Predictive validity of an animal model based on the three-hit theory of depression in the light of epigenetic changes

Doctoral (PhD) thesis

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Introduction

Depression and modelling in basic research

The prevalence of mood disorders rises steeply. Its most common form, major depression alone, affects more than 280 million people worldwide, according to data of WHO. In addition to the psychological impact, the disease also has cognitive, autonomic and somatic aspects. The diagnosis is complicated by the wide range of symptoms, which vary over a broad spectrum and fluctuate in severity over time. The long-term course of depression, which can last for years or decades, places a significant burden on society and the economy, besides the health care system. While the early onset of the illness results in a large loss on productive years and a drop in education rate. The importance of the disease is further underlined by the fact that the number of suicide attempts and completed suicides strongly correlate with the prevalence of mood disorders. Despite extensive research in the last decades to elucidate the etiology and pathomechanism, we still only know a few fragments of this enormously complex "puzzle".

There are difficulties even at the level of basic research and drug development because of the lack of an unequivocally accepted animal model. In order to assess the reliability of a newly developed animal model, it needs to be thoroughly tested. This is also true for the field of mood disorders. The animal models used in this research area must be evaluated according to three classical validity criteria. The basic principles of these were described by Willner. The first is construct validity, which means that the model must be based on known risk factors and triggers of the disease, while the second, face validity criterion postulates that the animal must show symptoms observed in human disease when exposed to these triggers. The third and perhaps the most difficult criterion to meet concerns the predictive validity of the model. According to this criterion, therapeutic procedures or drugs that have already been successfully used to treat the disease in the human must effectively improve the symptoms of animals. On this basis, the model can be used to test the effectiveness of future treatments. This is why it is called a predictive criterion, as such a model can "predict" the effectiveness of a future treatment in humans.

Drugs currently preferred in the pharmacotherapy of mood disorders, including the most commonly used selective serotonin reuptake inhibitors (SSRIs), have been tested in animal models based on the so-called monoamine theory. The concept of this is that the disease is caused by the dysfunction of the monoaminergic systems (serotonin, noradrenaline, dopamine). However, it has long been

known that this pharmacotherapeutical approach remains ineffective in almost one third of the cases. This convincingly illustrates that yet unknown mechanisms may lurk in the background of the disease beyond the well-characterized dysfunction of the monoamine systems. The currently accepted view is that depression is caused by hereditary and/or environmental factors. This concept is applied and complemented by the three-hit theory which our model is based on.

The three-hit theory

According to the three-hit theory, depression is caused by the co-existence of genetic, epigenetic and environmental factors. A number of potential genetic disorders have been identified in the background of mood disorders. Most of them are related to the control of monoamine biosynthesis or affect their receptors. Interestingly, there is also evidence in the literature for the common genetic background of depression and schizophrenia, or mood disorders and post-traumatic stress disorder (PTSD). In addition, a growing body of animal and human evidence supports the involvement of pituitary adenylate cyclase-activating polypeptide (PACAP) in depression. It is known that PACAP gene knockout mice exhibit depression-like behavior in tests. Heterozygous (HZ) animals were shown to exhibit reduced levels of PACAP in their brains. The use of HZ animals, in addition to the technical difficulties associated with gene knockout mice (*e.g.*: lower birth-, and higher death rates), can also be justified from a translational point of view: to our current knowledge only PACAP gene polymorphisms have been linked to human depression however no complete loss-of-function mutations have been identified in man. Besides the genetic factors, epigenetic effects have also been shown to contribute to the development of mood disorders. Epigenetic (*i.e.:* not affecting the DNA sequence *per se*, but involved in the regulation of gene expression) changes such as acetylation or methylation of histone proteins and methylation of the DNA may regulate the transcription of certain stress adaptation-recruited genes. The epigenetic pattern is mostly built up or transformed during the so-called vulnerable phases. The best known and most obvious is the perinatal period, especially the postnatal time. The significant negative life events suffered during this period of life are known to adversely affect stress adaptation and predispose to the development of mood disorders. Maternal deprivation is an animal model of early negative life events. Postnatal maternal deprivation is known to induce depression-like behavior in rodent models. The role of environmental stress in the pathogenesis of mood disorders has long been recognized. The widely

used animal model of stressors that affect humans in everyday life is chronic variable mild stress (CVMS). Based on the above, by subjecting PACAP heterozygous mice (first, genetic hit) to maternal deprivation after birth (second, epigenetic hit) and to CVMS later in life (third, environmental hit), a model consistent with the three-hit theory is obtained.

