MOLECULAR UNDERPINNINGS OF ANXIETY REGULATION: NOVEL INSIGHTS INTO THE ROLE OF THE PURINERGIC AND OXYTOCINERGIC SYSTEMS WITHIN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

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INTRODUCTION

Introduction

1. Positive and negative aspects of anxiety

Feelings are what make life worth living. They can be beautiful and pleasant like love and joy, but there are also many situations throughout our life that make us angry, mad or anxious. When we encounter fire, our naturally occurring anxiety of the flames protects us from harm. Anxiety is described as a psychological, physiological, and behavioural state that is induced by a threat (Steimer, 2002). Thus, normal anxiety is healthy and extends our lifespan. Being afraid of something is not necessarily an inborn characteristic trait and can be reversed in many cases, like fear of a snake (Nili et al., 2010). On the other hand, phobias, such as arachnophobia (fear of spiders), claustrophobia (fear of having no escape and being closed in), or acrophobia (fear of heights) are examples of how exaggerated anxiety can be maladaptive, affecting the life of those suffering from it. Anxiety can also turn out as a serious threat for mental health, for example the everyday ongoing disturbing worry of panic disorder patients to have yet another panic attack. In the modern world, so-called anxiety disorders are becoming more and more problematic and the number of persons concerned rises dramatically (Wittchen et al., 2011).

The term “anxiety disorder” summarizes a whole group of psychiatric diseases, including phobia, panic disorder, generalized anxiety disorder (GAD), social anxiety disorder, obsessive-compulsive disorder (OCD), and posttraumatic stress disorder (PTSD). Phobias are characterised by a persistent fear of an object or situation, typically disproportionally high compared with the actual threat. Panic disorder is diagnosed when a person suffers from sudden and repeated attacks of fear that last for several minutes or longer, namely panic attacks. Panic attacks can occur at any time and are characterized by the fear of disasters or

losing control over a situation, even when there is no real danger. GAD causes the patient to have consistent worries about everyday life tasks, even when there is little or no reason for it, resulting in a permanent fear to fail or to not make it through the day. Social anxiety disorder or social phobia is the fear of being judged by others and of being embarrassed. Affected persons feel extremely fearful and unsure around others. Even if they know that there is no need to, they cannot control their fear. Patients with OCD constantly feel the need to check and re-check things repeatedly, losing themselves in rituals by trying to gain control over their obsessions, which seriously affects their daily life. And, finally, PTSD is an anxiety disorder that might occur after seeing or living through a dangerous event. Persons concerned can be war veterans or survivors of physical or sexual assault, abuse, accidents, disasters and many other serious events, and even persons who suffer from a sudden, unexpected death of a loved one. The symptoms are flashbacks, bad dreams, feelings of strong guilt, depression, worry, or hyperarousal (NIMH).

In contrast to normal anxiety, the disordered variant lasts at least for months and can become more severe if no medication is applied. Although several treatment possibilities have already been discovered and are in use, the diversity of phenotypes of anxiety disorders demands for new therapeutic approaches incessantly.

2. Neurobiology of anxiety

Anxiety acts as a coping mechanism in dangerous situations and is therefore strongly associated with emotional processes as well as cognitive functions, such as learning and memory. Several neurotransmitter pathways are involved, including glutamate, γ-aminobutyric acid (GABA), serotonin, and norepinephrine. GABA is the primary inhibitory

neurotransmitter and its actions counterbalance those of the excitatory neurotransmitter glutamate. A down-regulation of the GABA system has been linked to the pathophysiology of anxiety disorders, and as such, GABA receptors are the known target of a number of pharmacological agents (Lydiard, 2003). In contrast, the excitatory glutamate neurotransmission is blocked by treatments to exert anxiolytic effects (Bergink et al., 2004). Furthermore, alterations in serotonergic neurotransmission have been implicated in the development of mood and anxiety disorders as well (Ressler and Nemeroff, 2000). In particular, the inhibitory serotonergic receptor 1a (5-HT1A) was revealed to be important, since a 5-HT1A knock-out (KO) leads to increased anxiety and stress responsivity (Heisler et al., 1998). Activation of the 5-HT1A autoreceptor leads to hyperpolarization of the serotonergic cell and thus self-inhibition as a form of negative feedback regulation of transmitter release (Celada et al., 2004). The norepinephrinic neurons of the locus coeruleus project to the forebrain, and play a critical role in fear response, stress, and arousal (Bremner et al., 1996). The central effects of norepinephrine are mediated via post-synaptic $\alpha 1$ and $\beta 1$ receptors and the pre-synaptic $\alpha 2$ receptor. The $\alpha 2$ receptor is an autoreceptor, similar to the 5-HT1A, and inhibits norepinephrine release pre-synaptically. Consequently, $\alpha 2$ receptor agonists reduce anxiety-like behaviour (Dell'Osso et al., 2010).

With the aid of functional magnetic resonance imaging, it was observed that distinct brain regions are activated in response to anxiety-inducing stimuli, including the amygdala, the hippocampus and the frontal cortex (Davidson et al., 1999). This confirmed earlier studies which had shown that fear and anxiety are mediated by several interrelated limbic structures: the amygdala, the septo-hippocampal system, and the hypothalamus (Charney and Deutch, 1996). The amygdala is composed of functionally and morphologically varying

subnuclei with complex connectivity. The basolateral amygdala (BLA) is primarily glutamatergic, whereas the central amygdala (CeA) mostly consists of GABAergic neurons (Tye et al., 2011). The BLA integrates processed information about the environment, is the locus for fear memory (LeDoux, 2000), and robustly projects to the CeA. CeA-projections to the hypothalamus and the brainstem mediate the anxiety response, such as avoidance of open spaces in rodents (Adhikari, 2014). The bed nucleus of the stria terminalis (BNST) modulates anxiety through processing of input from the amygdala and relaying the processed information to hypothalamic and brainstem structures. The BNST was therefore proposed to be part of the "extended amygdala" (Alheid et al., 1998). The regulation of anxiety by the amygdala is further expanded by interplay of BLA, medial pre-frontal cortex (mPFC) and ventral hippocampus (vHPC). It was suggested that contextual and sensory inputs from the mPFC and the vHPC are integrated by the BLA, which in turn regulates CeA and BNST, which activate downstream regions to control anxiety-related behaviour (Adhikari, 2014).

3. Drug treatment of anxiety disorders

Tricyclic antidepressants (TCAs) have been used in psychiatry since the 1950s and act by inhibiting the serotonin and norepinephrine reuptake from the synaptic cleft (Feighner, 1999; Ravindran and Stein, 2010). TCAs are able to reduce the number of panic attacks and decrease anticipatory anxiety and are therefore helpful in the acute treatment of panic disorder (Andersch et al., 1991). In addition, clomipramine, one of the most investigated TCAs, is regarded as the gold standard treatment for OCD. However, their side effects are multiple and diverse, including sedation, constipation, sexual dysfunction, and a high

possibility of a toxic overdose (Ravindran and Stein, 2010). These side effects have imposed the development for alternative drugs for the use of TCAs.

Other compounds that target the serotonin and norepinephrine pathways are monoamine oxidase (MAO) inhibitors and selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs). MAO is responsible for the degradation of serotonin and norepinephrine, and its inhibition leads to increased availability of both neurotransmitters in the synapse. SSRIs inhibit the reuptake of serotonin, whereas SNRIs inhibit the reuptake of both monoamines at the pre-synaptic site. SSRIs and SNRIs are considered as basic pharmacotherapy agents for each of the anxiety disorders (Ravindran and Stein, 2010). MAO inhibitors, SSRIs, and SNRIs have fewer side effects than TCAs. However, it was observed that there is an increased risk for suicidal thinking and behaviour in adolescents, resulting in a “black-box-warning” by the U.S. Food and Drug Administration on the labelling of those drugs (US Food and Drug Administration, 2007).

Anticonvulsant drugs are used for the treatment of different psychiatric diseases. They can differ in their chemical structure, and their mechanism of anxiolytic action is not completely elucidated yet. They are thought to reduce anxiety by decreasing the excessive neuronal activation within defined fear circuits in the brain. This was especially evidenced for pregabalin, which was shown to increase GABAergic inhibitory activity and reduce the release of excitatory neurotransmitters such as glutamate in several brain regions including the cortex, hypothalamus, amygdala, and hippocampus (Mico and Prieto, 2012).

Benzodiazepines are among the most commonly used drugs for the treatment of anxiety disorder, because of their efficacy, rapid onset of effect, and favourable side effect profile (Stevens and Pollack, 2005). They are positive allosteric modulators of the $GABA_A$ receptor

and therefore potentiate the effect of the inhibitory neurotransmitter GABA by increasing the frequency of chloride channel opening (Study and Barker, 1981). This inhibitory effect of the benzodiazepines is a double-edged sword: while low doses have anxiolytic and anticonvulsive effects, higher doses produce sedation, amnesia, and even unconsciousness (Saari et al., 2011). However, benzodiazepines provide a rapid and effective relief of symptoms, but adverse effects are, unfortunately, significant. Patients discontinuing benzodiazepine use may even experience uncomfortable withdrawal symptoms, which make a benzodiazepine therapy unfitting for individuals with a history of substance abuse (Ravindran and Stein, 2010).

The diverse risks and side effects of the established anxiolytic drugs provide the impetus for the continuation of basic research and the development of new therapeutic strategies to treat anxiety disorders. Some of the strategies concern the potential use of endogenous and exogenous modulators of glutamate and neuropeptide signalling. In particular, the putative anxiolytic activity of corticotropin-releasing factor (CRF) receptor antagonists, glutamate receptor antagonists, as well as of the neuropeptides oxytocin (OT), neuropeptide Y (NPY), vasopressin (AVP), neuropeptide S (NPS), and cholecystokinin (CCK) is currently under intensive survey (Mathew et al., 2008).

4. *In vivo* models of anxiety

Animal models were and still are an important aid for the research on psychiatric disorders. For anxiety research, many different KO models and animals selectively bred for extreme anxiolytic or anxiogenic phenotypes have been used. Consensus is that evidence from more than one behavioural test is required to make an animal model appropriate for the testing of

new anxiolytics, and, similarly, it is clear that no animal model will ever be able to combine all complex aspects of mood and anxiety disorders (Rotzinger et al., 2010).

One of the animal models for anxiety is represented by the high-anxiety behaviour (HAB) and low-anxiety behaviour (LAB) rats. These Wistar rats were selected for their anxiety levels, determined by their behaviour on the Elevated Plusmaze (EPM), and allocated to one of the two groups. Compared to LAB rats, HABs show high anxiety in a variety of tests, prefer passive coping strategies, and show signs of increased stress vulnerability (Landgraf and Wigger, 2003). Furthermore, female lactating HAB rats show a higher amount of maternal care and a heightened aggression towards a virgin intruder compared with LAB rats (Bosch, 2011). In contrast, male LAB rats display more aggressive behaviour towards an intruder than HAB males (Veenema and Neumann, 2007). Experimental evidence revealed overexpression and -release of AVP in the paraventricular nucleus (PVN) as underlying mechanism for the behavioural phenomena (Landgraf and Wigger, 2003).

Many behavioural tests of anxiety have been developed, so that researchers have the possibility to choose between tests for exploratory behaviour, social behaviour, reflexive fear responding, conflict behaviour as well as defensive behaviour (Rotzinger et al., 2010). The EPM and the Light-Dark-Box (LDB) are both tests that utilize anxiogenic stimuli of open spaces. It should always be taken into account that such exploratory tests are sensitive to changes in locomotion, which therefore should be observed as well, in order to obtain trustworthy results.

5. Regulation of anxiety by neuropeptides

5.1. The neuropeptide oxytocin

OT is a long-known and well-studied neurohypophysial hormone of nine amino acids. It is named after the "quick birth" which reveals its main function in the mammalian periphery: it is released during labour, facilitating birth itself as well as maternal bonding and lactation. Additionally, OT has a variety of central effects, including the regulation of anxiety-related behaviour, stress-coping, and multiple aspects of social behaviour (Neumann and Landgraf, 2012). OT, synthesised in two hypothalamic nuclei, the PVN and the supraoptic nuclei (SON), binds to one single OT receptor (OTR), which is a 389-amino acid polypeptide with seven transmembrane domains belonging to the class I G-protein-coupled receptor family (Gimpl and Fahrenholz, 2001). The receptor has relatively unselective binding capacities as the affinity for OT is only about tenfold higher than that for AVP – the second neurohypophysial hormone that differs from OT only in two amino acids. It was found that only two aromatic residues of the OTR need to be changed to allow full binding of AVP (Gimpl and Fahrenholz, 2001). OTR are functionally coupled to $G_{q/11}$ proteins and hence stimulate the activity of phospholipase C-β (PLCβ). Consequently, inositol triphosphate (IP3) and 1,2-diacylglycerol (DAG) are generated and trigger the release of calcium (Ca^{2+}) from intracellular stores and protein kinase C (PKC) activation, respectively (Gimpl and Fahrenholz, 2001). Ca^{2+}-induced processes can hereby include changes in gene transcription and protein synthesis. Strakova et al. showed that the rat OTR transfected into Chinese hamster ovary cells is coupled to both the $G_{q/11}$ and the $G_{i/o}$ proteins (Strakova et al., 1998). $G_{i/o}$ signalling generates an increase of intracellular Ca^{2+}, independent of the IP3 pathway (Hoare et al., 1999). In addition, OT can couple to G_s proteins that activate adenylate cyclase and increase cyclic

adenosine monophosphate (cAMP) production which leads to a sodium-dependent inward current (Alberi et al., 1997). Possibly, these various signalling pathways are differentially expressed in neuronal and peripheral tissue (Stoop, 2012).

5.2. Molecular mechanism of oxytocin's effect on anxiety

The axons of magnocellular OT neurons terminate in the posterior lobe of the pituitary gland, being part of the classic hypothalamic-neurohypophysial system (Brownstein et al., 1980). Vis-à-vis, parvocellular neurons of the PVN project to a series of brain regions. Consistent with this, microdialysis studies revealed the release of OT in the olfactory bulb, the dorsal hippocampus, the CeA, the septum, the nucleus of the solitary tract, the SON, and the PVN itself (Landgraf and Neumann, 2004; Neumann, 2007). OT has anxiolytic properties when released in brain regions involved in stress and anxiety regulation, such as the CeA and the PVN (Bale et al., 2001; Blume et al., 2008; Neumann, 2001; Neumann and Landgraf, 2012). Axons of hypothalamic OT neurons that project to the CeA lead to the activation of local inhibiting GABAergic circuits and thereby attenuate the fear response (Knobloch et al., 2012). Within the PVN, exogenously applied OT reduces anxiety-like behaviour in rats within 10 min after the application (Blume et al., 2008). A series of behavioural tests confirmed the anxiolytic effect of OT, among them the EPM, the LDB (Blume et al., 2008), the four-plate test, and the elevated zero maze (Ring et al., 2006). Endogenously released OT after successful mating exerts anxiolytic effects to at least 4 hours after mating in male rats, showing that a relatively short OT surge can lead to long-term anxiolysis (Waldherr and Neumann, 2007). Interestingly, intranasal application of OT in humans suppresses anxiety as shown in studies using the Trier Social Stress- and the Simulated Public Speaking Test (de Oliveira et al., 2012; Heinrichs et al., 2003). Male participants who received intranasal OT 50

min before stressor exposure reported a lower post-stress anxiety level (evaluated by a self-reporting questionnaire) than participants receiving placebo. The effect was even increased when they received social support by their best friend (Heinrichs et al., 2003).

Studies that focused on the elucidation of the molecular mechanism behind anxiolysis identified the mitogen-activated protein (MAP) kinase MEK1/2 as an important regulator of anxiety-like behaviour in the PVN of rats (Blume et al., 2008; Jurek et al., 2012). Since the OTR, being a G-coupled receptor, is not classically linked to the MAP-kinase system, it was suggested that the OTR transactivates the tyrosine kinase epidermal growth factor (EGF) receptor to subsequently activate the MAP-kinase cascade (Blume et al., 2008). This hypothesis was confirmed in a rat hypothalamic cell line by blocking the EGF receptor with the specific inhibitor AG1478 (Blume et al., 2008).

5.3. Further involvement of neuropeptides in anxiety

Neuropeptides of the brain are important modulators of physiology and behaviour. Their functions are mediated either via dendritic release or release at axonal terminals (Ludwig and Leng, 2006). Several neuropeptides have been shown to modulate the regulation of anxiety behaviour.

CRF and AVP are both regulators of the hypothalamic pituitary adrenal (HPA) axis activity and mediate central effects on emotional and cognitive behaviours. CRF is generally considered to have anxiogenic effects, but many studies utilizing CRF receptor 1 (CRFR1) antagonists have demonstrated that a stressor is necessary in order to see an anxiolytic effect of the antagonist (Deak et al., 1999; Heinrichs et al., 2002; Heinrichs et al., 1994; Schulz et al., 1996). The results of AVP administration are mixed. Septal injection of AVP leads to anxiolysis as determined on the EPM (Appenrodt et al., 1998). Injection of an V1b

receptor antagonist has anxiolytic properties on the EPM as well, although this observation was troubled by effects on locomotor activity (Liebsch et al., 1996). A review of the effects of SSR149415, a V1b antagonist, concluded that clear anxiolytic effects were only found in stressful situations (Griebel et al., 2003).

Additionally, the behavioural phenotype of HAB and LAB rats is correlated with AVP expression at the level of the PVN. Single nucleotide polymorphisms (SNPs) in regulatory structures of the AVP gene are the basis for the differential behavioural outcomes. Due to these SNPs, AVP is overexpressed and overreleased in the PVN of HAB rats (Landgraf et al., 2007b).

The most abundant neuropeptide in the brain, NPY, binds to five currently investigated receptors distributed in the central nervous system (CNS). Infusion of NPY, either *icv* or into the amygdala, has anxiolytic effects (Broqua et al., 1995; Heilig et al., 1989; Kokare et al., 2005). A specific NPY1 and 5 receptor agonist, given *icv*, showed dose-dependent anxiolytic effects as well, narrowing down the regulatory effect of NPY to those two receptor subtypes (Sorensen et al., 2004).

CCK, which primarily acts as a mediator of satiety, is another highly abundant neuropeptide in the brain and is found in cortex and limbic brain regions (Beinfeld et al., 1981). CCK was reported to induce anxiogenic effects, by activating the CCKB receptor in the BLA (Rotzinger and Vaccarino, 2003). CCKB agonists show anxiogenic effects in a number of tests. However, antagonists often have no effects on baseline anxiety behaviour, but instead modulate heightened states of anxiety (Rotzinger and Vaccarino, 2003; Wilson et al., 1998). The CCK fragment pentagastrin increases anxiety in the human social interaction test (McCann et al., 1994).

Only recently, NPS was discovered to increase wakefulness and arousal on the one hand, and to produce anxiolytic-like effects by reducing fear-responses on the other hand when administered centrally (Xu et al., 2004). NPS binds to a G-protein-coupled receptor and stimulates the mobilization of intracellular Ca^{2+} as well as activation of protein kinases (Pape et al., 2010). NPS receptor expression is found in brain regions involved in anxiety regulation, including the amygdala and the PVN. The neuropeptide is currently under intensive research and may provide new opportunities for clinical applications (Pape et al., 2010).

6. ATP as a neurotransmitter

Adenosine-triphosphate (ATP) is the most important energy source in the intra- and extracellular space. It is involved in cellular respiration, cell division, muscle contraction, and almost every other important process needed for the cell's wellbeing. In addition to this essential duty, ATP and its breakdown product adenosine were discovered to act as neurotransmitters in a wide variety of systems (Burnstock et al., 1970). ATP and adenosine bring about their effects through binding to two groups of receptors: P1 receptors for adenosine and P2 receptors, which have a high affinity for ATP (Figure 1) (Burnstock, 1978). The neurotransmitter ATP is released by exocytosis from nerve terminals (Bodin and Burnstock, 2001) or astrocytes (Lalo et al., 2014), but other transport mechanisms have also been proposed, including ATP binding cassette transporters, connexin hemichannels, and voltage-dependent anion channels (Fields and Burnstock, 2006).

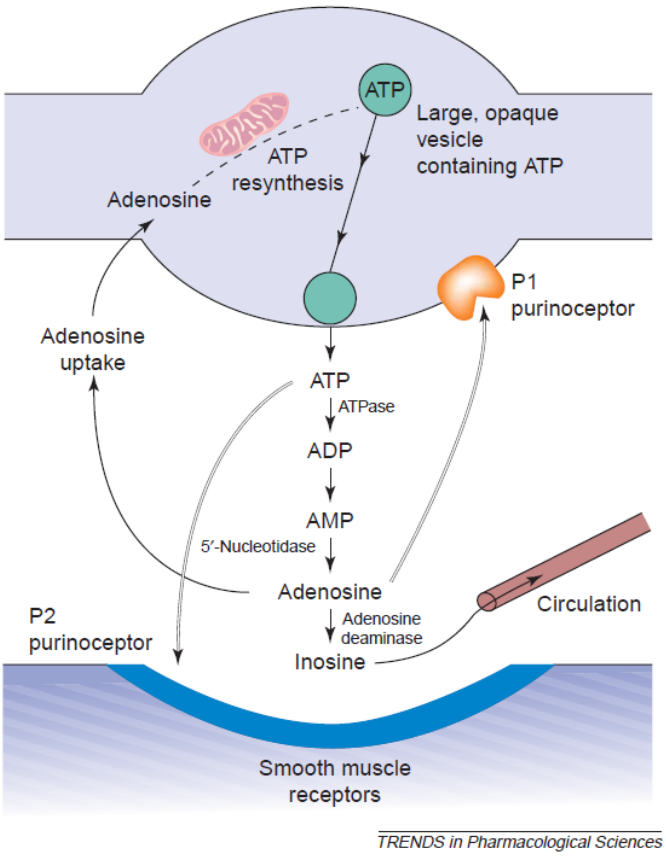

Figure 1: Purinergic transmission in a neuromuscular junction. Synthesis, storage, release, and inactivation of ATP are depicted (Burnstock, 2006a).

