Neuropeptides in stress reactivity

Role of enkephalin in response to chronic stress

Thesis

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Abbreviations list

ACTH: adrenocorticotropic hormone;

BLA: basolateral nucleus of the amygdala

BNST: bed nucleus of the stria terminalis

bp: base pairs

CeA: central nucleus of the amygdala

Cg: cingulate cortex

CMS: chronic mild stress

CNS: central nervous system;

CoA: cortical nucleus of the amygdala

CPu: caudate putamen

Crh: corticotrophin releasing hormone gene

CRH: corticotrophin releasing hormone

Crhbp: corticotrophin releasing hormone binding protein gene

CRHbp: corticotrophin releasing hormone binding protein

Crhr: corticotrophin releasing hormone receptor gene

CRHr: corticotrophin releasing hormone receptor

DMH: dorsomedial nucleus of the hypothalamus

DNA: deoxyribonucleic acid

DOP: delta opioid receptors

ELISA: enzyme-linked immunosorbent assay

FRCx: frontal cortex

Gr: type II glucocorticoid receptor gene

GR: type II glucocorticoid receptor

GREs: glucocorticoid response elements

HPA axis: hypothalamic-pituitary-adrenal axis

HPC: hippocampus

IL: infra-limbic cortex

KO: knockout

KOP: kappa opioid receptor

LC: locus coeruleus

MD: major depression

Mr: type I mineralocorticoid receptor gene

MR: type I mineralocorticoid receptor

MOP: mu opioid receptor

NAc: nucleus accumbens

Oprd1: delta opioid receptors gene

Oprk1: kappa opioid receptor gene

Oprm1: mu opioid receptor genePCR: polymerase Chain Reaction

PCx: parietal cortex

PDyn: prodynorphin gene

Penk: preproenkephalin gene

PFC: prefrontal cortex;

Pomc: pro-opiomelanocortin gene

PrL: pre-limbic cortex

PVN: paraventricular nucleus of the hypothalamus;

RNA: ribonucleic acid

Ucn: urocortin gene

UCN: urocortin

VMH: ventromedial hypothalamus

VP: vasopressin

VTA: ventral tegmental area

WT: wild-type

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Chapter 1: Abstract

The incidence of mood pathologies, such as chronic anxiety and major depression, is a serious health problem in modern societies. The knowledge about the molecular mechanisms involved in the development of these disorders is crucial for preventive and therapeutic approaches. Mood pathologies are complex, and rise from the interaction of genetic and environmental factors.

The experience of chronic stress in everyday life is a main environmental factor for the development of mood pathologies. Therefore, identification of the biological mechanisms involved in stress response and of molecular markers underlying the individual's vulnerability or resilience to stress-related mood pathologies is of crucial importance.

Enkephalin has been shown to play an important role in stress reactivity, leading to anxiolytic and antidepressant effects under acute models of stress. Thus, in the present study, we aimed to assess the role of enkephalin in stress reactivity under chronic stress conditions. In order to do that, we submitted constitutive preproenkephalin KO (*Penk* KO) and WT mice to five weeks of CMS protocol, followed by analysis of the baseline corticosterone levels, as well as anxiety and depression-like phenotypes, as measures of stress adaptation.

Our study revealed that constitutive *Penk* KO mice show a higher resilience to the behavioral and hormonal effects induced by CMS, when compared with WT animals. Exposure to CMS induced an increase in baseline corticosterone levels and anxiety and depressive-like phenotypes in the WT. In contrast, *Penk* KO animals exhibited resilience to the mentioned effects.

In addition, we showed that CMS induces an increase in the *Penk* gene expression in the paraventricular nucleus of the hypothalamus (PVN) of WT

mice. Therefore, enkephalin signaling pathways originating in the PVN are involved in the stress response to the CMS conditions, and may contribute to the vulnerability to the CMS effects observed in these animals.

Furthermore, gene expression analyses of the endogenous opioid, glucocorticoid and CRH systems, in WT and *Penk* KO mice, revealed several gene expression differences between these animals. Our findings might contribute to the insights of gene expression profiles underlying vulnerable and/or resilient phenotypes to CMS conditions.

Chapter 2: Introduction

2.1 Stress and stress response

Stress can be induced by any internal or external stimuli, which is perceived as a real or predicted threat to the organism´s homeostasis and survival [1–5]. As a consequence of perception of a potentially stressful condition, the organism triggers several physiological and behavioral alterations in order to adapt to internal or external changes and survive. Thus, the stress response is defined by the concept of "allostasis", i.e. maintenance of homeostasis through physiological and behavioral changes [3,5].

Stressful conditions may elicit an array of stress responses, depending on the type and duration of the stress stimuli [6–8]. Moreover, stress response rely on the activation of several processes and systems, such as metabolic pathways and the cardiovascular, endocrine and the central nervous (CNS) systems [1,7,8].

2.2 Physiology of the stress response: Health and pathological conditions

Stress response is usually a beneficial biological process, which, through "allostasis", allows adaptation to new conditions and survival [3,4]. However, "allostasis" requires the coordinated activation of several biological mediators involved in the stress response, such as hormones, cytokines or neurotransmitters, and mobilizes high energy resources [3–5]. Exaggerated and/or sustained stress response may be highly demanding to the organism, leading to a depletion of energetic resources and to an overuse and imbalance of several biological mediators, i.e. "allostatic overload" [5]. Therefore, stress response must be proportional to the intensity and duration of the stress stimuli. When not efficiently activated and/or terminated, it can be deleterious to the organism [4,5,9,10]. "Allostatic overload" leads to maladaptive stress response, and is associated with the onset of pathological conditions,

such as chronic anxiety, post-traumatic stress disorder or major depression (MD) [2,5,11–13].

2.3 Neurobiology of the stress response

Stress response is diverse in nature. Different stress stimuli may be perceived by distinct sensory pathways, leading to the activation of distinct circuits involved in the processing, integration, and execution of stress response. In a general manner, stress stimuli can be divided in two major groups: 1) systemic stressors, which have a strong physiological component and represent an immediate threat to organism´s homeostasis and survival; 2) processive stressors, which involve psychological components and require prior emotional and/or cognitive processing before inducing an effector stress response [6,8]. Systemic stressors include as example, hypoxia, dehydration or hemorrhage, while restraint and social stressors belong to processive stressors. In addition, some authors differentiate between processive stressors with only a psychological component, such as social stress, and processive stressors which have psychological and physical components, such as restraint and footshock stressors [7].

The CNS plays a major role in the integration and coordination of the stress response. Sensory pathways relay stress stimuli information to the CNS. Then, the CNS integrates it and sends output signals (e.g. neurotransmitters, hormones and cytokines) to distinct peripheral systems and within the CNS itself, triggering the activation of specific effector pathways involved in stress response [1,5]. CNS-mediated stress response involves several brain areas (e.g. hypothalamus, hippocampus and amygdala), neurotransmitter systems (e.g. GABA, glutamate and dopamine) and hormones (e.g. glucocorticoids and adrenaline)[1,7,8,13]. Activation of specific neuronal circuits during stress response is defined by the nature of the stress stimuli. Stress-regulatory neuronal circuits

can be divided in high-order and low-order processing circuits [6,8]. Perceived systemic stressors activate low-order processing stress neuronal circuits directly, which initiates an immediate stress response. These neuronal circuits include several hypothalamic (e.g. suprachiasmatic and paraventricular nuclei) and brain stem nuclei (e.g. raphe nuclei and locus coeruleus) [6,8]. On the other hand, high-order processing neuronal circuits, or limbic areas, are activated by processive stressors. Distinct sensory pathways convey stimuli information to the sensory cortex, leading to further activation of several limbic areas. Activation of these brain circuits initiates emotional and cognitive processes, which give a psychological meaning to the stimuli. This psychological load defines the specific activation of down-stream (intermediary and low order-sensory brain areas) neuronal circuits during stress response [6,8]. Hippocampus, prefrontal cortex (PFC) and amygdala are among the limbic stress-related neuronal circuits [6,8]. Limbic neuronal circuits have a special role in learned and anticipatory stress responses. These brain areas are highly sensitive and plastic to prior stress experiences. During stress exposure and response, they are modulated in order to retain information and associate it with the stressors *per se*. These cues may be remembered during posterior life experiences, enabling the organism to anticipate a stressful condition and initiate a faster stress response, therefore increasing adaptation and survival to challenges through life [7,8].

The CNS is also involved in the attenuation and/or termination of stress response. During stress response, the CNS triggers the activity of specific circuits (e.g. hippocampus-PVN), which restrain stress response. These allow the organism to attenuate and/or terminate stress response when adaptation is reached and/or stress stimuli are not present anymore [8,11,13]. In addition, CNS-mediated restraint of the stress response also avoids an imbalance in the

levels of biological mediators, which might have pathological consequences [1,5]. One of the most important CNS-mediated stress response restraint mechanisms is the glucocorticoid negative feedback (detail description in the following chapter 2.4) [1,5,13]. Chronic and/or uncontrollable stress conditions are believed to impair CNS-restraint mechanisms in stress response, and are associated with the development of pathological states, such as chronic anxiety and major depression [1,5,13].

Within the CNS, the paraventricular nucleus of the hypothalamus (PVN) is a central structure in the integration and modulation of the stress response. It integrates information about different stressors, perceived and processed by peripheral and/or other CNS brains areas [8,14,15]. The PVN is a heterogeneous structure, comprising several distinct functional subdivisions and neuronal populations involved in the control of the autonomic and hormonal stress response [6–8,14,16]. Medial parvocellular neurons, which produce corticotrophin releasing hormone (CRH), have a central role in the neuroendocrine stress response. These neurons, together with the anterior pituitary and adrenal glands, constitute the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is the main system responsible for the neuroendocrine stress response. The medial parvocellular CRH neurons project to the median eminence in proximity to the anterior lobes of the pituitary glands. Activation of CRH neurons by stressors leads to the release of CRH into the median eminence, stimulating the production and release of the adrenocorticotropic hormone (ACTH) by the pituitary glands. This in turn leads to the production and secretion of glucocorticoids by the adrenal glands [6,14,16] (Figure 2.1). Glucocorticoids are the major effector hormones in the stress response, modulating important and distinct physiological functions in the peripheral organs and in the brain [1,7,9,17]. In addition, the neuroendocrine stress response is also controlled by another

PVN subdivision: PVN magnocellular neurons, which, among others, express the neuropeptides vasopressin (VP) and oxytocin (OTX) and control the posterior part of the pituitary gland. These neurons are activated under more restricted psychological stress conditions, such as parturition and dehydration stress [18–20] (Figure 2.1). The PVN is also involved in the control of the autonomic stress response, which occurs via neuronal projections from the dorsal parvocellular subdivision. These neurons, which among others express the neuropeptide CRH, modulate the autonomic nervous system via projections to several brain stem nuclei, such as the core nucleus involved in noradrenergic signaling - locus coeruleus (LC) - and to the spinal cord [16,21–23] (Figure 2.1).

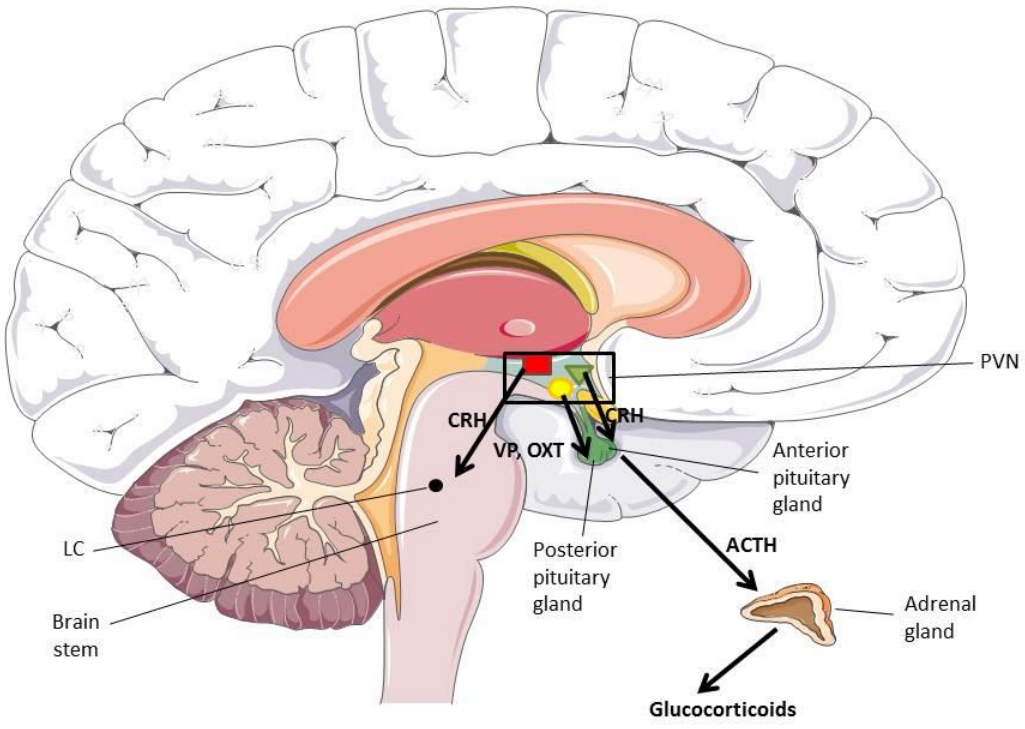


Figure 2.1 Subdivisions of the paraventricular nucleus of the hypothalamus (PVN) involved in the neuroendocrine and autonomic stress responses. PVN is composed of several subdivisions, where distinct neuronal populations are involved in the neuroendocrine and autonomic stress responses. CRH neurons at the medial parvocellular subdivision (green triangle) are the head of the hypothalamic-pituitary-adrenal (HPA) axis. Stimulation of these neurons leads to the production and secretion of corticotrophin releasing hormone (CRH), which in turn stimulates the production and secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. ACTH then leads to the production and release of glucocorticoids by the adrenal glands. Vasopressin (VP) and oxytocin- (OTX)-secreting neurons at the magnocellular subdivision (yellow circle) regulate the production of several hormones at the level of the posterior pituitary gland. VP and OTX magnocellular neurons are strongly involved in neuroendocrine response to osmotic and parturition stressors. CRH-expressing neurons of the dorsal parvocellular subdivision (red square) mediate the activation of the autonomous sympathetic nervous system during the stress response via projections to several brain stem nuclei, such as the noradrenergic locus coeruleus (LC). Brain and adrenal gland images adapted from www.servier.com.

2.4 Glucocorticoids in stress response

Glucocorticoids (mainly cortisol in humans and corticosterone in rodents) are secreted by the adrenal glands and released into the circulation. Glucocorticoid actions affect many peripheral organs and regulate distinct physiological functions, such as metabolism, growth, reproduction and immune responses [1,7,16]. Additionally, glucocorticoids can cross the blood brain barrier, thereby affecting the central nervous system [9,17]. Besides their role in the stress response, glucocorticoids are also involved in the regulation of appetite and sleep [24]. Glucocorticoid secretion is defined by two distinct, but intermingled patterns: 1) circadian cycle and 2) ultradian cycle. Circadian cycle of glucocorticoids secretion is characterized by slow and long oscillatory waves, which lead to relatively smooth variations in glucocorticoids levels during different time periods of the day. Two main extreme peaks of circadian

glucocorticoid secretion can usually be observed: a lower peak at the beginning of the resting phase and a higher peak at the beginning of the active phase [24–27]. In addition, secretion of glucocorticoids can occur in a fast and pulsatile fashion. These fast pulsatile secretions define the glucocorticoid ultradian rhythm. Pulsatile release of glucocorticoids is important for the maintenance of the glucocorticoid receptor responsiveness [25]. It is the frequency and amplitude, at which several pulsatile peaks occur, which delineate the circadian cycle of the glucocorticoids [24,25,27,28]. Therefore, high levels of glucocorticoids during the circadian cycle are due to an increase in the frequency and/or amplitude of the ultradian pulses, whereas low levels are a result of a decrease in the frequency and/or amplitude of pulsatile secretions (see figure 2.2 as example).

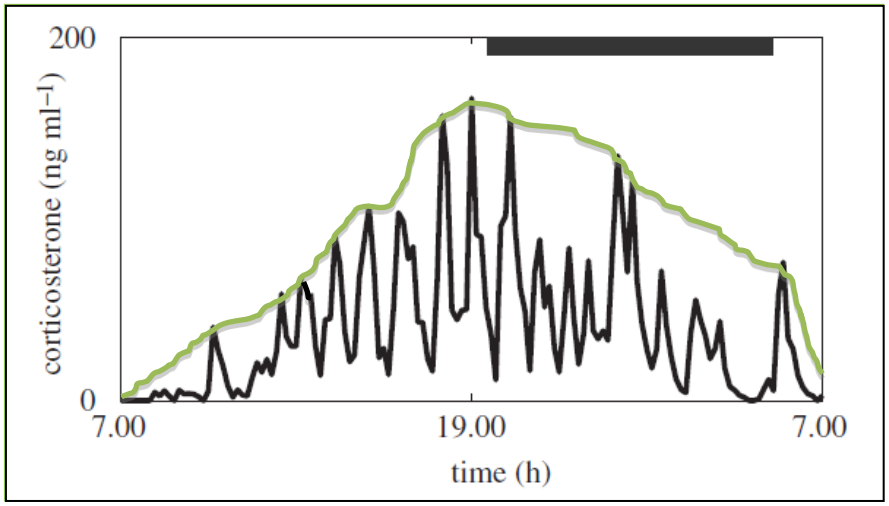


Figure 2.2 Ultradian and circadian cycles of baseline corticosterone levels.
Ultradian cycle is defined by a fast pulsatile secretion of glucocorticoids (black line). The number and amplitude of ultradian pulses delineate the circadian cycle of glucocorticoids secretion (green line). Figure adapted from Riedemann et al, 2010 [24] .

The effects of glucocorticoids are mainly exerted through two types of receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR and GR receptors are typically cytosolic receptors, but they

are also found in the cell membranes [29–32]. In the central nervous system, MR and GR display a distinct distribution. MR is only expressed in neurons and mostly in limbic areas, such as amygdala, PFC and hippocampus. GR is expressed in glial cells and neurons and widespread throughout the brain, with the highest expression in the PVN and hippocampus [29,33]. Both cortisol and corticosterone have a much higher affinity for the MR than for the GR. Therefore, under basal conditions, when glucocorticoid levels are relatively low, it is the activation of MR that occurs predominantly. On the other hand, the occupancy of GR receptors increases at a high scale when the levels of glucocorticoids increase. Increased glucocorticoid secretion occurs due to the stimulation of the adrenal glands by hypothalamic CRH, under stress conditions or due to other physiological functions, such as sleep and appetite stimulation [17,24,29].

In the brain, both fast and delayed effects of glucocorticoids can occur. Fast effects involve either facilitation or inhibition of neuronal cell signaling and are mediated by the activation of membrane bound receptors and G-protein coupled signaling pathways [29,32]. Activation of these receptors by glucocorticoids leads to the activation of intracellular pathways, which modulate the activity of several channels and receptors and the release of neurotransmitters. These effects are independent of alterations in gene expression [9,17,29,32]. On the other hand, delayed effects of glucocorticoids involve alterations in gene expression through the activation of cytosolic MR and GR receptors. Activation and binding of these receptors by glucocorticoids leads to the translocation of the receptors to the nucleus, where they bind to glucocorticoid response elements (GREs) and/or interact with certain transcription factors responsible for repression or stimulation of gene expression [9,17,29]. MR and GR both can bind to GREs, but only GR can interact

with transcription factors. Thus, both MR and GR are involved in the stimulation of gene expression, but only GR is responsible for repression of gene expression [17]. Therefore, GR and MR play different modulatory roles during the stress response. MR is implicated in the onset and maintenance of glucocorticoid-mediated effects in the stress response, while GR is involved in its termination [17].

As previously mentioned, when not efficiently activated and/or terminated, the stress response can be deleterious [3–5,9,12]. High and sustained corticosterone levels are associated with deleterious effects, such as deregulation of metabolic functions, immunosuppression [1] and decrease in neuronal proliferation, survival and plasticity [34–36]. An important feature of GRs is their role in mediating the glucocorticoid feedback mechanism. Too high and/or sustained levels of glucocorticoids activate GRs expressed in the PFC, hippocampus, PVN and pituitary gland, exerting an inhibitory effect on the HPA axis activity. Activation of the GRs leads to an inhibition of PVN parvocellular CRH neurons and/or pituitary gland activity, which results in a decrease in glucocorticoid secretion (Figure 2.3) [24,32,37].

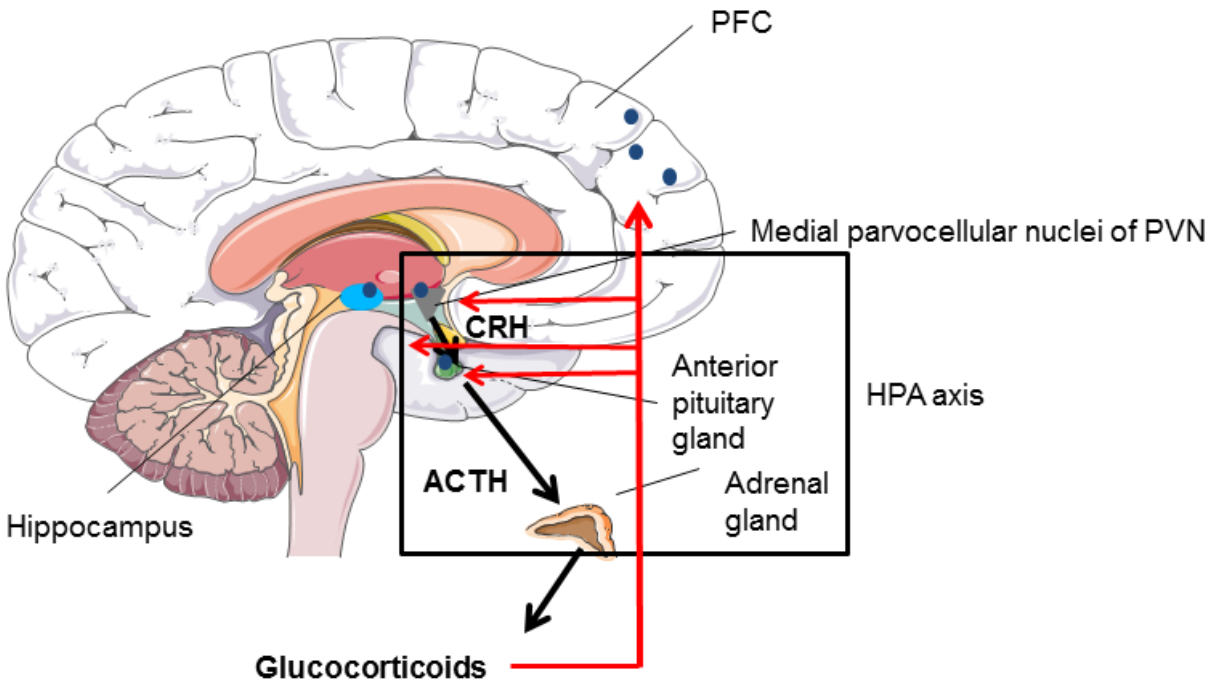


Figure 2.3 Stress-induced HPA axis activity and glucocorticoid-mediated negative feedback mechanism. Perception and processing of stress stimuli lead to the activation of the HPA axis. HPA axis activation begins with the stimulation of the PVN parvocellular CRH neurons. As a consequence, CRH is released and binds to the anterior pituitary gland receptors, stimulating the production and release of ACTH. In turn, ACTH stimulates the adrenal glands leading to the secretion and release of glucocorticoids. In order to avoid the deleterious effects of prolonged high levels of glucocorticoids, the latter also exert an inhibitory negative feedback action. Thus, when high levels of glucocorticoids are present, glucocorticoid receptors (GRs) (blue circles) expressed in the PVN, hippocampus, PFC and pituitary gland, are activated (red lines). Activation of these receptors leads to the inhibition of the parvocellular CRH neurons and/or of the pituitary gland activity, decreasing the production and release of more glucocorticoids. ACTH, adrenocorticotropic hormone. CRH, corticotrophin-releasing hormone. GR , glucocorticoid receptors. HPA axis, hypothalamic-pituitary-adrenal axis. PFC, prefrontal cortex. PVN, paraventricular nucleus of the hypothalamus. Brain and adrenal gland images adapted from www.servier.com.

