THE EFFECTS OF OXYTOCIN: REGULATION OF STRESS AND ANXIETY BY MEANS OF OXYTOCIN RECEPTOR-COUPLED SIGNALING CASCADES

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General Introduction

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1. Anxiety

1.1 Anxiety disorders

Anxiety is an adaptive, evolutionary conserved response to frightening stimuli. These stimuli often initiate a stress response, characterized by the activation of the hypothalamo-pituitary-adrenal (HPA) axis and subsequent secretion of adrenal steroids (Aguilera 1998). This leads to increased heart rate, vigilance, a decrease in feeding, and exploration of the environment (Korte 2001). However, if anxiety becomes excessive, it poses a threat to a person's or animal's mental well-being and then falls under the classification of anxiety disorders. About 18 % of the American adults and 14 % of EU citizens were affected by anxiety disorders in 2010 (Wittchen et al. 2011), which include panic disorders, obsessive-compulsive disorder, post-traumatic stress disorder, social anxiety disorder, and generalized anxiety disorder. Such high numbers imply the need for efficient medication. However, current treatments, such as the use of benzodiazepines and selective serotonin re-uptake inhibitors (SSRIs), are not fully satisfactory because of addictive effects, development of tolerance, and poor efficacy in a subset of patients. Therefore, new anxiolytic drugs are needed and unraveling the molecular mechanisms of anxiety regulation in the brain could contribute to the quest for proper medication. Current research in this field is devoted to finding genes that are important for anxiety and stress.

1.2 Anxiety-related genes

In recent years, some candidate genes have been found to be involved in the regulation of anxiety and, if dys-regulated, may cause anxiety disorders (Hovatta and Barlow 2008). These genes are involved, for example, in the regulation of G-protein signaling (Rgs2), GABA A receptor regulation (Gabra2), neurotrophic factor signaling (TrkB), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (Ppargc1a) signaling, opioid μ-receptor signaling (Opmr1) (Sokolowska and Hovatta 2013), or in HPA axis regulation and dendritic spine formation (corticotrophin releasing factor; CRF) (Maras and Baram 2012). Also, several other anxiety-relevant genes that code for neuropeptides are involved in the regulation of anxiety and stress, such as the neuropeptide Y (Giesbrecht et al. 2010), neuropeptide S (Ionescu et al. 2012), substance P (Ebner and Singewald 2006), nociceptin/orphanin FQ (Martin-Fardon et al. 2010), arginine-vasopressin (AVP), and oxytocin (OT) (Neumann and Landgraf 2012) to name a few. The plethora of neuropeptides regulating an organism's stress and anxiety response is necessary to fine-tune the response to the apparent stressor. It lies beyond the scope of this work to address all anxiety and stress-related neuropeptides; the focus will be therefore on CRF and OT. The transcriptional regulation of CRF was intensely studied in this work, because it has anxiogenic properties when it is released in the amygdala (Mountney et al. 2011). Furthermore, it is the first factor in the HPA axis and therefore an important stress-related neuropeptide.

Another factor that relates to this work is Rgs2, whose transcription is regulated by the neuropeptide OT (Okimoto et al. 2012), and, in a different context*, via* the CREB transcriptional coactivator 3 (CRTC3) (see below) (Song et al. 2010). It is thus possible that OT controls the expression of genes related to anxiety and stress by modulating a CRTC-

dependent pathway. This is one of the hypotheses that were tested in the work presented here.

1.3 Anxiety-related behavior in animal models

Although identification of genes involved in anxiety is useful, it has become more and more accepted over the years that one single gene is unlikely to be the regulator of complex emotions like anxiety. It is more likely the combination of psychological/environmental factors (stress), and the genetic predisposition that underlies the individual's susceptibility for developing anxiety disorders (Bakshi and Kalin 2000; Razafsha et al. 2013). Nevertheless, the genetic background is one important factor in the etiology of anxiety disorders and can be studied using a variety of methods like genome-wide expression profiling, proteomics, or quantitative trait locus mapping (Sokolowska and Hovatta 2013). To investigate the involvement of these genes in anxiety-like behavior several behavioral test paradigms, such as the elevated plus maze, open field test, light dark box (LDB), and novelty suppressed feeding test can be applied (Sokolowska and Hovatta 2013).

To assess the effects of OT on non-social anxiety-related behavior in my rat studies I monitored rats in the LDB. This test makes use of a conflict the rats will experience, i.e. the rats' exploratory drive versus the fear of open and bright spaces. Therefore, the more time a rat spends in the bright compartment of the LDB, the less anxious it is.

2. The neuropeptide oxytocin (OT)

In the search for endogenous anxiolytic factors, the neuropeptide OT was discovered to exert anxiolytic activity in the central amygdala (CeA) of rats in 2001 (Bale et al. 2001). It is produced only in two hypothalamic nuclei, the supraoptic and the paraventricular nuclei (PVN), from where it is distributed by axonal projections (Knobloch et al. 2011) and, more locally, dendritic release and diffusion (Landgraf and Neumann 2004; Ludwig and Leng 2006). OT, known as the "love, trust or cuddle hormone", is also available as a nasal spray, which has been reported to increase trust and to reduce cortisol levels during couple conflicts in humans (Kosfeld et al. 2005; Ditzen et al. 2009). When released in the brain, it has been well acknowledged for its regulation of reproductive behavior, such as maternal care and aggression, pair bonding, sexual behavior and anxiety-like behavior (Bosch et al. 2005; Bales et al. 2007; Waldherr and Neumann 2007). Further studies in humans revealed a role for OT in enhancing the individuals' social skills and to antagonize depression, post-traumatic stress disorder, autism, and other psychiatric illnesses. However, OT also stimulates aggression against group-outsiders, selfish behavior depending on the social context, personality, and gender (Bakermans-Kranenburg 2013) and can even be fear-enhancing (Guzman et al. 2013). The molecular underpinnings of this wide range of behavioral effects are rather unknown and under intense scientific investigation. To reveal some of these molecular mechanisms of OT actions was, therefore, in the focus of this thesis.

2.1 Central and peripheral OT release

Neuropeptides are amino acid assemblies that are synthesized on ribosomes in the cytoplasm, packed into Golgi vesicles and post-translationally modified in their transport vesicles. Neuronal dendrites are capable of synthesizing neuropeptides, such as OT, to allow fast dendritic release, while axonal synthesis could not be observed (Tiedge et al. 1999). Although peripheral release of OT from the neurohypophysis into blood is under the control of a variety of stimuli and neurotransmitters, noradrenaline (norepinephrine), histamine, and excitatory amino acids have been directly associated with peripheral release, especially in response to suckling. More recent studies revealed the involvement of these neurotransmitters in the regulation of central release of OT as well (for review see (Bealer et al. 2010)). Following an appropriate stimulus, OT is centrally released from either axon terminals as a rapid synaptic neurotransmitter (Dabrowska et al. 2011), or from dendrites, soma, or non-terminal axonal regions as a non-synaptic neuromodulator (Tobin et al. 2012). A neuromodulator is recognized by slow substance diffusion *via* the extracellular fluid, and binding to nearby or distant receptors in brain regions that are not necessarily connected *via* axonal projections. Interestingly, central release and peripheral release from neurohypophysial terminals into the blood stream can be either independent or coordinated, depending on the type of stimulus (Neumann and Landgraf 2012). Stimuli like birth, suckling, sexual activity, and a variety of stressors trigger coordinated central and peripheral release of OT, whereas other stressors, such as social defeat, exclusively trigger central release without altering peripheral levels (Engelmann et al. 1999; Landgraf and Neumann 2004). This differential release of a neuropeptide from separate compartments of a single neuron implies the need for fine-tuned regulatory mechanisms. Somato-dendritic, but not axon terminal release, is triggered by depolarization-induced release of calcium

(Ca^{2+}) from intracellular stores *via* voltage operated Ca^{2+} channels (VOCCs), which leads to the depolymerization of filamentous (F)-actin to monomer G-actin. F-actin is a barrier for Large Dense Core Vesicles (LDCV), which prevents exocytosis at the cell membrane, whereas depolymerized G-actin allows fusion of LDCV with the membrane and subsequent exocytosis of incorporated neuropeptides. Binding of OT to the OT receptor (OTR) leads to a rise in intracellular Ca^{2+} levels, which induces "priming" of the OT neuron for potentiated dendritic OT release (Ludwig et al. 2002). However, the physiological meaning of this self-potentiated dendritic release is not yet fully understood.

The classical action potential-driven axonal release from neurohypophysial terminals into the blood stream leads to the well-known peripheral effects of OT, like induction of labour or milk ejection.

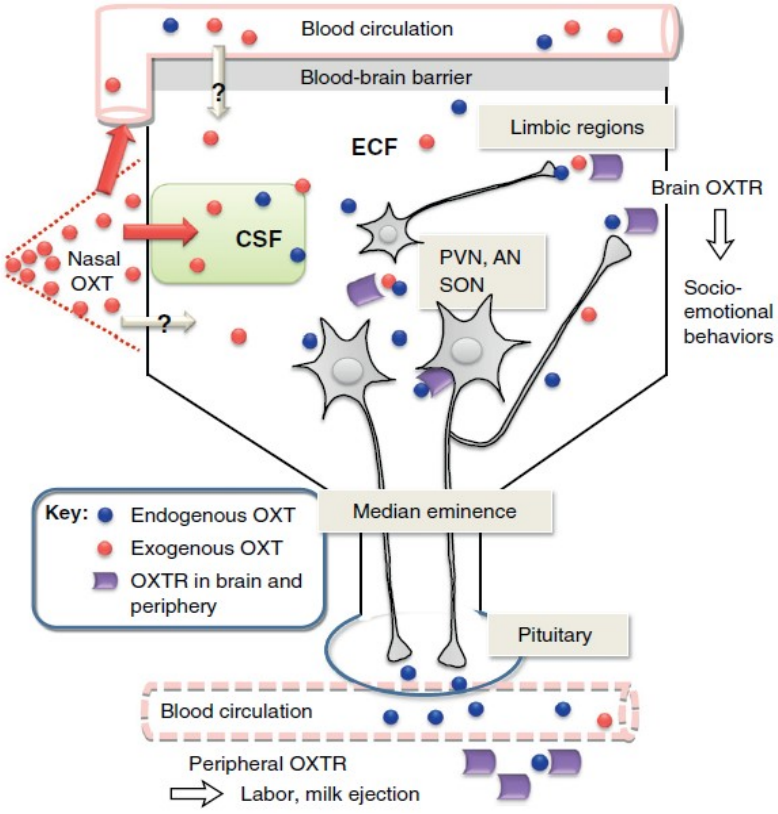


Figure 1 The brain OT system: neuronal projections, release, receptor-mediated effects, and external application. In brief, central release of OT as a neuromodulator occurs from dendrites and perikarya, in

addition to axon terminal release as a neurotransmitter. Central release can occur independently and simultaneously of peripheral release. Taken and adapted from (Neumann and Landgraf 2012)

2.2 The OTR and OTR-coupled signaling cascades

All of the effects that OT exerts on animal behavior and physiology, including its anxiolytic activity, are the consequence of its binding to the OTR and the subsequent induction of intracellular physiological responses. The OTR is a 7-trans-membrane domain class I G-protein coupled receptor (GPCR). The human OTR gene consists of 4 exons and 3 introns, and the promoter consists of a variety of species-dependent transcription factor binding sites. The OTR gene is differentially expressed in various tissues and under different physiologic conditions (virgin vs. post-partum), regulated by DNA methylation of a specific CpG site in the promoter (Mamrut et al. 2013). The sequence homology between the mammalian OTR and vasopressin receptor 1 (V1) is nearly 50%; between the OTR and V2R it is even lower (40%) (Gimpl and Fahrenholz 2001).

It should be noted that the expression of the OTRs at the cell membrane and their affinity for OT are both regulated. For example, OTRs can be desensitized upon permanent agonist stimulation. This process involves multiple mechanisms, and can occur at transcriptional, translational or protein levels. The fastest desensitizing mechanism includes phosphorylation of the receptor and subsequent ß-arrestin binding. This leads to uncoupling of the GPCR from its G-proteins and endocytosis of the receptor. It was found that 60 % of the OTR expressed in human kidney fibroblasts (HEK293 cells) is desensitized already after 5-10 min of agonist stimulation (Gimpl and Fahrenholz unpublished data). However, due to the artificial nature of this system (the OTR has been transfected into the cell), the

interpretation of the data has to be taken with care. Moreover, exposure of naturally OTR-expressing myometrial cells to OT for 20 hours lead only to an almost 10-fold reduction in OT binding, with no changes in the OTR protein level.

Although only one type of OTR exists, it can occur in a high or in a low affinity state. The OTR requires at least two essential components for high-affinity binding of OT: divalent cations, such as Mg^{2+} or Mn^{2+}, and cholesterol. These two components determine if the OTR displays the high-affinity state with a Kd > 1-50 nM, or the low affinity state with Kd > 100 nM. The conversion of the two affinity states is reversible. Cholesterol seems to stabilize the OTR for agonists in a high affinity state and acts, like the divalent cations, as an allosteric modulator (Gimpl and Fahrenholz 2001).

OTRs are functionally coupled mostly to $G_{q/11}\alpha$ class GTP binding proteins that stimulate together with Gβγ the activity of phospholipase C (PLC). This leads to the generation of inositol-3-phosphate (IP3) and 1,2-diacylglycerate (DAG). IP3 triggers Ca^{2+}-release from intracellular stores, whereas DAG stimulates protein kinase C (PKC), which phosphorylates downstream targets. The rise in intracellular Ca^{2+} stimulates the Ca^{2+}/calmodulin system that activates the myosin light chain kinase, which in turn leads to smooth muscle contraction, e.g. in myometrial cells during labour. However, the OTR does not exclusively bind the G_q-protein, but also G_i-protein subunits. This implies a differential downstream signaling of the activated OTR, since G_i-proteins inhibit adenylyl-cyclase, which reduces cAMP levels (Gimpl and Fahrenholz 2001; Busnelli et al. 2012).

Zhong and co-workers revealed the involvement of the trans-activated epidermal growth factor receptor (EGFR) in extracellular signal regulated kinase 1/2 (ERK1/2) activation by the myometrial OTR (Zhong et al. 2003). In the PVN, it is this pathway that mediates the

anxiolytic activity of OT (Blume et al. 2008). Blockade of the mitogen-activated protein (MAP) kinase “MAP ERK kinase 1/2” (MEK1/2) by the inhibitor U0126 attenuated the anxiolytic effect of OT in male rats, indicating a central role of MEK1/2 in the mediation of the anxiolytic effect of OT.

In the hippocampus, activation of OTRs lead to $G_{q/11}$ – IP3 activation, as well as EGFR-ERK1/2-mediated translational induction of the PKC subform PKMζ *via* the mammalian target of Rapamycin (mTOR) complex (Lin et al. 2012). The interplay between these cascades triggers long term potentiation (LTP) in the hippocampus, a mechanism also involved in the establishment of long-lasting spatial memory (Tomizawa et al. 2003). Blocking the MAPK cascade with the MEK inhibitor U0126 inhibited cyclic AMP responsive element binding protein (CREB) phosphorylation and subsequent spatial memory formation during motherhood (Tomizawa et al. 2003).

A global knockout of the OTR in male mice results in profound behavioral and physiological alterations such as impaired sociability and impaired preference for social novelty, impaired cognitive flexibility, increased aggression (Chini et al. 2008; Sala et al. 2011; Sala et al. 2013), but also obesity and dysfunction in body temperature when exposed to cold (Nishimori et al. 2008). In the ventral and dorsal striatum, it also has been reported that OTRs can occur as heterodimers coupled to a dopamine D2 receptor, which is thought to produce the rewarding effect of a social stimulus during pair bonding (Romero-Fernandez et al. 2013). This would increase the rich repertoire of intracellular responses to OTR activation, and makes very specific responses to particular stimuli possible.

In addition, our group found that OTR activation in hypothalamic neurons lead to the incorporation of transient receptor potential V2 (TRPV2) channels into the cell membrane

and a subsequent influx of Ca^{2+} from the extracellular space (unpublished data). This influx may increase cellular excitability, protein synthesis and neurotransmitter release *via* several Ca^{2+}-dependent signaling cascades. Activation of the TRPV2 channel is upstream of MEK1/2 phosphorylation, and it has therefore been postulated that blocking TRPV2 channels pharmacologically prevents the anxiolytic activity of OT.

2.3 OT and the regulation of stress responses and anxiety

It has long been established that physical as well as psychological stressors induce OT release into the peripheral circulation (Lang et al. 1983; Gibbs 1984). Later studies revealed the central release of OT in the hypothalamus and lateral septum upon an emotional stressor, which appeared to be independent from peripheral release (Engelmann et al. 1999). Furthermore, intra-cerebro-ventricular (icv) administration of a selective OTR antagonist (OTA) disinhibited basal and stress-induced (forced swim) HPA axis activity in male and female rats (Neumann et al. 2000), as indicated by increased adrenocorticotropic hormone (ACTH) and corticosterone release into the blood. Retrodialysis of the OTA in the PVN resulted in similar effects in unstressed males and females, locating OTs' effects on the HPA axis to this specific brain region. Interestingly, the expression of the main HPA axis activator, CRF, was more enhanced following a physical stressor (4 h of restraint) in the PVN of OT gene deficient male mice, when compared to wild type mice suggesting a central inhibitory role of OT in the regulation of CRF gene transcription (Nomura et al. 2003)

In 2004, Windle and co-workers infused OT icv at 1 or 10 ng over a 7-day period *via* an osmotic minipump in female, ovariectomized, estradiol-treated rats and subsequently subjected the rats to 30 min restraint stress. This treatment significantly reduced both basal

and restraint-induced plasma ACTH and corticosterone levels. CRF mRNA, which was measured by *in situ* hybridization in brain slices collected 210 min after the end of the stressor, increased in saline-treated restraint animals, and this increase was absent in OT-treated stressed rats (Windle et al. 2004). One year later, Ebner et al (2005) found that OT is released in the CeA and directs the stress-coping behavior toward a more passive coping style, as indicated by an increased floating time in the forced swim test. Blocking the OTR by an OTA likewise increased the release of excitatory amino acids (glutamate and aspartate) indicating an inhibitory effect of OT in the amygdala (Ebner et al. 2005). This was confirmed by a study that revealed that OT excites a population of GABAergic interneurons in the CeA, thereby reducing the activity of output neurons in the CeA that control the physiological expression of fear (Huber et al. 2005). The relation of axonal OT release in the CeA to fear responses has been investigated with the help of a sophisticated optogenetic approach, revealing decreased freezing responses in fear-conditioned rats upon blue-light-induced release of endogenous OT (Knobloch et al. 2011).

Besides regulating stress-coping and anxiety in the CeA, OT regulates sexual behavior and anxiety in the PVN of males and females. Endogenous, mating-induced release of OT in the PVN of male rats was found to be causally involved in mating-induced anxiolysis for up to 4 h (Waldherr and Neumann 2007). In addition, exogenous OT infused into the PVN of male rats also induces anxiolysis (Blume et al. 2008).

In contrast to the studies cited above, which suggest that central OT inhibits HPA axis activity, peripheral OT might be stimulatory at the level of the pituitary gland. Peripheral OT acts as ACTH and corticosterone secretagogue, increasing both basal and stress-induced plasma ACTH levels (Gibbs 1984; Petersson et al. 1999) after chronic (Ondrejcakova et al.

2010) or acute (Stachowiak et al. 1995) subcutaneous (s.c.) injection. Similar effects have been found *in vitro* in pituitary explants (Antoni et al. 1983).

2.4 Cross-talk between the OTR and CRFR coupled signaling in the periphery

What could be the physiological consequences of activation of the OTR and its downstream intracellular signaling pathways? One of the targets of the OT system appears to be the myometrial CRF system. An interesting study by Grammatopoulos et al (1999) investigated the crosstalk between the OTR and the CRF receptor (CRFR) in human myometrium at term (Grammatopoulos and Hillhouse 1999). They found that OT reduces CRF binding to the CRFR in human myometrial membranes in a time-dependent manner by reducing the affinity of the CRF receptor without affecting the number of receptors. The inhibitory effect of OT on CRF binding first appeared 15 – 20 min after the onset of OT stimulation. The maximum of CRF-binding inhibition (-50%) by OT was reached at 30 min and remained constant for at least 2 h. The effect was observed at OT concentrations between 1 – 100 nM OT. Further investigations in human myometrial cells suggested a role of PKC in CRF receptor desensitization. OTR binding activates the PLC pathway *via* G_q- proteins, which leads to rapid release of Ca^{2+}- ions from intracellular Ca^{2+} stores. Intracellular Ca^{2+} activates PKC and induces its translocation from the cytosol to the cell-membrane, where it phosphorylates the CRF receptor. Phosphorylation of the receptor leads to reduced receptor affinity for its agonist. It is interesting to note that this mechanism is only apparent in term, but not pre-term myometrium membranes. The authors speculate that CRF might influence myometrial contractility, and that OT antagonizes the relaxing effect of the activated CRFR. Additionally, studies in the pituitary gland describe opposite (i.e. potentiating) effects of PKC on CRF

receptor signaling (Cronin et al. 1986). It is not clarified to date how this highly dynamic interplay is regulated at the molecular level. It is tempting to speculate, however, that different sub-forms of PKC, like the PKMζ (Lin et al. 2012) are responsible for tissue and state (pre-term / term) specific effects.

3. Anxiety, stress, and its key regulator CRF

A physiological state that is intimately connected with anxiety is stress. A fearful stimulus will generally not only induce anxiety behavior, but also leads to neural and peripheral adaptations that enable an individual to cope with the stimulus; this is known as the stress response. Both OT and CRF are involved, and interact in anxiety behavior as well as in the stress response.

3.1 Definition of the term "stress"

It is essential for a living organism to maintain a constant inner environment. This ability to create an inner environment, independent from the external environment, was termed "homeostasis" by the American physiologist Walter Bradford Cannon (Cannon 1929, 1939). Disturbing homeostasis can be perceived as threat, provoking a response from the organism to regain a constant inner environment. It was the endocrinologist Hans Selye who termed the threat as "stressor" and defined stress as the "nonspecific response of the body to any demand placed upon it" (Selye 1936). Later studies revealed that Selye's definition of stress as "nonspecific" was not accurate (Pacak et al. 1998). The stress response is specific to the kind of stressor and depends on the organism's perception of the stressor, and the ability to cope with it (Goldstein and Kopin 2007).

3.2 Differentiation between physical, psychological, and cellular stress

For a better understanding of the work presented in this thesis, it is of importance to differentiate between physical, psychological and cellular stress, and the processing of each at the cellular level. The term “stress” is not always clearly defined in the literature, especially at the cellular level, which imposes the need to clarify and define terms that are used in this work. Severe physical stressors like cold, blood loss or injuries recruit the brainstem and hypothalamic regions, whereas the kind of stress we perceive every day and influences the organism’s homeostasis mostly, is of psychological nature, such as social embarrassment, examinations, or deadlines (Joels and Baram 2009). This physical/psychological stress is processed at a high level of different interacting networks in the brain and does normally not include damage to a single neuron. Cellular stress, to the contrary, imposes damage to single neurons and, as a consequence of an increasing imbalance in central networks, to the whole organism. This kind of stress can be caused by UV radiation, hypoxia, or chemical compounds (Chu et al. 2004). Cells respond differentially to these different kinds of stressors, however, one universal stress responsive protein is the MAPK family member “stress-activated protein kinases” (SAPK) and its most common member JNK has been reported to be activated in response to both cellular (e.g. glutamate-induced excitotoxicity) (Bogoyevitch et al. 2004) and psychological/physical stressors, such as forced swim stress in mice (Liu et al. 2004).

3.3 Regulation of the CRF neuron

Under basal conditions the CRF neuron is under inhibitory control by GABAergic interneurons, which act *via* GABA A receptors expressed on hypophyseotropic CRF neurons. If a stressor is perceived, depending on the type of stressor (blood loss, immune challenge, hypoglycemia), it is directly transmitted to the PVN *via* the brain stem and spinal cord projections to the PVN, or, in case of psychological stressors, integrated *via* limbic structures, such as the CeA, hippocampus, medial prefrontal cortex, or the bed nucleus of the stria terminalis (BNST). Additionally, CRF neurons in the BNST are innervated by oxytocinergic projections from magnocellular neurons of the PVN, thereby integrating stressors (CRF) and stress-protecting actions of OT to fine tune the organism's stress response. The BNST is a central relay station to integrate limbic inputs toward the PVN. The reciprocal innervation of CRF and OT neurons between PVN and BNST as described above is thought to be involved in balancing stress and affect (Dabrowska et al. 2011). Additionally, CRF neurons are under control of somatostatinergic / dopaminergic interneurons in the hypothalamus. Long-day photoperiods induce a depressive and anxiety-related behavior in nocturnal rats, *via* the induction of CRF release by somatostatinergic interneurons. These interneurons can switch their expression profile and start to synthesize dopamine, which down-regulates CRF release and plasma corticosteroids in response to a short-day photoperiod (prolonged active phase) (Dulcis et al. 2013).

Other neurotransmitters, such as dopamine, histamine, and acetylcholine are likely to modulate CRF neurons indirectly by modulating the activity of afferent neurons (Lee et al. 2008), for review see (Aguilera and Liu 2011)). Influences from the periphery are mediated by hormones such as angiotensin II, prolactin and leptin. Since these peptides cannot cross the blood-brain-barrier, specialized organs (circumventricular organs) receive these

hormonal signals and transmit them to central nuclei. Circumventricular organs, such as the subfornical organ, express the angiotensin II receptor to recognize peripherally released angiotensin II, which is the end product of the renin-angiotensin system, and transmit this stress-induced signal directly to the PVN. Another circumventricular organ, the choroid plexus, is involved in the uptake of prolactin, which results in hypo-responsiveness of the HPA axis during lactation (Blume et al. 2009).

3.4 Transcriptional regulation of the CRF gene

There is one gene encoding for CRF, which is located on the long arm of chromosome 8, with one promoter region, one intron (800 bp) and two exons (582 bp). The gene codes for the inactive pro-hormone. The pro-hormone is transcribed, translated, and processed into the active CRF peptide. There is a 97% homology of the promoter region between humans, mice, rats, and sheep, which suggests that signals leading to CRF gene transcription are highly conserved among mammals (King and Nicholson 2007). This allows for comparisons between animal and human hypothalamic response to stress. The human CRF gene promoter contains several response elements (RE) that, upon binding of their cognate factors, either activate or repress transcription of the CRF gene. There are three elements (metal-responsive transcription factor 1 RE, hybrid steroid RE, ecdysone RE) which activate gene transcription by inhibiting two repressive elements (Ying Yang RE, negative glucocorticoid RE (nGRE)). If these repressive elements are inhibited, the cyclic AMP RE (CRE) is free to bind CREB and its co-activators. There are also two canonical TATA-Box elements present in the CRF gene, only one of which seems to be involved in transcriptional regulation, the second one is thought to be silent (King and Nicholson 2007). The best studied inhibitory mechanism of CRF

transcription is the negative feedback regulation by glucocorticoids (GC). The activated HPA axis leads to the production and release of GCs, which cross the blood brain barrier to bind the GC receptor (GR) in the brain (hippocampus, PVN). This complex translocates to the nucleus and binds the nGRE in the CRF promoter. This inhibits the binding of the transcriptional machinery to the CRE region, thereby reducing CRF gene transcription (Jeanneteau et al. 2012).

Recent studies showed that the transcription of the CRF gene is also under the control of the CREB co-activator CRTC2. The molecular machinery employed is described in more detail in the CRTC section below. In brief, *in vitro* and *in vivo* studies revealed that CREB phosphorylation is essential but not sufficient for forskolin (FSK) or restraint stress-induced CRF gene transcription. The activation, i.e. dephosphorylation of the co-activator CRTC2 and its subsequent translocation to the nucleus is essential for a full transcriptional response (Liu et al. 2008; Liu et al. 2011).

3.5 Peripheral effects of CRF

CRF is the central regulator of the HPA axis, which mediates the organism´s response to a perceived stressor. The main regulatory brain region of the HPA axis is the hypothalamic PVN. The PVN contains the highest concentration of CRF neurons in the brain. It lies adjacent to the 3rd ventricle and consists of large magnocellular and smaller parvocellular neurons. The medial-dorsal parvocellular CRF neurons release CRF *via* the external zone of the median eminence into the hypophyseal portal blood capillaries to the adenohypophysis. These CRF-neurons co-express and co-release AVP together with CRF in response to a variety of psychological and physiological stressors into the portal circulation (Aguilera 1994, 1998;

Aguilera and Liu 2011). This increase serves as a trigger for the release of effector-hormones, such as ACTH, from the adenohypophysis into the blood stream. Interestingly, the circadian activation of the CRF neuron is delayed to the daily changes in circulating ACTH and corticosterone in rats (Watts et al. 2004), suggesting that HPA axis activity is driven by the release of CRF pool contents, followed by the transcriptional replenishment of the pools. During acute stress, CRF and AVP are rapidly released into the portal blood system to induce the full ACTH response. This release is followed by an increase of CRF gene transcription, and later on, AVP gene transcription (Herman et al. 1992; Ma et al. 1997). However, CRF is considered to be largely responsible for HPA axis activation, while AVP was reported not to be essential, but potentiating the organism's response to a stressor (Aguilera et al. 2008). Interestingly, other researchers favor a model of synergistic actions of AVP and CRF, instead of additive effects, to fully induce the stress response (Chen et al. 2008; Spiga et al. 2009). The binding of CRF to the CRF receptor 1 (CRFR1) expressed by the corticotrophe cells in the pituitary gland, activates adenylate cyclase, thus increasing cAMP levels. cAMP, in turn, activates protein kinase A (PKA), which translocates to the nucleus and up-regulates the expression of pro-opiomelanocortin (POMC). POMC can be cleaved in several active peptides, one of which is ACTH. The release of ACTH into the circulation will induce the synthesis and secretion of glucocorticoids (GC) upon binding to the melanocortin-2-receptor (Mc2r) expressed in the zona fasciculata of the adrenal cortex. The synthesis of GCs is rate-limited by the availability of the steroidogenic acute regulatory protein (StAR). The StAR enzyme stimulates the initial mitochondrial metabolism of cholesterol to pregnenolone by enhancing cholesterol transfer from the outer membrane to cytochrome P450 11A1 (P450scc) in the inner membrane (Stocco et al. 2001; Stocco et al. 2001; Jefcoate 2002). To initiate transcription of the StAR enzyme, the cooperative actions of multiple transcription

factors, including CREB (Clem et al. 2005) and its co-activator CRTC2 (Takemori et al. 2007; Liu et al. 2012), CCAAT enhancer- binding protein (C/EBP β), steroidogenic factor 1 (SF-1) (Manna et al. 2003; Manna et al. 2003) and GATA4 (Silverman et al. 2006) are required. The proximal region of the mouse StAR promoter contains three canonical 5' CRE half-sites (TGAC) that are bound by the activated CREB/ATF-1/CBP-p300/CRTC2 complex (Takemori et al. 2007; Liu et al. 2012).

3.6 Central effects of CRF

CRF plays an important role in the regulation of the HPA axis, but recent findings have shown that production and central release of CRF during stress influences neuronal structure and the functionality of the hippocampus and other brain regions. In fact, whereas mild or short-lasting stress enhances hippocampal function by augmenting synaptic plasticity, chronic stress impairs learning and memory by retraction of pyramidal cell dendrites, likely to be caused by the chronic presence of stress-induced CRF. The integrity of dendrites is only guaranteed if excitatory synapses are present on specialized structures called dendritic spines. The picture below represents an organotypic slice taken from a murine hippocampus and incubated with 100 nM CRF for 2 weeks. This treatment induces a significant loss of dendritic branching (Maras and Baram 2012).