The P2 receptors are further divided into two subgroups: the ionotropic P2X receptors (P2XR) and the metabotropic P2Y receptors (P2YR) (Burnstock and Kennedy, 1985). Additional subtypes of P2 receptors have been described, but will not be further discussed here: P2T receptors, selectively for ADP on platelets, P2Z receptors on macrophages, and P2U receptors that recognize pyrimidines (Gordon, 1986; O'Connor et al., 1991).

To date, eight G-protein-coupled P2Y receptor subtypes and seven P2X receptors have been defined (North, 2002). The responses to an ATP-stimulation can last from milliseconds to minutes, and even longer, since second messenger cascades can also induce changes in gene expression regulation (Khakh and North, 2012). The diversity of responses is further

increased by the fact that the receptors differ in their sensitivity to ATP. P2YR are already activated by ATP in the nanomolar range, but P2X7R requires hundreds of micromolar of ATP to open (Surprenant et al., 1996). Due to this diversity, purinergic signalling is very dynamic and accordingly, the expression of P2YR and P2XR throughout different tissues is variable. P2XR are present in multiple species, even in simple organisms like the eukaryote green algae *Ostreococcus tauri* (Fountain et al., 2008), underlining the importance of the purinergic system.

The ionotropic P2XR are ligand-gated ion channels permeable for cations, with their highest permeability for Ca^{2+} (Khakh and North, 2012). However, there is one exception from the rule: P2X5R is permeable to Cl^- (North, 2002). Each P2XR subunit assembles with two others into a trimeric channel, which can be either homomeric or heteromeric (Nicke et al., 1998). The receptors are believed to have three classical agonist binding sites (Browne et al., 2010), and binding of ATP on these sites leads to the opening of the channel (gating). The gating is divided into three phases: the activation phase, the desensitization phase and the deactivation phase (Coddou et al., 2011). Differences in activation and desensitization rates are what characterize the different receptor subtypes.

Purinergic signalling is an important system in the brain's neuroprotection, since it is involved in nervous tissue remodelling after trauma, stroke, ischaemia or neurodegenerative disorders (Burnstock, 2006a). In response to neuronal injuries, fibroblast growth factor, epidermal growth factor, and platelet-derived factor are released. Together with these factors, ATP stimulates astrocyte proliferation which leads to reactive astrogliosis (Burnstock, 2006a). In general, ATP acts as an important extracellular signalling molecule between neurons and glial cells in the CNS. Microglia, activated by ATP, release

inflammatory cytokines and tumour necrosis factor α – an overstimulation of this system can thus accelerate the neuronal damage caused by ischaemia, trauma or neurodegenerative diseases (Burnstock, 2006a). One of the most studied purinergic receptors is the P2X4 receptor (P2X4R).

6.1. P2X4 receptor

The detailed atomic anatomy of the receptor is known from studies in zebrafish and was described in the review of Khakh and North in 2012 very plastically as a resemblance to "a dolphin rising from the ocean surface" (Khakh and North, 2012) (Figure 2). One subunit is composed of two hydrophobic membrane-spanning segments and an extracellular loop mostly formed of β sheets. Three of those subunits curl around each other, building a trimeric channel that opens to let Ca^{2+} ions pass when ATP is bound (Khakh and North, 2012).

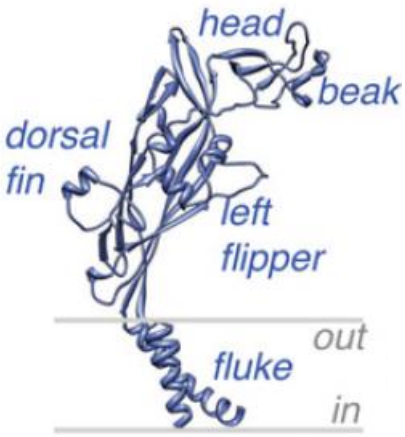

Figure 2: Crystal structure of the P2X4 receptor showing the two hydrophobic membrane-spanning segments ("fluke") and the extracellular loop (Khakh and North, 2012)

The P2X4R is the most widely distributed purinergic receptor throughout the CNS (Buell et al., 1996). P2X4Rs play a role in the regulation of multiple nervous functions, including

neuropathic pain (Tsuda et al., 2003), neuropeptide release (Lemos et al., 2012), and hippocampal plasticity (Baxter et al., 2011). In addition, P2X4Rs have been shown to modulate the functions of other neurotransmitter systems, such as N-methyl-D-aspartate (NMDA) glutamate receptors (Baxter et al., 2011) and $GABA_A$ receptors (Jo et al., 2011). In detail, a facilitation of NMDA synaptic current by insertion of NR2B subunits cannot be seen in P2X4R KO mice, suggesting a changed NMDA receptor composition (Baxter et al., 2011). Moreover, P2X4R directly interact with $GABA_A$ receptors via two residues in the C-terminus of the P2X4R. Electrophysiological experiments showed that the two receptors negatively interact with each other, which represents a form of short term synaptic plasticity (Jo et al., 2011). An involvement in the modulation of behaviour is therefore very likely. Indeed, P2X4R KO mice display less social interaction, and higher tactile sensitivity. In this study, no effect on anxiety-like behaviour was observed (Wyatt et al., 2013), which may be due to compensatory effects. In contrast, behavioural testing after P2X4R activation with the positive allosteric modulator ivermectin (IVM) produced anxiolytic-like effects on the EPM (Bortolato et al., 2013), indicating that P2X4R is involved in the modulation of anxiety-like behaviour. However, IVM has potentiating effects on $GABA_A$ receptors as well, so that further research on the role of P2X4R is necessary (Krusek and Zemkova, 1994).

7. Protein synthesis

One of the central dogmas of biology states that cellular processes, and therefore physiology and behaviour, directly or indirectly depend on the sequential transfer of biological information from DNA to RNA to protein via transcription and translation. Once the mRNA exits the cell nucleus, it is ready to be translated into a polypeptide chain by the ribosome in

the cell cytoplasm. This process requires the teamwork of non-coding RNAs (ribosomal RNAs and transfer RNAs (tRNAs)), the coding messenger RNA (mRNA) and a large number of proteins. These proteins are either ribosomal proteins or non-ribosomal proteins required for translation initiation (eIFs), for translation elongation (eEFs), or for translation termination (eTFs) (Meister, 2011).

The ribosome is responsible for the translation of the genetic code of the mRNA from 5' to 3' into the amino acid sequence and for the synthesis of the corresponding protein. It is composed of two subunits, in eukaryotes the large 60S and the small 40S ribosomal subunit. Protein synthesis starts with a methionine, which is loaded onto the inititator tRNA. The ribosome has three tRNA binding sites that accept the incoming tRNAs loaded with amino acids corresponding to the presented codon of the mRNA. Every new amino acid is incorporated into the growing peptide chain where it is bound to the previous amino acid by a peptide bond. The process of elongation is GTP-dependent and requires several eEFs. Among those, eEF2 is responsible for the release of the "old" tRNA, leaving a new space for the next tRNA (Figure 3). One mRNA is not only translated by one ribsosome but by several ribosomes (polyribosome) at the same time, allowing for fast and highly efficient protein synthesis (Meister, 2011). The translation is terminated by the presence of a stop codon, which leads to a release of the polypeptide from the ribosome.

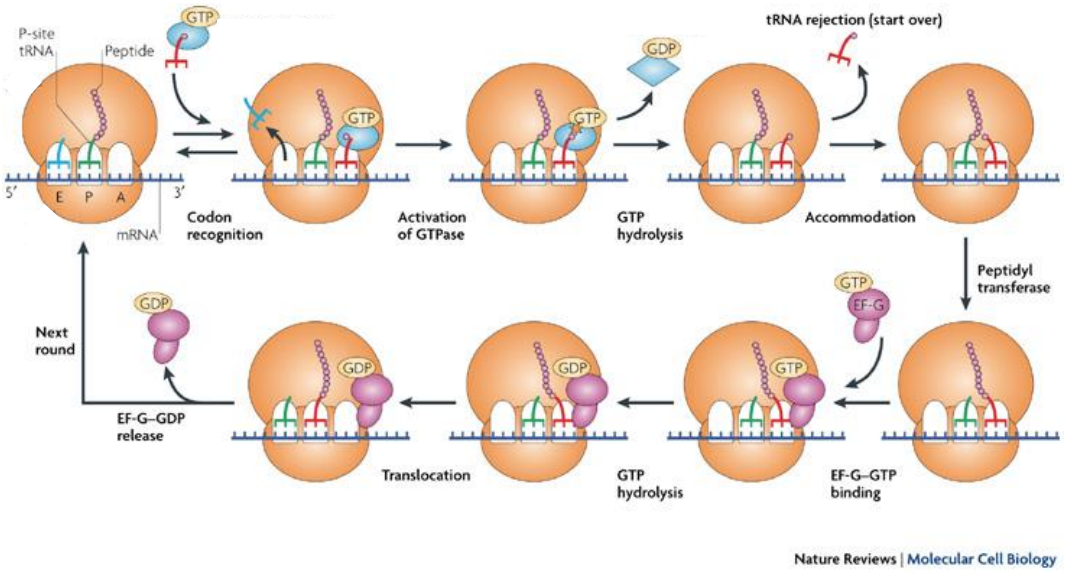

Figure 3: Illustration of GTP-dependent peptide chain elongation (Steitz, 2008).

Translation is a highly critical process with a need for precise regulation to make appropriate cellular reactions to environmental challenges possible. All three stages – translation initiation, elongation and termination – are subject to a fine-tuning involving several factors. Most prominently, proteins that interact with the initiation factor eIF4E, termed 4E binding proteins (4E-BPs), regulate the access of eIF4E and therefore translation initiation. eEF2, the important elongation factor, is part of the regulatory system of protein synthesis as well. Phosphorylation of eEF2 slows down translation, as seen for example when a cell enters mitosis. eEF2 phosphorylation is also important for local inhibition of translation: In neurons, mRNAs are stalled at the polyribosomal stage at synapses and after arrival of an action potential at the synapse, protein synthesis is rapidly transformed (Meister, 2011).

The mammalian target of rapamycin (mTOR) controls several components involved in the initiation and elongation of translation. One of those is the aforementioned eIF4E, which is regulated by the 4E-BPs. mTOR phosphorylates several sites of 4E-BP and decreases thereby

their inhibiting effects on the initiation factor. Furthermore, mTOR inhibits eEF2 kinase activity with positive effects on eEF2 itself (Wang and Proud, 2006).

8. MicroRNAs and the regulation of gene expression

8.1. General information

MicroRNAs are endogenous, non-coding RNAs, approximately 22 nt in length. They were discovered in 1993 by Lee et al. (Lee et al., 1993) in *Caenorhabditis elegans*, and it was rapidly understood that they exhibit base complementarity to mRNAs. Binding of a microRNA to its respective target RNA leads to a double-stranded RNA molecule and thus to RNA interference (RNAi) (Fire et al., 1998).

MicroRNAs are endogenously expressed in almost all eukaryotes with the exception of *Saccharomyces cerevisiae*. MicroRNA genes are transcribed by RNA polymerase II to pri-microRNAs, which are then further processed by a large microprocessor protein complex containing the RNase III enzyme Drosha and its partner DiGeorge syndrome critical region 8. The resulting product is a stem-loop-structured microRNA precursor (pre-miRNA) . The pre-miRNAs are transported from the nucleus into the cytoplasm via the export receptor exportin 5. In the cytoplasm, the mature microRNA is finalized by cleaving off the hairpin-loop of the pre-miRNA and unwinding of the microRNA duplex. The mature microRNA is incorporated into a microRNA-protein complex referred to as miRNP or RISC (RNA-induced silencing complex) (Meister, 2011). The miRNP includes the type III ribonuclease Dicer, the RNA-binding Argonaute proteins, and the adaptor protein transactivating response RNA-binding protein (O'Carroll and Schaefer, 2013).

The degree of complementarity between microRNAs and their target mRNAs determines the outcome of the binding of the miRNP to the target mRNA. A perfect or nearly perfect complementarity leads to the cleavage of the mRNA in an RNAi-like manner. This is the most common microRNA function in plants. In animals, however, a perfect complementarity is rare and microRNAs regulate the gene expression differently (Meister and Tuschl, 2004). Here, microRNAs associate with sequence elements in the 3' untranslated region (UTR) of the target mRNA. The target sites are only fully complementary to the nucleotides 2 – 8 of the microRNA, which is referred to as the seed sequence. The rest contacts only partially (Chen and Rajewsky, 2007). This way of binding does not result in RNA degradation but in the regulation of translation. Whether such regulation of translation affects translational elongation or initiation is still under debate (Meister, 2011). Either way, the level of total mRNA is not influenced by this interference, but rather the level of newly synthesised proteins. However, partial binding of a microRNA can also induce the deadenylation of the target RNA. The poly(A) tail is shortened, which leads to the initiation of RNA decay. Which pathway will be chosen is most likely dependent on the nature and sequence of the target mRNA, as well as the protein environment surrounding the microRNA target sites (Filipowicz et al., 2008; Meister, 2011).

8.2. MicroRNAs in brain and behaviour

The mammalian brain expresses almost 50 % of all identified microRNAs (Landgraf et al., 2007a), with specific expression profiles for specific brain regions. In addition, intraneuronal microRNA compartimentalization is common (O'Carroll and Schaefer, 2013). There are several microRNAs that are specialized on the expression in synapses or dendrites, like miR-125b, miR-128, miR-132, and miR-134 (Edbauer et al., 2010; Schratt et al., 2006; Siegel et al.,

2009). This indicates their important role in the regulation of local protein synthesis involved in synapse maturation and function. Indeed, microRNAs are seen as crucial regulators of neurogenesis, neurite outgrowth, synaptogenesis, and neural plasticity (Zhou et al., 2009).

The expression level of certain microRNAs is significantly higher than that of others. Some microRNAs, such as let-7, miR-124, and miR-128 are very strongly expressed in neurons, whereas others are only present in 1 – 2 copies/cell (O'Carroll and Schaefer, 2013). This fact should be taken into consideration when analysing microRNA expression in neurons, since it may lead to false conclusions about the significance of some microRNA changes. This diversity might be, amongst other reasons, rooted in the multiplicity of genes encoding a single microRNA. In other words, many of the brain-enriched microRNAs are encoded by more than one gene (Griffiths-Jones, 2006). This could serve as a back-up mechanism that ensures the expression of those important microRNAs even in the case of mutation of one gene. Secondly, the multiplicity of microRNA-encoding genes could support the interneuronal diversity of microRNA expression (O'Carroll and Schaefer, 2013).

Brain microRNAs are strongly involved in the development of neurological and psychiatric disease, making them an even more interesting target of investigation. Abnormal microRNA expression patterns were shown for patients suffering from schizophrenia, autism, Huntington's disease, Alzheimer's disease, and others (Abu-Elneel et al., 2008; Beveridge and Cairns, 2012; Johnson et al., 2008; Lukiw, 2007). However, it is not clear if these changes are cause or consequence of the specific neuronal alterations that characterize those diseases. Recent findings depict the involvement of microRNAs also in behavioural matters. Cocaine addiction (Hollander et al., 2010) as well as alcohol tolerance (Pietrzykowski et al., 2008) were shown to be controlled by specific microRNAs. Other studies observed a

regulation of fear extinction (Lin et al., 2011), or even the control of the circadian rhythm (Cheng et al., 2007). Most importantly for my studies, Haramati et al. found increased anxiety-like behaviour after Dicer ablation in the CeA. Furthermore, they identified miR-34c as a repressor of stress-induced anxiety (Haramati et al., 2011). Overexpression of this microRNA within the CeA induced anxiolytic behaviour after social defeat, thus marking microRNAs as promising targets for the development of novel treatments for anxiety disorders.

8.3. Regulation of the microRNA pathway

There are many ways how the microRNA pathway can be altered. Regulations can be of transcriptional, post-transcriptional or post-translational nature, and protein components of the pathway as well as the microRNAs themselves can be involved. Similar to genes that code for a protein, microRNA genes can be targets of gene expression regulation in response to developmental or environmental cues (O'Carroll and Schaefer, 2013). For example, regulation of gene expression in response to neuronal activity is an essential mechanism for neuronal adaptations like changes in synaptic strength and connectivity. miR-132 and miR-134 are two typical neuronal microRNAs involved in activity-dependent dendritic remodelling (Christensen et al., 2010; Hansen et al., 2010). MicroRNA turnover is an important and strongly regulated event in neurons, and the highly differing half-life of specific neuronal microRNAs plays an essential role. Studies where Dicer was eliminated showed that numerous microRNAs remained expressed even for months after the deletion of Dicer, while others became undetectable within days (Schaefer et al., 2007). This differential regulation of their turnover rate may control distinct neuronal functions, appears

to be activity-dependent, and can be activated by stimulation with neurotransmitters like glutamate (Krol et al., 2010).

Another way to regulate microRNA stability is to interfere at the level of the pre-microRNA. Enzymes that lead to a poly-uridylation of the pre-microRNA cause their degradation and thus the reduction of the corresponding mature microRNA (Heo et al., 2009).

Recently, a class of long noncoding RNAs was discovered that act as microRNA sponges in the cell and was termed “competing endogenous RNAs” (ceRNAs). ceRNAs can actively regulate microRNA activity, because they contain several consecutive microRNA binding sites (Cesana et al., 2011). In this way, it is believed that, especially in neurons where spatially separated zones of mRNA translation exist, local microRNA-mRNA-interactions are mediated. Thus, an activity-dependent induction of microRNA sponge expression could reduce target mRNA suppression, and hence increase their translation, because their inhibiting microRNAs would no longer be available (O'Carroll and Schaefer, 2013).

9. Aims and outline of the present thesis

The overall aim of my studies is to gather more fundamental knowledge about the molecular mechanisms that occur within the PVN controlling anxiety-related behaviour, supporting the role of this brain region for anxiety research. My experiments were carried out in the bigger context of developing new therapeutic approaches to help patients who suffer from anxiety disorders of any kind. In my studies, candidates for pharmacological treatment of anxiety disorders should give clear acute anxiolytic or anxiogenic effects in animal models of anxiety as well as in established behavioural tests in rodents. In addition, pharmacological

intervention should ideally lead to long-term effects to make treatments efficient. To achieve this, I addressed two main questions:

1. Can we determine a new target for future pharmacological intervention within the PVN that is anxiolytic with a rapid onset of effects?
2. Which intracellular processes are important to convert a rapid, acute anxiolytic effect into a sustained, long-term anxiolytic effect?

To answer these questions, three different studies were performed, each with a specific goal as described below.

9.1. Aim 1: Identification of a novel anxiolytic factor within the PVN of rats

To identify novel possible targets for elucidating the molecular mechanisms behind anxiety-related behaviour, we made use of a microarray that determined the differences of mRNA expression levels in an animal model for anxiety-related behaviour, the HAB and LAB rats. Subsequent quantitative PCR and Western blot analysis identified the P2X4R as a promising factor that could be implicated in the control of anxiety. This was confirmed in two tests for anxiety-like behaviour by pharmacological activation or inhibition of P2X4R, as well as by biochemical analysis.

9.2. Aim 2: Elucidation of the role of *de novo* protein synthesis in the mediation of the (long-term) anxiolytic effect of oxytocin

A single OT surge in the PVN has previously been shown to be anxiolytic for at least 4 h (Waldherr and Neumann, 2007). Therefore, OT was used to determine whether protein synthesis is involved in long-term anxiolysis. To this end, the activation of key factors of protein synthesis signalling (eEF2 and mTOR) was analysed by Western blot in hypothalamic

cells and tissue. *De novo* protein synthesis, induced by OT, was demonstrated by incorporation of a synthetic amino acid and detection of the labelled proteins with Click-chemistry. Finally, the role of protein synthesis in the control of anxiety-like behaviour by OT was assessed in male Wistar rats in which protein synthesis was inhibited pharmacologically prior to OT infusion in the PVN. Anxiety was analysed in two independent tests, the LDB and EPM, 30 min and 3 h, respectively, following OT administration to determine whether protein synthesis within the PVN mediates the early onset or is rather a stable substrate for long-term anxiolysis.

9.3. Aim 3: Determination of the control of microRNA expression levels by OT in the hypothalamus

One way of controlling protein levels in cells concerns the regulation of the bioavailability of corresponding mRNA. In collaboration with Prof. Dr. Gunter Meister, we generated Deep Sequencing libraries to show the influence of OT on microRNA expression in hypothalamic cells. The results were validated by means of Northern blot and qPCR and revealed, for the first time, that microRNA expression can be controlled by a neuropeptide of the PVN. The potential role of some microRNAs in anxiety-related behaviour and stress will be discussed with respect to the possible mRNA targets in the PVN.

MATERIALS & METHODS

Materials & Methods

1. Animals

Adult female or male HAB and LAB (250 - 300 g; local breeding colony; for selection procedures see (Neumann et al., 2005)) as well as rats non-selected for anxiety-related behaviour (hereafter: Wistar rats; 250 - 300 g; Charles River Laboratories, Germany) were housed under standard laboratory conditions (12 h light : dark cycle, 22 - 24 °C, lights on at 06:00 h, food and water *ad libitum*). Wistar rats were allowed to habituate for one week after arrival. All experiments were performed between 08:00 - 11:00 h. The studies were conducted in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Upper Palatinate, Germany.

2. Surgical Procedures

All surgical stereotaxic procedures were performed under isoflurane anaesthesia and semi-sterile conditions. Following surgery, rats received a subcutaneous injection of enrofloxacin (2.5 mg Baytril; Bayer, Germany). Rats were single-housed after surgery, handled daily to habituate them to the respective central infusion procedure and allowed at least seven days of recovery before undergoing behavioural testing.

