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

Doctoral (PhD) thesis

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List of abbreviations

ACTH – adrenocorticotropin
AFR – animal facility rearing
BSTov – bed nucleus of stria terminalis
cAMP – cyclic adenosine monophosphate
CeA – central nucleus of amygdala
CORT – corticosterone
cpEW - centrally projecting Edinger-Westphal nucleus
CRF – corticotropin releasing factor
CTRL – control
CVMS - chronic variable mild stress
DR - dorsal raphe nucleus
EDTA- ethylene-diamine tetra-acetic acid
FST – forced swim test
HPA – hypothalamus-pituitary-adrenal
5-HT – 5-hydroxytryptamine (serotonin)
HZ – heterozygous
KO – knock out
LDB – light-dark box
MANOVA – multifactorial analysis of variance
MBT – marble burying test
MD180 - maternal deprivation (180 minutes long)
MS15 – maternal separation (15 minutes long)
NDS – normal donkey serum
PACAP – pituitary adenylate cyclase-activating polypeptide
PBS – phosphate buffer saline
PCR – polymerase chain reaction
PND – postnatal day
PVN – paraventricular nucleus of the hypothalamus
SSD – specific signal density
TST – tail suspension test
Ucn1 – urocortin 1
vLS – ventral lateral septal nucleus
WT – wild type

Preface

"Mental pain is less dramatic than physical pain, but it is more common and also more hard to bear. The frequent attempt to conceal mental pain increases the burden: it is easier to say "My tooth is aching" than to say "My heart is broken." — C.S. Lewis, The Problem of Pain

Depression is a major burden on the affected person, on the immediate and extended family and the society as well. The great weight caused by the disease can be detected on families, on the extended society and more measurably on the economy.

The majority of human beings go through depressive episodes during their lifespans. Often depression is characterized as a "trending" disease of the high society of Western cultures. However, many recently published studies prove the opposite, depression affects people independently from the socioeconomic status all around the globe. Patients are unable or limited at best to function as a member of family, society and to provide for themselves. Depressive episodes often result in the death (not rarely due to suicidal activity) of the affected person. Societies are trying to cope with the results of depression to the best of their abilities.

One would expect that a disease with such a high impact on human life is well understood and researched to the point where it can be cured or at least treated effectively. Unfortunately, today this is not the case. Clinical psychiatry uses a pharmacological therapeutical approach which was developed decades ago and was found to be effective at only a fraction of the patients. Neuroscience is unable to present the complete pathomechanism of the disease at the moment. Moreover, it is unclear what the causes in the background are.

Present days neuroscientists are making discoveries that support the idea that depression is a multifactorial disease. Many people are prone to develop the disease but only under the "right" circumstances. It is imperative to discover all the factors behind depression in order to identify "attack points" where therapies may interrupt the development or reverse the process of the disease.

This PhD thesis focuses on the possibilities to develop a functional rodent model of major depressive disorder according to the Willnerian criteria. Such a model would be a great help to understand the disease itself and it would provide an important tool for development of

effective therapies. Moreover, the study attempts to uncover the long-term effects of early life events and genetic variations as possible factors of depression.

1. Introduction

1.1 Epidemiology of major depressive disorder (MDD)

MDD has a high impact on human life globally. According to the Global Burden of Disease Study (2013), 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. According to the World Mental Health (WMH) survey, MDD affects one in every six adults and the lifetime prevalence is approximately three times higher than the 12-month prevalence. MDD affects 350 million people globally (WHO 2017). According to Bromet's study the 12-month prevalence of the MDD is similar in high-, middle- and low-income countries, disproving the idea that it might be a disease of developed countries. In both genders, the median age of MDD onset is 25 years and the risk period ranges from adolescence to early 40s (Bromet et al. 2011). According to Seedat et al. (2009) after puberty, women have a twofold higher risk of developing MDD than men. Despite this fact, chances of recurrence and longer period MDD episodes are less common in women than measured at men (Eaton et al. 1997). A growing body of evidence suggests that negative sociocultural influences, such as absence of partner, negative life events, financial insecurity, and unemployment have a strong effect on the development of MDD (Bromet et al. 2011, Risch et al. 2009). Stressful early life events seem to increase the likelihood of developing MDD (Heim et al. 2012). Patients who have such background show more severe symptoms and tend to have a higher tendency for treatment resistance (Hovens et al. 2012).

The Diagnostic and Statistical Manual of Mental Disorders (DSM 5) defines MDD as a debilitating disease and characterizes it with multiple criteria, including depressed mood, markedly diminished interest in pleasure, considerable change in weight (both gain and loss), insomnia or hypersomnia, fatigue, loss of energy, feeling of worthlessness, diminished ability of concentration, recurrent suicidal intentions without a specific plan

etc. (For further details see DSM 5). MDD has a substantial impairing effect on various fields of the patient's life resulting sometimes in suicide (WHO 2017). Besides the psychiatric aspects, depression can be blamed for the increased likelihood of developing certain somatic diseases, such as various cardiac conditions, hypertension, stroke, diabetes, Alzheimer's disease and various types of cancer. Furthermore, depression increases the overall mortality and obesity (Penninx et al. 2013).

1.2 Genetic background of MDD

Because of all the serious effects on multiple aspects of human life, depressive diseases have been subjected to much research, including genetic studies. Since the 20th century (Winokur 1972; Dorzab et al. 1971; Winokur et al. 1969) it is known that MDD has a genetic background. Early studies have found evidence that first degree relatives of MDD patients show increased risk of developing the disease (Baker et al. 1972). More recent studies also publish similar results (Merikangas et al. 2014; Sullivan et al. 2000). In order to determine the heritability of MDD, large scale twin studies were carried out. Singh and coworkers published an extensive study on offspring of monozygotic twins discordant for MDD. They found association between depression of the patient and the descendant. Offspring with exposure to parental depression developed MDD more commonly than their unexposed cousins (Singh et al. 2011). Rice et al. (2005) also found evidence to the genetic background of major depressive disorder. Twin studies estimate the heritability of major depressive disorder between 40 (Kendler et al. 2006, Sullivan et al. 2000) and 70% (McGuffin et al. 2003). Several groups proved that the early onset and recurrent depression pose increased familial risk (Kendler et al. 2005, 1999; Bland et al. 1986; Weissman 1984).

There have been many attempts to identify chromosomal locations and common, structural and rare risk gene variants that are responsible for the onset of depressive disorders. Multiple genes have been implicated, mostly by genome wide association studies. Some of these genes and gene products in question may have a direct effect on the function of the hypothalamus-pituitary-adrenal (HPA) axis and other systems key to development of MDD. For instance, the protein PLCO is involved in the monoaminergic

neurotransmission in the brain (Sullivan et al. 2009). In addition, the gene product SLC6A15 participates in the transportation of neutral amino acids, moreover in the ACTH and cortisol secretion affecting memory and attention in MDD (Schuhmacher et al. 2013). The difficulties identifying genetic loci suggest that the genetic background of MDD is highly complex.

There is no doubt, that the monoaminergic systems show profound changes in MDD. For instance, a great body of evidence supports that the genetic polymorphism at the serotonin transporter (5HT-T) gene promoter region is linked to MDD (Caspi et al. 2003, for review see: Iurescia et al. 2016).

Besides the doubtless recruitment of main neurotransmitters (i.e. catecholamines) in MDD, the possible involvement of neuromodulators should not be neglected. Indeed, multiple studies described possible connection between the polymorphism of genes for corticotropin-releasing factor (CRF) (Chang et al. 2015), oxytocin (McQuaid et al. 2014), galanin (Wang et al. 2013; Unschuld et al. 2010,) and neuropeptide S (Okamura et al. 2007) and MDD. Importantly, single nucleotide polymorphism in the pituitary adenylate cyclase-activating polypeptide (PACAP) gene and depression were also linked by several studies (Lowe et al. 2015; Almli et al. 2013; Ressler et al. 2011; Hashimoto et al. 2010).

As one would argue based on the great variety of genetic alterations that major depressive disorder must be a genetic disease, so far, no consistent or replicable genetic effects have been revealed behind MDD. The inheritance pattern does not fit any Mendelian laws. It is evident that studies with enormously high number of patients are required to reveal the complete genetic background of MDD. Such a study is being carried out by the Psychiatric Genomics Consortium (http://www.med.unc.edu/pgc). The results of the latest molecular genetic studies were summarized by Smoller (Smoller et al. 2016).

1.3 Epigenetics

Epigenetics is a term describing dynamic DNA modifications. These changes control gene regulation and expression without altering the genetic sequence itself. Such modifications may involve methylation, hydroxymethylation. Histone modifications, such as acetylation, phosphorylation and methylation, are also known forms of epigenetic changes. The significance of some kinds of the modifications are less known, such as

ubiquitination, sulphonylation, and non-coding RNAs. Importantly, epigenetic changes are dynamically changing in accordance to the environmental stimuli, they may have a lasting effect and they can be inherited to further generations. This may contribute to an explanation of the heterogeneity of MDD and the controversial reactions of patients to medication. (For review see Vialou et al. in 2013)

Many studies found an extremely wide variety of epigenetic changes on stress relevant genetic material after stress exposure. Not surprisingly the main stress regulatory system, the HPA axis (see details later in chapter 1.6) may undergo epigenetic alterations after stress exposure. For instance, the altered epigenetics of the *crh* gene has been shown after stress exposure (Sterrenburg et al. 2011; Elliott et al. 2010). Methylation of the CRH receptor 1 gene (CRHR1) also undergoes changes (Wan et al. 2014) and glucocorticoid receptors (GR) play a crucial role in the negative feedback loop of the HPA axis. Hypermethylation of *NR3C1* (gene coding for GR) causes decrease in the GR expression in MDD (Webster 2002; Yehuda et al. 1993), while mineralocorticoid receptors (MR – encoded by NR3C2) play a role in the appraisal process and the onset of stress reaction (de Kloet 2005). The expression dynamics of MR is highly responsive to stress (Wu et al. 2013; Gesing et al. 2001; Lopez et al. 1998,) which is underlined by the epigenetic modifications on the NR3C2 gene (Perroud et al. 2014, Sober et al. 2010).

1.4 Significant early life events in the background of MDD

Many studies have found connection between significant early life events and the onset of MDD. 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 (for review see Li et al. 2015).

Besides psychiatric disorders, perinatal events are also known to cause various short- and long-term disturbances in cognitive, and other behavioral performances (Koehl et al. 2001). Postnatal stress is related to abnormal sensorimotor functioning later in life as well. Animal models of neonatal stress, like maternal separation, may provide important correlation with human psychiatric disorders, such as schizophrenia, drug addiction-abuse disorders, stress adaptation and depressive disorders (Brake et al. 2004, Kalinichev et al.

2003, 2002; Finamore et al. 2000; Ellenbroek et al. 1998). It has been shown that repeated separation of rat pups from their mothers increases behavioral fearfulness and HPA axis response to stress. Maternal separation has also been demonstrated to induce long-lasting changes in reactivity to a novel environment and morphine induced sensitization and tolerance (Kalinichev et al. 2002). Several neurochemical changes have been revealed after different paradigms of maternal separation, such as changes in the mesolimbic dopaminergic system and alterations in neuropeptide expression in the brain (Brake et al. 2004; Husum et al. 2002). Drastic separation (artificial rearing) leads to decreases in trophic factors and other plasticity markers in the brain (Burton et al. 2007). Neonatal maternal separation has been shown to induce several other changes, such as alterations in colon distension, neurogenesis, hypoxic ventilatory response and phase-shifting of circadian rhythms (Ren et al. 2007; Kinkead et al. 2005; Yoshihara et al. 2005; Mirescu et al. 2004; Ohta et al. 2003). However, contradictory data also exist. It has been shown that sucrose-reinforced and open-field behaviors in adults are not altered by maternal separation (Shalev et al. 2002). Other reports have also shown the lack of effect on locomotor activity after amphetamine injection or startle potentiation (De Jongh et al. 2005; Weiss et al. 2001). Also, Marmendal et al. (2004) found that maternal separation had no effect on offspring's behavior and brain opioid peptides, only a minor change in maternal care could be observed. No difference was reported in the response to NMDA-antagonist treatment or cocaine sensitization (Ellenbroek et al. 2005; Planeta et al. 2002). Even beneficial effects of maternal separation have been reported. Brief daily separation, for example, reverses prenatal diazepam-induced deficit in learning tasks (Cannizaro et al. 2005). Most studies examine effects of maternal separation in adulthood. Relatively little is known about immediate consequences of the neurobehavioral development of such early life experience. It has been reported that a single long-lasting (24 h) maternal deprivation retards the neurobehavioral development of newborn rats (Ellenbroek et al. 2005). A good indicator of postnatal neurodevelopment is the maturation of reflexes and motor coordination. The appearance of certain reflexes is influenced by various factors, such as nutritional state, genetic background, maternal care, environmental enrichment, toxic agents and stress (Segovia et al. 2008; Archer et al. 2007, 2002; Kostrzewa et al. 2007; Barros et al. 2006; Lavi-Avnon et al. 2004; Palomo et al. 2003; Beninger et al. 2002;

Eriksson et al. 2001; Smart et al. 1971a,b). Short-term neurofunctional outcome has been described to correlate with long-term functional deficits, which draws the attention to the predictive value and necessity of short term evaluation (Ten et al. 2003). Several effects of maternal deprivation are known to be gender-dependent, like light-enhanced startle, emergence latencies in the plus maze test, activity in a novel environment and ACTH response (Diehl et al. 2007; Slotten et al. 2006; De Jongh et al. 2005; Zimmerberg et al. 2004).

Our research group has previously described that perinatal monosodium glutamate treatment and hypoxic-ischemic injury delay the neurobehavioral development, while postnatal treatment with the trophic factor PACAP enhances it (Kiss et al. 2007, 2006, 2005; Lubics et al. 2005; Reglodi et al. 2003). The question arose whether the lack of PACAP might have significant influence on the neurobehavioral development, and to the reactivity of early life adversity.

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

In 1989 Arimura and his coworkers isolated a novel peptide from ovine hypothalamus, which, based on its stimulatory effect on the adenylate cyclase activity in the anterior lobe of the pituitary gland was designated as 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 (for more detailed see Chapter 1.6). PACAP positive terminals form synapses in the paraventricular nucleus of the hypothalamus (PVN) with CRF expressing neurons and modulate CRF expression (Hashimoto et al. 2011; Agarwal 2005; Legradi et al 1998). Furthermore, PACAP has a direct stimulatory effect on adrenocorticotropin (ACTH) release (Miyata et al. 1989). There were no PACAP receptors detected in the adrenal cortex, suggesting that PACAP's effect on the function of the HPA axis is driven by its central effect (Conconi et al. 2006, Shioda et al. 2000).

It was demonstrated that PACAP acts via several distinct signaling pathways in addition to the adenylate cyclase-induced routes (Vaudry et al. 2009). Besides HPA axis modulation PACAP is involved in numerous physiological processes, such as

thermoregulation (Banki et al. 2014), control of thyroid function (Bardosi et al. 2016; Egri et al. 2016), vasodilation (Vamos et al. 2014), barrier control (Maugeri et al. 2017, Wilhelm et al. 2014), gastrointestinal and urinary system regulation (Girard et al. 2016; Padua et al. 2016), cardiac neuronal excitability (Clason et al. 2016), synaptic transmission, neuronal protection (Pecoraro et al. 2017. Shioda and Nakamachi 2015) as well as pain transmission (Sundrum et al. 2017, Tuka et al. 2016; For a review see Reglodi et al. 2016).

In addition to its various physiological roles, PACAP has important functions in the embryonic development of various tissues, and it is also considered as a trophic factor during development and in the case of neuronal injuries (Kvarik et al. 2016b; Lindholm et al. 2016; Matsumoto et al. 2016; Vaczy et al. 2016; Watanabe et al. 2016; Shioda and Nakamachi 2015). Among others, it has been shown that the peptide is involved in the development of the nervous system and peripheral organs (Irwin et al. 2015; Irwin et al. 2015; Njaine et al. 2014; Uyttebroek et al. 2013; Watanabe et al. 2007)

Disruption of endogenous PACAP signaling affects normal development and plasticity after injuries. Animals lacking PACAP display altered cerebellar development: reduced thickness of the granular layers as well as delayed differentiation of granule cells have been described (Allais et al. 2007). Abnormal axonal arborization has been found in the dentate gyrus of PACAP knockout mice (Yamada et al. 2010). We have also shown that PACAP-deficient mice have an accelerated cerebral myelination and suggested an inhibitory effect of PACAP on myelination, possibly allowing time for axonal development, synapse formation, and neuronal plasticity (Vincze et al. 2011). Newborn mice show several metabolic changes, respiratory problems, and temperature sensitivity, possibly due to the abnormal development of brainstem regulatory pathways (Gray et al. 2001).

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, especially in challenged conditions (Kormos et al. 2016; Shibasaki et al. 2015; Lehmann et al. 2013; Gaszner et al. 2012; Reglodi et al. 2012, Ishihama et al. 2010; Marquez et al. 2009). The first observation reported explosive jumping behavior and subsequent studies have

reported on mice showing altered phenotypic signs of depression and anxiety (Hashimoto et al. 2001).

Altogether, these facts suggest that the development of the nervous system is severely affected by the lack of PACAP. The short-term neurofunctional outcome show correlation with long-term functional deficits, as described by Ten at al in 2003, but the early neurobehavioral development of PACAP knockout mice has not yet been evaluated.

1.6 Later life environmental factors behind MDD

Besides the genetic and epigenetic predisposition caused by early life adversities, stressful environmental influences may also contribute to the occurrence of depressive disorders.