2.5 The CRH system in the stress response

The CRH system includes several peptides - CRH and urocortins (UCN) 1-3, their receptors, CRH receptors 1 and 2 (CRHr1, CRHr2) and a CRH binding protein (CRHbp), which binds to the CRH and UCN peptides, reducing the amount of free peptides (Figure 2.4) [7,38–40]. The CRH peptide binds CRHr1 with higher affinity than CRHr2. UCN 2 and 3 bind CRHr2, whereas UCN 1 has equal affinity for both receptors. These peptides and their receptors are widely and differentially distributed throughout the CNS, including important areas involved in the stress response, such as amygdala, hippocampus, PFC and hypothalamus [7,11,39–41]. The CRH system is involved in the endocrine stress response via hypothalamic pathways and in behavioral and autonomic responses to stress via extra-hypothalamic areas, such as amygdala [7,42] and hippocampus [43]. Pharmacological and genetic studies showed that activation of CRHr1 has a stimulatory role in stress reactivity leading to anxiogenic effects [44–46], whereas activation of the CRHr2 is involved in the termination of the stress response, exerting anxiolytic effects [45,47,48].

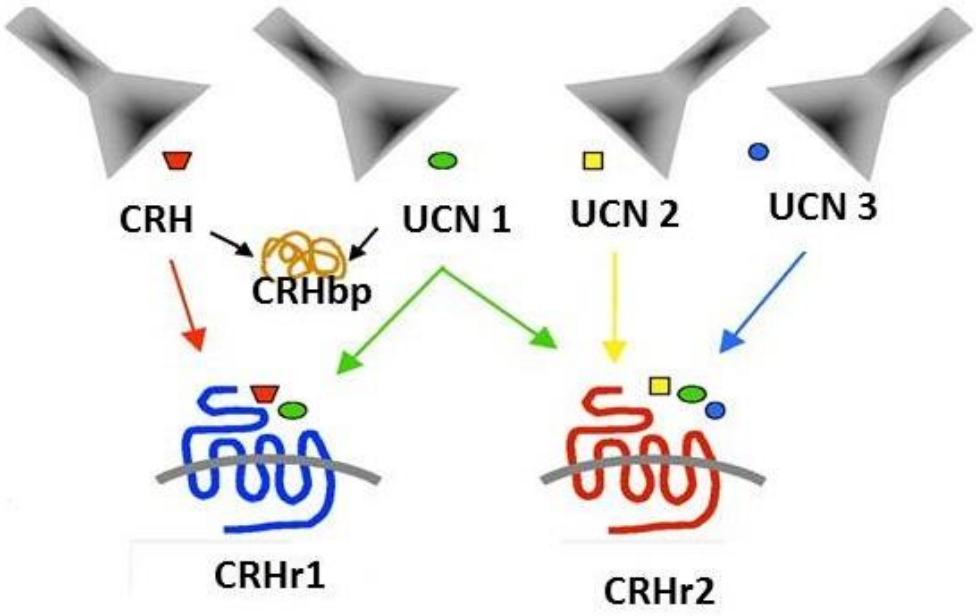


Figure 2.4 The CRH system: ligands and receptors. The CRH system is composed of 4 peptide ligands (CRH, UCN 1, 2 and 3) receptors (CRHr1 and 2), and the CRH binding protein, which binds to CRH and UCN 1. CRH, corticotropin releasing hormone. CRHbp, corticotropin releasing hormone binding protein. CRHr, corticotropin releasing hormone receptor. UCN, urocortin. Figure and legend adapted from Bale and Vale, 2004 [45].

2.6 The endogenous opioid system in stress response

The endogenous opioid system is composed of several G_i-protein coupled receptors - delta (DOP), mu (MOP) and kappa (KOP) opioid receptors - and their endogenous peptide ligands: β-endorphin, leu-enkephalin, met-enkephalin, dynorphin A and dynorphin B[49,50]. Endogenous opioid peptides are generated from precursor proteins, which are the pro-opiomelanocortin for β-endorphin, proenkephalin for leu-enkephalin and met-enkephalin and prodynorphin for dynorphin A and dynorphin B. These precursors are, in turn, encoded by prepro-opiomelanocortin (POMC), preproenkephalin (PENK) and preprodynorphin (PDYN) genes. Different opioid peptides have differential

binding affinities to opioid receptors. β-endorphin binds to MOP and DOP with equal affinity. Dynorphins bind almost exclusively to the KOP. Enkephalins bind both DOP and MOP, however, they have a higher affinity for DOP [49,50]. Endogenous opioid peptides and receptors have a widespread localization in brain regions involved in the regulation of stress reactivity, such as PVN, amygdala, hippocampus and PFC (Figure 2.5) [49].

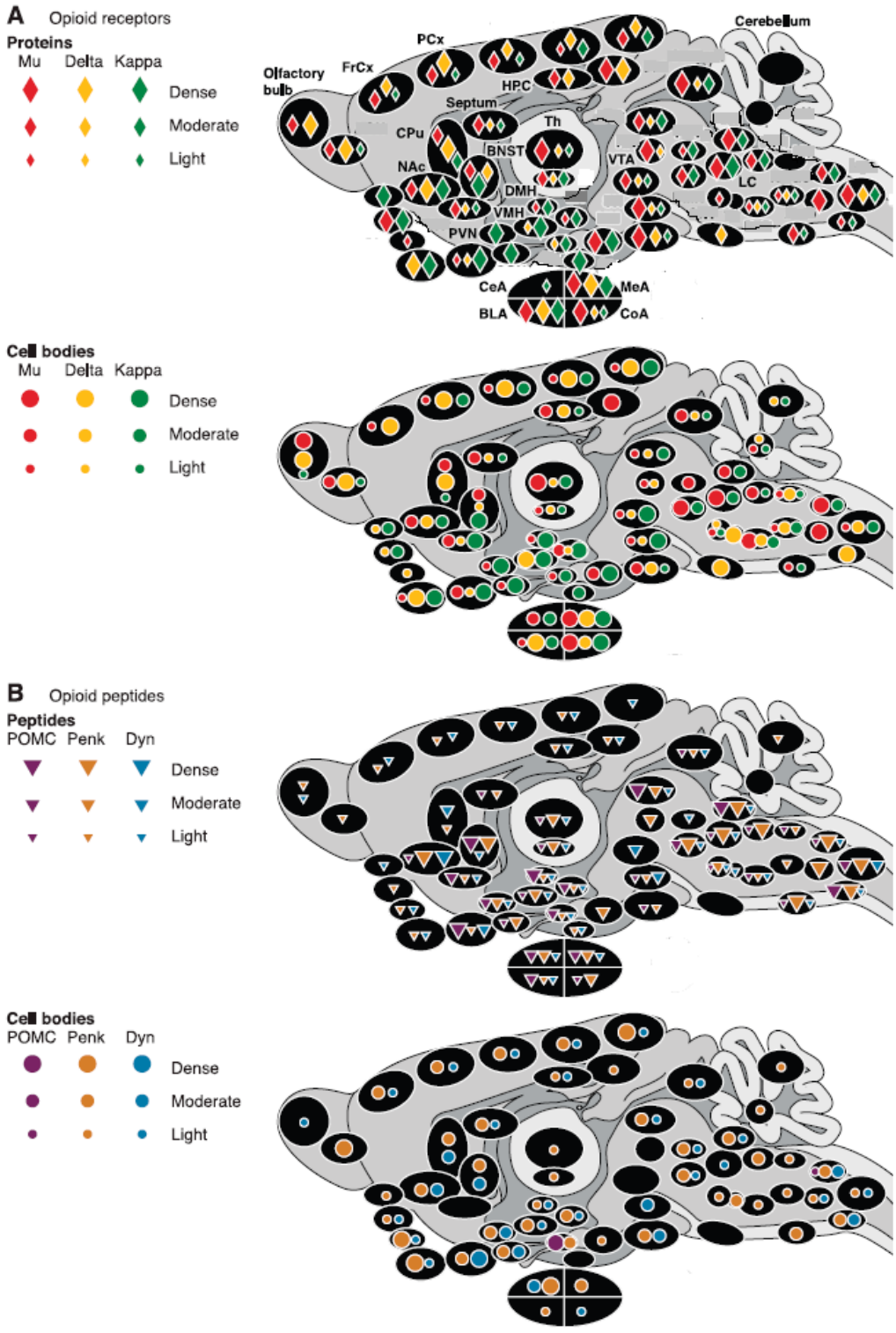


Figure 2.5 Anatomical distribution of the endogenous opioid system components in the rodent brain (rat and mouse). **A)** Opioid receptors: top figure - peptide expression; bottom figure - mRNA expression. **B)** Opioid peptides: top figure - protein expression; bottom figure - mRNA expression. BLA, basolateral nucleus, amygdala. BNST, bed nucleus of the stria

terminalis. CeA, central nucleus, amygdala. CoA, cortical nucleus, amygdala. CPu, caudate putamen. DMH, dorsomedial nucleus of the hypothalamus. FRCx, frontal cortex. HPC, hippocampus. LC, locus coeruleus. NAc, nucleus accumbens. PCx, parietal cortex. PVN, paraventricular nucleus, hypothalamus. VMH, ventralmedial nucleus, hypothalamus. VTA, ventral tegmental area. Figure and legend adapted from Le Merrer et al, 2009 (Le Merrer et al. 2009).

The potential role of the endogenous opioid system in stress response has been in the focus of stress-related research for some time now. In mice and rats, distinct stress conditions induce alterations in *Penk*, *Pdyn* and *Pomc* gene expression [50–53], as well as in the enkephalin, dynorphin and β-endorphin peptide levels [50]. Also, mRNA expression of opioid receptors [54–57], as well as their protein levels [50,58] and function [59] are altered after stress exposure. In addition, treatments with agonists and antagonists for the different opioid receptors lead to stress-related hormonal and behavioral alterations [50,60–65]. Finally, knockout (KO) mice for the different opioid peptides [66–68] and receptors [67,69–71] underline the importance of , and are an important tool to understand the role of, the endogenous opioid system in stress response,

2.6.1 Enkephalin, DOP and MOP in stress response

Previous studies showed that alterations in the *Penk* mRNA expression (Table 2.1) and enkephalin peptide levels (Table 2.2) in different brain areas of rodents can be induced by distinct stress conditions. For example, electroconvulsive shocks increase *Penk* mRNA in the hypothalamus [72] and chronic hypertonic stress in the PVN [73]. Fourteen days of isolation stress in rats decrease *Penk* mRNA levels in the caudate putamen (CPu) and in the nucleus accumbens (NAc) [74], but lead to its increase in the hypothalamus [75]. In the PVN, both acute [76,77] and chronic [76] immobilization stress induce an up-regulation of the *Penk* gene expression. Chronic immobilization stress also increases *Penk* mRNA expression in the hippocampus [52], but decreases it in the NAc [78]. In the

CPu, *Penk* mRNA levels increase after acute immobilization stress, but decrease after chronic immobilization stress [79]. Chronic unpredictable stress (CUS) induces an increase in the *Penk* mRNA expression in the NAc, central nucleus of the amygdala (CeA), PVN, ventral medial hypothalamus (VMH) and in the hippocampus [53]. In contrast, CUS induces a decrease in *Penk* mRNA in the basolateral nucleus of the amygdala (BLA) in rats [80]. Moreover, chronic social stress also induces a decrease in the *Penk* mRNA expression in the BLA [51].

Table 2.1 Stress-induced alterations in the *Penk* gene expression in rats. BLA, basolateral nucleus of the amygdala. CeA, central nucleus of the amygdala. CPu, caudate putamen. CUS, chronic unpredictable stress. NAc, nucleus accumbens. PVN, paraventricular nucleus of hypothalamus. VMH, ventral medial nucleus of the hypothalamus.

Brain area	Stress	*Penk* mRNA alterations	References
BLA	CUS	↓	Bérubé, Poulin, et al. 2013
	Chronic social defeat stress	↓	Bérubé, Laforest, et al. 2013
CeA	CUS	↑	Christiansen et al. 2011
CPu	Fourteen days of isolation stress	↓	Angulo et al. 1991
	Acute Immobilization	↑	Lucas et al. 2007
	Chronic immobilization	↓	Lucas et al. 2007
Hippocampus	Chronic immobilization	↑	Chen et al. 2004
	CUS	↑	Christiansen et al. 2011
Hypothalamus	Fourteen days of isolation stress	↑	Iglesias et al. 1992

	Electroconvulsive shocks	↑	Yoshikawa et al. 1985
Nac	Fourteen days of isolation stress	↓	Angulo et al. 1991
	Chronic immobilization	↓	Poulin et al. 2014
	CUS	↑	Christiansen et al. 2011
PVN	Chronic hypertonic stress	↑	Young & Lightman 1992
	Chronic immobilization	↑	Dumont et al. 2000
	Acute immobilization	↑	Dumont et al. 2000; Palkovits 2000
	CUS	↑	Christiansen et al. 2011
VMH	CUS	↑	Christiansen et al. 2011

In addition, stress-induced alterations of enkephalin peptide levels were also reported in several studies. For instance, enkephalin peptide levels are up-regulated by electroconvulsive shocks in the hypothalamus and CPu [72]. On the other hand, footschock stress leads to a decrease in peptide levels in the hypothalamus and CPu [81]. Forced swim stress decreases the levels of enkephalin peptide in the CPu in rats [81]. Fourteen days of isolation stress, in contrast, increase enkephalin peptide levels in the hypothalamus [75]. Chronic mild stress (CMS) leads to a decrease in the levels of the enkephalin peptide in the NAc of rats [82]. Acute social interaction stress increases the release of enkephalin peptide in the NAc. However, increased release of enkephalin in the NAc, mediated by acute social interaction stress, is abolished in animals previously submitted to CMS [83].

Table 2.2 Stress-induced alterations in the enkephalin peptide levels in rats. CPu, caudate putamen. CMS, chronic mild stress. NAc, nucleus accumbens.

Brain area	Stress	Enkephalin peptide alterations	References
CPu	Electroconvulsive shocks	↑	Yoshikawa et al. 1985
	Footschock	↓	Nabeshima et al. 1992
	Forced swim	↓	Nabeshima et al. 1992
Hypothalamus	Footschock	↓	Nabeshima et al. 1992
	Fourteen days of isolation stress	↑	Iglesias et al. 1992
NAc	CMS	↓	Dziedzicka-Wasylewska & Papp 1996
	Acute social stress	↑	Bertrand et al. 1997
	CMS + Acute social stress	=	Bertrand et al. 1997

Moreover, enkephalin receptors, MOP and DOP, were also shown to be modulated by stress. For instance, seventy-two hours of sleep deprivation in rats lead to a decrease in the number of MOP and DOP in the limbic system [84]. Foot-shock stress decreases, and water deprivation increases, MOP binding in the septum of rats [85]. Water deprivation increases DOP binding in the CPu and NAc in rats [85]. Acute and repeated social stress increase *Oprm1* mRNA expression in the ventral tegmental area (VTA) in rats [56,57]. Thus, stress effects on the expression of the *Penk, Oprm1 and Oprd1* genes, as well as peptide and

protein levels, may differ between different brain regions and are dependent on the nature of the stress stimulus (type and duration).

The importance of enkephalin and its receptors in stress response was also demonstrated in several pharmacological studies. Administration of exogenous enkephalin or enkephalin catabolism inhibitors induce an increase in enkephalin levels and ameliorate anxiety and depression – like phenotypes in mice and rats [65,86–89]. Moreover, agonist-induced activation of enkephalin receptors was shown to influence anxiety levels. Thus, agonists of DOPs have anxiolytic effects [63–65,90,91], while agonists of MOPs can induce both anxiogenic [64,92] and anxiolytic responses [93].

Finally, the importance of enkephalin, MOPs and DOPs in stress response was demonstrated using KO animals for the genes encoding the enkephalin peptide (*Penk* KO) and its receptors, MOPs (*Oprm1* KO) and DOPs (*Oprd1* KO). Constitutive *Penk* KO animals present high basal anxiety levels [66,94–97] and exacerbated anxiety and depression-like phenotypes after exposure to footshock stress [98]. Moreover, *Penk* KO mice show prolonged hormonal alterations in response to acute stress conditions [66]. In addition, mice with a specific down-regulation of the *Penk* gene in the CeA present a reduction in basal anxiety levels [99]. On the other hand, knock-down of the *Penk* gene in the BLA leads to anxiety-like phenotypes [80] (Table 2.3.).

Furthermore, constitutive *Oprd1* KO mice present basal anxiety and depressive-related phenotypes [100]. On the other hand, constitutive *Oprm1* KO mice show lower levels of anxiety and depression-related phenotypes, both under baseline [100,101] and after chronic social stress conditions [102].

Table 2.3 Anxiety and depressive-like phenotypes of *Penk* KO animals. CPu, caudate putamen. CMS, chronic mild stress. NAc, nucleus accumbens.

***Penk* gene modification**	**Species**	**Anxiety-like phenotypes**	**Depressive-like phenotypes**	**References**
Constitutive KO	Mice	Basal: ↑	Basal: =	Bilkei-Gorzo et al. 2004; Bilkei-Gorzo et al. 2008; Bilkei-Gorzó et al. 2008; König et al. 1996; Ragnauth et al. 2001
	Mice	Basal: =; After foot-shock stress : ↑	Basal: =; After foot-shock stress : ↑	Kung et al. 2010
BLA downregulation	Rats	Basal: ↑	Basal: =	Bérubé, Poulin, et al. 2013
CeA downregulation	Rats	Basal: ↓	Not accessed	Poulin et al. 2013

2.6.2 Dynorphin and KOP in stress response

Several studies implicate dynorphin and its main receptor, the kappa opioid receptor (KOP) [103], in hormonal and behavioral stress reactivity. For instance, alterations in dynorphin mRNA expression were reported in the PVN after immobilization stress [77], in the NAc after chronic unpredictable stress [53] and in the NAc and CPu after chronic social stress [51]. Moreover, activation of KOP was shown to increase anxiety-like behaviors [61] and ACTH secretion [104]. On the other hand, KOP antagonists reversed the effects of KOP activation in ACTH release [104] and reduced immobility time during repeated forced swim stress [105]. In addition, altered baseline and stress-induced hormonal status, as well as

altered anxiety and depressive-like phenotypes were reported in *Pdyn*-deficient mice (*Pdyn* KO). For instance, *Pdyn* KO mice presented reduced anxiety [68] and depressive-like behaviors [62], as well as reduced serum corticosterone levels [68]. However, some contradictory evidence has been reported: in other studies, increased anxiety levels and exacerbated stress-induced hormonal response have been demonstrated in these mice [66]. To sum it up, previous studies suggest that dynorphin/KOP signaling is involved in the modulation of anxiogenic, dysphoric and hedonic behaviors, with special relevance to stress sensitization to repeated stress conditions [106,107].

Dynorphin and enkephalin signaling pathways are co-localized in many stress-relevant brain regions. For instance, different opioid receptors (MOP, DOP and KOP) are expressed in the LC, but exhibit a distinct synaptic localization [108]. In another example, it was shown that enkephalin and dynorphin peptides are present in GABAergic neurons in the striatum, but within distinct GABAergic populations [109]. It is not entirely clear if and at which levels enkephalin and dynorphin pathways may interact, and how these interactions might influence stress responses. However, it is plausible that changes in enkephalinergic and/or dynorphinergic signaling may have reciprocal effects in stress reactivity.

2.7 Chronic mild stress protocol: an animal model to study stress-related mood disorders

Mood pathologies such as major depression (MD) and chronic anxiety occur more and more frequently in modern societies and are among the main health, social and economic issues affecting not only those suffering from these disorders but also the society as a whole [110,111]. A major problem in studying the biology of mood disorders is its complex etiology. Predisposition and susceptibility to the development of mood disorders originate from an interplay of genetic and environmental factors [2,11,112]. Animal models in which

genetic manipulation is combined with distinct environmental conditions have been generated in the last decades and have been of great help in understanding the biology of mood disorders [11,113–116].

The onset of major depression (MD) is highly correlated with environmental stress. Chronic and/or traumatic stressful life events have been shown to lead to MD episodes in genetically predisposed humans and animals [2,5,112,117,118]. Increased prevalence of chronic anxiety and MD pathologies in Western countries is believed to correlate with chronic stress conditions due to daily lifestyle rather than to traumatic stress events [4,112,118]. In everyday life, we constantly experience several stressors, such as sleep deprivation, abnormal feeding habits or social stress [4,119,120]. Each of the above mentioned mild stressors alone would certainly not lead to major impairments in a healthy organism. However, when experienced for prolonged periods of time and especially in an unpredictable way, such stressors can lead to cumulative alterations due to an exhaustion/imbalance (i.e. allostatic overuse) of the physiological components involved in stress coping and adaptation [3,5].

In order to study behavioral and molecular alterations due to the prolonged experience of daily life mild stressors in animals, Paul Willner and colleagues developed the chronic mild stress protocol (CMS) [121,122]. In this model, rats or mice are subjected to multiple mild stressors throughout the day, such as periodic sleep, food or water deprivation, restraint, wet or empty home cages and physical contact with an unknown conspecific animal, all applied in a semi-random way for long periods of time (4-8 weeks). CMS leads to certain behavioral and molecular alterations in rodents, which resemble those in patients suffering from MD and/or chronic anxiety [120,123–127]. Among the most commonly observed behavioral endpoints are anhedonia, despair and increased anxiety [120,123,126,127]. CMS also induces hormonal changes related to a

hyperactivation of the HPA axis, such as an increase in glucocorticoid secretion. Additionally, alterations in important neurotransmitters systems related to MD, such as CRH, serotonin, dopamine, noradrenaline, glutamate or GABA are also induced by the CMS protocol [124]. Furthermore, common antidepressant treatments applied in MD patients are also effective in rodents subjected to the CMS protocol [127]. Therefore, the CMS protocol fulfills the face validity (similarity to the disease symptoms), construct validity (similar etiology - genetic and/or environmental factors) and prediction validity (similar treatment responses in animals as in humans) criteria for a validated animal model to study human conditions [115,126].

2.8 Aims of the present study

In summary, the current literature underlines the important role of enkephalin in stress reactivity. Enkephalin peptide and mRNA levels are affected by distinct stressors and, in turn, alterations in enkephalin gene expression and peptide levels influence stress reactivity to several stress conditions (see Section 2.6 for details). For instance, previous studies from our group showed that *Penk KO* mice present higher basal anxiety levels [66,94,95] and exacerbated hormonal stress reactivity [66] to acute stress conditions compared with WT animals. However, stress reactivity under different stress conditions may rely on different stress-related circuits (see Section 2.3 for details). In addition, the literature suggests that the role of enkephalin in hormonal and behavioral stress reactivity may depend on the nature of the stress stimuli and/or on the stress-evoked neuronal circuits (see tables 2.1; 2.2 and 2.3). At the beginning of the present study, to our knowledge, only a few studies examined the role of enkephalin in stress reactivity under chronic unpredictable stress conditions (CMS or CUS) [82,83,128]. Recently, two new studies examined the effect of chronic unpredictable stress on *Penk* gene expression in distinct brain areas [53,80] and

how these alterations were correlated with vulnerability or resilience to stress conditions[80]. Therefore, the first aim of our project was to study the effect of enkephalin deficiency on stress reactivity after the CMS protocol. For that purpose, we submitted WT and constitutive *Penk* KO mice to the CMS protocol, followed by hormonal and behavioral evaluation. The second aim of our project was to examine how the CMS protocol affects the *Penk* gene expression in various brain areas with known functions in stress reactivity, such as the PVN, amygdala, hippocampus and PFC. Finally, our third aim was to investigate how the *Penk* gene deletion influences the gene expression of several other known stress response mediators, such as glucocorticoids and it receptors, the endogenous opioid peptide dynorphin and its receptor KOP, and the CRH system, in several brain areas, under basal (developmental) and CMS conditions. We believe that addressing these important questions will contribute to a better understanding about the role of enkephalin in stress response, and specifically under chronic stress conditions.