For analysis of local effects of pharmacological intervention within the PVN on anxiety-related behaviour, 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 mm and +2.1 mm lateral, DV: +6 mm below the surface of the skull, angle 10 °) (Blume et al.,

2008; Jurek et al., 2012; Paxinos G, 1998) through holes drilled in the skull and attached to two stainless-steel screws using dental cement.

The position of the cannulas was verified after the experiments and post-mortem. Blue ink was infused through the guide cannulas and brains were collected. 40-µm cryosections of the brains were stained with Nissl and correct cannula placement was verified with the aid of a rat brain atlas (Paxinos G, 1998).

For the analysis of cell physiological responses to OT in the PVN, rats were implanted with guide cannulas (21 G, 12 mm long) 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) (Blume et al., 2008; Paxinos G, 1998; Slattery and Neumann, 2010). The guide cannulas were kept feasible with dummy cannulas, which were removed and cleaned every day during the handling procedure. Rats received an infusion of 1 nmol OT/5 µl.

3. Cells

3.1. H32 cells

The immortalized foetal rat hypothalamic cell line H32 (Mugele et al., 1993) was cultured at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, USA) containing 10 % foetal bovine serum (FBS), 10 % horse serum and 1 % penicillin/streptomycin (Life Technologies). For experiments, cells were seeded in 10 cm petri-dishes at a density of 3 x 10^6 cells and grown to 80 % confluence overnight. Prior to stimulation, the medium was changed to serum-free DMEM containing 0.1 % bovine serum albumin (BSA; Sigma-Aldrich, Germany) and the cells were left undisturbed for 1 h.

3.2. Be(2)-M17 cells

The human neuroblastoma cell line Be(2)-M17 (European Collection of Cell Cultures, #95011816, UK, (Jurek, 2014)) was cultured at 37 °C and 5 % CO_2 in DMEM/F12 (1:1) (Life Technologies) containing 1.2 % L-glutamine, 15 % heat-inactivated FBS, 1.2 % non-essential amino acids, and 0.1 mg/ml gentamycin (Life Technologies). For experiments, 4 x 10^6 cells were seeded per 10 cm petri-dish and treated with 5 µM retinoic acid (Sigma-Aldrich) to initiate differentiation into neurons. After three days at 37 °C and 5 % CO_2, cells were ready for stimulation. On the day of the experiment, the cells were incubated in serum-free DMEM/F12 (+ 0.1 % BSA) for 1 h to reduce basal activation by any growth factors and steroids that might be present in the serum.

3.3. Primary hypothalamic neurons

Primary neurons were obtained from foetal Wistar rats on embryonic day 18. After decapitation of the foetuses, hypothalamic tissue was dissected and collected in ice-cold Hank's balanced salt solution (HBSS; Life Technologies) containing 0.1 mg/ml gentamycin. Hypothalami were digested for 45 min at 37 °C with 300 U/ml collagenase type 2 (Worthington, USA) diluted in HBSS supplemented with 4 mg/ml BSA (Sigma-Aldrich), 1 mg/ml glucose (Merck, Germany) and 0.2 mg/ml DNase (Sigma-Aldrich). The cell suspension was carefully filtered through a 40-µm cell strainer (BD Falcon, USA) and centrifuged at 200 x g for 10 min. The cell pellet was resuspended in DMEM/Ham's F12 containing 0.1 mg/ml gentamycin and 10 % heat-inactivated FBS. Cells were plated at a density of 3 x 10^6 cells/well in poly-D-lysine coated six-well plates (BD Falcon) and incubated for 24 h at 37 °C and 5 % CO_2. Medium was then changed to neurobasal medium (Life Technologies) containing B27 supplement (Life Technologies), 2 mM L-glutamine, 0.1 mg/ml gentamycin and 5 µM

ascorbic acid (Sigma-Aldrich). Cytosine arabinoside (Sigma-Aldrich) was added to a final concentration of 5 µM from day 4 onwards to prevent glial cell proliferation. On day 10, cells were prepared for stimulation by replacing the growth medium with supplement-free neurobasal medium containing 0.1 % BSA, and incubated for 1 h before the start of the experiment.

3.4. Cell stimulation

To reveal the regulatory effects of OT at the cellular level, OT was added to the serum-free medium and the cells were left undisturbed at 37 °C and 5 % CO_2 for different time periods as indicated in the results section. For signalling studies aimed to determine the pathway involved in OT-stimulated protein synthesis, the MEK1/2 inhibitor U0126 (final concentration 10 µM in 0.1 % DMSO) or the PKC inhibitor Gö6983 (final concentration 1 µM in 0.1 % DMSO; Sigma-Aldrich) were added to the cell medium 20 min before activating them with OT.

After incubation, cellular activity was stopped rapidly by cooling the petri-dishes on ice. Cells were lysed and total RNA or proteins were isolated as described below. RNA- and protein-expression levels were compared to respective vehicle-treated control groups and are shown as relative expression.

4. Behavioural studies

Rats received bilateral local infusions (0.5 µl/PVN) of the substance to be tested, and the effects of drug infusion were assessed on the EPM and in the LDB. The substances that were infused were OT (0.01 nmol in Ringer's Solution), anisomycin (23.5 µmol in HCl, adjusted to

pH 7.4), cytidine triphosphate (CTP; 4 and 40 nmol in Ringer's Solution) and 5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one (5-BDBD; 25 nmol in HEPES-buffered Ringer's Solution). To exclude effects of the dissolvent on behaviour, every treatment group was compared with a control group, given only the respective vehicle. The time period between infusions, as well as that between the last infusion and behavioural testing, varied according to the experiment (please see results section).

The test protocols for the EPM and the LDB were performed similarly to those previously described (Bosch and Neumann, 2008; Neumann et al., 2000; Pellow et al., 1985; Slattery and Neumann, 2010; Waldherr and Neumann, 2007). Briefly, the plus-shaped maze is made of two open (50 x 10 cm, 80 lux) and two closed (50 x 10 x 30 cm, 10 lux) arms surrounding a neutral square-shaped central zone (10 x 10 cm, 65 lux), elevated 80 cm above the floor. The percentage of time spent on the open vs. time spent on all arms is indicative of anxiety-related behaviour, while the number of closed arm entries is used to assess locomotion (Neumann et al., 2000). The duration of the test was 5 min.

The LDB setup consisted of two boxes; one lit box (40 x 50 cm, 350 lux; light box) and one dark box (40 x 30 cm, 70 lux). Light conditions in the light box were changed for HAB (85 lux) and LAB (1000 lux) studies, considering their extreme phenotypes. The floors in each box were divided into squares (10 x 10 cm) and the boxes were connected by a small opening (7.5 x 7.5 cm) enabling transition between the boxes. Rats were placed in the light box and line-crossings, time spent in each box, rearing, latency to enter the dark box and the latency to first re-enter the light box were assessed during the 5-min test live via a camera located above the box.

5. RNA studies

5.1. RNA extraction

RNA was extracted from cultured cells and from PVN tissue that had been dissected from the hypothalamus. The dissection was done in brains that were snap-frozen on dry-ice, and cut on a cryostat in 250 µm thick slices. The PVN was then punched out with the aid of a tissue puncher of 2 mm in diameter (Fine Science Tools, Germany), and expelled into an Eppendorf tube. To collect most of the PVN from a single animal, two consecutive punches were pooled. The punches were either kept frozen on dry ice or were directly lysed in the appropriate buffer for RNA-extraction.

Punches and cells were lysed in 1 ml of peqGold TriFast (peqLab, Germany) and kept on ice for the whole procedure to prevent degradation of RNA. The lysate was mixed with chloroform (200 µl in 1 ml lysate) and centrifuged for 20 min at 12000 x g and 4 °C. The upper aqueous phase containing the RNA was collected and transferred to a fresh cup. Next, the RNA was concentrated by precipitation in isopropanol overnight at -20 °C. Following centrifugation at 16000 x g, the RNA pellet was washed twice with 80 % ethanol, air-dried, suspended in RNase-free water, and solved at 70 °C for 5 min at 1000 rpm. RNA quantity and quality were determined at 260/280 nm using a NanoDrop spectrophotometer (Thermo Scientific, USA).

5.2. Microarray

Differential gene expression in the PVN of HAB and LAB rats were assessed using the microarray technique in collaboration with Dr. David von Schack and Dr. Robert H. Ring, Pfizer Inc., New York, USA.

HAB and LAB virgin female rats were sacrificed and the PVN was punched out. The frozen material was sent to the collaborators who performed the microarray. This method allows for a comparison of the mRNA expression profile in different samples. The extracted, fluorescently labelled cDNA hybridizes to their complementary sequences on a solid surface and is then scanned with a laser. Differential gene expression is revealed with a simultaneous, two-colour hybridization scheme: fluorescent probes with different colours were prepared from the two mRNA sources, mixed together, hybridized to a single array, and scanned separately (Schena et al., 1995).

5.3. Deep Sequencing

Deep Sequencing libraries were generated in collaboration with Prof. Dr. Gunter Meister, Department of Biochemistry I, University of Regensburg. To study the effects of OT on microRNA expression in rat hypothalamic cells, H32 and primary hypothalamic cells were stimulated with 250 nM OT for 30 min and 3 h, and total RNA was extracted according to the protocol described above (5.1). Subsequent steps were performed as described previously (Dueck et al., 2012). Briefly, total RNA was separated by size on a 12 % urea-polyacrylamide (PAA) gel and short RNAs (between 10 and 20 bp) were cut out and extracted from the gel. Isolated small RNA was ligated to a bar-coded, adenylated 3' adapter by a truncated T4 RNA ligase 2. Additionally, the RNA was enlarged by a 5' RNA adapter that was added in a second ligation step by T4 RNA ligase 1. The product was reverse transcribed and amplified by PCR (see below). The samples were run on a 6 % urea-PAA gel and the bands containing the ligation product were cut out of the gel and eluted overnight in 300 mM NaCl, 2 mM EDTA. After precipitation with ethanol overnight at -20 °C, samples were collected by centrifugation and dissolved in H_2O. The libraries were analysed on a Genome Analyzer GAIIx

(Illumina, USA) (Dueck et al., 2012), with the minimal length of a read set to 18 nucleotides, no mismatch was allowed. The reads for each microRNA were normalized against the total read number of the respective library (Dueck et al., 2014).

5.4. PCR and qPCR

Isolated RNA was reverse transcribed into cDNA. Random primers (3 µg/µl) and dNTPs (final concentration 0.5 mM; Life Technologies) were mixed with 1 µg total RNA and the mix was incubated for 5 min at 65 °C to anneal the primers. To start reverse transcription, FirstStrandBuffer, dithiothreitol (DTT; final concentration 5 mM), RNase OUT (40 U/µl) and the reverse transcriptase Super Script III (200 U/µl; Life Technologies) were added to a final volume of 20 µl. cDNA synthesis was performed at 42 °C for 50 min. The reaction was stopped by degradation of the enzyme at 70 °C for 15 min.

Primers were created with the open-source application PerlPrimer (Marshall, 2004). Wherever possible, primers were designed to span an intron/exon boundary to assure that no genomic DNA was amplified.

A regular reverse transcription PCR (RT-PCR) was always performed to test the specificity and efficacy of the primers, and to determine whether a particular gene product is expressed in hypothalamic tissue or cells. Following validation of the quality of the primers, cDNA (50 ng), 2 pmol forward and reverse primers (Metabion, Germany) and water were added to DreamTaq Master Mix (Thermo Scientific, Germany), containing dNTPs (final concentration 0.2 mM each) and DreamTaq™ polymerase, to a final reaction volume of 25 µl. Negative controls consisted of reactions where cDNA was substituted by H_2O, or samples where the reverse transcription had been omitted. The PCR was run for 40 amplification cycles with an initiating denaturation step at 95 °C for 5 min. Primer-annealing was

performed at 60 °C and elongation at 72 °C. The blockcycler (C1000 Thermal Cycler; BioRad, Germany) was programmed to run a final elongation for 10 additional min and to cool the reaction down to 4 °C. The PCR-products were then loaded onto a 1.5 % agarose gel. After electrophoresis, cDNA bands were detected with DNA Stain G (SERVA electrophoresis, Germany) and visualized with UV-light in the ChemiDoc XRS+ Imager (BioRad).

The method of real-time or quantitative PCR (qPCR) is essentially similar to that of RT-PCR. Differences are found in the detection method of the amplified products, and the analysis. qPCR was carried out with the 7500 Fast Real-Time PCR System (Applied Biosystems GmbH, Germany) and as detection dye SYBR Green I was used. SYBR Green binds to double stranded DNA and emits green light at 522 nm. The reaction mixture consisted of 10 µl QuantiFast SYBR Green PCR MasterMix (QIAGEN, the Netherlands), 4 µl RNAse-free water and each 2 µl of reverse and forward primers (4 pmol) and cDNA. Following amplification, a melting curve was made by slowly heating the sample from 60 °C to 95 °C, while constantly measuring the green fluorescence. This procedure gives the melting point of the amplified DNA. The detection of more than one melting point indicates the amplification of unspecific products, which was always verified on an agarose gel. Gene expression was quantified relative to the expression of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein L13a (Rpl) (Bonefeld et al., 2008)).

For validation of the Deep Sequencing on microRNAs in primary hypothalamic neurons, qPCR was used, with minor changes compared to the conventional qPCR described above. Mature microRNAs obtained from primary neurons stimulated with 250 nM OT or control cells were first modified by the addition of a polyA tail. To this end, RNA was treated with E-PAP buffer, MnCl (25 mM), ATP solution (10 mM) and E. coli Poly (A) Polymerase I (E-PAP; Poly (A) Tailing

Kit, Ambion/Life Technologies) (Hurteau et al., 2006). Next, the E-PAP treated total RNA was incubated with a dNTP mix and 100 µM Universal Reverse Transcription primer (URT-primer) – a primer that is composed of a complementary part to the added polyA tail, a polyT-sequence, and a universal sequence. After annealing for 5 min at 65 °C, the sample was reversely transcribed under the same conditions as described above.

The PCR was performed with a primer that is exactly complementary to the desired microRNA and a universal PCR primer that is complementary to the unique sequence of the URT primer. Quantitation of the reaction products is achieved using the intercalating dye SYBR Green with 18s ribosomal RNA as an internal standard (Hurteau et al., 2006).

P2X4R studies		
target	forward-primer 5'-3'	reverse primer 5'-3'
Casq2	CTC TGT CTC TAC TAC CAC GA	ATA CAG GCT TCC TTC TTC AC
Cckbr	TCC CTT CTC AAC AGC AGT AG	GCC AAC ACT CAT CAG AAA GA
Hcrt	CTC CAG ACA CCA TGA ACC TT	GTG CAA CAG TTC GTA GAG AC
Nrg1	GTC ATT ACA CTT CCA CAG CC	ATC ATA TTT CTT CTC CCG TAG CC
P2rx4	GTC CAG AGA TTC CTG ATA AGA C	GTA CCA GAT GTT GTT CTT TAC C
Trpm7	GCT GAA ATG TCC CAT ATC CC	GTA TGC CAA TGT GTT AAA CCA G
Trpv2	GAA ATC CTC TTT CTC CTT CAG	TGT GTA GTA AAG CAG GTT CAG
VIP	CGC CCT ATT ATG ATG TGT CC	CTG ATT CGT TTG CCA ATG AG
Rpl13A	ACA AGA AAA AGC GGA TGG TG	TTC CGG TAA TGG ATC TTT GC
Gapdh	TGA TGA CAT CAA GAA GGT GG	CAT TGT CAT ACC AGG AAA TGA G

protein synthesis studies		
target	forward-primer 5'-3'	reverse primer 5'-3'
OTR rat	CAG TAG TGT CAA GCT TAT CTC CA	AAG AGC ATG TAG ATC CAC GG
OTR human	AAG AGC AAC TCG TCC TCC TTT	ACA AAC ATA CGC CAT CAC CT

microRNA studies	
target	primer 5'-3'
rno-miR-124	TAA GGC ACG CGG TGA ATG CC
rno-miR-128	TCA CAG TGA ACC GGT CTC TTT
rno-miR-132	TAA CAG TCT ACA GCC ATG GTC G
rno-miR-212	TAA CAG TCT CCA GTC ACG GCC A
rno-miR-30a	TGT AAA CAT CCT CGA CTG GAA G
rno-miR-34c	AGG CAG TGT AGT TAG CTG ATT GC
18s rRNA	CGT AGG TGA ACC TGC GGA A
URT-Primer	AAC GAG ACG ACG ACA GAC TTT TTT TTT TTT TTT N
universal PCR Primer	AAC GAG ACG ACG ACA GAC TTT

Table 1: List of PCR-primers for P2X4R-, protein synthesis-, and microRNA-studies

Purity of the qPCR products was determined by analysing their respective melting curves and gel electrophoresis to ensure the correct amplicon size.

5.5. Northern Blot

To validate the results of the Deep Sequencing in OT-treated H32 cells, a Northern Blot was performed. To this end, 10 µg of total RNA extracted from both vehicle- and OT-treated H32 cells were loaded onto a 12 % PAA/urea gel and separated by size at 350 V for 1 – 2 h. As a size marker, ribooligonucleotides with a length of 19, 21 and 24 nt were labelled with ^{32}P prior to loading (Dueck et al., 2012). After gel electrophoresis, gels were stained with ethidiumbromide to visualize and record the amount and distribution of the RNA (Pall et al., 2007). Following that, the RNA was transferred to a nylon membrane (Hybond-N; Amersham/GE Healthcare, UK) for 30 min at 20 V.

To immobilize the small RNAs permanently on the membrane, the EDC cross-linking method was used. Immediately prior to use, a solution of 0.16 M l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma-Aldrich) was prepared in 0.13 M 1-methylimidazole (pH 8; Sigma-Aldrich). The membrane was placed on a Whatman-paper saturated with the EDC-solution

with the side onto which the RNA had been transferred facing up (Pall et al., 2007) and incubated for 1 h at 50 °C. Then, the membrane was washed with bidest H_2O to remove remaining EDC-solution and dried with a blow-dryer.

To visualize the desired microRNAs, complementary probes were labelled with ATP^{32}. For labelling, 20 pmol of the probe antisense to the respective microRNA were incubated with 20 µCi of ATP^{32} in a T4 PNK reaction (Fermentas/Thermo Scientific). Subsequently, the labelled probe was purified with a G-25 column (GE Healthcare), added to the membranes and allowed to hybridize with the blotted microRNAs at 50 °C overnight (Dueck et al., 2012). The next day, the membranes were washed three times at 50 °C to remove all unbound probes. The radioactivity of the bound probes was captured on a PhosphoScreen (Kodak, USA) and visualized by a PhosphoImager (BioRad). Blots were stripped by boiling in 0.1 % aqueous SDS for 45 min and were ready for hybridization with another microRNA probe (Lagos-Quintana et al., 2001).

6. Protein studies

6.1. Protein extraction

Proteins were isolated from fresh PVN punches and from cell cultures. The PVN punches were taken from fresh tissue, omitting freezing as was the case for RNA isolation. I found that proteins, especially the phosphorylated forms, suffered from the freezing procedure. Brains were therefore cut with a razor blade in 2 – 3 mm thin slices and the PVN was punched out with a tissue puncher.

A protein extraction kit (Active Motif, Belgium) was used to extract cytosolic and nuclear proteins out of H32-, Be(2)-M17-cells and PVN-punches. Briefly, cell pellets or punches were homogenized in hypotonic buffer containing protease- (Active Motif) and phosphatase-inhibitors (PhosSTOP; Roche, Switzerland) and incubated on ice for 15 min. After addition of 5 % detergent, the samples were vortexed and the process of dissolving the cell membrane was allowed to proceed for another 5 min. The cups were then centrifuged for 3 min at 14000 x g and the supernatant containing all released cytoplasmic proteins was transferred to a fresh cup. The pellet, still holding the intact cell nuclei, was resuspended in complete lysis buffer enriched with protease- and phosphatase-inhibitors, as well as 1 mM DTT (Active Motif). After incubation on ice for 30 min, the samples were again centrifuged at 14000 x g to collect all undigested material and the supernatant containing the nuclear proteins was transferred into another fresh cup.

To extract the complete proteome (*i.e.* cytosolic and nuclear proteins together) from primary hypothalamic neurons, another method was used to allow labelling of newly synthesized proteins. Cells of 4 wells (approximately 12 x 10^6 cells) were harvested in ice-cold phosphate buffered saline (PBS) and collected in Falcon tubes. After centrifugation at 2000 x g for 5 min, the supernatant was discarded and the pellet was carefully transferred to a fresh cup and resuspended in 100 µl lysis buffer containing 1 % sodium dodecyl sulphate (SDS; AppliChem, Germany). Benzonase Endonuclease (125 U; Sigma-Aldrich) was added to digest all disturbing nucleic acids. Samples were then excessively vortexed for 3 min and boiled for 10 min at 96 °C to achieve complete lysis. After centrifugation at 16000 x g for 5 min, all remaining debris was collected at the bottom of the cup and the supernatant was transferred into a fresh cup.

Following each extraction method, protein concentration was assessed with the colorimetric BCA protein assay kit (ThermoScientific), and compared to a standard curve obtained by dissolving several concentrations of BSA (2 mg/ml – 0.125 mg/ml) in the buffer in which the proteins had been dissolved.

6.2. SDS-PAGE and Western Blot analysis

For determination of basal (*i.e.* unstimulated) protein expression and phosphorylation levels, 30 µg of proteins were separated by their molecular weight on a 12.5 % SDS-gel for 2 h at 100 V. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Germany) for 1 h at 100 V. After blocking the membrane in the appropriate blocking solution (for details, please see Table 2) to cover all non-specific binding sites, the membrane was incubated with the diluted primary antibody under the conditions shown in Table 2, washed extensively in Tris-buffered saline with 0.001 % Tween-20 (TBST) to remove all unbound primary antibody and incubated with secondary antibody conjugated with horseradish peroxidase (HRP; Table 2; Cell Signaling, USA and Santa Cruz Biotechnology, USA). Following a second wash step to remove all unbound secondary antibody, the membranes were incubated for 1 min with ECL Detection mix (Amersham/GE Healthcare), and the protein/antibody complexes were then visualized by capturing luminescence with the ChemiDoc XRS+ Imager. The images were analysed with ImageLab software that was specifically created for the ChemiDoc Imager.