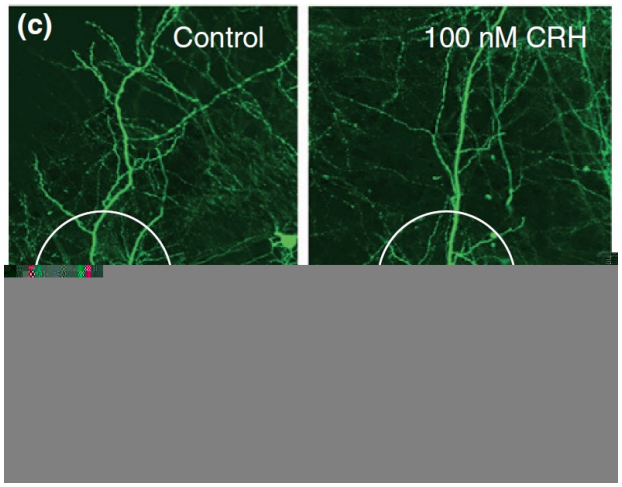


Figure 2 100 nM of CRF applied onto hippocampal organotypic slice cultures reduced dendritic complexity. Picture taken from (Maras and Baram 2012)

The number and functionality of dendritic spines is highly dynamic and can be influenced by neurotransmitters, hormones, and growth factors, which are in turn governed by environmental signals like stress (Maras and Baram 2012). Chronic stress, for instance, tends not only to cause dendritic retraction, but also inhibits neurogenesis and reduces survival of hippocampal neurons in a sex-dependent manner (Hillerer et al. 2013). In addition, acute (i.e. 30 min) restraint stress with subsequent intermittent tail stimulations increase spine densities in the hippocampal CA1 area of male rats (Shors 2009), while the same stimulus causes a decrease in hippocampal spine density in females. These sex-dependent stress-induced effects are also observed in the medial prefrontal cortex (mPFC), where stress decreases dendritic branching and length of pyramidal neurons in males, but increases these parameters in females (Garrett and Wellman 2009; Shansky and Morrison 2009). Stress-induced remodeling of layer V neurons in the mPFC has an impact on projections to subcortical regions like the BNST and amygdala, both being related to regulation of adaptive and maladaptive responses to stress (Gabbott et al. 2005; Conrad et al. 2011; Radley and Sawchenko 2011). The amygdala is most often linked to anxiety. However, recent human

and rodent studies revealed that the amygdala is more relevant to fear or short term anxiety responses, whereas the BNST is more involved in sustained anxiety (Davis et al. 2010; Leuner and Shors 2013). Intriguingly, the BNST is reciprocally connected with the PVN, with oxytocinergic projections toward the BNST, and CRF fibers originating in the BNST projecting toward the PVN (Dabrowska et al. 2013). This anatomical arrangement represents a direct morphological link of the CRF system with the oxytocinergic system. Considering the sex dependency of the stress response, reproduction-induced neuroplasticity (likely to be related to the oxytocinergic system), and the common brain regions these systems share, it is possible that OT is a sex-specific regulator of the CRF-driven stress response with major impact on the morphology of neurons in stress-related brain regions. It is tempting to speculate that the release of OT during stress serves as a modulator of CRF expression in a time-dependent manner, to prevent neuronal damage by large amounts of stress-induced CRF (see Results).

4. OTR-coupled signaling cascades and the regulation of CRF gene transcription

CREB and CRTC signaling is one example of how intracellular effectors of receptor activation can modulate gene expression. Today, literally hundreds if not thousands of such factors have been characterized, and some of them will be highlighted here as they might be involved in OT-mediated signaling and social behavior, or the control of CRF gene expression and stress responses.

Between 1994 and 2001, substantial progress has been made in the understanding of the mammalian MAP kinases. The first MAP kinase to be discovered was the extracellular signal regulated kinase 1 (ERK1), which is encoded by the MAPK3 gene. The closely related ERK2

(encoded by the MAPK1 gene) was identified in the same year. In the following years a plethora of mammalian MAPKs were identified, revealing an evolutionary highly conserved, and ubiquitously expressed enzymatic machinery in all eukaryotic cells. Mitogens, the first identified stimulators of MAPK signaling gave the protein family its name, although a diverse set of extracellular stimuli can activate MAPKs. Stimuli like hormones, growth factors, cytokines, GPCR regulated agents, transforming growth factor (TGF)-ß-related agents, and environmental stresses. As diverse as the stimuli are the effects on the cell physiology. MAPKs are involved in the regulation of gene transcription, protein biosynthesis, cell cycle control, apoptosis, and cell differentiation (Kyriakis and Avruch 2012).

The substantial core of MAPK signaling is a three-tiered module, in which activating signals are passed on as phosphorylation of specific motifs (Thr-X-Tyr) in the kinase subdomain VIII activation loop of the MAPK. The upstream kinases of MAPK are named dual specificity MAPK Kinase, MAP2K, MEK, or MKKs. These kinases are activated by Serine/Threonine (Ser/Thr) phosphorylation by a wide variety of MAPK kinase kinases (MAP3K) (Kyriakis and Avruch 2012).

MAPKs have remarkable substrate specificity. They phosphorylate Ser/Thr residues only if they are followed by a proline, and the presence of a MAPK docking site. Despite the high selectivity of the individual elements of the core signaling module, MAPK show promiscuous functions in related signaling cascades and are often regulated by a variety of extracellular signals. The question arose, how MAPKs maintain their signal/substrate specificity if the components are directed by proline and MAPK docking sites on the one hand, but can couple to multiple substrates on the other hand. The answer to this question lies in the function of scaffolding proteins, which sequester the elements of the cascade, bring them in

close physical contact, and thereby maintain signal specificity, and subcellular localization (Kyriakis and Avruch 2012; Roskoski 2012).

4.1 The ERK cascade

The highest upstream activators of the ERK cascade are receptor tyrosine kinases (RTKs) like the EGFR, which recruit the membrane associated proto-oncoprotein GTPase Rat sarcoma (Ras) *via* several possible intermediate signaling proteins (see Figure 3), depending on the subcellular localization of the cascade. Ras in turn phosphorylates the family of Raf MAP3Ks, such as Raf-1 (also known as c-Raf), A-Raf, and B-Raf. Raf-1 was first identified 1983 in murine retrovirus infected fibroblast cells, and named **V**irus-induced **R**apidly **A**ccelerated **F**ibrosarcoma (V-Raf). Later studies revealed the ubiquitous expression in all eukaryotic cells, so the gene's name was changed to cellular-Raf (c-Raf) (Osborne et al. 2011). C-Raf is the gatekeeper of the entire ERK cascade and therefore regulated at multiple steps. It contains an auto-inhibitory region, which physically blocks the kinase domain of the protein. Only GTP-bound Ras can compete with the auto-inhibitory block, leading to a conformational change and the relief of the kinase domain. Scaffolding proteins such as kinase suppressor of Ras (KSR) or 14-3-3 are either inhibitors or activators of Ras signaling, depending on their phosphorylation status. Non-phosphorylated 14-3-3 does not bind Ras, and this in turn is regulated by the TGF beta activated kinase 1 (TAK1) or the Protein phosphatase 1 (PP1) / Protein Phosphatase 2A (PP2A). Once c-Raf is in an active state, with its auto-inhibitory site deactivated, 14-3-3 can act as an activating scaffolding protein, by bringing Ras and its substrates (e.g. MEK1/2, BAD, adenylate cyclase and others) in close proximity (Osborne et al. 2011; Kyriakis and Avruch 2012). Interestingly 14-3-3 is also a scaffolding protein for

CRTCs, representing a direct spatial link between the MAPK cascade and the CRTCs (see CRTC section of the Introduction). MEK1 (also known as MAP2K1, MAPKK1, MKK1) and MEK2 are the direct downstream substrates of c-Raf. The protein MEK contains a phospho-domain in its C-terminus, and a trifunctional N-terminal sequence with an inhibitory segment, a nuclear export sequence, and an ERK binding site (Fischmann et al. 2009). Contrary to the complexity observed in c-Raf activation, MEK and ERK are simply activated by dual phosphorylation of their respective activating residues (Ser218 and Ser222 for MEK1; Ser222 and Ser226 for MEK2; Thr185 and Tyr 187 for ERK2 or Thr203, Tyr205 for ERK1). Interestingly, phosphorylation of Ser212 in MEK1 decreases the kinase activity of MEK *in vivo*, without interfering with the ERK2 binding or other activating phosphorylated residues (Gopalbhai et al. 2003). Other residues have been shown to be inhibitory as well, such as T286, which is phosphorylated by cyclin dependent kinase 5 (Cdk5). This kinase has several functions in cell proliferation, brain development and synaptic vesicle exocytosis. Furthermore, T292 in MEK1 is a substrate of ERK, which implies a negative feedback loop to reduce ERK activation by direct inhibition of MEK by ERK.

For many years, ERK was the only known substrate of MEK, only recently (2006-2010) new MEK targets were identified, such as PPARγ, MyoD, PI3K, or LIMKinase 1 in a variety of tissues (Brandt et al.; Jo et al.; Klemke et al.; Burgermeister et al. 2007). All these recent findings imply the possibility of MEK activation without subsequent ERK phosphorylation.

The distribution of the MAPK cascade in the rat brain has been studied intensively. MEK1 has been shown to be expressed in many brain regions, including the cortex, hippocampus, amygdala, bed nucleus of the stria terminals (BNST), lateral septum, hypothalamus (most notably in the PVN), cerebellum, and the brainstem (Flood et al. 1998).

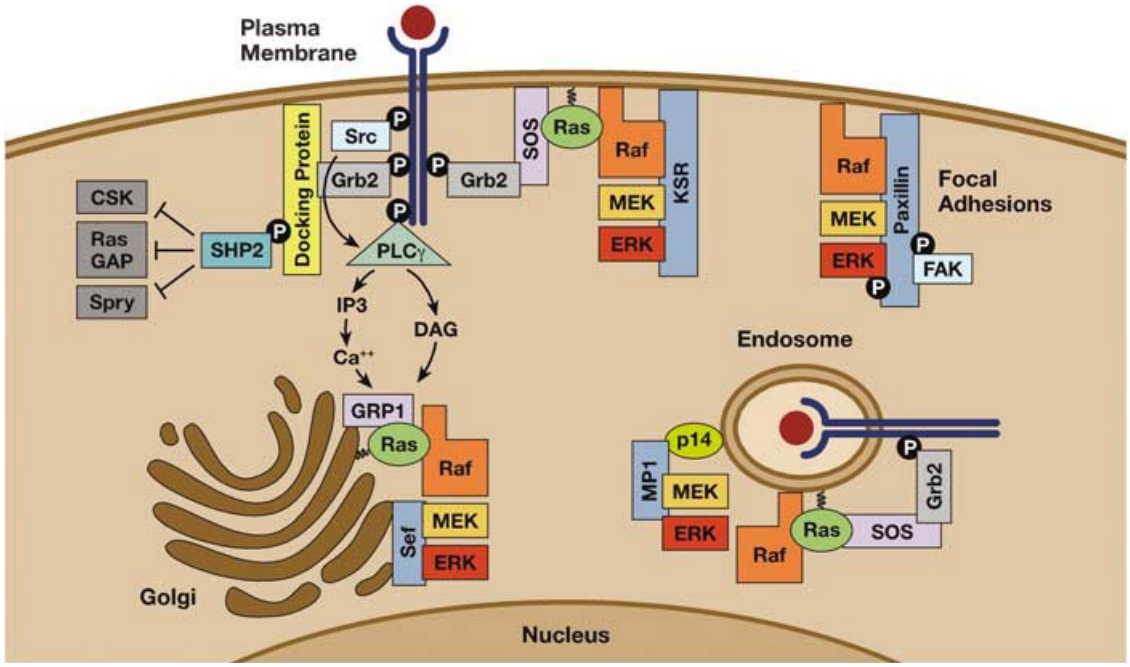


Figure 3 Receptor tyrosine kinase (RTK) to ERK signaling: Ras and ERK activation at various intracellular compartments. At the plasma membrane, activated RTKs promote Ras activation through the recruitment of growth factor receptor bound protein 2 / Son of sevenless (Grb2/Sos) complexes. Kinase suppressor of Ras (KSR) is an ERK scaffold that facilitates Ras dependent ERK cascade activation at the plasma membrane, whereas Paxillin directs ERK activation at focal adhesions. Active signaling complexes containing internalized receptors, Grb2/Sos and Ras are also found on endosomes. MEK partner-1 (MP1) is an MEK1/ERK1- specific scaffold that localizes to late endosomes through an interaction with the adaptor protein p14. Activated RTKs can also direct the activation of Golgi-associated Ras through a signaling route involving sarcoma (Src), Phospho-lipase-C (PLC)-γ and Ras-general receptor of phosphoinositides-1 (GRP1). Similar expression to fgf genes (Sef) is a Golgi-localized scaffold that recruits activated MEK and promotes ERK activation. Active ERK is retained on the Sef/MEK complex and confined to cytosolic substrates. The tyrosine phosphatase Src homology phosphatase-2 (Shp-2) is another effector of RTKs that positively regulates Ras signaling by antagonizing the ability of negative regulators, such as cellular-Src kinase (CSK), RasGAP and Sprouty, to access and downregulate critical enzymes involved in Ras activation. Picture taken from (McKay and Morrison 2007)

ERK1 and ERK2, two of the direct downstream targets of MEK1/2, transduce extracellular signals to the nucleus to regulate processes as diverse as proliferation, differentiation, apoptosis, and synaptic plasticity (Zhuang and Schnellmann 2006). When dephosphorylated, ERKs are basically incapable of catalyzing the transfer of phospho-groups (20 to 30 pmol min^{-1} mg^{-1}), mainly because interactions with cognate substrates are hindered by the

structural configuration of the activation loop of the kinase. MEK-mediated activation induces conformational changes that enhance the catalytic activities of the ERKs by about 5 orders of magnitude (5 µmol min^{-1} mg^{-1}) (Chang and Karin 2001; Kyriakis and Avruch 2012).

After being dually-phosphorylated, ERK1 dimerizes, which enables it to generate peak kinase activity, while a dual-phosphorylated monomer ERK1 is thought to maintain basal activity (Philipova and Whitaker 2005). However the nuclear translocation seems to be dimerization-independent, but is controlled by the rate of phosphorylation (Lidke et al.). These facts lead to the hypothesis that dual-phosphorylated ERK dimers, with the help of cytosolic scaffolding proteins that act as assembling platforms, target cytosolic proteins, and dual-phosphorylated monomers, that are released from these scaffolds, have nuclear targets. The release of ERK from its scaffolding proteins and the redistribution over cellular compartments, including the nucleus, cytosol, plasma membrane, intracellular vesicles, mitochondria and cytoskeletal components, enables contact with and subsequent phosphorylation of nearly 200 target proteins (Whitehurst et al. 2002; Yoon and Seger 2006). While there is evidence for a carrier-independent mechanism of nuclear entry of ERK2 (Whitehurst et al. 2002) by direct binding of ERK2 to a nuclear pore complex (Matsubayashi et al. 2001), the nuclear import of ERK1 is poorly studied. However, ERK1 seems to shuttle between cytosol and nucleus at a much slower rate than ERK2, and this difference is caused by differences in an N-terminal domain of ERK1. This N-terminal domain forms the main difference between ERK1 and ERK2; moreover, they share 85% of amino-acid sequence identity, and all other known functional domains (Marchi et al. 2008).

This shuttling of ERK is independent of its phosphorylation status, which might point toward a kinase-independent function of ERK in the nucleus. Indeed, recent studies discovered new

targets of ERK that are not necessarily modified by phosphorylation. ERK is involved in important cellular processes, such as chromatin remodeling, DNA transcription, and cell cycle regulation, acting independently of their kinase activities. In more detail, one example concerns the posttranslational modification of dual specificity phosphatase-6 (DUSP6) also known as MAPK phosphatase-3 (MKP-3), whose catalytic activity is increased 30 fold upon binding to ERK2. DUSPs specifically target MAPKs to fine tune their activity by dephosphorylation. Most DUSPs are encoded by inducible genes, the transcription of which is mainly regulated by MAPK themselves, or glucocorticoids. A second level of regulation is posttranslational modification of the phosphatase by its target. The binding of ERK2 to DUSP6 is sufficient to induce an allosteric conformational change that leads to the reorganization of the consensus acidic residue in the phosphatase, which results in enhanced catalytic activity. The specific recognition of ERK2 by MKP-3 is mediated by a conserved motif within the N terminal region of DUSP6 (Camps et al. 1998). Another non-canonical target that does not require kinase activity of ERK is poly (ADP-ribose) polymerase 1 (PARP-1), which is bound by phosphorylated ERK2, and activated without phospho-transfer activity. PARP-1 subsequently binds the transcription factor Elk-1, thus enhancing its transcriptional activity of genes such as c-fos. Acetylation of the core histones H3 and H4 is also enhanced in response to ERK2-mediated activation of PARP-1 (Cohen-Armon et al. 2007).

ERK2 is not only a signaling mediator between proteins, but also a DNA binding factor. High-throughput screening of the human protein-DNA interactome in HeLa cells identified ERK2 as a transcriptional repressor of interferon-γ (IFN-γ)–induced genes (Hu et al. 2009). Analysis of 82 genes that were increased in expression in response to knockdown of ERK2 revealed

G/CAAAG/C as the consensus sequence for the binding of ERK2. Within promoters, these ERK2-binding sites appear at around −90 base pairs, a typical distribution for many transcriptional regulators (Roy et al. 2000).

Besides these recently discovered non-canonical targets, ERK has its “normal” downstream signaling cascade substrates. The first identified substrate, which is exclusively phosphorylated by ERK1/2 and not by JNK or p38 (see p38 and JNK paragraph in the Introduction) was the ribosomal S6 Kinase (Rsk). This kinase derives its name from its kinase activity on the small ribosomal protein S6. However it does not represent the major kinase of the S6 protein, which is the S6K. Rsk is activated by ERK1/2, which phosphorylates the C-terminal catalytic domain. The activated catalytic domain auto-phosphorylates Rsk at Ser380, which provides a docking site for 3-phosphoinositide-dependent-kinase 1 (PDK1), which finally leads to full activation by phosphorylating Ser222 in the activation loop of the N-terminal domain (for review (Kyriakis and Avruch 2012). One factor that leads to MEK, ERK and Rsk activation in a hypothalamic neuronal cell line is AVP (Chen et al. 2009).

The shuttling of ERK and RSK is, among others, regulated by phosphoprotein enriched in Astrocytes-15 (PEA-15- or PED), a small protein (15 kDa) with an N-terminal death effector domain and an irregular C-terminal tail. The name suggests that it is expressed solely in astrocytes, but later studies revealed its ubiquitous expression in all neural cell types with a highly conserved structure among mammals (Araujo et al. 1993). The activation of PEA-15 and its control over ERK is tightly regulated. Under resting conditions, ERK is bound to PEA-15 in the cytosol. Upon EGFR-induced Ca^{2+} influx, activated Ca^{2+}-calmodulin dependent kinase II (CaMKII) phosphorylates the Ser116 residue of PEA-15, which allows the PKC mediated phosphorylation of Ser104. Dual-phosphorylation of PEA-15 releases ERK from

PEA-15 and allows its shuttling to the nucleus. The phosphorylation status of ERK is not altered by PEA-15 binding, so that PEA-15 restricts ERK activity to cytosolic targets, such as RSK or stathmins (Renault et al. 2003) (for the role of stathmins in behavior see Introduction JNK paragraph). The effects of PEA-15 on RSK are bi-functional. First, PEA-15 blocks nuclear accumulation of RSK after epidermal growth factor stimulation. Second, PEA-15 inhibits RSK2 kinase activity by 50 %. RSK1 is not bound by PEA-15 (Vaidyanathan and Ramos 2003; Vaidyanathan et al. 2007). The central importance of PEA-15 activity on MAPK cascades has been demonstrated by a PEA-15 knockout mouse line, which displayed altered CREB and c-fos mediated gene transcription activity. This results in a heightened stress reactivity and anxiety and impairment of spatial memory (Ramos et al. 2009).

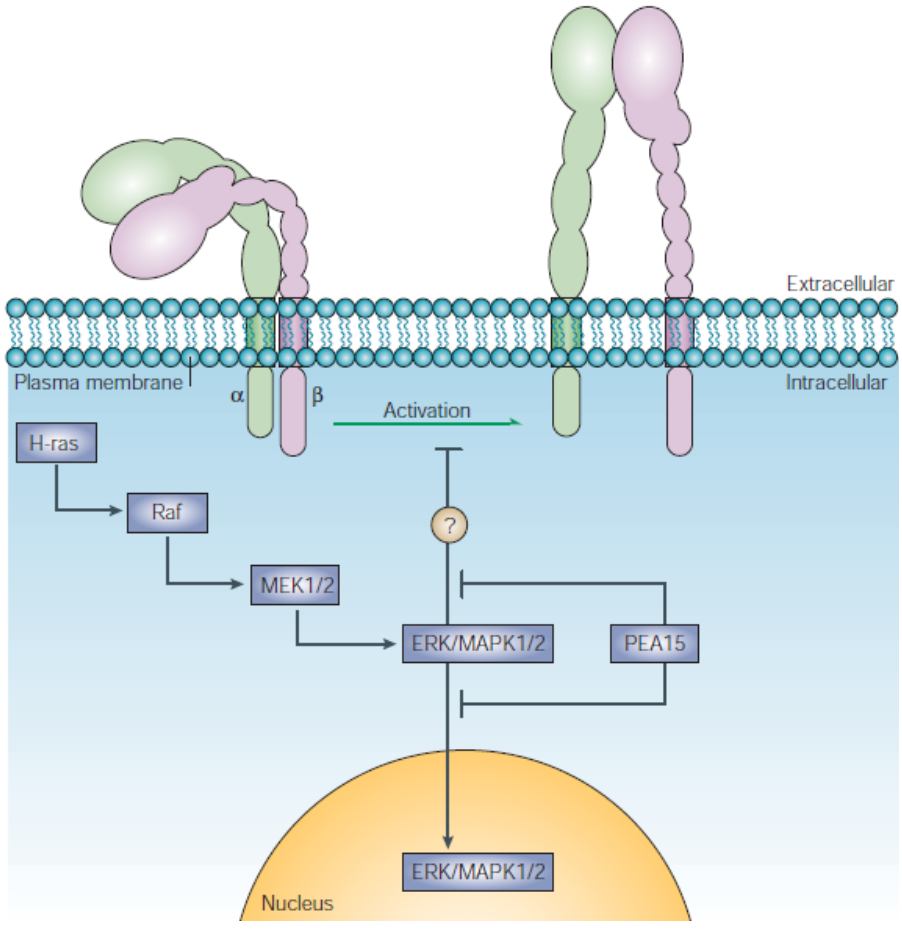


Figure 4 Depiction of the MAPK pathway and its inhibitory scaffolding protein PEA-15. Taken from (Kinbara et al. 2003)

Furthermore, the MAPK-interacting kinases (MNKs) are downstream ERK 1/2 targets. MNKs are involved in translational regulation, which occurs at the 5' methylguanosine cap structure. The eukaryotic initiation factor (eIF)-4E tethers mRNA onto a scaffolding protein, and with the help of other co-factors, unwinds the secondary structure of the mRNA, which allows the ribosomal complex to scan the mRNA. The exact role of eIF-4E phosphorylation by MNKs in transcriptional regulation is still under debate. However, directly related to eIF-4E phosphorylation is another interesting translational repressor binding protein (4E-BP1), which interrupts the eIF-4E interaction with its co-factors, but dissociates from the translational complex upon phosphorylation induced by the mammalian target of rapamycin (mTOR) (Sonenberg and Gingras 1998). Studies have shown a co-localization of activated (i.e. phosphorylated) mTOR and oxytocinergic neurons of the PVN (Lembke et al.) in the context of energy balance in fasted rats. mTOR is a protein central to the regulation of protein translation, and has shown to be involved in mediating the fast antidepressant effects of NMDA antagonists by synapse formation (Li et al.). The central role of mTOR in the regulation of mRNA translation, its regulation by MAP Kinases (see Figure 5) and its co-localization with OT in the PVN, identifies the possibility for a role in mediating the effects of OT on protein synthesis (Devost et al. 2008), and hence sustained anxiolytic effects.

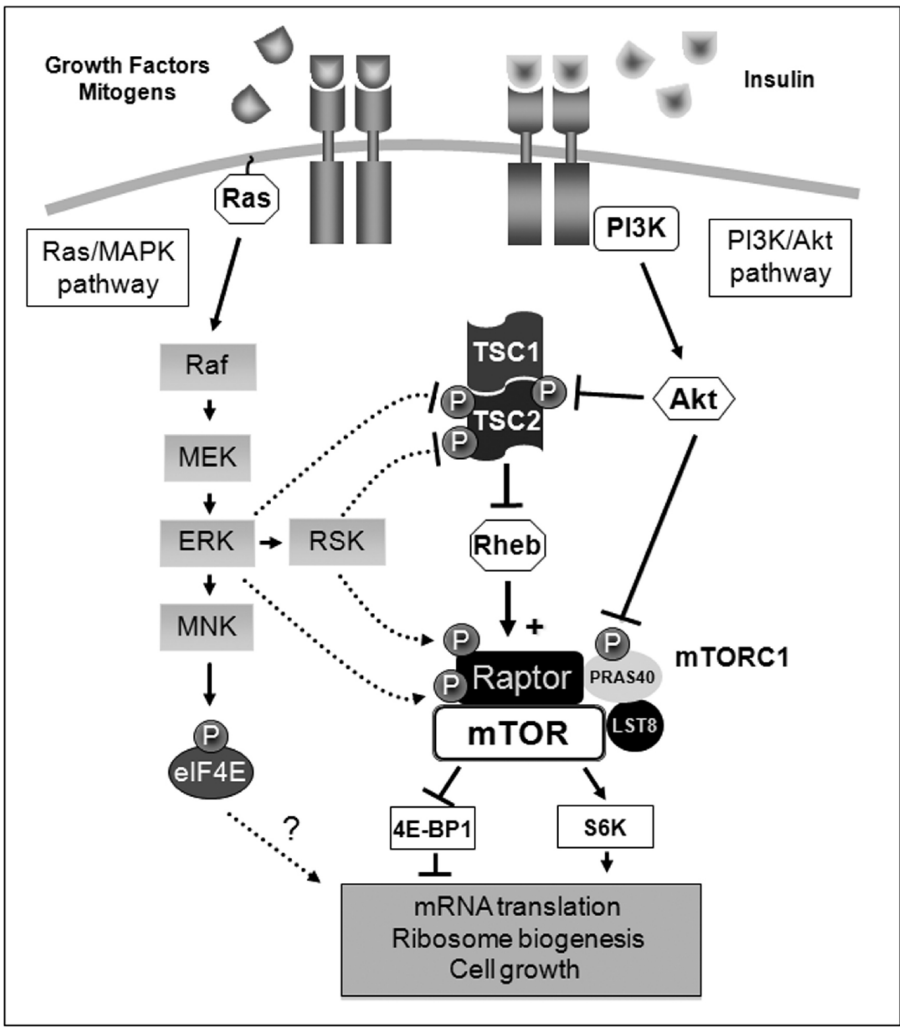


Figure 5 Schematic representation of the molecular mechanisms employed by the Ras/MAPK pathway to regulate mTORC1 signaling. Activation of the Ras/MAPK pathway stimulates mTORC1 activity through the coordinated action of the kinases ERK1/2 and Rsk on the tuberous sclerosis (TSC) protein complex upstream mTORC1, and on Raptor, an important partner of mTOR within mTORC1. Taken from (Carriere et al. 2011)

Another family of ERK substrates are the Mitogen- and Stress-activated Protein Kinases (MSKs). MSKs can be phosphorylated by ERK1/2 and p38 (for p38 see respective chapter in Introduction), and are therefore mediators of many physiological and pathological stimuli. Their most acknowledged activity lies in the phosphorylation of CREB and nuclear factor κB (NF- κB).

Recent studies revealed an additional role for MSKs in histone phosphorylation, which leads, just like histone acetylation, to chromatin relaxation and facilitated binding of proteins

involved in transcription. It has been reported that MSKs were necessary for EGF-induced Histone 3 (H3) Ser10 phosphorylation of the cellular-**F**inkel-Biskis-Jinkins murine **o**steo**s**arcoma virus homolog (c-Fos) - promoter (Duncan et al. 2006). MSKs are predominantly found in the nucleus and can partly be relocated to the cytosol upon GC signaling. It is therefore suggested that MSK1 contributes to the anti-inflammatory actions of GCs, by impairing transcription of genes related to inflammatory processes by nuclear export of MSKs. Gene transcription is regulated by transcription factors, of which CREB is the most studied one. The influence of MSKs on CREB and its result on gene transcription will be described in the CREB paragraph of the Introduction and in the Results section.

4.2 P38 and JNK

P38α was the first isolated isoform of the stress-, inflammatory cytokines, and endotoxin-induced MAPK p38. There are four p38 genes (p38α to p38δ), however, p38 is a MAPK often overseen and poorly studied. There is evidence, that p38 disrupts BDNF-induced TrkB signaling, thereby inhibiting long term potentiation (LTP) (Tong et al.), but the effects of p38 are often studied in relation with other members of the MAPK family, because exclusive p38 activation is rarely seen (Chang 2012). The downstream targets of p38 are mainly MNK1/2, which regulates the activity of eIF-4E, and MSK1/2, which is the main kinase of the transcription factor CREB (see CREB paragraph in the Introduction).

The dominant stress-induced MAPK is the stress activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK). It is named after its substrate, the transcription factor c-Jun, short for cellular–ju nana, which is Japanese for 17 and refers to the avian sarcoma virus 17, from which the protein has been originally isolated. C-Jun recognizes and binds to the enhancer

heptamer motif 5'-TGA(C/G)TCA-3'. It activates gene expression through direct interaction with enhancers, and has structural similarities with the activating protein 1 (AP1) (Bohmann et al. 1987). There are three main isoforms, JNK1, JNK2, and JNK3, encoded by the MAPK 8, 9, and 10 genes, respectively. Each JNK isoform contains the typical MAPK Thr-X-Tyr phospho-acceptor loop in its kinase subdomain VIII. The JNK specific phosphorylation sites are Thr183 and Tyr185 (for review (Kyriakis and Avruch 2012). The JNK1 and JNK2 isoforms are ubiquitously expressed, while JNK3 is almost exclusively found in the brain and testis. Upstream of JNK are the MAP2Ks MKK4 and MKK7. MKK4 is localized in the cortex, hippocampus, amygdala, BNST, thalamus, hypothalamus, cerebellum, and brainstem of the adult rat central nervous system (Flood et al. 1998), and was shown to co-activate p38, while MKK7 is a specific upstream kinase of JNK, expressed at a much lower level, especially in the liver (Nishina et al. 1999). However, whereas MKK4 is present in cell body, dendrites and axons of neurons, MKK7 is exclusively detected in the nucleus. JNK is phosphorylated by MKK4/7 upon stressful stimuli in an isoform-dependent manner. It has been shown that JNK2/3 are strongly activated by trophic withdrawal, while JNK1 phosphorylation remained constant (Coffey et al. 2002). Constant JNK1 activity is related to the stability of microtubule associated protein, which influences dendritic and axonal outgrowth. It is therefore highly possible that MKK4 plays a prominent role in regulating the establishment of functional neural circuits in the brain *via* JNK1 (for review see (Wang et al. 2007). The interplay between MKK7 and JNK can be disrupted by activated (hormone-bound) GR. It was shown before that GC can repress the activation of the transcription factor activator protein 1 (AP-1), and this repression is likely mediated *via* physical interaction with JNK through a hormone-regulated docking site located in the ligand-binding domain of the GR. Thus, stress-induced GCs can interrupt JNK-mediated stabilization of microtubules, which is a necessary

prerequisite for axonal branching and therefore neuronal plasticity and memory formation. In fact, GCs are not the only regulators to induce dendritic and axonal outgrowth *via* JNK. Jeanneteau et al could show that BDNF-induced MAPK phosphatase-1 (MKP-1; also known as dual specificity phosphatase-1 (DUSP-1)) activation (*via* TrkB-ERK signaling) leads to JNK dephosphorylation, microtubule destabilization (*via* stathmin dephosphorylation), and subsequent axonal branching (Jeanneteau et al.). Stathmins, the downstream effectors of JNK, have been shown to be involved in the establishment of social and parental behavior in the basolateral amygdala of female rats (Martel et al. 2008). This pathway shows one possibility of how intracellular cascades can, by influencing cytosolic target proteins, change neuronal circuits and thereby the behavior of an animal.