Chapter 3: Materials and methods

3.1 Animals

Male WT and *Penk* KO homozygous mice on a C57BL/6J genetic background were used in this study. Mice with a deletion of the *Penk* gene [96] were crossed with C57BL/6J mice for more than 10 generations in order to obtain *Penk* KO homozygous mice on a pure C57BL/6J background [94]. WT mice were originally obtained from a commercial breeder (Janvier, France) and bred at our animal facility. At postnatal day 21, pups were weaned and housed in groups of 4-5 animals. Starting at the age of 6 weeks, WT and *Penk* KO male mice were individually housed until the end of the experimental procedure. Rooms were maintained at 23°C, under a 12:12 hours inverted light cycle with *ad libitum* access to water and food, except when animals were food or water restricted during CMS. To avoid possible maternal care effects, homozygous WT and *Penk* KO gestating females were housed together from the first week of gestation until the end of the lactation period. All experiments followed the guidelines of the German Animal Protection Law.

3.2 Genotyping

DNA from a tail biopsy was extracted by the *hot shot* lysis method [129]. Amplification of the DNA for genotyping was performed by adding a master mix solution (GreenTaq (Promega), RNA free water, 0.5 µl E31 Primer (Metabion) - GCATCCAGGTAATTGGCAGGAA-, 0.5 µl neoRL Primer (Metabion) -CAGCAGCCTCT GTTCCACATACACTTCAT- and 0.5 µl E1R Primer (Metabion) - TCCTTCACATTCCAGTGTGC-) to the DNA samples, followed by 40 cycles of amplification. The products were separated by electrophoresis. A band of 700 bp was amplified for WT and of 550 bp for *Penk* KO mice (Figure 3.1).

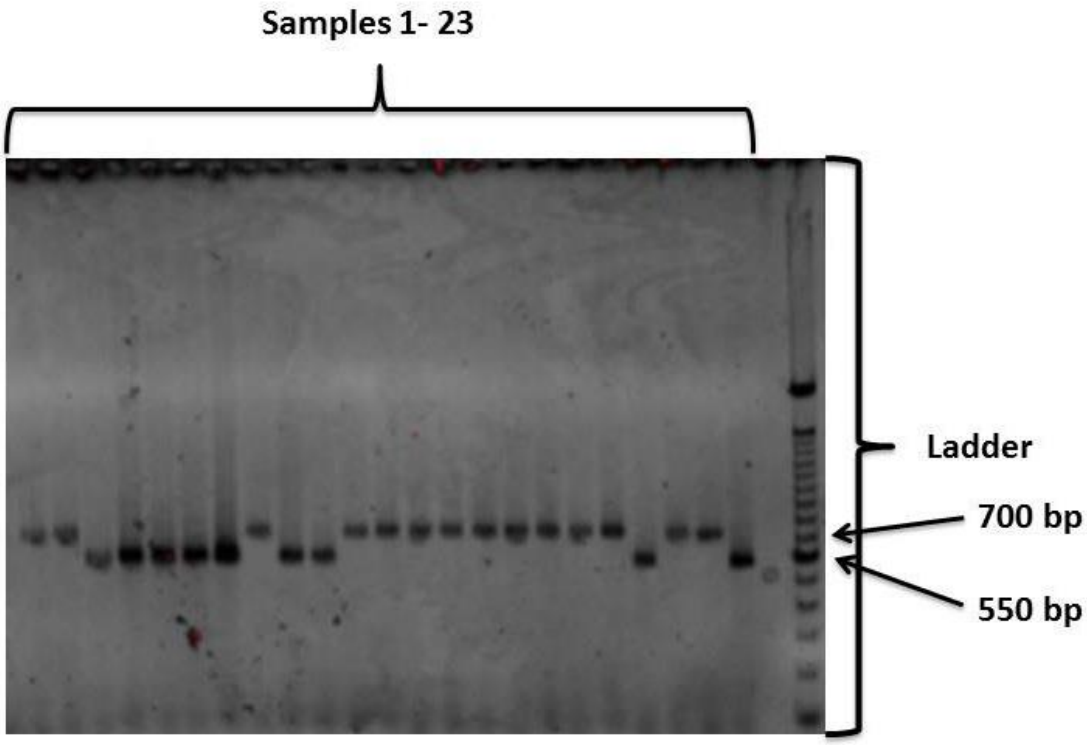


Figure 3.1 Genotyping of WT and *Penk* KO mice with tail DNA. PCR products were separated in an agarose gel electrophoresis, showing fragments with the size of 700 bp for WT mice (lanes 1-2, 8, 11-19, and 21-22) and of 550 bp for *Penk* KO mice (lanes 3-7, 9-10, 20 and 23).

3.3 Experimental design and CMS procedure

WT and *Penk* KO mice were submitted to the CMS protocol, which is a validated animal model to study chronic stress-related pathologies, such as major depression (MD) [124,126,127]. The experiment was carried out using two cohorts of mice due to the large number of animals necessary for the study. The first cohort consisted of 8 WT control, 8 WT CMS, 8 *Penk* KO control and 8 *Penk* KO CMS animals. The second cohort consisted of 15 WT control, 14 WT CMS, 11 *Penk* KO control and 10 *Penk* KO CMS animals. After two weeks of individual housing, WT and *Penk* KO animals from the CMS groups were submitted to 5 weeks of CMS consisting of the following stressors: 1 hour

restraint stress, 1 hour social stress (4 animals/ cage), 1-3 hours of stroboscopic lights, 4-8 hours tilted cages, 8-12 hours wet bedding, 8-12 hours cage without bedding, 24 hours light or dark periods, 18-24 hours food deprivation followed by 30 minutes of inaccessible food and 18-24 hours water deprivation followed by 30 minutes of exposure to an empty bottle. The different stressors were applied randomly in order to avoid adaptation to expected stress conditions. Additionally, some stressors, such as food and water deprivation, empty bottle and inaccessible food and lights on and off, were alternated from week to week (Table 3.1).

Table 3.1 Schedule and stressors applied in the chronic mild stress protocol for one week period .

	Morning	Afternoon	Overnight
Monday	Restraint (1h)	Stroboscopic lights (3h)	Tilted cages
Tuesday	Social stress (1h)	Wet sawdust	Wet sawdust
Wednesday	Stroboscopic lights (3h)	Water or food deprivation	Water or food deprivation
Thursday	Empty botlle or inacessible food (30 min)	Social stress (1h)	Cage without sawdust
Friday	Social stress (1h)	Restraint (1h)	
Saturday	Sucrose preference test	Sucrose preference test	Sucrose preference test
Sunday	Lights on/ off	Lights on/ off	Lights on/ off

WT and *Penk* KO mice from control groups were handled twice a week during the 5 weeks of the CMS protocol. Animals were left undisturbed for a period of 24 hours once a week, while the sucrose preference test was performed. At the end of the CMS procedure, animals were tested in a battery of behavioral

tests to assess general exploratory and locomotor activity, anxiety and depression-related phenotypes. Behavioral tests were performed in the following order: last sucrose preference test at day 1; open field test at day 3; 0-maze test at day 4; light-dark box test at day 5; forced swim test at day 7 (Figure 3.2).

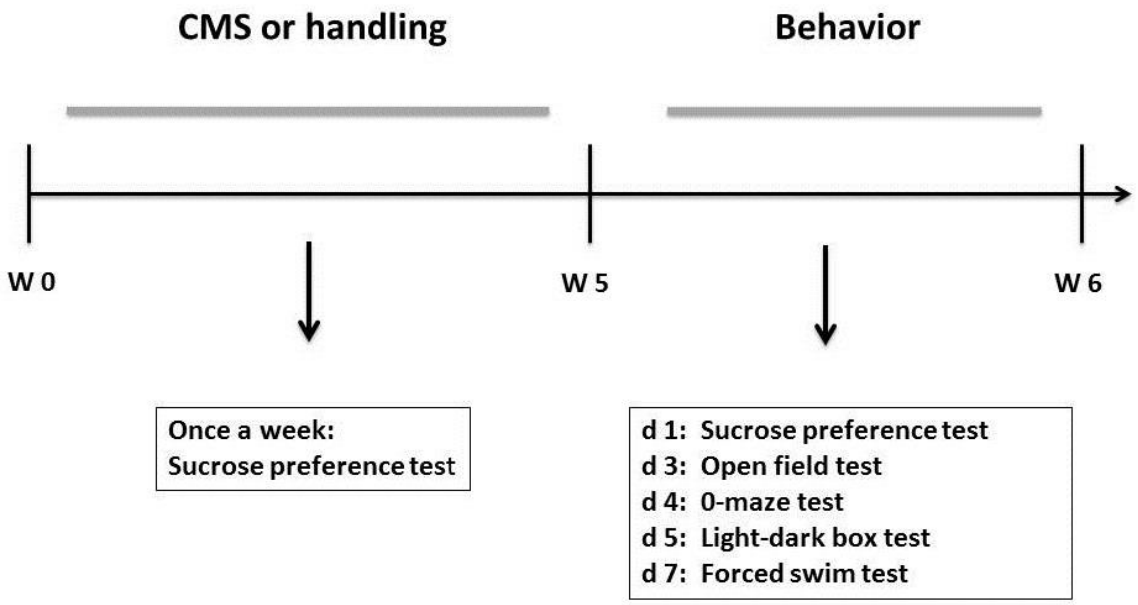


Figure 3.2 Timeline of the experimental procedure. WT and *Penk* KO animals were submitted to 5 weeks of CMS (CMS groups) or to handling (control groups). In week 6, all animals were tested in a battery of behavioral tests as indicated in the scheme. 24 hours after the last behavioral test, animals were killed and the brains removed for gene expression analyses. CMS = chronic mild stress; w = week; d = day.

3.4 Behavioral analyses

All behavioral experiments were performed during the dark phase of the light cycle.

Open field test

Animals were tested for exploratory and locomotor activity in the open field test. The test apparatus consists of an arena of 45*45*22 cm. The arena was dimly illuminated (20 lux at the floor level). Animals were placed in one of the corners and allowed to explore the arena for 10 minutes. The activity of the animals was recorded using a system of infrared beams connected to a computer. The total distance travelled (m) was analyzed with the Actimot 2 software (TSE Systems GmbH, Germany).

0-maze test

Anxiety-related behavior was tested in the 0-maze test. The test takes part in a round arena, elevated 38 cm above the ground, and composed of four areas: two areas with walls (15 cm high) and two with only a short rim (1 cm) (open areas). Animals were tested for 5 minutes with 400 lux of illumination. Time spent in the open areas (s) was evaluated with the Ethovision software (Noldus).

Light-dark box test

Anxiety-related behavior was further evaluated in the light-dark box test. The test apparatus is divided in two areas, a dark one (15*45*22 cm) and a lit one (30*45*22 cm) with 1000 lux illumination at the floor level. Both areas are connected by a 6*6 cm passageway. Each animal was placed in the center of the lit area and allowed to explore the apparatus for 10 minutes. The activity of the animals was recorded using a system of infrared beams connected to a computer. Time spent in the lit area (s) was analyzed with the Actimot 2 software (TSE Systems GmbH, Germany).

Sucrose preference test

Individually housed animals were first habituated to two drinking water bottles. Habituation to the sucrose solution was done within one week before the CMS protocol was initiated. Therefore, one water bottle was exchanged by a bottle filled with 1 % sucrose solution for 48 hours. The position of the bottles was changed after 24 hours. Animals presented preference for the sucrose bottle, independently of the side at which was positioned. The sucrose preference test was performed once a week during the 5 weeks of CMS by again the two bottles, one containing 1% sucrose solution and the other water, for 24 hours. Sucrose preference was calculated as follows: Sucrose preference = [Sucrose intake/ (sucrose intake + water intake)] * 100. Sucrose and water intake were calculated by subtracting the final weight (after 24 hours) from the initial weight of the bottles.

Forced swim test

Animals were tested for despair behavior in the forced swim test. During 6 minutes, animals were placed in a Plexiglas cylinder (10 cm internal diameter, 50 cm high) filled with 26-28°C water (30 cm height). Immobility time was measured during the last 4 minutes of the test as a measure of despair behavior using a stopwatch. An animal was judged to be immobile when it remained floating in the water, making only movements necessary to keep its head above the water.

3.5 Hormonal measurements: corticosterone metabolites in feces

At the second day after the end of the CMS protocol, WT and *Penk* KO mice were housed in new clean home cages. 24 hours later, feces samples at the home cages were collected and frozen at -80° C until further analyses of corticosterone levels. For corticosterone metabolites analyses, feces were

unfrozen, placed on open petri dishes and dried in an oven at 37°C for 1 hour. Subsequently, samples were grinded into a powder with the help of a pestle, weighted and used for corticosterone metabolites extraction followed by quantification by an ELISA assay for corticosterone. Corticosterone metabolites were extracted with 1 ml of ethanol per 100 mg of feces powder, followed by 30 minutes of vigorous shaking at room temperature (RT) and 30 minutes of centrifugation at 5000 rpm at RT. Subsequently, 450 µl of the supernatant were collected and dried in a speedvac (Speedvac Savant, Thermo Scientific) for 1 hour at 35°C. Pellets were frozen at -20°C in a desiccator to avoid hydration of the pellets. On the following day, corticosterone ELISA assays were performed following the manufacturer's instructions (Arbor assays: catalog number KO14-H5).

3.6 Molecular analyses

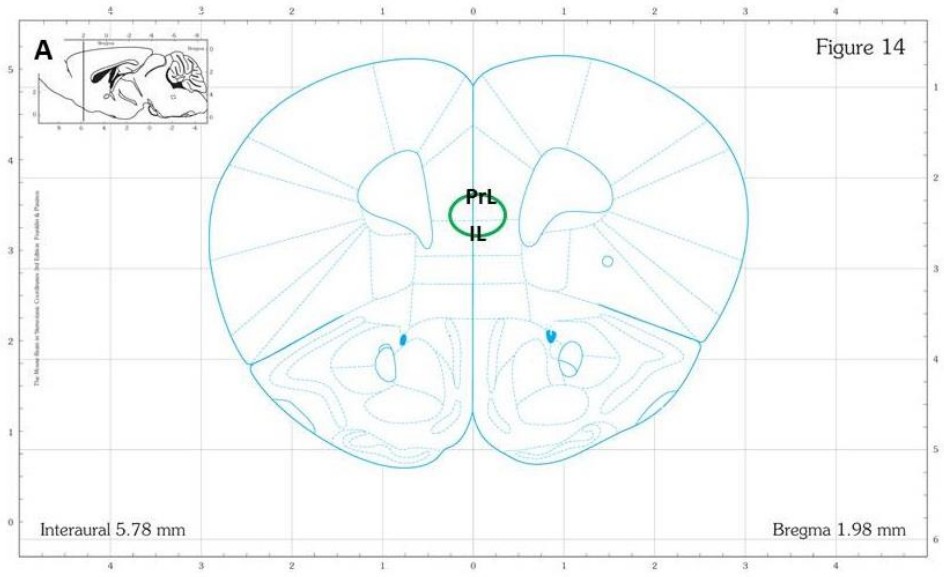
To evaluate the effect of CMS exposure in the modulation of the endogenous opioid system, the CRH system and the glucocorticoid receptors, mRNA levels of *Penk*, *Oprd1*, *Oprm1*, *Pdyn, Oprk1*, *Mr*, *Gr*, *Crh*, *Ucn 2*, *Ucn 3, Crhr1*, *Crhr2*and *Crhbp* were measured in the PFC, PVN, amygdala and hippocampus of WT and *Penk* KO mice (Table 3.2). The decision of which target genes should be measured in the analyzed brain areas was based in the gene expression patterns described in the literature [40,49,130]

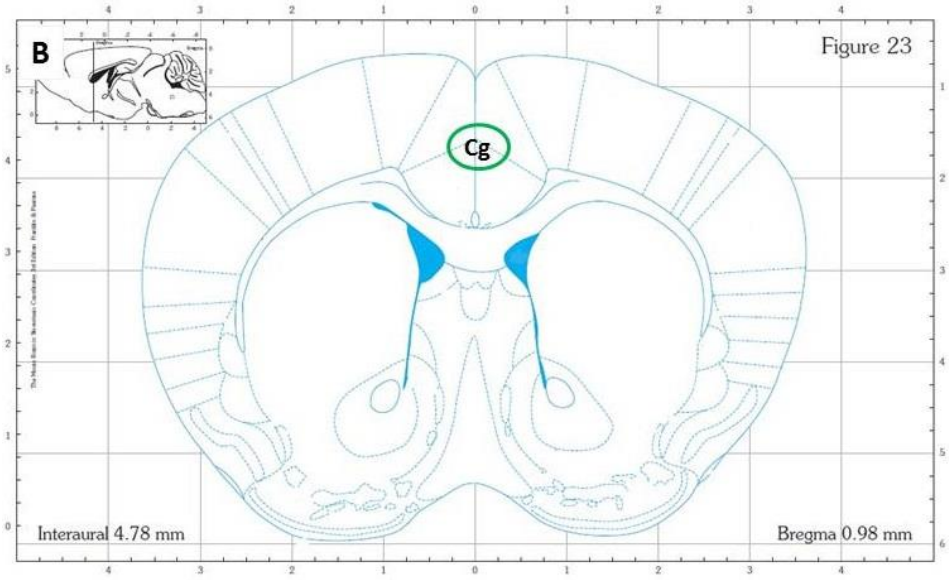
Table 3.2 Names and symbols for the analyzed genes.

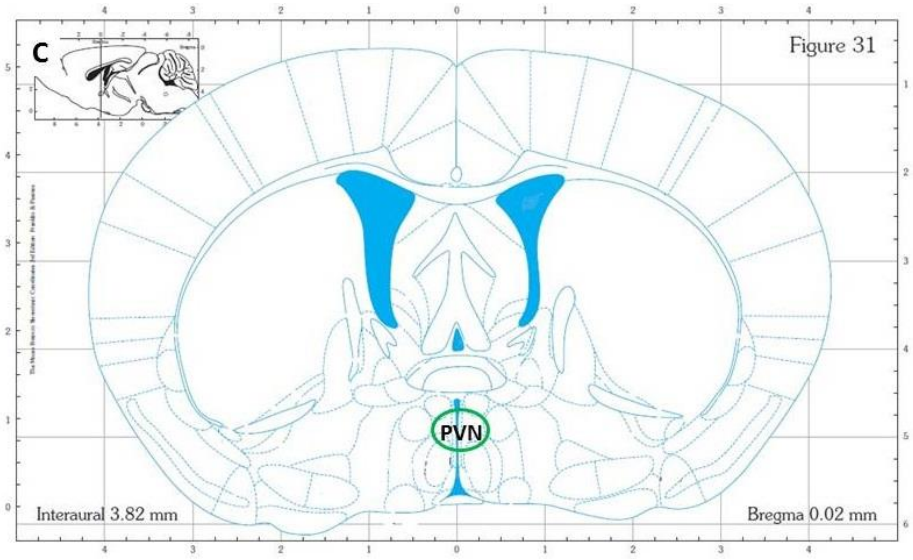
Gene name	Gene symbol
Preproenkephalin	*Penk*
Delta opioid receptor	*Oprd1*
Mμ opioid receptor	*Oprm1*
Preprodynorphin	*Pdyn*
Kappa opioid receptor	*Oprk1*
Glucocorticoid receptor type 2	*Gr*
Glucocorticoid receptor type 1	*Mr*
Corticotrophin releasing hormone	*Crh*
Urocortin 2	*Ucn2*
Urocortin 3	*Ucn3*
Corticotrophin releasing hormone receptor 1	*Crhr1*
Corticotrophin releasing hormone receptor 2	*Crhr2*
Corticotrophin releasing hormone binding protein	*Crhbp*

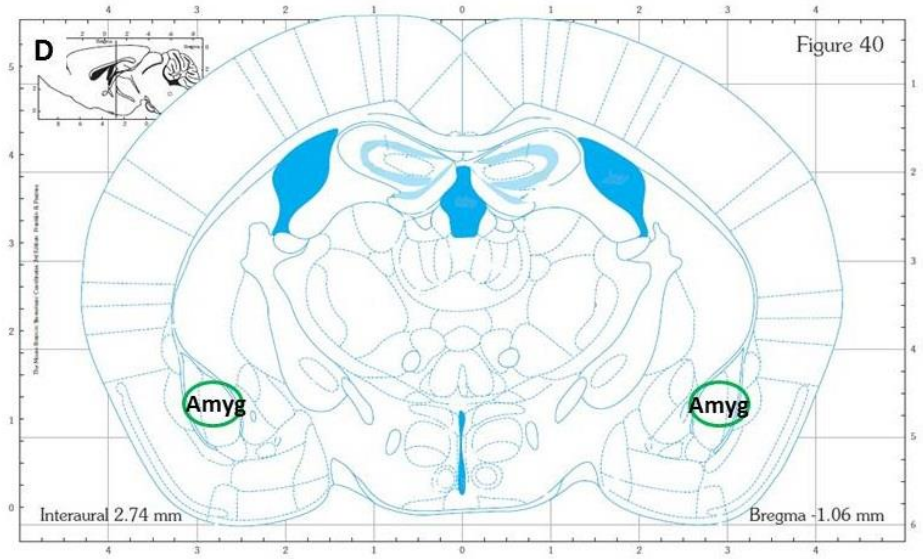
Brain tissue samples

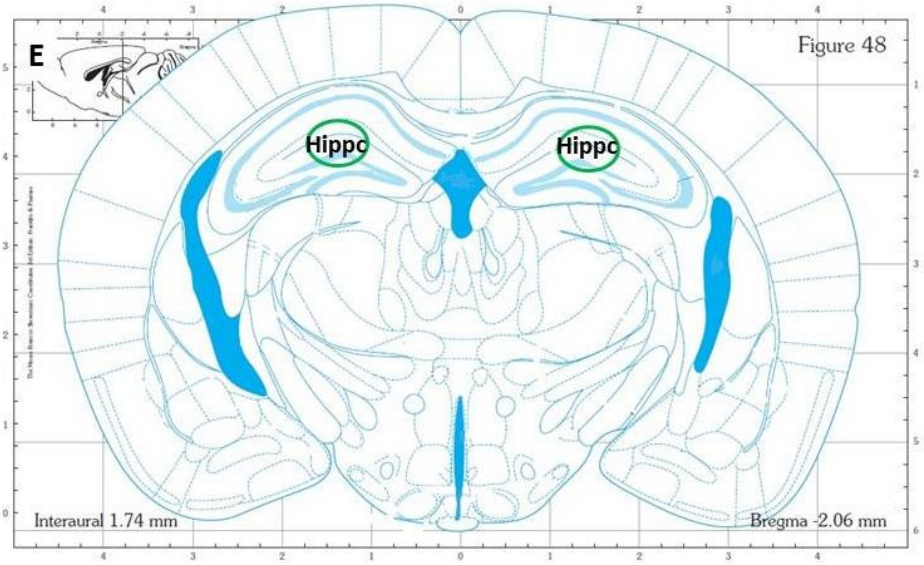
Twenty-four hours after the last behavioral test, animals were sacrificed by cervical dislocation. Brains were removed, snap frozen in ice-cold isopentane, and stored at -80°C. In order to isolate the brain areas of interest, brains were slowly warmed up to -20°C and kept at this temperature. The brains were then cut in 1 mm slices using a metal matrix for mouse brains (Zivic). PFC, PVN, amygdala and hippocampus were isolated by the punching technique using a 12G punching needle, according to the coordinates of the mouse atlas [131]. Eppendorf tubes containing the isolated brain areas were stored and kept at -80°C until further molecular analyses (Figure 3.3).