The body is continuously challenged by noxious factors, which endanger the equilibrium. Sellye (1936) defined the nonspecific response of the body to such threatening demand as stress. Stressful stimuli are conveyed to the limbic system. Such effects exert a higher-order control on stress response, which is primarily orchestrated by the HPA axis (Tsigos et al. 2002). CRF produced by the parvocellular PVN controls the release of adrenocorticotropin (ACTH) from the anterior lobe of pituitary. ACTH regulates the glucocorticoid response [i.e. corticosterone, (CORT) in rodents] from the adrenal cortex (for reviews see: Silverman and Sternberg 2012). The HPA axis reactivity is often affected in mood disorders at CRF level (Hartline, et al. 1996; Nemeroff, et al. 1984). Early life events (i.e. maternal deprivation, MD) may cause long lasting changes in the activity of CRF neurons (Desbonnet, et al. 2008, Plotsky, et al. 2005,). Such alterations are accompanied by elevated ACTH and CORT levels (Plotsky, et al. 2005) and cognitive impairment (Aisa, et al. 2007) in the rat.

In humans there is a wide variety of documented cases in which stressful life events were blamed for the onset of depressive episodes. Such events may be 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 (Li et al. 2016; Kessler et al. 1997). Based on these findings many models of major depressive disorder utilize environmental stress as triggering mechanism of the psychopathology. The most common form of it is the chronic variable mild stress (CVMS). Such a model was first applied by Katz and his colleagues (Katz et

al. 1981) and further improved by many authors, including Willner et al. (1992). As of 2015 there are over 1300 published studies using a version of the chronic mild stress and the number is exponentially increasing (for review see Willner 2016). In such models rodents are chronically exposed to unpredictable micro-stressors. This treatment results in a number of behavioral changes, such as decreased response to rewards, which correlates to one of the core symptoms of depression, anhedonia.

1.7 Animal model for MDD based on the three hit theory

In the last decades numerous models and theories (for instance monoaminergic, neurotrophin, and glucocorticoid theories) have been elaborated to explain the neurobiological alterations in stress-related mood disorders (Farkas et al. 2016; Kormos and Gaszner 2013). More recently the three hit concept of resilience and vulnerability became more widely accepted: genetic factors (hit 1), early life environmental challenges (hit 2) and late-life environmental stress (hit 3) increase the risk of depression (Daskalakis, et al. 2013; de Kloet, et al. 2007).

As detailed earlier in Chapter 1.5., PACAP has regulatory role in stress adaptation response (for reviews see: Eiden et al. 2017, Lind et al. 2017, Hammack and May 2015; Kormos and Gaszner 2013; Pinhasov, et al. 2011; Stroth et al. 2011; Hammack, et al. 2010; Norrholm et al. 2005; Agarwal et al. 2005). Mice lacking the functional PACAP gene (knock out, KO) on various genetic background have been generated and extensively studied on their stress adaptation (Kormos et al. 2016; Mustafa et al. 2015; Gaszner et al. 2014; Lehmann et al. 2013; Kormos and Gaszner 2013; Gaszner et al. 2012; Tsukiyama et al. 2011; Stroth and Eiden 2010; Hashimoto et al. 2009; Hashimoto, et al. 2001). It has to be pointed out that the effect of PACAP deficiency is also a function of the mouse strain used (Hattori et al. 2012).

PACAP KO mice on CD1 background used also in our laboratory show marked abnormalities in their psychomotor activity, circadian CORT rhythm, hippocampal glucocorticoid receptor mRNA expression and body temperature (Hashimoto et al. 2009). The behavioral assessment on mood status in naive CD1 PACAP KO mice revealed anxiolytic phenotype (Gaszner et al. 2012; Hashimoto et al. 2009, 2001; Girard et al. 2006) associated with depression-like anomalies in forced swim test (FST) (Gaszner et al. 2012;

Hashimoto et al. 2009, 2009). Interestingly, the CVMS exposure in these mice dramatically reduced the depression-like phenotype (Kormos et al. 2016).

Non-stressed PACAP KO mice on the C57BL/6J × 129SvEv hybrid background also exert reduced anxiety in the elvated plus maze and slightly reduced depression-like phenotype in the FST (Hattori et al. 2012), however the behavioral phenotype of those mice upon chronic stress is to date not known. To the contrary, C57BL/6 N PACAP mutants were shown to exert normal anxiety and depression levels if they had not been exposed to stress. Social defeat however caused reduced anxiety and depression levels in C57BL/6 N PACAP KO mice (Lehmann et al. 2013). Regardless the genetic background PACAP deficiency blunts the function of the HPA axis both at the level of PVN-CRF neurons and CORT release in acute (C57BL/6 N mice: Stroth and Eiden 2010, CD1 mice: Tsukiyama, et al. 2011; Gaszner, et al. 2012) and chronic models such as social defeat (C57BL/6N mice: Lehmann, et al. 2013), repeated restraint stress (C57BL/6N mice: Mustafa, et al. 2015) and CVMS (CD 1 mice: Kormos et al. 2016). The altered stress response, and depression-like phenotype found in PACAP mutants on CD1 background suggested that these animals could be used in a new model for depression based on the three hit theory (for review see also: Farkas et al. 2016).

Extrahypothalamic CRF systems such as the oval nucleus of the bed nucleus of stria terminalis (BSTov) and central nucleus of amygdala (CeA) also play significant roles in stress and depression (for reviews see: Waters, et al. 2015; Kovács 2013; Kehne and Cain 2010; Carrasco and de Kar 2003). For instance, lentiviral overexpression of CRF induced depression-like phenotype in the BST in mice (Regev, et al. 2011). Interestingly, PACAP containing nerve fibers were found in the BSTov (Hannibal 2002). More specifically, PACAP fibers innervate CRF neurons in the BSTov (Kozic, et al. 1997). This enables interaction between PACAP and extrahypothalamic CRF through PAC1 receptors with behavioral significance (for reviews see: Kormos and Gaszner 2013; Hammak, et al. 2010).

The CRF neuropeptide family member urocortin 1 (Ucn1) is expressed mainly in the centrally projecting Edinger-Westphal nucleus (cpEW) (Janssen and Kozicz 2013; Vaughan et al. 1995). Ucn1 neurons show selective sensitivity to various types of acute stressors (Gaszner, et al. 2004; Kozicz, et al. 2001), which is blunted by MD in the rat

(Gaszner, et al. 2009) and in PACAP KO mice (Gaszner, et al. 2012). Chronic stress exposure results in a constantly high c-Fos expression in Ucn1 neurons in wild type (WT) mice (Korosi, et al. 2005). CVMS induces FosB expression in murine Ucn1 neurons, which is blunted in PACAP KO mice (Kormos et al. 2016). Tree shrews react to chronic stress with a reduction of Ucn1 neuron count (Kozicz, et al. 2008a). Interestingly, upregulation of Ucn1 mRNA in female suicide victims was demonstrated (Kozicz, et al. 2008b).

Serotonin (5-HT) is a monoamine neurotransmitter expressed mainly in the dorsal raphe nucleus (DR) (for reviews see: Holmes 2008; Carrasco and de Kar 2003; Graeff, et al. 1996). The 5-HT cells of DR innervate the hippocampus, medial prefrontal cortex, septum, extended amygdala and the basal ganglia (Ma, et al. 1991; Steinbusch, et al. 1981). 5-HT-DR neurons were found to be sensitive to acute (Bouwknecht, et al. 2007) and chronic stress in the rat (Lim, et al. 2015). Perinatal stress affects the functioning of 5-HT systems in mice (Akatsu, et al. 2015; Yajima, et al. 2013), rats (van der Doelen, et al. 2014, Ohta, et al. 2014) and in humans also (Knaepen, et al. 2014). Malfunctioning of 5-HT systems is associated with depression and anxiety (Challis and Berton 2015) as also shown in PACAP KO mice (Kormos, et al. 2016; Hashimoto, et al. 2001,).

2. Aims of the studies

2.1. Maternal deprivation study

The aim of this project was to describe the neurobehavioral development, early motor coordination and early open-field activity in Wistar rat pups 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.

Our hypothesis in this work was that rats subjected to maternal deprivation will show delayed neurobehavioral development, poorer motor coordination and will show altered behavior in the open-field.

2.2. PACAP mutant mouse development study

The early neurobehavioral development of PACAP KO mice has not yet been evaluated. The aim of the PACAP KO 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.

Based on the various aspects of development that are effected by PACAP in this part of the study we hypothesized that the partial or complete lack of endogenous PACAP will have an impairing effect on the early postnatal development.

2.3. Depression model study

In the third chapter of this PhD thesis describes a study where we aimed to validate a mouse model for the three hit theory of depression based on the classic criterion system by Willner. Offspring of PACAP HZ pairs (hit 1) were subjected to maternal deprivation (hit 2), and later adult mice were subjected to CVMS (hit 3) *vs.* controls.

Our hypothesis was that mice subjected to all three hits would develop maladaptive changes at a) physical, b) behavioral c) endocrinological and d) functional-morphological levels.

3. Materials and methods

3.1. Laboratory animals

3.1.1. 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. The method of creating and maintaining of the in-house-bred PACAP KO mice was published earlier by Hashimoto et al. (2001), Gaszner et al. (2012) and Kormos et al. (2016). Briefly, mice were backcrossed for 10 generations with CD1 mice. By mating PACAP KO and WT mice a HZ generation was established. Offspring were weaned on the 21st postnatal day (PND). Male and female mice were separated and littermates were housed in the same cage. Tail tissue sampling was

performed to obtain DNA samples for genotyping. Tail clipping took place after the behavioral analysis in the developmental studies. In the depression model study, the tail samples were collected on PND 70. The offspring's genotype was determined by PCR. Genotyping was performed using Phire Animal Tissue Direct PCR Kit (Thermo Fischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Primer sequences used for the detection of wild type and PACAP KO DNA signatures were 5'-ACC GAA AAC AAA TGG CTG TC-3' (sense) and 5'-GGT CCA CAA AGT ATA TCT GTG CAT TCT-3' (antisense) for PACAP WT, and 5'-ATC TCC TGT CAT CTC ACC TTG CTC CT-3' (sense) and 5'-GAA GAA CTC GTC AAG AGA GGC GAT AG-3' (antisense) for KO mice. The PCR reaction was run at 98^{o}C for 5 min; followed by 36 cycles of 98^{o}C 5 sec; 61^{o}C 5 sec; 72^{o}C 20 sec; and finally 72^{o}C 1 min. After agarose gel-electrophoresis gels were stained with Sybr Green I (Thermo Fischer Scientific, Waltham, MA, USA). DNA bands on gel photos were evaluated using confirmed controls in the PCR reaction (heterozygous control, no template control).

3.1.2 Wistar rats

Six litters of in-house bred Wistar rats (n=10±1 pups per litter) were used. Animals were cross-fostered PND 1, to minimize litter differences. Pups were weaned after 4 weeks of age. Both male and female offspring were included into the study.

3.1.3. Housing conditions and ethical approval

Animals were housed in temperature and humidity controlled 12 h light-dark cycle environment (lights on at 6 am) in standard polycarbonate cages (for rats: 425X266X185 mm; for mice: 365X207X140 mm) 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. Animals were provided *ad libitum* with standard rodent chaw and drinking water. The number of animals, used in the experiments, was kept to the statistically acceptable minimum in order to avoid the unnecessary utilization of laboratory animals. All procedures applied in this protocol were studied and approved by the Ethical Committee on Use of Laboratory Animals at the University of Pécs [Depression model study: permission No: BA02/2000/25/2011 and BA02/2000-39/2016, PACAP mutant mouse development study: BA02/2000-15024/2011,

Maternal deprivation study: BA02/2000-20/2006) in agreement with the directive of the European Communities Council in 1986, and with the Law of XXCIII, in 1998, on Animal Care and Use in Hungary.

3.2 Experimental design

3.2.1 Maternal deprivation study in the rat

Maternal deprivation involved daily removal of the dam from the nest in a randomized manner, on PND 1–14 between 8 and 11 am. The same person removed dams in a semi-randomized order from their home cages and placed them into a separate cage; pups were put together into a container lined with home cage nesting material, in an incubator at 32 °C from PND 2 to 8 and at 30 °C from PND 8 to 14 [maternally deprived (MD) rats, *n* = 16 males, 19 females]. After 180 min of maternal separation, pups were returned to their dams. Control rats were only shortly handled, for the duration of the neurobehavioral testing (*n* = 12 males, 13 females).

3.2.2 PACAP transgenic mouse development study

46 pups derived from six litters of PACAP HZ pairs were cross-fostered. Genotyping revealed that these litters consisted of 11 WT, 22 HZ and 13 KO animals. Only animals reaching weaning age were included in the study. Throughout the PACAP KO mouse development study, the development of both genders were observed.

3.2.3 Depression model study

Seventy PACAP HZ female mice were mated with PACAP HZ males. 37 litters born within a 36 hours' time period were selected for this experiment and cross fostered on PND 1. Three main groups based on the quality of maternal care were formed: 12 litters were kept under standard animal facility rearing conditions (AFR); 12 litters were separated from their dam daily for 15 minutes (MS15) on PND 1-14; 13 litters were deprived by separating them from their dams for 180 minutes (MD180) on PND 1-14. Only male offspring (AFR: n=56; MS15: n=58; MD180: n=44) were further used. These subjects were divided into two subgroups: control group *vs.* mice exposed to

CVMS (see details below). Finally, considering quality of maternal care, stress factor and genotype, 18 groups of mice were created, with four to nineteen subjects. Due to the Mendelian ratios and the reduced adaptation ability of KO mice (see also Farkas et al. 2016) the group size in this experiment was limited to four mice as minimum. Since statistical tests are sensitive to differences in group sizes, in total ninety-eight mice were included into the assessments to compare groups consisting of four to six mice shown in Table 3.1.

AFR						MS15						MD180					
CTRL			CVMS			CTRL			CVMS			CTRL			CVMS		
WT	HZ	KO	WT	HZ	KO	WT	HZ	KO	WT	HZ	KO	WT	HZ	KO	WT	HZ	KO
6	6	4	6	6	5	6	6	5	6	6	4	6	6	4	6	6	4

Table 3.1. Experimental setup and sample sizes (n) per groups in the depression model study.

3.2.3.1. Experimental timeline of our three hit model.

PACAP heterozygous pairs of mice were mated (**C** – day of conception). The day of birth (**DOB**) was defined as postnatal day (**PND**) zero. On PND 1 litters were cross fostered (CF). Between PND1 and PND14 15 minutes maternal separation (**MS15**) or 180 minutes maternal deprivation (**MD180**) was applied on the selected litters. The third group was left undisturbed and handled according to the routine animal facility protocol: animal facility rearing (**AFR**). On PD70 tail clipping was performed for genotyping. On PD120 the male offspring in AFR, MS15 and MD180 groups were equally assigned to chronic variable mild stress (**CVMS**) and control (**CTRL**) groups (see also Figure 3.1). The CVMS consisted of daytime and nighttime stressors. Daytime stressors: shaker (the cage was placed on a laboratory orbital shaker set to 60 RPM for two hours), tilted cage (the cage was placed obliquely in 45 degrees angle for 3 hours), dark room (the cages were placed in a completely dark room for 3 hours). Nighttime stressors: wet bedding (animals were kept overnight in cages where the nesting material was soaked with tap water), social isolation (subjects were kept

overnight in separate cages), group holding (no stressor was applied). The CTRL groups were subjected to behavior tests between PD131 and PD134 (one test per day): marble burying (**MB**), tail suspension test (**TST**), light-dark test (**LD**) and forced swim test (**FST**). The same tests were conducted in CVMS groups between PD134 and PD137 and were considered as daytime stressors. On PD138 animals were euthanized and transcardially perfused. (Farkas et al. 2016). See Figure 3.1

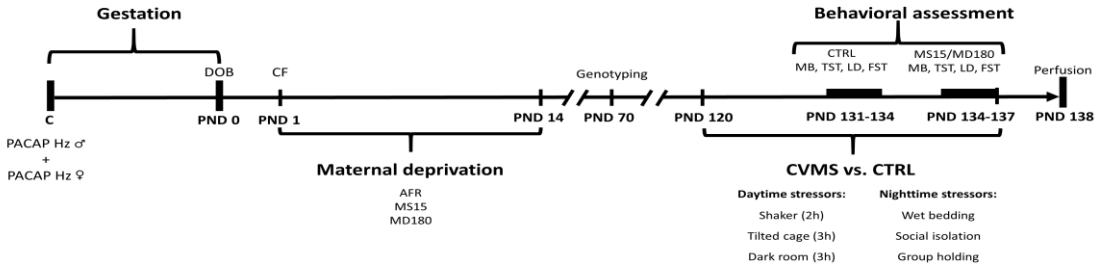

Figure 3.1 Experimental setup of our three hit model.

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

Examinations of neurobehavioral development were started on the first postnatal day and were carried out daily between 12 and 15 PM until PND 21. Neurobehavioral maturation and development of motor coordination were tested in littermates, based on a battery of tests used in rats and adapted to mice (Kvarik et al. 2016a; Horvath et al. 2015b; Kiss et al. 2009, 2013; Farkas et al. 2009; Lubics et al. 2005). The genotyping of the animals was not yet done at the time of the behavioral analysis, so the investigator performing the behavioral testing was not aware of the genotype of the mice.

3.3.1 Physical signs

Body weight was recorded every day until 3 weeks of age, then weekly until 5 weeks of age. Inspections were made for maturation of physical characteristics such as eye opening, incisor eruption and ear unfolding.

3.3.2 Neurological reflexes

Pups were tested for the following neurological signs and reflexes: (1) *Surface righting reflex*: rats were placed in supine position and the time in seconds to turn over to prone position and place all four paws in contact with the surface was recorded. (2) *Negative geotaxis*: animals were placed head down on an inclined grid (45°) of 30 cm. The hind limbs of the pups were placed in the middle of the grid. The day they began to turn around and climb up the board with their forelimbs reaching the upper rim was observed. In cases the animal did not turn around and climb up the board within the observed 30 s, the test was considered negative. From the day of the appearance of the negative geotaxis, the time in seconds to reach the upper end of the board was recorded daily. (3) *Crossed extensor reflex*: the left rear paw was pinched and the animal was observed for the extension of the right leg. The day of disappearance of the crossed extensor reflex in its pure form, when it was replaced by a more complex behavioral response, was noted. (4) *Sensory reflexes*: the ear and the eyelid were gently touched with a cotton swab and the first day of the ear twitch reflex and the contraction of the eyelid were recorded. (5) *Limb placing*: the back of the forepaw and hind paw was touched with the edge of the bench while the animal suspended, and the first day of lifting and placing the paws on the table was noted. (6) *Limb grasp*: the fore and hind limbs were touched with a thin rod, and the first day of grasping onto the rod was recorded. (7) *Gait*: the animals were placed in the center of a white paper circle of 13 cm in diameter, and the day they began to move off the circle with both forelimbs was recorded. In cases the animal did not leave the circle for 30 s, the test was considered to be negative. From the day of the appearance, the time in seconds to move off the circle was recorded daily. (8) *Auditory startle*: the first day of the startle response to a clapping sound was observed. (9) *Air righting*: subjects were dropped head down onto a bed of shavings from a height of 50 cm, and the day of first landing on four feet was recorded.