Following visualization of the bands, the blots were stripped to remove bound antibody complexes (Re-Blot Plus Strong Solution 10x; Millipore, Germany) and blocked twice for 10 min with the appropriate blocking solution. Next, the blots were incubated with antibodies either against the total form of the protein for phosphorylation level studies or antibodies

against the protein serving as the loading control. Wash steps, incubation with secondary antibody and detection were carried out as described above.

P2X4R studies	
P2X4R #APR-002 (Alomone Labs)	blocking in 5 % MP, 2 h AB 1:2000 in 5 % MP o/n 4 °C
phospho-MEK1/2 (Ser217/221) #9154S (Cell Signaling)	blocking in 5 % BSA/50 mM NaF, 1 h AB 1:1000 in 5% BSA/50 mM NaF o/n 4 °C
MEK1/2 total #9122S (Cell Signaling)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C
phospho-p44/42 MAPK (Thr202/Tyr204) #9101 (Cell Signaling)	blocking in 5 % BSA/50 mM NaF, 2 h AB 1:1000 in 5 % BSA/50 mM NaF o/n 4 °C
p44/42 MAPK total #9102 (Cell Signaling)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C
phospho-CaMKI #sc28438 (Thr177) (Santa Cruz)	blocking in 1 % MP/50 mM NaF, 1 h AB 1:500 in 1 % MP/50 mM NaF o/n 4 °C
CaMKI total #sc33165 (Santa Cruz)	blocking in 1 % MP, 2 x 10 min AB 1:500 in 1 % MP o/n 4°C
phospho-CaMKIIα (Thr286) #sc12886R (Santa Cruz)	blocking in 2 % MP/50 mM NaF, 1 h AB 1:250 in 2 % MP/50 mM NaF o/n 4 °C
CaMKII total #sc13082 (Santa Cruz)	blocking in 2 % MP, 2 x 10 min AB 1:500 in 2 % MP o/n 4°C
phospho-eEF2 (Thr56) #2331 (Cell Signaling)	blocking in 5 % MP/50 mM NaF, 2 h AB 1:1000 in 5 % MP/50 mM NaF o/n 4 °C
eEF2 total #2332 (Cell Signaling)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C

Protein synthesis studies	
phospho-eEF2 (Thr56) #2331 (Cell Signaling)	blocking in 5 % MP/50 mM NaF, 2 h AB 1:1000 in 5 % MP/50 mM NaF o/n 4 °C
eEF2 total #2332 (Cell Signaling)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C
phospho-mTOR (Ser2481) #09343 (Millipore)	blocking in 5 % BSA/50 mM NaF, 1.5 h AB 1:1000 in 5 % BSA/50 mM NaF o/n 4 °C
mTOR total #2983 (Cell Signaling)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C
phospho-p90RSK (Ser380) #9341 (Cell Signaling)	blocking in 5 % BSA/50 mM NaF, 2 h AB 1:1000 in 5 % BSA/50 mM NaF o/n 4 °C
biotin #D5A7 (Cell Signaling)	blocking in 5 % BSA, 1 h AB 1:1000 in 0.1 % BSA 30 min RT
NPY5R #ab43824 (abcam)	blocking in 5 % MP, 2 h AB 1:500 in 5 % BSA o/n 4 °C

Loading controls and secondary antibodies	
β-tubulin #2146 (Cell Signaling)	blocking in 5 % MP, 2 x 10 min AB 1:1000 in 5 % MP 2 h RT
actin #sc1616 (Santa Cruz)	blocking in 5 % MP, 2 x 10 min AB 1:500 in TBST o/n 4 °C
GAPDH #ab9485 (abcam)	blocking in 5 % BSA, 2 x 10 min AB 1:1000 in 5 % BSA o/n 4 °C
2nd anti-rabbit IgG HRP-linked #7074 (Cell Signaling)	AB 1:1000 in TBST 30 min RT
2nd donkey anti-goat IgG HRP-linked #sc2020 (Santa Cruz)	AB 1:5000 in 5 % MP 30 min RT

Table 2: List of antibodies and their respective blocking and incubation conditions

6.3. Labelling, tagging and affinity purification of newly synthesized proteins

To determine if OT activates *de novo* protein synthesis in hypothalamic cells, primary neurons were starved from methionine in methionine-free medium (Life Technologies) for 1 h. Cells were then treated with 250 nM OT and simultaneously with 50 µM of the labelling reagent L-azidohomoalanine (AHA; Life Technologies), a synthetic amino acid that serves as a surrogate for methionine which is actively incorporated in every newly built protein (Dieterich et al., 2007). After 3 h, the reaction was stopped by placing the wells on ice and

cells were collected in ice-cold PBS. Cells were pelleted by centrifugation at 2000 x g for 5 min and lysed in 100 µl lysis buffer containing 1 % SDS and 125 U Benzonase Endonuclease. Proteins were extracted according to the protocol for complete proteome extraction described above (6.1). 200 µg of the labelled proteins were then used for the copper-catalysed "click" reaction between the azide-containing AHA and the corresponding alkyne-containing detection reagent (Dieterich et al., 2007). To this end, 100 µl Click-iT reaction buffer including 40 µM of the detection reagent biotin alkyne (Life Technologies) were mixed with the proteins and H_2O was added to a final volume of 160 µl. This reaction was then supplied with 10 µl 40 mM $CuSO_4$, 10 µl Click-iT reaction buffer additive 1 and 20 µl buffer additive 2 solutions (Life Technologies). The mixture was rotated end-over-end for 1 h at room temperature (RT). Next, proteins were extracted with a chloroform-methanol precipitation to free the protein material from salt and detergent. To this end, 600 µl methanol were added to 200 µl of the reaction mix and vortexed briefly. After the addition of 150 µl chloroform and 400 µl H_2O, the samples were centrifuged for 5 min at 16000 x g, and the upper aqueous phase was removed, leaving the interface layer containing the proteins intact. The layer was washed twice with 450 µl methanol, and the resulting pellet was resuspended in PBS (pH 7.4) to a concentration of 1 µg/µl and stored on -20 °C until further use.

To separate and enrich the biotinylated, that is the newly synthesized proteins, from the non-biotinylated, 200 µg proteins were incubated with 10 µl streptavidin-coupled magnetic beads (Dynabeads M-280 Streptavidin; Invitrogen/Life Technologies) for 5 h with gentle rotation of the tubes during which the biotinylated proteins will bind to the beads. The cups were then placed on a magnet (DynaMag; Life Technologies) for 2 – 3 min to collect all

magnetic protein-coated dynabeads at the wall of the cup and the supernatant was discarded. The coated beads were washed 5 times with PBS containing 0.05 % SDS and were finally resuspended in 30 µl of the saline.

6.4. Dot Blot

To compare the amount of newly synthesized proteins in the OT-treated cells with that in control cells, 10 µl of both samples prepared like described above (6.3) were spotted onto a nitrocellulose membrane. Non-specific sites were blocked by soaking the membrane in 5 % BSA in TBST for 1 h. Following this, the membrane was incubated with anti-biotin antibody (Table 2; Cell Signaling) in 0.1 % BSA in TBST for 30 min. Following a wash step, the membrane was then incubated with secondary antibody conjugated with HRP for 30 min. The blot was washed again to remove all unbound antibody and detection was carried out like described above for Western Blots.

6.5. Immunohistochemistry

Adult Wistar rats (250 - 300 g) were used to determine whether P2X4R co-localizes with AVP and/or OT. The rats were killed by asphyxiation with CO_2 and perfused through the aorta with 4 % paraformaldehyde (PFA; Sigma-Aldrich) in PBS (pH 7.2). The brains were removed and post-fixed in 4 % PFA in PBS for 3 h before transferring the brains to 30 % sucrose in PBS. Brains were kept in the solution until they sank to the bottom. Then, the brains were rapidly frozen, cut in 40 µm slices and kept free-floating in PBS at 4 °C.

Immunohistochemistry for P2X4R was performed using a rabbit polyclonal antibody against the P2X4 receptor (#AB5226, Merck Millipore) and mouse monoclonal antibodies against AVP and OT (p38 and p41 respectively, both were generous gifts of Dr. Gainer, NIH, USA). To block non-specific binding sites, the slices were pre-incubated in 10 % normal goat serum

(NGS; Vector Laboratories, USA) in PBS/0.3 % Triton X 100 (PBST) for 2 h at RT. Next, the slices were incubated with an antibody mix of anti-P2X4R (1:800) and anti-AVP (1:500) or anti-OT (1:500) in 2 % NGS in PBST overnight at 4 °C. The next day, the slices were extensively washed with PBST and incubated with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:400 in 1 % NGS in PBST; Invitrogen/Life Technologies) for 2 h at RT and protected from sunlight. After a second wash step, horse anti-mouse conjugated with DyLight 594 (1:200 in PBST; Vector Laboratories) was added and again incubated for 2 h. A 1:300 solution of the nuclear dye 4,6-diamidino-2-phenylindol (DAPI; Invitrogen/Life Technologies) was used to stain the nuclei.

The P2X4R antibody was raised against a purified peptide of rat P2X4R protein corresponding to amino acids 370-388. The specificity of all primary antibodies was assessed by pre-incubation with their respective antigens (1 µg), and by omission of the first antibody. In all cases, no immunoreactivity was observed. Immunoreactivity persisted when the anti-OT and anti-AVP antibodies were pre-incubated with AVP and OT, respectively. The characteristics of the OT- and AVP-antibodies are further described in the original publications (Ben-Barak et al., 1985; Whitnall et al., 1985).

7. Statistics

Behavioural data were analysed using either a Student's *t*-test, ONE-WAY ANOVA followed by LSD post-hoc-test or TWO-WAY ANOVA followed by LSD post-hoc-test. qPCR and Western Blot data were analysed using either a Student's *t*-test or ONE-WAY ANOVA followed by Bonferroni's post-hoc-test. The software package SPSS (Version 19.0; IBM, Germany) was used, data represent mean + SEM and significance was accepted at $p < 0.05$.

RESULTS

Results

Part I: P2X4R as a regulator of anxiety in the PVN of Wistar rats

Experiment 1: Validation of a microarray study showing differences in gene expression profile in the PVN of female virgin HAB and LAB rats

To identify novel proteins in the PVN that might play a role in anxiolysis, I first analysed the gene expression differences that were found in a microarray comparing virgin female rats selectively bred for low and high levels of anxiety-like behaviour, the LAB and HAB rats, respectively. Over 2800 genes were identified to be differentially expressed in the two breeding lines ($p < 0.05$) (Slattery, personal communication). Among those, the microarray revealed the up-regulation (≥ 1.5-fold) of the levels of 175 mRNAs and the down-regulation (≥ -1.5-fold) of 262 mRNAs in virgin LAB females compared with virgin HAB females. Of particular interest was the 1.7-fold up-regulation of *p2rx4* mRNA in LABs compared with HABs, as it was found earlier that the influx of extracellular Ca^{2+} via transient receptor potential cation channel subfamily V member 2 (TRPV2) into neurons has an anxiolytic effect (Van den Burg et al., unpublished). We thus hypothesised that Ca^{2+} influx through P2X4R might bring about the same effect.

qPCR analysis confirmed the up-regulation of *p2rx4* mRNA in the PVN with a 2.7-fold change in LAB females compared with HAB females ($p = 0.002$, $n = 6 - 7$), and an even higher regulation in males (3.6-fold, $p < 0.001$, $n = 8$; Figure 4).

Western Blot analysis of HAB and LAB PVN tissue confirmed the P2X4R up-regulation in female (1.8-fold change, $p = 0.001$, $n = 5$) and male (1.9-fold change, $p < 0.001$, $n = 7$) LAB rats compared with HAB rats at the protein level (Figure 5). These results establish a robust

difference between the two breeding lines in the expression of an important regulator of purinergic signalling within the PVN. These results not only confirm the microarray findings, but extend them by showing that the up-regulation in LABs is not sex-dependent and, importantly, that it is true at the protein level.

In addition to *p2rx4*, mRNA levels of other selected targets from the microarray were determined, again both in male and female rats. The genes were chosen based on their involvement in the regulation of cellular Ca^{2+}-levels (*Trpv2, Trpm7, Casq2*), anxiety (*Cckbr*), cell growth and differentiation (*Nrg1*), and neuropeptidergic character (*Hcrt*, *VIP*) (Table 3). The validation via qPCR confirmed half of the microarray results, but not the regulation of *Casq2*, *Hcrt*, and *VIP*, which were either found to be not regulated between HAB and LAB rats (*Casq2*, *VIP*) or up-regulated instead of down-regulated (*Hcrt*). These findings can serve as a basis for further research regarding gene expression changes between HAB and LAB and the potential physiological and behavioural consequences.

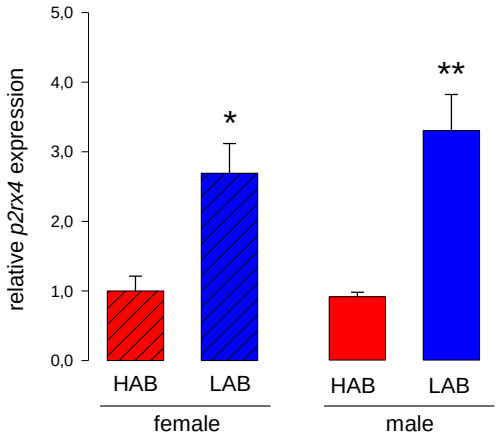

Figure 4: Relative expression of *p2rx4* mRNA in female (left; n = 6, 7) and male (right; n = 8) HAB and LAB rats. *p2rx4* levels were normalized to *gapdh* and are shown as relative expression to HAB *p2rx4*. Plot represents means + SEM; Student's *t*-test, * $p < 0.05$, ** $p < 0.001$.

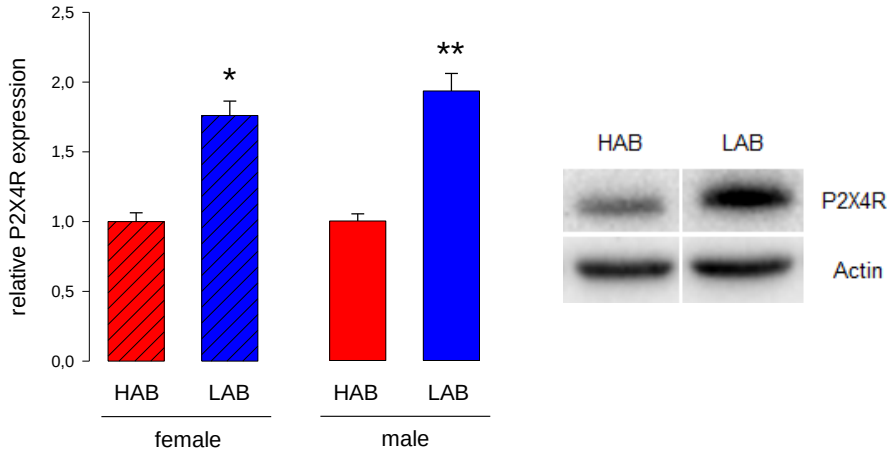

Figure 5: Relative protein expression of P2X4R in female (left; n = 5) and male (right; n = 7) HAB and LAB rats. P2X4R levels were normalized to actin and are shown relative to HAB P2X4R expression. A representative blot of male PVN tissue is shown on the right side. Plot represents means + SEM; Student's *t*-test, * $p < 0.05$, ** $p < 0.001$.

Microarray validation via qPCR of selected female LAB vs HAB gene differences			
Main characteristic	Gene	Microarray	qPCR
Ca^{2+} regulation	Casq2	↑	↔
	P2rx4	↑	↑
	Trpv2	↓	↓
	Trpm7	↑	↑
Anxiety regulation	Cckbr	↓	↓
Cell growth/differentiation	Nrg1	↑	↑
Neuropeptides	Hcrt	↓	↑
	VIP	↓	↔

Table 3: qPCR validation of the HAB LAB microarray results. Half of the gene expression changes were reproducible, providing additional data for further research using HAB and LAB rats as a basis.

Experiment 2: Immunostaining of the ATP-receptor P2X4R in the hypothalamus of rats

Next, I sought to determine whether P2X4R was expressed in OT- and/or AVP-positive neurons in the PVN, as both neuropeptides have been implicated in anxiety within the PVN (Blume et al., 2008; Wigger et al., 2004).

Fluorescent immunostaining of rat brain slices with an anti-P2X4R antibody showed a strong and widely distributed expression of P2X4R throughout the PVN (Figure 6A). Immunoreactivity was found in the cytosol, and was punctate (Figure 6B), suggesting a location at the membrane and in lysosomes, consistent with data from previous studies showing this type of distribution for macrophages, microglia, and endothelial cells (Qureshi et al., 2007). Co-staining with an anti-OT antibody revealed that a subpopulation of 56 % of OT-positive cells expressed P2X4R (Figure 7A). Additionally to the positively correlated neurons, cells expressing P2X4R can be OT-negative and vice versa (Figure 7B). Less pronounced P2X4R immunoreactivity was found in AVP-positive cells (32 %; Figure 7C). Thus, P2X4R signalling is at least in part associated with the anxiolytic OT-system as well as the anxiogenic AVP-system, favouring a role of P2X4R in the control of anxiety-like behaviour.

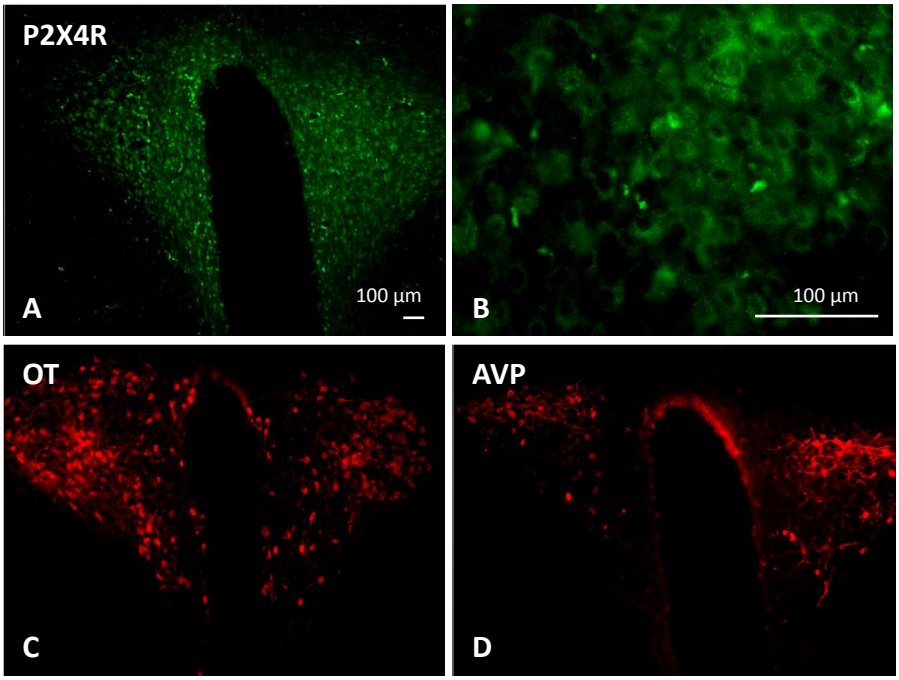

Figure 6: Staining of P2X4R (green, A and B), OT (red, C) and AVP (red, D) in consecutive 40 µm slices of the PVN of Wistar rats. B illustrates the distribution of P2X4R in the cells.

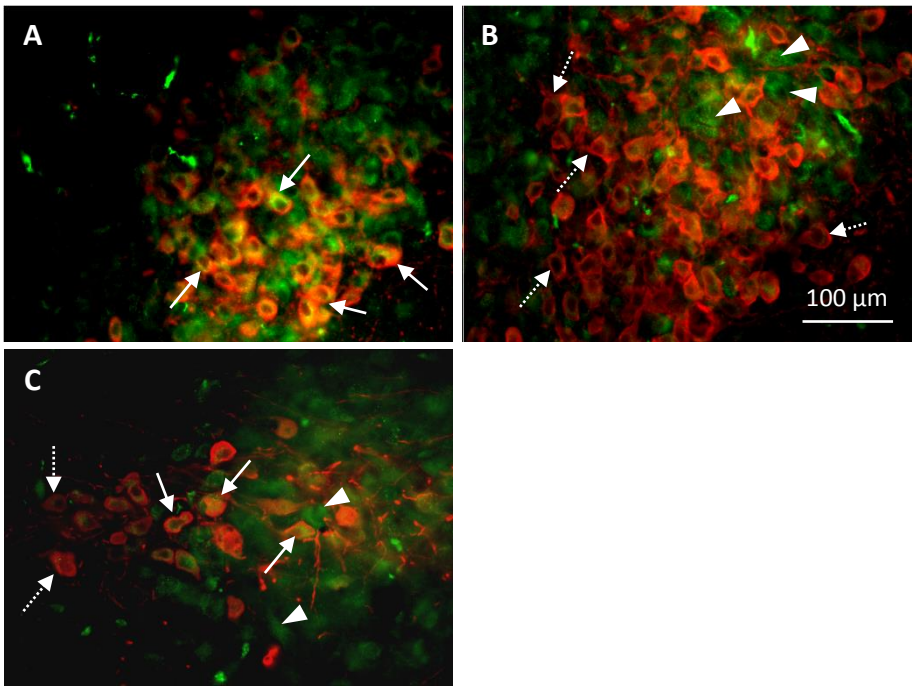

Figure 7: Co-staining of P2X4R (green) and OT (A + B, red) or AVP (C, red) in the PVN. A: White arrows point at cells co-expressing P2X4R and OT. B: Note that some OT cells do not express P2X4R (dashed arrows) and vice versa (arrow heads). C: White arrows point at cells co-expressing P2X4R and AVP, some AVP cells do not express P2X4R (dashed arrows) and vice versa (arrow heads).