A
Figure 14
PrL
IL
Interaural 5.78 mm
Bregma 1.98 mm


B
Figure 23
Cg
Interaural 4.78 mm
Bregma 0.98 mm


C
Figure 31
PVN
Interaural 3.82 mm
Bregma 0.02 mm


D
Figure 40
Amyg
Amyg
Interaural 2.74 mm
Bregma -1.06 mm


E
Figure 48
Hippc
Hippc
Interaural 1.74 mm
Bregma -2.06 mm

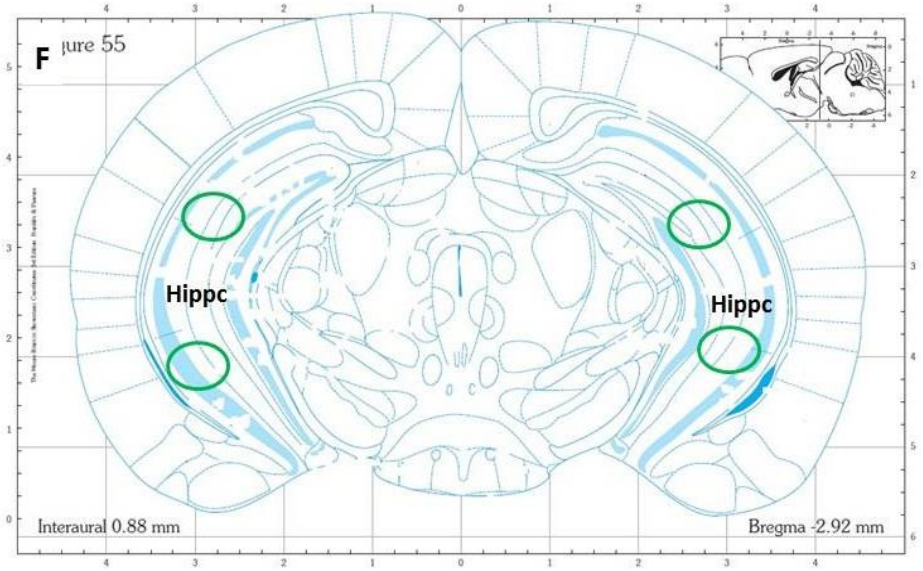

F
ure 55
Hippc
Hippc
Interaural 0.88 mm
Bregma -2.92 mm

Figure 3.3 molecular analyses, brains were cut in 1 mm slices according to the coordinates of the mouse atlas (Paxinos & Franklin 2007). PFC (PrL, IL, Cg) (**A, B**), PVN (**C**), amygdala (amyg)(**D**) and hippocampus (hippc)(**E, F**) were isolated by the punching technique using a 12G needle.

RNA extraction

RNA extraction was performed by adding 1 ml Trizol reagent per hippocampus sample and 800 µl for PVN, PFC and amygdala. The samples were then transferred to magnalyser tubes and homogenized with a Precellys machine (Peqlab). After homogenization, samples were incubated for 5 minutes at RT. Subsequently, 200 µl of chloroform was added and samples were mixed by vortexing, incubated for 3 minutes at RT and centrifuged for 15 minutes at 12000 rpm at 4°C. The upper aqueous phase was then transferred to a new tube and RNA was precipitated by adding 500 µl of isopropanol. Samples were incubated for 10 minutes at RT and centrifuged for 15 to 20 minutes at 12000 rpm at 4°C.

The supernatant was discarded, the pellet washed twice in 1 ml 75% ethanol followed by 10 minutes of 8000 rpm centrifugation at 4°C. Afterwards, the supernatant was discarded and the RNA pellet was dried on a thermoplate for 5 minutes at 55°C. Dried pellets were dissolved in RNA-free water (hippocampus: 15 µl, amygdala and PFC: 6 µl, PVN: 5 µl). Optical densities at 260 nm and 280 nm were measured using a Nanodrop spectrometer. RNA concentration was evaluated by optical density measurements at 260 nm. Purity of RNA was assessed by the ratio between the optical densities of 260 nm and 280 nm (ratio = 260/ 280). RNA samples with ratios less than 1.85 were rejected due to possible contamination with proteins.

Gene expression analysis

cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR Kits (Invitrogen Corp., Carlsbad, CA, USA) with oligodeoxynucleotide T (dT) primers according to the manufacturer's instructions. Total RNA (20 ng for hippocampus and 15 ng for PFC, PVN and amygdala) was used as starting material. mRNA expression of the target genes was determined in triplicates by custom TaqMan® Gene Expression Assays (Applied Biosystems, Darmstadt, Germany). Each TaqMan® assay reaction consisted of 4 µl cDNA, 5 µl TaqMan® universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 0.5 µl Custom TaqMan® Gene Expression Assay (primer) (Applied Biosystems, Darmstadt, Germany) (Table 3.3) and 0.5 µl of RNA-free water. Samples were processed in a 7500 Real-Time PCR Detection System (Applied Biosystems, Darmstadt, Germany) with the following cycling parameters: 95°C for 10 minutes (hot start), 40 cycles at 95°C for 15 seconds (melting) and 60°C for 1 minute (annealing and extension). Analysis was performed using the 7500 Sequence Detection Software version 2.2.2 (Applied Biosystems, Darmstadt, Germany) and data were obtained as a function of threshold cycle (CT). For relative quantification (RQ) of the gene expression, the mean CT values of the triplicates of each target gene and of the housekeeping gene GAPDH were calculated, followed by subtraction of the mean CT values of the GAPDH gene to the mean CT values of each target gene (ΔCT values). Next, the power of all ΔCT values was calculated based on the formula power = 2^-ΔCT [132]. Finally, RQ values, presented as fold change of the WT control group, were obtained by the division of the power value of each sample by the mean power value of the WT control group.

Table 3.2 Names, symbols and primers for the analyzed genes by TaqMan Gene Expression Assays.

Gene name	Gene symbol	Primer used at the TaqMan assay
Glycerinaldehyd-3-phosphat-Dehydrogenase*	*Gapdh*	Mm99999915_g1
Preproenkephalin	*Penk*	Mm 01212875_m1
Delta opioid receptor	*Oprd1*	Mm00443063_m1
Mμ opioid receptor	*Oprm1*	Mm01188089_m1
Preprodynorphin	*Pdyn*	Mm 00457573_m1
Kappa opioid receptor	*Oprk1*	Mm01230885_m1
Glucocorticoid receptor type 2	*Gr*	Mm01241596_m1
Glucocorticoid receptor type 1	*Mr*	Mm01188089_m1
Corticotrophin releasing hormone	*Crh*	Mm01293920_s1
Urocortin 2	*Ucn2*	Mm01227928_s1
Urocortin 3	*Ucn3*	Mm00453206_s1
Corticotrophin releasing hormone receptor 1	*Crhr1*	Mm00432670_m1
Corticotrophin releasing hormone receptor 2	*Crhr2*	Mm00438303_m1
Corticotrophin releasing hormone binding protein	*Crhbp*	Mm01283832_m1

* Housekeeping gene

3.7 Statistical analyses

Hormonal and behavioral data from the two-cohorts was first analyzed by multifactorial ANOVA taking in account cohort number as an additional between-subjects factor. Interaction effects between cohort number and CMS or genotype were not detected. Therefore, data from the two cohorts were pooled. Accordingly, corticosterone measurements and the behavioral data, except from the sucrose preference test, were analyzed by two-way ANOVA, with CMS and genotype as between-subjects factors. Post-hoc analyses were performed using the Bonferroni test. Sucrose preference test data were analyzed with three-way ANOVA with repeated measures, with CMS and genotype as between-subjects factors and week as within-subject factor. Post-hoc analyses for the sucrose preference test data were performed with the Fisher LSD test.

Gene expression data, except for the *Penk* gene, were analyzed by two-way ANOVA, with CMS and genotype as between-subjects factors. Post-hoc analyses were performed using the Bonferroni test. *Penk* gene mRNA expression in WT mice were analyzed with the Mann-Whitney U test. Statistical significance was set at $p < 0.05$.

Chapter 4: Results

4.1 Cohort effect

In order to evaluate if behavior and hormonal data from the two cohorts of animals could be analyzed together, i.e. pooled, we first performed statistical analyses including cohort as between-subjects factor. Open field, O-maze, light dark box and corticosterone levels data were analyzed by three-way ANOVA with CMS, genotype and cohort number as between-subjects factors. Sucrose preference test data were analyzed by four-way ANOVA with repeated measures, with CMS, genotype and cohort number as between-subjects factors and week as within-subject factor. For the forced swim test, only data from the first cohort of animals is available in the present study. Data from the second cohort of animals was not possible to evaluate due to problems with the video recordings. Interaction effects between cohort number and CMS or genotype were not detected (tables 4.1-4.5). Therefore, data from the two cohorts were pooled.

Table 4.1 Statistical analyses of the open field test by three- way ANOVA.

Open field	**F (1, 74)**	**P**
Genotype	0,1236	0,726199
CMS	6,3927	0,013591
Cohort	1,4824	0,227272
genotype*CMS	0,9239	0,339592
genotype*cohort	1,4959	0,225188
CMS*cohort	2,3457	0,129893
genotype*CMS*cohort	0,0253	0,874135

T**able 4.2** Statistical analyses of the O-maze test by three- way ANOVA.

O-maze test	**F (1, 73)**	**p**
Genotype	0,3652	0,547532
CMS	9,7477	0,002573
Cohort	5,1757	0,025843
genotype*CMS	2,0503	0,156448
genotype*cohort	0,3668	0,546613
CMS*cohort	1,7568	0,189152
genotype*CMS*cohort	0,0038	0,950902

Table 4.3 Statistical analyses of the light-dark box test by three- way ANOVA.

Light-Dark box test	**F (1, 73)**	**P**
Genotype	0,1082	0,743136
CMS	1,7316	0,192320
Cohort	1,3607	0,247205
genotype*CMS	0,0056	0,940558
genotype*cohort	0,4889	0,486655
CMS*cohort	1,5328	0,219660
genotype*CMS*cohort	0,8984	0,346326

Table 4.4 Statistical analyses of the sucrose preference test by four- way ANOVA.

Sucrose preference test	**F (1, 68)**	**P**
Genotype	0,324	0,571328
CMS	7,030	0,009966
Cohort	0,197	0,658595
genotype*CMS	3,043	0,085616
genotype*cohort	1,173	0,282693
CMS*cohort	0,698	0,406441
genotype*CMS*cohort	0,000	0,988827
	F (5, 340)	**P**
WEEK	1,859	0,100910
WEEK*genotype	0,495	0,779775
WEEK*CMS	2,918	0,013541
WEEK*Cohort	1,674	0,140107
WEEK*genotype*CMS	0,658	0,655540
WEEK*genotype*Cohort	0,109	0,990278
WEEK*CMS*Cohort	1,459	0,202779
WEEK*genotype*CMS*Cohort	0,258	0,935831

Table 4.5 Statistical analyses of the corticosterone measurements by three- way ANOVA.

Corticosterone	F (1, 62)	P
Genotype	**0,8774**	**0,352553**
CMS	**2,2832**	**0,135858**
Cohort	**7,7765**	**0,007020**
genotype*CMS	**21,4288**	**0,000019**
genotype*cohort	**0,2923**	**0,590701**
CMS*cohort	**0,2562**	**0,614560**
genotype*CMS*cohort	**0,1794**	**0,673367**

4.2 CMS effects on the behavior of WT and *Penk* KO mice

WT and *Penk* KO mice were tested in a battery of behavioral tests to evaluate the effects of the CMS protocol on locomotor activity, anxiety and depression-like behaviors.

CMS induces an overall hyperactivity in both WT and *Penk* KO mice.

General exploratory and locomotor activity was evaluated as total distance travelled in the open field test (n = 18-23/ group) (Figure 4.1). CMS induced an overall increase in the locomotor activity in both WT and *Penk* KO mice, revealed by a CMS main effect ($F_{(1, 78)} = 5.110$, $p < 0.05$), but no significant results in the Bonferroni post-hoc test. There was neither a significant genotype effect ($F_{(1, 78)} = 0.03712$, n.s.) nor a significant CMS*genotype interaction ($F_{(1, 78)} = 0.6320$, n.s.)

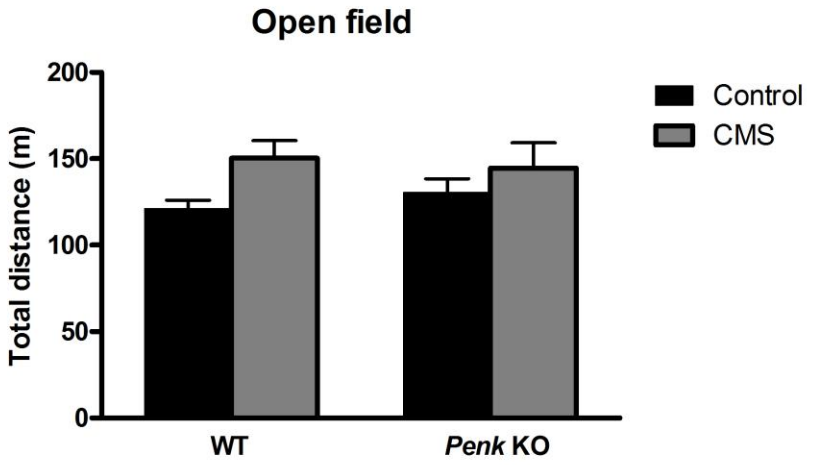


Figure 4.1 General exploratory and locomotor activity were evaluated by the total distance travelled (m) in the open field test. CMS induced an overall hyperactivity in WT and *Penk* KO mice, revealed by a significant CMS main effect. Data expressed as mean ± S.E.M.

CMS increases anxiety levels in WT but not in *Penk* KO mice.

Anxiety levels were assessed in the O-maze and light-dark box tests (n = 18-22/ group). In the 0-maze test, time in the open areas was inversely correlated to anxiety levels. Results from the O-maze test, revealed that CMS induced an increase in the anxiety levels (CMS effect: $F_{(1, 77)} = 8.008$, $p < 0.01$) in WT but not in *Penk* KO mice (Bonferroni post-hoc test: WT control vs. WT CMS: $p < 0.01$; *Penk* KO control vs. *Penk* KO CMS: p n.s.) (Figure 4.2 A). No significant results for a genotype effect ($F_{(1, 77)} = 0.1147$, n.s.) or for a CMS*genotype interaction ($F_{(1, 77)} = 1.817$, n.s.) were observed. In the light-dark test, time in the lit area was also inversely correlated to anxiety levels. No significant results were observed for CMS ($F_{(1, 77)} = 0.9640$, n.s.), genotype ($F_{(1, 77)} = 0.3533$, n.s.) or for a CMS*genotype ($F_{(1, 77)} = 0.1477$, n.s.) interaction in this test (Figure 4.2 B).

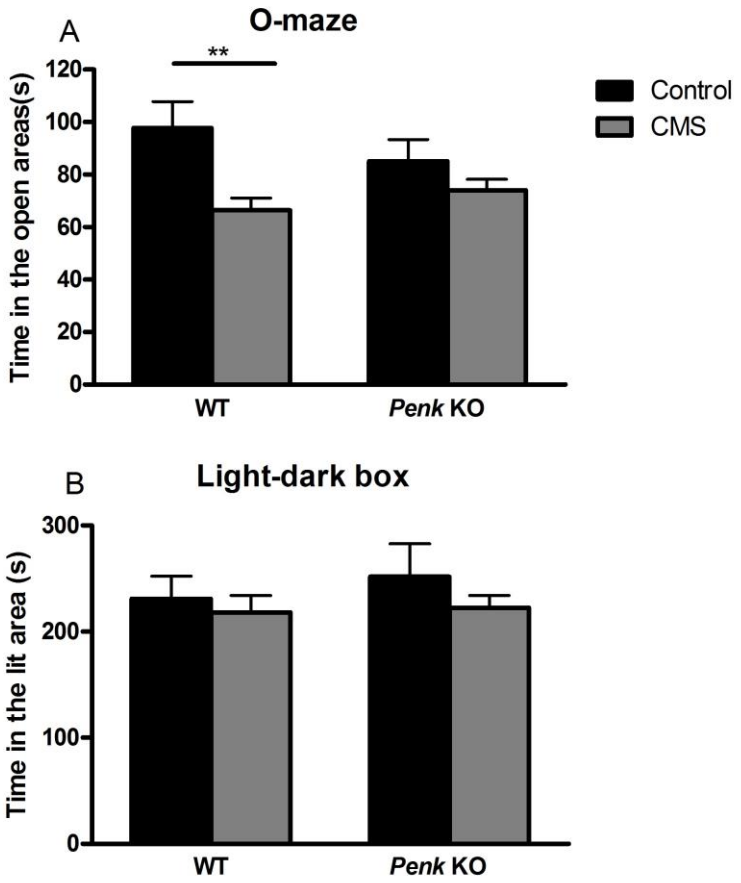


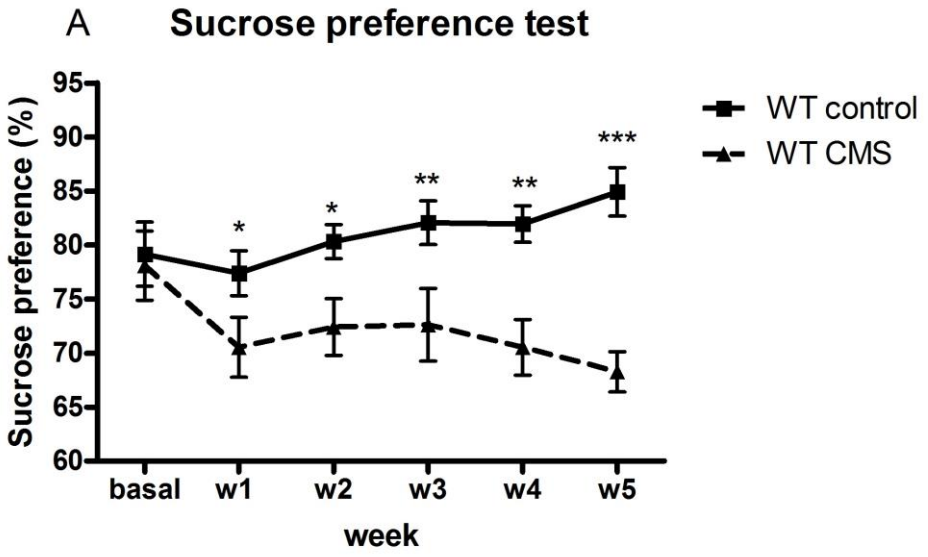
Figure 4.2 Anxiety levels were assessed in the 0-maze and light-dark box tests. In the 0-maze (**A**), analysis revealed a significant main CMS effect in WT mice, shown by a decrease in the time that CMS animals spent in the open parts of the maze. In the light-dark box test (**B**), there were no differences in the anxiety levels between the experimental groups. Data expressed as mean ± S.E.M. **p < 0.01, using two-way ANOVA followed by Bonferroni test.

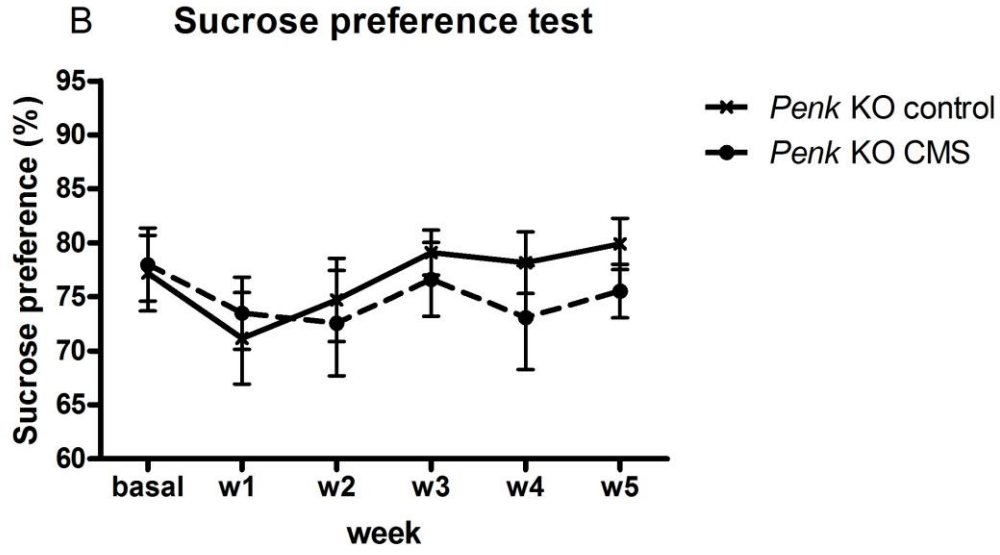
CMS induces anhedonia in the sucrose preference test and increases despair levels in the forced swim test in WT, but not in *Penk* KO mice.

Anhedonia and despair levels were evaluated as measures of depression-like behaviors. Anhedonic levels were assessed in the sucrose preference test (n = 17-22/ group), where a state of anhedonia is reflected through a decreased preference for the sucrose solution. Repeated measures three-way ANOVA for WT and *Penk* KO mice revealed that CMS induced anhedonia (CMS effect ($F_{(1, 72)} = 6.704$, $p < 0.05$) in WT but not in *Penk* KO mice (Fisher's LSD post-hoc test: WT control vs. WT CMS: $p < 0.05$; *Penk* KO control vs. Penk KO CMS: p n.s.)

(Figures 4.3 A and B). In addition, a significant week*CMS interaction was observed ($F_{(5, 360)} = 2.660$, $p < 0.05$), indicative that CMS effects in the sucrose preference were different along the several weeks of CMS submission. Fisher's LDS test showed a significant CMS*week effect in WT animals between week 1 and week 5 (Figures 4.3). No significant genotype ($F_{(1, 72)} = 0.128$, n.s.), CMS*genotype ($F_{(1, 72)} = 2.915$, n.s.), week*genotype ($F_{(5, 360)} = 0.366$, n.s.) or week*genotype*CMS ($F_{(5, 360)} = 0.563$, n.s.) effects were observed.

Despair behavior was measured in the forced swim test (n = 8/ group). Despair behavior is correlated with an increase in immobility time. No significant results for genotype ($F_{(1, 28)} = 0.01431$, n.s.) or CMS*genotype interaction ($F_{(1, 28)} = 3.709$, n.s.) effects were observed. However, a significant CMS effect ($F_{(1, 28)} = 5.497$, $p < 0.05$) was observed. Bonferroni post-hoc test revealed a significant CMS-induced increase in the immobility time in WT (WT control vs. WT CMS: $p < 0.05$), but not in *Penk* KO mice (*Penk* KO control vs. *Penk* KO CMS: p n.s.) (Figure 4.3 C).


A
Sucrose preference test
Sucrose preference (%)
95
90
85
80
75
70
65
60
basal
w1
w2
w3
w4
w5
week
*
*
**
**
***
WT control
WT CMS


B
Sucrose preference test
Sucrose preference (%)
95
90
85
80
75
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basal
w1
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week
Penk KO control
Penk KO CMS

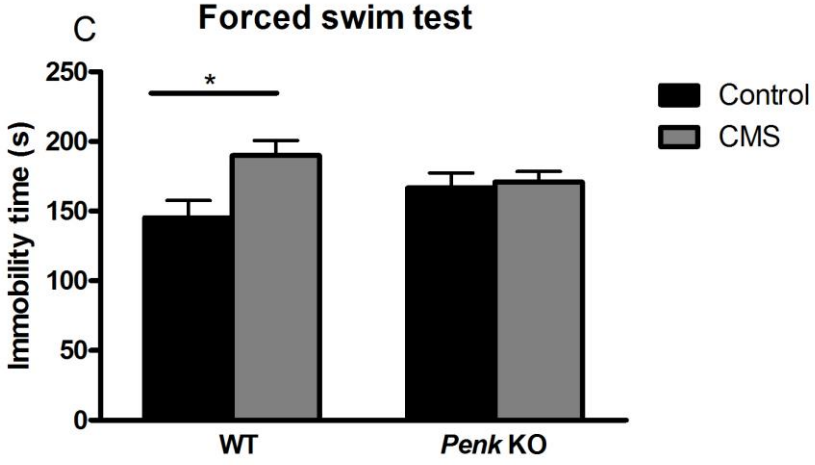

C
Forced swim test
Immobility time (s)
250
200
150
100
50
0
*
WT
Penk KO
Control
CMS

Figure 4.3 Anhedonia was measured in the sucrose preference test for WT (A) and for *Penk* KO mice (B). Five weeks of CMS led to a decreased preference for the sucrose solution (anhedonia) in WT but not in *Penk* KO mice. Data expressed as mean ± S.E.M. * p <0.05; ** p < 0.01, *** p < 0.001, using three-way ANOVA with repeated measurement followed by Fisher´s LSD test. Despair behavior was measured in the forced swim test (C). CMS led to an increase in the immobility time (despair) in WT but not in *Penk* KO mice. Data expressed as mean ± S.E.M. * p <0.05, using two-way ANOVA followed by Bonferroni test.