3.3.3 Motor coordination tests

Pups were tested for motor coordination twice a week between 2 and 5 weeks of age.

3.3.3.1. Grid-walking and footfault test:

Animals were placed on a stainless steel grid floor (20cm×40cm with a mesh size of 4 cm2) elevated 1 m above the floor. For a 1-min observation period, the total number of steps was counted. The number of footfault errors, when the animals misplaced a forelimb or hindlimb that it fell through the grid, was also recorded during a 1-min period.

3.3.3.2. Walking initiation

Animals were placed on a horizontal surface in the center of two concentric circles with diameters of 10 and 45cm (inner and outer circles). The time taken to move off the circles was recorded.

3.3.3.3. Rope suspension test:

Animals were suspended by both their forepaws on a horizontal, 4-mm-diameter nylon rope, stretched horizontally 40cm over a foam pad. The time the animals could hang on the rope was recorded (max. 30 s).

3.3.3.4. Inclined board test:

Animals were placed on a wooden board, and the board was gradually elevated by 5°. The maximum angle at which the animals could maintain position on the inclined board for 5s was recorded.

3.3.3.5. Rotarod test:

Animals were tested on a commercially available treadmill with diameter of 14cm for small animals, attached to a rotating motor. The test was performed at a speed of 13 rpm. The pups were placed on the rotating drum and the time the animal could stay on the rotarod was measured (max. 2min).

3.3.4. Open-field activity

Animals in the maternal deprivation study were also observed for locomotor behavior in an open-field at 3–5 weeks of age as previously described (Kiss et al. 2007; Lubics et al. 2005; Reglodi et al. 2003). After acclimatization to the environment, pups were placed in an open-field consisting of a 42 cm×42cm box with 21cm high walls around. The floor was divided into 8×8 areas. Subjects were placed individually in the center,

always facing the same direction, and were video-recorded for 5 min. Recordings were evaluated in a blinded fashion. The following parameters of locomotor activity were measured: distance traveled, rearing, the time spent in the center.

3.4 Depression model study

3.4.1. Chronic variable mild stress

Animals were subjected to CVMS between PD 120-138. Various challenges in a completely random fashion were applied as mid-day (between 10am and 2pm) and overnight (between 6pm and 7 am) stressors to avoid habituation. The mid-day stress paradigm consisted of the following stressors: a) tilted cage: the subjects were placed in a cage which was tilted in a 45 degrees angle for 2 hours b) shaker: the cage with the animals was placed on an orbital laboratory shaker, set to a 60 rpm speed for 2 hours. c) dark room: the animals were placed in a completely dark room for 3 hours. As mid-day stressor of the CVMS paradigm behavior tests were carried out. For control groups tests took place 5-8 days prior perfusion in order to avoid the acute stress effects. This battery of tests consisted of marble burying test (MBT), tail suspension test (TST), light-dark test (LDT) and forced swim test (FST) (see also Farkas et al. 2016).

The overnight stress paradigm consisted of a) social isolation: subjects were placed individually in a separate cage b) wet bedding: the mice were placed in a standard sized cage which was lined with nesting material moisturized with 150ml tap water. The overnight stress was initiated at 8pm and was terminated at 8am on the following day. c) On some nights animals were left undisturbed (group holding). After stress exposure, subjects were returned to their original home cages with fresh nesting material.

3.4.2. Behavioral tests

The following battery of tests was performed in the depression model study integrated into the CVMS protocol (see also ref. Farkas et al.2016). After all tests animals were placed back to their home cages.

3.4.2.1. Marble burying

Each animal was placed in a cage (30X30X28 cm) where on the top of the bedding 24 colored marbles were placed. Throughout the test, the animals had no access to food or water. The results were expressed as the number of marbles buried (at least to the 2/3 of the marble sphere was covered) during the 30 minutes test session (Gaszner et al. 2012; Njung'e and Handley 1991).

3.4.2.2. Tail suspension test

Mice were suspended 50 cm above a table by their tail with adhesive tape for 6 minutes. The suspension was designed in a way that subjects were unable to reach and hold on to the suspension platform. The duration of the test was 6 minutes. The process was videotaped and analyzed. The last 4 minutes period was assessed by measuring the cumulative time mice spent immobile (Steru et al. 1985).

3.4.2.3. Light-dark test

This test was performed in a box (40X20X27 cm) divided into two equal compartments. Between the two parts there was a 7X7cm large aperture. One compartment was painted white and lit with a 100W lamp while the other one was black and dark. The animals were placed in the lit part of the box facing the aperture. Each animal was videotaped for 5 minutes. The number of entering the dark compartment, the time spent there, the number of transitions and the number of peaks and aborted transitions into the lit compartment were evaluated.

3.4.2.4. Forced swim test

The test was carried out as described originally by Porsolt et al. (1977) and modified by (Ghasemi et al. 2009). Briefly, mice were placed into transparent glass cylinders (diameter 11.5 cm, height 25 cm) filled with 23°C tap water to 19 cm. Subjects were videotaped for 6 minutes. The recording was analyzed and the time spent immobile was measured in the last 240 seconds. All

behavioral tests were evaluated manually by a person who was unaware of the identity of mice.

3.4.3. Transcardial perfusion and sample collection

For the depression model study, in the morning of PND138 mice were placed on fresh bedding. Between 9 am and 12 pm animals were sacrificed with a high dose of urethane (2.4 g/kg) intraperitoneally. All animals in one cage were injected within 2 minutes. After losing consciousness they were weighed. Then, the chest cavity and the heart were opened and 1ml blood was collected in a pre-chilled syringe. Blood clotting was prevented by 50 µl 7.5 m/m% ethylene-diamine tetra-acetic acid (EDTA) solution.

After blood sampling, a cannula was introduced into the ascending aorta via the left ventricle. Animals were first perfused by 20 ml of ice-cold 0.1 M phosphate buffered saline (PBS, pH: 7.4) followed by 150 ml 4% paraformaldehyde solution in Millonig buffer (pH 7.4) for 15 minutes. Blood samples were transferred into pre-chilled vials and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and stored at -20°C in 40 µl aliquots for CORT radioimmunoassay.

3.4.4. Histological sample preparation and immunocytochemistry

Following perfusion, adrenal glands were collected and weighed. Brains were removed and postfixed at 4°C for 72 hours. In total ninety-eight brains (n=4-6/group) were coronally sectioned using Leica VT1000 S vibratome (Leica Biosystems, Wetzlar, Germany). Four series of 30 µm sections were made and stored in anti-freeze solution (20% ethylene glycol, 30% glycerol and 0.1 M sodium phosphate buffer) at -20°C until further use. The consequent labeling was performed as published earlier by Kormos et al. (2016). A short summary is given below.

3.4.5. Free floating double immunofluorescence for CRF and FosB

Sections at the plane of the anterior commissure containing the BSTov were selected. They were rinsed 6 x 10 minutes with PBS. Subsequently, heat induced epitope retrieval was applied in citrate buffer at 90°C for 10 minutes. Then, sections were transferred to 0.5% Triton X-100 solution for 60 minutes, followed by 5% normal donkey serum (NDS, Jackson Immunoresearch, Europe Ltd. Suffolk, UK) treatment. Thereafter, the sections were placed into a mixture of primary antibodies: rabbit anti-FosB diluted to 1:250 (Santa Cruz, sc-48, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-CRF diluted to 1:150 (Santa Cruz, sc-1759) in 5% NDS for 72 hours at 4°C. After 2x15 minutes washes in PBS, the slides were placed into a 5 % NDS, containing Cy3-conjugated donkey anti-rabbit serum (1:800) (Jackson) and a biotinylated donkey anti-goat serum (1:1000) (Jackson) for 24 hours at 4°C. After repeated washes in PBS Alexa 488-conjugated streptividin (1:2000) (Jackson) in PBS was applied for 3 hours. Finally, the sections were rinsed 2x15 minutes in PBS, mounted on gelatin covered slides, air dried and covered with a solution containing PBS and glycerol (1:1).

3.4.6. Free floating double immunofluorescence for Ucn1 and FosB

Sections of cpEW at the rostral midbrain were selected at the level of superior colliculus. After 6x10 minutes washes in PBS sections were permeabilized by 0.5% Triton X-100 for 60 minutes. Then, the sections were treated with 2% NDS for 60 minutes. Subsequently, a cocktail of primary antibodies in 2% NDS was applied for 48 hours at 4°C containing rabbit anti-FosB 1:250 (Santa Cruz, sc-48 Santa) and goat anti-Ucn1 (1:175) (Santa Cruz, sc-1825). After 2X15 minutes PBS washes a mixture of secondary antibodies with Alexa 488-conjugated donkey anti goat (1:200) and Cy3 conjugated donkey anti rabbit serum (1:800) (Jackson) for 24 hours was used. Finally, sections were washed, mounted and cover slipped exactly as described in the CRF-FosB labeling chapter.

3.4.7. Free floating double immunofluorescence for 5-HT and FosB in the DR

Caudal midbrain sections containing the DR cut at the level of the inferior colliculus were selected. The staining protocol was similar used for Ucn1 and FosB labeling, except that here a mouse monoclonal primary antibody for 5-HT (1:10.000) (gift from Dr. Lucienne Léger, Université Claude Bernard, Lyon, France) and an Alexa 488 conjugated donkey anti mouse (1:200) (Jackson) secondary antiserum was used.

3.4.8. 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. The confocal aperture was set to 80µm. Scanning was performed with a 20X objective to obtain image of 1024X1024 pixel resolution. The excitation and emission spectra of the respective fluorophores were set according to the built-in settings of the Fluo-View software (Fv10-ASW; Version 0102). 488 nm and 550 nm beam wave lengths were used for excitation (at 100% intensity) of Alexa 488 (emission peak for detection: 525 nm) and Cy 3 (emission peak for detection: 570 nm) respectively. Images of respective channels were stored both individually, and superimposed to evaluate co-localization of fluorescent signals. Red and green virtual colors were used.

Cells were counted in non-edited digital images by an experienced observer who was blinded to the identity of the files. Quantification was performed on five digital images for each brain area. All cells were counted on the cross section surface of the respective nuclei. The average cell count value of each animal was used for statistical analysis.

For densitometry, ImageJ software (version 1.42., NIH, Bethesda, MD) was used. The intensity of immunofluorescence was measured in 10 cell bodies per slide for CRF, Ucn1 or 5-HT. For the measurements five non-edited images of five sections per animal were used. The area of interest was determined manually at cytoplasmic areas of neurons. The signal density measurements were corrected for the background signal measured next to the area expressing the antigen in question. The specific signal density (SSD) was expressed in arbitrary units. The average SSD value was

calculated in case of each animal, by averaging data obtained in 10 neurons of 5 sections. Sections of the BSTov were used to measure the CRF density, Ucn1 density was detected in the cpEW, 5-HT was observed in the DR according to (Paxinos and Franklin 2003).

3.4.9. Immunofluorescence controls

Our CRF antibody (Santa Cruz, sc-1759) was raised against a C terminus peptide fragment of CRF of human origin. Its specificity and sensitivity on mouse brain tissue was previously tested (Kormos et al. 2016). According to the supplier (http://datasheets.scbt.com/sc-1759.pdf), the specificity of this antibody was verified by Western blot. The omission or replacement with non-immune sera of either primary or secondary antisera prevented the labeling. Preabsorption with the synthetic blocking peptide (Santa Cruz, sc-1759-P) completely abolished the labeling in the BSTov.

The specificity of the goat anti-Ucn1 antibody was verified earlier (Gaszner et al. 2009; Bachtell, et al. 2003). The antibody was raised against a C terminus peptide fragment of rat Ucn1 (Santa Cruz, sc-1825). Preabsorption experiments and the omission/replacement of Ucn1 serum abolished the immunosignal in all cases.

The specificity and sensitivity of the monoclonal mouse 5-HT antiserum was tested earlier (Kormos et al. 2016; Leger et al. 2001; Leger et al. 1998).

3.4.10. CORT radioimmunoassay

The method used for quantification of the CORT titer in the collected blood samples, was published previously (Gaszner et al. 2009; Gaszner et al. 2004). In this study, 40µl blood serum samples were assayed using ^{3}H-corticosterone (12,000 cpm; 90–120 Ci/mmol, NET-399; PerkinElmer, Boston, MA) and CS-RCS-57 antiserum (Jozsa et al. 2005). Inter- and intra-assay co-efficients of variation were 9.1% and 6.5%, respectively.

3.5. Statistical analysis

3.5.1. Maternal deprivation study

Data are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Dunn's post hoc analysis. Results were considered significant when $p < 0.05$.

3.5.2. PACAP transgenic mouse development study

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by Fisher's post hoc analysis. Results were considered significant when $p < 0.05$.

3.5.3. Depression model study

All data were expressed as mean and standard error of the mean for each experimental group. Homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cohran 1989) and normality (Shapiro–Wilk test; Shapiro and Wilk 1965) were tested for all data groups. When data were not normally distributed, a square root mathematical transformation was applied. Data were assessed by multifactorial analysis of variance (MANOVA) followed by Fisher's *post hoc* test (Statistica 8.0 - StatSoft, Tulsa, OK) (alpha= 5%).

4. Results

4.1. Maternal deprivation study

In this chapter I summarized the findings on the neurobehavioral development of rats upon maternal deprivation compared to animals with normal maternal care history. The changes of the somatic development and the appearance/disappearance of neurological reflexes are detailed here. Furthermore, a description of the changes detected by motor coordination and behavioral tests is provided.

4.1.1. Somatic development

The average body weight of MD rats was not different from control rats either in males or females at the beginning of the observation period (Figure 4.1A and B). However, 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 (Figure 4.1A).

Although male MD rats still weighed more on the 4th (74.6±4.6 and 84.3±4.1 g, control and MD rats, respectively) and on the 5th weeks (119.7±4.3 and 136.1±6.3 g, control and MD rats, respectively), differences were no longer significant. Control and MD female rats did not show differences in weight either at later ages (68.7±4.6, 66.1±4.1 and 104.7±4.3, 110.1±6.3 g in control and MD females, on the 4th and 5th weeks, respectively). Statistical analysis of the daily body weight revealed a significant gender difference in MD rats only: while weight of control male and female rats were significantly different only starting after the 4th week, MD males weighed significantly more than MD females from the 4th day throughout the whole observation period ($p < 0.01$). Statistically significant gender difference was observed in controls only on week 5. Further signs of somatic development, such as the day of eye opening, incisor eruption and ear unfolding were not significantly different in MD rats and controls, or between genders (Figure 4.1C and D, columns 1–3).

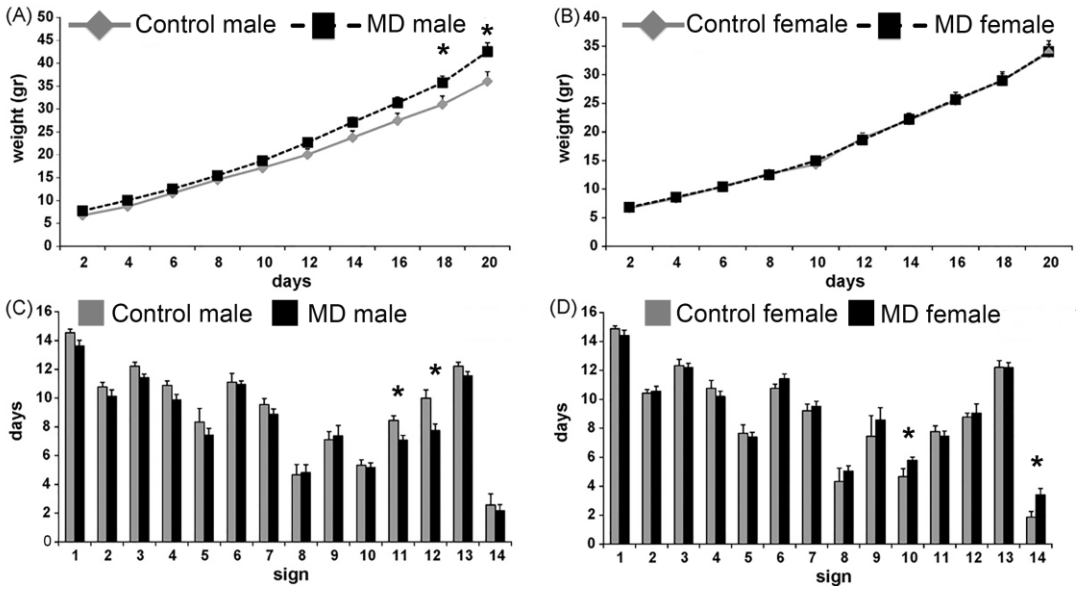

Figure 4.1.

Average body weights (mean ±S.E.M.) of male (A) and female (B) MD and control rats. *$p<0.05$ versus control group. Day of appearance (mean±S.E.M.) of physical and neurological signs in male (C) and female (D) control and MD pups. The day of appearance of the following signs are represented: (1) eye opening; (2) incisor eruption; (3) ear unfolding; (4) negative geotaxis; (5) disappearance of crossed extensor reflex; (6) ear twitch reflex; (7) eyelid reflex; (8) forelimb placing reflex; (9) hindlimb placing reflex; (10) forelimb grasp reflex; (11) hindlimb grasp reflex; (12) gait reflex; (13) auditory startle reflex; (14) air righting reflex. *$p<0.05$ versus control group.