Experiment 3: Effects of the regulation of P2X4R within the PVN on anxiety-like behaviour in Wistar rats

To determine whether P2X4R activation in the PVN of rats led to anxiolysis, male rats non-selected for anxiety-related behaviour (hereafter: Wistar rats) were infused with 4 nmol and 40 nmol/0.5 µl CTP bilaterally into the PVN and were tested on the EPM 10 min later. CTP was chosen as it has a higher specificity for P2X4R than for other P2XRs and is currently the most selective P2X4R agonist available for *in vivo* use (Soto et al., 1996). The higher dose (40 nmol) of CTP significantly increased the % time spent on the open arms of the EPM ($F_{2,43}$ = 3.358, p = 0.044, n = 13 – 19) compared to vehicle-treated rats (Veh) (Figure 8A). CTP did not affect locomotion, as the number of closed arm entries was not altered by either dose (Figure 8B). A pilot study with the positive allosteric modulator of P2X4R, IVM (10 mg/kg

body weight *ip*, (Khakh et al., 1999)), showed a slight trend (p = 0.083) to increase the % time spent on the open arms (Figure 8C).

Based on the EPM results, the higher dose of CTP (40 nmol/0.5 µl) was chosen for the rest of the studies. This concentration of CTP increased the % time spent on the open arms of the EPM in female rats (p = 0.03, n = 6), as it did in males, again without affecting locomotion (Figure 9A). The anxiolytic properties of 40 nmol of CTP were corroborated in another test for anxiety-like behaviour, the LDB. Rats treated with CTP spent significantly more time in the light compartment of the LDB compared to the Veh-treated group (p = 0.01, n = 10 - 13), without altering the number of line-crossings in the dark compartment, indicating that CTP did not induce changes in locomotion (Figure 9B).

As CTP is not a specific agonist for the P2X4R, experiments to confirm the specific role of P2X4R in the control of anxiety-like behaviour were required. The specific P2X4R antagonist 5-BDBD was infused bilaterally into the PVN (25 nmol/0.5 µl) (Balazs et al., 2013; Fisher R, 2005) 10 min prior to CTP, and behaviour was measured in the LDB 10 min later. Pre-treatment with 5-BDBD abolished the anxiolytic effect of CTP ($F_{3,39}$ = 2,884, p = 0.048, n = 8 – 13), while 5-BDBD did not alter locomotion (Figure 10), demonstrating that anxiolysis induced by purinergic signalling in the PVN depends on P2X4R.

In a separate experiment, rats were treated intra-PVN with the endogenous P2XR agonist ATP (2 nmol/0.5 µl). This concentration corresponded to the applied CTP concentration, since the EC_{50} of CTP is 20 times higher than that of ATP for the P2X4R (Coddou et al., 2011). The aim of this experiment was to determine if other ATP-responsive receptors in the PVN can add to the anxiolytic effect of a P2X4R-activation and to further illustrate the specific involvement of P2X4R in the regulation of anxiety. ATP had no effect on the % time spent on

the open arms of the EPM, thus the activation of other P2X and P2Y receptors in addition to P2X4R did not recapitulate the anxiolytic effect observed in the CTP-treated animals. Pre-treatment with 5-BDBD was without effect (Figure 11) and I could recapitulate the fact that 5-BDBD itself does not affect anxiety-related behaviour. However, it was not determined whether a higher dose of ATP would be sufficient to mimic the anxiolytic effect of CTP.

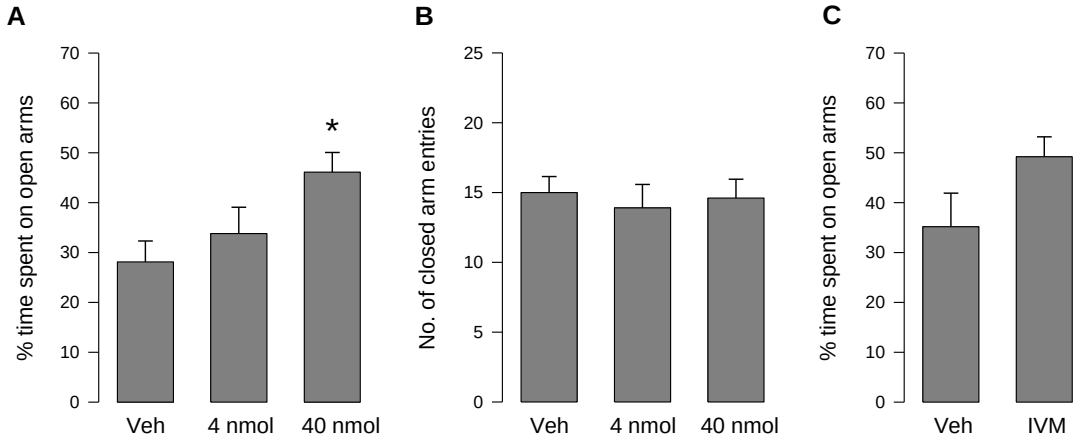

Figure 8: Dose-dependent anxiolytic effect of CTP in male Wistar rats infused with either 4 or 40 nmol/0.5 µl CTP ($p = 0.016$ vs Veh following LSD post-hoc test; n = 14, 19, 13). EPM test occurred 10 min after the infusion, levels of anxiety-related behaviour are indicated by the % time spent on the open arms (A) and locomotion by the number of closed arm entries (B). C: Effect of an *ip* injection of IVM 10 min prior to the EPM. IVM tends to increase the % time spent on the open arms ($p = 0.083$; n = 6, 8). Plots represent means + SEM; ONE-WAY ANOVA followed by LSD post-hoc-test, * $p < 0.05$.

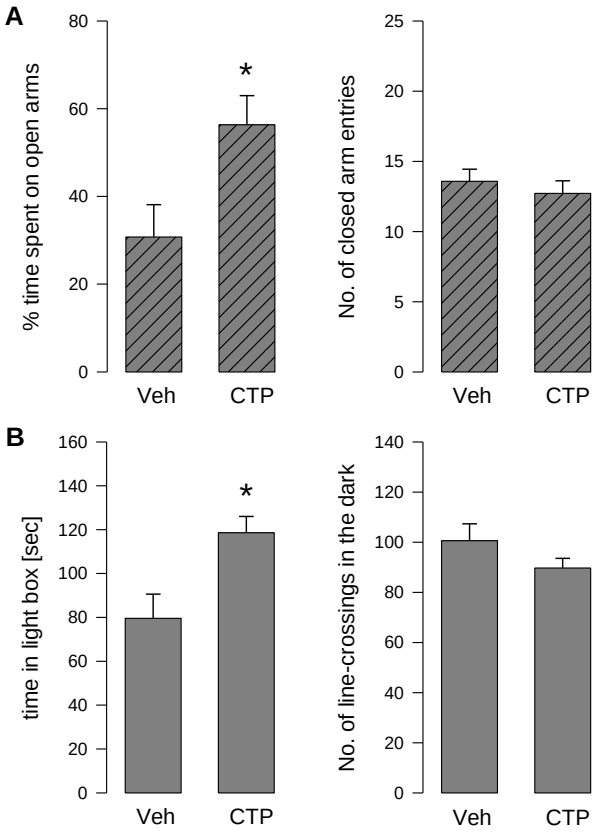

Figure 9: Anxiolytic effect of 40 nmol CTP is independent of sex: female Wistar rats spent more time on the open arms (A; n = 6). Anxiolytic effect of CTP is reproducible in the LDB (B; n = 10, 13). Both tests showed that locomotion is not affected. Plots represent means + SEM; Student's *t*-test, * $p < 0.05$.

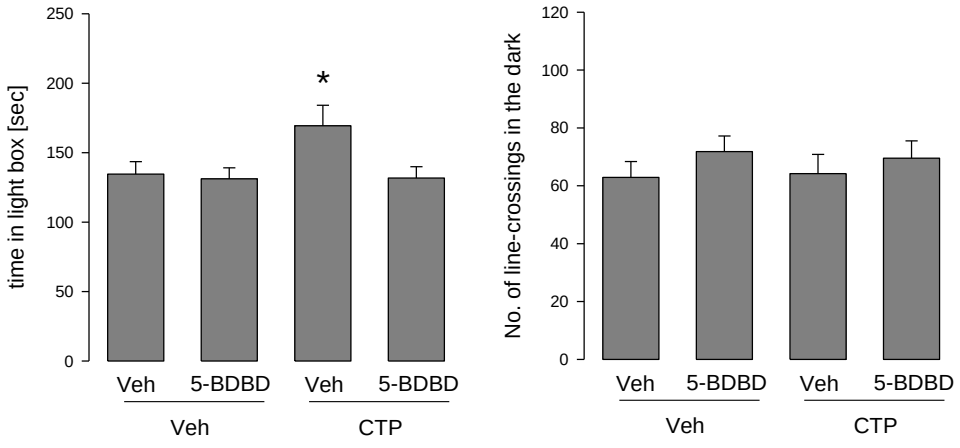

Figure 10: Pre-treatment with 25 nmol/0.5 µl 5-BDBD abolished the anxiolytic effect of 40 nmol/0.5 µl CTP in male rats (n = 13, 8, 13, 9). Plots represent means + SEM; TWO-WAY ANOVA followed by LSD post-hoc-test, * $p < 0.05$ vs Veh/Veh and vs 5-BDBD/CTP.

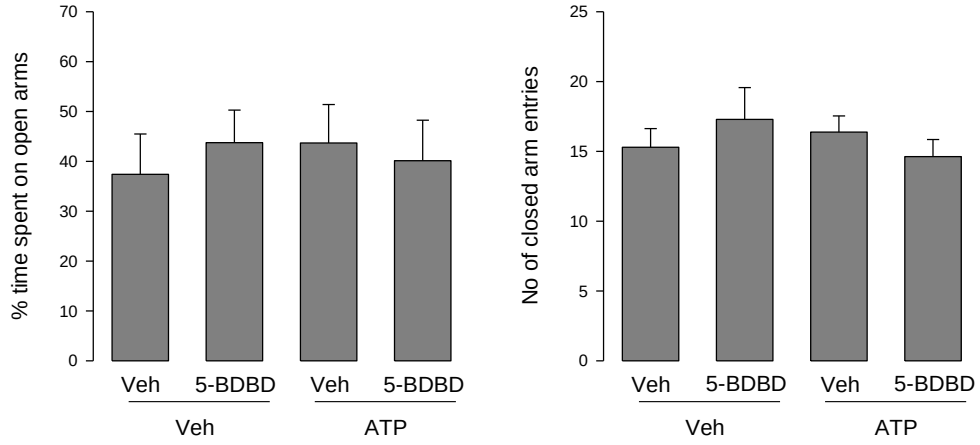

Figure 11: Local infusion of 2 nmol/0.5 µl ATP and pre-treatment with 5-BDBD was without effect on anxiety-like behaviour (n = 7, 7, 8, 8). Plots represent means + SEM.

Experiment 4: Effects of the regulation of P2X4R within the PVN on anxiety-like behaviour in HAB and LAB rats

As CTP exerted anxiolytic effects that were mediated by the P2X4R in Wistar rats, I next determined whether the two extreme phenotypes of HAB and LAB rats could be, at least partially, reversed by activating or inhibiting, respectively, P2X4R in the PVN. Indeed, CTP-treated male HAB rats (40 nmol/0.5 µl) spent significantly more time in the light compartment of the LDB ($p = 0.008$, $n = 8 - 10$), without affecting locomotion (Figure 12). On the other hand, LAB rats with extremely low inborn anxiety spent less time in the light compartment following a 25 nmol/0.5 µl 5-BDBD infusion compared with the Veh-group ($p = 0.043$, $n = 11$; Figure 13). Together, these results showed that the differential expression of P2X4R in HAB and LAB rats, as observed in the microarray and qPCR data, is reflected in their more and less anxious phenotype, respectively.

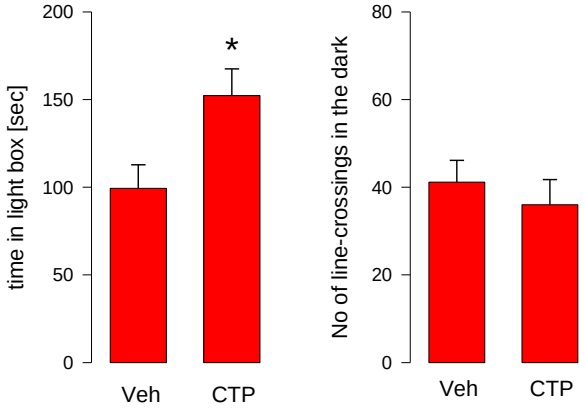

Figure 12: HAB rats spent more time in the light compartment of the LDB following a CTP (40 nmol) infusion bilaterally into the PVN, without influencing locomotion (n = 8, 10). Plots represent means + SEM; Student's *t*-test, * $p < 0.05$.

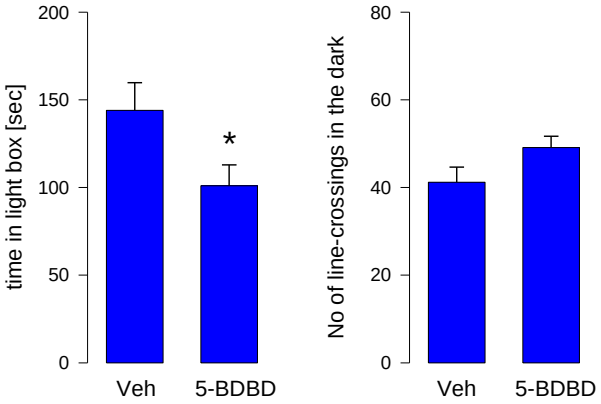

Figure 13: LAB rats spent less time in the light compartment of the LDB following a 5-BDBD (25 nmol) infusion bilaterally into the PVN, without influencing locomotion (n = 11). Plots represent means + SEM; Student's *t*-test, * $p < 0.05$.

Experiment 5: Elucidation of possible signalling cascades in hypothalamic cells following P2X4R activation

To identify the intracellular mechanisms downstream of P2X4R activation that are involved in anxiolysis, it was necessary to assess the recruitment of several enzymatic cascades in PVN tissue from male and female Wistar rats that had received 40 nmol/0.5 µl CTP in Ringer's

solution in both the left and right PVN. The cascades were chosen based on their Ca^{2+}-dependency as well as their known involvement in the mediation of OT-induced anxiolysis.

Levels of phosphorylated, and thus active, CaMKI and CaMKIIα in male rats were not altered 10 min after CTP treatment (n = 6 – 7; Figure 14). Comparison of total CaMKI and CaMKIIα levels with those of the housekeeping proteins β-tubulin and GAPDH revealed no changes in both CaMKs tested. The results suggest that neither CaMKI nor CaMKIIα were involved in the signalling of P2X4R, and hence, are not necessary for the anxiolytic effects of P2X4R activation.

However, the levels of phosphorylated eEF2 decreased significantly in the PVN of both male ($p = 0.011$, n = 6 - 7) and female ($p = 0.042$, n = 6 - 9) rats after the CTP infusion (Figure 15). A decrease of the phosphorylated form of the protein synthesis factor indicates an increased rate of protein synthesis and might be correlated with the behavioural effect of CTP. A similar phenomenon was observed after treatment with another mediator of anxiolysis in the PVN, the neuropeptide OT (see results for part II for details).

The MAP kinases MEK1/2, which are known to be crucial for the acute anxiolytic effect of OT in the PVN (Blume et al., 2008; Jurek et al., 2012) and ERK1/2 showed different patterns of regulation in males and females. While pMEK1/2 ($p = 0.024$) and pERK1/2 ($p = 0.019$ pERK1 vs GAPDH, $p = 0.009$ pERK2 vs GAPDH) were significantly down-regulated in male rats (Figure 16), CTP up-regulated pMEK1/2 in females ($p = 0.002$, Figure 17). Phosphorylated ERK1/2 in females was not altered (Figure 17). Considering, that CTP acts anxiolytically in both genders, it is highly likely that these MAP kinases are not involved in the anxiolytic effect of the P2X4R.

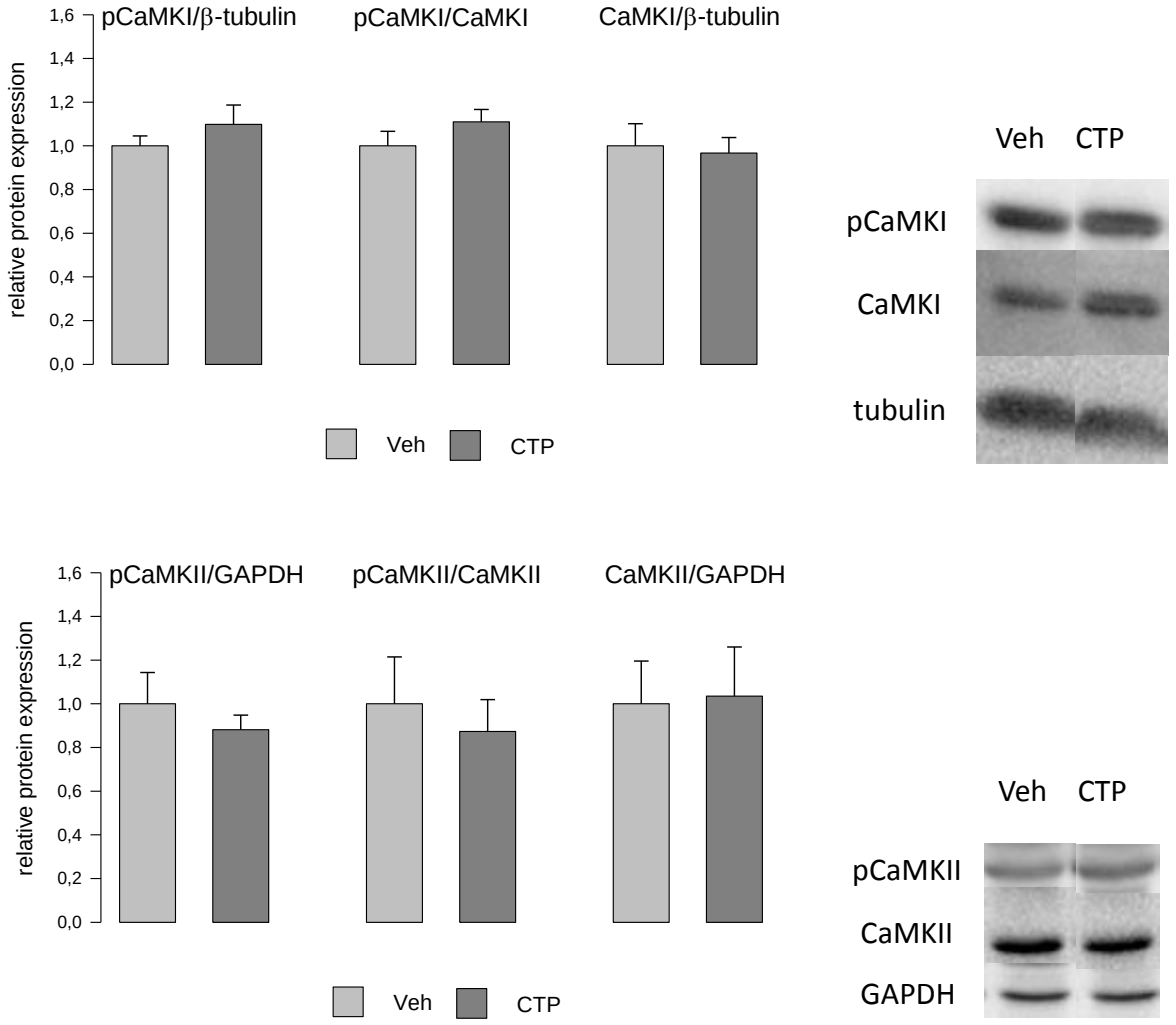

Figure 14: Relative protein expression of the phosphorylated forms of CaMKI (Thr177) and CaMKIIα (Thr286) in the PVN of male rats 10 min after CTP treatment compared to the respective total CaMK and the loading control (β-tubulin and GAPDH, respectively; n = 6, 7)). Plots represent means + SEM. Representative blots are shown on the right side.