4.3 CMS effects in the baseline corticosterone levels of WT and *Penk* KO mice

In order to evaluate the impact of the CMS protocol on the basal activity of the HPA axis, baseline corticosterone levels from WT and *Penk* KO mice were analyzed in feces collected after the termination of the 5 weeks of the CMS protocol.

CMS induces an increase in the baseline corticosterone levels in WT but not in *Penk* KO mice.

Corticosterone levels in feces, produced within 24 hours, were measured at the end of the CMS protocol in WT and *Penk* KO mice (n = 17-18/ group) (Figure 4.5). We found neither CMS ($F_{(1, 66)} = 2.15$, n.s.) nor genotype ($F_{(1, 66)} = 0.98$, n.s.) effects, but a significant CMS*genotype interaction ($F_{(1, 66)} = 20.47$, $p < 0.0001$). Post-hoc analyses showed that CMS significantly increased the corticosterone levels in WT animals (WT control vs. WT CMS: $p < 0.001$), but not in *Penk* KO mice (*Penk* KO control vs. *Penk* KO CMS: p n.s.). Moreover, post-hoc analyses also revealed that WT control animals present lower corticosterone levels compared with *Penk* KO control animals ($p < 0.05$), and that, in opposition, WT CMS animals present higher corticosterone levels than *Penk* KO CMS ($p < 0.001$).

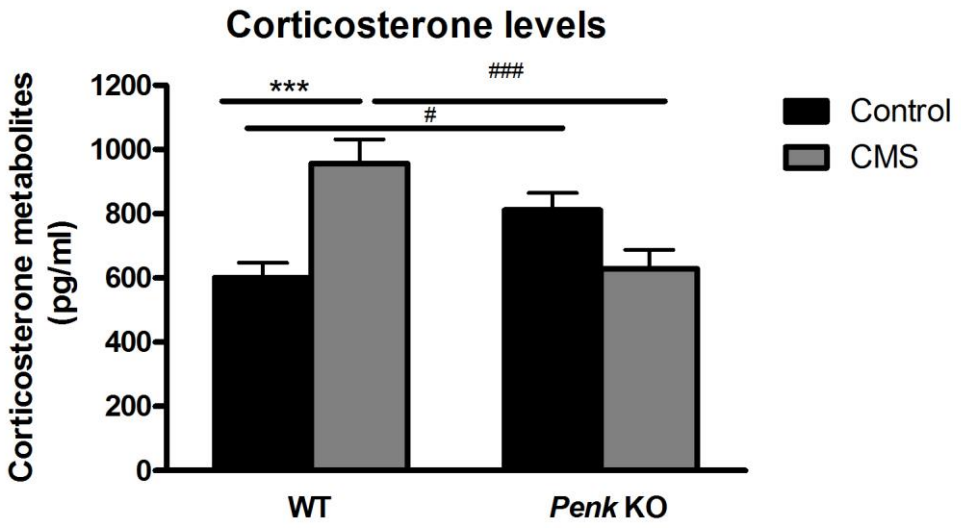


Figure 4.5 Measurement of corticosterone levels in feces, produced within 24 hours, after 5 weeks of CMS. CMS led to a significant increase in the corticosterone levels in WT mice, but not in *Penk* KO mice. Corticosterone levels in the control groups were higher in *Penk* KO compared to WT animals, while in the CMS groups, corticosterone levels were higher in WT than in *Penk* KO mice. Data are expressed as mean ± S.E.M. $^{\#}$ $p < 0.05$; $^{\#\#\#}$ and *** $p < 0.001$. * comparison between control and CMS groups. $^{\#}$ comparison between WT and PENK KO groups. Two-way ANOVA followed by Bonferroni test.

4.4 Gene expression analyses

To evaluate the effects of the CMS protocol on the gene expression of genes with a putative important role in CMS-induced stress reactivity, mRNA levels of several genes were evaluated in the PFC, PVN, amygdala and hippocampus of WT and *Penk* KO mice.

4.4.1 Gene expression of the opioid system

CMS increases *Penk* gene expression in the PVN of WT mice.

CMS induced an increase in the *Penk* mRNA levels in the PVN (n = 5-6/ group) (U = 3.000; p< 0.05), without affecting the *Penk* mRNA levels in the PFC (n = 8/ group) (U = 18.00, n.s.), amygdala (n = 5-7/ group) (U = 12.00, n.s.) and hippocampus (n = 7/ group) (U = 13.00, n.s.) (Figure 4.6).

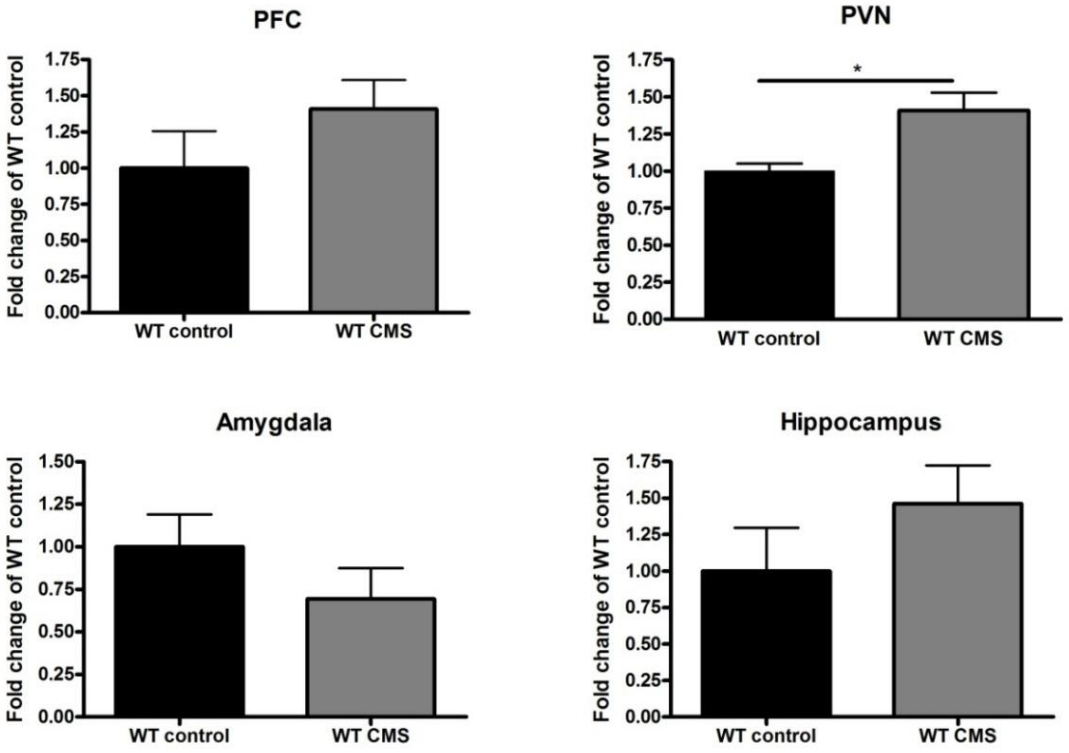


Figure 4.6 *Penk* gene mRNA expression was measured in the PFC, PVN, amygdala and hippocampus of WT control and WT CMS mice. CMS led to a significant increase of mRNA expression in the PVN. No significant changes were observed in the other brain regions analyzed. Data are expressed as fold change relative to the WT control group. * $p < 0.05$, using Mann-Whitney U test.

Penk KO mice present lower *Oprd1* gene expression in the hippocampus than WT animals.

mRNA expression of the *Oprd1* gene was measured in the PFC (n = 8/group) , amygdala (n = 4-6/group), and hippocampus (n = 6-7/group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.7). *Oprd1* mRNA is not present in the PVN [49], and therefore not was measured in this brain area. In the PFC, no significant results were observed for CMS ($F_{(1, 28)} = 0.2688$, n.s.), genotype ($F_{(1, 28)} = 0.7598$, n.s.), or CMS*genotype ($F_{(1, 28)} = 0.05575$, n.s.)

effects. In the amygdala, CMS ($F_{(1, 18)} = 0.2954$, n.s.), genotype ($F_{(1, 18)} = 3.723$, n.s.) and CMS*genotype ($F_{(1, 18)} = 0.09430$, n.s.) effects were not significant either. In the hippocampus, *Penk* KO mice presented an overall lower *Oprd1* mRNA expression compared with WT mice, revealed by a main genotype effect ($F_{(1, 21)} = 0.09430$, $p < 0.05$), but no significant results in the Bonferroni post hoc test. No significant effects for CMS ($F_{(1, 21)} = 2.109$, n.s.) or CMS*genotype ($F_{(1, 21)} = 0.1441$, n.s.) were observed in the hippocampus.

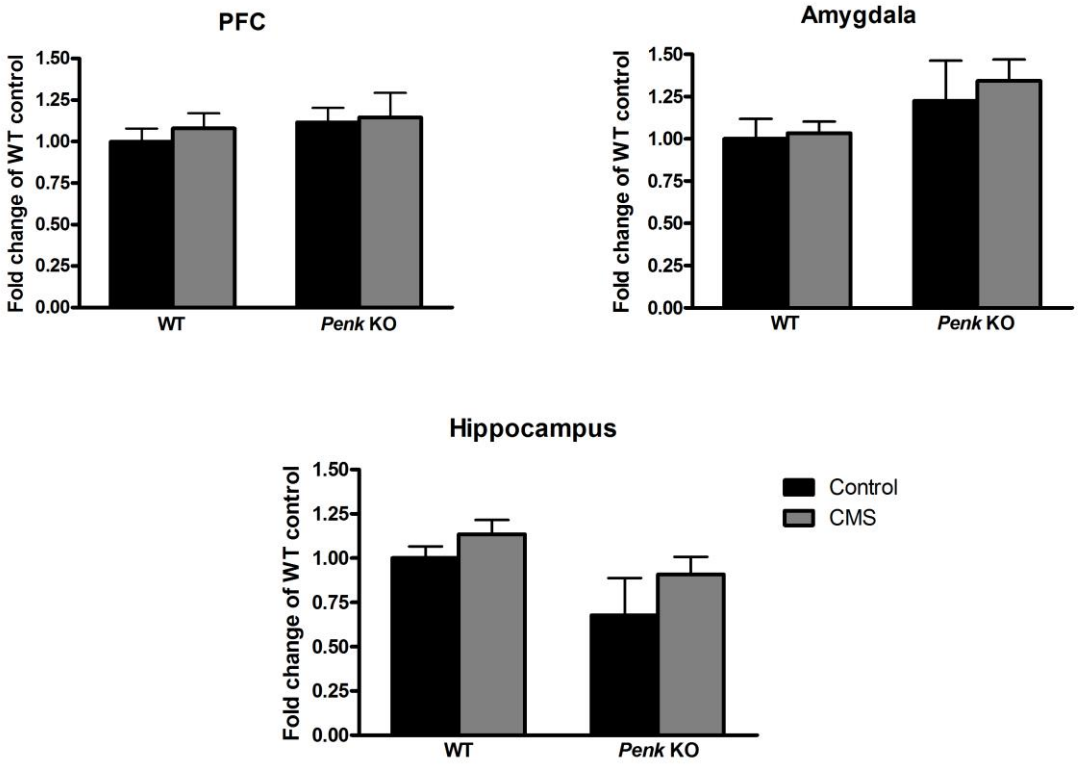


Figure 4.7 *Oprd1* mRNA expression was measured in the PFC, amygdala and hippocampus of WT and *Penk* KO mice from control and CMS groups. Statistical analyses showed a significant main genotype effect in the hippocampus, due to an overall lower gene expression in *Penk* KO mice compared with WT animals. No significant results were observed in the other brain areas analyzed. Data are expressed as fold change relative to the WT control group.

CMS and *Penk* gene deletion do not influence *Oprm1* gene expression.

Oprm1 mRNA expression was measured in the PFC (n = 8/ group), PVN (n = 6/ group), amygdala (n = 5-7/ group) and hippocampus (n = 7/ group) of WT and

Penk KO mice from control and CMS groups (Figure 4.8). No significant results were observed in the PFC (CMS: $F_{(1, 28)} = 0.4536$, n.s.; genotype: $F_{(1, 28)} = 0.2622$, n.s.; CMS*genotype interaction: $F_{(1, 28)} = 0.2283$, n.s.), PVN (CMS: $F_{(1, 19)} = 3.251$, n.s.; genotype: $F_{(1, 19)} = 1.084$, n.s.; CMS*genotype interaction: $F_{(1, 19)} = 0.3875$, n.s.), amygdala (CMS : $F_{(1, 19)} = 0.1012$, n.s.; genotype $F_{(1, 19)} = 2.221$, n.s.; CMS*genotype interaction: $F_{(1, 19)} = 0.5662$, n.s.) or in the hippocampus (CMS: $F_{(1, 24)} = 0.7720$, n.s.; genotype: $F_{(1, 24)} = 3.057$, n.s.; CMS*genotype interaction: $F_{(1, 24)} = 2.362$, n.s.).

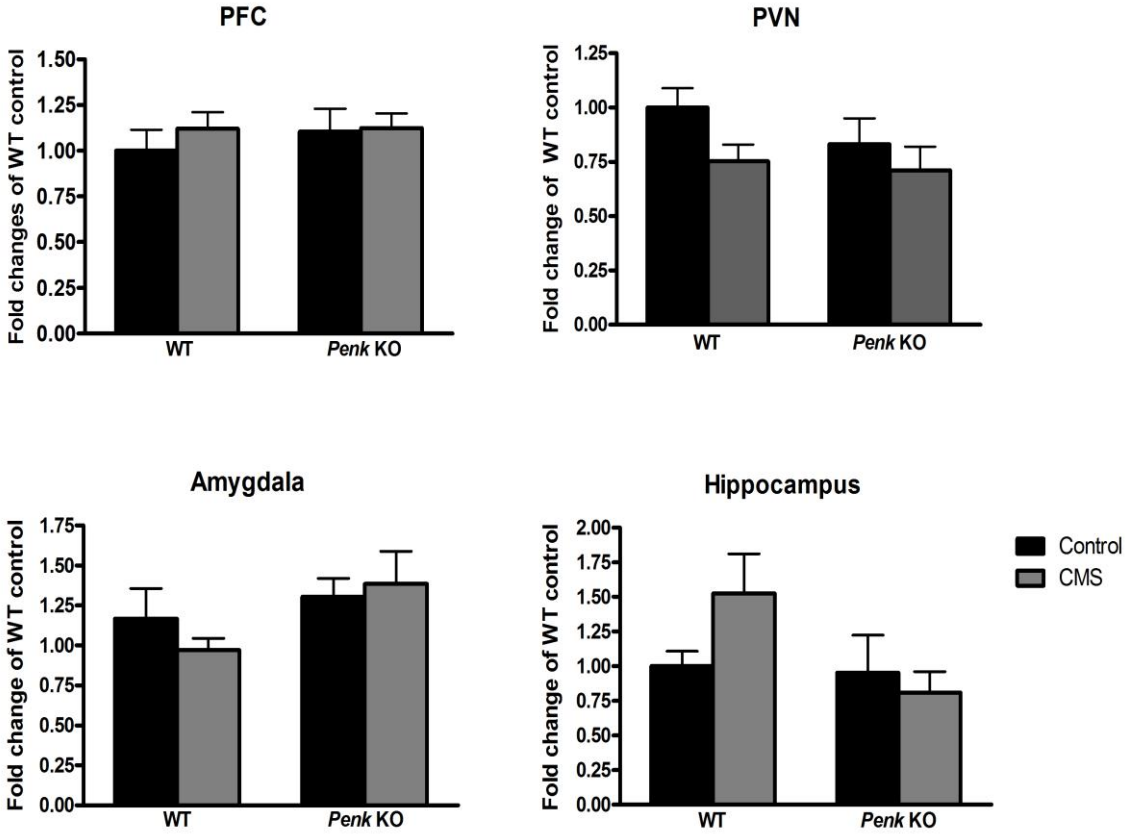


Figure 4.8 *Oprm1* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus of WT and *Penk* KO mice from control and CMS groups. Statistical analyses showed no significant results in any of the brain areas. Data are expressed as fold change relative to the WT control group.

Penk KO mice present opposite alterations in the _Pdyn_ gene expression in the PVN and amygdala compared with WT mice.

Pdyn mRNA expression analyses in the PFC (n= 8/ group), revealed no significant results for CMS ($F_{(1, 27)} = 1.925$, n.s.), genotype ($F_{(1, 27)} = 2.182$, n.s.) or CMS*genotype ($F_{(1, 27)} = 1.925$, n.s.). In the PVN (n= 6/ group) lower Pdyn mRNA levels in *Penk* KO CMS compared to WT CMS animals were revealed by a significant genotype effect ($F_{(1, 19)} = 7.810$, $p < 0.05$) and significant results in the Bonferroni post-hoc test (WT CMS vs. *Penk* KO CMS: $p < 0.05$). Neither the CMS effect ($F_{(1, 19)} = 1.438$, n.s.) nor the CMS*genotype interaction ($F_{(1, 19)} = 1.353$, n.s.) were significant. In the amygdala (n= 5-7/ group) was observed a significant main genotype effect ($F_{(1, 18)} = 6.031$, $p< 0.05$), but the Bonferroni post-hoc test was not significant. Thus, suggesting an overall increase in the Pdyn mRNA levels in the amygdala of *Penk* KO mice compared with WT animals. No significant results were observed for a CMS effect ($F_{(1, 18)} = 0.4434$, n.s.) or CMS*genotype interaction ($F_{(1, 18)} = .3578$, n.s.). In the hippocampus (n= 7/ group) no significant results were observed for a CMS ($F_{(1, 24)} = 0.1542$, n.s.), genotype ($F_{(1, 24)} = 1.059$, n.s.), or CMS*genotype interaction ($F_{(1, 24)} = 0.4674$, n.s.) effects (Figure 4.9).

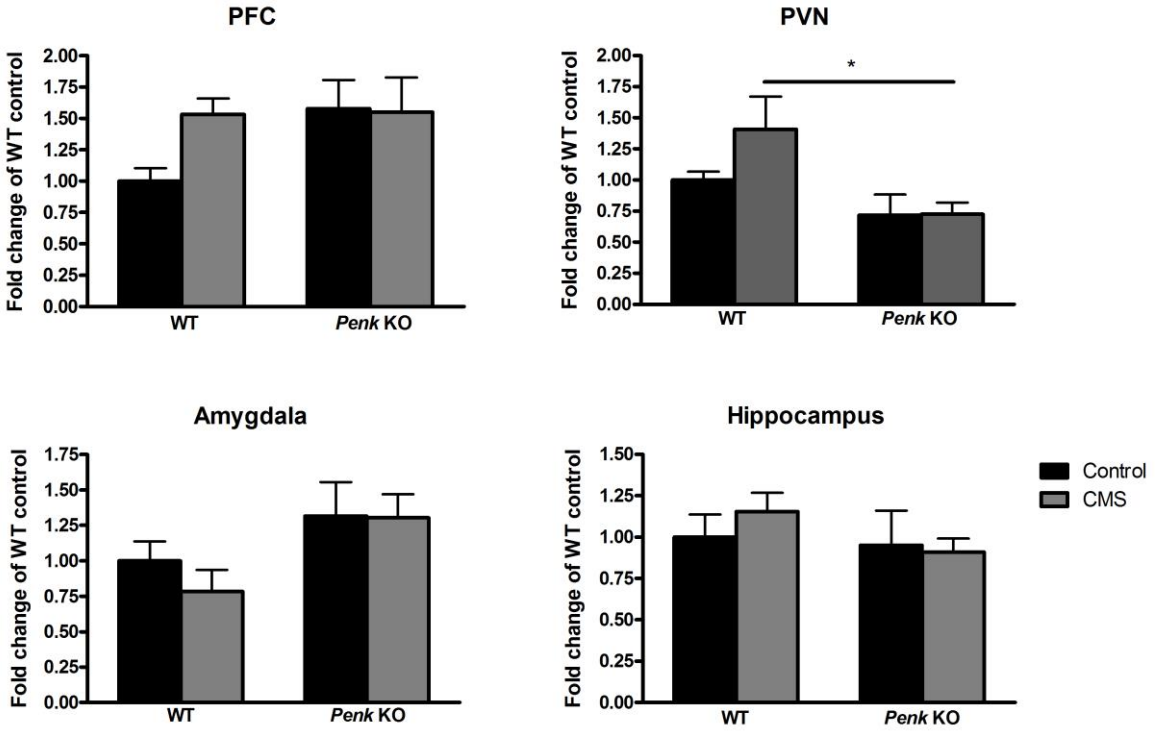


Figure 4.9 *Pdyn* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus of WT and *Penk* KO mice from control and CMS groups. Statistical analyses revealed significant genotype effects in the PVN and in the amygdala. In the PVN, significant main genotype effect and Bonferroni results, revealed lower gene expression in *Penk* KO CMS mice compared with WT CMS mice. In the amygdala, significant main genotype effect but not significant results in the post-hoc test show an overall higher gene expression in the *Penk* KO mice compared with WT animals. Data are expressed as fold change relative to the WT control group. * $p<0.05$, using two-way ANOVA followed by Bonferroni test.

CMS increases *Oprk1* gene expression in the PVN in *Penk* KO, but not in WT mice

Oprk1 mRNA expression was measured in the PFC (n = 7-8/ group), PVN (n = 5-6/ group), amygdala (n = 4-6/ group) and hippocampus (n = 6-7/ group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.10). In the PFC, no significant results for CMS ($F_{(1, 27)} = 1.112$, n.s.), genotype ($F_{(1, 27)} = 0.03308$, n.s.), or for CMS*genotype ($F_{(1, 27)} = 0.2725$, n.s.) effects were observed. In the

PVN, a significant CMS effect was observed ($F_{(1, 19)} = 9.368$, $p < 0.01$), which was due to an up-regulation in the *Oprk1* gene expression in *Penk* KO CMS animals (WT control vs. WT CMS: p n.s.; *Penk* KO control vs. *Penk* KO CMS: $p < 0.05$). No significant results were observed for genotype ($F_{(1, 19)} = 0.6654$, n.s.) or CMS*genotype ($F_{(1, 19)} = 0.5016$, n.s.) effects. No significant results were observed in the amygdala (CMS: $F_{(1, 18)} = 3.129$, n.s.; genotype: $F_{(1, 18)} = 3.559$, n.s.; CMS*genotype interaction: $F_{(1, 18)} = 0.1106$, n.s) or hippocampus (CMS: $F_{(1, 20)} = 2.807$, n.s.; genotype: $F_{(1, 20)} = 1.298$, n.s.; CMS*genotype interaction: $F_{(1, 20)} = 1.582$, n.s.).