4.3 The transcription factor CREB

Cyclic AMP responsive element binding protein (CREB) is a transcription factor that promotes genome-wide changes in gene expression if activated by hormonal (GPCR) stimuli, Ca^{2+}-influx, growth factors, osmotic stress and ultraviolet irradiation. CREB-regulated gene expression, such as that of c-fos, BDNF, neuronal nitric oxide synthase (nNOS), somatostatin, peroxisome proliferator-activated receptor γ (PPARγ) co-activator 1α (PGC1α), B cell lymphoma 2 (Bcl-2), the StAR enzyme and CRF (Yamamori et al. 2004; Sasaki et al. 2011), is the molecular basis of adaptive behavioral changes that occur in response to a changing environment, learning and memory in mature organisms, as well as cell proliferation and differentiation in a range of cell types of developing vertebrates (Bonni et al. 1999; Riccio et al. 1999; Walton et al. 1999).

CREB was first discovered 1987 as a gene transcription activating, DNA binding protein in the promoter sequence of the somatostatin gene (Montminy and Bilezikjian 1987). The binding region is named “cyclic AMP responsive element” and concerns typically either palindromic (TGACGTCA) or half site (TGACG or CGTCA) sequences. A small number of variants have been described since then. There are about 750.000 palindromic or half sites in the human genome, but the large majority is silenced by disruptive cytosine methylation within the CRE. Non-methylated CREs in the promoter of active genes appear in about one quarter of the human genome (~ 5000 genes). CREB typically contains an amino-terminal transactivation domain (TAD) and a C-terminal basic Leucine zipper (bZIP) DNA binding and dimerization domain. Upon phosphorylation, CREB forms either homodimers with itself, or heterodimers with the other members of the CREB family, including ATF and CREM. The TAD consists of a central kinase inducible domain (KID) and a cAMP responsive glutamine-rich domain (Q2 domain), which enhances transcription by interacting with the “TATA-box binding protein associated factor 4” (TAF4), a component of the basal transcription factor IID (TFIID). In contrast to the Q2 domain, the KID domain is activated in response to cAMP and Ca^{2+} through a phosphorylation-dependent mechanism. In general, binding of a ligand to a GPCR activates the stimulatory subunits of the G-protein (α, β, γ), which in turn activate the membrane bound adenylate-cyclase (AC) to synthesize cAMP from ATP. cAMP binds to the 2 regulatory subunits of the PKA, thereby releasing the catalytic subunits of PKA. The catalytic subunits translocate to the nucleus by passive diffusion and phosphorylate nuclear CREB at Ser133 in its KID domain. Phosphorylation at Ser133 promotes association of the KID with the KIX domain of the co-factor CBP/p300, which enhances CREB target gene expression by acetylating histones, and recruiting RNA polymerase II complexes.

In neurons, trans-synaptic release of glutamate triggers Ca^{2+}-influx through N-methyl-D-aspartate (NMDA) receptors and L-type voltage sensitive Ca^{2+} channels (L-VSCC). This elevated Ca^{2+}-level is responsible for activating nuclear CaMKIV, which phosphorylates CREB at Ser133 at an early phase, but also, by activating the MAPK pathway, to prolong Ser133 phosphorylation *via* RSK (Wu et al. 2001).

The estimated number of CREB molecules per cell (40.000) (Hagiwara et al. 1993) is in far excess of the number of putative CREB target genes (5000) (Zhang et al. 2005). Additionally, many different stimuli lead to CREB Ser133 phosphorylation. To maintain stimulus / substrate specificity, a set of co-activators and/or stimulus-specific CREB modulations that direct activated CREB molecules to the promoter of specific target genes has been hypothesized. Indeed, the group of Greenberg discovered a mechanism, how a specific stimulus (Ca^{2+}-influx) can activate a subset of CREB regulated target genes by Ca^{2+}-dependent phosphorylation of the Ser142 and Ser143 in the KID domain of CREB by CaMKII. Triple phosphorylation of CREB (Ser133, Ser142, and Ser143) induces selective Ca^{2+}-dependent gene transcription, different from that of monophosphorylated CREB at Ser133, by disrupting the binding of CBP to CREB *via* the KIX (CBP) - KID (CREB) domains, which allows gene transcription only of genes that are not dependent on the presence of CBP. Interestingly, phosphorylation of the Ca^{2+}-dependent Ser142/143 seems to follow slower kinetics compared to the rapid Ser133 phosphorylation (see Figure 6 and (Kornhauser et al. 2002)), a mechanism that might prolong CREB activation independent of CREB Ser133 phosphorylation.

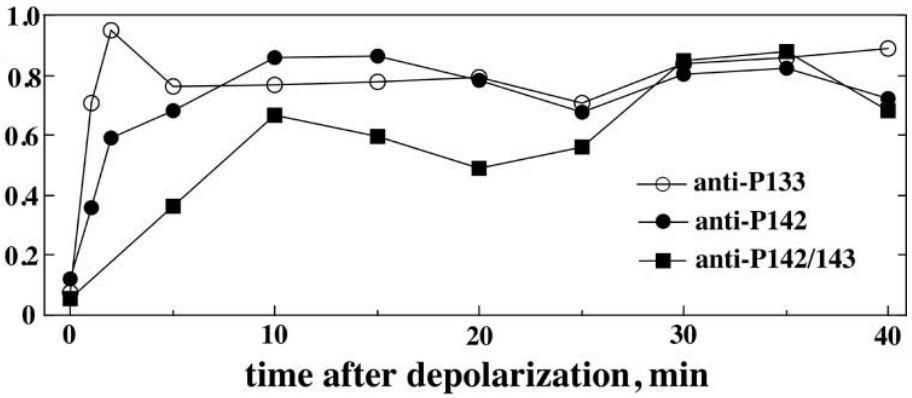


Figure 6 Distinct kinetics of depolarization-induced phosphorylation of CREB at Ser133, Ser142, and Ser142/Ser143. Values within each experiment were normalized to the highest value (maximum level = one), and mean values across experiments are plotted for anti-P133 (open circles), anti-P142 (closed circles), and anti-P142/143 (closed squares) signals. Each data point represents at least three independent experiments. adapted from (Kornhauser et al. 2002)

The physiological importance of the MAPK mediated CREB phosphorylation for spatial memory was revealed by Tomizawa and co-workers (Tomizawa et al. 2003) by icv injection of OT in virgin mice, which improved spatial learning and enhanced CREB phosphorylation by MAPK (MAPK inhibitors blocked these inductions). Conversely, an OT antagonist inhibited the improved spatial memory, LTP, and CREB Ser133 phosphorylation.

4.4 The CRTC family of CREB co-activators

In 2003 Conkright and co-workers screened the human genome for potent CRE activators, and found the cyclic AMP regulated transcriptional co-activators (CRTC) 1 and CRTC2. A third co-activator CRTC3 was identified by BLASTP search of the GENBANK NR database. The group named these co-activators originally “transducer of regulated CREB activity (TORC), but growing confusion with the mammalian target of rapamycin complex (mTORC) convinced the authors to rename the co-activators to CREB regulated transcriptional co-activator (CRTC) [also referred to as mucoepidermoid carcinoma translocated protein

(MECT)]. CRTC1 is predominantly expressed in the brain, while CRTC2 and CRTC3 are ubiquitously expressed (Conkright et al. 2003). CRTCs potentiate CREB activity *via* a phospho-Ser133- independent mechanism by binding CREB *via* its bZIP domain, thereby enhancing the association of CREB with the TAFII130 component of the TFIID and the CREB binding protein (CBP) as well as the histone acetyltransferase paralogue p300 (Ravnskjaer et al. 2007). This association was shown to be enhanced by lithium, thereby in part explaining the mood modulatory activity of lithium by changing CREB dependent gene transcription (Heinrich et al. 2013). All three CRTCs have a similar modular structure: an N-terminal CREB binding domain (CBD), a central regulatory domain (REG), a splicing domain (SD), and a C-terminal TAD (Luo et al. 2012).

A second function of CRTCs is the regulation of alternative splicing. Studies revealed the binding of CRTC to the spliceosome factor non-POU domain –containing octamer-binding (NONO, also known as p54NRB), to regulate alternative splicing of TATA box-less genes. It may be even a mutual process, insofar that CRTCs have no effect on transcription from TATA-less promoters, but are competent to promote alternative splicing of these genes. Alternative splicing induced by CRTCs does not occur in promoters with a TATA-box, like the CRF gene (King and Nicholson 2007).

13 years ago, it has been discovered that the PKA-mediated phosphorylation of CREB on chromatin templates was relatively inefficient, compared to the phosphorylation over naked templates, i.e. DNA without any proteins bound to it. This effect was reversed by acetylation of nucleosomes, which increases the accessibility to chromatin (Michael et al. 2000). A very recent study solved the problem by revealing the recruitment of arginine methyltransferase 5 (PRMT5) by CRTC2. PRMT5 is a type II methyltransferase that symmetrically mono- or di-

methylates arginine residues in histone 3. CRTC2 is activated by cAMP, translocates to the nucleus, recruits PRMT5 to relevant target gene promoters, which increases nucleosome clearance over CREB binding sites (Tsai et al. 2013) by arginine2-di-methylation of the H3. This opens the chromatin structure and allows the phosphorylation of CREB by PKA.

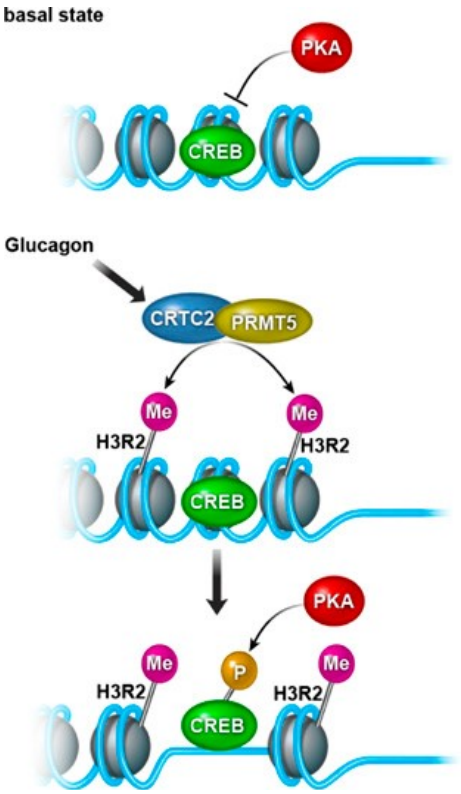


Figure 7 Model of gene activation by concerted action of CRTC2 and PRMT5 to allow CREB phosphorylation by PKA. Under basal conditions CREB over CREB-binding-sites is not accessible for PKA. An extracellular signal induces cAMP production, which activates CRTC2, which in turn recruits PRMT5 to the target promoter region. PRMT5 induces nucleosomal clearance over the target region, by di-methylation of arginine2 on Histone3, which allows for CREB phosphorylation and subsequent gene expression. taken and adapted from (Tsai et al. 2013)

The group of Greti Aguilera could prove the involvement of CRTCs in the transcriptional regulation of the CRF gene. CRTC1, 2, and 3 are translocated to the nucleus upon stimulation with the adenylate cyclase stimulator FSK in 4B cells and primary hypothalamic neurons, where they bind to CREB to activate transcription at the CRF promoter (Liu et al. 2010). Further studies on the physiological relevance of this mechanism revealed CRTC2

immunoreactivity in magno- as well as parvocellular regions of the hypothalamic PVN. This hypothalamic CRTC2 was found to be mostly cytosolic under basal conditions, but translocates to the nucleus under stressful conditions (restraint stress). Translocation only occurred in parvocellular CRF neurons (Liu et al. 2011). Distribution studies revealed specific expression of CRTC subforms in brain nuclei, with CRTC1 being the most abundantly expressed subform in the forebrain, while CRTC2 was found to be located in discrete nuclei as the PVN, SON and suprachiasmatic nucleus. CRTC3 expression was strong in the ependyma and pia mater, whereas being low in the PVN (Watts et al. 2011). Subsequent studies revealed SIK2 as the upstream inhibitor under basal conditions, whereas induction of SIK1 limits transcriptional activation of CRF expression (Liu et al. 2012).

4.5 Salt-inducible kinase (SIK)

Under basal conditions, CRTCs are sequestered in the cytosol by phosphorylation-dependent binding to the scaffolding protein 14-3-3. This phosphorylation is mainly maintained by the salt inducible kinase (SIK). This kinase was first identified as an enzyme induced in the adrenal glands of rats fed with a high-salt diet (Wang et al. 1999). The three subforms (SIK 1-3) belong to the family of AMP-activated protein kinases (AMPKs), which are known to be involved in energy homeostasis (Winder and Hardie 1999). SIK1 expression in the hippocampus is involved in the development of cortical neurons by regulating CRTC1 (Feldman et al. 2000; Li et al. 2009). Sasaki et al (2011) found high levels of SIK2 in neurons and that a decrease in SIK2 is accompanied by the dephosphorylation of CRTC1. Interestingly, the decrease of SIK2 was shown to be induced by CaMKI / IV phosphorylating Thr484 in the SIK2 protein, which leads to proteasomal degradation. The CaMKIV activation

seems to be dependent on NMDAR subtype NR2A-mediated Ca^{2+}-influx (Sasaki et al. 2011). In this way, CaMKIV can activate CREB regulated gene transcription *via* two separate mechanisms: First, *via* NR2A mediated Ca^{2+}-influx, CaMKIV phosphorylates SIK2, thereby leading to its degradation. This inhibits CRTC1 phosphorylation and maintains its nuclear activity. Second, CaMKIV phosphorylates CREB at Ser133 directly to activate its transcriptional activity. In addition, CaMKIV can also phosphorylate CBP and thereby stimulate CREB-dependent transcription (Hardingham et al. 1999; Impey et al. 2002). A Ca^{2+}-independent inhibitory mechanism is represented by SIK2 phosphorylation at Ser587 by cAMP-induced PKA activity. This phosphorylation leads to SIK2 inhibition, but in contrast to Thr484 phosphorylation, this is not followed by proteasomal degradation.

4.6 Calcineurin

Calcineurin (CaN) is a Ca^{2+}-activated protein phosphatase, also known as protein phosphatase 3 (PP3). Calcineurin consists of two subunits: a catalytic subunit A, and a regulatory subunit B. It was shown to be involved in the dephosphorylation (activation) of the CRTCs. This dephosphorylation is Ca^{2+}-dependent, and the expression of TrpV6 was shown to induce CaN activity. However, other Ca^{2+} channels might also play a role in activating CaN through Ca^{2+}-influx from the extracellular space. This increase in intracellular Ca^{2+} leads to binding of Ca^{2+} to calmodulin, and this complex then activates CaN *via* direct interaction with the regulatory subunit (Yamashita et al. 2000). Upon Ca^{2+}-induced activation of calcineurin, CRTCs are dephosphorylated and are free to translocate to the nucleus, where they bind CREB at its bZIP domain. CRTC itself, like all other transcriptional co-activators, has no DNA binding activity.

4.7 Pin1 and its interactions with ERK1/2 and CRTC2

Pin1 is a peptidyl-prolyl *cis/trans* isomerase (also known as NIMA interacting protein), that is bound to activated MEK2, which acts as a scaffolding protein. Upon activation by MEK2, Pin1 has different effects. It has a role in prolonging MAPK signaling by inhibiting the negative feedback phosphorylation of Ras (Dougherty et al. 2005), thereby sustaining MAPK cascade activity, but has also been shown to inhibit ERK1/2 phosphorylation *via* a Rho GTPase-activating protein in the context of cell migration (Pan et al. 2010). This finding is of special interest for the work presented in this thesis, because it provides one possible explanation for the lack of ERK activation in spite of activated MEK1/2 in the PVN upon OT treatment. Pin1 not only inhibits ERK phosphorylation, it also promotes the translocation of CRTC2 out of the nucleus and into the cytosol, thereby inhibiting the interaction with CREB (Nakatsu et al. 2010). This leads to transcriptional down-regulation, comparable to the inhibition of CRF gene transcription described in the results section of this thesis.

5. Aim of the present thesis

Anxiety-related disorders, caused by chronic and/or psychosocial stress, have become a major burden in our modern society, and its impact on our health system is constantly increasing (Wittchen et al. 2011). For effective treatment, detailed understanding of the molecular machinery that regulates the perception and processing of stressful cues and anxiety is urgently needed.

The neuropeptide OT has received substantial attention during recent years, due to its anxiolytic properties revealed in the PVN and amygdala and to its apparent stress-reducing activity. As outlined above, OT exerts many other behavioral and physiological effects, so

before OT can be of routine pharmacy-therapeutic use for the treatment of anxiety disorders, detailed research must unravel how OT brings about its anxiolytic and stress-protective effects. This not only concerns the cell types and brain regions involved, but also the molecular mechanisms at the intracellular level that are at the basis of these effects. Especially the latter has not received much attention in the scientific community, because of the complexity of intracellular signaling and subsequent control of gene expression and neuronal activity.

The work presented in this thesis aimed to unravel some of the intracellular signaling pathways that mediate the anxiolytic and stress-inhibiting effects of OT in the PVN. In the first part of my experiments, I aimed to reveal MAPK signaling pathways that are activated following OTR activation in male as well as in female rats at different phases of reproduction (virgin, pregnant, lactating). The role of some of these signaling pathways in the regulation of anxiety will be shown. In the second part, I will describe my efforts to identify a specific intracellular signaling pathway, which is activated by OT and reduces the expression of CRF during stress.

The data presented in this thesis reflect the complexity and specificity of intracellular signaling pathways that are coupled to the OTR. This might serve as a basis for future identification of intraneuronal factors that specifically mediate the anxiolytic and anti-stress effects of OT. These factors would make promising candidates for the development of specific medication to treat anxiety disorders and to reduce the negative consequences of stress.

Materials & Methods

Materials & Methods

1. Animals

Adult female or male Wistar rats (Charles River, Germany, 220–300 g body weight at the beginning of the experiment) were housed under standard laboratory conditions in groups of 3 to 4 (12 h light : dark cycle, 22 – 24 °C, lights on at 06.00 h, food and water ad libitum). All experiments were performed between 08:00 – 11:00, approved by the government of the Oberpfalz, Germany, and performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, Bethesda, MD, USA.

For pregnancy related experiments or primary neuron isolation, mating with a sexually-experienced male (300 – 350 g) was performed after at least one week of habituation. Confirmation of pregnancy was accomplished by observing the presence of sperm in vaginal smears and designated as pregnancy day 1.

1.1 Surgical procedures

All surgical stereotaxic procedures were performed under isoflurane anesthesia and semi-sterile conditions on pregnancy day (PD) 14, lactation day (LD) 2 and in age-matched virgin females and males. Following surgery, rats received an s.c. injection of antibiotic (0.03 ml enrofloxacin; 100mg/1ml Baytril, Bayer). Animals were allowed to recover before undergoing behavioral or protein phosphorylation assessment six days after surgery (i.e. PD 20 or LD 8). Rats were single-housed after surgery and handled daily to habituate them to

the respective central infusion procedure and to avoid non-specific stress responses during the experiment.

For analysis of the hypothalamic MAPK pathway in virgin, pregnant and lactating female rats under the influence of icv vehicle, OT (Sigma), or MEK1/2 inhibitor (U0126, Sigma) infusion, an indwelling icv guide cannula (stainless steel, 21 G, 12 mm long) was stereotaxically implanted 2 mm above the right lateral ventricle (AP: −1.0 mm bregma, ML: +1.6 mm lateral, DV: +1.8 mm below the surface of the skull; (Paxinos and Watson 1998)) as previously described (Blume et al. 2008; Slattery and Neumann 2010).

To determine the effect of OT on hypothalamic CRF expression, MAPK activation, anxiety-related behavior, and CRTC translocation, vehicle, OT (Sigma) or (Thr^4 Gly^7)-OT (TGOT) (Bachem) were infused through an indwelling icv guide cannula (stainless steel, 23 G, 12 mm long) that was stereotaxically implanted 2mm above the third ventricle (AP: +0.9 mm bregma, ML: -1.2mm lateral, DV: +5 mm below the surface of the skull, Angle: 10°; (Paxinos 1998; Baird et al. 2008; Roy et al. 2008).

For analysis of local effects of OT within the PVN on anxiety-related behavior, indwelling bilateral guide cannulas (stainless steel, 23 G, 12 mm long) were implanted 2 mm above both the left and right PVN (AP: -1.4 mm bregma, ML: -1.8 and +2.1 mm lateral; DV: +6 mm below the surface of the skull; angle 10°; (Paxinos and Watson 1998; Blume et al. 2008), and anchored to two stainless-steel screws using dental acrylic. The guide cannulas were kept viable with dummy cannulas, which were removed daily and cleaned during the handling procedure.

1.2 Light-dark box

To assess the effects of OT, TGOT, and MEK inhibitor infusions bilaterally in the PVN / 3rd ventricle of males or virgin / lactating female rats on anxiety-related behavior, the animals were tested in the LDB seven days after the implantation of the guide cannulas (i.e. LD 8 or equivalent in age-matched virgins) and 10 min after the last drug infusion. The LDB test was performed as previously described (Waldherr and Neumann 2007; Slattery and Neumann 2010). Briefly, the LDB setup consisted of two compartments; one lit compartment (40 x 50 cm, 350 lux; light box) and one dark compartment (40 x 30 cm, 70 lux). The floors in each compartment were divided into squares (10 x 10 cm) and the compartments were connected *via* a small opening (7.5 x 7.5 cm). Rats were placed in the dark compartment, as described in (Jurek et al. 2012)). In later studies of CRF gene expression, rats were placed in the light compartment first, and latency to first light compartment re-entry, as well as line-crosses, time spent in each compartment, and rearings were assessed during the 5-min test on-line *via* a camera located above the box by an observer blind to treatment. The time spent in the light box by the vehicle/vehicle group was set to 100 % for each experiment.

1.3 Restraint Stress

To analyze the influence of icv OT or TGOT on basal and stress-induced CRF expression and CRTC translocation, four groups of rats were used in separate experiments: (1) a vehicle (Ringer) / no stress group, (2) an OT or TGOT / no stress group, (3) a vehicle / stress group, and (4) an OT or TGOT / stress group. OT and TGOT are equally effective at the concentration applied (1 nmol in 2 µl; compare (Blume et al. 2008; Jurek et al. 2012) with (Lukas et al. 2011; Viviani et al. 2011) and have a similar affinity for the OTR (Manning et al.

2008)). TGOT was used in some of the experiments to exclude the contribution of the AVP receptors to the observed effects. The drugs or their vehicle were infused 10 min prior to the restraint stress procedure, and the animals were returned to their home cage during this period. Then, the animals were restrained in a Plexiglas column (12 cm diameter) with ventilation holes, and were kept there for 10, 15, or 30 minutes. The non-stressed controls were time-matched to accommodate for the time of the stressor. Immediately after restraint, rats were decapitated, trunk blood was collected and the brains were dissected and quickly frozen on dry-ice and stored at -80 °C until cryo-sectioning for CRF expression. The brains were cut at a thickness of 250 µm, and the PVN isolated with a tissue puncher (Fine Science tools 1.8 mm diameter; (Jurek et al. 2012)). RNA was extracted by addition of 1 ml of TriFast Gold (PeqLab) to the isolated tissue. Tissue dispersion was achieved by extensive vortexing. The lysate was then either frozen at -80°C (for RNA isolation), or immediately processed (for protein isolation). For CRTC translocation analysis, non-frozen, freshly isolated hypothalami were dissected, the PVN punched out, and transferred to ice-cold lysis buffer (see below). In addition, cortical tissue served as a control for brain region specific effects of stress and OT.

1.4 Verification of cannula placements

After the experimental procedure, the animals were sacrificed. For verification of the placement of icv cannula, following dissection of the hypothalamus, the rest of the brain was snap-frozen in isopentane cooled to -32°C by dry-ice. Localization of the cannula tract was then performed using 40-µm cryosections stained with Nissl and with aid of a rat brain atlas (Paxinos and Watson 1998). For PVN cannula verification, blue dye was infused into the PVN as described above, then the brain was snap-frozen in isopentane and histological

assessment performed as described for the icv cannulas above. Additional verification of cannula placement for the 3rd ventricle is the fact that TGOT and AVP infusions into neighboring nuclei (i.e. the ventromedial hypothalamus, PVN) at concentrations we used for icv infusions (1nmol/2 µl) would lead to immediate severe overdose-effects.

2. Cell culture and stimulation

2.1 Primary hypothalamic neurons

Fetal Wistar rats, embryonic d 18, were used to obtain primary hypothalamic neurons. Fetal rats were rapidly removed from 18-d pregnant rats after CO_2 sedation and decapitation. Fetuses were decapitated and hypothalamic tissue dissected and collected in ice-cold buffer (pH 7.4, containing 137mM NaCl, 5mM KCl, 0.7 mM Na_2HPO_4, 25 mM HEPES buffer, and 100 µg/ml gentamycin). Tissues were then digested for 1.5 h with collagenase type 2 (1 mg/ml; Worthington, Lakewood, NJ) dissolved in the buffer mentioned above, supplemented with 1 mg/ml glucose, 4 mg/ml BSA, and 0.2 mg/ml desoxyribonuclease. The cell suspension was filtered through a 40-µm cell strainer (BD Falcon, Heidelberg, GER) centrifuged at 200 g for 10 min. Cells were consecutively washed twice in dispersion buffer and once in plating medium (DMEM/F12, 100 µg/ml gentamycin, and 10% heat-inactivated fetal bovine serum). Cells were plated at a density of 1 x 10^6 cells/well in six-well plates coated with poly-L-lysine. After 24 h in the presence of serum, cultures were maintained for eight additional days in neurobasal medium containing B27 supplement (Life Technologies, Inc., Invitrogen). Cytosine arabinoside (Sigma) was added to a final concentration of 5 µM from day 4 onwards to prevent glial cell proliferation. On day 10, growth medium was replaced by supplement-free neurobasal medium containing 0.1% BSA (protocol adapted from (Liu et al. 2008)). After

1h of pre-incubation in this medium, cells were incubated with or without forskolin (FSK, 1µM, Sigma) and/or the OTR agonist TGOT (10 nM Bachem) for the periods of time indicated in the results section. Following this, the cells were harvested and RNA was isolated as described below.

2.2 H32 cells

The hypothalamic cell line H32 (Mugele et al. 1993) was cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Gaithersburg, MD, USA) containing 10% fetal bovine serum, 10% horse serum and 1% penicillin/streptomycin (Life Technologies, Inc.) at 37 °C and 5% CO_2. Prior to the experiments, the cells were transferred to 100 mm plates at a density of 3×10^6 cells per plate. 24 h later, the medium was changed to serum-free medium containing 0.1% BSA for 2h. To determine the signaling pathways mediating the effects of OT, cells were incubated in the presence or absence of FSK (1 µM) and/or TGOT (10 nM). After incubation for the time periods indicated in the results section and figure legends, cytosolic and nuclear proteins were extracted for Western blot analysis for CRTC2/3 and pCREB levels as described below.

2.3 Be(2)-M17 cells

The human neuroblastoma cell line Be(2)-M17 (European Collection of Cell Cultures, #95011816) was cultured in DMEM/F12 (1:1) (Invitrogen/GIBCO) containing L-glutamine, 2,435 g/L sodium bicarbonate, 15 % heat-inactivated foetal bovine serum (GIBCO), 1 % non-essential amino acids (GIBCO), and 0.1 mg/ml Gentamycin (GIBCO 15750-060) at 37 °C and 5 % CO_2. Three days before the experiment the cells were allowed to differentiate to neurons

by adding retinoic acid (Sigma) to a final concentration of 5 µM. On the day of experiment, the cells were incubated in serum-free DMEM/F12 (+ 0.1 % BSA) for 2 h to reduce basal activation of gene transcription initiated by any growth factors and steroids that might be present in the serum. The TGOT (10 nM) and forskolin (50 µM) stimulation protocols of the Be(2)-M17 cells were similar to those described for H32 cells, as were those for protein or RNA isolation.

3. Western blot analysis of protein phosphorylation and nuclear translocation

Cytosolic and nuclear proteins were extracted using a protein extraction kit (Active Motif, Rixensart, Belgium). Briefly, single hypothalamic tissue blocks were homogenized in 300 µl hypotonic buffer (supplemented with 0.1 mM DTT, 0.1 mM detergent, phosphatase and protease inhibitors as included in the kit), and incubated on ice for 15 min. Following centrifugation (10 min, 850 g, 4 °C), the supernatant containing cytosolic proteins was collected. To ensure complete lysis, the pellet was resuspended in 200 µl of hypotonic buffer (supplemented only with phosphatase and protease inhibitors) and incubated on ice for 15 min. Then 50 µl / ml of detergent was added, the mixture vigorously vortexed and centrifuged (3 min, 14,000 g, 4 °C). The supernatant was collected and pooled with the supernatant collected earlier to an end volume of 500 µl. The pellet was washed once with ice-cold phosphate-buffered saline (PBS), to wash away any remaining cytosolic proteins, and resuspended in 100 µl complete lysis buffer, supplemented with DTT, and phosphatase and protease inhibitors, as indicated by the manufacturer. The samples were incubated on ice for 30 min, then vortexed, centrifuged (10 min, 14,000 g, 4 °C), and supernatants

containing nuclear proteins collected. The protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

It should be noted that the vast differences in the protein content of hypothalamic tissue blocks, tissue punches, Be(2)-M17 cells and H32 cells brings about the need to apply different protein extraction kits. Nuclear extracts from Be(2)-M17 cells and PVN tissue punches were prepared using ice-cold NE-PER Nuclear and Cytosolic Extraction Reagent (Pierce, Rockford, IL), with added 100 x HALT Protease and Phosphatase Inhibitor (Thermo Scientific), according to the manufacturer's protocol. Western blot analysis was performed for CRTC2, CRTC3, pCREB, CREB , Lamin A, ß-tubulin, Ras, histone deacetylase 1, as previously described (Liu et al. 2008) with the following modifications. For CRTC3, 15 µg of cytosolic or nuclear extract were loaded with a 4x Laemmli Loading Dye (2.0 ml 1M Tris-HCl pH 6.8; 0.8 g SDS; 4.0 ml 100% glycerol; 0.4 ml 14.7 M β-mercaptoethanol; 1.0 ml 0.5 M EDTA; 8 mg bromophenol blue) and separated in a 6% Tris-glycine gel (Invitrogen). To detect CRTC2 from Be(2)-M17 lysates 30 µg of protein were loaded onto the gel. Gels were run until the 50 kDa marker (prestained protein ladder, Fermentas, Inc., Glen Burnie, MD, USA) ran off the gel, to separate phospho- from dephospho-CRTCs. All other proteins were separated on a 10 % Tris-glycine gel. Following electrophoresis, proteins were transferred to a polyvinyl-difluoride membrane (GE Amersham) and blocked in Tris-buffered saline with 0.1% tween (TBS-T) + 5% milk powder or BSA for 1 h at room temperature. After the transfer, the remaining gels were stained with Coomassie-Blue dye to control for even loading and transfer of proteins.

