and Stress-Hormone Level Increase during the Early Postnatal Period. *Brain Sci,* vol. 11, pp. Doi: 10.3390/brainsci11040444
Torok, B., Sipos, E., Pivac, N. et al. (2019) Modelling posttraumatic stress disorders in animals. *Prog Neuropsychopharmacol Biol Psychiatry,* vol. 90, pp. 117-133. Doi: 10.1016/j.pnpbp.2018.11.013
Zelena, D., Demeter, K., Haller, J. et al. (2017) Considerations for the use of virally delivered genetic tools for in-vivo circuit analysis and behavior in mutant mice: a practical guide to optogenetics. *Behav Pharmacol,* vol. 28, pp. 598-609. Doi: 10.1097/FBP.0000000000000361