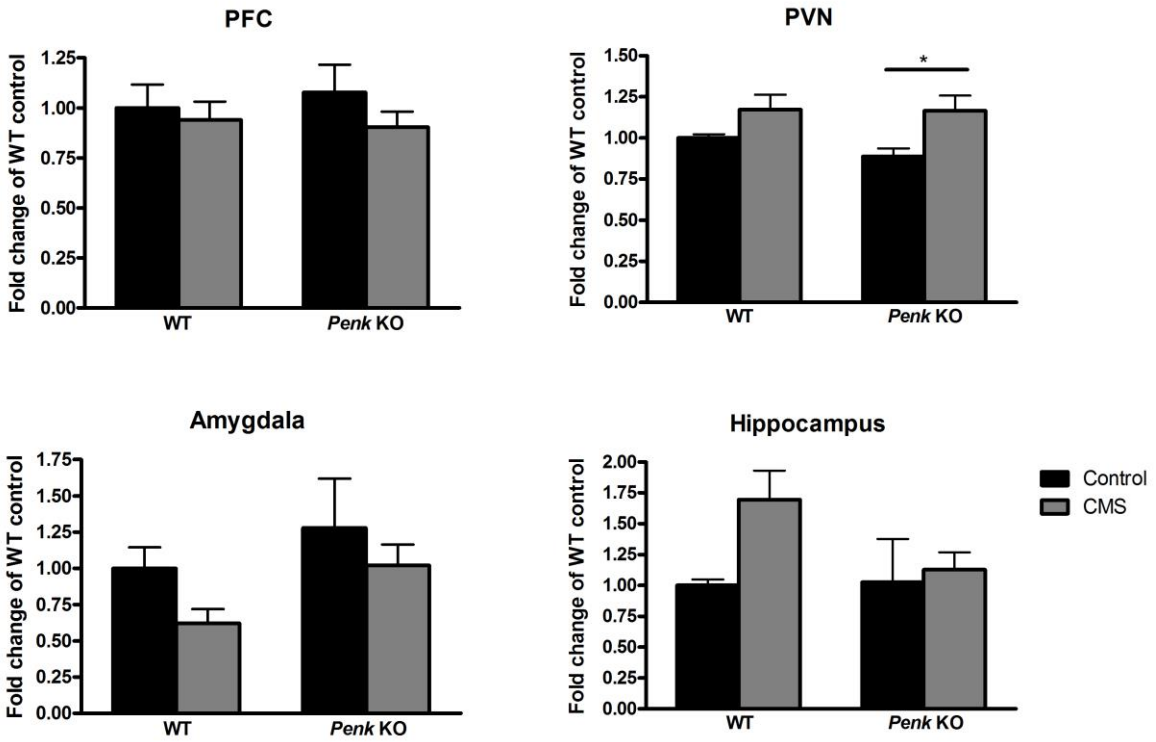


Figure 4.10 ***Oprk1*** mRNA expression was measured in the PFC, PVN, amygdala and hippocampus of WT and *Penk* KO mice from control and CMS groups. Statistical analyses revealed a significant CMS effect in the PVN, which was due to an up-regulation in the *Kor* gene expression. A Bonferroni post-hoc test showed significant results for *Penk* KO, but not for WT mice. Data are expressed as fold changes relative to the WT control group. * $p<0.05$, using two-way ANOVA followed by Bonferroni test.

4.4.2 Gene expression of glucocorticoid receptors

CMS decreases *Gr* gene expression in the PVN in WT, but not in *Penk* KO mice.

Gr mRNA expression was measured in the PFC (n = 6-7/ group), PVN (n = 5-7/ group), amygdala (n = 7/ group) and hippocampus (n = 7/ group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.11). In the PVN, a significant CMS effect was observed ($F_{(1, 21)} = 6.324$, $p < 0.05$), and post-hoc analysis showed a significant reduction of *Gr* mRNA levels in WT mice after CMS (WT control vs. WT CMS, $p< 0.05$) but no changes in *Penk* KO mice. No significant genotype effect ($F_{(1, 21)} = 1.719$, n.s.) or CMS*genotype interaction ($F_{(1, 21)} = 3.960$, n.s) were observed. In the PFC, amygdala and hippocampus, no significant CMS ($F_{(1, 21)} = 0.1448$, n.s.; $F_{(1, 24)} = 0.4408$, n.s.; $F_{(1, 24)} = 1.095$, n.s.), genotype ($F_{(1, 21)} = 0.3547$, n.s., $F_{(1, 24)} = 2.478$, n.s.; $F_{(1, 24)} = 1.841$, n.s.) or CMS*genotype ($F_{(1, 21)} = 0.03623$, n.s.; $F_{(1, 24)} = 0.1912$, n.s.; $F_{(1, 24)} = 0.007452$, n.s.) effects were observed.

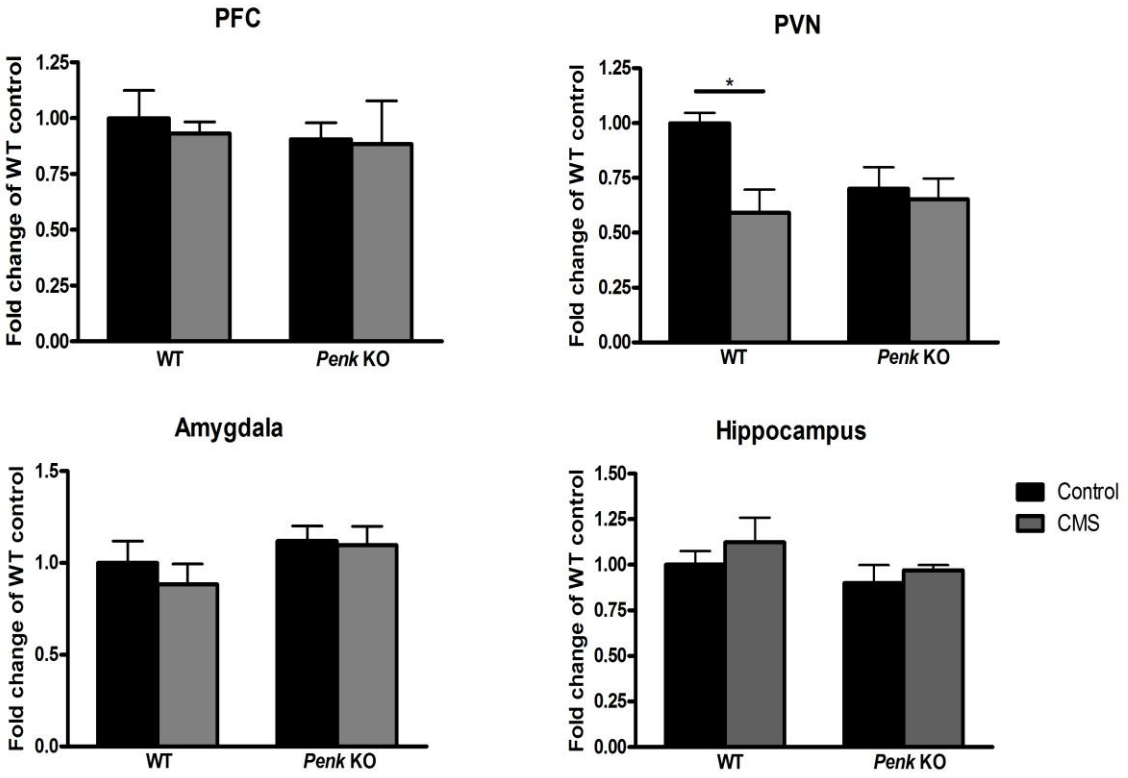


Figure 4.11 *Gr* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus. Statistical analyses showed a significant decrease in the mRNA levels in the PVN of WT mice after CMS. No other significant results were observed in any of the analyzed brain areas. Data are presented as fold change relative to the WT control group. * $p < 0.05$. Two-way ANOVA followed by Bonferroni test.

CMS and *Penk* gene deletion did not influence *Mr* gene expression.

mRNA expression of the *Mr* gene was measured in the PFC (n= 6-7/ group), amygdala (n= 7/ group) and hippocampus (n= 7/ group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.12). MR mRNA is not present in the PVN [130] and therefore not was measured in this brain area. No significant results were observed in the PFC (CMS effect: $F_{(1, 22)} = 0.3723$, n.s.; genotype effect: $F_{(1, 22)} = 0.09656$, n.s.; CMS*genotype interaction: $F_{(1, 22)} = 1.178$, n.s.), amygdala (CMS effect; $F_{(1, 24)} = 01922$, n.s.; genotype effect: $F_{(1, 24)} = 0.1075$, n.s.; CMS*genotype interaction: $F_{(1, 24)} = 0.8023$, n.s.); or hippocampus (CMS

effect: $F_{(1, 22)} = 1.210$, n.s.; genotype effect: $F_{(1, 22)} = 4.228$, n.s.; CMS*genotype interaction: $F_{(1, 22)} = 1.744$, n.s.).

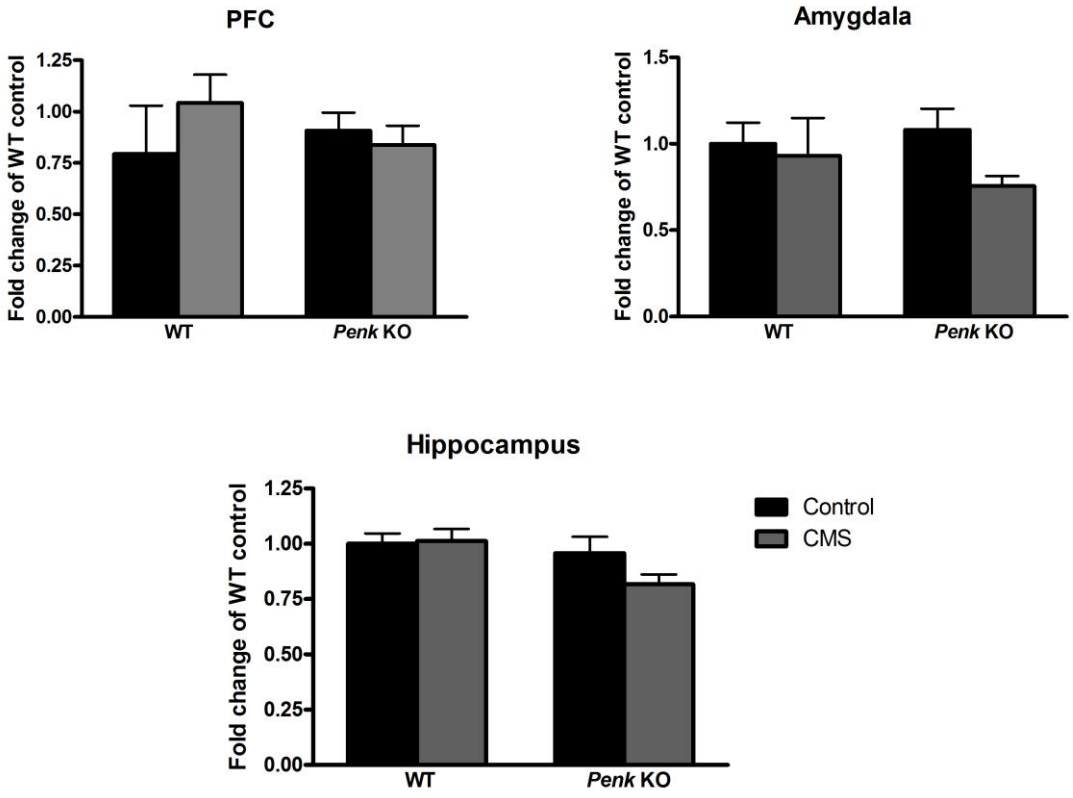


Figure 4.12 *Mr* mRNA expression was measured in the PFC, amygdala and hippocampus. Statistical analyses showed no significant results. Data are presented as fold change relative to the WT control group.

4.4.3 Gene expression of the CRH system

Penk KO animals present higher _Crh_ gene expression in the amygdala than WT mice.

Crh mRNA expression was measured in the PFC (n = 6-7/ group), PVN (n = 6-7/ group), amygdala (n = 6-7/ group) and hippocampus (n = 6/ group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.13). No significant results were observed in the PFC (CMS: $F_{(1, 22)} = 0.002649$, n.s.; genotype: $F_{(1, 22)} =$

0.2744, n.s.; CMS*genotype interaction: $F_{(1, 22)} = 0.08536$, n.s) or in the PVN (CMS: $F_{(1, 21)} = 3.452$, n.s.; genotype effect: $F_{(1, 21)} = 0.7814$, n.s.; CMS*genotype interaction: $F_{(1, 21)} = 1.440$, n.s.). In the amygdala, a significant genotype effect ($F_{(1, 22)} = 10.60$, $p < 0.01$) was observed, and the Bonferroni post-hoc test revealed a significant increase in the gene expression in *Penk* KO CMS mice compared with WT CMS animals (WT CMS and *Penk* KO CMS: $p < 0.05$). No significant results were observed for CMS effects ($F_{(1, 22)} = 0.2331$, n.s.) or CMS*genotype interaction ($F_{(1, 22)} = 0.3147$, n.s.). In the hippocampus, no significant results for CMS ($F_{(1, 20)} = 3.455$, n.s.), genotype ($F_{(1, 20)} = 1.194$, n.s.) or CMS*genotype interaction ($F_{(1, 20)} = 2.142$, n.s.) effects were observed.

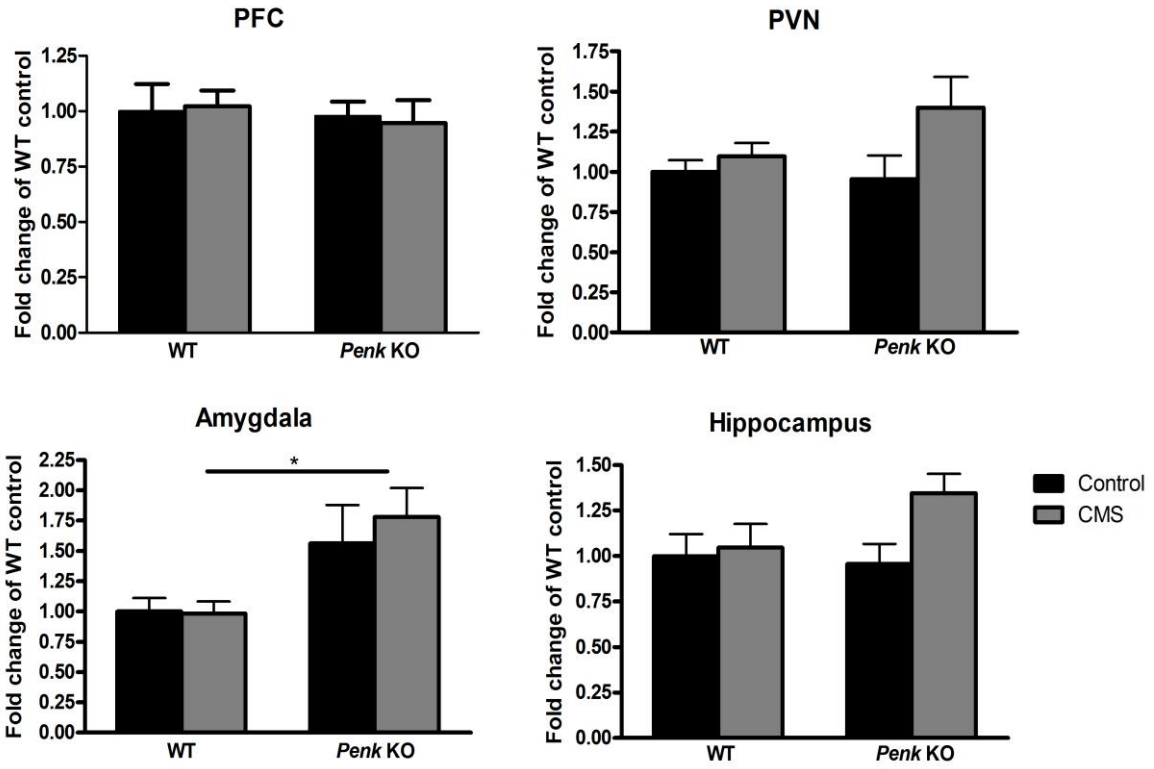


Figure 4.13 *Crh* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus. Statistical analyses revealed higher *Crh* gene expression in the amygdala of *Penk* KO CMS mice compared with WT CMS mice. No other significant results were observed in any of the analyzed brain areas. Data are expressed as fold changes relative to the WT control group. * $p<0.05$, using two-way ANOVA followed by Bonferroni test.

CMS and *Penk* gene deletion do not influence *Ucn* 2 and 3 gene expression.

Accordingly to the literature [40] mRNA expression for urocortins is absent in the PFC and hippocampus. Furthermore, the literature also shows that in Ucn2 mRNA is expressed in PVN, and Ucn3 mRNA in the amygdala. Therefore, in the present study Ucn2 mRNA expression was measured in the PVN (n = 5-7/ group) and *U*cn3 mRNA expression in the amygdala (n =6-7/ group) (Figure 4.14). No significant results were observed for the *Ucn* 2 gene expression in the PVN (CMS: $F_{(1,\ 21)}$ = 0.09422, n.s.; genotype: $F_{(1,\ 21)}$ = 1.173, n.s.; CMS*genotype interaction: $F_{(1,\ 21)}$ = 0.9791, n.s.), or for the *Ucn* 3 gene expression in the amygdala (CMS: $F_{(1,\ 21)}$ = 0.3115, n.s.; genotype: $F_{(1,\ 21)}$ = 0.9901, n.s.; CMS*genotype interaction: $F_{(1,\ 21)}$ = 0.3772, n.s.).

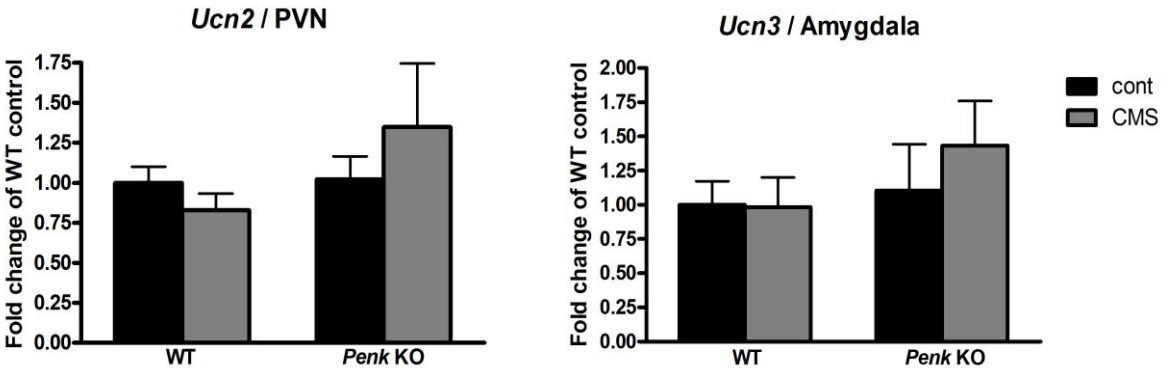


Figure 4.14 mRNA expression of *Ucn*2 was measured in the PVN and of *U*cn3 in the amygdala. Statistical analyses showed no significant results. Data are expressed as fold changes relative to the WT control group.

CMS and *Penk* gene deletion affect CRHr1 gene expression

Crhr1 mRNA expression was measured in the PFC (n = 7/ group), PVN (n = 6-7/ group), amygdala (n = 6-7/ group) and hippocampus (n = 5-6/ group) of WT and *Penk* KO mice from control and CMS groups (Figure 4.15). In the PFC, a significant CMS*genotype interaction ($F_{(1,\ 24)}$) = 5.178, $p < 0.05$) was observed, and the Bonferroni post-hoc test revealed a significant increase in the mRNA

levels in WT animals after CMS conditions (WT control vs. WT CMS: $p < 0.05$). No significant results were observed for CMS ($F_{(1, 24)} = 1.238$, n.s.) or genotype ($F_{(1, 24)} = 0.1024$, n.s.) effects.

No significant results were observed in the PVN (CMS: $F_{(1, 23)} = 1.306$, n.s.; genotype: $F_{(1, 23)} = 0.2494$, n.s.; CMS*genotype interaction: $F_{(1, 23)} = 3.797$, n.s.); or in the amygdala (CMS: $F_{(1, 22)} = 0.5772$, n.s.; genotype: $F_{(1, 22)} = 0.01376$, n.s.; CMS* genotype interaction: $F_{(1, 22)} = 1.662$, n.s.). In the hippocampus, a significant genotype effect was observed ($F_{(1,19)} = 5.807$, $p<0.05$.), and the Bonferroni post-hoc test showed significantly lower mRNA levels in the *Penk* KO control group compared to WT control ($p < 0.05$). No significant results for CMS effect ($F_{(1, 19)} = 0.01555$, n.s.) or CMS*genotype interaction ($F_{(1, 19)} = 2.121$, n.s.) were observed.

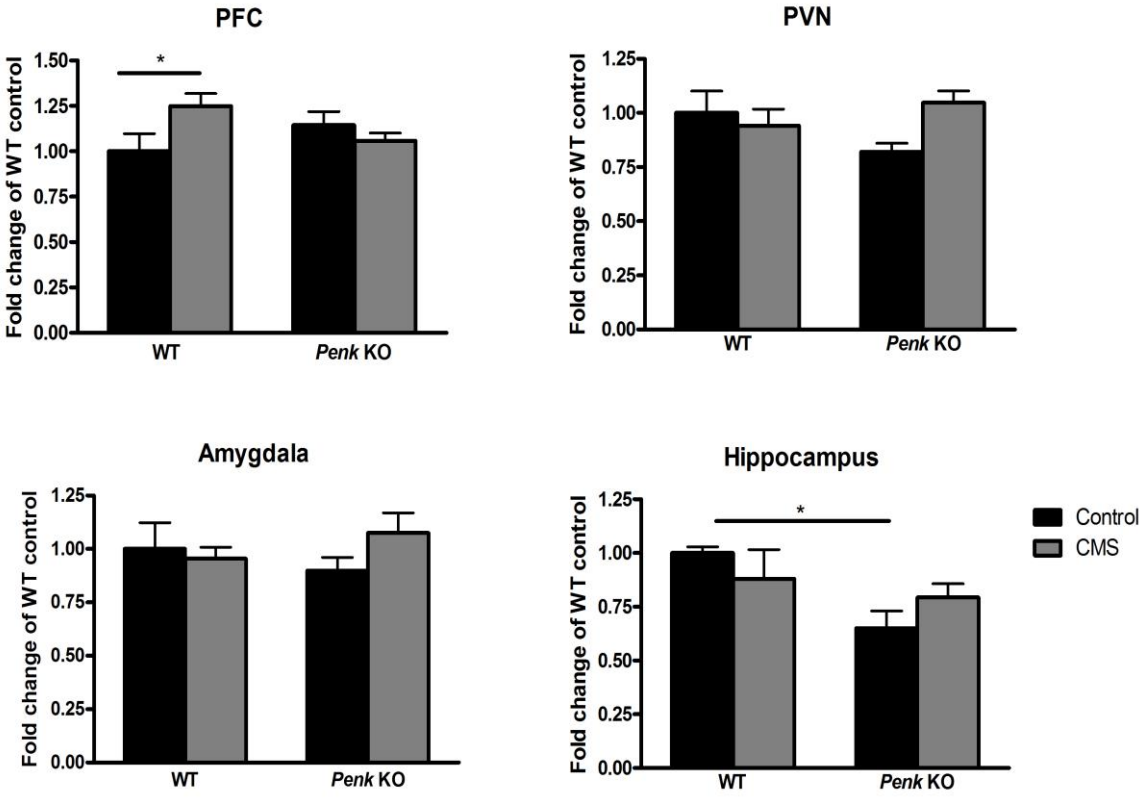


Figure 4.15 *Crhr1* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus. Statistical analyses revealed a significant CMS*genotype interaction in the PFC, which was due to an increase in the mRNA levels after CMS in WT mice. Furthermore, a significant genotype effect was observed in the hippocampus, due to a lower mRNA expression in *Penk* KO mice compared with WT mice under control conditions, but not after CMS. No other significant results were observed in any of the analyzed brain areas. Data are expressed as fold changes relative to the WT control group. * $p<0.05$, using two-way ANOVA followed by Bonferroni test.

Penk KO mice present an overall lower *Crhr2* gene expression in the amygdala than WT animals.