Target	Company	Dilution	Diluents
Anti CRTC2	Calbiochem/EDM	1:12.000	In TBS-T
Anti-CRTC3	Abcam ab91654	1:2000	In TBS-T
Anti-phospho-CREB	Millipore #06-519	1:5000	in 5% BSA
Anti-CREB	Cell signaling #9197	1:1000	In 5% milk powder
Anti-Lamin A/C	Active Motif #39287	1:5000	In TBS-T
Anti-ß-tubulin	Cell signaling #2146	1:1000	In 5% milk powder
Anti-Ras	Abcam ab52939	1:5000	In TBS-T
Anti-HDAC1	Santa Cruz sc6298	1:1000	In TBS-T
Anti- GAPDH	Abcam ab9485	1:5000	in 2% BSA
Anti-TATA-box bp	Abcam ab63766	1:2000	in 5% BSA
Anti-p38	Abcam ab32557	1:5000	in TBS-T
Anti-pMSK	Abcam ab81294	1:2000	in 5% BSA
Anti-pMEK 1/2	Cell Signaling #9154S	1:2000	in 5% BSA
Anti-MEK 1/2	Cell Signaling #9122S	1:2000	in 5% BSA
Anti-pERK 1/2	Cell Signaling #9101	1:5000	in 5% BSA
Anti-ERK 1/2	Cell Signaling #9102	1:5000	in 5% BSA
Anti-pCaMKIIα	Santa Cruz sc-12886-R	1:1000	in TBS-T
Anti-PEA-15 (Ser104)	Cell signaling #2776	1:1000	in 5% BSA
Anti-PEA-15 (Ser116)	Millipore #07-865	1:1000	in 5% BSA
Anti-PEA-15	Cell signaling #2780	1:1000	in 5% milk powder
Phospho-c-Jun (Ser63)	Cell signaling #2361S	1:5000	in 5% BSA
pElk-1 (Ser383)	Cell signaling #9181	1:5000	in 5% BSA
p-mTOR (Ser2481)	Millipore #09-343	1:3000	in 5% BSA
mTOR	Cell signaling #2983	1:1000	in 5% milk powder

Table 1 Antibodies used for western blotting with optimal dilution factor and diluent

The blots were blocked with TBS-T containing either 2-5% milk powder or BSA (depending on the antibody) for 2 hours at room temperature with gentle agitation. Next, the primary antibodies were added, and incubated at the respective dilution (Table 1) overnight at 4°C with gentle agitation. After washing and incubating with the appropriate horseradish peroxidase labeled secondary antibody (horseradish-peroxidase linked rabbit anti rat (Cell signaling) or donkey anti goat (Santa Cruz)), immunoreactive bands were visualized using enhanced chemiluminescence plus detection system and film exposure (Liu et al. 2008), or with the ChemiDoc XRS+ Imager (Bio-Rad). The intensity of the bands on the film was quantified using ImageJ (NIH). Images obtained with the ChemiDoc XRS+ were analyzed with the included software. Following imaging, blots were stripped with a mild stripping solution (Millipore), or 0.2 N NaOH and assayed for HDAC1 / Lamin A, Ras, GAPDH, TATA box binding protein, ß-tubulin, or the respective non-phospho-protein antibody as loading controls.

4. RNA isolation and qPCR

Punched PVN tissue from the stress experiments were treated with 1 ml TriFast Gold (PeqLab), and RNA was isolated according to the protocol provided by the manufacturer with some modifications. Briefly, the aqueous supernatant obtained from chloroform precipitation with TriFast was added to an RNeasy Mini Kit (Qiagen) column, washed, treated with RNase free DNase (according to the manufacturers protocol, Qiagen), washed and eluted with nuclease free water. RNA content was determined by means of a NanoDrop photospectrometer (Liu and Aguilera 2009).

To isolate RNA from the stimulated cells, the medium was aspirated off, and 1 ml of TriFast Gold Reagent (PeqLab) was added to the 6-well plates (primary cells) or petri-dishes (Be cells), respectively. The remaining cells were scraped off from the petri-dishes by means of a sterile cell scraper and collected in RNase free 1.5 ml tubes. From here on, the protocol used for RNA isolation was similar to that described for brain punches.

300 ng of total RNA per sample were used for reverse transcription into cDNA using Super Script III First strand Synthesis System for RT-PCR (Invitrogen). Relative quantification of CRF hnRNA levels was performed using SYBR Green (QuantiFast Qiagen), using ribosomal protein L13A (Rpl13A) and cyclophilin A (CycA) as housekeeping genes (Bonefeld et al. 2008). Specificity of the qPCR was assured by omitting reverse transcription and by using ddH_2O as template. As the results obtained using Rpl13A and CycA yielded similar results, only those for Rpl13A are shown. The PCR protocol consisted of an initial denaturation step of 5 min at 95 °C, followed by 50 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 45 s. At the end of the protocol a melting curve was generated to confirm the specificity of the primers. All samples were run in triplicate.

Target name	Sequence	species	Amplicon size
Rpl13A forward	5'ACAAGAAAAAGCGGATGGTG3'	rat	167 bp
Rpl13A reverse	5'TTCCGGTAATGGATCTTTGC3'	rat	
CycA forward	5' AGCACTGGGGAGAAAGGATT3'	rat	248 bp
CycA reverse	5'AGCCACTCAGTCTTGGCAGT3'	rat	
CRF hnRNA forw	5' TCAATCCAATCTGCCACTCA3'	rat	154 bp
CRF hnRNA rev	5'TAAGCTATTCGCCCGCTCTA3'	rat	
CRF hnRNA forw	5'AAGACAACCTCCAGAGAAAG3'	human	324 bp
CRF hnRNA rev	5'CATCCCAGCTACTATTGTAATC3'	human	
Rpl13A forward	5'TGGCTAAACAGGTACTGCTGG3'	human	284 bp
Rpl13A reverse	5'CCGCTTTTTCTTGTCGTAGGG3'	human	
OTR forward	5'AAGAGCAACTCGTCCTCCTTT 3'	human	215 bp
OTR reverse	5'ACAAACATACGCCATCACCT3'	human	
AVPR1a forw	5'CGCCTTTCTTCATCATCCAG3'	human	135 bp
AVPR1a rev	5'AGTCCTTCCACATACCCGTA3'	human	
AVPR1b forw	5'GGAGGGTAAGGGTTGGAGTT3'	human	128 bp
AVPR1b rev	5'GGAGGGTAAGGGTTGGAGTT3'	human	
CRF ChIP forw	5'- AGTCATAAGAAGCCCTTCCA-3'	human	92 bp
CRF ChIP rev	5'- CAACACTGAATCTCACATCCA-3'	human	

Table 2 Primer sequences with corresponding amplicon size and species

5. siRNA transfection

Be(2)-M17 cells were transfected with 33 nM small interfering RNA (siRNA) and Lipofectamine RNAiMAX Reagent (Invitrogen), diluted in OptiMEM to inhibit CRTC2 and CRTC3 synthesis. siRNA specific for CRTC2 and the negative control Silencer #5 were

purchased pre-designed from Ambion/Life Technologies (Darmstadt, Germany). The human oligonucleotide sequences were 5'-CUAUAGUCCUGCCUACUUAtt-3' (sense) for CRTC2, and 5'-GCACAUCAAGGUUUCAGCAtt-3' (sense) for CRTC3. After 12 h, transfection medium was removed and replaced by DMEM/F12 (supplemented with 0.1% sterile-filtered BSA) for 2 h. The cells were then stimulated with FSK and TGOT for 60 min, and gene and protein expression were analyzed as described above. Verification of knockdown was performed by RT-qPCR for CRTC2 and CRTC3 mRNA, and at the protein level by applying a RIPA-Buffer (Sigma; added with HALT Protease- and phosphatase inhibitor (100x, Thermo Scientific)) to the cells to extract whole cell protein lysates. These were immuno-stained by western blotting (described above) with the respective CRTC antibodies.

6. Chromatin immunoprecipitation (ChIP)

To investigate if CRTC3 binds the CRF promoter *in vitro*, Be(2)-M17 cells (7 x 10^6 cells) were cultured in 75 cm^2 flasks (Sarstedt, Nümbrecht, Germany) in growth medium supplemented with 5 µM retinoic acid for 3 days until cell number reached 20 x 10^6 cells. Cells were then stimulated with FSK and TGOT for 10 min, as described above. The chromatin IP protocol was carried out as described previously (Lichtinger et al. 2007) with some modifications. Briefly, the stimulation medium was removed and replaced by 10 ml of 1% formaldehyde solution for 10 min at room temperature. Then, glycine was added to a final concentration of 125 mM and incubated for 5 min at room temperature to stop fixation. Fixed cells were washed with ice cold 1x PBS supplemented with 1mM PMSF (Sigma) and harvested with 5 ml of the same solution. The cell suspension was centrifuged for 8 min at 370 g and 4 °C, and the pellet was frozen at -80 °C until lysis in cell lysis buffer (10mM HEPES, 85 mM KCl, 1 mM

EDTA, 1% NP-40). Next, the nuclei were sonicated 8 times for 10 sec (30 sec sample cooling between each sonification step) at output control 2 (Branson Sonifier 250) in nuclear lysis buffer (50 mM Tris/HCl, 1% SDS, 0,5% Empigen BB, 10 mM EDTA, 1 mM PMSF, 1x protease inhibitor (Roche)) to produce 0.2 to 1 kbp DNA fragments of chromatin. Immunoprecipitation was performed with either 4 µg of CRTC3 antibody (Abcam, ab91654), rabbit IgG (Qiagen) as negative control, and polymerase II antibody as positive control (Qiagen) at 4°C under rotation overnight. DNA-protein-antibody complexes were collected using Protein A Sepharose beads, washed, eluted, reverse cross linked, treated with Proteinase K and cleaned up with Qiaquick PCR purification columns (Qiagen). Immunoprecipitated CRF promoter was quantified using quantitative real-time PCR with primers designed to amplify the human CRF promoter region containing the cyclic AMP responsive element (CRE) (forward: 5'- AGTCATAAGAAGCCCTTCCA-3'; reverse: 5'-CAACACTGAATCTCACATCCA-3').

7. Statistical analyses

Signaling and behavioral data were analyzed using either a one-way or a two-way analysis of variance (ANOVA, factors reproductive state x treatment, factors treatment 1 x treatment 2). Behavioral data is expressed in comparison to vehicle-treated groups of both virgins and lactating dams, as the experiments were performed separately. Any overall statistical differences, which were set at $P < 0.05$, were further analyzed using a Fisher's or Student Newman-Keuls *post-hoc* test. Separate parametric t-test between two groups or non-parametric Mann-Whitney U tests (MWU) were performed. Data are expressed as group mean ± S.E.M. Statistical analyses were performed using SPSS for Windows (version 16; SPSS Inc, Chicago, IL, USA) and Sigma Plot (version 11.0; Systat Software Inc.).

Results

Results

Part I: Differential effects of oxytocin on MAPK activity

[partly adapted from: Jurek B, Slattery DA, Maloumby R, Hillerer K, Koszinowski S, et al. (2012) Differential Contribution of Hypothalamic MAPK Activity to Anxiety-Like Behavior in Virgin and Lactating Rats. PLoS ONE 7(5): e37060. doi:10.1371/journal.pone.0037060]

Author's contribution to study cited above:

Jurek: study design, protein isolation, SDS Page, Western Blot, Data analysis,, writing the first draft of the manuscript

Slattery: study design, brain isolation in all experiments, performing behavioral experiments, data analysis, writing the manuscript

Maloumby: infusion of compounds

Hillerer and Koszinowski: preliminary experiments

Neumann: study design

van den Burg: study design, writing the manuscript, data analysis

all other experiments that were not part of the publication were planned, conducted, and analyzed by the author

Introduction to Part I

The c-Raf – MEK1/2 – ERK1/2 mitogen-activated protein kinase (MAPK) pathway is one of the most important and best-studied intracellular signaling pathways. This pathway conveys extracellular signals to intracellular effectors *via* activation of a variety of cell membrane receptors, and hence is responsible for a battery of effects (for detailed description see General Introduction). In the brain, ERKs are strongly activated by synaptic stimulation, and are essential for the induction and maintenance of synaptic plasticity that is thought to underlie memory and learning (Davis and Laroche 2006). Furthermore, ERKs have been shown to regulate social behaviors, including social memory and aggression, particularly *via* ERK2 (Satoh et al.). These effects overlap, at least partially, with those reported for the neuropeptide OT, and indeed it has been shown that OT activates the MAPK pathway within the hypothalamic PVN *via* transactivation of the EGFR to regulate anxiety in male rats (Blume et al. 2008). Interestingly, the related nonapeptide, AVP, has been shown to activate the MAPK pathway *in vitro* (Chen et al. 2008) and also *in vivo* (Du et al. 2008; Oligny-Longpre et al. 2012). However, it has been repeatedly demonstrated that exogenous central administration of AVP has an anxiogenic effect in the amygdala (Landgraf and Neumann 2004) and that its expression level within the PVN correlates negatively with anxiety-related behavior (Murgatroyd et al. 2004; Bunck et al. 2009).

In females, the brain OT system is particularly active during the peripartum period with elevated rates of synthesis of the neuropeptide and its receptor, enhanced local release, and receptor binding within limbic and hypothalamic regions (for review see (Brunton and Russell 2008; Slattery and Neumann 2008; Hillerer et al. 2011)). Such high brain OT levels are important to induce anxiolysis during lactation (Carter et al. 2001; Windle et al. 2004). Also, OT enhances spatial memory in the hippocampus of lactating rats, thought to improve the

recollection of locations where food and water are present, and thus to reduce the time the mother needs to spend finding resources and leaving the pups unattended (Tomizawa et al. 2003). This effect of OT on spatial memory depends on the activation of ERK, and one of its downstream effectors, the transcription factor CREB (Tomizawa et al. 2003). This example, together with the anxiolytic effect described above in males, shows the importance of ERK1/2 and its kinase MEK1/2 as intracellular mediators of the behavioral effects of OT signaling. Further, it shows that the MAPK signaling pathway is recruited during lactation in the hippocampus, and that its recruitment depends on OT. Moreover, given lactation-associated anxiolysis, and the role of both OT and MAPK in anxiety, this pathway may be necessary for this effect. However, the roles of MEK1/2 and ERK1/2 in the PVN of females as effectors of anxiety-related behavior have not been reported to date. This is nevertheless of particular importance considering the mood changes that frequently occur at peripartum in humans ((Hillerer et al. 2011) and references therein), and the reported pro-social and anxiolytic effects of OT in males and females, in rats as well as in humans (Landgraf and Neumann 2004; Kosfeld et al. 2005; Blume et al. 2008; Labuschagne et al. 2010; Meyer-Lindenberg et al. 2011; Viviani et al. 2011).

Therefore, the present data is concerned with the central question of whether MAPK pathway activity within the PVN of female rats is necessary for an anxiolytic phenotype. To address this, we employed two distinct approaches: (1) acute pharmacologically-induced (i.e. application of exogenous OT), and (2) long-term physiologically-induced (i.e. lactation) anxiolysis and assessed their effects on the MAPK pathway within the PVN (adapted from (Jurek et al. 2012).

Outline of experimental protocols part I

Experiment 1. Effect of intra-PVN infusion of OT on anxiety-related behavior in virgin and lactating rats, and effects of local pre-treatment with the MEK1/2 inhibitor, U0126

The following experiment was performed in order to (i) unravel the anxiolytic effects of OT, infused into the PVN, as a function of the reproductive status of female rats, and (ii) determine the role of the MEK1/2 – ERK1/2 signaling cascade in the anxiolytic effect of both OT and lactation. Thus, conscious virgin and lactating rats received two subsequent bilateral PVN infusions. They were pre-treated with either vehicle (0.5 µl; 10 % DMSO in Ringer solution, pH 7.4, Braun) or the MEK inhibitor U0126 (0.5 nmol/0.5 µl) bilaterally within the PVN 5 min prior to infusion of either vehicle, or OT (0.01 nmol/0.5 µl). This experimental design gives four different groups: vehicle/vehicle, vehicle/OT, U0126/vehicle or U0126/OT. After each infusion, the cannulas were kept in place for 30 s to allow local substance diffusion. Anxiety-related behavior was assessed in the light-dark box (LDB) 10 min later. The doses were chosen based on previous studies (Blume et al. 2008).

Experiment 2. Determination of basal and OT-induced MEK1/2 and ERK1/2 activation within the hypothalamus of virgin, pregnant, and lactating rats

To assess the impact of reproductive status under basal and acute icv OT conditions on hypothalamic MEK1/2 and ERK1/2 activation, conscious well-handled virgin, pregnant (PD20), and lactating (LD8) rats received either an acute vehicle (5 µl; Ringer solution, pH 7.4, Braun) or OT (1 nmol) infusion. Ten minutes later, rats were decapitated and the

hypothalami dissected, immediately frozen, and stored at -20°C until further processing. The hypothalamus was dissected rostrally at the level of the optic chiasm, and caudally through the median eminence. Laterally, the lateral ventricle was used as a landmark to remove the most lateral parts of the hypothalamus. Dorsally, the third ventricle represented the top most level of the PVN-enriched region. The suprachiasmatic and remaining part of the supraoptic nuclei were also removed. This results in a hypothalamic tissue block containing mostly the PVN with some bed nucleus of the stria terminalis, medial preoptic area, and the anterior hypothalamic nuclei (Paxinos and Watson 1998).

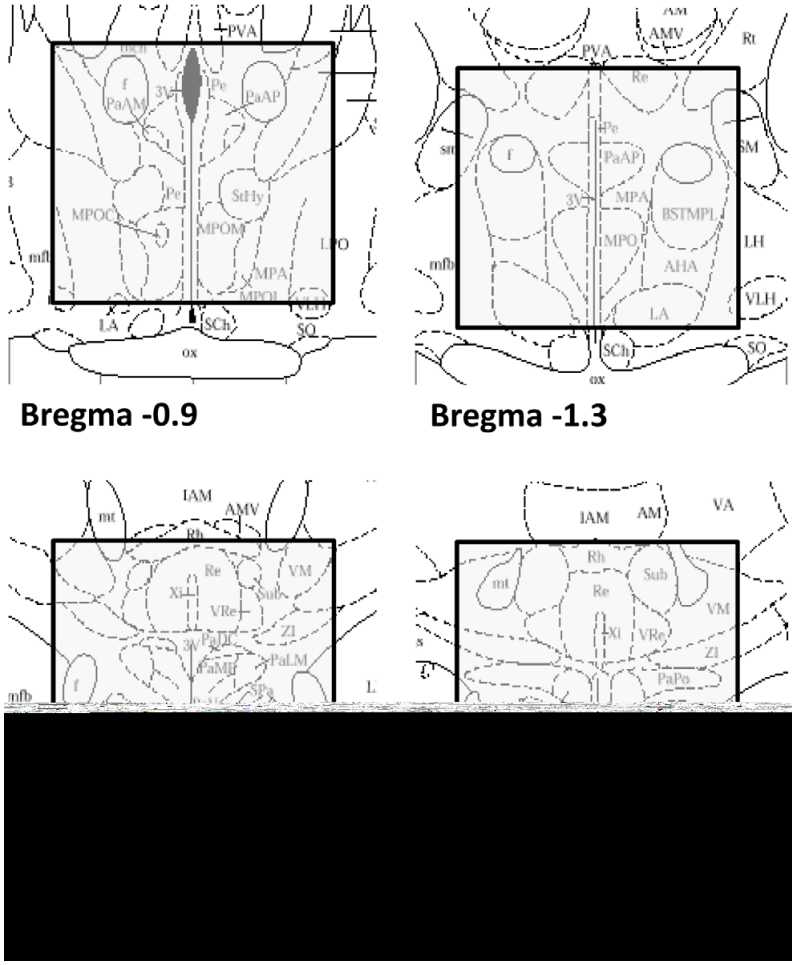


Figure 8 Schematic representation of PVN-enriched hypothalamic tissue that was extracted for western blot analyses. Scale bar: 1 mm; adapted from (Jurek et al. 2012)

Experiment 3. Effects of blockade of MEK1/2 activity on basal and OT-induced ERK1/2 activation within the hypothalamus

To assess if icv pre-treatment with the MEK1/2 kinase inhibitor, U0126, reduced basal or OT-induced ERK1/2 activation in virgin or lactating (LD8) rats, the following groups were compared: vehicle/vehicle, U0126 (1nmol)/vehicle, vehicle/OT (1 nmol) or U0126/OT. The icv infusions were 10 min apart, and 10 min after the second infusion, the hypothalami were isolated as described in Experiment 2.

Experiment 4. Assessment of activated signaling cascades and transcription factors within the PVN of male or virgin female rats, induced by an intra-PVN, lateral ventricle, or 3rd ventricle infusion of OT or TGOT, and consequences for anxiety-like behavior

4.1 Effects of several application protocols on the intracellular signaling were assessed and compared to gain insight into intracellular processes coupled to the OTR. (i) Activation of cytosolic MEK1/2 and ERK1/2 in males 10 min after either icv (1nmol / 5µl) or intra-PVN (0.01 nmol / 0.5 µl) infusion of OT or vehicle was measured and compared by western blotting. (ii) To prevent tissue damage in the PVN caused by the application cannula or pressure of the liquid infused, which might interfere with MAPK activation, we applied TGOT (1nmol / 2µl, dissolved in Ringer solution, pH 7.4, Bachem) or vehicle (Ringer solution, Braun) to the adjacent 3rd ventricle, allowed substance diffusion for 30 s, returned the animals to their home-cage and decapitated them after 10 min. A precise isolation of the hypothalamic PVN, using a tissue puncher (1.8 mm diameter, Fine science tools) with fresh, non-frozen tissue was applied, which reduces the protein concentration obtained, but

excludes possible contamination from nearby hypothalamic nuclei. TGOT was used to exclude non-specific VP-receptor-activation effects on MEK1/2 and CaMKII, as well as to assess ERK1/2 and PEA-15 activation. In addition, PEA-15 activation, mTOR and Elk-1 phosphorylation by 250 nM OT was assessed in hypothalamic H32 cells.

4.2 After the characterization of intracellular signaling pathways that are activated by OT, we assessed whether they influence anxiety-like behavior when stimulated by an infusion of TGOT into the 3rd ventricle. 10 min after the infusion of TGOT or vehicle, male rats were tested in the LDB for anxiety-like behavior as described above.

Experiment 5. Assessment of sex-specific differences in the pathway leading to CREB phosphorylation by 3rd ventricle infusion of OT

To determine whether the intracellular pathway that leads to the phosphorylation of CREB are differentially recruited by OT in male and female Wistar rats, we infused OT (1nmol / 2µl) or vehicle into the 3rd ventricle of rats of both sexes, isolated the PVN after 10 min as described above, and assessed protein phosphorylation in the hypothalamic lysates by western blotting.

Results Part I

Experiment 1. Effect of intra-PVN infusion of OT on anxiety-related behavior in virgin and lactating rats, and effects of local pre-treatment with the MEK1/2 inhibitor, U0126

In virgin rats, ANOVA did not reveal a significant effect of either bilateral infusion of U0126 ($F_{(1,29)}$ = 2.21; p = 0.15) or OT ($F_{(1, 29)}$ = 3.59; p = 0.068) directly into the PVN on the relative time spent in the light compartment of the LDB. However, separate analyses revealed a significant anxiolytic effect of an OT infusion (MWU; p = 0.009 versus Veh/Veh group; Figure 9A) while there were no significant differences between the three other groups. This effect was not paralleled by altered locomotor activity as the number of line-crosses in the dark compartment did not differ between the treatment groups (data not shown).

In contrast to the effect observed in virgins, a significant effect of MEK1/2 inhibition on anxiety was observed in lactating rats ($F_{(1,32)}$ = 11.3, p = 0.002). Specifically, U0126 treatment increased basal anxiety in lactating rats (p < 0.05 versus Veh/Veh, Figure 9B) without altering locomotor activity (data not shown). Interestingly, bilateral intra-PVN infusion of synthetic OT did not affect anxiety in lactation ($F_{(1,32)}$ = 3.08; p = 0.089; Figure 9B) (adapted from (Jurek et al. 2012)).

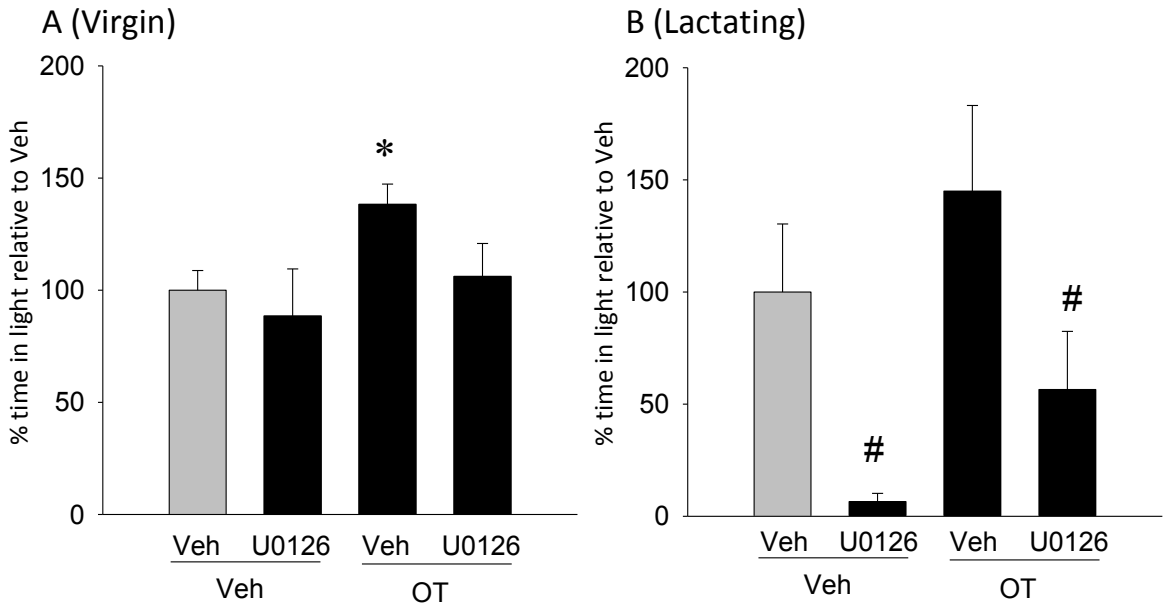


Figure 9 The effects of acute bilateral PVN administration of vehicle (Ringer solution; Veh), or OT (0.01 nmol/0.5ul) after intra-PVN pre-treatment with either vehicle (DMSO) or the MEK 1/2 inhibitor, U0126 (0.5 nmol/0.5ul) on time spent in the light compartment of the LDB in (A) virgin rats and (B) lactation day 8 rat dams. Data represent mean ± SEM (n = 7 – 12 per group). Two-way ANOVA was performed followed by Fisher's LSD post-hoc tests where appropriate. * $p < 0.05$ compared with vehicle treatment and # $p < 0.05$ compared with respective virgin group (adapted from (Jurek et al. 2012)).

Experiment 2. Determination of basal MEK 1/2 activation within the PVN in virgin and lactating rats

To determine whether reproductive status altered hypothalamic MEK1/2 activation within the PVN, we analyzed phosphorylated MEK1/2 (pMEK1/2) levels relative to total MEK and protein content in virgin and lactating (LD8) rats. pMEK1/2 levels appeared to be 24 ± 7% higher in the cytosolic fraction of lactating than in virgin rats ($p = 0.04$), and this applied to both, the pMEK/MEK and the pMEK/GAPDH ratio (Figure 10). The MEK/GAPDH ratio was similar in both groups, indicating that the increased pMEK1/2 concentration is due to increased phosphorylation, rather than to increased MEK synthesis during lactation. There were no effects of reproductive state on nuclear MEK phosphorylation or content (data not shown) (adapted from (Jurek et al. 2012)).

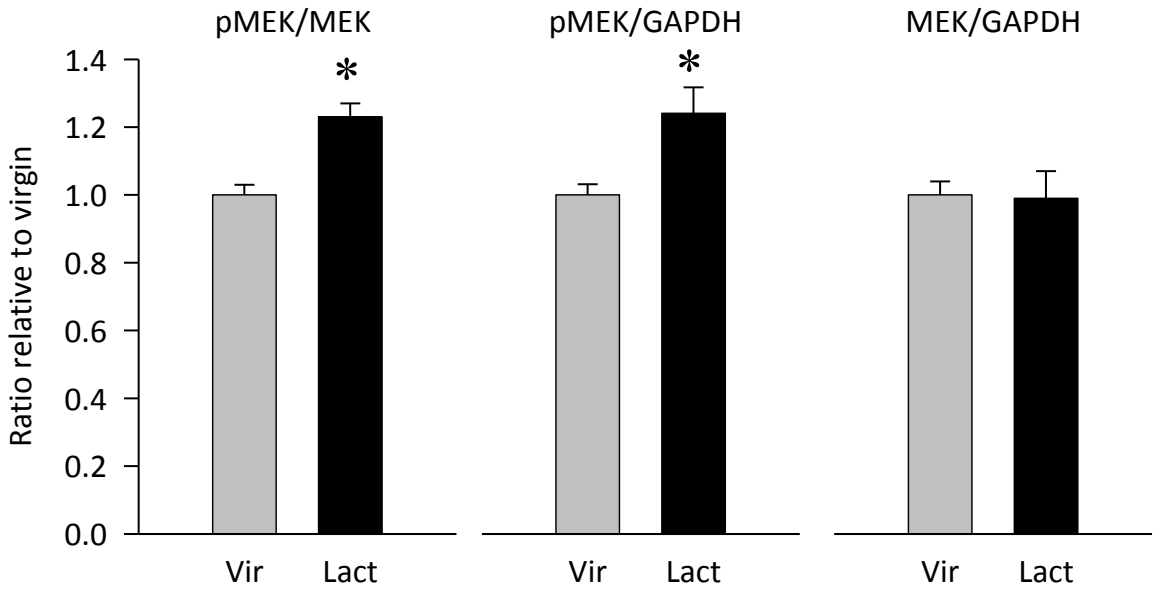
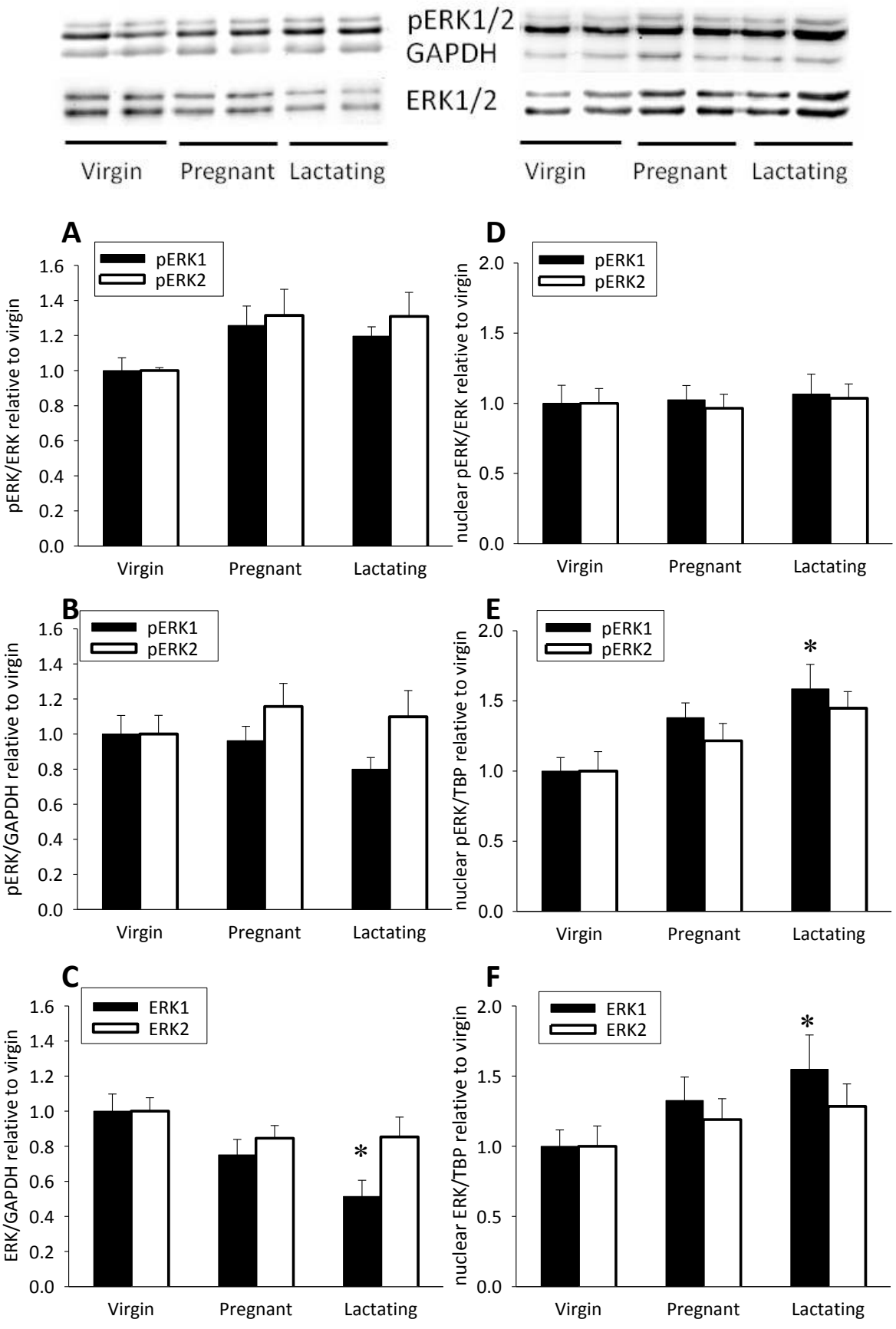


Figure 10 MEK phosphorylation as function of reproductive status in female rats. Representative blot of cytosolic pMEK and MEK from PVN protein extracts from virgin and lactating rats. Cytosolic pMEK levels were higher in the PVN of lactating rats when compared with total MEK and total protein (as measured by GAPDH amount), whereas total MEK levels were similar in both groups. Data relative to virgin control value of 1. Mann-Whitney U-test, * $p < 0.05$. (adapted from (Jurek et al. 2012))

Experiment 2.1. Determination of basal ERK1/2 activation within the PVN in virgin, pregnant, and lactating rats

Cytosolic pERK1, but not pERK2, levels relative to both loading controls (GAPDH and β-tubulin; the latter not shown) tended to decrease in late-pregnant rats, and this effect reached significance in lactating rats (to 51.4% relative to virgin; $F_{(2,17)} = 6.65$; $p = 0.009$; Figure 11A).


pERK1/2
GAPDH
ERK1/2
Virgin
Pregnant
Lactating
Virgin
Pregnant
Lactating
A
pERK1
pERK2
pERK/ERK relative to virgin
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Virgin
Pregnant
Lactating
D
pERK1
pERK2
nuclear pERK/ERK relative to virgin
2.0
1.5
1.0
0.5
0.0
Virgin
Pregnant
Lactating
B
pERK1
pERK2
pERK/GAPDH relative to virgin
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Virgin
Pregnant
Lactating
E
pERK1
pERK2
*
nuclear pERK/TBP relative to virgin
2.0
1.5
1.0
0.5
0.0
Virgin
Pregnant
Lactating
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ERK1
ERK2
*
ERK/GAPDH relative to virgin
1.6
1.4
1.2
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Virgin
Pregnant
Lactating
F
ERK1
ERK2
*
nuclear ERK/TBP relative to virgin
2.0
1.5
1.0
0.5
0.0
Virgin
Pregnant
Lactating

Figure 11 ERK phosphorylation status in cytosolic (left) and nuclear (right) protein fractions from PVNs of virgin, pregnant, and lactating rats. Above; representative cytosolic and nuclear blots. A, ERK phosphorylation in the cytosol tended to increase during pregnancy and lactation when corrected for the amount of total kinase, although not significantly. B, Cytosolic pERK levels did not change during peripartum, when corrected for total protein amount (as measured by GAPDH levels). C, Cytosolic ERK1 concentration decreased during lactation, when compared with total protein amount. D, Nuclear pERK/ERK ratio is constant over the peripartum period. E, pERK1 levels were elevated in the nuclear fraction of lactating animals, when corrected for total amount of protein (as measured by TBP levels). F, Nuclear total ERK1 levels were elevated in lactating rats. ANOVA was performed followed by Bonferroni´s post-hoc tests (adapted from (Jurek et al. 2012)).