Main brain areas involved in stress adaptation

The role of higher limbic territories, such as the prefrontal cortex (PFC) and the hippocampus in mood regulation and in the pathogenesis of depression has been demonstrated. The role of the PFC as a cognitive, decision-making and emotional center has been described not only in animal-, but also in numerous human studies including functional magnetic resonance imaging, deep brain stimulation and case reports on PFC-injured patients. The PFC is also known to send efferent fibers to other important sites of mood regulation, such as the ventral tegmental area (VTA), amygdala and hippocampus. The latter is perhaps the most studied brain area in depression. Stress is known to affect hippocampal synaptic plasticity, brain-delivered neurotophic factor (BDNF) production (a protein responsible for synaptic maturation of neurons) and neuronal activity, thus contributing to the development of mood disorders. Its neuronal connections are extremely complex, most importantly, in addition to innervating the PFC and the extended amygdala, it is also involved in the direct regulation of the hypothalamic-pituitary-adrenal (HPA) axis (the endocrine regulator of the stress response). The amygdala, especially its central nucleus (CeA) and the related oval subdivision of bed nucleus of stria terminalis (ovBNST) accommodate prominent centers of the CRH system. Besides regulating the stress response of HPA axis, CRH neurons are both known to express PACAP. The role of brainstem monoamine systems in relation to mood disorders is also known. The mesencephalic dopaminergic VTA, as the center of the mesocorticolimbic pathway, is a key component of the reward system. A rate-limiting enzyme in the dopamine production is the tyrosine hydroxylase (TH), that has long been used as a tool to investigate VTA activity. The periaqueductal gray matter, and within that the centrally projecting Edinger-Westphal nucleus (cpEW) and its urocortin1 (UCN1) containing neurons, also play an important role in the pathomechanism of depression. This is supported by a number of experiments in various species including studies of cpEW samples on suicide victims. Importantly, these cells are also known to express PACAP. The raphe nuclei, including the dorsal raphe (DR), are centers of the

serotoninergic (5-HT) system, which is also linked to the extrahypothalamic CRH systems and cpEW. Serotonergic signaling has also been shown to be of crucial importance in the regulation of mood and thus in the pathomechanism of mood disorders.

Objective and hypothesis

The Willner's construct and face validity criteria of our animal model based on the three-hit theory have been previously demonstrated by our research team. The aim of our present project was to test the predictive validity of the model. We hypothesized that treatment with fluoxetine, the SSRI-type antidepressant most commonly used in the pharmacotherapy of mood disorders, reverses depression-like behavior in animals exposed to the three hits, demonstrating the predictive validity of our model. To confirm this, our animals were subjected to behavioral tests, physical and endocrinological parameters were measured, and functional-morphological investigations were performed in the centers which have been implicated in the pathomechanism of mood disorders as described above: PFC, hippocampus, extended amygdala, VTA, cpEW and DR. We hypothesized that the hits as risk factors interact with each other and with fluoxetine treatment as reflected by altered CRH, TH, UCN1 and 5-HT immunoreactivities as well as changes in H3 histone acetylation and FOSB neuronal activity patterns.

Methods

Experimental animals and experimental design

In this experiment we used male mice derived from 17 litters from our in-house-bred PACAP HZ CD1 mice. Half of the litters were subjected to three hours of maternal deprivation during the first two weeks after birth (MD180), when the animals were separated from their dams for three hours and were kept warm by a heating plate. While the other half of the mice were reared in normal animal housing conditions throughout the entire period (AFR). On day 70 after birth animals were genotyped using tail samples. Only PACAP HZ mice were used in the subsequent part of the experiment. At four months of age, half of the animals of both MD180 and AFR main groups were exposed to CVMS for two weeks. Here we randomly applied both daytime (cage tilt, shaker, dark room) and nocturnal (wet bedding, social isolation, group holding) stressors. The other half of the

animals in the main groups served as controls. These four groups (AFR-CVMS and AFR-control moreover MD180-CVMS and MD180-control) were further subdivided based on treatment. Half of the animals received daily intraperitoneal fluoxetine injections while the other half received vehicle. Thus, a total of eight groups were used, with 4-6 animals per group. The *in vivo* experiments were approved by the National Food Chain Safety Office (approval number: BAI/35/51-122/2016).