Crhr2 mRNA expression was measured in the PVN (n = 5-7/ group), amygdala (n = 7/ group) and hippocampus (n= 5-6/ group) (Figure 4.16). *Crhr2* mRNA is not present in the PFC [40] and therefore not was measured in this brain area. In the PVN, no significant results were observed for CMS ($F_{(1, 19)} = 0.6117$, n.s.), genotype ($F_{(1, 19)} = 0.05640$, n.s.) or CMS*genotype interaction ($F_{(1, 19)} = 0.1529$, n.s.) effects. In the amygdala, an overall lower gene expression in the *Penk* KO animals compared with WT mice was revealed by a significant main genotype effect ($F_{(1, 23)} = 5.417$, $p< 0.05$) but no significant results with the Bonferroni post-hoc test. No significant results were observed for CMS ($F_{(1, 23)} = 0.3691$, n.s.) or for CMS*genotype interaction ($F_{(1, 23)} = 1.180$, n.s.) effects. In the hippocampus, no significant results were observed for CMS ($F_{(1, 19)} =0.05558$, n.s.), genotype ($F_{(1, 19)} = 2.046$, n.s.) or for CMS*genotype interaction ($F_{(1, 19)} = 0.5131$, n.s.) effects.

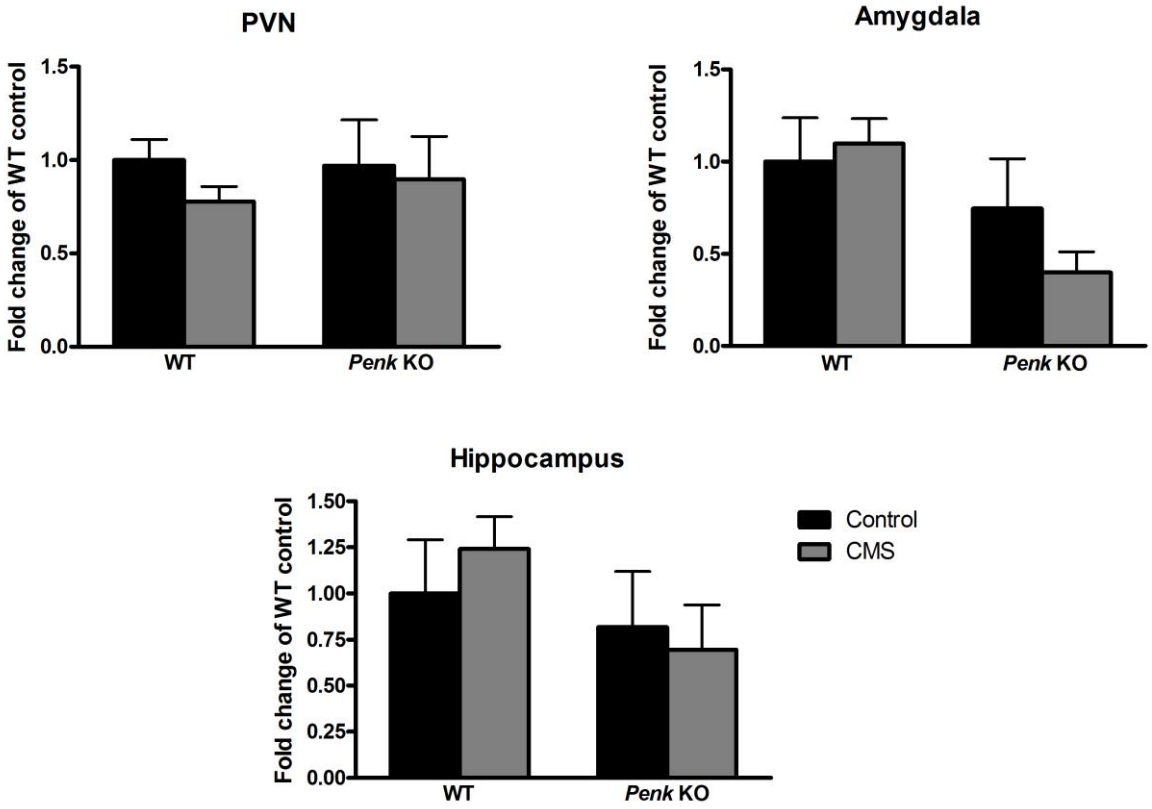


Figure 4.16 *Crhr2* mRNA expression was measured in the PVN, amygdala and hippocampus. Statistical analyses revealed a main genotype effect in the amygdala, with an overall decrease of the gene expression in *Penk* KO mice compared with WT animals. No other significant results were observed in any of the analyzed brain areas. Data are expressed as fold change of WT control group.

CMS has opposite effects in the *Crhbp* gene expression in the hippocampus of WT and *Penk* KO mice.

Crhbp mRNA expression was measured in the PFC (n = 6-7/ group), PVN (n = 7/ group), amygdala (n = 6-7/ group) and hippocampus (n = 5-6/ group) (Figure 4.17). No significant results were found in the PFC (CMS: $F_{(1, 23)} = 0.05937$, n.s.; genotype: $F_{(1, 23)} = 1.710$, n.s.; CMS*genotype interaction: $F_{(1, 23)} = 0.4282$, n.s.), PVN (CMS: $F_{(1, 23)} = 0.3860$, n.s.; genotype: $F_{(1, 23)} = 1.574$, n.s.; CMS*genotype interaction: $F_{(1, 23)} = 0.1258$, n.s.) or amygdala (CMS: $F_{(1, 22)} = 0.03198$, n.s.;

genotype: $F_{(1, 22)} = 0.6848$, n.s.; CMS*genotype interaction: $F_{(1, 22)} = 1.147$, n.s.). In the hippocampus, was observed a significant main CMS*genotype interaction ($F_{(1, 19)} = 7.453$, $p<0.05$), but no significant results with post-hoc analyses. These results show that the CMS has an opposite overall effect in WT and PENK KO mice. No significant results were observed for CMS ($F_{(1, 19)} = 0.07945$, n.s.) or genotype ($F_{(1, 19)} = 0.01876$, n.s.) effects.

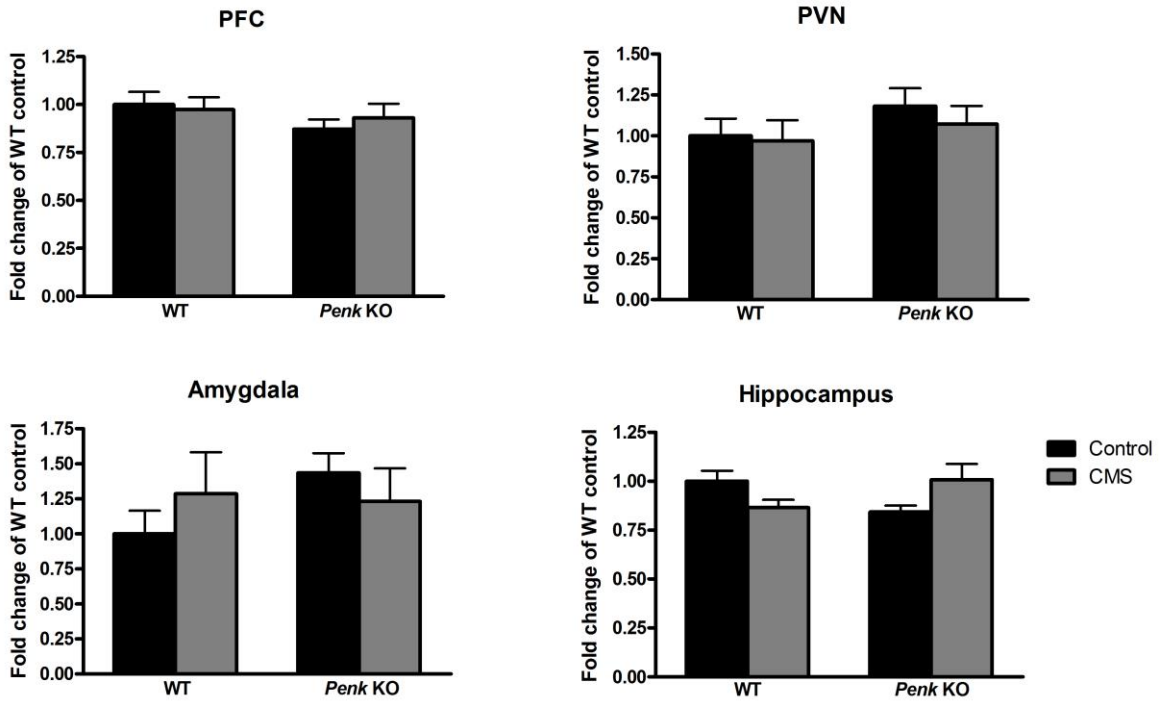


Figure 4.17 *Crhbp* mRNA expression was measured in the PFC, PVN, amygdala and hippocampus. Statistical analyses revealed a main CMS*genotype interaction in the hippocampus, showing that CMS induces opposite changes in the gene expression in WT and *Penk* KO mice. No other significant results were observed in any of the analyzed brain areas. Data are expressed as fold change relative to the WT control group.

Chapter 5: Discussion

The present study was designed to investigate the role of enkephalin in stress reactivity under chronic stress conditions and in the susceptibility to chronic stress-related mood pathologies. Therefore, we submitted WT and constitutive *Penk* KO mice to the chronic mild stress (CMS) protocol, followed by hormonal, behavioral and gene expression analyses.

Modulation of enkephalin levels under several stress conditions, as well as its potential role as an anxiolytic and/ or antidepressant compound, was previously shown in several studies [65,86,89] (see details in chapter 2.7). Moreover, mice lacking the *Penk* gene (*Penk* KO) exhibited enhanced basal anxiety levels [66,94–96], but no basal depression-related phenotype [133].Nevertheless, *Penk* KO mice showed an increased vulnerability to developed anxiety and depression-related phenotypes followed submission to foot-shock stress [98]. Thus, we hypothesized that *Penk* KO mice would show a higher vulnerability to anxiety and depression-related phenotypes when submitted to a CMS protocol.

5.1 Behavioral reactivity of WT and *Penk* KO mice

To investigate the impact of the *Penk* gene deletion in stress reactivity to CMS conditions, WT and *Penk* KO mice were submitted to a CMS protocol, followed by behavioral testing to assess anxiety and depressive-like phenotypes.

5.1.1 Basal behavioral phenotype of WT and *Penk* KO mice

Under basal/unstressed conditions (control groups), we detected similar levels of anxiety and depression-like behaviors between *Penk* KO and WT mice. Although these findings are in contrast to some of the previously published data [66,94–96], they can be readily explained by the distinct experimental conditions. Mice from the present study were single–housed for

8 weeks, submitted to an extensive period of handling, and the same animals were tested in multiple behavioral paradigms. In previous studies [66,94–96], *Penk* KO animals were group-housed and naïve for each behavioral test. It is well known that group- versus single-housing [134] as well as test-test interactions [135,136] strongly influence behavioral phenotypes. Furthermore, previously, our group showed that the basal phenotype of *Penk* KO mice is particularly sensitive to environmental conditions [95] . In that study, *Penk* KO mice from the same colony but housed in two different facilities presented distinct anxiety-like behavior in the O-maze test, while showing enhanced anxiety levels compared with WT mice. This arose because *Penk* KO animals from one facility spent less time in the open areas of the maze, while *Penk* KO mice from the other facility showed a generalized freezing behavior in both open and closed areas of the maze, indicative of an extreme anxiety/fear. Furthermore, re-analyses of previous data from our group (supplementary figure 1) revealed that *Penk* KO mice are more sensitive to social housing conditions than WT mice. While housing conditions do not affect anxiety levels of WT animals, *Penk* KO mice show enhanced anxiety levels when housed with conspecifics, but not after two weeks of single-housing. Moreover, a similar basal anxiety-like phenotype between *Penk* KO and WT mice was also previously shown by Kung et al. [98],in animals group-housed and tested in a battery of behavioral tests. In the present study, to avoid variability in stress reactivity due to the hierarchic status occurring in group-housed animals [59,137], and to minimize the number of experimental animals, we used individual-housed animals, which were tested in a battery of behavioral paradigms.

To sum up, the present and previously data suggest that the basal anxiety phenotype of *Penk* KO mice is highly sensitive to environmental and experimental conditions, such as housing, handling and behavioral testing.

5.1.2 Behavioral phenotype of WT and *Penk* KO mice after submission to 5 weeks of CMS

In accordance with the literature, CMS increased anhedonia in the sucrose preference test, increased despair status in the forced swim test and increased anxiety levels in the O-maze test in WT mice [120,123,124,127]. In stark contrast, CMS did not induce anxiety or depression-like phenotypes in *Penk* KO mice, which showed no significant alterations in their anhedonic, despair or anxiety levels.

Since the behavioral outcome in the forced swim and in the O-maze tests may be influenced by differences in locomotor and/or exploratory activity [138,139140], the animals were also tested in the open field test under dim light conditions (20 lux). Exploratory drive and/ or locomotor activity were similar between WT and *Penk* KO mice from control groups. CMS induced a similar slight increase in the activity of mice of both genotypes. CMS-induced hyperactivity was also described by others [138,139]. Therefore, we exclude that differences in the exploratory drive and/or locomotor activity may influence the distinct anxiety- and depression-like phenotypes observed between WT and *Penk* KO mice after CMS.

Thus, the present results show a higher resistance of the *Penk* KO mice to the detrimental behavioral effects of CMS, which is indicative of a resilience-like phenotype.

5.2 Corticosterone regulation in WT and *Penk* KO mice

Glucocorticoids (cortisol in humans and corticosterone in rodents) are the main mediators of the neuroendocrine stress response. Their production and secretion is driven by the HPA axis. CRH-parvocellular PVN neurons stimulate the production of ACTH by the pituitary gland, which in turn stimulates the production of glucocorticoids by the adrenal glands [6,7,25] (see details in section 2.4). Increased levels of glucocorticoids are essential during several physiological functions, such as in the sleep/wake cycle, and in the stress response [3,17,25]. However, sustained higher glucocorticoid levels have deleterious effects for the brain and peripheral organs[5,17]. Alterations in baseline levels of corticosterone suggest a poor control of secretion, which may lead to dysregulation of several physiological functions and an increase in the susceptibility to maladaptive stress responses [5,12,17,141]. Both genetic and environmental factors might induce long-term alterations in the regulatory mechanisms controlling glucocorticoid secretion [5,12,17,141]. Baseline hyperactivity of the HPA axis, is a feature in several pathologies, such as anxiety and major depression [12,17], and is also a common endpoint of the CMS protocol in rodents [34,142]. Therefore, we investigated gene-environment effects of *Penk* gene deletion and of CMS on the baseline levels of glucocorticoids (see detailed method in chapter 3.5).

5.2.1 Regulation of baseline corticosterone secretion under control conditions

Our results show that baseline corticosterone levels in control animals were higher in *Penk* KO than in WT mice. These results contrast with previous data from our group [66], in which naïve *Penk* KO animals present lower baseline levels of corticosterone than WT mice. This difference can be most likely explained by the differential sampling procedures. In the previous study [66],

corticosterone levels were measured from blood samples at a single time point, while in the present study the measurements were done from feces collected over a period of 24 hours. Glucocorticoid baseline levels display circadian fluctuations (over a period of 24 hours) [24,28],[25,143] and also a second type of fast, pulsatile fluctuation (45 to 60 min interpulse interval), i.e. in an ultradian pattern [24,25,28,29,144]. Therefore, a 24 hours sampling procedure is a more reliable marker for evaluation of general corticosterone secretion and avoids putative misleading results due to inter- and/or intra-group mismatching of circadian and ultradian cycles. Furthermore, this method allows the detection of alterations which may occur in several specific time points [140], and/or small continuous alterations, which individually are below detection threshold, but are indicative of a general alteration in baseline glucocorticoid secretion [145]. In addition, collection of feces samples is non-invasive and allows to collect samples during long periods of time from the same animals without disturbing them and, therefore without interfering with baseline secretion levels [146].

5.2.2 Regulation of baseline corticosterone secretion in CMS animals

Analyses of baseline corticosterone levels after CMS revealed an increase in WT mice, but not in *Penk* KO animals. Furthermore, after CMS *Penk* KO mice have lower baseline glucocorticoid levels when compared to WT mice. Increased baseline corticosterone levels in WT mice after CMS suggest a CMS-induced chronic hyper-activation of the HPA axis indicative of a maladaptive neuroendocrine stress response. In contrast, the lack of significant CMS induced alterations in baseline corticosterone levels in *Penk* KO mice, suggests a more robust neuroendocrine system, which is indicative of better stress copying mechanisms and resilience.

Together, behavioral and hormonal data show that *Penk* KO mice can cope better with CMS than WT animals. The resilience of *Penk* KO mice is surprising and the opposite of our initial hypothesis. These results suggest that a decrease in enkephalin signaling contributes to a better adaptation to CMS conditions and/or that the constitutive deletion of the *Penk* gene leads to developmental compensations, which have a protective effect under CMS-conditions.

5.3 Regulation of the *Penk* gene by CMS in WT mice

To scrutinize possible brain areas on which enkephalin functions as mediator of the stress response under CMS conditions, we measured the levels of the *Penk* gene in WT animals before and after CMS, in brain areas known to be involved in the behavioral and/or hormonal stress response, i.e. amygdala, hippocampus, PFC and PVN [8,14].

Penk gene expression was altered only in the PVN, where CMS induced an increase in the *Penk* gene expression. Similar results were shown after exposure to other stressors, such as hypertonic saline injections [20,73,147,148], immobilization [76,77,149] and chronic variable stress [53]. Thus, our results suggest that enkephalin produced in the PVN is an important modulator of the stress response to CMS.

The PVN is considered a central station for stress integration, where several neuronal populations, distributed among several subdivisions, are involved in the control of behavioral and neuroendocrine alterations during the stress response [14,16] (see details in chapter 2.3). Enkephalinergic cell bodies are present in both parvocellular and magnocellular subdivisions of the PVN, with the majority of PVN enkephalinergic cells localized in the parvocellular subdivision [20,21,73,77,147,150,151]. Enkephalin produced in the PVN may be released locally [152], where it can bind to MOP and DOP expressed in different PVN-

neuronal populations [153–157]. Moreover, PVN-enkephalinergic projections can release enkephalin in other brain regions involved in stress response or in the modulation of physiological functions commonly affected by stress conditions [158,159].

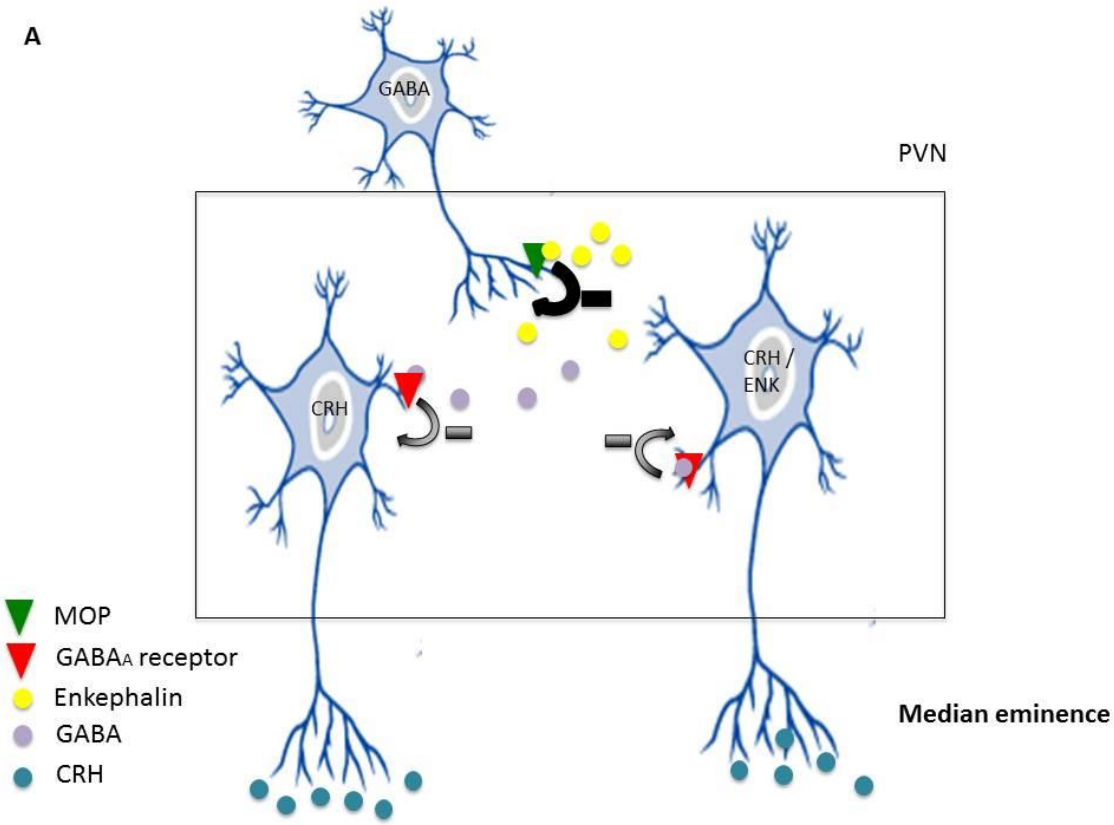
Importantly, a sub-population of PVN parvocellular CRH-expressing neurons, positioned at the head of the HPA axis [6,7,14], co-express enkephalin [150,151,160], suggesting enkephalin as a putative co-modulator of the neuroendocrine stress response. Enkephalin released by these neurons may influence the neuroendocrine stress response via distinct mechanisms. These neurons project to the median eminence, where enkephalin released by the axon terminals can activate opioid receptors expressed at the pituitary glands [153,161,162] and/or at the hypophysiotropic neuronal terminals [147,153,163,164]. In addition, these neurons may release enkephalin somatodendritically in the PVN [152,157], where opioid receptors are present in important neuronal populations involved in the modulation of stress response [157]. Indeed, GABAergic neurons, which form synapses with CRH and CRH/enkephalin parvocellular neurons, express pre-synaptic MOP receptors, and the activation of these receptors results in presynaptic inhibition of GABA release (LTD GABA). Therefore, decreasing the GABAergic modulatory role onto the neuroendocrine parvocellular neurons [157], which is of high importance to restrain the hyperactivity of the HPA axis[165,166]. During stress conditions, GABA is believed to exert an inhibitory action, decreasing the firing of CRH neuroendocrine neurons, and consequently, corticosterone secretion [15,166–169]. Thus, a CMS-induced increased expression of the Penk gene and increase release of enkephalin peptide in the PVN, might increase the activation of MOP at the GABAergic neurons. This would decrease the GABAergic tone in the PVN neuroendocrine CRH neurons, contributing to the stress-induced

hyperactivation of the HPA axis and increased corticosterone secretion (figure 5.1). In line with this hypothesis, decreased GABAergic tone in the PVN and increased corticosterone levels were previously shown after CMS conditions in WT mice [169]. Moreover, in our study, animals lacking enkephalin present a reduction in corticosterone secretion compared with WT mice, after exposure to CMS conditions.

In summary, our results show that CMS induced an increase in the *Penk* gene in WT mice, suggesting that PVN-enkephalin signaling is a strong candidate in the modulation of stress response under CMS conditions. Not excluding the possible influence of other enkephalin-signaling neuronal pathways in the observed phenotype of WT mice after CMS exposure, we discussed a hypothetical model through which an increase in enkephalin release, locally in the PVN, could contribute to the CMS-induced maladaptive stress response in these animals. Future studies need to evaluate GABA levels, GABAergic tone, and the effects of GABA in neuroendocrine CRH neurons, in the PVN of WT and *Penk* KO mice to solidify this speculative mechanism.

5.4 Gene expression analyses in WT and *Penk* KO animals

To obtain further insights into the molecular mechanisms contributing to the distinct behavioral and hormonal phenotypes between WT and *Penk* KO mice after CMS, we evaluated gene expression profiles of other known mediators involved in the stress response, namely components of the endogenous opioid, glucocorticoid and CRH systems, in WT and *Penk* KO mice.