In parallel, the nuclear pERK1 content increased in lactating rats as measured relative to the TATA box binding protein (TBP) loading control (1.6-fold increase relative to levels in virgins; $F_{(2,17)}$ = 5.68; p = 0.016; Figure 11E). This was accompanied by a similar increase of the ERK1/TBP ratio (1.5-fold increase; $F_{(2,17)}$ = 4.17; p = 0.04; F). The ratio of pERK1/ERK1 was, in contrast, constant in both cellular compartments (Figure 11A, D), indicating that in lactating rats, but not in late-pregnant rats, ERK1 is phosphorylated, and subsequently translocated to the nucleus (adapted from (Jurek et al. 2012)).

Experiment 2.2. Determination of OT-induced MEK and ERK1/2 activation within the PVN in virgin and lactating rats

Infusion of OT (1 nmol icv) increased pMEK levels, relative to total MEK and protein content, by 24 ± 9 % in the cytosolic fraction of PVN tissue of virgin rats (p = 0.049; Figure 12). Surprisingly, MEK activation was not accompanied by increased ERK1/2 phosphorylation in virgins (Figure 12A). In lactating rats, where brain OT activity is already high, pMEK levels were decreased in response to icv OT (-27 ± 9 %; p = 0.049 versus lactating, vehicle-treated rats; Figure 12B). Again, the icv OT infusion did not alter pERK1/2 levels in both fractions.

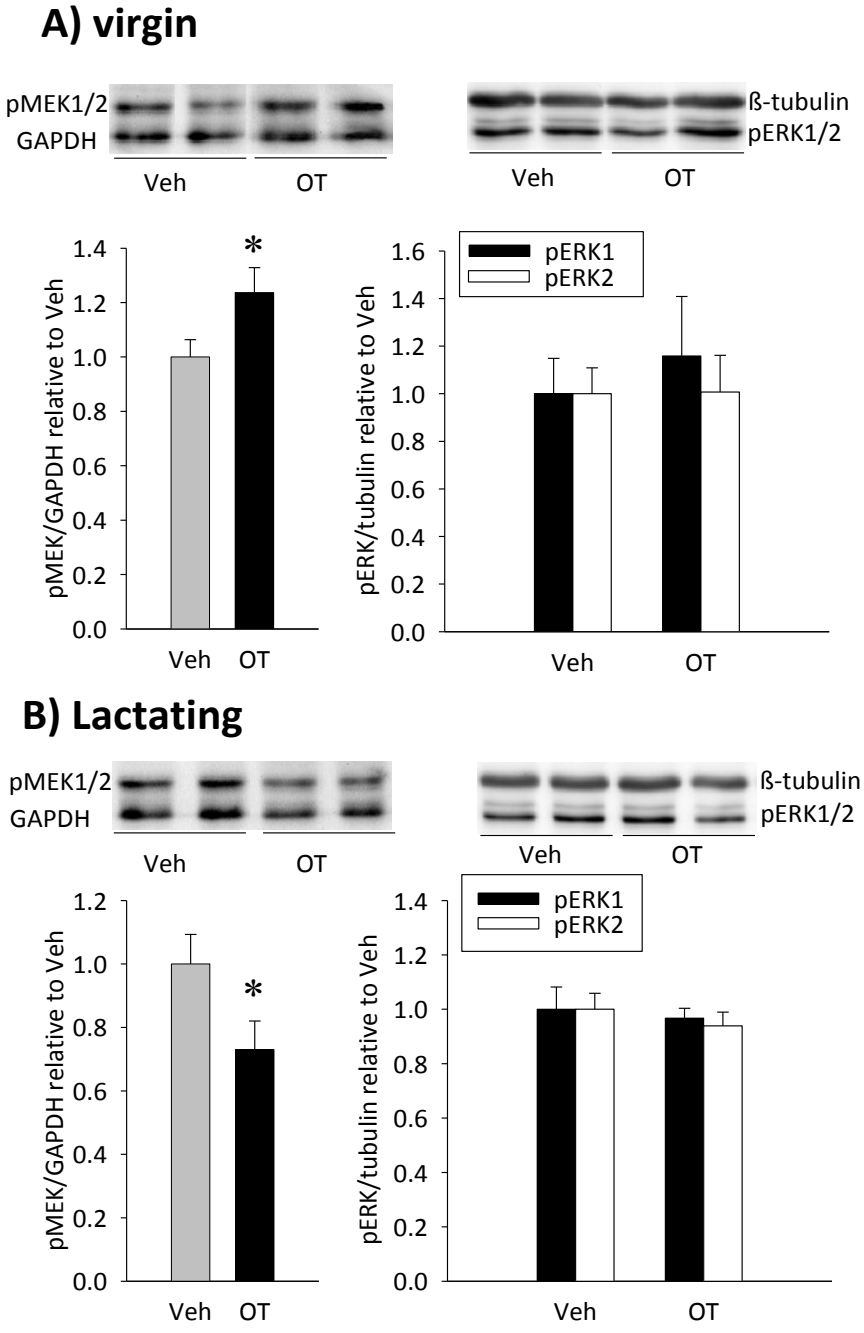

Figure 12 The effect of acute icv administration of OT on MEK and ERK phosphorylation in the cytosolic fraction of PVN tissue of virgin and lactating rats. In virgins, OT induced the phosphorylation of MEK, but not ERK, while in lactating rats OT induced dephosphorylation of MEK, without affecting pERK levels. Total MEK and ERK levels did not change (not shown). Data are relative to Veh groups for each reproductive state. MWU-test, * $p < 0.05$. (adapted from (Jurek et al. 2012))

Experiment 3. Effects of blockade of MEK1/2 activity on basal and OT-induced ERK1/2 activation within the hypothalamus

As the OT-induced phosphorylation of MEK1/2 appeared to be uncoupled from the phosphorylation of ERK1/2, we inhibited the activity of pMEK1/2 pharmacologically with U0126 (1 nmol in DMSO, icv) to determine whether pMEK1/2 exerts ERK1/2 phosphorylation activity in the PVN of female rats. Treatment with U0126 lowered basal pERK1/2 concentrations to a similar extent in both virgin and lactating rats to 52.9% (pERK1) and 58.4% (pERK2) of control levels ($p = 0.019$; Veh; DMSO treated rats; Figure 13), indicating that pMEK1/2 is indeed a kinase of ERK1/2 in the PVN of female virgin and lactating rats – under basal conditions (adapted from (Jurek et al. 2012)).

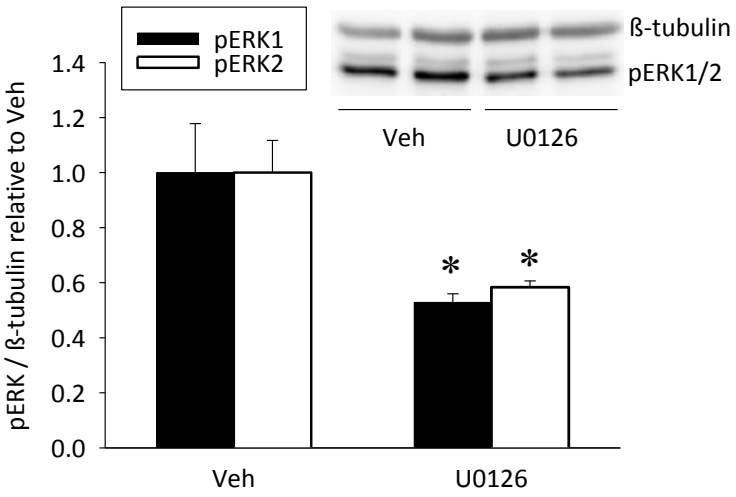

Figure 13 The effect of acute icv administration of U0126 on ERK1/2 phosphorylation in the cytosolic fraction of the PVN of lactating rats. U0126 reduced phosphorylation of both ERK1 and ERK2; highly similar results were obtained in virgin animals (not shown). Data are expressed relative to respective Veh control group. MWU-test, * $p < 0.05$. (adapted from (Jurek et al. 2012)).

Experiment 4. Activated signaling cascades within the PVN of male rats induced by an OT infusion into the lateral ventricle, versus a bilateral PVN- infusion

To assess if the uncoupling of MEK1/2 from ERK1/2 is sex-specific and/or depends on the infusion site, we infused OT in either the lateral ventricle or bi-lateral into the PVN of male rats and analyzed the phosphorylation status of hypothalamic MEK1/2 and ERK1/2. Infusion of OT into the lateral ventricle increased the level of pMEK1/2 1.5 fold when compared to vehicle infused rats ($p < 0.05$, Figure 14A). An intra-PVN infusion increased pMEK1/2 levels in the PVN almost 1.6 fold ($p < 0.05$) with no changes in the total amount of MEK, pointing toward an increased phosphorylation/activation of already present MEK with no changes in protein synthesis. Surprisingly, like in females, OT infusion in the lateral ventricle or the PVN did not produce a measureable increase of pERK1/2 levels in the PVN, nor did it influence the total amount of ERK1/2.

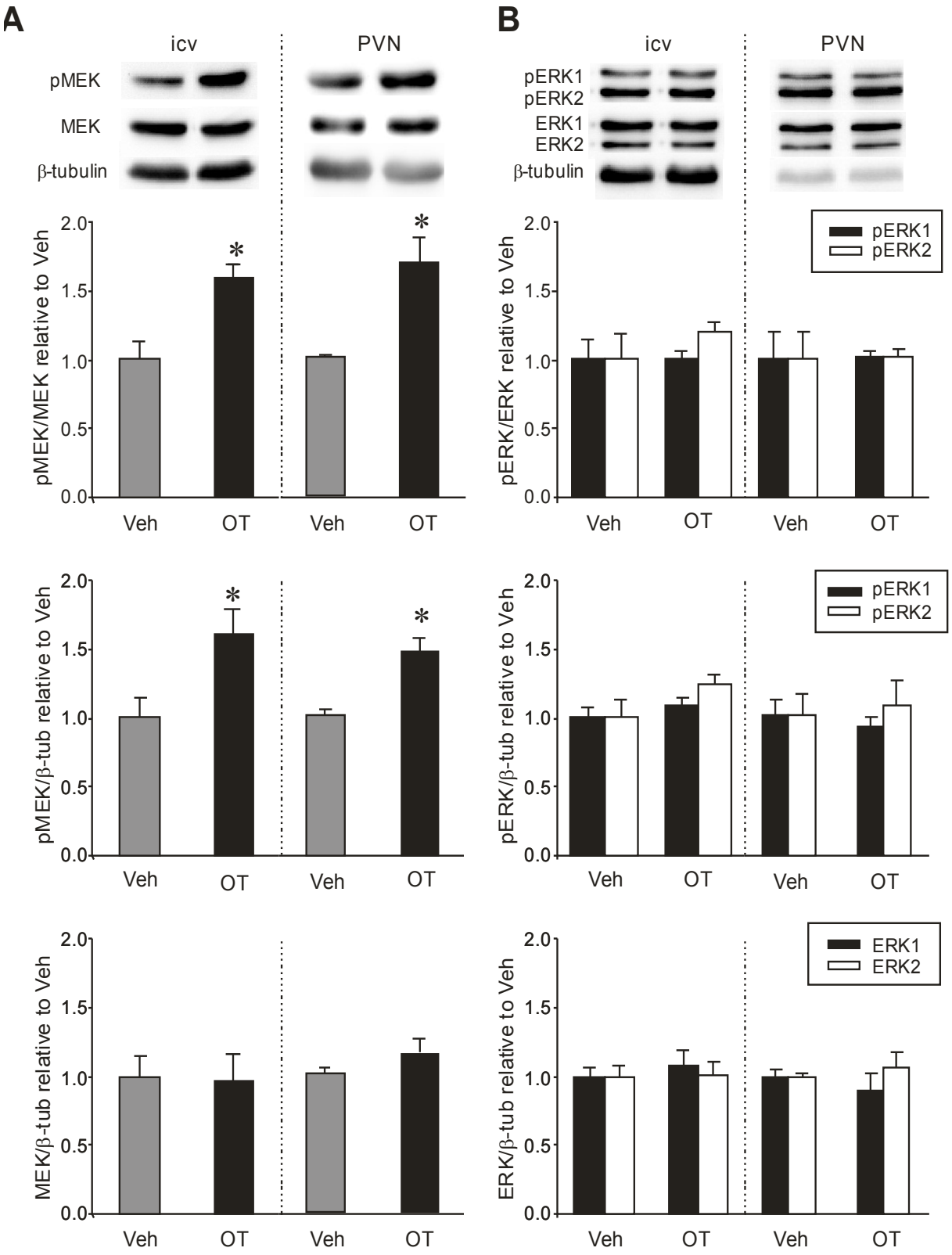

Figure 14 Activated MAPK cascades by an OT infusion into the lateral ventricle versus a bi-lateral intra-PVN infusion in male rats. A, OT phosphorylates MEK1/2 when infused into the lateral ventricle (1nmol/5µl) or bi-lateral intra-PVN (0.01 nmol/0.5 µl), whereas (B) activation of ERK1/2 is absent in icv as well

as in intra-PVN infused hypothalami. Data are expressed relative to respective Veh control group. Tub, ß-tubulin. t-test, * $p < 0.05$.

Experiment 4.1. Activated signaling cascades within the PVN of male rats induced by a TGOT infusion into the 3rd ventricle.

As OT infusion in male rats leads to MEK activation, but not ERK1/2 phosphorylation, we investigated if binding of OT to the VP receptor plays a role in the uncoupling of MEK from ERK. To address this issue, rats were infused with the highly specific OTR agonist TGOT (1nmol/2µl) into the 3rd ventricle to determine protein phosphorylation. Comparable to virgin females, OTR activation by TGOT induced MEK and also CaMKII phosphorylation (Figure 15A,B) with an almost 2 fold increase compared to vehicle infused rats ($p = 0.001$ for pMEK, $p = 0.047$ for pCaMKII n = 7-8). Again, the ratio of pERK / ERK in the cytosol or nucleus was not affected by OTR activation (data not shown).

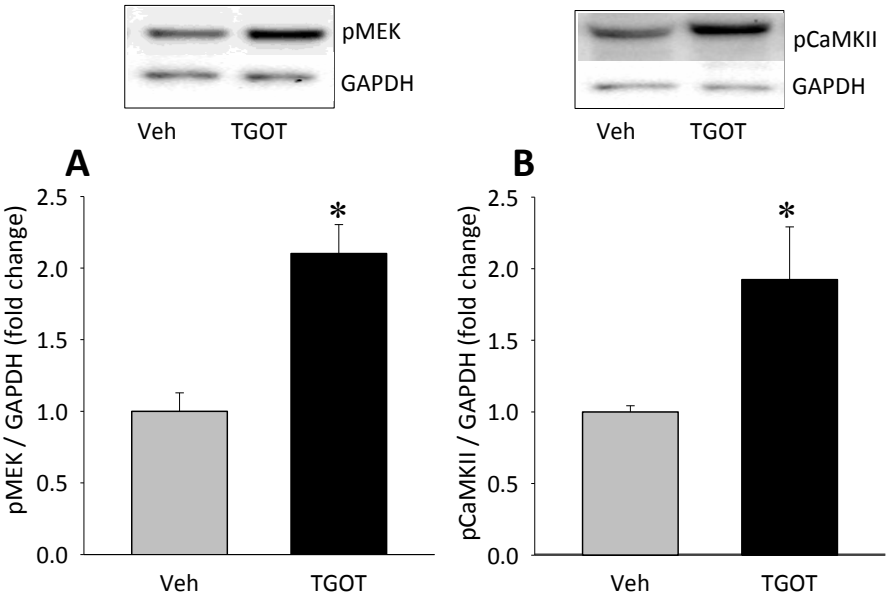

Figure 15 TGOT (1 nmol / 2 µl) infused into the 3rd ventricle of non-stressed male rats (TGOT) increases pMEK (A) and pCaMKII (B) in the cytosolic fraction of PVN homogenates. Representative western blot show the TGOT-induced increase of pMEK and pCaMKII relative to Veh controls. Phosphorylated protein was normalized to GAPDH. n = 7-8 in all groups;*$p < 0.05$ versus Veh

To further study the lack of involvement of pERK1/2 in OTR mediated signaling, we monitored the phosphorylation status of the scaffolding protein PEA-15 upon OT stimulation *in vivo* and H32 cells. OTR activation induces CaMKII (Figure 15B) and PKC phosphorylation (Gimpl and Fahrenholz 2001)**,** two kinases that phosphorylate PEA-15, a necessary pre-requisite for the release of pERK1/2 from PEA-15 and nuclear translocation of the ERKs. In line with the apparent lack of ERK1/2 phosphorylation (despite the activated MEK1/2) we found no increased phosphorylation of PEA-15 at Ser116 (CaMKII) in virgin female rats 10 min after infusion of OT (1 nmol / 5 µl) into the lateral ventricle (Figure 16). PEA-15 phosphorylation is regulated hierarchical with Ser116 phosphorylation preceding Ser104 phosphorylation. Thus, a lack in Ser116 phosphorylation indicates no subsequent Ser104 phosphorylation.

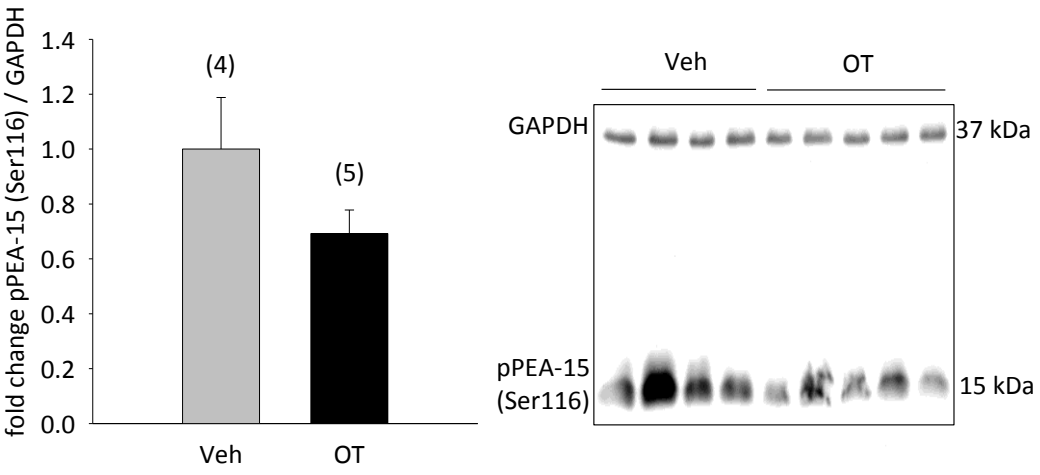

Figure 16 OT (1 nmol / 5 µl) infused into the lateral ventricle of non-stressed female virgin rats (OT) did not affect pPEA-15 (Ser116) in the cytosolic fraction of PVN homogenates (p = 0.152). Original western blot showing Veh vs. OT-induced pPEA-15 values (lower band, 15 kDa) normalized to GAPDH (upper band, ~37 kDa). The membrane was immunostained simultaneously with both antibodies. n = 4-5

Further, to exclude effects of OT at later time points, PEA-15 phosphorylation was monitored in H32 cells after 10, 20, 30, or 60 min of OT (250 nM) stimulation (Figure 17). In conclusion, OT was ineffective in phosphorylating PEA-15 *in vivo* and *in vitro* for up to 60 min.

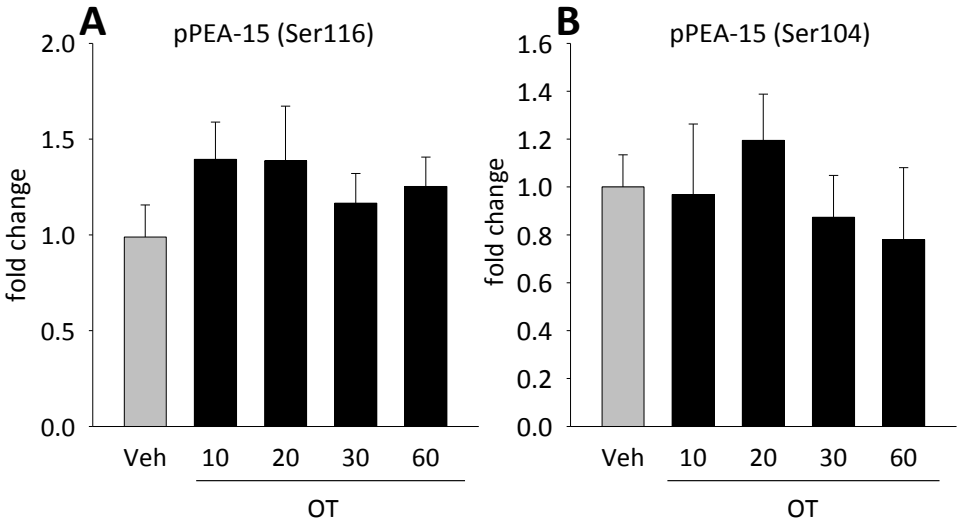

Figure 17 Time course of PEA-15 phosphorylation induced by OT (250 nM) at (A) Ser116, and (B) Ser104 in H32 cells. OT stimulation has no significant effect on the phosphorylation of PEA-15 at Ser116 or Ser104. Data are expressed as mean ± SEM, normalized to total PEA-15, n = 4 in all groups

Additional evidence for our model of OT-induced MEK, but not ERK activation and its inhibited nuclear translocation is represented by the lack of phosphorylation of the ERK-activated transcription factor Elk-1 after OT stimulation in H32 cells (data not shown).

Another important downstream target of the MAP kinase pathway is the central regulator of protein synthesis, mTORC. This complex unifies literally every signaling cascade discussed in this thesis, to regulate *de novo* protein synthesis. We assessed whether OT activates this complex by phosphorylation, in order to regulate protein synthesis, which might be the molecular underpinning of OTs long term anxiolytic effect. Due to the many phosphorylation sites of the mTOR complex, we determined the activation status by monitoring the autophosphorylation-site. All activating signals lead *via* different mechanisms to the

phosphorylation of this specific Ser2481, and therefore to the activation of the complex. To our surprise, we could not detect any influence of OT on mTOR phosphorylation, *in vitro* (250 nM; 30 min stimulation of H32 cells), nor *in vivo* (Martinetz et al., manuscript in preparation), thereby excluding mTOR as downstream target of OTR activation.

Nevertheless, OT could influence protein synthesis by activating the eukaryotic elongation factor 2 (eEF2), and indeed, time dependent activation of this regulating factor by OT in an *in vitro* assay was found in our lab (Martinetz et al., manuscript in preparation).

Experiment 4.2. Assessment of the anxiolytic effect of TGOT infused into the 3rd ventricle

Previous work has shown that an OTR antagonist infused into the lateral ventricle has no effects on anxiety-like behavior (Neumann et al. 2000). As OT binds the vasopressin 1A receptor with high affinity, and AVP is anxiogenic, its anxiolytic activity may have been masked by simultaneous activation of both receptors. We therefore assessed whether the highly specific OTR agonist TGOT reduces anxiety-like behavior when infused into the 3rd ventricle. However, TGOT, applied at 1 nmol / 2 µl did not modulate any anxiety or locomotor-related behavior parameter measured on the LDB (data not shown), supporting the view that OT needs to be secreted in specific brain regions (PVN and amygdala) to bring about anxiolysis.

Experiment 5. Assessment of sex-specific differences in the pathway leading to CREB phosphorylation by 3rd ventricle infusion of OT

Infusion of OT into the 3rd ventricle of virgin female rats did not activate the CREB-regulating MAPK p38 (Figure 18A), whereas in males a slight, yet significant increase of phosphorylated p38 could be detected (Figure 18B, p=0.028, n=7). p38 is one of several upstream kinases of MSK1/2, and accordingly, MSK1/2 appeared activated in the PVN of males, and, somewhat surprisingly, in females (1.5-fold in males, p=0.009, and 3.5-fold in females, p=0.029, n=5-7). It is thus possible that MSK1/2 is activated by pp38 in males and by a yet unknown factor in females. The downstream effector of MSK1/2 is the transcription factor CREB, which was found to be activated by OT in males ((1.5 fold, p=0.011, n=7)) and females (2.3 fold, p=0.02, n=4)).

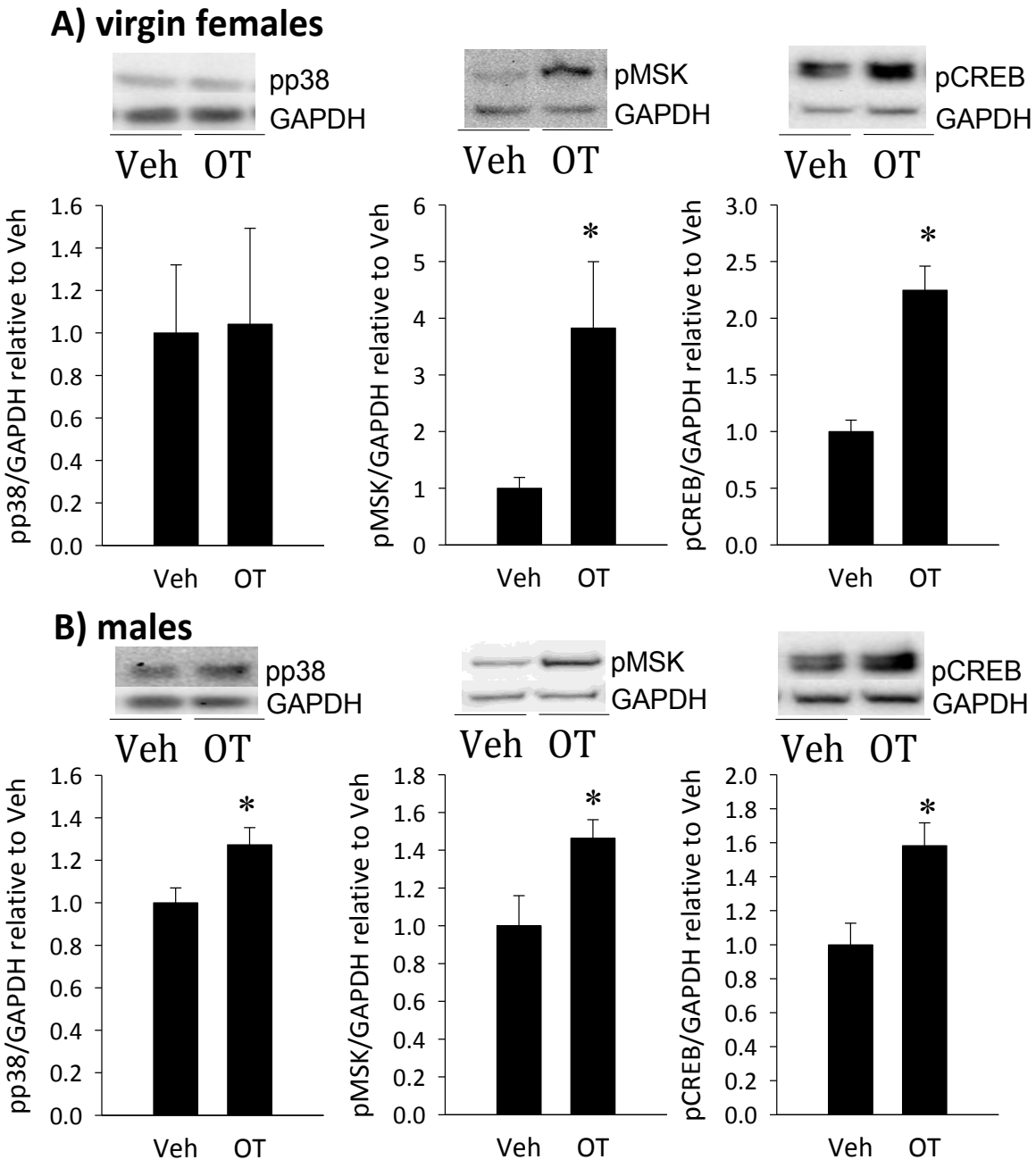

Figure 18 Sex specific activation of MAPK pathways leading to CREB phosphorylation. A, Infusion of OT into the 3rd ventricle of virgin female rats does not activate the MAPK p38, whereas phosphorylation levels of MSK1/2 and its downstream transcription factor CREB increase 3,5 fold or 2,2 fold, respectively. B, In contrast to females, p38 could be the OT-induced upstream kinase of MSK, which in turn phosphorylates CREB. Data relative to respective control value of 1 (t-test, * $p < 0.05$, n=4-7)

Summary Part I

The data presented in Part I reveal a tonic activation of MEK1/2 and ERK1 within the PVN during lactation, but not during late pregnancy or in virgin animals. OT infused into the PVN is anxiolytic and activates MEK1/2 in virgin rats. Blocking the activity of MEK1/2 in virgin rats prevented the anxiolytic activity of OT, and caused even a profound anxiogenic phenotype in lactating rats (in the absence of exogenous OT). Anxiolysis was exclusively observed when OT was applied into the PVN of virgin rats, whereas lateral or 3rd ventricle infusions increased hypothalamic pMEK1/2 levels in males and females, but was without effect on anxiety-related behavior. Surprisingly, OT infusion in lactating rats reduced MEK1/2 phosphorylation and was without effect on anxiety-related behavior, suggesting a highly dynamic and context-dependent role for MEK1/2 in OT-induced behavior. Moreover, the OT-activated MEK1/2 in virgin female and male rats displayed no kinase-activity for ERK1/2, providing evidence for a yet unknown downstream target of OTR-coupled MEK1/2 in the hypothalamus. The lack of ERK1/2 phosphorylation is supported by the observation of inactive PEA-15 and the ERK1/2 downstream target Elk-1. Furthermore, activation of the canonical p38 - MSK1/2 - CREB pathway in males, or a similar but p38-independent pathway in females, could provide one molecular mechanism of OT-induced gene transcription. CREB mediated CRF transcription and OT's role in its regulation will be addressed in Part II.