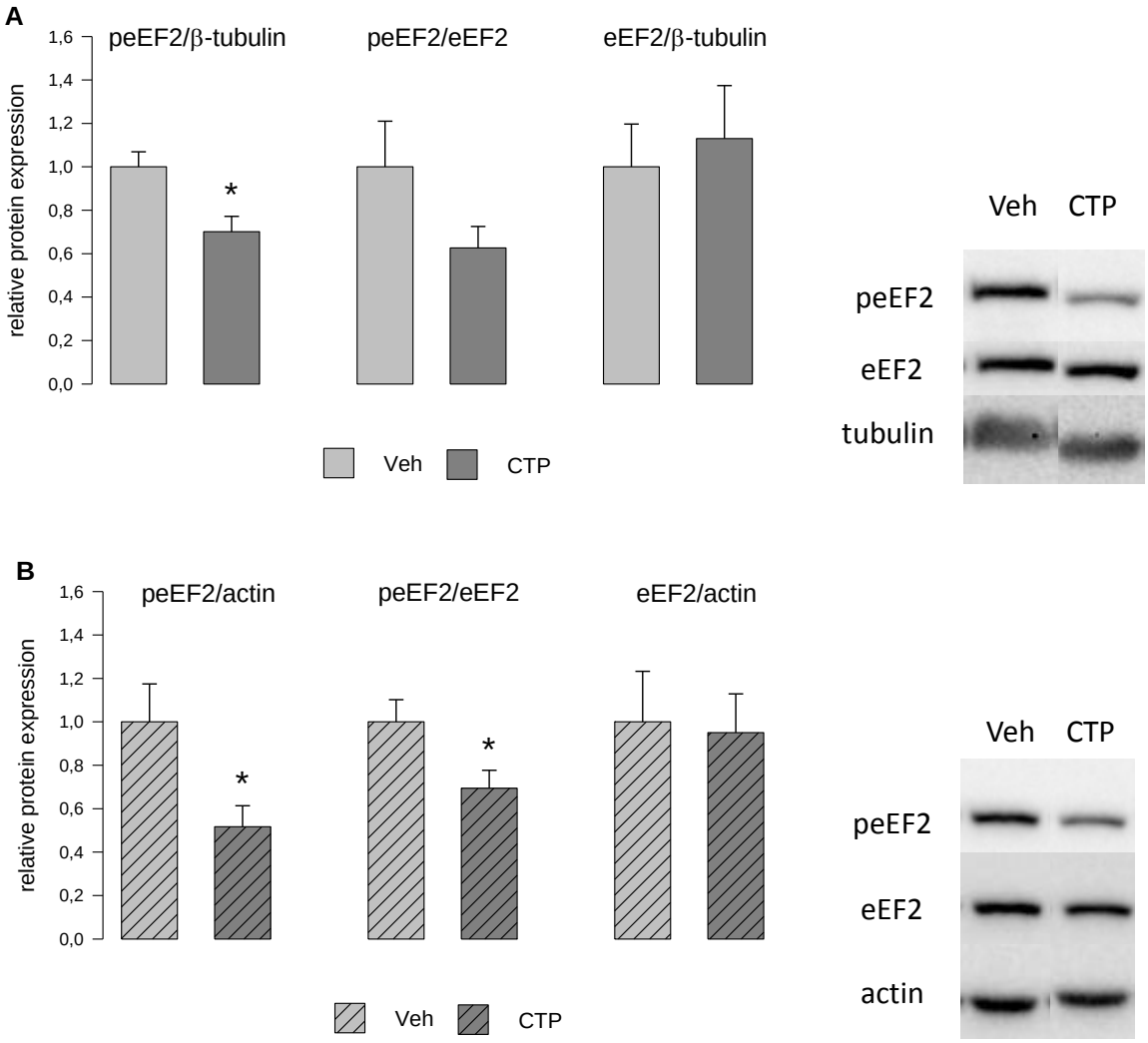

Figure 15: Relative protein expression of phosphorylated eEF2 (Thr56) in the PVN of male (A; n = 6, 7) and female (B; n = 6, 9) rats 10 min after CTP treatment compared to total eEF2 and the loading control (β-tubulin and actin, respectively). Plots represent means + SEM; Student's *t*-test, * $p < 0.05$. Representative blots are shown on the right side.

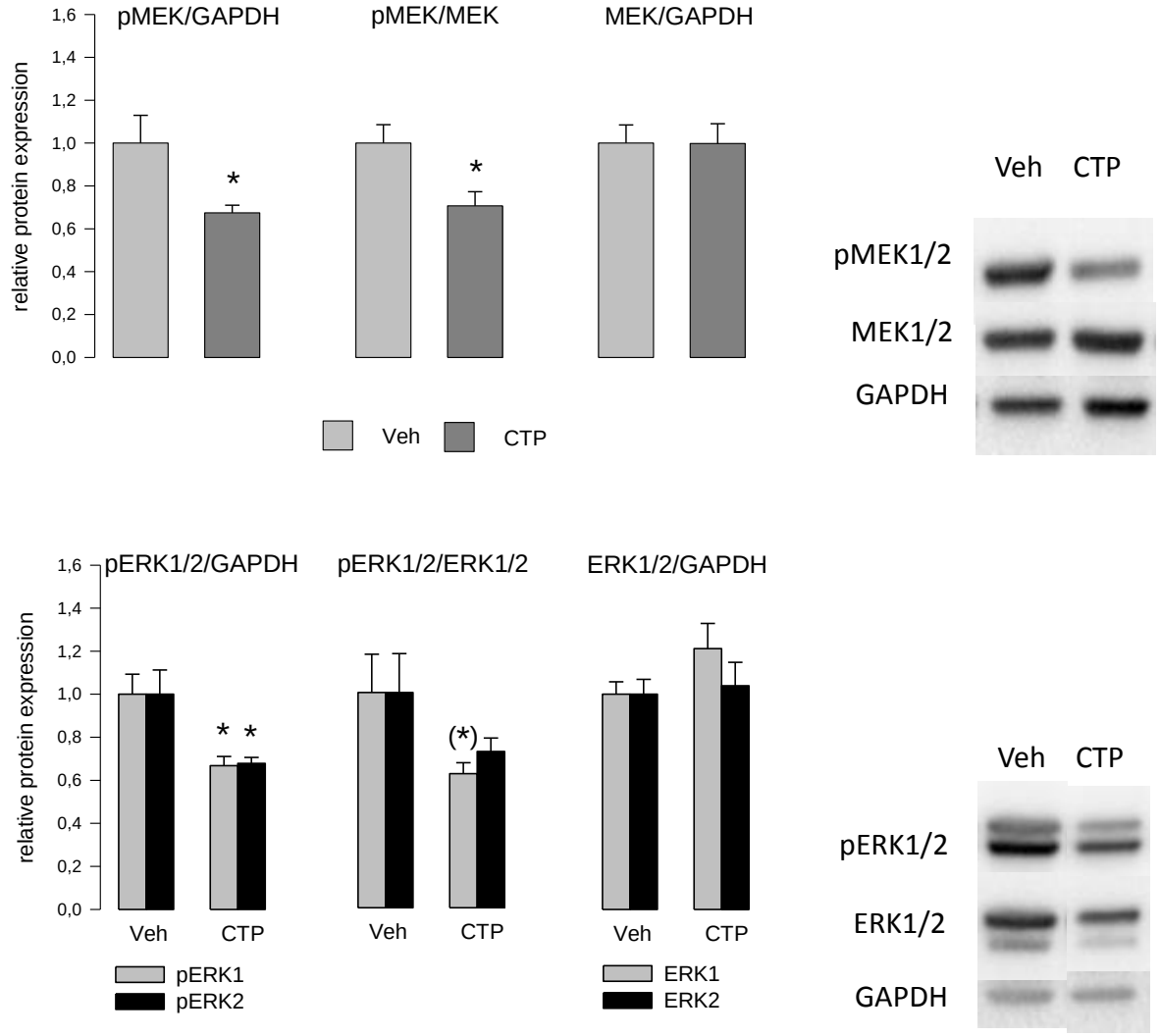

Figure 16: Relative protein expression of phosphorylated MEK1/2 (Ser217/221) and ERK1/2 (Thr202/Tyr204) in the PVN of male rats 10 min after CTP treatment compared with total MEK1/2 and ERK1/2, and the loading control GAPDH (n = 6, 7). Plots represent means + SEM; Student's *t*-test, * $p < 0.05$, (*) $p = 0.052$. Representative blots are shown on the right side.

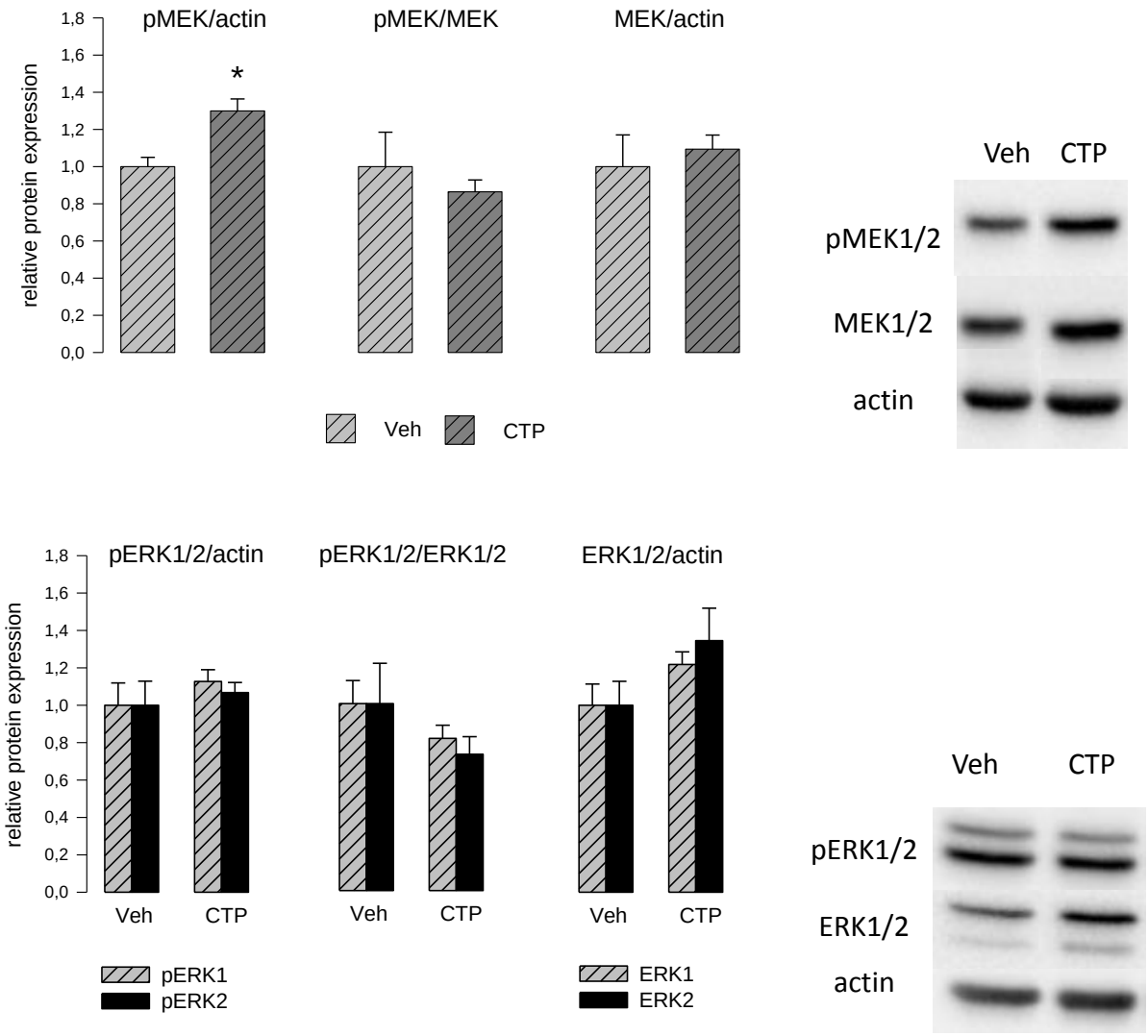

Figure 17: Relative protein expression of phosphorylated MEK1/2 (Ser217/221) and ERK1/2 (Thr202/Tyr204) in the PVN of female rats 10 min after CTP treatment compared with total MEK1/2 and ERK1/2, and the loading control actin (n = 6, 9). Plots represent means + SEM; Student's *t*-test, * $p < 0.05$. Representative blots are shown on the right side.

Part II: Oxytocin activates protein synthesis in the rat hypothalamus

Experiment 1: Effect of OT on key modulators of protein synthesis

The anxiolytic effect of intra-PVN OT occurs already 10 min after the activation of the OTR (Blume et al., 2008), and can last up to 4 h following endogenous release via mating (Waldherr and Neumann, 2007), raising the question about the molecular mechanism behind the long-lasting anxiolytic effect. One possibility is that OT stimulates the cell to produce new proteins that can prolong the anxiolytic effect. To test this hypothesis, the activation of key modulators of protein synthesis in the cell (mTOR (Wang and Proud, 2006), p90RSK (Volarevic and Thomas, 2001) and eEF2 (Kaul et al., 2011)) were investigated following OT treatment.

H32 cells treated with 250 nM OT (Grund, unpublished) showed no alterations neither in mTOR- nor p90RSK-phosphorylation 10, 30 and 60 min after OT treatment compared with the vehicle-treated cells (Figure 18).

In contrast, levels of phosphorylated eEF2 were significantly down-regulated 10 min after the OT pulse ($F_{3,15} = 4.352$, $p = 0.021$, $n = 2 - 9$) compared with total eEF2 levels and returned to normal after 30 – 60 min (Figure 19). The *in vivo* studies showed a similar but slightly shifted pattern of regulation with a significant decrease of peEF2 10 min after *icv* infusion of OT (1 nmol/5 µl) ($F_{2,28} = 3.919$, $p = 0.32$, $n = 4 - 15$) (Figure 19).

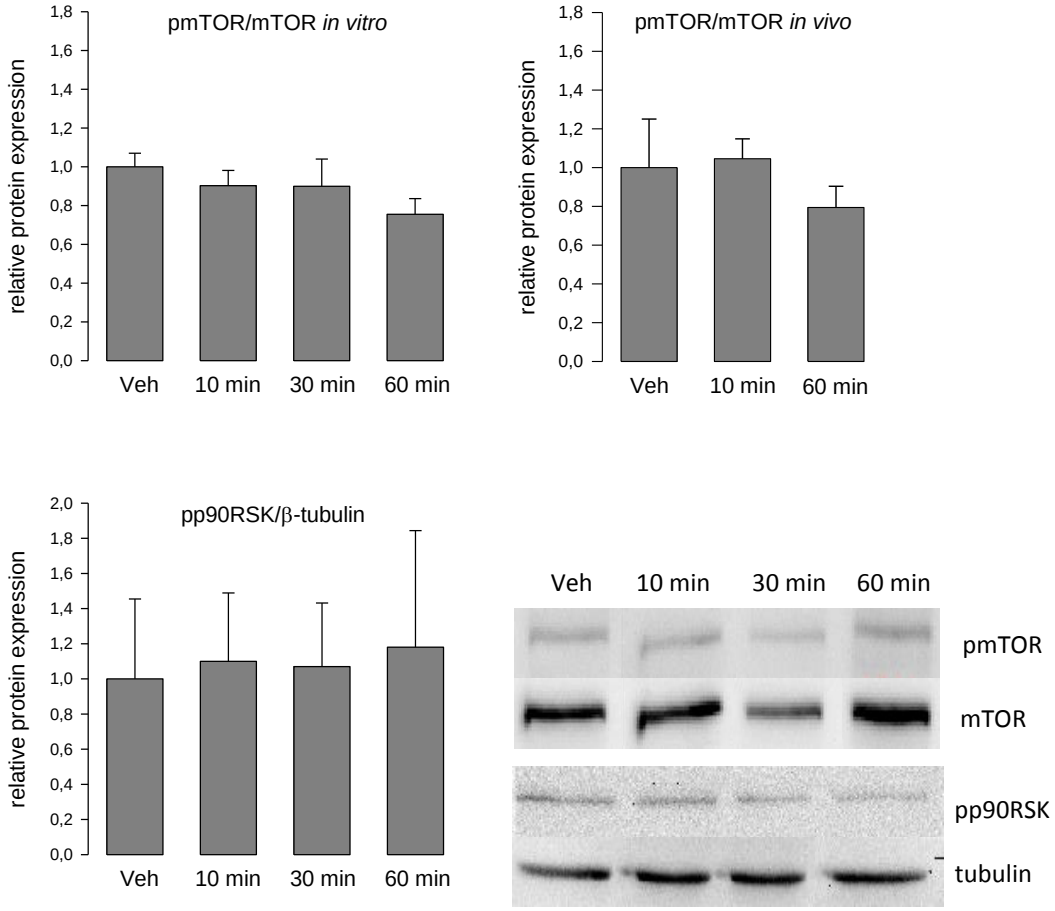

Figure 18: Relative protein expression of pmTOR *in vitro* (n = 8, 4, 3, 4) and *in vivo* (n = 11, 10, 5) and pp90RSK (n = 5, 4, 4, 3). OT had no effect on the phosphorylation level of those proteins, independent of the time point. Plots represent means + SEM. Representative *in vitro* blots are shown at the bottom right.

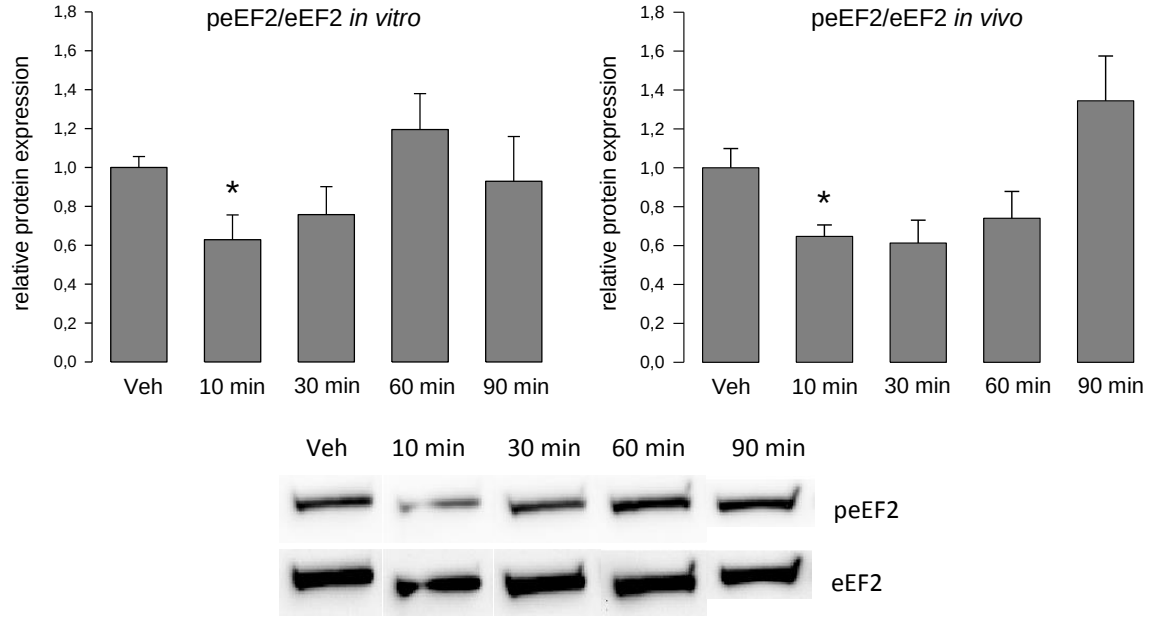

Figure 19: Relative protein expression of phosphorylated eEF2 *in vitro* (left; n = 9, 4, 4, 3, 3) and *in vivo* (right; n =15, 10, 4, 6, 5). OT induced a significant decrease of peEF2 after 10 min in cells and PVN compared to total eEF2. Plots represent means + SEM; ONE-WAY ANOVA followed by Bonferroni's post-hoc-test, * $p < 0.05$. A representative *in vitro* blot is shown below.

Experiment 2: Elucidation of the signalling pathway for the OT-mediated eEF2-activation in the rat's hypothalamus

Next, I determined the intracellular pathway involved in the rapid (*i.e.* within 10 min) dephosphorylation, and therefore activation, of eEF2. Initial experiments with H32 cells failed, and this turned out to be caused by a loss of OTR expression in these cells (Figure 20). I continued my experiments with Be(2)-M17 cells that express the OTR, and showed a similar dephosphorylation of eEF2 when stimulated with OT (Figure 21).

As MEK1/2 was shown to be critical for the anxiolytic effect of OT (Blume et al., 2008), I first tested whether an inhibition of these kinases with the inhibitor U0126 (10 µM) prevented the dephosphorylation of eEF2 following a stimulus of OT. Application of U0126 in the cell culture medium reduced the levels of phosphorylated eEF2, to a similar extent as OT (n = 2 –

3; Figure 22). There were no clear effects (additive or reversal) when U0126 and OT were both present in the cell culture medium, making MEK1/2 a less likely candidate for the control of OT-stimulated eEF2 dephosphorylation.

In contrast, pre-treatment of the cells with the PKC-inhibitor Gö6983 (1 µM) 20 min prior to the OT stimulation abolished the dephosphorylating effect of OT on eEF2 significantly ($F_{3,9}$ = 5.697, p = 0.018, n = 3 - 4). The inhibitor alone had no effect on the phosphorylation status of eEF2 (Figure 23). This suggested that OT activates the PKC pathway to dephosphorylate and thus activate eEF2 and hence protein synthesis.

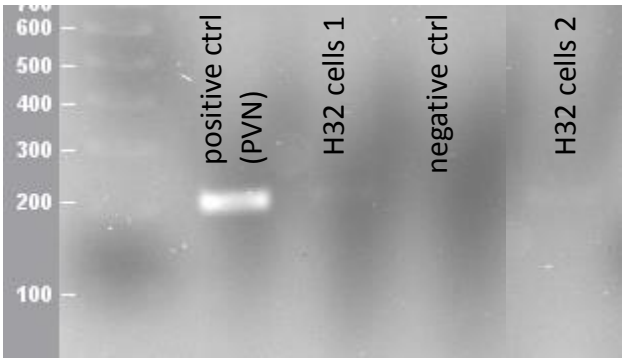

Figure 20: Agarose gel showing the very low expression of OTR in two samples of H32 cell line and a sample of PVN tissue as positive control. H32 cells seemed to lose significance for OT-studies.

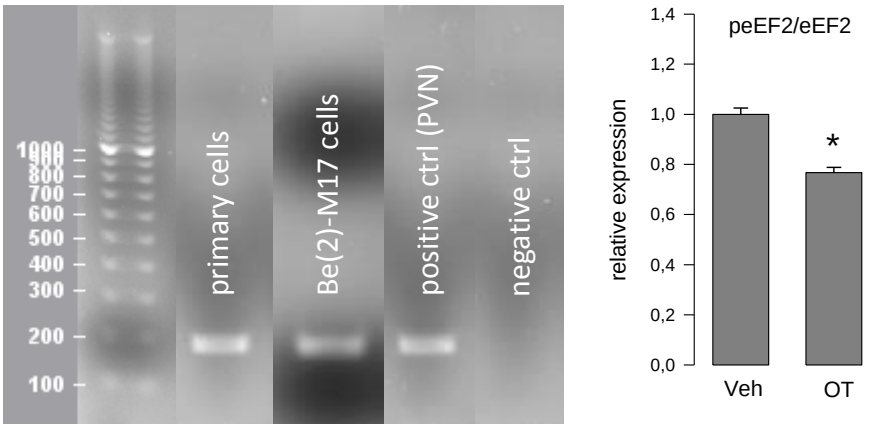

Figure 21: Agarose gel showing the expression of OTR in Be(2)-M17 cells, as well as in primary hypothalamic rat cells. Bar chart demonstrates the dephosphorylation of eEF2 10 min after OT-treatment in Be(2)-M17 cells (n = 4). Plot represents means + SEM; Student's *t*-test, * $p < 0.05$.