4.1.2. Neurological reflexes

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: most reflexes appeared approximately 1 day earlier, but results were significantly different only in cases of hindlimb grasping reflex and gait reflex (Figure. 4.1C). In MD female animals, some reflexes appeared earlier, while others later than in controls, but significant delays were only observed in cases of forelimb grasp and air righting reflexes (Figure 4.1D). The daily performance of righting reflex, negative geotaxis and gait reflex was not markedly different between groups (Figure 4.2). All groups showed a manifest improvement in the reflex performances throughout the observation period. Male MD rats improved parallel with control rats, with no significant differences at any time point (Figure 4.2A), while there was a significant delay in righting reflex performance in female MD rats on day 4 (Figure 4.2B). No differences were found in the performance of negative geotaxis in either gender group, only a tendency of enhanced performance in MD rats could be observed (Figure 4.2C and D). Similarly, no difference was found between MD and control groups in the performance of gait reflex (Figure 4.2E and F). Gender differences were revealed only in the gait reflex: MD males performed significantly better than MD females on days 8, 10 and 16.

4.1.3. Development of motor coordination

The only motor coordination test which revealed a significant difference was the footfault test in male rats (Figure. 4.3A and B). Although pups did not take significantly more steps on the elevated grid (Figure 4.3A), the percentage of footfaults was significantly less in male MD animals on the 3rd week (Figure 4.3B). A tendency could also be observed on the 4th week, but results were no longer significant. Only a tendency to make more mistakes could be observed in female MD rats compared to controls, but differences were not statistically significant. No significant differences could be observed in any other motor coordination tests, such as the walk initiation test (Figure 4.3C and D), rotarod test (Figure 4.3E and F), rope

suspension and inclined board test (data not shown). No difference was found between the performances of control males and females, and MD males and females.

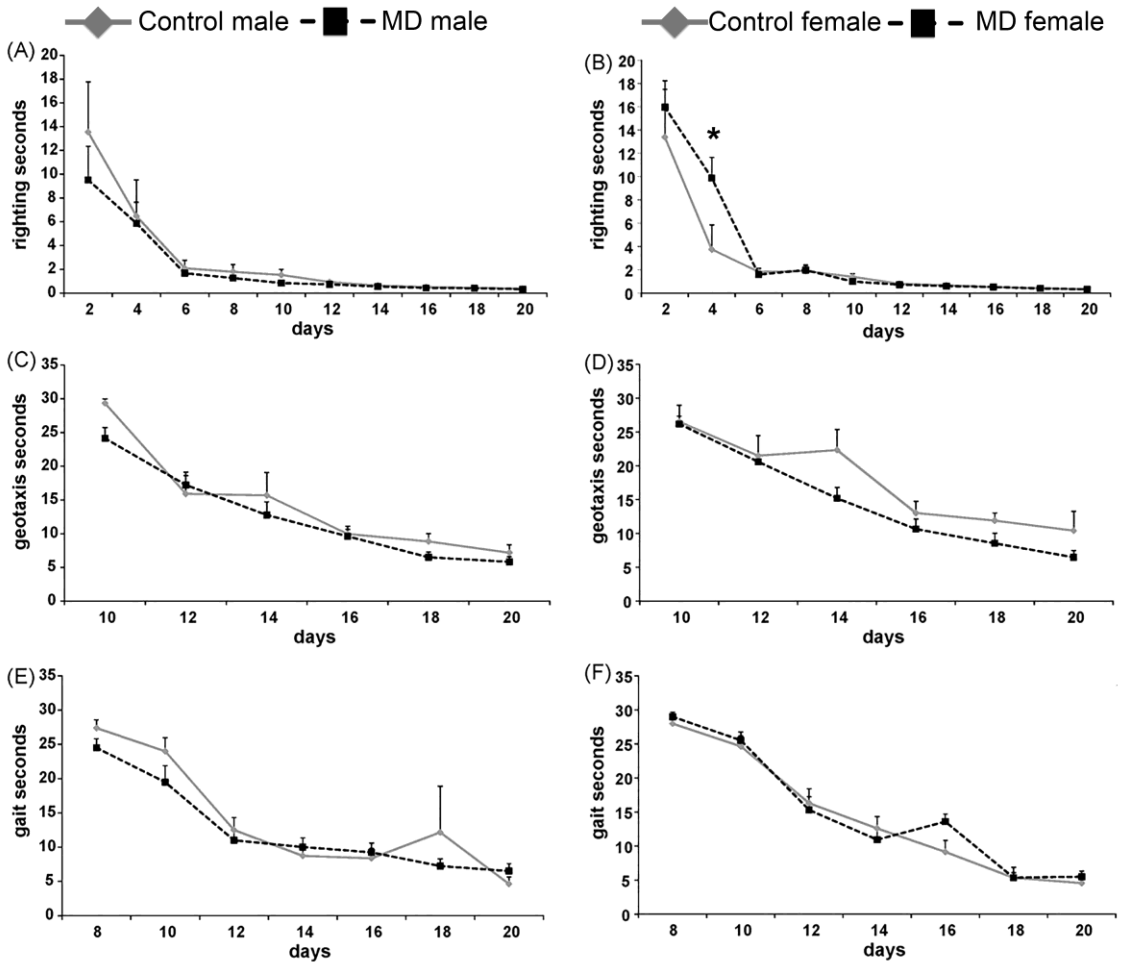

Figure 4.2.

Daily performance of surface righting (A and B), negative geotaxis (C and D) and gait (E and F) reflexes of control and MD pups. A, C and E show data from male rats, B, D and F data of female rats. Results are expressed as mean±S.E.M. *p<0.05 versus control group.

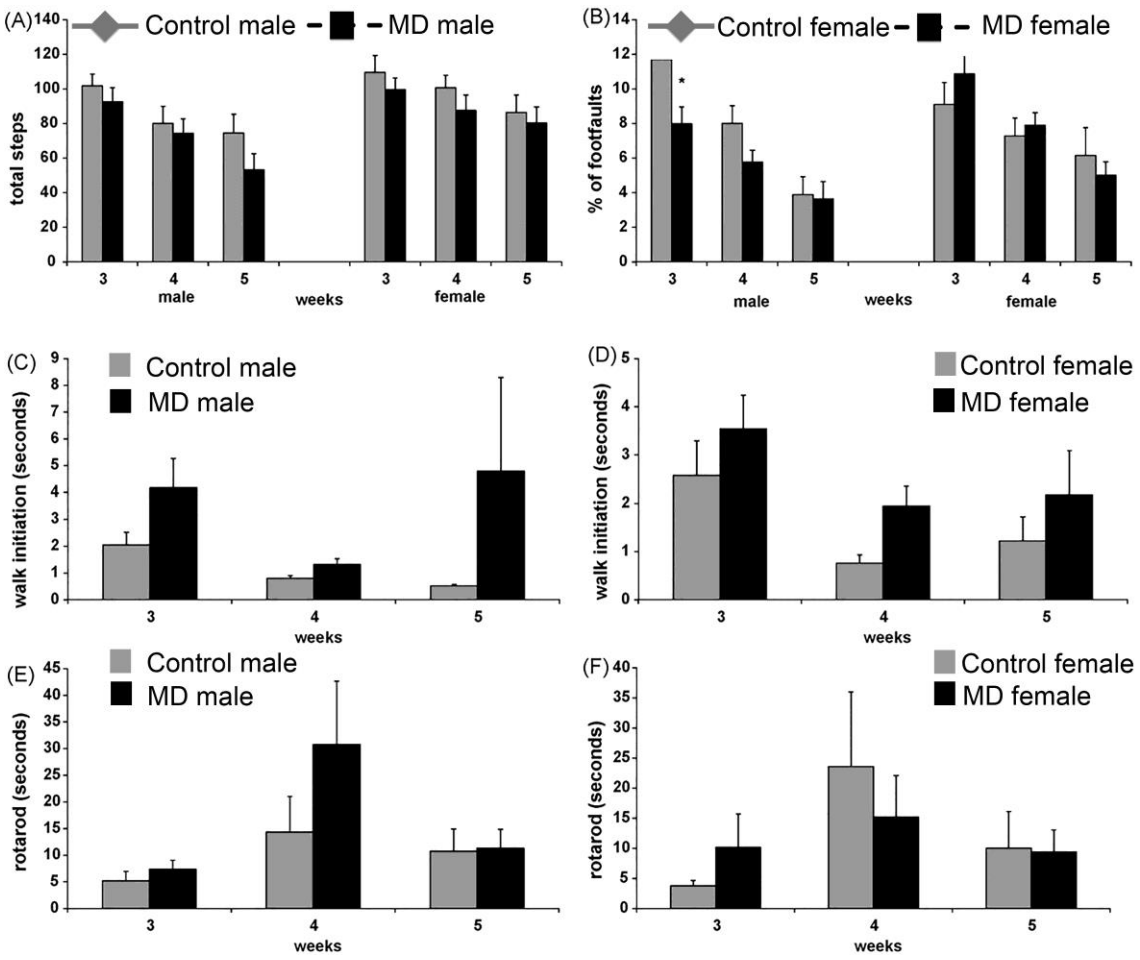

Figure 4.3

Footfault test of control and MD pups on postnatal weeks 3–5. Total steps (A) and footfaults expressed as percentage of total steps (B). Time to perform the walk initiation test in male (C) and female (D) rats, and the time to stay on the rotating rod in male (E) and female (F) pups. All data are expressed as mean±S.E.M. **p*<0.05, compared to control rats.

4.1.4. Open-field behavior

We found no statistical difference in the distance covered and the number of rearings by male and female control and MD rats at 3, 4 and 5 weeks of age (Figure 4.4A and B). These two signs measure the horizontal and the vertical activity of the animals, respectively. Although a slight tendency of hypoactivity was observed in the male rats, results were not significant between control and MD males. Female animals covered more distance, and it was significant between female MD and male MD rats at 4 and 5 weeks of age. The time spent in the center is a sign of less anxiety, but it was not different in maternally separated pups. Although a tendency of spending more

time in the center could be observed in female MD animals, it was different only from male MD rats at 4 and 5 weeks of age, but not from controls (Figure. 4.4C).

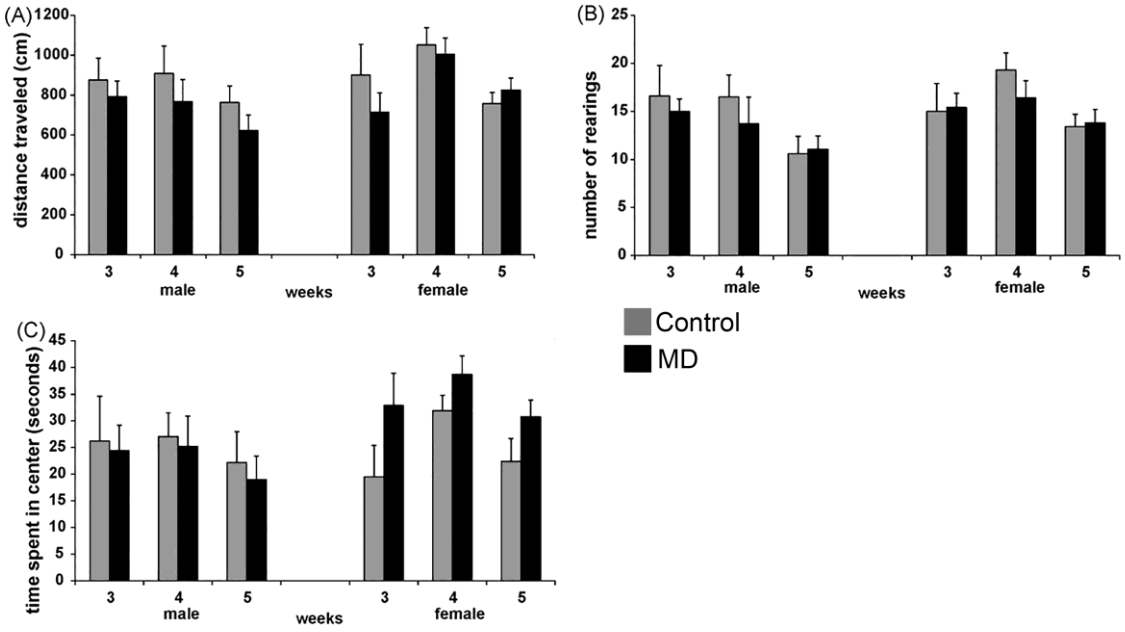

Figure 4.4.

Open-field activity in male and female control and MD rats on postnatal weeks 3–5. Total distance traveled (A), total number of rearings (B) and the time spent in the center of the arena (C) are represented as mean±S.E.M.

4.2. PACAP mutant mouse development study

Subchapter 4.2 summarizes my findings of the development of PACAP transgenic mice, subjected to maternal deprivation versus members of control litters which received normal maternal care. I gathered information regarding the somatic and neurological development, including motor coordination, of the subjects. In this study both male and female wild type, heterozygous and knock out mice were used.

4.2.1. Somatic development

The average body weight of WT mice showed a continuous gain throughout the first 3 weeks (Figure 4.5). There was no significant difference between female and male mice in any group. Birth weight showed no difference between any of the groups. The weight gain of PACAP KO mice, both males and females, was slower than in

WT mice. Interestingly, however, the slowest weight gain was observed in mice partially lacking PACAP (HZ mice) (Figure 4.5A).

Since there was no significant difference between male and female animals during the first 3 weeks, we also analyzed pooled data of the weight change from WT, HZ and KO mice between days 1-7, 1-14, 1-21, 7-14, 7-21 and 14-21 (Figure 4.5B). Results show that weight gain in all time intervals was fastest in the WT group, except for the first week, when KO mice had a slightly, but significantly, faster weight gain. Comparing results of the first, second, and third weeks, we can conclude that the dynamics of weight gain did not change in the WT or HZ groups: the HZ mice had a significantly slower weight gain throughout the entire observation period. Homozygous KO mice had a more variable weight gain patterns: while animals during the first week had a faster weight gain, they slowed down during the second week. Altogether, our data clearly demonstrate a slower weight gain of heterozygous PACAP animals. Signs of somatic development, such as the day of eye opening and ear unfolding, did not show any significant difference between groups (Table 4.1).

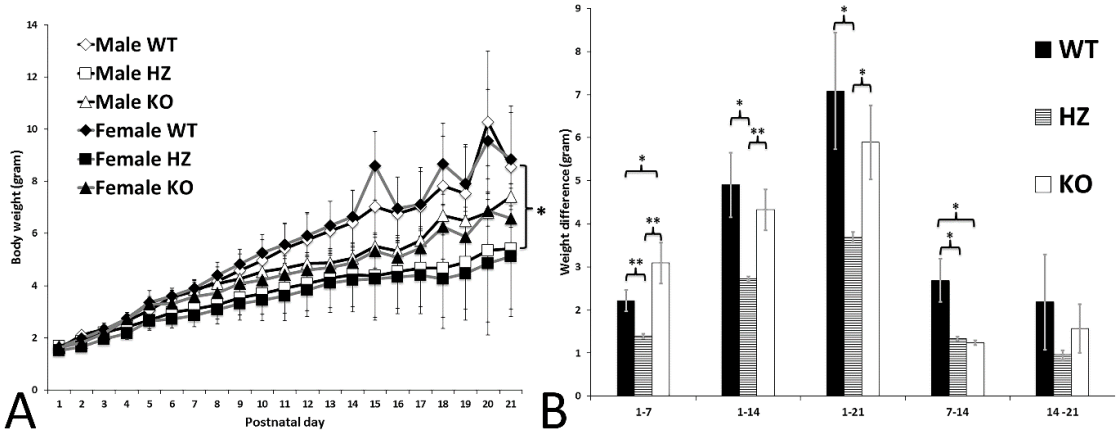

Figure 4.5.

Daily weight measurements (A). Data are expressed in average gram ± S.E.M. of wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice. *p<0.05, **p<0.01, versus gender-matching WT group. Weight changes between distinguished postnatal days (B). The first 3 column groups represent differences in weight gain between day 1 and the end of first (1-7), second (1-14) and third weeks (1-21). The other 3 groups represent data between other time intervals: during the second week (7-14), during the second and third weeks (7-21) and during the third week (14-21). Data are expressed in average gram ± SEM. *p < 0.05, **p<0.01 (Two-way ANOVA Dunn's post hoc test).

		Eye opening	Ear unfold	Crossed extensor reflex	Ear twitch	Eyelid reflex	Forelimb placing	Hind limb placing	Hind limb grasp	Auditory startle
Male	WT	13.50 (±0.29)	13.00 (±0.58)	6.50 (±0.96)	10.00 (±0.00)	10.75 (±0.48)	5.25 (±0.75)	11.00 (±1.00)	9.33 (±0.33)	12.75 (±0.48)
	HZ	13.77 (±0.12)	12.62 (±0.31)	5.92 (±0.21)	9.15 (±0.19)	10.62 (±0.31)	6.00 (±0.45)	11.6 (±0.60)	8.77 (±0.54)	12.23 (±0.26)
	KO	13.67 (±0.17)	12.11 (±0.26)	7.22 (±0.40)	10.56 (±0.58)	11.00 (±0.24)	5.67 (±0.60)	11.00 (±0.32)	7.89 (±0.48)	12.22 (±0.15)
Female	WT	13.43 (±0.30)	12.29 (±0.36)	7.00 (±0.53)	9.57 (±0.30)	10.50 (±0.22)	5.57 (±0.84)	7.67 (±1.33)	8.33 (±1.17)	12.14 (±0.26)
	HZ	14.22 (±0.22)	12.78 (±0.28)	6.78 (±0.22)	9.67 (±0.24)	11.13 (±0.35)	6.44 (±0.82)	11.57 (±1.19)	9.78 (±0.32)	12.89 (±0.2)
	KO	14.00 (±0.00)	12.67 (±0.67)	7.50 (±0.50)	10.00 (±0.58)	10.50 (±0.50)	6.67 (±0.33)	10.00 (±2.00)	8.00 (±2.08)	12.33 (±0.33)

Table 4.1.
Summarized data of developmental signs (appearance of eye opening, ear unfolding) and reflexes (disappearance of crossed extensor reflex and appearance of ear twitch, eyelid reflex, forelimb placing, hind limb placing, hind limb grasp, auditory startle) without significant differences between genders or genotypes. The data are expressed in average postnatal days and ± S.E.M.