Part II: Stress and oxytocin; molecular mechanisms that regulate CRF gene expression

(Adapted from: **Benjamin Jurek**, David A. Slattery, Ying Liu, Inga D. Neumann, Greti Aguilera, Erwin H. van den Burg (2013) Oxytocin regulates CRF gene transcription *via* altered translocation of CREB-regulated transcription coactivator 3 (CRTC3), in preparation)

Author's contribution:

Jurek: study design, protein isolation, SDS Page, Western Blot, primary cell isolation and culturing, cell culture with cell lines H32 and Be(2)-M17, cell stimulations, knock down by siRNA, Chromatin IP, data analysis in all experiments, writing the manuscript

Slattery: study design, brain isolation in *in vivo* Experiments, writing the manuscript

Liu: technical help with primary neuron isolation, cell culture and western blotting at NIH

Aguilera: study design, technical help with primary neuron isolation at NIH, writing the manuscript

Neumann: study design, writing the manuscript

van den Burg: study design, writing the manuscript

Introduction to Part II

The neuropeptide CRF, synthesized in the PVN, is the main activator of the HPA axis during stress. Following its release into the median eminence, CRF stimulates the release of ACTH from the pituitary gland. CRF-producing neurons are under negative feedback from glucocorticoids to prevent the detrimental effects of long-term HPA axis activity. CRF cells are further regulated by other factors during stress, including the neuropeptide OT (Neumann et al. 2000; Windle et al. 2004). Known for its role in reproduction in the periphery, central OT promotes prosocial behavior and dampens the neuroendocrine stress response (de Kloet et al. 2005; Neumann and Landgraf 2012). Both elevated endogenous levels of OT protein and mRNA, as observed during the peripartum period (Neumann et al. 2000; Hillerer et al. 2011), as well as chronic infusion of synthetic OT (Windle et al. 2004), reduce the stress-induced ACTH response in rats. However, former studies have reported potentiating effects of peripheral OT on CRF (Antoni et al. 1983) or stress-induced ACTH release (Gibbs et al. 1984; Petersson et al. 1999; Ondrejcakova et al. 2010); thus, the role of OT in the control of CRF synthesis and release remains enigmatic. Intriguingly, OT is released within the PVN during or immediately after stress (Engelmann et al. 1999), and OTRs are located on magnocellular CRF cells within this region (Dabrowska et al. 2013). Thus, it is feasible that OT can influence CRF synthesis and release directly within the PVN, and hence HPA axis-activity.

One of the early hallmarks of CRF cell activity during stress is increased CRF gene transcription *via* a cAMP/PKA-dependent mechanism; presumably to replenish protein

stores within the cells (Aguilera and Liu 2011). The downstream binding of pCREB to a cAMP-responsive element in the promoter is accompanied by binding of one or more of the three known CRTCs to CREB. Under basal conditions, CRTC is phosphorylated and resides in the cytoplasm, bound to the scaffolding protein 14-3-3. Following its dephosphorylation, CRTC translocates to the nucleus where it binds CREB *via* its bZIP domain and recruits CBP/p300 to the CRF promoter for gene transcription to commence (Conkright et al. 2003; Liu et al. 2011).

While it remains unknown whether OT influences CRF gene transcription, in the hippocampus both OT and stress induce the phosphorylation of CREB (Tomizawa et al. 2003), which led us to hypothesize that OT regulates CRF expression by controlling the translocation and, therefore, nuclear availability of the CREB co-factor CRTC2 or 3. Besides CRTC3, the related CRTC2 has been shown to play an important role in the regulation of CRF gene transcription (Liu et al. 2011), but to our knowledge, it is not clear to which extent CRTC2 and CRTC3 share their modulating potential on CRF gene transcription *in vivo*. Therefore, the aim was to determine whether OT, concomitant to the reduction of stress-induced ACTH release, may inhibit CRF gene transcription through phosphorylation of one or more of the CTRCs.

Outline of experiments part II

Experiment 6. Assessment of the effect of exogenous OT, applied in the 3rd ventricle, on CRF gene expression and plasma ACTH

Male Wistar rats were infused with OT (1nmol / 2µl, 3rd ventricle) or vehicle (Ringer), and stressed by restraint 10 min later as described above, and PVN tissue and trunk blood were collected for CRF gene expression and ACTH concentration, respectively. Primers targeting the rat CRF intron (hnRNA), or the spliced intron-less variant (spanning exon-intron junctions; mRNA) were used to determine transcript levels by RT-qPCR. Blood samples for ACTH measurement were collected in ice-chilled 1 ml EDTA-tubes. After centrifugation for 15 min at 3000 g, plasma (supernatant) was stored at -80°C until analyzed. ACTH levels were determined by an ELISA-assay specific for human ACTH (#SG51041; IBL, Hamburg) according to the manufacturers' protocol. The colorimetric readout signal was determined using an Optima FluoStar plate reader.

Experiment 7. Effect of TGOT on restraint stress-induced CRTC translocation and CREB phosphorylation

Male rats were infused with TGOT (1nmol / 2µl, 3rd ventricle) or vehicle (Ringer) as described in Experiment 7 and in the restraint stress protocol. PVN punches from non-frozen tissue (as described in Experiment 4) were treated to obtain separate cytosolic and nuclear protein lysates to determine levels of CRTC2 and CRTC3 in both fractions, and pCREB in the nuclear fraction.

In addition to CRTCs, we assessed the contribution of the JNK – c-Jun signaling pathway to the regulation of the CRF gene (King and Nicholson 2007). We hypothesized a modulatory cross-talk between OTR signaling and the JNK pathway to regulate CRF gene transcription in rats during stress. Rats were placed for 5 or 10 min on an elevated, inescapable platform (25 cm diameter, 75 cm height), located at 50-70 cm distance from their home-cage. This procedure is established in our laboratory as a mild stressor to activate the HPA axis. Control animals were left undisturbed in their home-cage to control for the duration of the stressor. Brains were removed and the hypothalamic tissue isolated as described in Experiment 2 for western blot analysis with the respective JNK antibodies. Detailed assessment of the cross-talk between OT and JNK signaling was carried out in H32 cells, stimulated with TNFα (50 ng/ml) or anisomycin (5 µM) as potent stimulators of JNK signaling, and OT (10 -1000 nM).

Experiment 8. Effects of TGOT on FSK-induced CRF hnRNA, CREB phosphorylation, and CRTC2/3 translocation in primary neurons, and H32, and Be(2)-M17 cells

To study TGOT's effects on CRTC translocation and CRF gene expression in more detail we made use of primary neurons, H32 cells, and the human neuroblastoma cell line Be(2)-M17. Cells were cultured and stimulated as described above in the *material and methods* paragraph with FSK (Sigma) at 10, 50, or 75 µM, dissolved in 0.1 % dimethylsulfoxide (DMSO), or vehicle (0.1 % DMSO in cell culture medium), and TGOT at 10 or 100 nM concentration. Time course experiments were carried out in a reverse manner, beginning the incubation period at the longest time point (240, 90, or 60 min), and adding drugs when appropriate to the remaining cell culture dishes, so that all cells (including non-treated controls) were serum-starved for the same amount of time and harvested all at once (time

point 0). To allow for comparisons between independent experiments, the values of CRF expression, and cytosolic and nuclear CRTC2 and CRTC3 content are expressed relative to the maximum level of the Veh + FSK group in each experiment.

Experiment 9. siRNA knockdown of CRTC3 in Be(2)-M17 cells reduces FSK-induced CRF transcription, but also blocks the TGOT-induced negative effects on CRF hnRNA

Be(2)-M17 cells were pre-incubated with specific CRTC2, CRTC3, or negative control oligo #5 siRNA as described above in the siRNA transfection paragraph. After 12 h of incubation at 37°C and 95% O_2 / 5% CO_2, cells were treated with FSK and TGOT for 1h. Half of the petri-dishes were used for RNA isolation to assess CRF hnRNA, as well as CRTC2 and 3 mRNA levels, whereas the other half was used for protein isolation to determine the efficiency of the knockdown of CRTC2 and CRTC3 at the protein level.

Experiment 10. TGOT attenuates FSK-induced binding of CRTC3, but not CRTC2 to the CRF promoter

To determine whether changes in CRTC2 and CRTC3 translocation correlate with altered CRTC2 and 3 occupancy of the CRF promoter, we immuno-precipitated chromatin from FSK + TGOT (10 min) stimulated Be(2)-M17 cells with a CRTC2 or CRTC3 antibody as described above in the ChIP paragraph and analyzed the obtained chromatin fragments by RT-qPCR with CRF-promoter specific primers. Other known anxiety-related gene promoters, which also contain a CRE region, served as controls.

Results Part II

Experiment 6. Assessment of the effect of exogenous OT, applied in the 3rd ventricle, on CRF gene expression and plasma ACTH

Vehicle treated / restraint stressed (Veh-RS) rats displayed a rapid increase in CRF hnRNA in the PVN with maximal levels observed between 10 and 15 min of restraint, which returned to near-basal levels by 30 min. OT delayed this stress-induced response, with peak CRF hnRNA values found in oxytocin treated / restraint stressed (OT-RS) rats at 15 min and a slower decrease to basal levels (two-way ANOVA; treatment x time $F_{(3;48)}$= 195,4; p= 0.001, Figure 19A). After 10 min of stress, OT-RS rats showed a reduced CRF hnRNA level compared with Veh-RS controls (p = 0.001), whereas at the 30 min time point, CRF hnRNA levels of OT-RS rats were higher than both basal levels and those of Veh-RS animals (p = 0.001). There was no difference in CRF hnRNA expression within the PVN between oxytocin treated / non-stressed (OT-NS) and Veh-NS rats, indicating that OT has no effect on basal CRF expression.

As CRF controls ACTH release from the pituitary gland, plasma ACTH levels were measured as a rough reflection of CRF release. In parallel to the delayed increase in CRF hnRNA expression, OT likewise tended to delay the plasma ACTH response to restraint stress (two-way ANOVA; treatment x time $F_{(3;36)}$= 3.4; p = 0.032, Figure 19B). In the Veh-RS group, plasma ACTH concentrations peaked at 10 min and remained elevated until the end of the stressor. In the OT-RS rats, plasma ACTH tended to be lower at 10 min (p = 0.065 versus Veh-RS), and peaked at 15 min of restraint, thus being significantly higher (p = 0.035) than those in Veh-RS animals. At 30 min, ACTH levels of Veh-RS and OT-RS treated rats did not differ (p

= 0.109). Thus, OT, when infused into the 3rd ventricle prior to the onset of restraint stress, acutely delays the increase of CRF gene transcription following restraint stress, whereas effects on HPA axis activity, as measured by trunk blood ACTH concentration, are negligible.

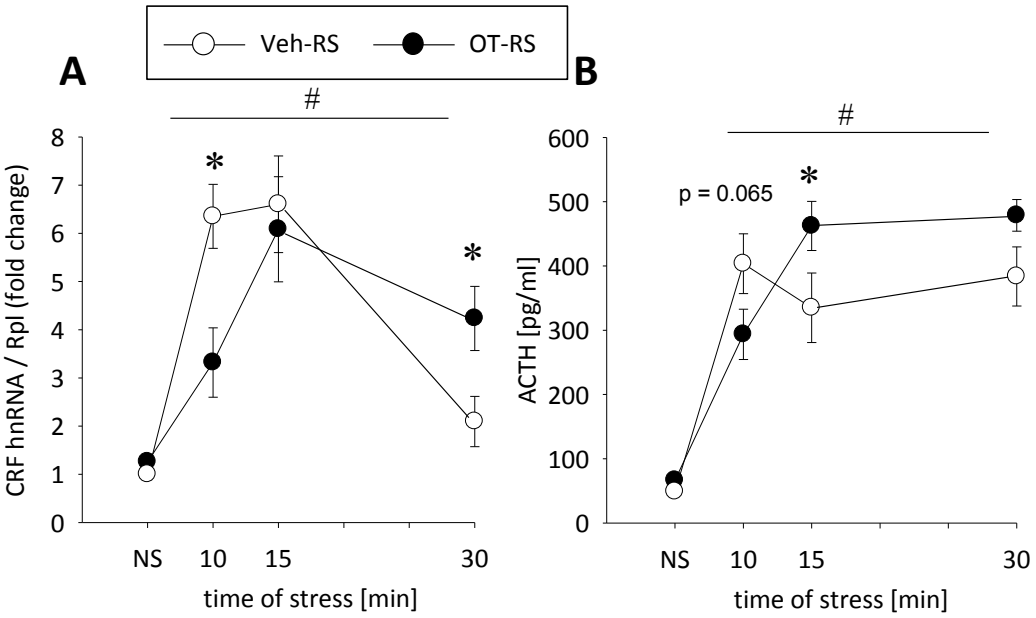

Figure 19 OT (1 nmol / 2 µl infused into the 3rd ventricle) delays restraint stress-(RS)-induced CRF gene transcription in the PVN (A) and plasma ACTH response (B). A, The stress-induced increase in normalized CRF hnRNA levels (Veh-RS) is delayed by an OT infusion 10 min earlier (OT-RS), as is the subsequent return to basal levels. B, OT tends to delay the increase in plasma ACTH levels induced by restraint stress, and establishes slightly higher peak levels. Under basal conditions, icv OT (OT-NS) was ineffective in all of the experiments. Data are mean ± SEM. * $p < 0.05$ versus Veh-RS group at the respective time point, # $p < 0.05$ versus respective NS group, n = 4 (10 and 15 min), or n = 8 (NS, 30 min) for all figures.

In contrast to the findings above, there were no changes in CRF mRNA levels during the time period studied (30 min) in any of the groups tested (Figure 20). Changes may occur at later time points, as processing of hnRNA into mRNA takes longer than 30 min (Ma et al. 1997).

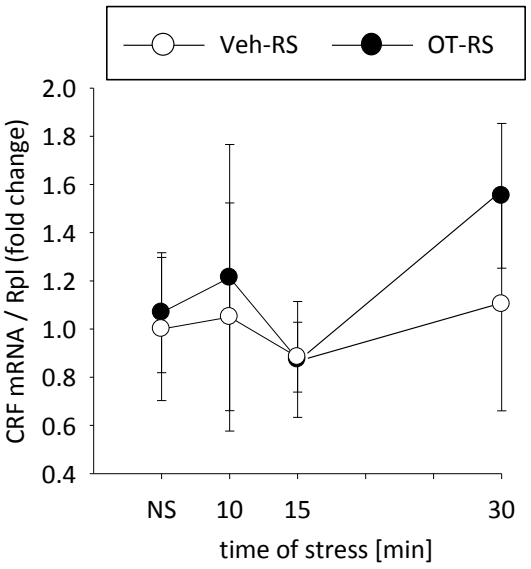

Figure 20 CRF mRNA levels remained constant and were not affected by both, OT (1 nmol / 2 µl infused into the 3rd ventricle) or restraint stress-(RS) in the PVN. Data are mean ± SEM, n = 4 (10 and 15 min), or n = 8 (NS, 30 min).

Experiment 7. Effect of TGOT on restraint stress-induced CRTC translocation and CREB phosphorylation

To study the mechanism underlying the effects of OT on CRF gene expression *in vivo*, we examined the translocation of CRTCs following 10 min of restraint stress. As expected, stress tended to decrease cytosolic levels of CRTC2 (not significant) and CRTC3 ($p = 0.007$) compared to Veh-NS rats (Figure 21A, C). This reduction was mirrored in the nuclear compartment, where CRTC2 and CRTC3 levels were significantly increased ($p = 0.03$ for both; Figure 21B, D). This provides clear evidence for stress-induced activation of CRTC2 and CRTC3 and their subsequent nuclear translocation within the PVN.

Pre-treatment of the stress group with TGOT (TGOT-RS) prevented the reduction of cytosolic levels of CRTC2 and CRTC3 significantly compared to RS rats (Figure 21A, C) and, accordingly, decreased nuclear levels of CRTC2 (not significant) and CRTC3 ($p = 0.026$) compared to Veh-RS (Figure 21B, D). These data suggest that TGOT inhibits the stress-induced activation, and

subsequent translocation, of predominantly CRTC3 and to a lesser extent of CRTC2. This effect on CRTCs was shown to be region-specific, as no effect of TGOT or stress, on CRTC2 or CRTC3 translocation was found in the cortex (data not shown; for region specific CRTC expression levels see (Watts et al. 2011)).

As CRTCs are co-factors of CREB signaling, we assessed whether TGOT inhibits the stress-induced pCREB phosphorylation. Icv infusion of TGOT did not influence the increase of pCREB following 10 min of restraint stress, suggesting that OT regulates CRF gene expression through modulation of CRTC translocation, rather than CREB phosphorylation (Figure 21E, F).

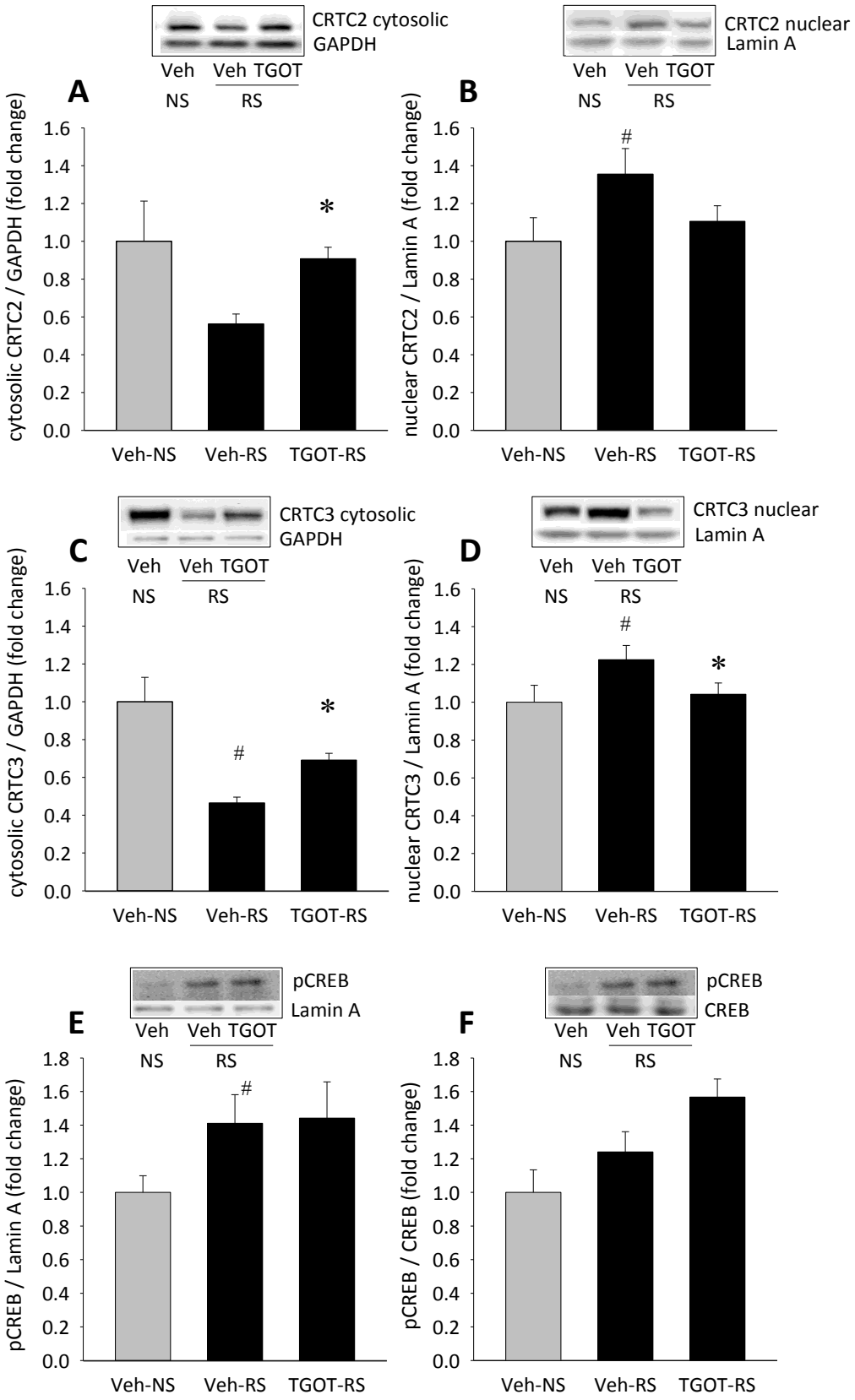

Figure 21 TGOT prevents the RS-induced translocation of CRTC2 and CRTC3 from the cytosol to the nucleus without modifying stress-induced phosphorylation of CREB (pCREB) in PVN protein extracts. TGOT (1nmol/2µl, 3rd ventricle) prevents the RS-induced decrease in cytosolic CRTC2 (A), the simultaneous increase in nuclear CRTC2 (B) the decrease in cytosolic CRTC3 levels observed after RS (C) and the simultaneous increase in

nuclear CRTC3 levels (D). CRTC2/3 levels were normalized for total protein levels by GAPDH (A,C), or Lamin A (B,D). The RS-induced increase in nuclear pCREB is not influenced by TGOT when normalized for total protein (E) or total CREB (F) levels. Data are mean ± SEM. *p < 0.05 versus Veh-RS; #p < 0.05 versus Veh-NS, n = 5-7 in all groups.

Involvement of JNK in the stress response

Another factor that binds the CRF promoter to regulate its gene expression is the transcription factor c-Jun, whose upstream kinase is the stress activated MAP kinase JNK. We found in a preliminary experiment that even a mild stressor (elevated platform stress) increased the level of phosphorylated JNK1 to 135% ± 1.6% (Figure 22) in the hypothalamus of male rats compared to non-stressed animals, indicating an active role in the regulation of the stress response. The related JNK2 and JNK3 were not activated by the stressor, indicative by the very faint bands above pJNK1.

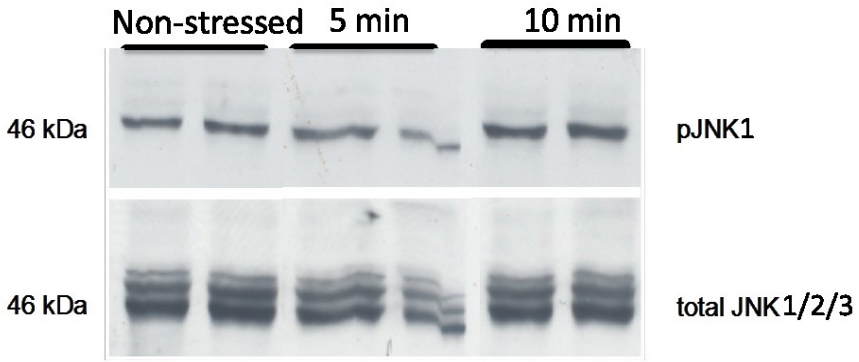

Figure 22 Representative Blot revealing a 135 ± 1.6% increase of phosphorylated JNK1 in the hypothalamus of male rats after 10 min of elevated platform stress. The level of total JNK1/2/3 was not altered over the time period measured.

To further study the crosstalk between the activated OTR and JNK – c-Jun signaling we made use of the rat hypothalamic cell line H32.

Anisomycin, a strong activator of JNK signaling induced JNK phosphorylation (211 ± 27 % at 10 min; 785 ± 32 % at 20 min), whereas TGOT alone had no effect. Also, TGOT did not alter anisomycin-induced pJNK levels. In addition to the lack of effect on JNK, we found no influence of TGOT on the phosphorylation of JNK's downstream transcription factor c-Jun (Figure 23).

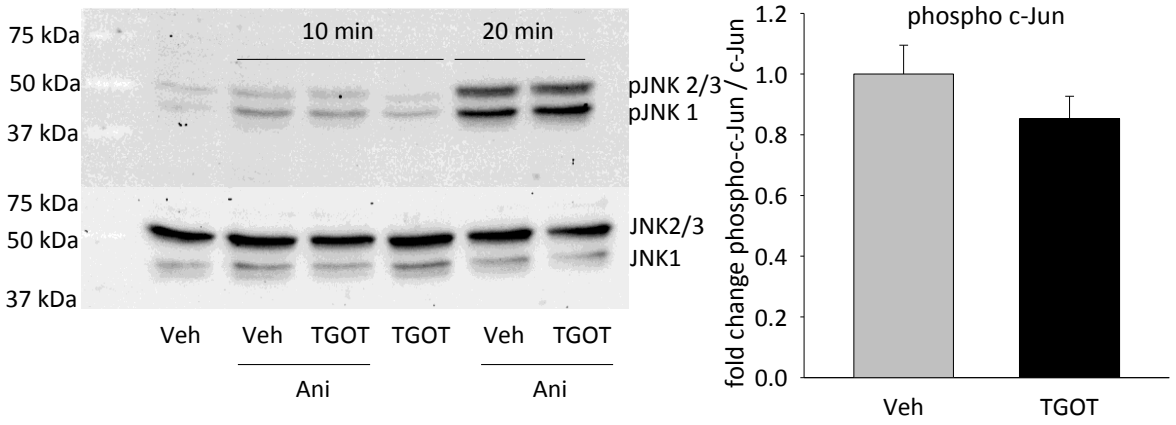

Figure 23 TGOT does not alter basal or anisomycin-induced pJNK1/2/3, or phospho-c-Jun levels in H32 cells **A**, Representative western blot of TGOT (10 nM) and anisomycin (5µM)-induced JNK1/2/3 phosphorylation. Anisomycin stimulation for 10 or 20 min induces prominent JNK1/2/3 phosphorylation, whereas TGOT has no effect on basal pJNK or on anisomycin-induced pJNK-levels at 10 or 20 min time point. The level of total JNK remained constant in the cytosol. B, In line with the lack of effect on JNK, TGOT treatment (20 min, 10 nM) did not induce c-Jun phosphorylation in the nuclear compartment. Data are expressed as mean ± SEM, normalized to total JNK (A), or total c-Jun (B), n = 4.

Taken together, our *in vivo* results suggest that OT delays the peak of CRF transcription activity, and that this effect might be mediated by the regulation of CRTC3, and partly CRTC2, translocation, while cross-talk between the OTR and the stress-activated JNK pathway is unlikely.

Experiment 8. Effects of TGOT on FSK-induced CRF hnRNA, CREB posphorylation, and CRTC2/3 translocation in primary neurons and H32 cells

To study the effects of OT and TGOT on CRF gene transcription and CRTC translocation in more detail, we made use of rat primary hypothalamic cells, and the rat hypothalamic cell line H32 (Mugele et al. 1993).

Stimulation of rat embryonic d18 primary hypothalamic neurons with FSK (1 µM) and/or TGOT (10 nM) revealed a striking similarity in the pattern of CRF hnRNA expression compared with adult rat hypothalamic PVN tissue *in vivo*. FSK induced a rapid increase of CRF hnRNA levels, which peaked at 20-30 min ($p < 0.001$ for both time points), and returned to near-basal levels ($p = 0.061$) at 90 min. The presence of TGOT in the incubation medium reduced the FSK-induced CRF hnRNA-peak to 74 ± 4.6 % at 30 min ($p = 0.002$). Also, TGOT prolonged the duration of FSK-induced CRF gene expression, as CRF hnRNA levels were still elevated at the 90 min time point (Figure 24A; two-way ANOVA $F_{(5,84)}= 4.6$; $p < 0.001$).

The protein yield of the primary cultures was too low to analyze CRTC2 and CRTC3 translocation and CREB phosphorylation by western blotting. Therefore, we made use of the immortalized rat hypothalamic cell line H32 (Mugele et al. 1993), because of their hypothalamic origin and expression of endogenous OTR. Although these cells have lost CRF gene expression, the CREB-CRTC signaling pathway that is known to regulate CRF gene expression (Liu et al. 2011), and pathways that are coupled to the OTR, are still intact (Blume et al. 2008). In H32 cells, we found no effect of TGOT on the FSK-induced, transient CREB phosphorylation, peaking at 10 min and returning to baseline 20 min after the incubation started (Figure 24B). Likewise, FSK-induced nuclear CRTC2 levels were not affected by TGOT

during the first 60 min after administration (Figure 24C), but a further increase in nuclear CRTC2 was found 90 min after the application of both compounds ($p = 0.047$).

In sharp contrast, TGOT had profound effects on the FSK-stimulated increase in nuclear CRTC3 levels of H32 cells (Figure 24D). FSK alone augmented CRTC3 in the nucleus with a peak at 10 min after the onset of FSK stimulation ($p < 0.001$), and maintained nuclear CRTC3 levels elevated during the 90 min of incubation ($p < 0.001$). Treatment of H32 cells with TGOT prevented the fast peak that was induced by FSK alone at 10 min ($p = 0.007$), but steadily increased nuclear CRTC3 levels until their maximum was reached at 60 min at a level that was about 1.4-fold higher than the peak levels induced by FSK alone after 10 min ($p < 0.001$) (two-way ANOVA, $F_{(6,64)}= 5.3$; $p < 0.001$). Next, a rapid drop during 30 min annuled the differences between FSK- and FSK + TGOT-induced nuclear CRTC3 levels at 90 min. Comparable to the lack of effect of TGOT alone on CRF hnRNA in primary neurons (Figure 24A), we found no effect of TGOT without FSK on nuclear CRTC2 and CRTC3 levels (Figure 24C,D).

The data from the restraint stress experiments, together with those obtained in cell culture, suggest that OT controls CRF expression through inhibited nuclear translocation of CRTC3 during stress at early time points, but not under basal (NS or without FSK) conditions.