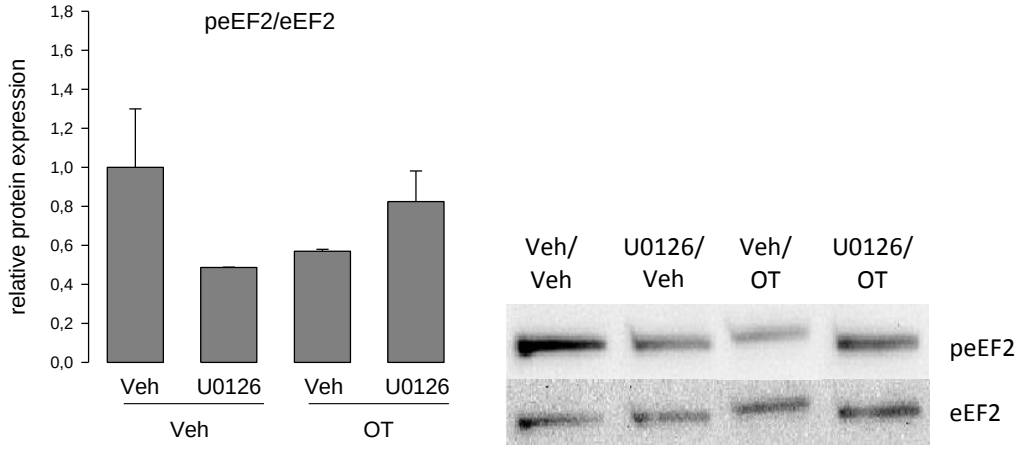

Figure 22: Relative protein expression of peEF2. Treatment with the MEK1/2 inhibitor U0126 alone dephosphorylated eEF2 (n = 3). Plot represents means + SEM. A representative blot is shown on the right side.

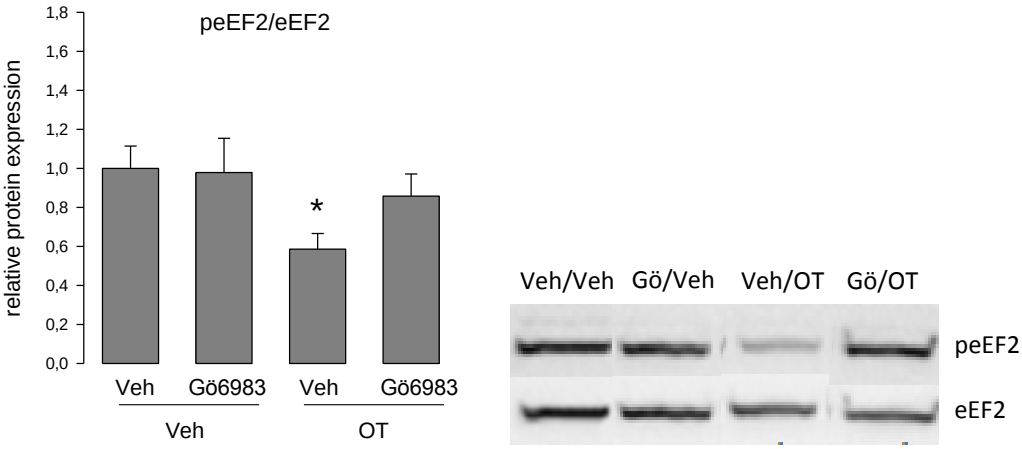

Figure 23: Relative protein expression of peEF2 compared to total eEF2. The PKC-inhibitor Gö6983 abolished the dephosphorylating effect of OT, while having no effect on the phosphorylation of eEF2 by itself (n = 3, 3, 4, 3). Plot represents means + SEM; TWO-WAY ANOVA followed by LSD post-hoc-test, * $p < 0.05$ vs Veh/Veh and vs Gö6983/OT. A representative blot is shown on the right side.

Experiment 3: Verification of enhanced protein synthesis in the hypothalamus after OT-treatment

Given that OT activated an important factor controlling protein synthesis, eEF2, it seemed likely that OT-stimulated cells produce new proteins. To test this hypothesis, primary

hypothalamic cells were starved from methionine. After 1 h, the synthetic amino acid L-AHA was added to the medium and the cells were stimulated with 250 nM OT (or Veh). L-AHA gets incorporated in every newly built protein and can be detected afterwards with a Click-reaction. The isolated proteins were separated into L-AHA containing and non-containing proteins by immunoprecipitation and analysed by dot blot.

Incorporation of the synthetic amino acid L-AHA, and therefore protein synthesis, was greatly enhanced in primary hypothalamic cells 3 h after stimulation with 250 nM OT as compared with vehicle-treated cells ($p = 0.01$, $n = 3$; Figure 24 C). The incorporation of L-AHA started already after 30 min and was enhanced in OT-treated cells ($n = 2$, Figure 24), indicating an early start of *de novo* protein synthesis, stimulated by OT.

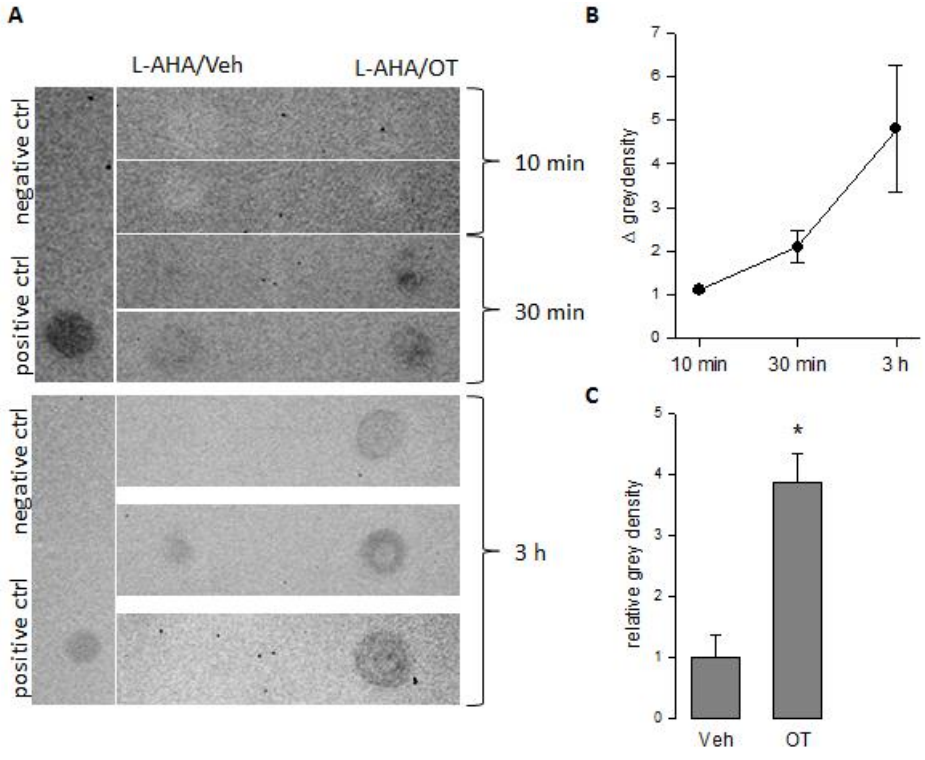

Figure 24: A: Dot blot analysis of seven separate labelling experiments in primary cells, 10 min, 30 min, and 3 h after OT stimulation. B: The difference in the grey density of Veh- and OT-treated samples increases time-dependently. C: The grey density in the OT-treated sample is significantly higher compared to Veh after 3 h. Plot represents means + SEM; Student's *t*-test, * $p < 0.05$.

Experiment 4: NPY5R as selected protein target for the regulatory properties of OT

I validated in a previous study that *icv* OT infusion in Wistar rats leads to a differential gene expression 30 min after the stimulus (Martinetz, 2010). One of the regulated genes, *npy5r*, was selected to be analysed on protein level, due to the protein's anxiolytic properties when activated by central administration of a specific receptor agonist (Sorensen et al., 2004).

Central *icv* infusion of 1 nmol/5 µl OT increased the expression of NPY5R in the PVN of male Wistar rats 30 min ($p = 0.018$, $n = 3 – 4$) and also 3 h ($p = 0.006$, $n = 7$) after the infusion (Figure 25). The NPY5R expression level in the hippocampus, which served as a control region for NPY5R expression, was not altered. These results underline my previous finding that OT enhances *npy5r* mRNA expression in the PVN 30 min after an *icv* OT infusion (Martinetz, 2010), and revealed that these changes are longer-lasting, which suggests a functional link between the two neuropeptidergic systems.

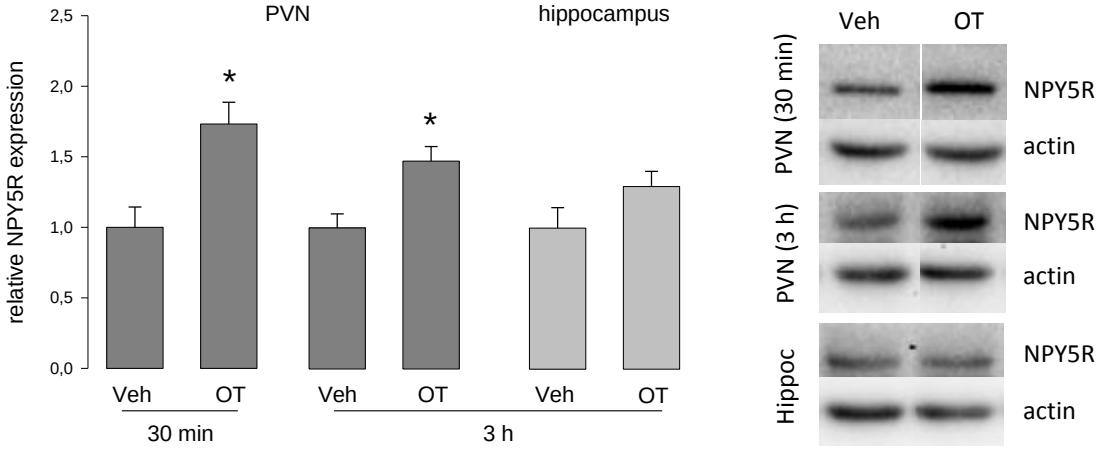

Figure 25: Relative protein expression of NPY5R 30 min (n = 3, 4) and 3 h (n = 7) after central infusion of 1 nmol OT normalized to actin levels. OT increased the level of NPY5R in the PVN but NPY5R expression in the hippocampus was not altered after 3 h. Plot represents means + SEM; Student's *t*-test, * $p < 0.05$. Representative blots are shown on the right side.

Experiment 5: Inhibition of protein synthesis in the PVN and the effects on the anxiolytic property of OT

Having established the capacity of OT to induce protein synthesis, I assessed whether protein synthesis is a mediator of the anxiolytic effect of OT in the PVN 30 min and 3 h after the stimulus. Male Wistar rats received bilateral PVN infusion of 23.5 µmol/0.5 µl of the general protein synthesis inhibitor anisomycin (Qi and Gold, 2009) or its vehicle (HCl, adjusted to pH 7.4), followed 20 min later by OT (0.01 nmol/0.5 µl) or its vehicle (Ringer's solution). Rats were tested for their anxiety-like behaviour 30 min and 3 h after the last infusion in the LDB and on the EPM, respectively, for 5 min.

Rats treated with OT spent significantly more time in the light compartment of the LDB 30 min after the infusion ($F_{3,54} = 7.807$, $p < 0.001$; $p = 0.047$ vs Veh/Veh, n = 14 – 15, Figure 26) compared with the Veh group, indicating that the acute (10 min) anxiolytic effect of OT within the PVN (Blume et al., 2008) is still present after 30 min. Pre-treatment with anisomycin 20 min before the OT infusion prevented the effect of OT ($p < 0.001$ vs Veh/OT), while having no effect alone. No treatment altered locomotion. These observations indicate that, after 30 min, the anxiolytic effect of OT in the PVN is dependent on newly synthesised proteins.

Rats that were tested 3 h after the last infusion on the EPM showed a decrease in anxiety-like behaviour stimulated by OT as well, represented by a significantly higher % time spent on the open arm of the EPM ($F_{3,34} = 6.412$, $p = 0.001$, $p = 0.005$ vs Veh/Veh, n = 8 - 11; Figure 27). Former observations of a long-term anxiolytic effect of endogenous OT (Waldherr and Neumann, 2007) could therefore be replicated with the exogenous infusion. However, pre-treatment with anisomycin before the OT infusion also led to a significant increase in the %

time spent on the open arm (p = 0.005 vs Ani/Veh). An infusion of anisomycin alone again had no effect on anxiety-like behaviour. The number of closed arm entries was lower in the Ani/OT-group compared with Ani/Veh, which may suggest that the treatment affected locomotion. However, it is likely that the great difference in % time spent on the open arms between those two groups forms the basis of this observation. The total number of arm entries, which can serve as a measurement for locomotion, too (Pellow et al., 1985), was not changed throughout the groups.

Inhibition of protein synthesis in the PVN did therefore not affect the long-term anxiolytic effect of OT, suggesting that this effect is mediated via other mechanisms or other brain regions, for example the central amygdala (Knobloch et al., 2012).

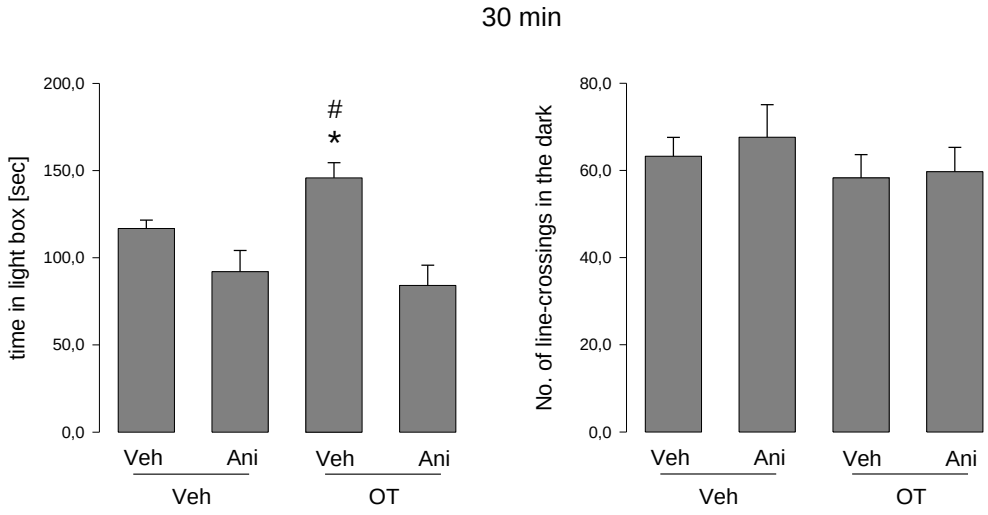

Figure 26: Pre-treatment with 23.5 µmol/0.5 µl anisomycin abolished the anxiolytic effect of OT in male rats (n = 14, 15, 14, 15). Plots represent means + SEM; TWO-WAY ANOVA followed by LSD post-hoc-test, * $p < 0.05$ vs Veh/Veh, $^{\#}$ $p < 0.001$ vs Ani/OT.

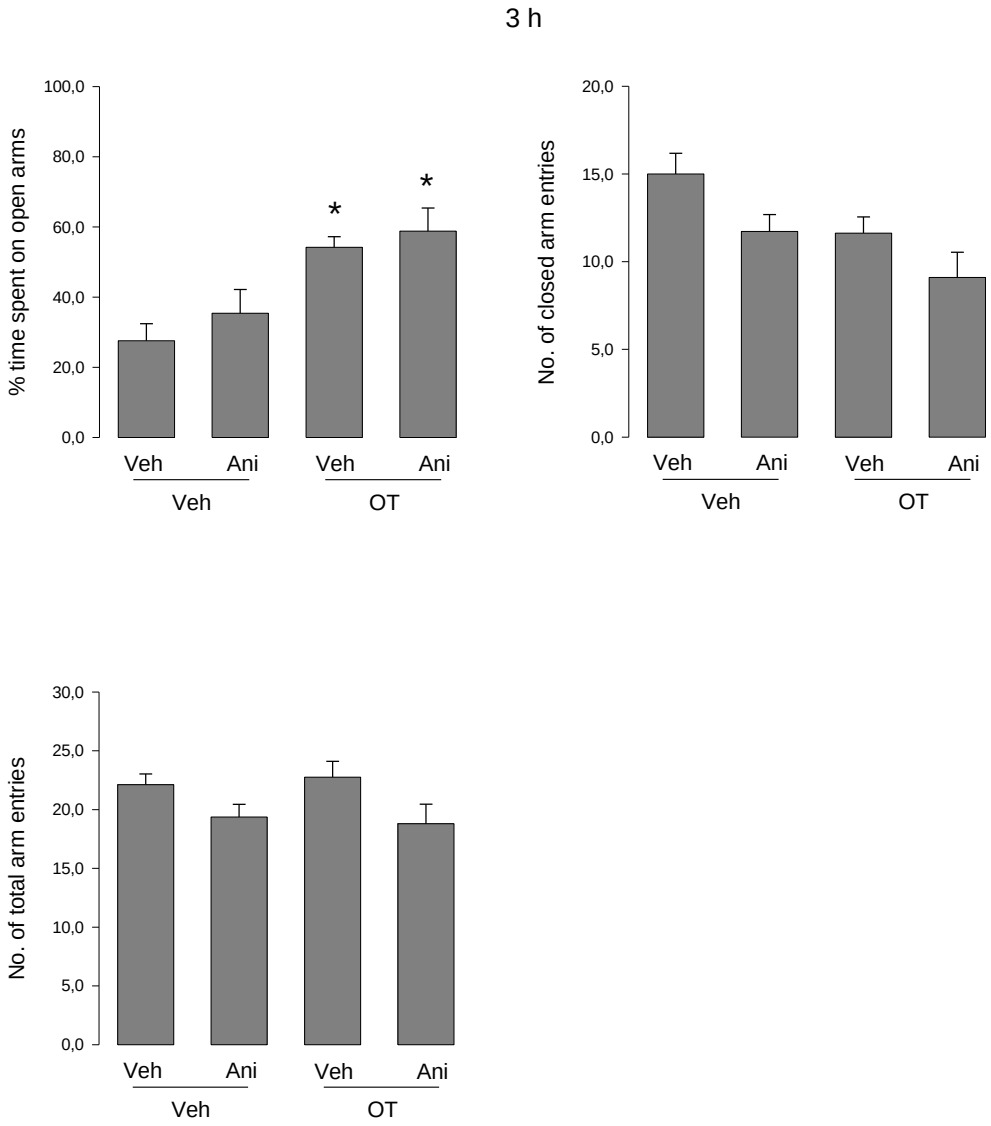

Figure 27: Intra-PVN infusion of 0.01 nmol/0.5 µl OT remained anxiolytic 3 h after the injection. Pre-treatment with 23.5 µmol/0.5 µl anisomycin did not block the effect of OT. The number of closed arm entries indicated a treatment effect on locomotor activity, which was not replicated in the number of total arm entries (n = 9, 11, 8, 10). Plots represent means + SEM; TWO-WAY ANOVA followed by LSD post-hoc-test, * $p < 0.05$ vs respective Veh.

Part III: Oxytocin has regulatory effects on the microRNAome of the hypothalamus

Experiment 1: Determination of the expression of microRNAs in the hypothalamus with and without OT-treatment via Deep Sequencing

In addition to changing protein synthesis rate directly, it is also possible that OT exerts its long-term anxiolytic effects through the regulation of post-transcriptional events in the cell. One of these events is the prevention of mRNA translation by RNA-inhibiting microRNAs. To study the possible influence of OT on this process, the expression of microRNAs in OT-treated cells was compared to that of vehicle-treated cells.

Total RNA was isolated and separated on a 12 % PAA gel, and the short RNAs with a size of approximately 20 nucleotides were cut out of the gel (Figure 28). Thereafter, 3'- and 5'-adapters were ligated to the short RNAs and the product was amplified and run on a 6 % PAA gel to separate the successfully ligated products from the empty constructs (Figure 29). The cDNA was cut out of the gel and used for the Deep Sequencing.

The resulting sequences produced by the Deep Sequencing were sorted and annotated to the miRBase database (Kozomara and Griffiths-Jones, 2011). Over 300 different microRNAs were detected and identified in H32 cells and over 450 in primary hypothalamic cells. MicroRNAs with an abundance < 0.001 % of total reads in both samples (Veh and OT) were excluded from further analysis, because it was assumed that their regulatory role within the cell is negligible. This selection criterion led to a total number of 217 detected microRNAs in H32 cells and 158 in primary cells. The two sample types showed a great variability in their regulation profile as demonstrated in the heatmap illustration (Figure 30), which may be, at least in part, a result of the comparison of homotypic (cell line) with heterotypic cells

(primary cells). Although the total number of microRNAs was smaller in the primary cell sample (158) than in the H32 cell sample (217), the percentage of microRNAs that were more than 1.5-fold up- or down-regulated by OT was higher in primary cells (42.4 %) than in H32 cells (37.3 %). Furthermore, the percentage of up-regulated microRNAs relative to the total number of microRNAs after OT-treatment was higher in the H32 cell sample (33.6 %) than in the primary cell sample (16.5 %), but the percentage of down-regulated microRNAs was lower in the H32 cells (3.7 %) compared to the primary cells (26.0 %) (Figure 31). MicroRNAs have the ability to inhibit the translation of their respective target mRNAs and therefore are indirect regulators of protein synthesis. Depending on the function of the target mRNA/protein, microRNAs can have activating or inhibiting effects on cellular processes.