4.2.2. Neurological reflex development

Regarding reflex development, we did not observe significant differences between groups in the day of appearance of ear twitch and eyelid sensory reflexes, in fore- and hindlimb placing, hindlimb grasp and auditory startle reflexes or in the disappearance of the crossed extensor reflex (Table 4.1). Examining forelimb grasp reflex, we observed a significantly delayed appearance of more than 2 days of this reflex in female HZ mice compared to female WT and KO (Figure 4.6). Air righting reflex appearance showed a similar pattern: it was delayed only in female HZ mice, compared to female WT and KO animals (Figure 4.7). Gait initiation reflex appearance was also delayed in female HZ mice compared to their WT mates (Figure 4.8). In males, only a tendency was observed. In the elevated grid, homozygous mice

took significantly more steps than WT or HZ animals, but there was significant difference between WT and HZ groups (Figure 4.9). Calculating the number of foot faults on the grid, we observed that 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 (Figure 4.10A). On week 4, significant differences were observed in male mice: both HZ and KO mice made more mistakes than WT animals, while there was a similar tendency but no significant difference between groups in females (Figure 4.10B).

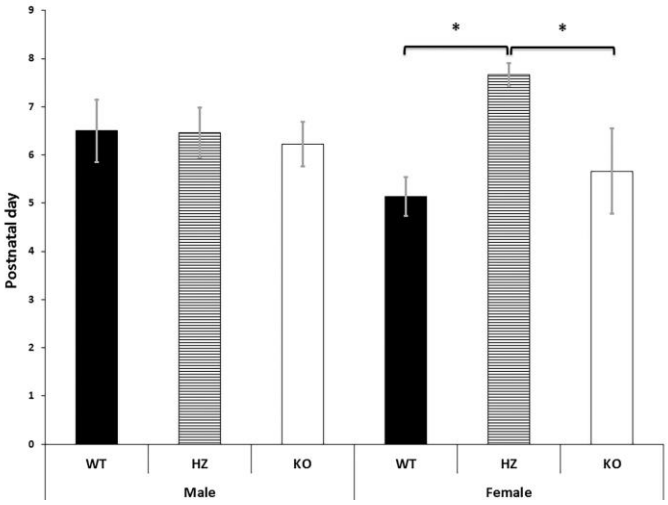

Figure 4.6.
Appearance of forelimb grasp reflex in wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice. Data are expressed in average postnatal days ± SEM.*p<0.05. (Two-way ANOVA, Dunn's post hoc test * p<0.05)

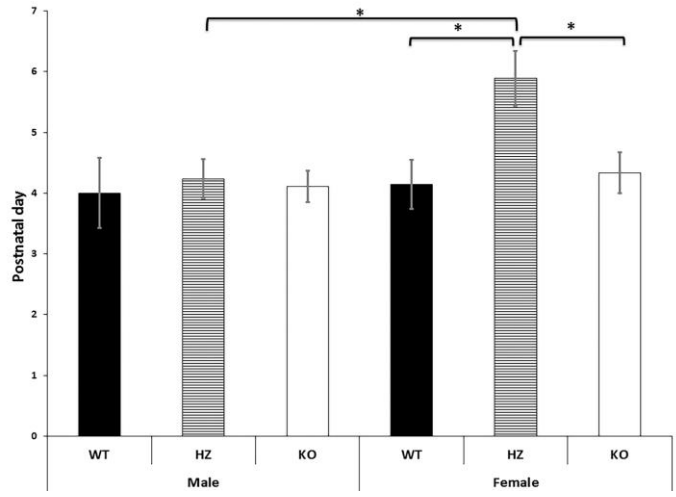

Figure 4.7.
Appearance of air right reflex in wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice. Data are expressed in average postnatal days ± S.E.M.*p<0.05 (Two-way ANOVA, Dunn's post hoc test * p<0.05).

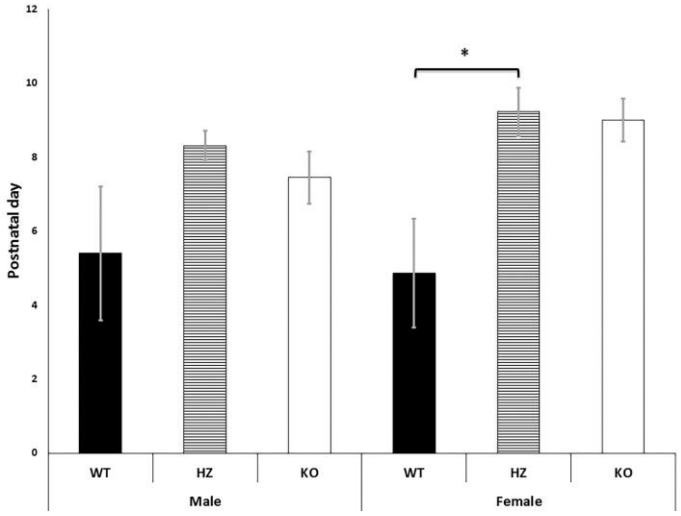

Figure 4.8.

Appearance of gait initiation activity in wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice. Data are expressed in average postnatal days ± S.E.M.*p<0.05. (Two-Way ANOVA, Dunn's post hoc test * p<0.05)

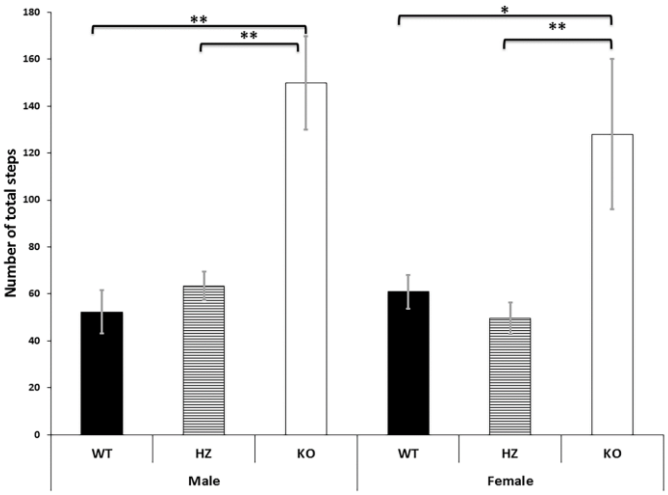

Figure 4.9.

Number of total number of steps in wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice. Data are expressed in average postnatal days ± S.E.M. (Two-way ANOVA, Dunn's post hoc test * p<0.05; **p<0.01)

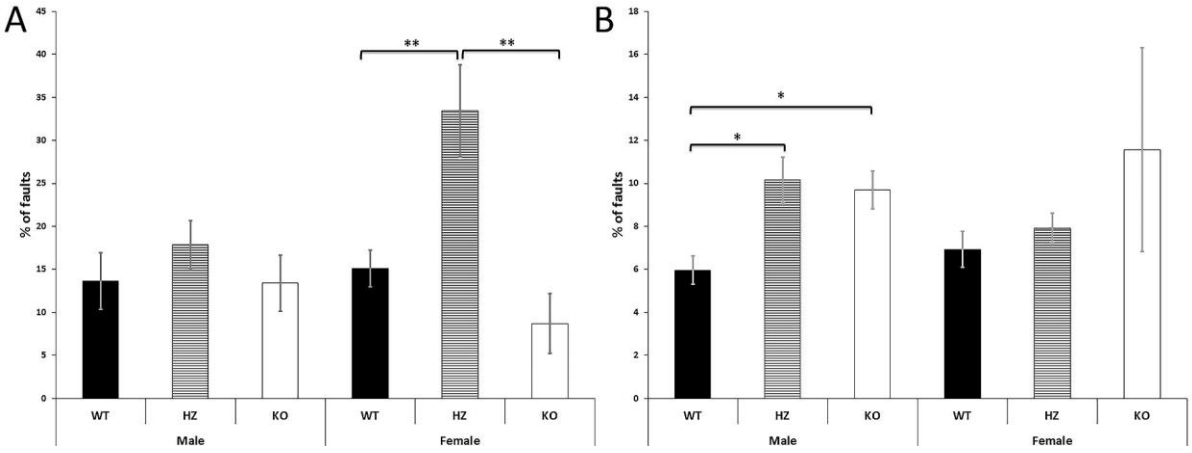

Figure 4.10.

Footfaults on the elevated grid given as percentage of total steps in wild type (WT), heterozygous (HZ) and homozygous (KO) PACAP deficient mice on week 3 (A) and week 4 (B). Data are expressed in average postnatal days ± S.E.M. **p<0.01. (Two-way ANOVA)

4.3. Depression model study

In this chapter, we provide a detailed description of the physical and morphological changes caused by the practical application of the three hit theory of depression. The model's validity was tested by measurements of physical (i.e. body- and organ weight), endocrinological (i.e. CORT titer) and behavioral tools (i.e. FST, TST, LD and MDT) as detailed below. In this chapter, we also demonstrate our neuromorphological results in BSTov-CRF, cpEW-Ucn1 and DR-5HT areas in male PACAP WT, HZ and KO mice upon CVMS vs. controls with the history of various maternal care qualities (i.e. AFR, MS15, or MD180).

4.3.1. Body and adrenal weight data

CVMS exposure in WT ($p<0.0005$) and HZ ($p<0.000005$) AFR animals caused a statistically significant decrease in their body weight compared to the AFR controls (Figure 4.11A, compare bars 'a' and 'b' to bars 'd' and 'e'). In MS15 mice, CVMS decreased the body weight in all genotypes [WT: $p<0.005$; HZ: $p<0.00005$; KO: $p<0.005$ (see bars 'g', 'h' and 'i' vs. 'j', 'k' and 'l')]. CVMS exposure on MD180 HZ mice *vs.* controls had a significant decreasing effect on body weight (compare bars 'n' and 'q'; $p<0.05$). [MANOVA main effect: stress ($F_{17,98}=43.28$; $p<0.0001$). Interactions: maternal care x stress ($F_{17,98}=6.66$; $p<0.005$), stress x genotype ($F_{17,98}=3.46$; $p<0.05$)].

Adrenal gland weight measurements proved the effectivity of our CVMS paradigm (MANOVA: $F_{17,98}=4.443$ $p<0.05$), 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 ($p<0.05$; see Figure 4.11B, compare bars 'b' and 'e').

4.3.2. Serum CORT titer

Plasma CORT values further corroborated the efficacy of our CVMS model WT and HZ animals with AFR history reacted to CVMS with a CORT increase by 78% (Figure 4.11C, compare bars 'a' and 'd'; $p<0.005$;) and 76.7% (see bars 'b' vs. 'e'; $p<0.005$), respectively, while KO mice showed negligible change (bars 'c' and 'f';

p=0.95). CVMS exposure of MS15 mice caused a 67.01% rise of CORT level in HZ (bars ‘h’ and ‘k’; $p<0.05$) and 99.13% in KO animals (bars ‘i’ and ‘l’; $p<0.01$), while the 54.41% CORT elevation in WT animals did not reach statistical significance ($p=0.10$).

Both MD180 KO and WT animals reacted with a significant CORT increase (bars ‘m’ and ‘o’ vs ‘p’ and ‘r’; $p<0.05$). Surprisingly, MD180 HZ animals did not react in terms of CORT value (bars ‘n’ and ‘q’; $p=0.31$). [MANOVA: main effect: stress ($F_{17,98}=32.89$; $p<0.000001$); interactions maternal care x genotype ($F_{17,98}=2.51$; $p<0.05$), maternal care x stress x genotype ($F_{17,98}=3.13$; $p<0.05$).]

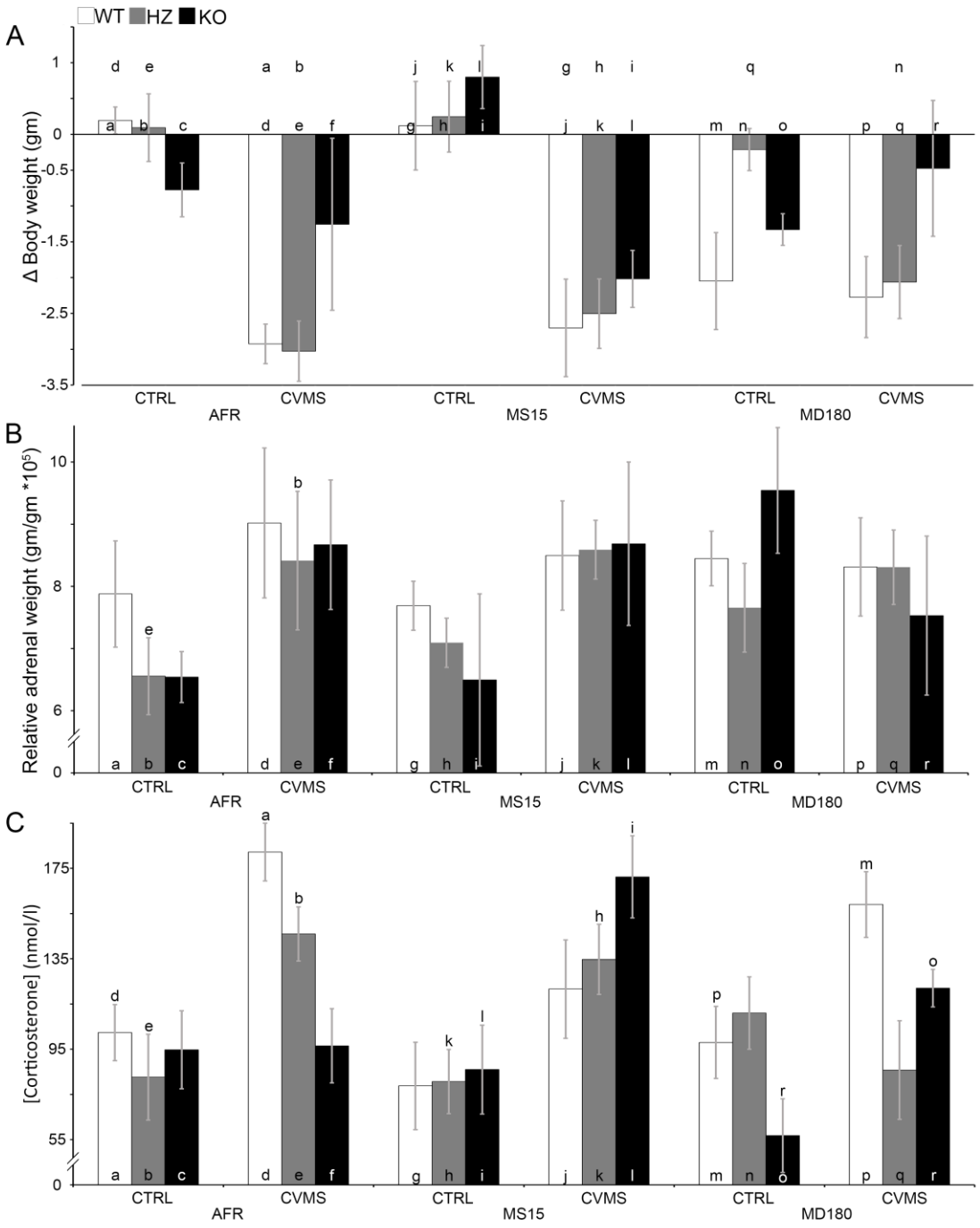

Figure 4.11.
Summary of physical and endocrinological parameters to test the effect of stress. White bars: wild type (WT), gray bars: PACAP heterozygous (HZ), black bars: PACAP knock out (KO) animals. (A) Bodyweight change, expressed in gram (gm), during the period of chronic variable mild stress (CVMS). (B) Total adrenal tissue weight corrected to the bodyweight, expressed in gram per bodyweight gram (10^{-5} gm/gm). (C) Blood corticosterone titers (nmol/l). AFR: animal facility-reared, MS15: 15 minutes maternal separation, MD180: 180 minutes of maternal deprivation. CTRL: control animals (i.e. not CVMS exposed). Lettering at the top of bars represent the most relevant significant statistical differences between pairs of groups according to the *post hoc* tests ($p<0.05$).