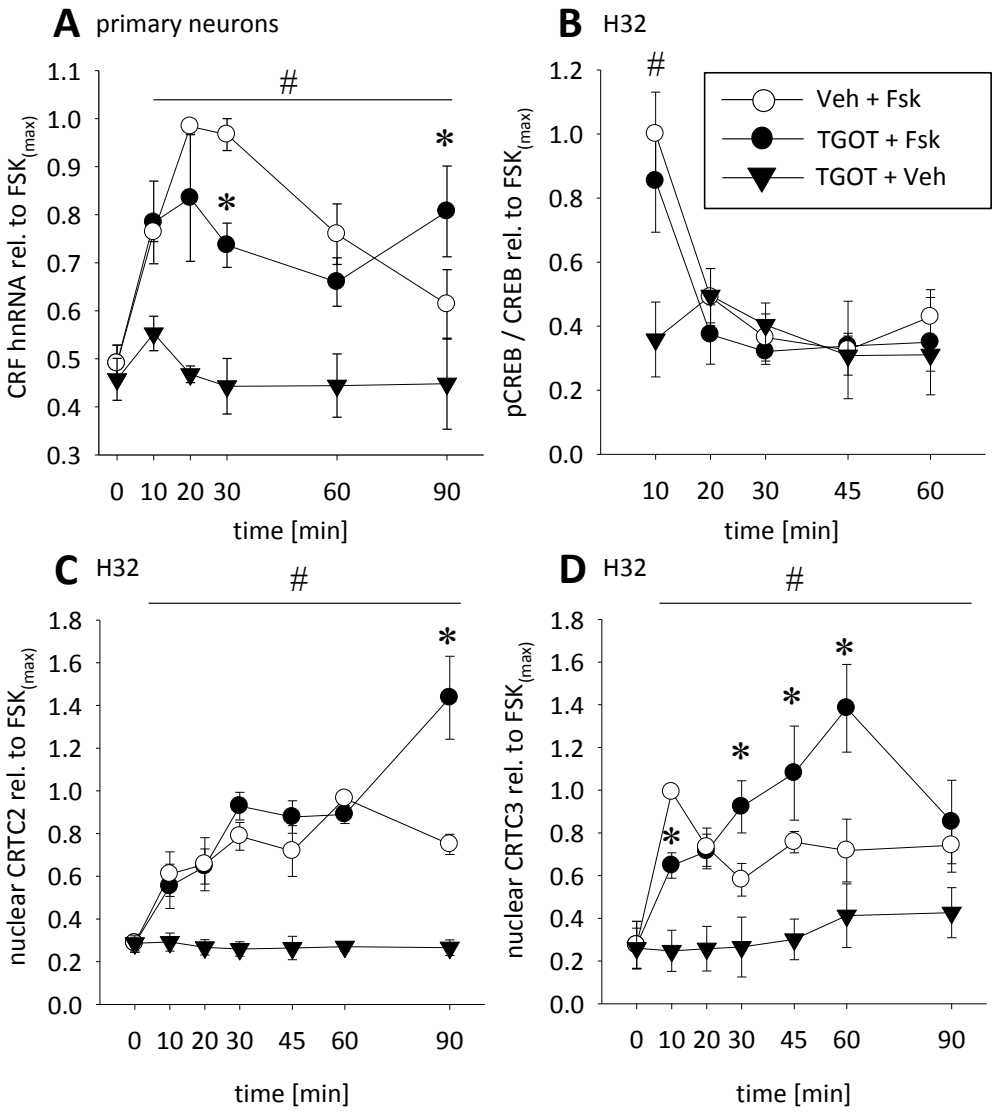

Figure 24. TGOT (10 nM) reduces the FSK (1µM)-induced CRF hnRNA levels in primary cells, and delays FSK-induced increase of nuclear CRTC3 levels in H32 cells. A, In primary hypothalamic cells, the CRF hnRNA levels that are augmented by FSK peak between 20 and 30 min of incubation; peak levels were set to 1 to make comparisons across multiple independent experiments possible in this and subsequent figures. TGOT reduces the magnitude of the CRF hnRNA response, and delays the return to basal levels. B, TGOT does not affect the FSK-induced increase of CREB phosphorylation. Data was normalized with total CREB. C, TGOT has no effect on the FSK-induced increase of normalized (to Lamin A) nuclear CRTC2 levels until 60 min of incubation. D, TGOT delays the FSK-induced normalized (to Lamin A) nuclear CRTC3 levels, but increases these to 1.4 fold higher at 60 min as compared to the maximum response to FSK alone at 10 min. Two-way ANOVA was performed followed by Student-Newman-Keuls post hoc test. * $p < 0.05$ versus Veh + FSK; # $p < 0.05$ versus respective 0 min time point (Veh + FSK, TGOT + FSK groups). n= 5-8

Experiment 8.1. Forskolin (FSK) and TGOT dose response and time course experiments in human Be(2)-M17 cells

To study the effects of OT on CRTC translocation in more detail, we made use of the human neuroblastoma cell line Be(2)-M17. First, we assessed the presence of OTR, V1a, and V1b mRNA in the cell line. As depicted in Figure 25 Be(2)-M17 cells express the OTR (215 bp amplicon), but also V1a (135 bp amplicon) and, although very faint, V1b receptors (128 bp amplicon). To exclude activation of the vasopressin receptors by OT, we used from there on the specific OTR agonist TGOT exclusively. For this initial experiment we used RNA isolated from Veh or OT treated cells to compare receptor mRNA levels after treatment. As expected, we found no alterations in receptor mRNA after a 60 min incubation with 250 nM OT. In line with literature (Gimpl and Fahrenholz 2001), this preliminary result suggests that gene expression of the OTR is not altered by 60 min of OT treatment. However, receptor desensitization and/or internalization upon TGOT treatment may occur and further experiments to validate the initial results are needed.

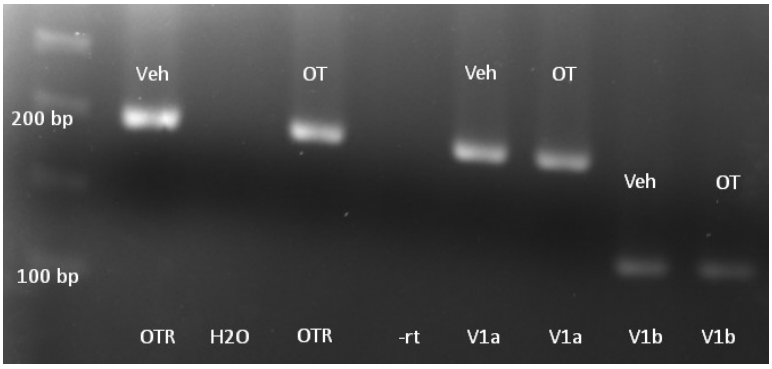

Figure 25 Agarose-gel displaying qPCR amplicons of vehicle-or OT stimulated Be(2)-M17 cells. mRNA was isolated from stimulated cells (Veh; Ringers, or OT 10 nM for 1h) and analyzed using specific primer pairs for the human OTR, V1a and V1b receptor.

In initial experiments, lower doses of FSK (1-10 µM) failed to induce reproducible CRF responses. Three independent dose-response experiments using 10, 50, and 75 µM of FSK (for comparable doses see (Sala et al. 2000; Liu et al. 2010; Heo et al. 2013)) were performed

and 60 min stimulation with 50 µM of FSK induced the maximal and most reliable CRTC3 ($p < 0.001$) translocation from cytoplasm (Figure 26C) into the nucleus (Figure 26D, $p < 0.001$) and CREB phosphorylation (Figure 26F, $p < 0.001$).

To address if the different affinity states of the OTR are involved in this response (Gimpl and Fahrenholz 2001), we compared two doses of TGOT (10 nM versus 100 nM) in their effectiveness to reduce FSK-induced CRF hnRNA levels (Figure 26E). Indeed, FSK increased CRF hnRNA levels after 60 min of incubation, and this was significantly reduced by either dose of TGOT with no apparent dose dependency (one-way ANOVA $F_{(4;29]}= 12.3$; $p = 0.001$). Therefore, 10 nM TGOT was used throughout the rest of the experiments.

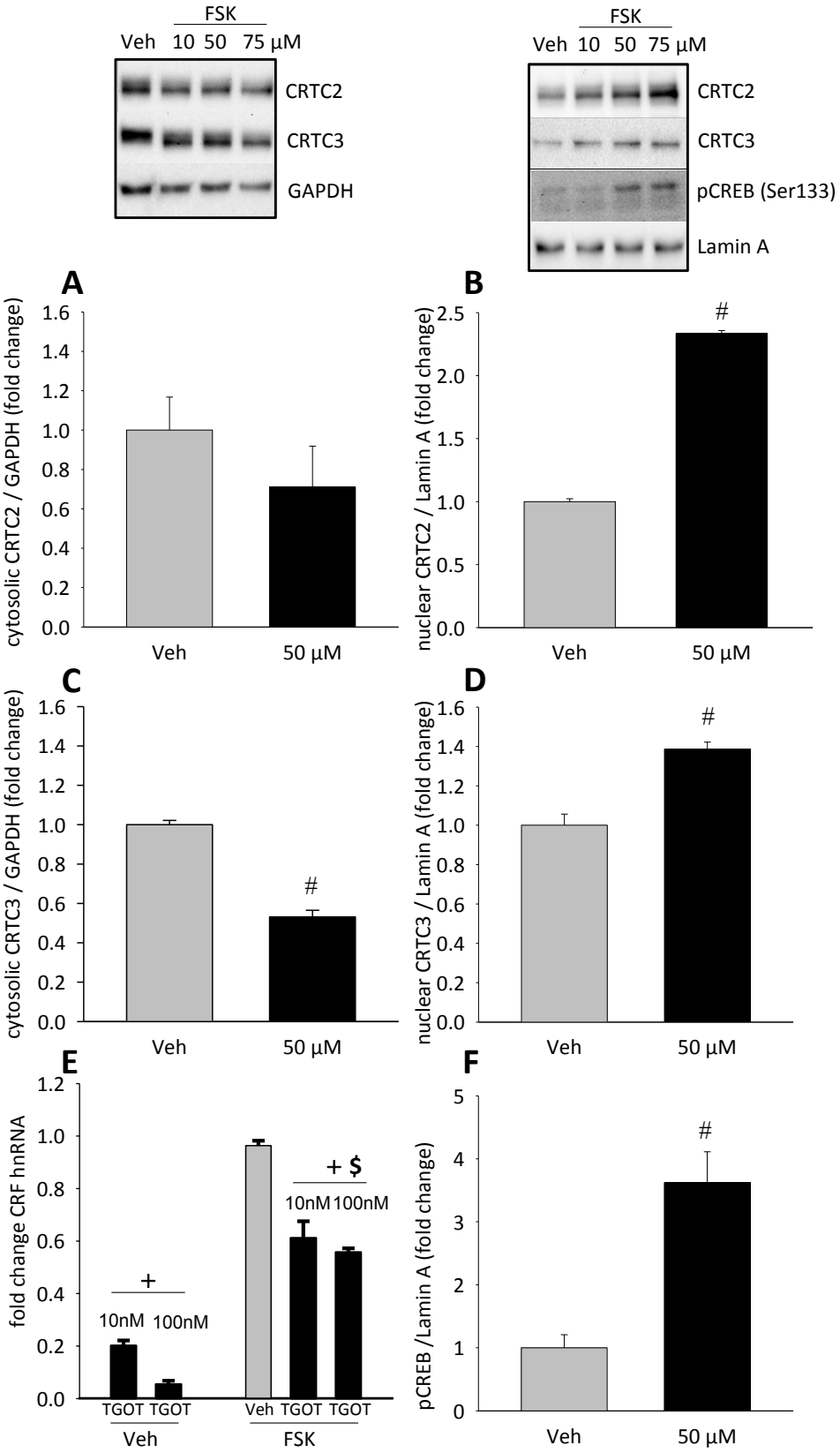

Figure 26. Dose response experiment to determine the optimal dosage of FSK and TGOT-induced stimulation of CRTC2 and CRTC3 translocation, CREB activation, and CRF hnRNA expression in Be(2)M17 cells. Be(2) M17 cells were treated with 10, 50, and 75 µM FSK for 60 min. For reasons of clear arrangement 10 and 75 µM data are not shown. A,B, 50 µM of FSK did not alter cytoplasmic CRTC2 content significantly (A, p =

0.201) but increased the nuclear content of CRTC2 (B, $p < 0.001$). C,D, 50 µM FSK induced nuclear translocation of CRTC3 ($p < 0.001$ for cytoplasmic decrease, $p = 0.001$ for nuclear increase). Cytoplasmic proteins were normalized by ß-tubulin, nuclear proteins were normalized by Lamin A. E, 10 nM and 100 nM TGOT were equally effective in attenuating the FSK-induced increase of CRF hnRNA. No effect of TGOT on CRF hnRNA levels was apparent in the absence of FSK. F, 50 µM FSK increased pCREB levels about 3.5 fold ($p = 0.001$). Data are expressed as mean ± SEM. # $p < 0.05$ versus Veh group; + $p < 0.05$ versus Veh + FSK group, $ $p < 0.05$ versus TGOT + Veh group; n = 3

Experiment 8.2. Time course of FSK-induced CRF hnRNA levels and CRTC2/3 translocation in Be(2)-M17 cells

A temporal analysis showed that stimulation of Be(2)-M17 cells with 50 µM of FSK augmented CRF hnRNA levels, which reached their maximum after 60 min and remained elevated until at least 90 min. Baseline levels were reached at 240 min (data not shown). TGOT affected FSK-induced CRF hnRNA (two-way ANOVA $F_{(3,41)}= 4.4$; $p = 0.01$) reaching lower levels at 60 min ($p < 0.001$) and 90 min ($p = 0.005$) after the onset of the incubation. Incubation with TGOT alone was without effect on CRF hnRNA levels at any of the time points measured (Figure 27A). The FSK-induced phosphorylation of CREB was not affected by TGOT (Figure 27B), again suggesting that OT does not control CRF expression by modulation of CREB phosphorylation.

Although FSK increased nuclear CRTC2 levels ($p = 0.039$) after 10 min of incubation, we did not find statistical differences between the effects of FSK on the one hand, and FSK and TGOT together on the other (two-way ANOVA, $F_{(3,31)}= 2.3$; $p = 0.103$). It is to note that the FSK-induced CRTC2-response is relatively short-lived (30 min) in comparison to H32 cells (at least 90 min), suggesting a minor importance of CRTC2 in the regulation of CRF gene transcription in Be(2)-M17 cells. However, TGOT tended to delay the increase of FSK-induced CRTC2 levels in the nucleus, resulting in a delay of the maximum response from 10 to 30 min after the onset of the incubation (Figure 27C). This was more evident for CRTC3 (two-way

ANOVA; $F_{(4,52)}$= 2,56; p = 0.05) where TGOT significantly prevented FSK-induced increase of nuclear CRTC3 levels during the early phase of the response (p = 0.016), so that the maximum response was not reached until 60 min of incubation (Figure 27D).

Thus, the effects of TGOT on FSK-induced changes in CRF hnRNA levels, CRTC2 and CRTC3 translocation, and CREB phosphorylation in the human Be(2)-M17 cells, are strikingly similar to those observed in the rat hypothalamus, and rat hypothalamic cells. Therefore, we concluded that the Be(2)-M17 cells are a suitable model to further study the modulatory role of OT on CRTC translocation and the control of CRF gene expression.

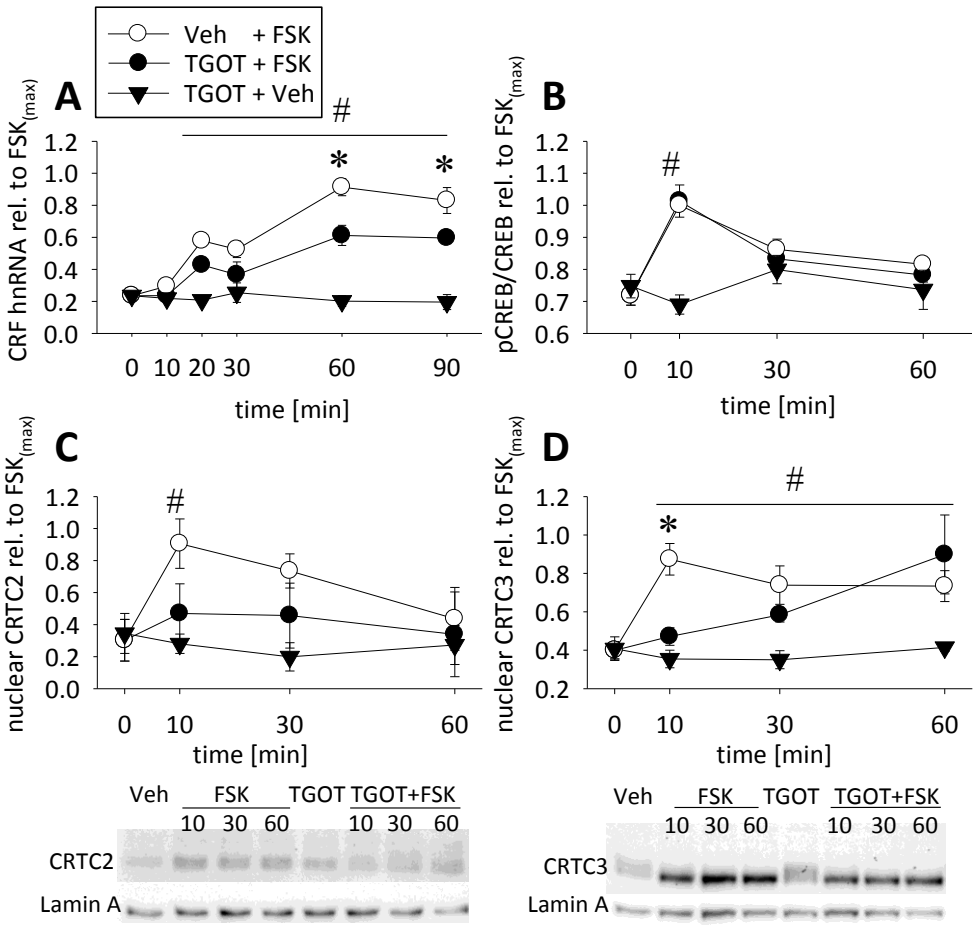

Figure 27. TGOT delays FSK-induced CRF hnRNA and CRTC3 translocation in Be(2)-M17 cells. A, CRF hnRNA levels peaked at 60 min, and remained elevated until 90 min after the onset of FSK. TGOT attenuated the FSK-induced increase at 60 (p < 0.001) and 90 min (p = 0.05) significantly until it paralleled FSK-levels at 120 min (not shown) and declined similarly to FSK to near basal levels at 240 min (not shown). TGOT was without effect in the absence of FSK. B, pCREB levels peaked at 10 min in FSK stimulated cells, with or without TGOT.

This peak level returned to almost basal at 30 min, revealing no influence of TGOT on FSK-induced CREB phosphorylation. pCREB levels were normalized by total CREB and Lamin A (not shown). The amount of total CREB remained constant over time. C, Nuclear CRTC2 levels peak at 10 min after FSK treatment ($p = 0.039$) and return to basal at 30 min, whereas TGOT was without effect. D, FSK-induced nuclear CRTC3 levels peaked at 10 min ($p = 0.009$), and were delayed by TGOT until 60 min ($p = 0.016$), where both reached similar levels. TGOT without FSK had no effect on CRTC3 levels. Data are expressed as mean ± SEM. Data are relative to maximum response to FSK incubation to make comparison across experiments possible. Representative Western blots are shown collectively. * $p < 0.05$ versus Veh + FSK group; # $p < 0.05$ versus respective 0 min time point; n = 5-8

Experiment 9. siRNA knockdown of CRTC3 in Be(2)-M17 cells reduces FSK-induced CRF transcription, but also blocks the TGOT-induced negative effects on CRF hnRNA.

To determine whether CRTC2 and/or CRTC3 are necessary for the inhibition of CRF gene expression by OT, we down-regulated the expression of CRTC2 and CRTC3 by transfecting the Be(2)-M17 cells with siRNA oligonucleotides. The CRTC2 siRNA construct down-regulated both CRTC2 and CRTC3 mRNA levels by 60 % ($p = 0.002$) and 20 % ($p = 0.019$), respectively. The CRTC2 mRNA downregulation was mirrored at the protein level (64 %), whereas CRTC3 protein levels did not change. The CRTC3 siRNA construct specifically reduced both CRTC3 mRNA and protein levels by 60 %. The non-specific control siRNA oligonucleotide was without effect (Table 3).

Target specificity of CRTC2 and CRTC3 siRNA

	mRNA		protein	
	CRTC2 % change (SEM)	CRTC3 % change (SEM)	CRTC2 % change (SEM)	CRTC3 % change (SEM)
CRTC2 siRNA	-59 (5.3)	-22 (20.5)	-64.6 (5.5)	+7.1 (15.7)
CRTC3 siRNA	-19.5 (3.6)	-61.8 (0.8)	-8.4 (0.6)	-67.6 (0.6)

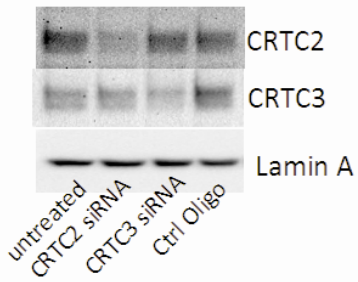

Table 3 Target specificity of the CRTC2/3 siRNA toward mRNA and protein levels. Data are expressed as mean ± SEM. SEM are put in parentheses. n = 3

Statistical analysis revealed a significant effect of TGOT + FSK treatment ($F_{(3,27)}$ = 33.93; p < 0.001; Figure 28), and of pre-treatment with siRNA constructs ($F_{(3,27)}$ = 17.4; p < 0.001). Student-Newman-Keuls *post hoc* analysis revealed a significant reduction in stimulated CRF hnRNA levels by TGOT in 3 groups, i.e. in untreated (p < 0.001), siCRTC2- (p = 0.005) , and control oligo (p = 0.018) pre-treated cells. In contrast, cells that were pre-treated with the CRTC3 siRNA construct revealed a less pronounced FSK-induced rise in CRF hnRNA (50% of untreated or control oligo pre-treated cells, p < 0.001). Importantly, TGOT treatment was without effect on CRF transcript levels in CRTC3 siRNA transfected cells. Due to the remaining three-fold increase of CRF hnRNA levels in FSK-treated cells, we can exclude a floor effect in the TGOT + FSK group. This shows that CRF gene transcription relies on both CRTCs, and that the effect of TGOT on CRF gene expression depends exclusively on CRTC3, rather than on CRTC2.

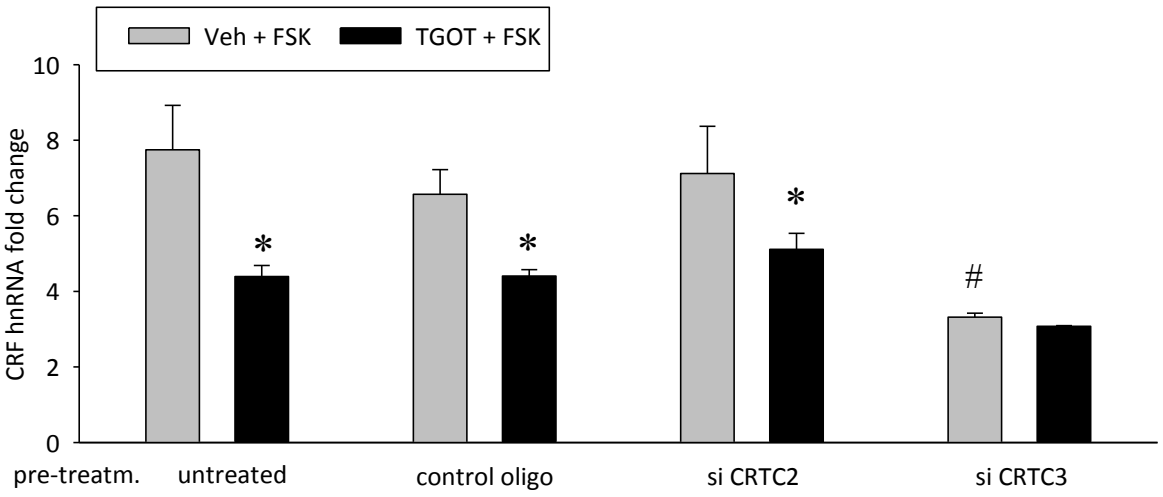

Figure 28 Knockdown of CRTC3 reduces FSK-induced CRF hnRNA in Be(2)-M17 cells, and prevents the additional negative effect of TGOT on the FSK-induced increase. Treatment of Be(2)-M17 cells with FSK + TGOT for 60 min decreased FSK-induced CRF hnRNA as expected (p = 0.004). Pretreatment with a negative control oligonucleotide had no significant effect on FSK or FSK + TGOT induced CRF hnRNA levels. Pretreatment of the cells with CRTC2 siRNA tended to decrease FSK-induced CRF hnRNA levels, compared to cells with no pre-treatment (untreated), whereas TGOT retained its negative effect on FSK-induced CRF hnRNA

(p = 0.016). CRTC3 siRNA reduced the FSK-induced CRF hnRNA level significantly about 40 % (3 fold above basal; p = 0.003)), whereas TGOT had no additional negative effect on the FSK-induced CRF hnRNA levels (p = 0.774). Data are expressed as mean ± SEM; * p < 0.05 versus respective Veh + FSK; # p < 0.05 versus untreated Veh + FSK; n = 5-8

In summary, our data reveals the central role of CRTC3 in CRF gene expression regulation in Be(2)-M17 cells, and that OT attenuates CRF gene expression specifically *via* this factor. However, compensatory mechanisms between CRTC2 and CRTC3 cannot be ruled out, and might account for the apparent lack of effect of the CRTC2 knock down on CRF hnRNA levels, as well as the observed weak expression of CRTC2 protein in Be(2)-M17 cells by means of western blotting.

Experiment 10. TGOT attenuates FSK-induced binding of CRTC3, but not CRTC2 to the CRF promoter

To demonstrate that OT affects CRTC3 binding to the promoter of the CRF gene, we performed ChIP analyses in Be(2)-M17 cells. We showed that FSK induced binding of CRTC2 and CRTC3 to the CRF promoter (Figure 29A,B; one way ANOVA, $F_{(3,15)}$ = 10.7; p = 0.001 for CRTC2; $F_{(3,19)}$ = 4.4; p = 0.02 for CRTC3). TGOT (10 min, 10 nM) prevented FSK-induced CRTC3 (p = 0.028), but not CRTC2, binding to the CRF promoter. TGOT in the absence of FSK was without effect. This supports the hypothesis that OT mediates the downregulation of CRF transcription during stress *via* CRTC3, but not CRTC2.

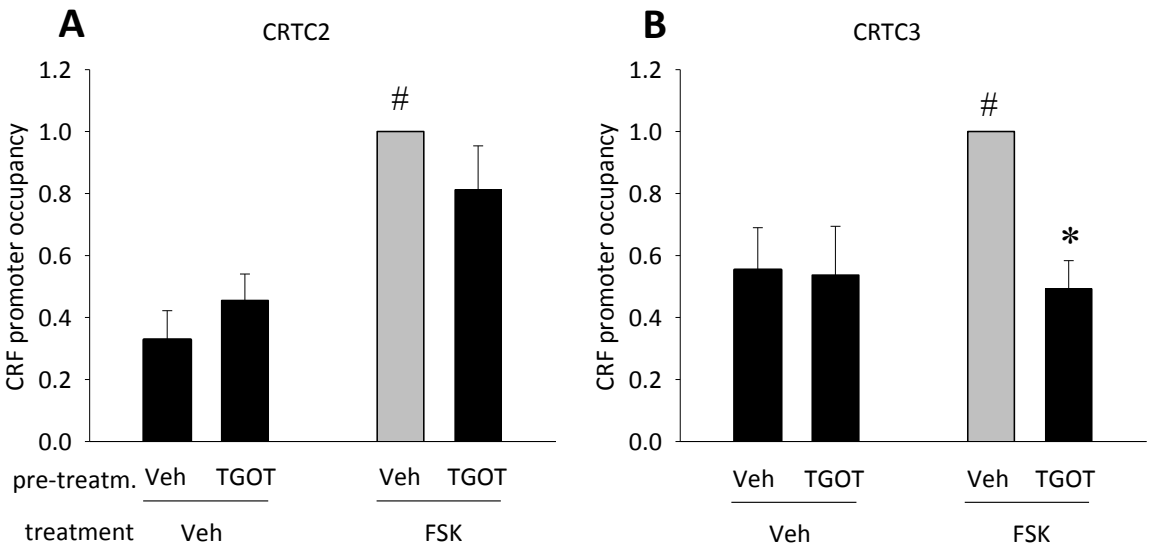

Figure 29 Chromatin Immunoprecipitation revealed that TGOT decreases FSK-induced binding of CRTC3 to the CRF promoter, but not that of CRTC2. A, FSK (10 min, 50 µM, gray bar) induced a rapid increase of CRTC2-CRF promoter-binding ($p = 0.002$), whereas TGOT treatment had no influence on CRTC2 binding activity B, Binding of CRTC3 to the CRF promoter was greatly enhanced by FSK ($p = 0.014$), but reduced to basal (vehicle) levels by treatment with TGOT ($p = 0.028$). TGOT in absence of FSK was without effect on the activity of the CRF promoter. Data are expressed as mean ± SEM, * $p < 0.05$ versus Veh + FSK group, # $p < 0.05$ versus Veh-Veh group; n = 4

Preliminary data reveals a potential influence of activated CRTC3 on other anxiety-related and CRE-regulated genes. The opioid receptor µ, which has been linked to PTSD, might be activated by FSK, and influenced by the presence of OT. The somatostatin gene has been shown to be co-expressed and released with CRF (Dulcis et al. 2013) and might also have an effect on the release of CRF itself. The PPARγ subunit 1c has been associated with anxiety disorders in humans (Sokolowska and Hovatta 2013) and might contribute to the regulation of stress and anxiety. Interestingly, the promoter regions of these three genes were also immuno-precipitated in our ChIP experiment, indicating that activated CRTCs have bound these genes and altered their gene expression. However, the nature of this regulation, and its significance, still has to be determined in continuative studies.

Discussion

General Discussion

In this thesis I have shown some of the intracellular pathways that are coupled to an activation of the OTR by its ligand, the neuropeptide OT. Also, the roles that these signaling cascades play in emotion (anxiety) and physiology (stress) have been clarified by studying the consequences of acute high OT levels in the PVN of rats. For one of these pathways, the CREB pathway, it has become clear how an extracellular signal is translated into a molecular response at the gene transcription level. Characterization of another pathway, centered around MAPK signaling, has provided insight into how an extracellular stimulus gives rise to a multitude of intracellular biochemical responses that are necessary to bring about behavioral changes. It is also interesting to note that these responses are highly specific, as for instance demonstrated by a mismatch of MEK1/2 and ERK1/2 phosphorylation. Such specificity could make the development of anxiolytic or stress-protective medication easier when a highly specific factor has been identified. On the other hand, it might prove to be very challenging to target a specific intracellular pathway that is composed of common factors that influence many neuronal processes, but is unique in the assembly of these factors. On top of that, I have found that the physiological status of an individual, here the reproductive status of female rats, has a profound influence on how an animal (or a human being) will respond to a particular pharmaceutical treatment.

1. OT and MAPK signaling in the PVN

We found that MEK1/2 activity is necessary for the anxiolytic effect of OT in the PVN, although this is only the case when OT is infused into the PVN, and not into the ventricular system. This is rather surprising, considering that following both infusion protocols, MEK1/2

is phosphorylated to more or less the same extent. Apparently, (i) other factors are needed for anxiolysis, or (ii) the MEK1/2 molecules that are phosphorylated by OT infused into the PVN are different from the ones that are activated following icv infusion. Which of these two scenarios applies is not known at the moment. It is, however, possible that the differential effects of MEK1/2 phosphorylation depend on the final OT concentration within the PVN. When OT is infused directly into the PVN at 0.01 nmol / 0.5 µl, its concentration will be in the µM range (taking into account that the concentration infused is 20 µM and that the volume of the PVN is approximately 1 mm^3 (based on Paxinos atlas)). This would imply that the OTR in the high and the low affinity state will be activated. When OT is infused into the ventricular system, only a small portion of the OT molecules will likely be able to enter the PVN, even when 1 nmol of OT is infused. This would mean that only the high affinity OTRs are activated. One could hypothesize that MEK1/2 signaling that is coupled to the OTRs in the high affinity state is not involved in the anxiolytic effect of OT, whereas MEK1/2 coupled to the OTRs in the low affinity state are. In support of this, I found that icv infusion of OT or TGOT altered CRTC3 translocation and CRF expression, which was mimicked by stimulation of hypothalamic cells with low nM doses of TGOT and OT. This suggests that OT exerts its effects on CRF gene transcription *via* activation of OTRs in the high affinity state.

My observation that MEK1/2 is activated upon an infusion of OT or TGOT into the PVN or icv suggests that OTR activation is followed by transactivation of the EGFR, as has been shown in other cell types (Zhong et al. 2003). This therefore seems to be a common mechanism of relaying the extracellular OT stimulus to an intracellular response.

1.1 Downstream targets of OT-induced MEK1/2

What is surprising, however, is that MEK1/2 activation is not accompanied by ERK1/2 activation in both male and female rats. This is quite remarkable, as ERK1/2 were considered to be the only targets of MEK1/2. Interactions of MEK with its substrates (and upstream kinases) are organized by several scaffolding proteins including KSR (Stewart et al. 1999), MP1 (Pullikuth et al. 2005), and disc-large (Maiga et al. 2011). It seems reasonable to assume that MEK1/2, being part of a large signaling complex anchored to scaffolding proteins, could physically interact with substrates other than ERK1/2 as well. Indeed, in human monocytes, a MEK2 – PI3Kδ pathway has been identified that operates independently from ERK, and serves to stimulate the production of an endogenous interleukin 1 receptor antagonist (Brandt et al.). Other proteins that directly interact with MEK1/2 are the TGF-β receptor type II in a lymphoma cell line (Chen et al. 2011), and tumor suppressor WOXI in lysosomes of Jurkat cells (promotes apoptosis in T cell leukemia (Lin et al. 2011)). Also, MEK has been found to translocate to and from the nucleus, where it directly phosphorylates the transcription factor MyoD in differentiating myoblasts, thus influencing gene transcription (Jo et al. 2011). Finally, MEK has been shown to bind the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), then to export it out of the nucleus, and hence, to reduce PPARγ-controlled gene expression (Burgermeister et al. 2007).