A
GABA
PVN
CRH
CRH / ENK
MOP
GABAA receptor
Enkephalin
GABA
CRH
Median eminence

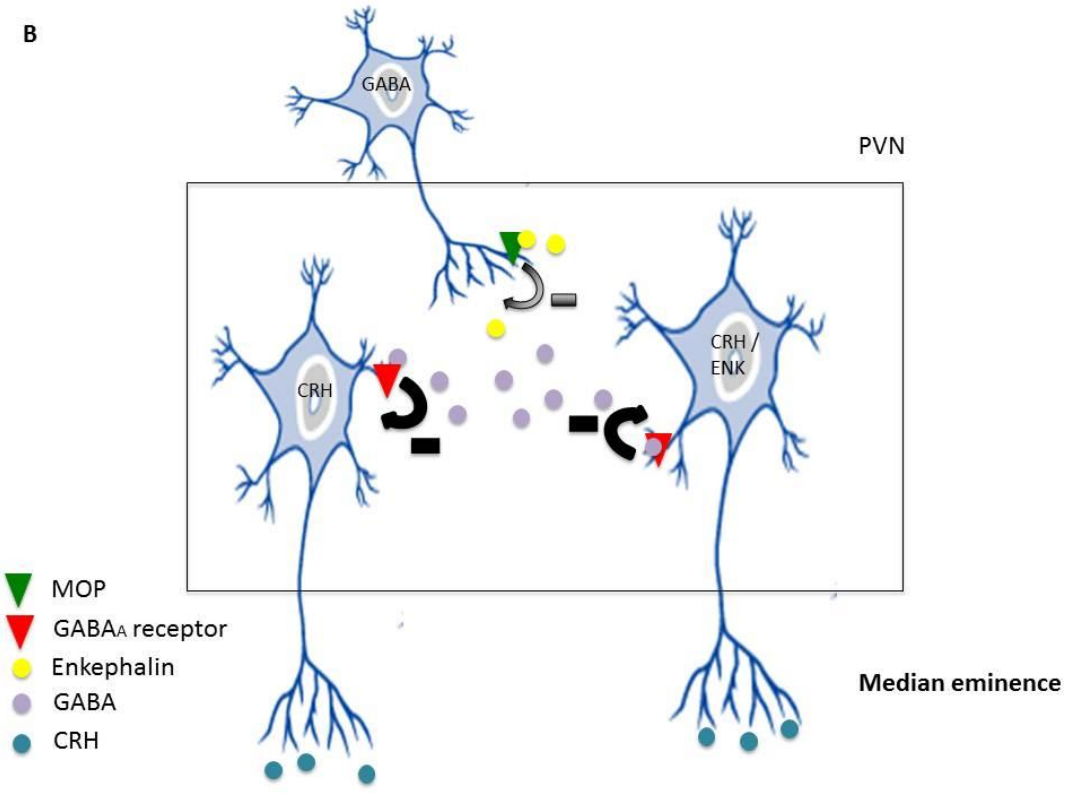

B
GABA
PVN
CRH
CRH / ENK
MOP
GABAA receptor
Enkephalin
GABA
CRH
Median eminence

Figure 5.1 Enkephalin signaling in the PVN: modulation of CRH neuroendocrine neurons. **(A)**. Parvocellular CRH neurons within the PVN are under inhibitory control by GABA. Presynaptic MOPs expressed in GABAergic neurons may be activated by enkephalin, inhibiting GABA release, leading to a decrease in the inhibitory tone onto CRH neurons. Thus, increased enkephalin signaling in the PVN observed under CMS conditions may contribute to hyper-excitation of CRH neuroendocrine neurons, leading to increased corticosterone secretion and thus, anxiety- and depression-related behavioral phenotypes. **(B)** In contrast, lack of enkephalin may contribute to the maintenance of the inhibitory GABAergic tone onto CRH neurons, restraining the activity of CRH neurons under CMS conditions, which could mediate the resilience to CMS.

5.4.1 mRNA expression of the *Oprd1* and *Oprm1* genes

Enkephalin binds and activates DOP and MOP [49,50]. Therefore, alterations in the gene expression levels of these receptors may be translated into altered number of functional receptors at the cell surface, which in turn may influence the role of enkephalin in stress reactivity. Analyses of *Oprd1* and *Oprm1* gene expression revealed only a main genotype effect in the *Oprd1* gene expression in the hippocampus, with lower *Oprd1* gene expression in the hippocampus of *Penk* KO mice compared to WT animals. No other alterations in the *Oprd1* or *Oprm1* gene expression were detected. Our results are not in agreement with a previous study on which *Penk* KO mice presented equal levels of *Oprd1* gene expression in the hippocampus compared with WT animals [174]. The discrepant results can be explained by the different experimental conditions. While in the previous study, animals were naïve, in the present study, animals were submitted to handling and tested in several behavioral paradigms before gene expression evaluation, conditions which are known to influence gene expression [175,176]. In addition, the use of different evaluation methods, mRNA expression vs. binding-affinity measurements may also explain the observed differences.

In the hippocampus, DOP receptors are mainly present presynaptically in parvalbumin and somatostatin -positive GABAergic interneurons, which form inhibitory synapses with glutamatergic pyramidal neurons [177–179]. Activation of DOP receptors is associated with a facilitation in hippocampal excitability, via indirect disinhibition of glutamatergic pyramidal cells [177–179]. Sustained increase in hippocampus excitability is believed to contribute to the deleterious effects of the chronic stress on hippocampal structure and functioning [34,123,180]. Thus, a decrease in hippocampal DOP expression in the *Penk* KO mice can attenuate a CMS-induced hippocampal excitability and contribute to a higher resilience to the deleterious effects of CMS in these animals.

5.4.2 mRNA expression of the *Pdyn* and *Oprk1* genes

The involvement of the endogenous opioid system in stress response and mood disorders is not confined to enkephalin and its receptors (DOP and MOP). Several studies support the role of dynorphin and its main receptor, the kappa opioid receptor (KOP) [103], in the modulation of stress response and susceptibility to pathophysiological conditions, such as anxiety and major depression [107,181,182]. Furthermore, long-term deletion of the *Penk* gene may lead to developmental compensatory mechanisms in other components of the endogenous opioid system [174,183–185]. Therefore, expression of the *Pdyn* and the *Oprk1* genes was analyzed in WT and *Penk* KO mice from control and CMS groups in the PFC, hippocampus, amygdala and PVN.

Our results revealed an overall up-regulation of the *Pdyn* gene expression in the amygdala of *Penk* KO compared with WT mice. Furthermore, we observed a down-regulation of the *Pdyn* gene in the PVN of *Penk* KO versus (vs.) WT animals following CMS. Finally, results from the *Oprk1* gene expression

revealed that CMS induced a significant upregulation of the *Oprk1* gene in the PVN of *Penk* KO mice.

Pdyn mRNA in the amygdala, is mainly expressed in the central nucleus (CeA) [182]. Therefore, we will restrict our discussion to the influence of dynorphin signaling pathways related with dynorphin synthesized in the CeA. Dynorphin synthesized in the CeA can be locally released by dendrites and/or axon terminals [181,182], where it can bind to KOP expressed presynaptic in GABAergic neurons [186], leading to inhibition of GABA release, locally or in CeA-GABAergic efferents to other brain areas [14]. Activation of KOP in the CeA is suggested to mediate fear conditioned responses but not anxiety [187]. Nevertheless, KOP activation in the CeA may influence pathways with opposite regulatory effects in stress response. As examples, while a KOP-mediated decrease GABAergic tone from the CEA to the BST may inhibit PVN activity, a KOP-mediated decrease GABAergic tone from the CEA to the raphe nuclei might lead to an increase activation of the PVN [6,14]. In addition, the effects of CeA dynorphinergic neurons can influence other brain regions, to which these neurons project [188,189]. For instance, CeA send dynorphinergic projections to the locus coeruleus (LC), the key brain area where noradrenaline is synthesized. These neurons are suggested to inhibit the excitatory effects of the CRH peptide at the noradrenergic neurons, leading to possible anxiolytic effects [188,189]. Therefore, an up-regulation of Pdyn gene in the amygdala of *Penk* KO mice could decrease the activity of the noradrenergic system in the LC, contributing to an attenuation of stress reactivity. This is in line with the observed phenotype of *Penk* KO CMS mice in the present study.

To sum up, an upregulation of the *Pdyn* gene in the amygdala may attenuate or exacerbate stress response, depending on which pathways dynorphin is involved. Thus, further studies are needed to clarify how a CMS-induced *Pdyn*

gene expression in the amygdala of *Penk* KO mice influences the CMS-induced reactivity.

In the PVN, *Pdyn* expressing neurons are mainly localized at the magnocellular subdivision, where these neurons are involved in the modulation of the neurohypophysial system and co-express vasopressin (VP) [20,190,191]. PVN-dynorphin is believed to attenuate the activity of several neuropeptides, such as VP locally[152,182,190], or at the level of the median eminence (neurohypophysial neurons) [18,20,161,192]. Moreover, KOP are expressed in several neuronal populations within the PVN [152]. Activation of KOP in the PVN was shown to have an important role in the modulation of the HPA axis, leading to an increase in ACTH and corticosterone secretion [104,193–195]. In summary, dynorphin-KOP signaling in the PVN is suggested to contribute to an activation of the HPA axis activity. Therefore, a decrease in *Pdyn* expression in the PVN of *Penk* KO mice can contribute to an attenuation of the hormonal stress response, which is in line with our data. On the other hand, an increase in the *Oprk1* gene expression in the PVN of *Penk* KO CMS mice, can contribute to an increase activation of the HPA axis and corticosterone secretion under CMS conditions. However, an increased activation of the HPA axis in *Penk* KO mice is not supported by our present results, which show that these animals present no alterations in corticosterone levels after CMS, and even have lower corticosterone levels than WT CMS mice. One factor accounting for that, might be a counterbalancing effect due to a decrease in the expression of PVN-*Pdyn* gene expression also observed in these animals.

Overall, our data suggest that the resilient phenotype of *Penk* KO mice under CMS conditions may be related with alterations in the dynorphin-KOP signaling, namely in the PVN, between these animals and WT mice.

5.4.3 mRNA expression of the *Gr* and *Mr* genes

The role of glucocorticoids in the modulation of cellular processes under basal and stress conditions does not only depend on the hormonal levels, but also on the expression profile of GR and MR receptors [196]. Furthermore, glucocorticoids have auto-regulatory mechanisms to avoid a sustained increase in corticosteroid levels and consequent deleterious effects [17,37,197]. Activation of glucocorticoid receptors in the hippocampus, PFC and PVN contribute to an attenuation of the HPA axis activity, while in the amygdala it has an opposite effect [198]. Generally, sustained high glucocorticoid levels, due to stress or to injection of exogenous glucocorticoids, leads to a down-regulation of glucocorticoid receptors[199]. However, differential stress protocols and/or treatment with exogenous glucocorticoids may lead to distinct alterations in the gene expression of glucocorticoid receptors [165,200–202]. For instance, repeated restraint stress during one week led to an increase *Gr* mRNA in the PVN, while 3 weeks had no effect[200]. In another study, chronic variable stress led to a reduction in the PVN *Gr* mRNA[165]. The results of the present study show a significant CMS induced decreased GR mRNA expression in the PVN in WT animals. GR receptors in the PVN are expressed in several neuronal populations, which are important modulators of the HPA axis activity, such as CRH-expressing parvocellular neurons [203–205]. Activation of GR in these neuronal populations inhibits the HPA axis activity, via a direct negative feedback mechanism [6,165]. Thus, CMS-induced reduction of GR in the PVN of WT mice may mediate a deficit in the inhibitory control of corticosteroid secretion, which is supported by the observed increased baseline corticosterone levels after CMS in these animals. On the other hand, a lack of alterations in the PVN-GR expression in *Penk* KO mice after CMS,

suggest a more robust HPA axis regulatory mechanism in these animals. This is in line with the observed lower baseline corticosterone levels in *Penk* KO animals compared with WT mice after CMS conditions.

5.4.4 mRNA expression of the different components of the CRH system

The CRH system plays an important role in stress response [40,44,124,206,207]. Alterations in the CRH system signaling may lead to differential stress responses and susceptibility to develop mood disorders [40,44,124,206,207]. The activation of CRHr1 by the CRH and UCN I peptides, or by exogenous agonists was shown to lead to anxiogenic effects and to stress hyper-sensibility [45]. In addition, mice overexpressing the *Crh* gene present increased basal anxiety phenotypes and increased baseline corticosterone secretion [140]. On the other hand, activation of CRHr2 via UCN 1, 2 and 3 or exogenous agonists is suggested to be involved in the termination of the stress response [45].

Elements of the CRH system and the endogenous opioid system are co-expressed within several neuronal circuits involved in stress response [77,151,160,189]. Moreover, it was shown that several types of stressors induce alterations in both the CRH and opioid systems in the same brain regions [77]. In addition, genetic manipulation of the CRH system leads to gene expression alterations in components of the endogenous opioid system [208]. Therefore, we hypothesized that the deletion of the *Penk* gene may influence gene expression in the CRH system under basal and/or CMS conditions.

5.4.4.1 mRNA expression of the *Crh*, *Ucn* and *Crhbp* genes

We observed a higher expression of the *Crh* gene in the amygdala of *Penk* KO CMS compared to WT CMS animals. Increased *Crh* levels in the amygdala is associated with anxiogenic states and HPA axis hyperactivity [209–212],which was not observed, in *Penk* KO mice, in our study. A possible explanation for that can be related with an intermingled signaling between enkephalin and CRH

within the amygdala or in brain areas which receive amygdalar CRH projections, on which the lack of enkephalin masks the anxiogenic role of higher CRH levels.

In addition, we observed an interaction effect in the *Crhbp* gene expression in the hippocampus, where WT CMS animals present a decrease and *Penk* KO CMS mice an increase in the gene expression. *Crhbp* overexpression is associated with anxiolytic, and *Crhbp* deletion with anxiogenic effects [45]. CRHbp is a water soluble protein, which binds to CRH and UCN 1 peptides, competing with the CRHr1 [213]. Thus, CRHbp is an important modulator of the CRH system, reducing the concentration of CRH and UCN 1 free peptides [45,213],and in consequence the activation of CRHr1. Increase activation of CRHr1 in the hippocampus, under chronic stress, is associated with deleterious effects in neuronal functioning and morphology [43,212]. Therefore, increased levels of CRHbp in the hippocampus of *Penk* KO mice may lead to a reduced activation of CRHr1, contributing to the stress resilience observed in these animals.

5.4.4.2 mRNA expression of the *Crhr1* and *Crhr2* genes

Our results revealed an interaction effect in the regulation of the *Crhr1* mRNA in the PFC, due to a CMS-induced upregulation of *Crhr1* mRNA in the PFC of WT but not in *Penk* KO mice. Alterations in the *Crhr1* mRNA in the PFC of rats and mice, after CMS conditions were reported in other studies [211,214–216]. However, in these studies, *Crhr1* mRNA expression decreased rather than increased. Furthermore, depressed suicide victims exhibited a decrease in CRH binding sites[217]. Decrease in *Crhr1* mRNA or binding sites is believed to be a compensatory mechanism to attenuate the increased PFC *Crh* gene expression and CRH peptide levels also observed in depressed suicide victims [218]. We have no explanation why we observed opposite results to the ones

described in the literature. Nevertheless, an up-regulation of *Crhr1* mRNA can increase the CHR signaling, contributing to pro-depressive states [211,214–216,218], as we observed in WT CMS mice in our study.

Moreover, we observed a down-regulation in the *Crhr1* gene expression in the hippocampus of *Penk* KO control animals compared with control WT mice. CRHr1 is widely expressed among hippocampal pyramidal neurons [219–221] but also in GABAergic interneurons [222]. CRH-CRHr1 signaling was shown to be necessary for normal hippocampal development and maturation[43]. Furthermore, brief and moderate increase in CRH-CRHr1 signaling was shown to improve hippocampal plasticity, supporting learning and memory [224–226]. On the other hand, severe and/or chronic stress conditions lead to long lasting activation of CRH-CRHr1 signaling, which was shown to be associated with neuronal atrophy and spine loss[43]. Therefore, lower baseline levels of CRHr1 might lead to abnormal development and maturation of hippocampal neurons, which can contribute to impairments in stress response regulation, since the hippocampus has an important regulatory role in stress reactivity [14,227,228]. In opposition, lower baseline levels of CRHr1 might have a protective effect during chronic stress conditions, attenuating exacerbated hippocampal excitability, which would be in line with the observed phenotype in *Penk* KO mice in our study.

In addition, we observed an overall down-regulation in the *Crhr2* gene expression in the amygdala of *Penk* KO mice compared to WT animals. CRHr2 is suggested to have an important role in stress response termination, mainly in the attenuation of exaggerated and/or sustained activation of stress response mechanisms [40,45]. Therefore, lower levels of CRHr2 may contribute to an impairment of stress response termination and increase susceptibility to the deleterious effects of chronic stress [40,45]. In our study *Penk* KO mice

present lower expression of CRHr2 gene in the amygdala, but these animals do not present an impairment in stress response termination. It is possible that the lack of enkephalin in Penk KO mice prevent an exacerbated activation of stress response mechanisms, masking the putative effects of lower levels of CRHr2 signaling in the termination of stress response.

Overall, differences in the gene expression of the CRH system components, due to both developmental and environmental conditions, in WT and *Penk* KO mice, may support the distinct phenotype between these animals under CMS. Therefore, it is important further research on the interaction between the CRH system and enkephalin signaling in stress reactivity under CMS conditions.

5.4.5 Summary of gene expression results

We identify enkephalin with origin in the PVN as a mediator in stress response to CMS. Furthermore, we discuss a putative mechanism by which an increase *Penk* gene expression in the PVN can contribute to the prodepressive and anxiogenic CMS-induced phenotype in WT mice, but absent in *Penk* KO animals, in the present study.

In addition, we identify several differences in the gene expression of the endogenous opioid, glucocorticoid and CRH systems, between WT and *Penk* KO mice, before and after CMS conditions. In line with the observed resilient phenotype of *Penk* KO mice, we found a decrease *Pdyn* in the PVN, a decrease gene expression of *Crhr1* in the hippocampus, an increase *Crhbp* also in the hippocampus, and the lack of alterations in the *Gr* expression in the PVN, of *Penk* KO mice. On the other hand, not in line with the resilient phenotype observed in *Penk* KO mice, we observed an increase *Crh* and decrease *Crhr2* expression in the amygdala of *Penk* KO mice, suggesting that the lack of

enkephalin masks the expected increase stress susceptibility associated with these alterations.

To sum up, however not a complete "black and white" picture, our gene expression analyses in CMS- resilient *Penk* KO and susceptible WT mice, might give an important contribute to the identification of gene expression profiles associated with a better stress copying mechanisms to CMS conditions.

5.5 Technical considerations

Constitutive *Penk* KO animals: Constitutive KO animals are very useful models in order to identify the contribution of specific molecules in several behavioral phenotypes and physiological functions. However, several limitations accrue from these models. First, the constitutive deletion of a gene may lead to developmental compensations, which may be difficult to interpret and/or to discriminate from the environmental effects. Secondly, the constitutive deletion of a gene makes impossible to distinguish the specific role of a molecule in distinct signaling pathways, both within the brain and peripheral tissues, where molecules may have distinct functional relevance depending on the expression site [229]. Thus, future studies should rely on more specific models such as conditional and inducible KO mice [229].

Tissue isolation for gene expression analysis: The Palkovits punching technique is widely used for extraction of brain tissue samples because is an easy and fast execution method [230]. However, this procedure does not allow the isolation of distinct subnuclei or subdivisions of small brain areas, and even less of specific cell populations. Therefore, misleading results may occur, due to possible differential regulations of a gene between different sub nuclei, subdivisions or cell populations within a brain area. Therefore, techniques such as laser capture microdissection are a necessary step to understand the

role of a specific molecule in different cell populations or subdivisions, which may undergo different physiological functions [231].

Gene expression vs. protein expression: Gene expression measurements might give a good picture of how and if a specific molecule is involved in the modulation of a function. However, mRNA quantification does not account to post-transcriptional and post-translational modifications, which may lead to significant differences between the mRNA expression and protein/ peptide levels [232]. Furthermore, assuming a positive correlation between gene expression and protein synthesis, gene expression do not allows identification of the brain areas where protein expression levels are changed (locally and/ or in other brain areas, which receive projections from the brain area where the cell bodies are localized). Thus, in future studies, western-blot and/ or immunohistochemistry must be also performed to correlate alterations in the gene expression with alterations in protein levels, and identification of brain areas where the alterations in gene expression will be reflected.

Chapter 6: Summary/Conclusions

The current literature highlights an anxiolytic and antidepressant effect of enkephalin. In the present study, we suggest that under certain stress conditions, namely CMS, enkephalin signaling might have the opposite effect. Our key finding is that constitutive *Penk* KO mice show a higher resilience to the deleterious behavioral and hormonal effects induced by CMS when compared with WT animals. Importantly, while WT mice present exacerbated anxiety- and depression-like phenotypes and increased levels of corticosterone after 5 weeks of CMS, no alterations were observed in the *Penk* KO mice.

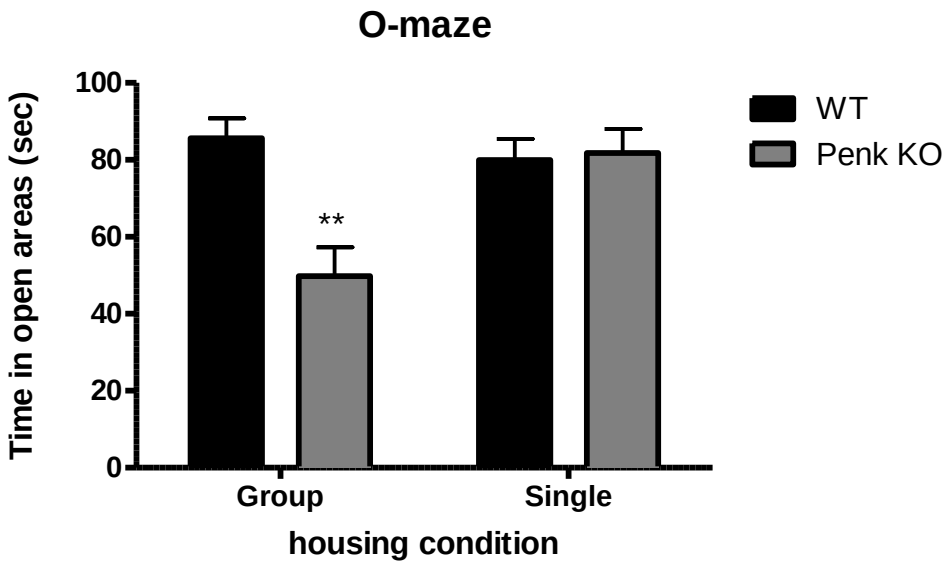
Furthermore, our study shows that *Penk* gene expression is up-regulated in the PVN of WT mice after CMS conditions. Because these animals showed vulnerability to CMS conditions, this suggests a putative involvement of PVN-enkephalin in the deleterious effects of CMS.

Overall, our data underlines the importance of enkephalin in stress response. Yet, in opposition to what was shown by others under distinct stress conditions, the lack of enkephalin favors, rather than impairs the adaptation to CMS conditions. Therefore, we emphasize the need to study the role of enkephalin under distinct stress conditions, as well as its role in several signaling pathways.

In addition, we show several differences in the gene expression of the endogenous opioid, glucocorticoid and CRH systems, between WT and *Penk* KO mice, which may account to the differential phenotype between these animals to CMS. Therefore, contributing to the identification of gene expression profiles involved in the resilience and vulnerability to CMS-induced pathologies

7 Supplementary data

7.1. Supplementary figure 1



Supplementary figure 1 housing effects in the anxiety levels of WT and *Penk* KO mice tested in the O-maze test. *Penk* KO mice under group-housed conditions present an increase in the anxiety levels in the o-maze test (decrease time in the open areas of the maze), compared with wild type mice. *Penk* KO mice individual-housed for a period of two weeks do not show differences in the anxiety levels tested in the o-maze test, compared with WT mice. Data expressed as mean ± S.E.M. ** p <0.01, using two-way ANOVA followed by Bonferroni test (data published at supplementary material at Melo I. et al, 2014)[233] .

Chapter 8: References

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