4.3.3. Behavioral assessment

4.3.3.1. Forced swim test

The effect of maternal care quality on depression level was proven when AFR CTRL WT and MD180 CTRL WT animals were compared (see Figure 4.12A, bars 'a' and 'm'; $p<0.01$). Upon CVMS exposure, HZ mice with MD180 history showed a robust, 130% increase in their immobility time (compare bars 'n' and 'q'; $p<0.005$). In AFR and MS15 HZ mice the immobility time did not change significantly upon CVMS (bars 'b' *vs* 'e' and 'h' *vs* 'k'). AFR WT animals reacted with a significantly increased immobility time to CVMS (see bars 'a' and 'd'; $p<0.05$). Interestingly, AFR KO animals showed a paradoxical decrease of immobility time after CVMS exposure (compare bars 'c' to 'f'; $p<0.001$). [MANOVA: main effect: genotype ($F_{17,98}=16.70$; $p<0.001$); interactions: maternal care x stress ($F_{17,98}=3.24$; $p<0.05$), maternal care x genotype ($F_{17,98}=5.21$; $p<0.001$), stress x genotype ($F_{17,98}=4.63$; $p<0.05$), maternal care x stress x genotype ($F_{17,98}=3.84$; $p<0.01$)]

4.3.3.2. Tail suspension test

CVMS did not influence the immobility time of WT and HZ animals with MD180 history. On the contrary, KO animals decreased their immobility time significantly ($p<0.05$), by 60.9% (data not shown). [MANOVA: main effects: genotype ($F_{17,98}=88.23$; $p<0.001$), interactions: maternal care x genotype ($F_{17,98}=26.53$; $p<0.05$) and stress x genotype ($F_{17,98}=55.54$; $p<0.01$)]

4.3.3.3. Light-dark test

- *Time spent in lit compartment.* The comparison of genotypes within the MD180 CTRL group revealed that KO animals had a higher anxiety level than WT (Figure 4.12B, bars 'o' and 'm'; $p<0.05$) and HZ (see bars 'o' and 'n'; $p<0.01$) mice. The assessment of the effect of maternal care quality on anxiety level in CVMS exposed KO mice revealed that severe maternal deprivation caused increased anxiety compared both to AFR (compare bar 'f' to 'o'; $p<0.05$) and MS15 (bars 'l' and 'r'; $p<0.005$) KO mice. In the MD180 group

CVMS exposure caused a statistically significant, 103% increase in case of the KO animals compared to their MD180 CTRL counterparts (see bar 'o' and 'r'; $p<0.001$). The MD180 CVMS KO animals spent more time in the illuminated chamber compared both to AFR CVMS (see bars 'r' and 'f'; $p<0.05$) and MS15 CVMS KO mice (see bars 'r' and 'l'; $p<0.05$). [MANOVA: interactions: maternal care x stress ($F_{17,98}=5.17$; $p<0.01$), stress x genotype ($F_{17,98}=5.11$; $p<0.01$), care x stress x genotype ($F_{17,98}=3.56$; $p<0.05$)]

- *Transitions.* In the AFR CTRL group the KO animals showed a significantly higher transition number compared to their WT (Figure 4.12C, bars 'c' and 'a'; $p<0.05$) and HZ (Figure 4.12C, bars 'c' and 'b'; $p<0.05$) counterparts. Exposure to CVSM did not cause any significant changes in the AFR group. In the MS15 CTRL group the KO animals showed higher transition values compared to the WT group (see bar 'g' and 'i'; $p<0.05$). In the MD180 group CVMS significantly decreased the transition number of KO mice (compare bar 'o' to bar 'r'; $p<0.05$). The MD180 CVMS KO animals showed a significantly lower transition value compared to MD180 CVMS WT (bars 'p' and 'r'; $p<0.01$) and HZ mice (bars 'q' and 'r'; $p<0.01$). [MANOVA: interactions: maternal care x genotype ($F_{17,98}=31.63$; $p<0.05$), stress x genotype ($F_{17,98}=41.12$; $p<0.05$).]
- *Aborted transitions.* The number of aborted transitions was also counted. No statistically valuable effects of the examined factors or their interactions were found (data not shown).

4.3.3.4 Marble burying test

After CVMS exposure AFR animals of all genotypes showed a robust and statistically significant increase in the number of buried marbles (Figure 4.12D, WT: bars 'a' and 'd', $p<0.0001$; HZ: bars 'b' and 'e', $p<0.0001$; KO: bars 'c' and 'f', $p<0.0001$). In the MS15 CTRL group WT animals buried 71.97% (bars 'g' and 'a'; $p<0.05$) while the HZ animals hid 135.54% (bars 'h' and 'b'; $p<0.001$) more marbles than their AFR CTRL counterparts. In case of the KO animals the number of buried marbles did not change. Similarly, to the AFR group, the WT and HZ

animals with MS15 history showed a similar reaction to CVMS. WT animals buried 46.29% (bars ‘g’ and ‘j’; $p<0.05$), HZ animals 45.45% (bars ‘h’ and ‘k’; $p<0.01$) more marbles than their MS15 CTRL counterparts. In the CVMS KO group no change was detected. In the MD180 CTRL group WT animals buried significantly more marbles than their AFR CTRL WT counterparts (compare bars ‘m’ and ‘a’; $p<0.05$). Other significant differences between control groups across AFR, MS15 and MD180 were not found.

After CVMS exposure the MD180 WT animals did not show any change. MD180 HZ animals hid 225% more marbles after CVMS exposure (see bars ‘n’ and ‘q’; $p<0.001$). In KO animals, CVMS superimposed to MD180 history did not affect the count of hidden marbles. [MANOVA: main effects: stress ($F_{17,98}=448.04$; $p<0.0001$), genotype ($F_{17,98}=62.31$; $p<0.01$); interaction: maternal care x stress ($F_{17,98}=115.15$; $p<0.0001$).]

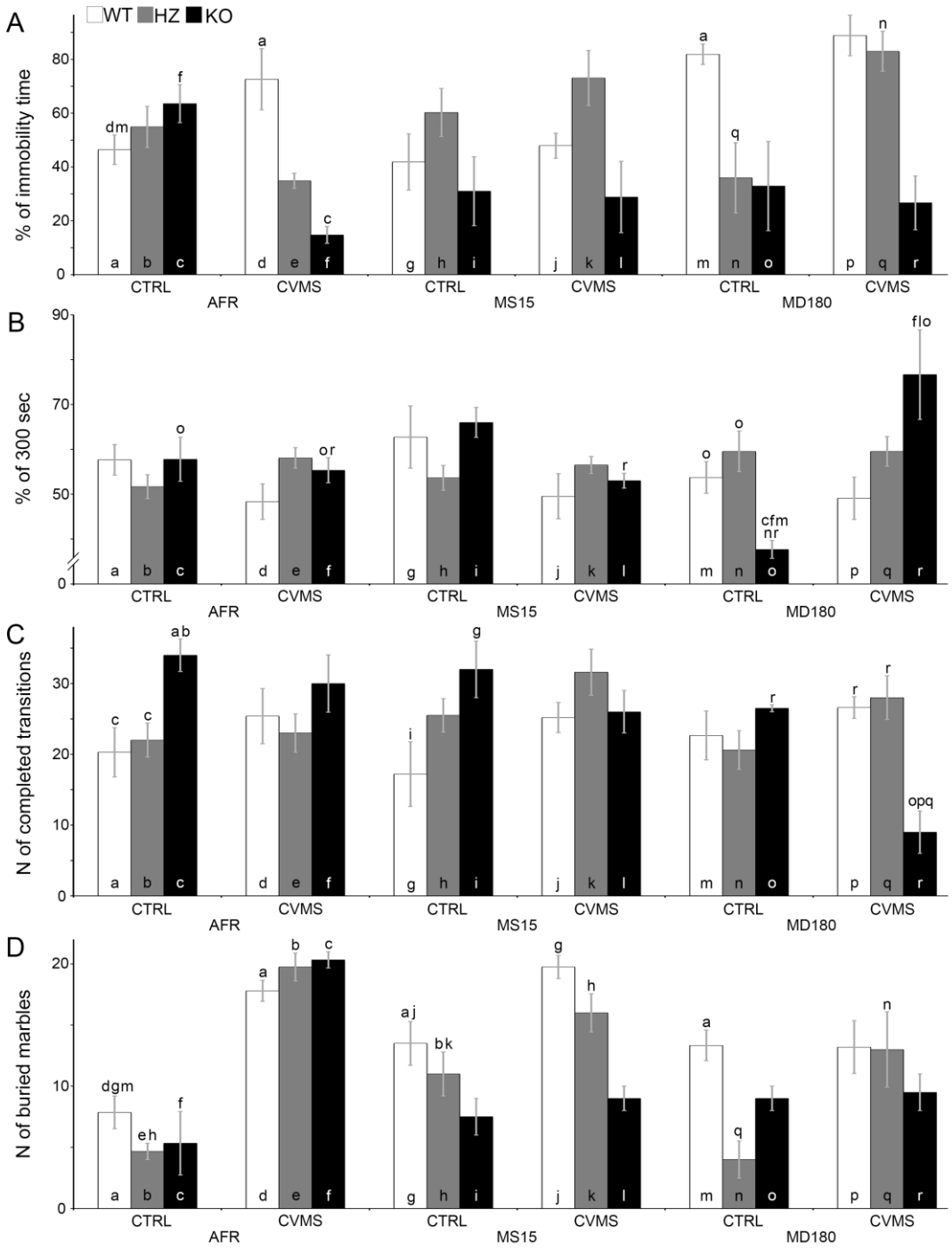

Figure 4.12.
Summary of behavioral test results. **(A)** Immobility time in forced swim test. **(B)** Time spent in the illuminated chamber of the light-dark box test (LD). **(C)** Number of transition between the two compartments of the LD apparatus. **(D)** Number of marbles hid in the marble burying test for anxiety. White bars: wild type (WT), gray bars: heterozygous (HZ), black bars: knock out (KO) animals. AFR: animal facility-reared, MS15 and MD180 handling with 15 and 180 minutes of maternal separation. CTRL: control animals no chronic variable mild stress (CVMS) exposure. Lettering at the top of bars represent the most relevant significant statistical differences between pairs of groups according to the *post hoc* tests ($p<0.05$).

4.3.4. Morphological results

4.3.4.1. Corticotropin-releasing factor in the BSTov

4.3.4.1.1. CRF – FosB co-localization in the BSTov

In the AFR group, the CVMS exposure caused a significant increase in the CRF-FosB co-localization both in the WT (Figure 4.13C, compare bars ‘a’ and ‘d’; $p<0.05$) and HZ (compare bars ‘b’ and ‘e’; $p<0.01$) animals.

Mice with MS15 history showed similar dynamics upon CVMS both in WT (compare bars ‘g’ and ‘j’; $p<0.05$) and HZ (compare bars ‘h’ and ‘k’; $p<0.005$) genotypes. MD180 CTRL WT (see bars ‘a’ and ‘m’; $p<0.01$) and HZ (bars ‘b’ and ‘n’; $p<0.01$) animals showed significantly higher control values compared to their AFR CTRL counterparts.

In the MD180 CTRL HZ animal group, we detected a significantly higher colocalization level compared to their MS15 CTRL HZ groups (compare bars ‘n’ and ‘h’; $p<0.05$). KO animals did not show any changes in the CRF-FosB cell count in the BSTov. [MANOVA: maternal care ($F_{17,98}=4.38$; $p<0.05$), stress ($F_{17,98}=10.21$; $p<0.005$) and genotype ($F_{17,98}=6.39$; $p<0.005$).]

4.3.4.1.2. Number of CRF immunoreactive neurons in the BSTov

Post hoc comparisons did not prove the significant effect of CVMS on CRF cell counts in any pairs of respective groups. Interestingly, the CRF positive cell count in the MD180 CTRL WT animals was significantly higher than in AFR CTRL WT (Figure 4.13D, compare bars ‘a’ and ‘m’; $p<0.01$) and in MS15 WT mice (compare bars ‘g’ and ‘m’; $p<0.01$). Similarly, MD180 CTRL HZ (bars ‘b’ and ‘n’; $p<0.05$) and KO (‘c’ and ‘o’; $p<0.05$) animals had more CRF cells in the BSTov, than their AFR CTRL counterparts. [MANOVA: main effects: maternal care ($F_{17,98}=17.85$; $p<0.0001$), stress ($F_{17,98}=4.66$; $p<0.05$)]

4.3.4.1.3. CRF SSD in the BSTov

In the AFR and MD180 group no significant change was observed after CVMS exposure. CVMS exposure in MS15 WT mice resulted in the increase of CRF SSD

by 32.56% (Figure 4.13E, see bars ‘g’ and ‘j’; $p<0.01$) in line with HZ animals with a rise by 25.55% (compare bars ‘h’ and ‘k’; $p<0.005$). MS15 KO animals after CVMS exposure did not show statistically significant alteration. The MD180 CTRL WT animals showed significantly higher signal density compared to AFR CTRL (compare bar ‘m’ to ‘a’; $p<0.0001$) and MS15 CTRL (compare bars ‘m’ and ‘g’; $p<0.005$) animals. [MANOVA: main effects: maternal care ($F_{17,98}=43.88$; $p<0.05$), stress ($F_{17,98}=97.02$; $p<0.005$)]

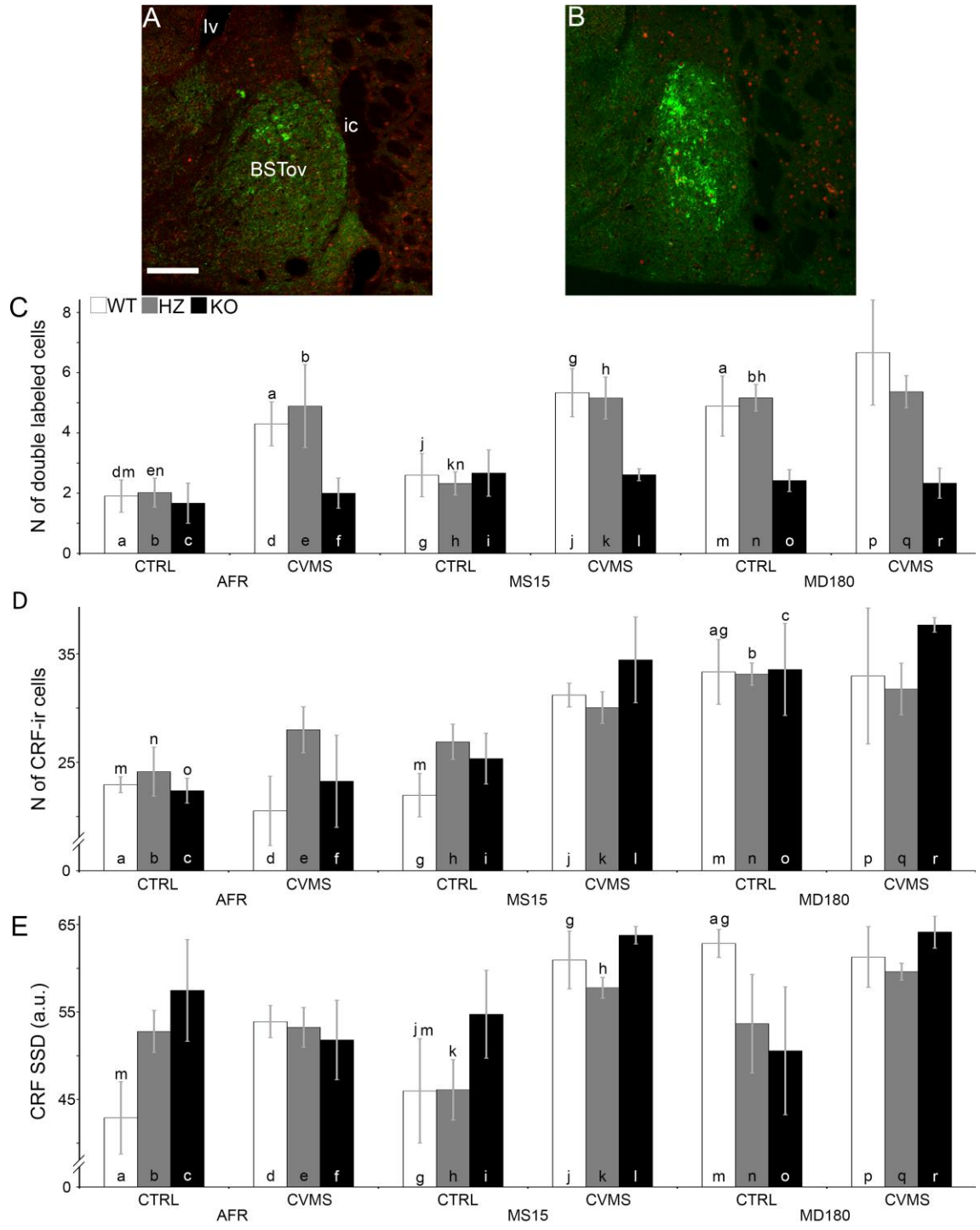
A
lv
ic
BSTov
B
C
WT HZ KO
N of double labeled cells
CTRL
CVMS
AFR
MS15
MD180
D
N of CRF-ir cells
E
CRF SSD (a.u.)

Figure 4.13.