Behavioral tests

Our experimental animals were subjected to behavioral tests after the stress period. To assess the anxiety level, we performed marble burying test (MBT) and light-dark box test (LDT). In case of MBT 24 marbles were placed on the litter. More buried marbles refer to a higher anxiety level. While at the LDT, less time spent in the light compartment of the device with dark and light boxes indicates higher anxiety levels. To determine the level of depression, tail suspension test (TST) and forced swim test (FST) were performed. Both tests are based on that the animals are not restricted in their movement, but cannot escape from the situation, however try to do so. The mice that spend less time trying to escape exhibit a higher immobility time and therefore a higher level of depression.

Perfusion and sample preparation

On the 140th day of the experiment, our animals were anesthetized with urethane and their chest was opened. After opening the left ventricle, blood samples were taken to determine the animals' corticosterone (CORT) levels, then a cannula was introduced into the aorta where saline was first injected to rinse the blood vessels, and consecutively, animals were fixed with 4% paraformaldehyde. Their adrenal glands and thymus were removed and weighed. After removal, brains were postfixed and coronal sections were made using a vibratome. Four series of 30 μm thick sections were stored in antifreeze solution at -20°C until staining.

Immunlabelings

Free-floating double immunolabelings were performed in CeA, ovBNST, VTA, cpEW and DR, where the second antibody always targeted FOSB. Thus, the following double stainings were performed: dual labeling for CRH-FOSB in CeA and ovBNST, TH-FOSB in VTA, UCN1-FOSB in cpEW and 5-HT-FOSB staining in DR. Immunofluorescence was examined and digitized using an Olympus Fluoview 1000 confocal microscope. Histological photographs were subjected to cell counting and densitometry for CRH, TH, UCN1 and 5-HT. Labeled cells and their co-localization with FOSB was also investigated. FOSB immunolabeling was performed in the PFC and the three main subregions of the hippocampus [i.e. cornu ammonis 1 (CA1), CA3 and gyrus dentatus (DG)], using diaminobenzidine (DAB) chromogen. In addition, acetyl-lysine H3 histone (H3K9ac) DAB labelling was also performed in all the regions listed above. DAB-labeled H3K9ac and FOSB immunohistochemical preparations were evaluated and digitized using a Nikon Microphot FXA microscope with a Spot RT camera, followed by cell counting.

Statistics

Statistical analysis was carried out using Statistica software. Data beyond the two sigma range were excluded from the analysis. The normality of the data was tested using the Shapiro-Wilk test, while the homogeneity of variance was tested using Bartlett's Chi-squared test. The data were subjected to a multiple-way analysis of variance (MANOVA) followed by Tukey *post hoc* tests ($\alpha<5\%$). To explore deeper relationships between data series, a Spearman's rank correlation test was also performed.

Results and discussion

Physical and endocrine parameters

The efficacy of CVMS was confirmed by a reduction in body weight gain during the second week of stress exposure. Maternal deprivation was also associated with a reduction in body weight gain, indicating that the model was successful in interfering with the epigenetic pattern during the vulnerable phase of early life. To further demonstrate this experimentally, we extended our

morphological work by a semiquantitative determination of an epigenetic marker, H3K9ac. Relative adrenal and thymus weight data further supported the overall efficacy of maternal deprivation as an epigenetic stressor, as shown by MANOVA. The CORT studies showed that SSRI treatment slightly reduced corticosterone levels in subjects which suffered both maternal deprivation and stress. The phenomenon, that the HPA axis over-activity can be normalized by antidepressants, is well known.

Evaluation of the behavioral tests

In MBT, we found that regardless of the quality of maternal care animals exposed to stress hid fewer marbles after fluoxetine treatment. This result clearly supports the predictive validity of our model, as fluoxetine treatment effectively reduced the anxiety level of mice. A similar trend in the effect of fluoxetine was observed in LDT in AFR animals, while maternal deprivation completely abolished the effect of fluoxetine. This further emphasizes that aversive early life events significantly affect stress adaptation and response to antidepressant treatment. TST showed that SSRI treatment strongly reduced depression levels in AFR animals. Maternal deprivation, on the other hand (even without CVMS), reduced the efficacy of fluoxetine treatment. When exposed to all risk factors, the animals were no longer able to adapt. These behavioral results both further strengthen the predictive validity of our model and highlight the importance of personalized therapy in the treatment of mood disorders. TST showed higher sensitivity in our model than the FST. FST found smaller differences between groups and higher depression levels after fluoxetine treatment in AFR, stressed mice. This second finding is against all other results. Data from other laboratories have also pointed higher sensitivity of the TST, while recently more and more respected experts are of the opinion that the FST should be used no longer to measure depression levels.