Recently it has become clear that MEK2 can even play an active role in the dephosphorylation of ERK1/2, rather than their phosphorylation. During cell migration, activated MEK2 serves as a scaffolding protein and binds a RhoGTPase, which, in turn, interacts with the isomerase Pin1. Pin1 dephosphorylates ERK1/2 (Nakatsu et al. 2010; Pan et al. 2010) and, intriguingly, the expression of Pin1 is upregulated during lactation (DA Slattery, unpublished data), a time when the OT system is highly active. Thus, OT induces the

activation of MEK1/2-dependent pathways in the PVN; pathways that possibly involve substrates that are different from ERK1/2. If so, it seems reasonable to assume that these substrates are mediators of the anxiolytic effect of OT, which we showed to depend on MEK1/2 in male and female (virgin) rats. Blocking the kinase activity of MEK1/2 with U0126 in the PVN of female, virgin rats revealed no effects on basal anxiety-like behavior. However, U0126 did prevent the anxiolytic activity of exogenous OT. Similar results have been found in male rats (Waldherr and Neumann 2007; Blume et al. 2008).

Again, this shows that MEK1/2 plays a role in anxiety only when it is recruited by the OTR. In other words, there seems to be a certain differentiation in the intracellular pathways that contain MEK1/2. The function of MEK1/2 depends on what pathway it is in, and which substrates it can phosphorylate or which downstream signaling factors it can bind. Scaffolding proteins are likely the organizers of such differentiation, as I have argued above.

1.2 Effects of OT on ERK phosphorylation

The only effect of OT on pERK I observed was the apparent translocation of pERK1 (but not pERK2) to the nucleus in lactating animals. This translocation might accommodate the changes at the gene expression level necessary to induce neuroplasticity and stable rewiring of the neural circuitries that occur in lactation, resulting in physiological, psychological, and behavioral adaptations. Thus, it has consistently been reported that the PVN undergoes major morphological alterations during the peripartum period. This has been associated with increased OT activity, both in the supraoptic nucleus and PVN (Theodosis and Poulain 1989; Oliet 2002; Theodosis 2002). The differential effect of reproductive status on ERK1 and ERK2 adds to the notion that these two closely related MAP kinases exert separate functions in

the brain. It has for long been believed that ERK1 and ERK2 are redundant, but elegant experiments employing genetic strategies (such as the generation of knockdown, knockout, and conditional knock-out mice) have revealed especially ERK2-specific effects on learning and memory (Satoh et al. 2007), as well as on the regulation of complex behavior, including social behavior (Satoh et al. 2011). Although ERK1 was reported not to influence learning and memory (Selcher et al. 2001), a later study using ERK1 knockout mice revealed a specific role of ERK1 in synaptic plasticity and drug addiction in the striatum (Mazzucchelli et al. 2002). Therefore, the translocation that we observed in lactating rats may, at least in part, underlie the plasticity seen within the PVN of lactating dams, in addition to the anxiolysis.

The lack of ERK1/2 phosphorylation when OT is infused icv or directly into the PVN is in contrast to an earlier report that describes a 28% (± 23% SEM) increase of ERK1/2 phosphorylation (Blume et al. 2008). This study further mentioned a lack of effect on ERK1/2 phosphorylation when AVP, instead of OT, was infused icv. Although there is no convincing explanation for the effects reported by these authors, they appear somewhat surprising, considering that AVP is known to induce ERK1/2 phosphorylation in astrocytes (Du et al. 2008), hypothalamic cells ((10nM AVP) (Chen and Aguilera 2010)), and even in V2-expressing cultured kidney cells and *in vivo* (Oligny-Longpre et al. 2012). Also, in the paper by Blume et al., the *in vitro* OT concentration used to stimulate H32 cells was 1 µM, by far exceeding the dissociation constant (Kd) of the OTR (1-100 nM), which is almost sure to activate the V1 receptors expressed by these cells (Chen et al. 2009). To circumvent the non-specific binding of OT to the AVP receptors, I have used the highly specific OTR agonist TGOT. Like OT *in vivo* and at low concentrations (1-10 nM) in H32 cells, TGOT did not induce ERK1/2 phosphorylation. Whatever the cause may be for the different results presented here and the previous study by Blume et al., it is clear that MEK1/2, in general, is able to

phosphorylate ERK1/2 in the PVN, as blocking MEK1/2 activity by U0126 reduced the non-stimulated pERK1/2 content in the PVN of virgin female rats. The MEK1/2 – ERK1/2 module is, however, not recruited by OTRs.

To complicate things further, ERK can reside in the nucleus and affect several intracellular processes without being phosphorylated and independently from its kinase activity. These processes include cell cycle control by inactivation of the regulatory protein retinoblastoma (Rb), activation of the transcription factor Elk-1, and even binding to ERK-responsive elements on the DNA can occur (for review see Rodriguez and Crespo 2011). A prerequisite for these activities is nuclear translocation, which is unlikely to happen following stimulation of hypothalamic neurons with OT, since the phosphorylation state of PEA-15 did not change in our experiments, so that ERK1/2 remains sequestered in the cytoplasm.

1.3 Effects of the reproductive status on MAPK signaling and anxiety

The anxiolytic effect of endogenous brain OT during the peripartum period has been well-documented in female rats (Windle et al. 1997; Neumann et al. 2000; Bosch et al. 2005). My observation that an acute, bilateral OT infusion into the PVN of lactating rats did not augment anxiolysis further might indicate that during lactation maximum anxiolysis has already been reached, as endogenous OT levels are already high (Slattery and Neumann 2008). Thus, one might expect that blocking the activity of MEK1/2 in lactating rats would be anxiogenic, and this turned out to be indeed the case. I like therefore to propose that during lactation, an OTR– MEK1/2 – anxiolysis pathway is active.

Interestingly, while icv OT infusion resulted in increased phosphorylation of MEK1/2 in the PVN of virgin rats, OT *reduced* MEK1/2 phosphorylation in lactating rats. This decrease of

phosphorylated MEK1/2 was not accompanied by altered anxiety-like behavior, indicating that OT can control MEK1/2 differentially, depending on the reproductive status of female rats. This also demonstrates, once again, that pMEK1/2 is not necessarily coupled to anxiety-like behavior, even when high OT concentrations are present in the PVN. Even more, as OT *reduces* pMEK1/2 levels in the PVN of lactating rats, one might predict that OT recruits additional factors in these animals that lead to inhibition of MEK1/2.

1.4 Differences in transcriptional regulation in males and females

Active MAPK signaling influences the activity of nuclear transcription factors, like the transcription factor CREB and its cofactor CRTC. Although the PVN undergoes major reorganization during the peripartum period (Theodosis 2002), the signaling cascades that induce CREB activation are relatively stable (Shaywitz and Greenberg 1999). One of these is the MEK1/2-ERK1/2 pathway. Upon phosphorylation ERK1/2 translocates to the nucleus, activates p90rsk or MSK1/2, which phosphorylate CREB to ultimately activate gene transcription. However, the lack of ERK1/2 activity I observed in males and virgin females excludes this possibility. A related MAPK, p38, is able to activate MSK1/2 as well. Indeed, I found p38 to be phosphorylated by an OT stimulus in males. The three-tiered p38-MSK-CREB cascade might translate the OT signal into changes in gene transcription, while phosphorylated MEK1/2 affects cytosolic targets. This complex adaptation seen in males is probably absent in lactating females, where the MEK1/2-ERK1/2 pathway is constantly elevated, thereby enabling CREB activation by ERK-p90rsk or ERK-MSK1/2. The observation of sex-specific recruitment of intracellular pathways is supported by other studies that reveal a differential regulation of CRF, by sex-specific DNA methylation in the PVN upon chronic

mild stress (Sterrenburg et al. 2011), or sex-specific phosphorylation of TrkB-ERK1/2 in the hippocampus (Hill et al. 2013).

In summary, the data suggest that hypothalamic CREB is activated by p38-MSK1/2 in males, while females (especially lactating females) activate CREB *via* the activated MEK1/2-ERK1/2, or *via* a yet unknown pathway.

One common, sex-independent mechanism of transcriptional regulation represents the histone kinase activity of MSK1/2. MSK1/2 has been shown to phosphorylate histone 3, which induces transcriptional activity (Dyson et al. 2005; Duncan et al. 2006). However, histone phosphorylation *via* MSK1/2 upon OT treatment has not been investigated in this work, nor has it been studied elsewhere (Vermeulen et al. 2009).

2. OT release and its influence on the stress response

As previous studies have shown, OT dampens HPA axis activity when central exogenous or endogenous OT levels are high during a long period of time, as observed during the peripartum period (Neumann et al. 2000; Windle et al. 2004; Hillerer et al. 2011). Accordingly, it appeared that the CRF mRNA levels in the PVN are reduced during this time (Walker et al. 2001). Conversely, in OT knock-out mice, CRF mRNA levels are elevated (Nomura, Saito et al. 2003). However, here we focused on the acute effects of OT, for example such as seen following successful mating in male rats (Waldherr and Neumann 2007). It is important to consider that OT can be released during or immediately after stress in the PVN (Wotjak et al. 1998); although the exact timing of this elevation in available OT is not precisely known. I propose that OT release during stress inhibits CRF gene expression,

either to reallocate energy away from investment processes (when OT is released during stress), or as a negative feedback mechanism (when OT is released immediately after the stress). However, the latter is less likely, because in our study OT levels are already high before the onset of a stressor, so that negative feedback mechanisms are not yet operational.

Another function of OT release during stress might be to reduce stress *via* social buffering (Smith and Wang 2013). When female monogamous prairie voles were restraint stressed, the presence of their male partner reduced anxiety-like behavior and plasma corticosterone levels during recovery, compared to females that had to recover in social isolation. This effect was mimicked by bilateral intra-PVN infusion of a high dose of OT (100ng/200nl/side) in females that had to recover without their partner. Moreover, the beneficial effect of the partner could be blocked with an OTR antagonist, suggesting a role for OT in the reduction of the stress response in a social context. Whether this social stress-buffering effect of OT is related to the inhibition of CRF expression is currently not known.

However, the delay of CRF expression that I observed in the hypothalamus may not only influence the HPA axis, but also other physiological and behavioral effects of stress. CRF neurons in the PVN make synaptic contacts with neurons, both CRF-positive and negative, in the PVN (Liposits et al. 1985). Some of these contacts involve reciprocal connections between CRF and OT cells, suggesting that the effects of OT on CRF expression may be direct (Dabrowska et al. 2011). Also, CRF neurons project to various brain regions including the ventral tegmental area (Rodaros et al. 2007), hippocampus, and the locus coeruleus (Koegler-Muly et al. 1993), where CRF has been shown to be released during stress (Valentino and Van Bockstaele 2008). These neurons are a distinct population from those

that project to the median eminence to regulate pituitary ACTH release (Reyes et al. 2005). CRF release in these brain regions during stress modifies the local microcircuitry, and hence the processing of information. This has best been studied in the hippocampus where CRF release induces long-term potentiation or dendritic spine retraction, hyperexcitability and seizures - depending on the severity of the stressor (Maras and Baram 2012). OT released during or immediately after stress may play a role in dampening these detrimental effects *via* the CREB/CRTC3 pathway.

2.1 Regulatory mechanisms of CRF gene transcription

The regulation of gene transcription as it appears at the CRF gene is controlled at multiple levels, i.e. a variety of transcription factors and co-factors has to act in concert to activate their specific targets. CRF is mainly regulated by the transcription factor CREB. One remarkable observation was that OT influences the stress- or FSK-induced translocation of the CREB Co-activator CRTC3, but not CRTC2, as mirrored by the reduced binding of CRTC3 to the CRF promoter. Data from rats *in vivo*, primary hypothalamic rat cells, an immortalized rat hypothalamic cell line (selected for OTR expression), and human neuroblastoma cells that were differentiated to neurons all point in the same direction: less stress-induced CRF gene expression, in parallel to delayed CRTC3 translocation, induced by the presence of OT. It is especially convincing that the phenomenon occurs in rat as well as human cells, in adult PVN tissue punches as well as embryonic rat hypothalamic cells. This shows that the mechanism applies no matter what the age of the animal is, and operates both in rats and in humans. The only difference between the rat and human cells was that the potentiating effect of OT on CRF gene transcription and CRTC translocation observed in rat cells at late time points,

was absent in the differentiated Be(2)-M17 human neurons. This might be explained by the origin of the cells from bone marrow, where intracellular mechanisms, which would regulate CRF expression in hypothalamic neurons, could be absent. Although the human Be(2)-M17 cells reacted comparable to rat hypothalamic cells *in vitro* and *in vivo* at early time points, suggesting similar regulating mechanisms across species, we cannot exclude species specific differences between rats and humans at later time points. Nevertheless, the strong similarity in the early phase of the stress (or FSK)-induced CRF gene expression and CRTC3 translocation between the rat and human cells made it possible to assess whether CRTC3 is necessary for the effects of OT on the stress-induced increase of CRF gene expression in the human cells. Indeed, the knockdown of CRTC3, but not CRTC2, prevented the inhibitory effects of OT, and the ChIP analysis demonstrated that OT significantly reduced the binding of CRTC3 to the CRF promoter.

Furthermore, it was interesting to note that both, OT and stress lead to CREB phosphorylation. While restraint stress appeared as a strong activator of CREB phosphorylation, OT induced a rather moderate effect on pCREB. The stress-induced phosphorylation of CREB at Ser133 appeared transient, peaking 10 min after the onset of the stimulus and coinciding with the maximum nuclear CRTC3 levels, which is followed by CRF gene transcription. Interestingly, when both, OT and stress were applied to the animals, OT revealed no effect on stress-induced pCREB levels, but reduced CRF hnRNA levels at early time points. We observed the same effects *in vitro,* when the cells were stimulated with TGOT alone or in combination with FSK. Our observation that OT (or TGOT) alone leads to increased pCREB levels, whereas OT in the presence of stress has no effect, strengthens our conclusion that OT mediates its inhibitory effects on CRF gene transcription *via* a factor other than pCREB, and that was found to be CRTC3. While CREB and CRTC are both required

for CRF gene transcription to occur, it should be noted that CRTC3 levels plateaued for a rather long time period, in contrast to the sharp and short-lived CREB Ser133 phosphorylation. It has been found, however, that the binding of CREB to the TAFII130 component of the transcription factor IID complex is independent of CREB Ser133 phosphorylation, whereas binding of the complex to the CRE is potentiated by CRTCs (Riccio et al. 2006; Altarejos and Montminy 2011). In addition, other phospho-acceptor sites of CREB, such as the Ca^{2+}-influx-dependent Ser142 and Ser143 residues, which are phosphorylated with slightly slower kinetics than Ser 133 (Kornhauser et al. 2002), may sustain the activity of CREB.

Other factors, such as the JNK-activated transcription factor c-Jun, also play a crucial role in the transcriptional regulation of the CRF gene. c-Jun activation appears in distinct brain regions in a stress- and species-dependent manner (Liu et al. 2004), and is one factor that binds the CRF promoter to induce gene transcription (King and Nicholson 2007). We found that acute mild stress, such as the elevated platform, induces JNK1 phosphorylation in the hypothalamus of male rats. We hypothesized that OT might interfere with the JNK-c-Jun pathway to influence CRF gene transcription. By inhibiting JNK signaling, OT could decrease phospho-c-Jun levels and subsequently reduce CRF gene transcription. However, no interactions between OTR-coupled signaling cascades and the JNK pathway were found, which suggests a central role of the CRTCs in the OTR-induced regulation of CRF gene transcription. Also, it has been proposed that the regulatory elements on the CRF promoter are hierarchically regulated (King and Nicholson 2007). To induce CRF gene expression, the activation of the c-Jun binding site can be overcome by other factors that bind the CRE, like CREB or CRTCs, so that CRF gene expression is attenuated, or enhanced. However, c-Jun binding is necessary for CRF gene expression, regardless of CREB-CRTC binding to the

promoter. This might be one explanation for the lack of effects of OT on CRF gene expression under basal, non-stressed conditions.

2.2 Other potential targets of CREB/CRTC signaling

We have shown in this study that OT delays CRF transcription specifically *via* the CREB coactivator CRTC3 during stress. This intracellular arrangement may not be unique to the control of the CRF gene, but could likewise apply to the regulation of other anxiety- and stress-related genes by OT. For example, the expression of Rgs2 is known to be regulated by CRTC3 in human adipose tissue (Song et al. 2010). In the rodent brain and human myometrium cells Rgs2 is downstream of OTR activation (Park et al. 2002; Okimoto et al. 2012) and is likely to mediate anxiolytic effects as seen in rodents (Oliveira-Dos-Santos et al. 2000; Lifschytz et al. 2012). There are more anxiety- and stress-related genes (Sokolowska and Hovatta 2013) that harbor a CRE region in their promoter, including PPARγ-coactivator-1α, TrkB, and opioid receptor mu1 (B. Jurek, unpublished observation) and could, thus, be regulated by OT and CRTC3 as well.

In summary, this work reveals a delaying effect of OT on CRF gene transcription *via* the CREB coactivator CRTC3, but not *via* CRTC2. Although both factors get activated by stressors, OT exclusively interferes with CRTC3. This finding complements hitherto existing research by underlining the potent regulatory function of CRTC3 in CRF transcription, which makes it a promising protein to target for the treatment of diseases related to a dysregulated HPA axis. However, changes in gene transcription are long term adaptations, hardly influencing the acute stress response that appears from seconds to minutes. It lies upon future studies to determine if and how OT influences the acute stress response as well.

Summary - concluding remarks

The work presented in this thesis first and foremost reveals the specificity of intracellular signaling pathways that are coupled to the OTR in the PVN. This specificity does not only refer to the factors involved in each of these, but also reflects the unique recruitment of intracellular pathways depending on the physiological condition of the animal. I found profound differences in OT-dependent intracellular signaling when comparing female rats in different reproductive states (virgin, pregnant, or lactating), and when comparing pathways coupled to CRF expression in stressed and non-stressed male rats. Sex-specific differences in CRF expression in the PVN can partly be caused by differential expression of CREB-pathway associated enzymes, such as CBP and the p300/CBP associated factor (PCAF) (Sterrenburg et al. 2011), and this adaptability of the system is likely to account not only for different sexes, but also for different reproductive states.

The question remains if the results of my work can be translated to humans, and can contribute to the understanding of anxiety or stress-related diseases. To some extent, the evolutionary conservation of the emotion anxiety and its related pathways certainly allows a cross-species comparison of genes involved in the regulation of anxiety between humans and rodents by means of genome wide expression profiles (Sokolowska and Hovatta 2013). For instance, human studies have associated OT with social cognition and social anxiety, and recent studies revealed sex-specific differences in the neural (activity in amygdala, striatum, and hippocampus) and behavioral responses (tested in the social “Prisoner’s Dilemma” game) to intranasal OT (Rilling et al. 2013). However, given that OT is involved in a wide

array of processes in the brain, and considering sex-specific and reproductive state-dependent effects as described above, treatment with OT might cause unwanted side effects. Therefore, it is of crucial interest if the involvement of pMEK1/2 in the processing of the emotion anxiety represents a target that is specific enough for the pharmacological treatment of anxiety disorders, or if targeting MEK1/2 would cause severe side effect due to the widespread cellular use of this kinase. Downstream targets of MEK1/2 may be more specifically related to anxiety and the processing of stress. One of these stress- and OT-specific targets could be CRTC3. We do not know how CRTC3 activation is coupled to the OTR, and if the activating pathway includes MEK1/2, however, it might be a factor to target for the treatment of anxiety disorders. Drugs that target brain proteins, such as CRTC3, are in general unspecific for distinct brain regions, whereas brain region specificity is of central importance for the regulation of distinct behaviors. Possibly, the distinct expression of CRTCs in only a subset of brain regions (Watts et al. 2011) may contribute to the avoidance of unwanted side-effects of CRTC-targeting drugs. However, side-effects cannot be completely excluded, as CRTC3 is also expressed in the periphery and has been shown to be involved in the regulation of energy homeostasis (Song et al. 2010), mitochondrial biogenesis (Than et al. 2011) and the production of interleukin 10 (MacKenzie et al. 2013). Although the related CRTC1 is expressed exclusively in the brain, Liu and co-workers suggested that it, if at all, only plays a minor role in the control of CRF gene transcription (Liu et al. 2010).

In conclusion, the findings in humans, in combination with the results of my work in rodents, emphasize the need to fully characterize the effects of OT in males and females, at different physiological states, and at multiple doses, before a widespread clinical application can be legitimated.

Perspectives

To fully understand the effects of OT it is crucial to find the downstream target of phosphorylated MEK1/2 in the hypothalamus. This cytoplasmic or nuclear target protein could be the sought-after cellular regulator that initiates a change in anxiety-related behavior. This issue could be addressed by immunoprecipitations of OT-induced hypothalamic pMEK1/2 and subsequent identification of the proteins physically bound to it by mass spectrometry.

Also, the precise molecular mechanism that OT recruits to control CRF release in parvocellular CRF neurons (these neurons do not express the OTR (Dabrowska et al. 2013)) remains somewhat enigmatic. With action potential-mediated release of endogenous OT being blocked with tetrodotoxin, the effects of specific OTR agonists (TGOT) and antagonists (Manning compound, OTR antagonist) could be tested for effects on CRF neuron intrinsic excitability, glutamate and GABA synaptic inputs, but also noradrenergic synaptic modulation.

Related to this question is the nature of the target neurons or brain nuclei of the OTR expressing cells, which may be part of a pathway that regulates the physiological expression of fear. Target candidates would be the locus coeruleus (mediating sympathetic effects during stress (Benarroch 2009)) ventral tegmental area (influences social behavior (Tang et al. 2014) in press), and the nucleus of the solitary tract that is known to receive projections from the central amygdala (Zardetto-Smith and Gray 1990; Roozendaal et al. 1999) and to project, *via* noradrenergic neurons, to the BNST. The amygdala is involved in the processing

of anxiety (Knobloch et al. 2011; Viviani et al. 2011) and is, as the BNST (Dabrowska et al. 2011), reciprocally innervated with the PVN, forming a regulatory circuit that influences the perception and processing of stimuli that lead to stress and anxiety. The method of choice would be the expression of channel rhodopsin 2 (ChR2) in OT neurons by stereotaxic injection of a ChR2/tdTomato (red fluorescence)-expressing, cre-sensitive adeno-associated virus into the PVN of OT-cre mice. These mice are then crossed with CRH-green-fluorescence protein (GFP) mice to produce a mouse that expresses ChR2 and tdTomato in OT neurons and GFP in CRH neurons. This allows the recording of the response of CRH neurons to optical stimulation of OT release and to test for sensitivity to OTR and $GABA_A$ receptor antagonists.

Addressing these open questions will contribute to the understanding how stress and anxiety are regulated at the cellular level and how this is translated into behavioral and physiological responses.

Abbreviations

ANOVA	analysis of variance
AVP	Arginine-Vasopressin
BNST	bed nucleus of the stria terminalis
Ca^{2+}	calcium
CBP	CREB binding protein
c-Fos	cellular- Finkel-Biskis-Jinkins murine osteosarcoma virus homologue
c-Jun	cellular- Ju Nana (jap. for 17)
CaMK	Ca^{2+}/Calmodulin activated kinase
CeA	central amygdala
ChIP	chromatin immuno precipitation
CNS	central nervous system
CRE	Cyclic Adenosylmonophosphat responsive element
CREB	Cyclic Adenosylmonophosphat responsive element binding protein
CRF	Corticotropin releasing factor
CRFR	CRF receptor
CRH	Corticotropin releasing hormone
CRTC	CREB transcriptional co-activator (also see TORC)
DMEM	Dulbecco´s modified eagle medium
ERK	extracellular signal regulated kinase

EPM	elevated plus-maze
FSK	forskolin
GAPDH	Glycerinealdehyde-3-phosphat-dehydrogenase
HPA	hypothalamic-pituitary-adrenal
hnRNA	heteronuclear ribonucleic acid
icv	intracerebroventricular
ip	intraperitonal
JNK	c-Jun-N-terminal kinase
LDB	Light Dark Box
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated ERK Kinase
mRNA	messenger ribonucleic acid
MSK	Mitogen and stress activated kinase
mTOR	mammalian target of rapamycin
MWU	Mann-Whitney-U-test
OT	oxytocin
OTR	oxytocin receptor
PEA-15	Phosphoprotein-enriched in Astrocytes- 15 kDa
PKA	protein kinase A
PKC	protein kinase C
PVN	paraventricular nucleus
P38	protein 38 kDa
Ras	rat sarcoma
RE	responsive element

Rgs2	regulator of G-protein coupled signaling 2
RS	restraint stress
RT-qPCR	quantitative real time Polymerase chain reaction
s.c.	subcutan
SEM	standard error of the mean
Ser/Thr	serine/threonine
SIK	salt-inducible kinase
siRNA	small interfering RNA
SON	supraoptic nucleus
TBP	TATA-Box binding protein
TBS-T	Tris-buffered saline - Tween
TGOT	Thr^4-Gly^7-oxytocin
TORC	transducer of regulated CREB activity (see CRTC)
U0126	MEK-inhibitor
Veh	vehicle
V1a/b	Vasopressin receptor 1a/b
V2	Vasopressin receptor 2

German summary - Deutsche Zusammenfassung

Das Neuropeptid Oxytocin (OT) nimmt nicht nur Einfluss auf grundlegende physiologische Vorgänge wie zum Beispiel Kontraktion der Uterusmuskulatur oder das Einschießen der Muttermilch, sondern moduliert auch grundlegende Verhaltensweisen wie Angst, mütterliches oder soziales Verhalten und die Stressantwort des Körpers. Dabei ist die Art der Modulation stark kontextabhängig. So wird OT oft als angstlösend beschrieben (Bales et al. 2004; Blume et al. 2008; Jurek et al. 2012), wohingegen es in bestimmten Gehirnregionen (Septum) Angstverhalten verstärken kann (Guzman et al. 2013). Darüber hinaus vermindert zentrales endogenes OT die basale und stressinduzierte Ausschüttung von ACTH (Neumann et al. 2000), obgleich es in der Peripherie und in Kombination mit bestimmten Stressoren die Ausschüttung potenzieren kann (Petersson et al. 1999; Ondrejcakova et al. 2010). Um diese divergierenden Effekte verstehen zu können, ist es essentiell die molekularen Faktoren zu kennen, die diese Effekte hervorrufen.

In der vorliegenden Arbeit befasse ich mich mit eben diesen molekularen Faktoren, die – abhängig vom Kontext – ein bestimmtes Verhalten als Reaktion auf Umweltreize hervorrufen. Die Bindung von OT an den Oxytocinrezeptor (OTR) rekrutiert Signalkaskaden von proteinaktivierenden Kinasen, mithilfe derer Informationen an verschiedene Zellkompartimente weitergegeben werden können. Zum einen werden so schnelle Effekte im Zytoplasma der Zelle vermittelt (neuronale Erregbarkeit, Ausschüttung von

Neuropeptiden), zum anderen werden Signale in den Zellkern transloziert, die Einfluss auf die Genaktivität nehmen und somit längerfristige Effekte hervorrufen können.

Wie sich zeigte, vermindert eine Infusion von OT direkt in den Paraventrikulären Nukleus (PVN) des Hypothalamus weiblicher, unverpaarter Tiere deren Angstverhalten in Abhängigkeit der Aktivität der MAP Kinase MEK1/2. Überraschenderweise fanden wir keine Aktivierung der nachfolgenden Kinase ERK1/2. Dies ist außergewöhnlich, da bis dato keine anderen Zielproteine von MEK1/2 im Hypothalamus bekannt sind. Jedoch scheint MEK1/2 von zentraler Bedeutung für den angstlösenden Effekt von OT zu sein, da eine Inhibierung der Kinase den Effekt von OT blockiert.

Im Gegensatz zu unverpaarten Weibchen wirkt sich eine OT Infusion in den PVN laktierender Tiere vermindernd auf die Aktivierung von MEK1/2 aus, welches in diesem Fall wieder mit der nachfolgenden Kinase ERK1 gekoppelt ist, ohne weiter Einfluss auf das Angstverhalten zu nehmen.

Diese Daten zeigen zum einen, dass MEK1/2 nur als Vermittler von verschiedenartigen extrazellulären Signalen (wie zum Beispiel OT) dient, seine Aktivität aber keine Rückschlüsse auf den Auslöser oder die Wirkung zulässt. Zum anderen wird gezeigt, dass die laut Lehrbuch starre Abfolge von MEK1/2 auf ERK1/2 nicht immer zutreffend ist. Vielmehr zeigt sich die hohe Dynamik der Rezeptor-gekoppelten Signalkaskaden, wobei verschiedene Signale unterschiedliche Effekte über die gleichen Signalkaskaden vermitteln können.

Ein Schwerpunkt dieser Arbeit liegt in der Regulation des Gens für den *Corticotropin releasing factor* (CRF). CRF ist ein entscheidender Aktivator der Hypothalamus-Hypophysen-Nebennieren-Achse (engl. *HPA axis*) und damit eine maßgebliche Kontrollinstanz in der

Stressantwort des Körpers. Die Transkription dieses Gens wird unter anderem durch den Transkriptionsfaktor CREB und dessen verschiedene Co-Aktivatoren gesteuert. Einer dieser Co-Aktivatoren ist CRTC3. Wie ich zeigen konnte, verändert OT die Verfügbarkeit von CRTC3 im Zellkern. Dies wiederum beeinflusst die Transkription von CRTC3-kontrollierten Genen, wie zum Beispiel CRF. In diesem Fall wirkt OT nur als Modulator, da es ausschließlich unter Stressbedingungen seine Wirkung entfaltet, auf basale CRF Transkription aber keinerlei Einfluss nimmt. Eine Ursache hierfür könnte die hierarchische Regulation der Gentranskription sein. Damit CREB und seine Co-Aktivatoren die Transkription initiieren können, muss zuerst der stressinduzierte Transkriptionsfaktor c-Jun an den CRF Promotor binden. Unter basalen (ungestressten) Bedingungen wäre somit c-Jun nicht aktiv und CREB/CRTC können die Transkription nicht modulieren. Unter Stressbedingungen bindet c-Jun an den Promotor, was CREB/CRTC erst ermöglicht die Gentranskription zu potenzieren oder abzuschwächen.

Der Transkriptionsfaktor CREB wird durch verschiedene Signalkaskaden aktiviert, unter anderem durch den klassischen MAP Kinase MEK-ERK Signalweg. Da ERK1 nur in laktierenden Tieren aktiv ist, und ERK2 in keiner der Experimente nachweisbare Aktivität zeigte, stellt sich die Frage wie CREB durch OT aktiviert wird. Die Phosphorylierung der Proteinkinasen p38 und MSK1/2 im PVN von männlichen Ratten deutet auf eine ERK1/2-unabhängige CREB-Aktivierung hin. Unveröffentlichte Daten unserer Gruppe zeigen zudem eine Aktivierung der CaMKIV, die ebenfalls Kinase-Aktivität für CREB aufweist.

Ebenso konnte ich nachweisen, dass OT spezifisch den CREB Co-Aktivator CRTC3 rekrutiert. Dieser Co-Aktivator grenzt das Wirkspektrum des aktivierten CREB auf bestimmte Gene ein, indem nur solche Gene abgelesen werden für deren Transkription eben jener CRTC3

benötigt wird. Umgekehrt können andere Signale, welche ausschließlich CREB aktivieren, und CRTC3 inaktiv belassen, nicht die gleichen Gene aktivieren wie OT. Weitere Co-Aktivatoren von CREB (CBP, p300, TAF4, CRTC1/2) erhöhen dessen Zielspezifität durch die enormen Kombinationsmöglichkeiten einen Gen-spezifischen Transkriptionskomplex zu bilden.

Zusammengefasst habe ich mit dieser Arbeit dazu beigetragen einen Teil des Signalweges, ausgehend vom aktivierten OTR über zytoplasmatische Proteinkinasen bis hin zu veränderter Genaktivität durch nukleäre Transkriptionsfaktoren, aufzuklären. Die Kenntnis all dieser Faktoren, sowie das Verständnis ihrer Rolle in der Entstehung und Regulation von Stress und Angst, ist eine grundlegende Voraussetzung zur erfolgreichen und zielgerichteten Behandlung von stressinduzierten Angsterkrankungen.

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Author´s declaration - Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als die angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

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Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, Dezember 2013

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(Benjamin Jurek)