4.3.4.2. Urocortin 1 in the cpEW

4.3.4.2.1 Ucn1 – FosB co-localization in the cpEW

In the AFR group CVMS caused a significant growth in the Ucn1-FosB co-localization in WT (Figure 4.14F, compare bars 'a' and 'd'; $p<0.01$) and HZ (see bars 'b' and 'e'; $p<0.001$) animals compared to CTRL counterparts. Mice with MS15 history showed higher Ucn1-FosB baseline level in case of WT (see bars 'a' and 'd'; $p<0.001$) and HZ mice ('b' and 'e'; $p<0.001$) when compared to AFR CTRL animals. In MS15 CTRL KO mice the increase did not reach the significant statistical value ($p=0.054$). CVMS exposed animals with MS15 history failed to show significant changes compared to control counterparts in terms of Ucn1-FosB cell count. CVMS in WT and HZ MD180 mice did not cause significant changes in the Ucn1-FosB cell counts. Interestingly, KO animals reacted to CVMS by a 48% rise in Ucn1-FosB expressing neuron count, although this remained just under the significant value (compare bars 'o' and 'r'; $p=0.06$). The comparison of CVMS exposed MD180 mice revealed that KO mice showed 2.8 times higher Ucn1-FosB cell counts than WTs (see bars 'p' and 'r'; $p<0.0001$), while the difference compared to HZ was 2.10 times (see bars 'q' and 'r'; $p<0.01$). [MANOVA: main effects: maternal care ($F_{17,98}=10.05$; $p<0.001$), stress ($F_{17,98}=8.50$; $p<0.005$), genotype ($F_{17,98}=8.46$; $p<0.001$); interaction: maternal care x stress ($F_{17,98}=5.54$; $p<0.01$)]

4.3.4.2.2. Ucn1 specific signal density

In the AFR CTRL group the KO animals showed a significantly higher Ucn1 SSD than AFR CTRL WT counterparts (Figure 4.14G, compare bars 'a' and 'c'; $p<0.05$). CVMS in AFR animals decreased the Ucn1 SSD by 22.53% in HZ animals (compare bars 'b' and 'e'; $p<0.01$). The AFR CVMS KO animals showed a significantly higher SSD level compared to their HZ (see bars 'e' *vs.* 'f'; $p<0.01$) and WT (columns 'd' *vs.* 'f'; $p<0.05$) counterparts. In CTRL HZ animals with MS15 history a significantly lower Ucn1 SSD was observed compared to their AFR CTRL HZ counterparts (see bars 'b' and 'h'; $p<0.05$). After applying CVMS in MS15 mice, the Ucn1 SSD value decreased in KO mice only (bars 'i' and 'l'; p

<0.05). MD180 WT and HZ animals, subjected to CVMS, did not display any significant changes in Ucn1 SSD when compared to their MD180 CTRL counterparts CVMS resulted in 46% increase in the Ucn1 SSD in the MD180 KO group compared to the MD180 CTRL KO animals (compare columns ‘o’ and ‘r’; $p<0.05$). The Ucn1 SSD in MD180 CVMS KO animals (bar ‘r’) was higher than that of the MD180 CVMS WT (bar ‘p’; $p<0.05$) and HZ (bar ‘q’; $p<0.05$). [MANOVA: main effects: maternal care ($F_{17,98}=4.82$; $p<0.05$), stress ($F_{17,98}=4.70$; $p<0.05$), genotype ($F_{17,98}=11.90$; $p<0.0001$); interaction: maternal care x stress ($F_{17,98}=5.59$; $p<0.01$)]

4.3.4.2.3. Number of Ucn1 immunoreactive cells in cpEW

In WT AFR animals, CVMS decreased the number of Ucn1 positive cells (Figure 4.14H see bars ‘a’ and ‘d’; $p<0.01$). In HZ and KO animals, this decrease did not reach the significant level ($p=0.16$, $p=0.07$, respectively). Similarly, in mice with MS15 history CVMS reduced Ucn1 cell count in WT mice (compare bars ‘g’ and ‘j’; $p<0.01$), while this change did not reach the statistical value of significance in HZ or KO mice ($p=0.058$, $p=0.58$, respectively). Interestingly, MD180 CTRL WT mice have significantly less Ucn1 neurons in the cpEW than their HZ (compare bars ‘m’ and ‘n’; $p<0.005$) and KO (see bars ‘m’ and ‘o’; $p<0.05$) counterparts. In line with this, CVMS exposed PACAP KO mice with MD180 history have 43.5% higher Ucn1 cell counts than WT counterparts (see bars ‘p’ and ‘r’; $p<0.05$). [Significant main effects revealed by MANOVA: stress ($F_{17,98}=14.49$; $p<0.0001$), genotype ($F_{17,98}=5.04$; $p<0.01$)]

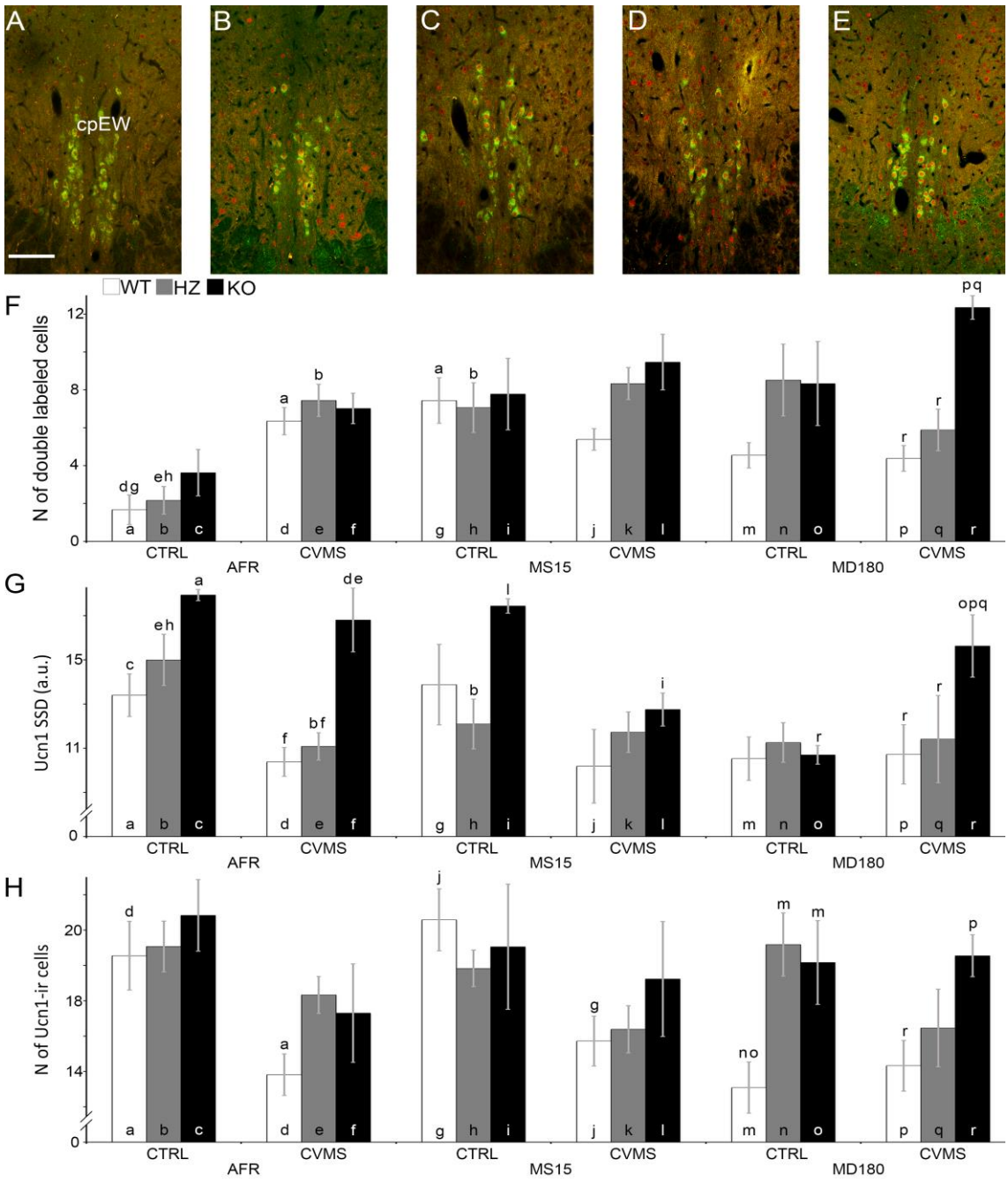
A
B
C
D
E
cpEW
WT HZ KO
F
N of double labeled cells
G
Ucn1 SSD (a.u.)
H
N of Ucn1-ir cells
CTRL
CVMS
AFR
MS15
MD180

Figure 4.14.

4.3.4.3. Serotonin in the DR

4.3.4.3.1 Serotonin specific signal density

Comparisons between AFR groups revealed no significant differences. KO mice with MS15 history showed lower 5-HT SSD than respective WT and HZ mice (Figure 4.15G, compare pairs of bars 'g' and 'i'; $p<0.02$ and 'h' and 'i'; $p<0.03$). Interestingly, in CTRL KO mice with MD180 history the 5-HT SSD was similar to that of WT and HZ mice, and higher than in MS15 CTRL KO mice (see bars 'o' and 'i'; $p<0.01$). [MANOVA: main effects: maternal care ($F_{17,98}=5.13$; $p<0.01$), genotype ($F_{17,98}=3.61$; $p<0.05$); interactions: maternal care x stress ($F_{17,98}=3.55$; $p<0.04$) maternal care x genotype ($F_{17,98}=2.58$; $p<0.05$)]

4.3.4.3.2 Co-localization of 5-HT and FosB positive immunoreactivity in DR

In the AFR group CVMS exposure increased the number of co-localizing cells significantly only in WT animals (Figure 4.15H, compare bars 'a' and 'd'; $p<0.05$). In the MS15 group the control values of WT and HZ animals were significantly higher than those of AFR CTRL WT (see bars 'a' and 'g'; $p<0.05$) and HZ mice (bars 'b' and 'h'; $p<0.05$). The MS15 animals did not react significantly to CVMS exposure in any genotypes. 5-HT-FosB cell count of the MD180 CTRL group in comparison to their AFR counterparts both in WT (see bars 'a' and 'm'; $p<0.0001$) and HZ (see bars 'b' and 'n'; $p<0.00005$) animals showed significantly elevated basal values. CVMS exposure in the MD180 group decreased the number of 5-HT-FosB co-expressing cells in all three genotypes. The number of activated 5-HT neurons was reduced in WTs by 68.08%, (compare bars 'm' and 'p'; $p<0.005$); in HZ mice by 71.52%, (compare bars 'n' and 'q'; $p<0.0005$) and in KO animals by 92.26%, (compare bars 'o' and 'r'; $p<0.00005$). [Statistically significant results by MANOVA: main effect: stress ($F_{17,98}=55.42$; $p<0.05$); interactions: maternal care x stress ($F_{17,98}=245.87$; $p<0.0001$), maternal care x genotype ($F_{17,98}=26.35$; $p<0.05$), stress x genotype ($F_{17,98}=37.45$; $p<0.05$)]

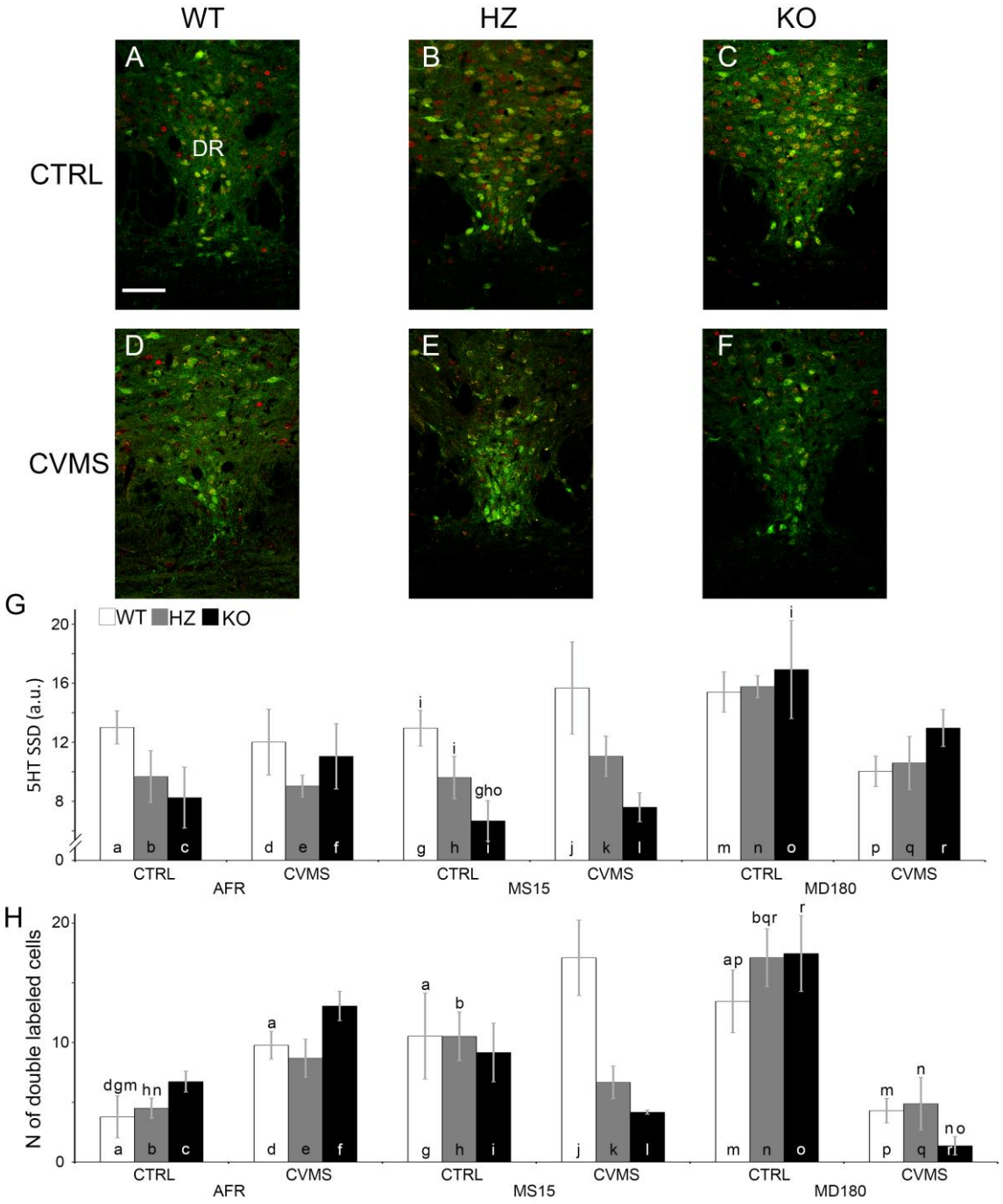
WT
HZ
KO
A
B
C
DR
CTRL
D
E
F
CVMS
G
WT HZ KO
5HT SSD (a.u.)
20
16
12
8
0
a b c d e f g h i j k l m n o p q r
i
i
gho
i
CTRL
CVMS
CTRL
CVMS
CTRL
CVMS
AFR
MS15
MD180
H
N of double labeled cells
20
10
0
dgm hn
a
a b
bqr r
ap
m
n
no
CTRL
CVMS
CTRL
CVMS
CTRL
CVMS
AFR
MS15
MD180

Figure 4.15

4.3.4.3.3. Serotonin positive cell number in the DR

In the MS15 group the serotonin positive cell number decreased when comparing CTRL HZ and CVMS HZ animals ($p<0.05$). A similar tendency in the MD180 group was observed, however changes did not reach significance. MANOVA found only the main effect of stress ($F_{17,98}=4.49$; $p<0.05$) significant.

5. Discussion

The above detailed results obtained in the a) maternal deprivation study in rats, b) in the developmental study on PACAP mutant mice and c) in our depression model study in PACAP mutant mice based on the three hit concept of human MDD will be assessed in three separate sections as follows:

5.1 Maternal deprivation study

Here we described the neurobehavioral development of male and female newborn rats subjected to maternal deprivation. The results revealed that a 3-h-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.

Others have studied the early neurobehavioral effects of maternal deprivation previously. Such studies were carried out under different experimental conditions, a single 24-h-period paradigm, which is a more drastic form of maternal separation (Ellenbroek et al. 2005). Deprived rats showed delayed eye opening, reduction in bodyweight, delayed emergence of walking and rearing, increased latency in negative geotaxis and retarded emergence of the behavioral response to amphetamine. Our results are only partially in accordance with these observations: the development of some reflexes were delayed, but only in females, while in males, the opposite was observed. We did not find reduction of body weight, nor delayed eye opening. In

contrast, male MD rats weighed more than controls, similarly to the findings of Slotten (Slotten et al. 2006), who also found that MD rats weighed more in a paradigm similar to ours. The discrepancy between observations may be related to the different types of maternal separation: a single, 24-h-long deprivation has more profound effects than a daily, but brief (i.e. 180 mins) separation used in the present study. The authors also draw our attention to the importance of the used protocol, as well as other environmental conditions, such as room temperature (Ellenbroek et al. 2005). Other studies also emphasize that different results obtained following maternal separation may depend on separation conditions, such as time of day, ambient temperature, length of separation, genetic background and gender of pups (Slotten et al. 2006; Yamazaki et al. 2005; Ellenbroek et al. 2000; McIntosh et al. 1999). Regarding the open-field activity, most studies have examined it in adult rats after postnatal maternal separation. It has been reported that the overall horizontal activity is higher in adult MD rats, while vertical activity is not different (Slotten et al. 2006; Marmendal et al. 2004). Others have reported increase in both horizontal and vertical activity (Kalinichev et al. 2002). In contrast, a single, 24-h-long deprivation caused significantly lower rearing activity in 2-week-old pups (Ellenbroek et al. 2005). We found that the overall activity was not changed in MD rats, although a tendency to move less could be observed in male animals. Our observation that females generally move more is in accordance with previous descriptions in adults (Slotten et al. 2006). Also, time spent in the center of the arena or at the walls is mostly related to anxiety. Less time spent in the center may indicate higher levels of anxiety. Maternal deprivation has been shown to be related to higher anxiety later in life, however, in line with a study by Zimmerberg and Kajunski (Zimmerberg et al. 2004) we did not observe any difference between MD rats and controls in this measure at such an early life. The overall effect of maternal deprivation was not as drastic in the present model as in our previous postnatal treatments. Earlier we have described that postnatal toxic and hypoxic injuries induce a pronounced delay in most developmental signs examined in this study (Kiss et al. 2007, 2005; Lubics et al. 2005). Most drastic changes have been observed in perinatal asphyxia (Kiss et al. 2008). These previous studies show that the used neurobehavioral testing is suitable to detect severe damages. Our present observations indicate that maternal deprivation does

not induce such robust changes in early neurodevelopment.

Many aspects of gender differences are being studied in various fields of neuroscience, and results from these experiments could help to find more insight into sex-differences in the occurrence, prevalence and severity of stress-induced disorders. Several effects of maternal deprivation have been described to be gender dependent while others are similar in males and females (Ellenbroek et al. 1998). For instance, light-enhanced startle is severely disrupted in female, but not in male rats (De Jongh et al. 2005). In addition males had shorter emergence latencies in the plus maze test than control rats, and exhibited lower activity in a novel environment after maternal deprivation (Slotten et al. 2006). ACTH response is higher in male rats exposed to elevated plus maze test after maternal separation than in females (Wigger et al. 1999). In the present study, males showed a subtle enhancement in some neurodevelopmental signs, while females displayed a slight delay in others, indicating that the female nervous system does not adapt to MD in contrast to males. Interestingly, stress-related disorders examined often by the rodent MD model, show gender differences in humans as well (Da Silva et al. 1999; Desai et al. 2000; López-Gallardo et al. 2008). However, in the present study we have found that males and females react only slightly differently to early maternal separation, we believe that those differences might be important as they could be the earliest signs of gender-dependent changes leading to the long-lasting sex-dependent effects of maternal separation.

5.2 PACAP mutant mouse development study

In this part of the research, I described the neurobehavioral development of male and female PACAP mutant mice. Considering physical signs, we found that PACAP-deficient mice had slower weight gain throughout the observation period. Interestingly, mice partially lacking PACAP (heterozygous) weighed significantly less than did homozygous mice. There was no difference between male and female mice during the first 3 weeks. Other signs were also more severely affected in the HZ mice than in the homozygous mice. Interestingly, incisor teeth erupted earlier in mice lacking PACAP. On the elevated grid, homozygous 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 (Hashimoto et al. 2001).