Histological examination of the PFC

In the PFC CVMS reactivity of the FOSB was influenced by both maternal care quality and SSRI treatment. The FOSB is a neuronal activity marker widely used in stress research, reflecting the cellular response to a stimulus that requires modulation at the level of gene transcription. The

antibody we used in this study recognizes both full-length FOSB and its splice variant, deltaFOSB. Considering the shorter half-life of full-length FOSB and the fact that in this study mice were perfused one day after the last stress exposure, the detected FOSB protein signal should correspond to the delta isoform, which reflects chronic neuronal activation. The phenomenon that chronic stress exposure increases FOSB immunoreactivity in the PFC is well known. Importantly, we observed here that when CVMS exposure was applied following maternal deprivation, neither CVMS nor fluoxetine treatment increased FOSB activity in the PFC. This phenomenon may have a great translational significance, as FOSB-related transcriptional changes are increasingly being used to determine therapeutic efficacy in models of depression. In our study of histone acetylation, we found that in AFR mice fluoxetine treatment was associated with reduced acetylation, but when mice were subjected to MD180, we saw a lower level of acetylation that was no longer affected by fluoxetine treatment. This suggests that fluoxetine therapy and early-life stress interact in the PFC, so we found an evidence again, that exposure to aversive early life events affects the response to antidepressant treatment.

Histological examination of the hippocampus

As maternal deprivation was associated with very low, almost undetectable FOSB signal in all hippocampal regions examined, we must conclude that SSRI treatment had little space to reduce FOSB immunoreactivity. This observation may possibly be explained by a technical error that prevented the immune signal, but since PFC sections of the same animals in the same staining procedure gave reliable signal, we propose that the low hippocampal FOSB cell counts in MD180 mice are true area-specific observations. The dynamics of hippocampal histone acetylation differed from that observed in the PFC. MD180 reduced histone H3 acetylation in all hippocampal regions. CVMS, on the other hand reduced H3K9ac immunoreactivity in vehicle-treated AFR mice in all regions tested, whereas fluoxetine treatment increased acetylation in mice that had previously undergone CVMS. Our present study shows that in CVMS-exposed mice that also underwent MD180, the acetylation-enhancing effect of fluoxetine was abolished in the CA1 and was inverted into a reducing effect in the CA3 and DG. This suggests in our model that the efficacy of antidepressants may depend on the number of risk factors and the epigenetic status carried by the

animal. This may ultimately underline the importance of an individualized therapeutic approach in the treatment of depression to increase the efficacy of pharmacotherapy.

Histological examination of ovBNST

Contrary to our previous results, CVMS exposure did not induce significant neuronal activation in ovBNST in AFR animals, which may be explained by the mild stress effect caused by daily intraperitoneal injections of the vehicle animals. However, after maternal deprivation the magnitude of the response to the same stress was reduced in both CRH and FOSB cell numbers. This supports the interpretation of our behavioral assays that we see maladaptation in MD180 animals. Our correlational analyses confirmed that levels of anxiety (in MBT) and depression (in TST) are inversely proportional to the FOSB positivity of ovBNST/CRH cells. Similarly, an increase in CRH/FOSB cell number induced by fluoxetine treatment was seen in most groups, which was associated with a strong anxiolytic and antidepressant effect in behavioral tests. CRH specific signal density (SSD) measurements revealed very low ovBNST/CRH levels in all deprived groups, suggesting that maternal deprivation reduces CRH levels in these cells.

Histological examination of CeA

Activation of CeA by stress is a well-known phenomenon. In our model, we also found elevated CRH-FOSB levels and concomitantly higher CRH-density, suggesting increased activity. Post-stress fluoxetine treatment decreased the number of CRH positive neurons. The effect of maternal deprivation fundamentally rearranged the neuronal activity pattern, as fluoxetine treatment decreased both CeA activity and CRH content, and stress neither activated the nucleus upon MD180, nor did it increase the number of CRH-producing cells. This observation again highlights that significant negative life events experienced early in life cause maladaptive changes in neuronal stress adaptation, which may contribute to the differences in behavioral tests. The negative correlation between the CeA/CRH SSD and the number of marbles hidden in the MBT demonstrates a strong anxiolytic effect of fluoxetine after stress, providing further evidence for the predictive validity of our model.