It has been shown earlier that heterozygous mice show a ~70% reduction of PACAP level (Hashimoto et al. 2001). Previous studies using homozygous and/or heterozygous mice have described very different results depending on the tissues studied and the experimental conditions (Reglodi et al. 2012). In heterozygous mice, it can be expected that the spectrum of symptoms/lesions fall between wild-type and homozygous mice or resemble either homozygous or wild-type mice. In the case of PACAP, some studies have shown that mice partially lacking PACAP have normal phenotype, possibly due to the successful compensation of the lower levels of the peptide by other factors and/or by the upregulation of the PACAP receptors. For instance, tear secretion in young male and female mice was severely reduced in homozygous mice but was not affected in heterozygous mice, only in older females (Nakamachi et al. 2016).

Other studies have found that the partial lack of PACAP results in a phenotype similar to the one observed in complete lack of PACAP. For example, it has been found that heterozygous mice show increased vulnerability to certain insults (Nakamachi et al. 2010). It has been described in a model of excitotoxicity in the retina (Endo et al. 2011), where the degree of degeneration was similar to that seen in homozygous mice (Atlasz et al. 2016; Szabadfi et al. 2012). Also, in ischemia/reperfusion injury, we found that the degree of injury was similar in mice completely or partially lacking PACAP (Laszlo et al. 2015; Szakaly et al. 2011). Ohtaki and coworkers demonstrated that infarct volume and neurological deficits were approximately 25% higher in both homozygous and heterozygous PACAP-deficient mice in a focal cerebral ischemia model, with no significant difference between heterozygous and homozygous animals (Ohtaki et al. 2006). In heterozygous mice, injury volume was larger, the number of degenerated neuronal cells was significantly higher, and the recovery was slower than in wild-type mice following contusion of the spinal cord (Tsuchikawa et al. 2012). It can be suggested that in these injuries, endogenous PACAP is part of the endogenous protective machinery; thus, partial lack of PACAP also results in increased sensitivity and a lesion similar to that seen in mice with complete lack of PACAP (Reglodi et al. 2012). The third possibility is that heterozygous mice show a phenotype between wild types and homozygous mice. In the heart, homozygous PACAP-deficient mice showed

a high mortality after doxorubicin treatment with only about 20% survival, and heterozygous animals also displayed higher mortality with about 50% survival rate, compared to wild-type mice (90% survival) (Mori et al. 2010). Similar pattern was observed with tear secretion in aging mice: levels were reduced already in heterozygous mice and further reduced in mice with complete lack of PACAP (Nakamachi et al. 2016).

Interestingly, 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. In the present study, we found that some reflexes appeared later than normal, and weight gain was slower than in wild-type mice. These results are in agreement with studies showing a delay in development in the case of complete or partial lack of neurotrophic factors. The complex behavioral pattern is influenced by many factors, the alterations of which are not known in the case of gene-deficient mice. Besides, individual components can be independently altered and differentially influenced by factors, like competition, physical strength, etc., as part of the complex behavioral phenotype, similarly to what has been described in brain-derived neurotrophic factor-deficient mice (Olsen et al. 2013).

The explanation in these cases may be that a complete lack of PACAP may induce the upregulation of other trophic factors compensating for the genetic defect of PACAP, but the partial lack does not induce these factors. The nature of compensatory mechanisms is still not elucidated, even though several attempts have been made. Monoaminergic transmitters, serotonin, and dopamine do not show altered expression in lack of PACAP (Ogawa et al. 2005). It has been hypothesized that up regulation of VIP, the peptide most closely related to PACAP, may compensate the lack of endogenous PACAP. However, in spite of this logical expectation, data could not confirm this suggestion (Girard et al. 2006). A reciprocal relationship between enriched environment and PACAP has been described: enriched environment leads to upregulation of PACAP (Horvath et al. 2015a), and it can also compensate the behavioral alterations in PACAP knockout mice, suggesting that environmental conditions can activate trophic factors that trigger similar pathways as PACAP (Takuma et al. 2014; Ishihama et al. 2010). Although the exact mechanism explaining the present

findings is not known at the moment, we suggest that trophic factor-related pathways are upregulated in homozygous PACAP-deficient mice, partially compensating for the complete lack of the trophic effects of PACAP during development, but it is less so in heterozygous PACAP knockout mice. Our preliminary investigations in this issue show that several signaling pathways involved in the development of hard tissues (cartilage, bone, tooth) are disturbed in both KO and HZ mice.

5.3. Depression model study

Simple animal models based on genetic, epigenetic or environmental approaches did not become equivocally accepted (Mill and Petronis 2007). In this study we aimed to validate an animal model for the three hit theory of resilience and vulnerability (de Kloet et al. 2007). 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) (Gaszner et al. 2012; Hashimoto et al. 2009, 2001). 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 (Gröger et al. 2016; Authement et al. 2015; Jawahar et al. 2015). Finally, CVMS, as a third hit, superimposed to the genetic predisposition and history of MD may precipitate the symptoms.

The efficacy of this new model was tested by physical, endocrinological, behavioral and functional morphological tools.

5.3.1. The efficacy of the CMVS paradigm

Bodyweight change is a commonly used physical parameter to assess the efficacy of stress exposure (Kormos et al. 2016; Harris et al. 2015; Rabasa et al. 2015; Boleij et al. 2014; for a review see: Harris 2015). 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 an effective indicator of stress level [as reviewed by (Bali and Jaggi 2015)]. 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 (Kormos et al. 2016; Mustafa et al. 2015; Lehman, et al. 2013; Tsukiyama et al. 2011; Stroth and Eiden 2010). An interesting novel finding of this study is that if PACAP KO mice receive an exposure to short or severe MD180, their CORT response to CVMS becomes normal. This data awaits further experimentation. One hypothetical explanation for this phenomenon could be that maternal deprivation may have inhibited myelination (Carlyle et al. 2012) in our animals. As PACAP KO pups show premature myelin formation resulting in reduced neuronal plasticity (Vincze et al. 2011), the effect of MD180 could have been beneficial on HPA axis plasticity. Importantly, the CVMS-induced CORT response became disrupted in PACAP HZ mice with the MD180 history suggesting HPA axis maladaptation.

5.3.2. Behavioral considerations

Due to the reduced adaptation ability of PACAP KO mice the sample size of behavioral tests was relatively low in this study (see also Farkas et al. 2016). Nevertheless, despite the limitations, FST appeared to be more sensitive than TST in this experiment. The effectivity of CVMS exposure was further supported by FST, as stressed AFR WT mice showed longer immobility time. This finding falls in line with our earlier work (Kormos et al. 2016). MD180 effectively increased the depression-like behavior in WT mice which is in line with work of others (MacQueen et al. 2003). Importantly, to cause increased depression-like phenotype, CVMS exposure was required in MD180 HZ mice, supporting the model value of this group.

The depression-like phenotype of non-stressed PACAP KO mice on CD1 background was demonstrated in our earlier (Gaszner et al. 2012; Hashimoto et al. 2009) and present work also. However, this depression-like behavior was not observed in C57BL/6 N PACAP KO (Lehmann et al. 2013) and C57BL/6J × 129SvEv hybrid PACAP KO mice, it seems that upon stress this strain difference disappears. Our mice showed dramatically reduced depression-like behavior both in FST (Kormos et al. 2016) and TST upon CVMS. Our current results are also in good agreement with the findings of Lehman et al. (2013) obtained in C57BL/6 N PACAP KO mice subjected to social defeat.

MBT and LD tests for anxiety were performed to support the model's validity. 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 (Gaszner et al. 2012), 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. In line with the work of Akillioglu (Akillioglu et al. 2015) using elevated plus maze and that of Wang (Wang et al. 2012) in LD, MD180 history caused increased anxiety levels in WT mice. 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.

As summarized in Table 5.1 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.

	AFR			MD180					
	CVMS			CTRL			CVMS		
	WT	HZ	KO	WT	HZ	KO	WT	HZ	KO
TST (Immobility %)	-	-	↑	-	-	-	-	-	↓
FST (Immobility %)	↑	-	↓	↑	-	-	↑	↑	-
MB (marbles buried)	↑	↑	↑	↑	-	-	↑	↑	-
LD (Time in lit compartment)	-	-	-	-	-	↓	-	-	↑
LD (Number of transitions)	-	-	-	-	-	-	-	-	-
BW change	↓	↓	-	↓	-	-	↓	↓	-

Table 5.1
Summary of behavioral and body weight changes in response to the presence of different number of hits in the three hit model. Data were compared to the results of AFR CTRL WT mice (i.e. zero hit mice). Symbols „↑" indicate significant increase ($p < 0.05$), „↓" refer to significant decrease ($p < 0.05$), „-" means no significant differences based on statistics ($p > 0.05$). Dotted line indicates groups with two hits, continuous line indicates the groups which suffered all three hits. Absence of line indicates one hit. Note that the MD180 HZ animals show increased depression-like behavior in forced swim test (FST), increased anxiety in marble burying test (MBT) and reduced bodyweight (BW). These phenomena supporting depression do not occur in KO mice with three hits. LD: light-dark box test.

5.3.3. Morphological findings

5.3.3.1. BSTov CRF

CRF neurons in the BSTov are heavily innervated by PACAP containing nerve fibers (Koves et al. 2016; Kozicz et al. 1997; Koves et al. 1994). The functional relationship between PACAP and CRF in the BSTov is also known. For instance, CVMS increases the PAC1 receptor mRNA expression in the BSTov. Furthermore, site-specific PACAP infusion into the BSTov increases anxiety (Hammack et al. 2009, 2010) in line with the effect of the local CRF overexpression (Regev et al. 2011). PAC1 receptor antagonism has opposite effect (Roman et al. 2014). Our histological results in this study further support the involvement of BSTov CRF neurons in mood control. First, in line with our recent study (Kormos et al. 2016), 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 SSD 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 (Hashimoto et al. 2001) together with the MD180 history and CVMS exposure contributes to the observed phenotype. These findings further support the validity of our model. 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. Supporting this, it has been shown in the rat that lesions in the anterior BST reduce the PVN and HPA axis activity (Choi et al. 2007).

5.3.3.2. Urocortin1

Our findings that CVMS increased the FosB activity of Ucn1 neurons of WT mice is in accordance with earlier studies on mice (Korosi et al. 2005). Most recently we found that PACAP KO mice did not react to CVMS in terms of FosB and Ucn1 peptide expression (Kormos et al. 2016) which we could replicate in the present study. Maternal deprivation has a long lasting effect on the acute stress sensitivity of Ucn1 neurons as demonstrated earlier in rat (Gaszner et al. 2009). 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. The relatively low depression level in FST may be explained by the over-activity of the Ucn1 neurons. This is supported by the facts, that Ucn1 KO mice have impaired adaptation ability to chronic stress (Zalutskaya et al. 2007). Moreover, reduced Ucn1 cell count was found in brain samples of chronically stressed tree shrews (Kozicz et al. 2008). Low anxiety measures in LDT may be explained by the higher activity of the Ucn1

system as in an Ucn1 null mouse strain increased anxiety was demonstrated (Vetter et al. 2002). In our CVMS exposed MD180 KO mice the increased signaling through CRF2 receptors may have conveyed an anxiolytic effect (for a review see Kormos and Gaszner 2013). However, this finding awaits further experimentation by CRF2 receptor antagonist treatments.

5.3.3.3. Serotonin

Earlier studies (Kormos et al. 2016; Gaszner et al. 2012; Hashimoto et al. 2001) 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, which is in contrary to our latest study (Kormos et al. 2016). This inconsistency might be explained by the different CVMS protocol applied in the present study. A battery of behavioral tests, applied on the last four days of the CVMS paradigm, may have exerted a more potent FosB inducing effect in the DR (this study) than the mild stressor exposures in our recent work (Kormos et al. 2016). A similar explanation applies why we did not see the increased 5-HT content of control KO mice in this study. Although these mice were not exposed to the CVMS protocol, they underwent the four behavioral tests which may have abolished the higher basal 5-HT content of DR as shown in (Kormos et al. 2016). The effect of short and long term maternal separation on the serotoninergic system was described by other laboratories earlier when the expression of tryptophan-hydroxylase 2 was assessed (Gardner et al. 2009). The higher 5-HT content in our MD180 mice is well supported by the finding that the mRNA expression of the rate limiting enzyme for 5-HT synthesis was found to be higher in animals with MD180 history (Gardner et al. 2009). 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.

6. Conclusions and future perspectives

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. As it is widely accepted that maternal deprivation causes long-lasting changes in the epigenome shaping adaptation ability (van Bodegom et al. 2017, Otte et al. 2016) we concluded that the behavioral alterations might develop in the later postnatal period. 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. In accord with the findings of others who examined other growth factors (Olsen et al. 2013.) we found a somewhat slower weight gain and delay in the neurobehavioral development of PACAP KO mice. A great body of evidence suggests that PACAP KO mice show mild phenotypical anomalies only, but these mice are known to display markedly reduced adaptation capacity to noxious environmental effects (for review see: Reglodi et al. 2012).

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. This approach was based on the widely accepted three hit concept of human depressive disorder.

The assessment of our new model based on the classic Willnerian criteria (Willner 1984) revealed that a) the construct validity was successfully achieved in PACAP HZ mice. b). The face validity criterion was supported by two out of the four behavioral tests: PACAP HZ mice with MD180 history show increased depression and anxiety levels. Bodyweight change of these mice was in good agreement with the behavioral findings. Functional-morphological tools show that in HZ mice with MD180 history,

the FosB expression was elevated, and it remains unresponsive to CVMS in CRF and Ucn1 neurons. Interestingly, 5-HT neurons show reduced 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 (1984).

The reversibility of behavioral, physical, endocrinological and functional morphological alterations by selective serotonin reuptake inhibitor treatment are in progress. Based on the currently available preliminary results (Gaszner T et al., in preparation) it seems that the reversal of the observed anomalies is only partial. This suggests that individual differences in the vulnerability should also be taken into consideration. This supports the fourth, population validity criterion proposed by Schmidt et al. (2010). According to this, symptoms of the examined disease should occur in that fraction of the exposed animal population in which proportion humans develop the disease if they had been exposed to the risk factors. An alternative interpretation of the partial response also might support the validity of our model: the currently available pharmacological therapeutic approach fails or does not provide the required results. If the anti-depressant therapy resistant phenomena in our mouse model resemble to clinical cases without improper remission requires further research.

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.

7. 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. **This study is the first to show that 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.**

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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)
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Figure 4.13.
Summary of morphological results in the oval division of the bed nucleus of the stria terminalis (BSTov). **(A)** and **(B)**: Immunofluorescent microphotographs of corticotropin releasing factor (CRF) (green) and FosB (red) double labeling in the BSTov of an **(A)** animal facility reared (AFR) control (CTRL) PACAP heterozygous (HZ) and **(B)** an AFR chronic variable mild stress (CVMS) exposed HZ mouse. **(C)** The number (N) of CRF and FosB double positive cells in the BSTov. **(D)** Summary of changes in CRF positive cell count in BSTov. **(E)** Summary of changes in CRF specific signal density (SSD) (mean ± SEM). White bars: wild type (WT), gray bars: heterozygous (HZ), black bars: knock out (KO) animals. Lettering at the top of bars represent the most relevant significant statistical differences between pairs of groups according to the *post hoc* tests ($p<0.05$). MS15: 15 minutes maternal separation, MD180: 180 minutes of maternal deprivation lv: lateral ventricle; ic: internal capsule. Bar: 100 μm

Figure 4.14.
Summary of morphological results in the centrally projecting Edinger-Westphal nucleus (cpEW). **(A-E):** representative images of Ucn1 (green) and FosB (red) double immunofluorescence. **(A)**: Animal facility reared (AFR) control (CTRL) PACAP heterozygous (HZ) mouse **(B)**: AFR chronic variable mild stress (CVMS) exposed HZ mouse. Note that CVMS exposure induced FosB (red) immunoreactivity in the nuclei of Ucn1 (green) neurons. **(C)**: Representative image of a CTRL HZ mouse with 180 minutes maternal separation history (MD180). Note that MD180 exposure increased FosB immunoreactivity without CVMS exposure. **(D)**: MD180 and CVMS exposed HZ mouse. **(E)**: MD180 and CVMS exposed PACAP knockout (KO) mouse. Note that the strongest FosB immunoreactivity was found in this group. **(F):** Histogram depicts the number (N) of Ucn1 and FosB co-expressing neurons in the cpEW. **(G)**: Histogram showing the magnitude of the Ucn1 specific signal density (SSD) in arbitrary units (a.u.). **(H)**: Histogram with the count of Ucn1 neurons in the cpEW. White bars: wild type (WT), gray bars: HZ, black bars: KO animals. MS15: 15 minutes maternal separation. WT: wild type. Lettering at the top of bars represent the most relevant significant statistical differences between pairs of groups according to the *post hoc* tests ($p<0.05$). Bar: 100μm

Figure 4.15.
Summary of morphological results in the dorsal raphe nucleus (DR). (A-F): representative images of the DR in serotonin (5-HT, green) FosB (red) double labeled preparations in maternally deprived (MD180) mice exposed to chronic variable mild stress (CVMS) *vs.* non-CVMS exposed controls (CTRL). Note that CVMS reduced the 5-HT signal in all groups, in addition CTRL FosB cell counts were higher than in CVMS exposed mice. Histogram (G) depicts the magnitude of the 5-HT specific signal density (SSD) in arbitrary units (a.u.). (H): Histogram depicts the number (N) of Ucn1 and FosB co-expressing neurons in the DR. White bars: wild type (WT), gray bars: heterozygous for the PACAP gene (HZ), black bars: PACAP knockout (KO) animals. AFR: animal facility-reared, MS15: 15 minutes maternal separation. Lettering at the top of bars represent the most relevant significant statistical differences between pairs of groups according to the post hoc tests ($p<0.05$). Bar: 100μm