Histological examination of the VTA

The effect of maternal deprivation in this nucleus was similar to that observed in the extended amygdala. The 40% reduction in TH SSD in offspring that suffered maternal deprivation is consistent with the results of other research groups. These data further emphasize that significant negative life events experienced at a young age cause long-term changes in stress adaptation and responsiveness to antidepressant therapy. Our H3K9ac results showed that only fluoxetine treatment increased the rate of acetylation in animals. This effect of fluoxetine may increase the sense of reward by activating the mesolimbic reward pathway, thus alleviating typical symptoms of depression such as anhedonia and lack of motivation.

Histological examination of cpEW

The response of UCN1-producing neurons to fluoxetine treatment was consistent in all groups: they showed reduced UCN1 content. This, in correlation with the MBT results, establishes the predictive validity of our model and supports the importance of cpEW in anxiety-related disorders. Early life stress did not affect the number of urocortinergic cells, but reduced their FOSB activity and UCN1 content. This is consistent with the results of acetylation of histone proteins and the correlation between acetylation and UCN1 density, where lower acetylation was associated with lower UCN1 SSD. The further correlation between histone acetylation measured in cpEW and CRH SSD measured in ovBNST supports the role of the urocortinergic system in the regulation of BNST and HPA axis.

Histological examination of DR

DR/5-HT neurons are important regulators of the HPA axis and limbic brain areas in mood regulation. Previously observed behavioral abnormalities in PACAP KO mice were mainly attributed to alterations in the serotoninergic system. Here, we saw in AFR mice that stress reduced both DR/5-HT cell numbers and 5-HT SSD, but found elevated cell numbers following maternal deprivation. In MD180 suffered mice, stress *per se* did not affect DR/5-HT, whereas fluoxetine decreased DR/5-HT cell counts. This further confirms the long-term importance of early-life

aversive life events in the stress adaptation response and mood regulation. The number of buried marbles in MBT showed a negative correlation with DR/5-HT cell count and 5-HT SSD. This provides convincing evidence that there is a real link between morphological and behavioral findings. This is also consistent with the well-known importance of serotonergic systems in mood regulation. In case of acetylation, stress did not induce any change in mice with the history of maternal deprivation, but administration of fluoxetine after CVMS increased histone acetylation in DR. The strong negative correlation between 5-HT-positive cell number and DR H3K9ac cell number again highlights the importance of epigenetics in mood regulation. The strong correlation between the number of 5-HT cells and the number of ovBNST/CRH and cpEW/UCN1 cells demonstrates a functional link between these centers.

Connection between behavioral and functional-morphological results

In this study, we performed functional neuroanatomical studies in several limbic centers and found that each of the brain areas investigated may have contributed to the altered mood state. The activity of ovBNST and CeA CRH correlated negatively, whereas cpEW/UCN1 and DR/5-HT correlated positively with the levels of anxiety observed in MBT and depression recorded in TST. This suggests that they have opposite effects in regulating mood state. In the VTA, histone acetylation correlated with FST behavior, suggesting that this area contributes to the long-term development of mood state.

Summary

In our experiments, we demonstrated the reproducibility of our previous results in relation to the construct and face validity of the mouse model based on the three-hit theory. Further, we also extended the experimental setup with fluoxetine treatment, a widely used drug in human therapy. As a new achievement, we demonstrated the predictive validity of our model, as fluoxetine treatment was shown to reduce anxiety and depression levels in animals. The obtained behavioral results correlated well with the changes observed in the examined brain areas, providing further evidence for the influence of higher limbic centers, CRH-, and urocortinergic systems on HPA axis, thus supporting their role in stress adaptation. The results of the serotoninergic system, and especially their close correlations with behavioral outcomes, underline the key role of serotoninergic neurotransmission and raphe nuclei in mood regulation. In turn, the VTA results illustrate that a possible malfunction of reward pathways may also play an important role in the pathogenesis of mood disorders. Our epigenetic studies clearly demonstrate that early life (epigenetic) stress and the negative life events suffered at that time fundamentally influence both the stress adaptation mechanism, setting the stage for an eventual later mood disorder and the response to antidepressant treatment.

Publications contributing to this dissertation

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Cummulated impact factor of these publications: 12,263

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