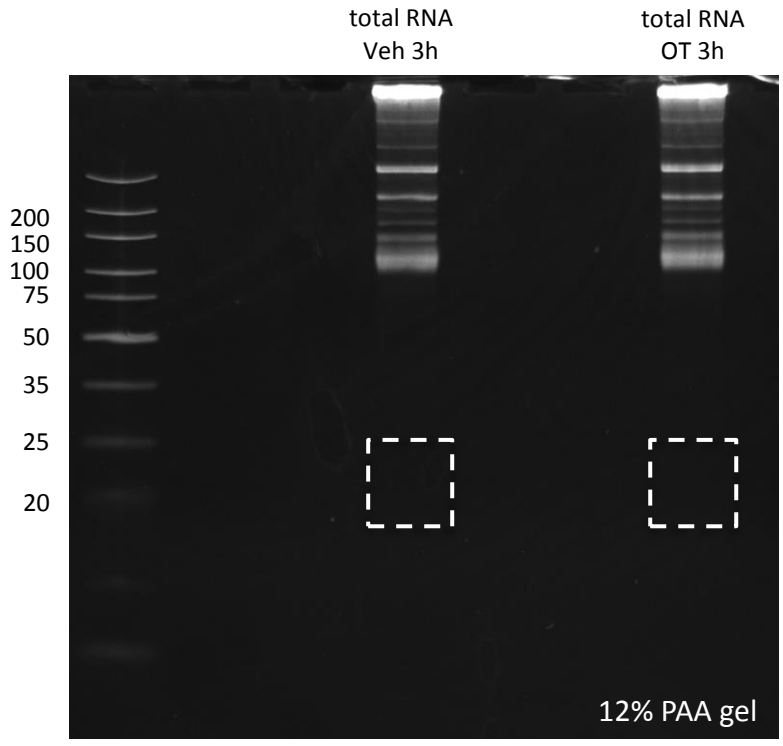

Figure 28: Representative PAA gel showing the separated RNAs. The gel was cut out at 20 nucleotides (dashed line) and short RNAs were used for further analysis.

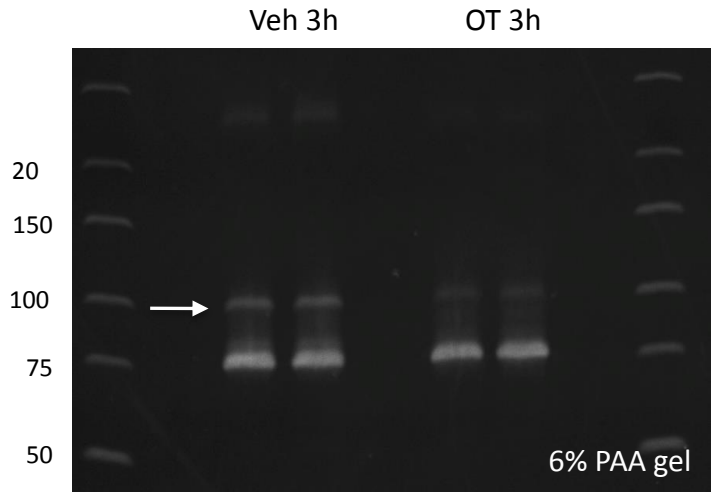

Figure 29: Representative PAA gel showing the PCR product after ligation of 3'- and 5'-adapters to the short RNAs. The band at 100 bp (white arrow) was cut out and used for further analysis; the lower band represents the empty construct without short RNAs. Pictures were kindly provided by Anne Dueck, Department of Biochemistry I, University of Regensburg.

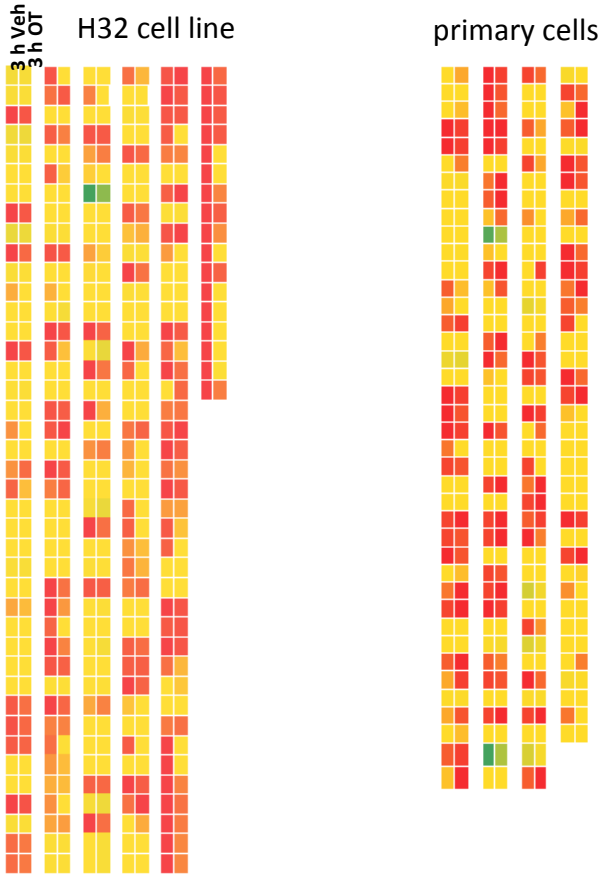

Figure 30: Heatmap reflecting the results for both Deep Sequencing in H32 cells (left) and primary hypothalamic cells (right) 3 h after OT stimulation. Colours represent the % of total reads with green as the highest percentage and red as the lowest. Each square represents one microRNA and Veh- and OT-samples are seen alongside to each other. The heatmap illustrates the high variability between the two cell populations.

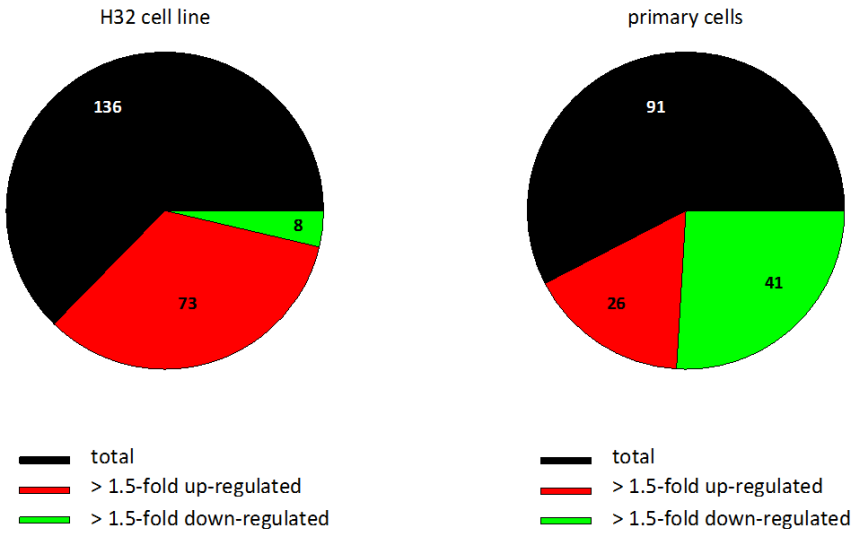

Figure 31: Pie chart showing the ratio of regulated microRNAs (red and green) to total (black). Not only differs the number of total microRNA between the cells, but also the ratio up- to down-regulated microRNAs (9:1 in H32 cells, 1:1.6 in primary cells).

Experiment 2: Validation of the Deep Sequencing

I next attempted to validate the Deep Sequencing results of H32 cells via Northern Blot. The strong sequencing regulation observed in H32 could not be replicated via Northern Blot (Figure 32). This may be due to possible mutations occurring in the cell line H32, which probably caused the partial loss of OTR (Figure 20). The microRNAs that were validated were chosen because of their strong up-regulation seen in the Sequencing (miR22: 6.8-fold, miR24: 2.6-fold, miR29a: 2.0-fold, miR29b: 26.6-fold, miR301a: 8.5-fold and miR193: 229.1-fold).

Due to these difficulties, the Deep Sequencing was repeated in primary hypothalamic cells (I verified that they expressed the OTR, Figure 21), which gave a new set of results that were validated with qPCR adapted for microRNAs as described in the methods section. The new

validation method was chosen because this greatly reduced the amount of required RNA and thus the number of animals sacrificed for the primary cell isolation. The regulation of five selected microRNAs was successfully recapitulated by qPCR 3 h after the OT stimulation. All five were significantly up-regulated in OT-treated primary cells compared to Veh-treated cells ($p < 0.05$, $n = 6 – 9$) (Figure 33). The microRNAs that were validated in this approach were not only chosen based on their regulation detected in the Sequencing, but also based on literature pointing those microRNAs out as interesting targets (Haramati et al., 2011; Lawson et al., 2013; Tognini and Pizzorusso, 2012). In addition, miR30a was identified in the Deep Sequencing as one of the most abundant microRNAs with 3.1 % of the total number of microRNAs in the Veh-treated sample and 2.4 % in the OT-treated sample and might therefore play a greater role in the regulation of cellular processes.

Preliminary target prediction with TargetScan (Release 6.2, (Garcia et al., 2011)) revealed several interesting targets of the OT-activated microRNAs. Among the hundreds of predicted target mRNAs are several protein phosphatases, ion channels, ubiquitin peptidases, and neurotransmitter receptors, as well as other interesting proteins like methyl CpG binding protein 2 (MeCP2), vasoactive intestinal peptide (VIP), and eukaryotic elongation factor-2 kinase (eEF2K), on which I will focus in more detail in the discussion section.

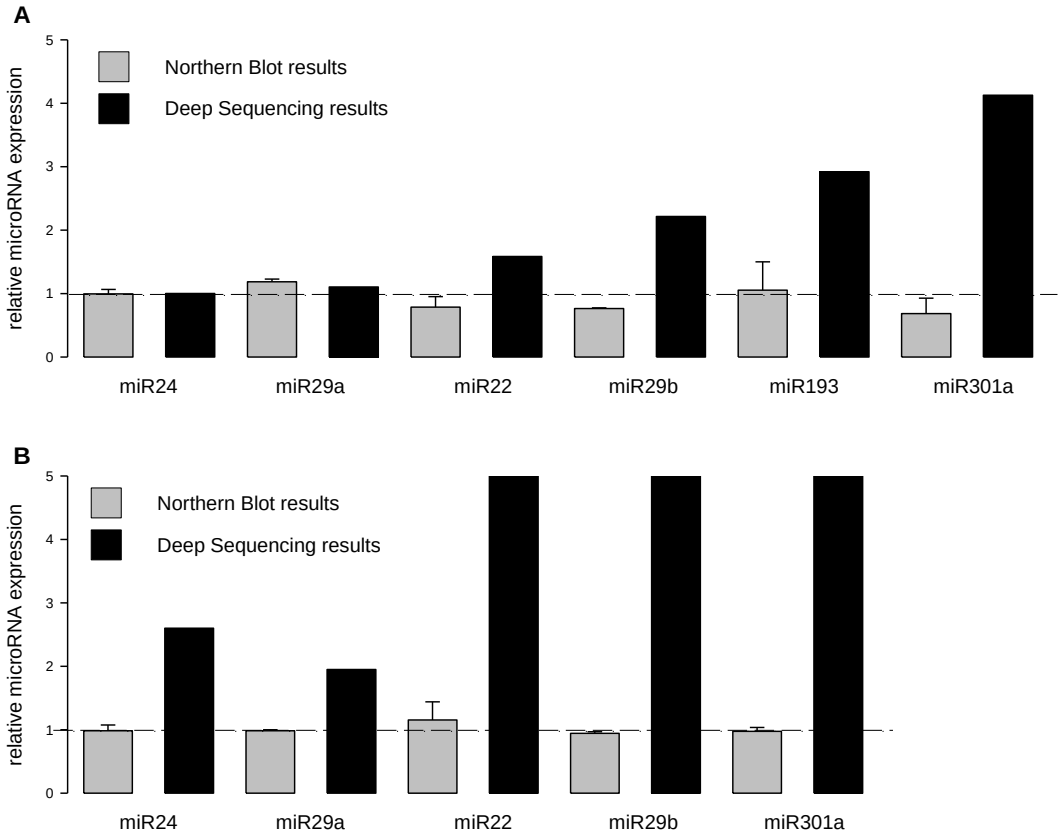

Figure 32: Validation of Deep Sequencing via Northern Blot. H32 cells were treated with 250 nM OT for 30 min (A) or 3 h (B). Dashed line illustrates the microRNA expression level of vehicle-treated cells. MicroRNA levels were normalized to U6 RNA. Black bars show the expression levels of the respective microRNAs measured after OT stimulation in the Deep Sequencing. Plots represent means + SEM.

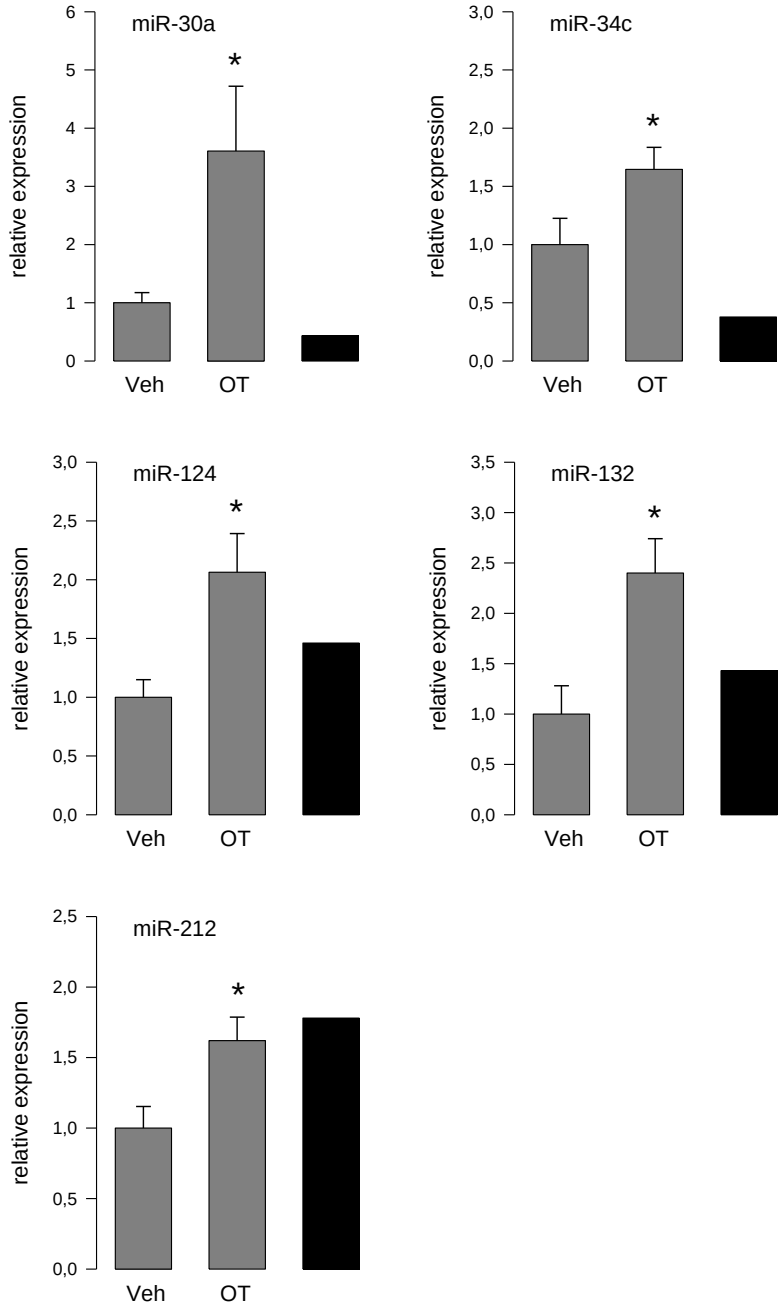

Figure 33: Validation of selected microRNAs after Deep Sequencing in primary hypothalamic cells. All microRNAs were significantly up-regulated in their expression level 3 h after OT stimulation of the cells, and were thus not always reflecting the result of the Deep Sequencing (black bar). (miR30a: $p = 0.048$, $n = 8, 9$; miR34c: $p = 0.045$, $n = 7, 9$; miR124: $p = 0.013$, $n = 9$; miR132: $p = 0.006$, $n = 8, 9$; miR212: $p = 0.019$, $n = 6, 7$) Plots represent means + SEM; Student's *t*-test, * $p < 0.05$.

DISCUSSION

Discussion

In the present study, I advanced our understanding of the regulation of anxiety-like behaviour in the PVN. First, I revealed a novel mediator of anxiety-related behaviour, the ATP-receptor P2X4R, which is a member of the purinergic system. Furthermore, I found that the established anxiolytic factor OT stimulates protein synthesis in the PVN, not for long-term anxiolysis as anticipated, but rather for mid-term (30 min) anxiolysis. OT-induced protein synthesis depended on a typical intracellular pathway via PKC and eEF2, but might also be influenced by the up- and down-regulation of several microRNAs. The regulation of microRNA expression is a completely novel role of OT, and the first example of a neuropeptide of the PVN controlling the expression of microRNAs. The results presented in this thesis reflect, *in toto*, the importance of the purinergic and oxytocinergic systems in the PVN of rats in the control of anxiety-related behaviour, mark these systems as potential targets for treatment of anxiety disorders in the future, and emphasize the importance of the PVN as one of the key brain regions in anxiety regulation.

Part I: P2X4R as a regulator of anxiety in the PVN of Wistar rats

Higher P2X4R expression in LAB compared with HAB rats

In the quest for endogenous regulators of anxiety in the PVN, I made use of rats with either high or low inborn anxiety levels, the HAB and LAB rats, respectively. While analysing the results of a microarray that had been performed before the start of my research project, I found that the expression of P2X4R was significantly higher in the PVN of LAB rats as compared with that in HAB rats. The comparison of LAB and HAB PVN tissue at the mRNA

and protein level confirmed this result, clearly demonstrating that male as well as female LAB rats express significantly more P2X4R than HAB rats. As a ligand-gated ion channel, the P2X4R opens in response to ATP binding and permits Ca^{2+}-influx. Therefore, higher P2X4R expression in LAB rats would make the PVN cell more amenable to ATP-stimulation, which leads to greater Ca^{2+}-influx, and activation of intracellular processes. Importantly, in a previous study, Van den Burg and colleagues revealed that extracellular Ca^{2+}-influx is a crucial factor for OT-mediated anxiolysis within the PVN of rats (Van den Burg et al., unpublished). Ca^{2+} seems to be involved in anxiety regulation in other brain regions as well. The Ca^{2+}-dependent phosphatase calcineurin, for instance, was recently shown to have significant impact on anxiety and depression in the amygdala (Mineur et al., 2014), the prefrontal cortex (Yu et al., 2013) and the hippocampus (Zhu et al., 2011). A difference in receptor expression as prominent as in the case of P2X4R between HAB and LAB indicates a fundamental role of the receptor in the behavioural expression of fear and anxiety.

P2X4R is expressed by OT- and AVP-positive neurons in the PVN

As I hypothesized P2X4R to be involved in anxiety regulation, I sought to determine whether P2X4R is co-localised with the OT system in the PVN. Indeed, 56 % of the OT-positive cells expressed P2X4R, as well as 32 % of the AVP-positive cells. The remainder of the cells that express P2X4R could be neurons that produce CRF, or glial cells. Indeed, P2X4R is expressed by astrocytes (Franke et al., 2001; Kukley et al., 2001), microglia (Xiang and Burnstock, 2005), and neurons (Guo et al., 2009).

The neuropeptides OT, AVP, and CRF are involved in the regulation of anxiety, but with opposing effects. More specifically, while OT is known for its anxiolytic properties within the PVN (Blume et al., 2008; Jurek et al., 2012), AVP and CRF are anxiogenic. Intra-PVN treatment of HAB rats with an AVP V1 receptor antagonist decreased anxiety (Wigger et al., 2004), and in Wistar rats, AVP was shown to act anxiogenic following systemic administration of the receptor agonist desmopressin (Mak et al., 2012). Activation of the CRF/CRFR1 system within the mPFC and the BNST increases anxiety-like behaviour (Miguel et al., 2014; Tran et al., 2014). The co-localisation of the P2X4R with at least OT and AVP makes it possible that these neuropeptides are instrumental in the anxiolytic effect of P2X4R. Interestingly, Denda et al. observed an enhanced OT-release following stimulation of keratinocytes with ATP. The detected OT-release occurred 10 min after ATP-stimulation, was Ca^{2+}-dependent, and could be blocked by application of 2',3'-0-(2,4,6-trinitrophenyl) adenosine 5-triphosphate (TNP-ATP), an inhibitor of P2X1R, P2X3R, P2X2/3R, P2X4R and P2X7R (Coddou et al., 2011; Denda et al., 2012). One could assume that a similar scenario occurs in the PVN, and that P2X4R activation might lead to local OT-release, OTR-activation, and anxiolysis. However, this scenario might not apply, as this is not in line with the intracellular signalling pathways that are differentially activated in males and females (see below and results section). The anxiolytic effect of CTP is similar in both genders, yet MAPK signalling is unique to each of the sexes, whereas the short-term anxiolytic effect of OT depends, in males as well as in females, on MEK 1/2 activation (Blume et al., 2008; Jurek et al., 2012).

The immunohistochemical staining further revealed that the P2X4R has a somatic localisation in a granulated fashion, which suggests a temporal internalisation and storage of

the receptor in lysosomes, similar to the receptor distribution in microglia, endothelial cells, and macrophages (Qureshi et al., 2007). Thus, the cell can alter the amount of available active P2X4R at the cell membrane according to the incoming stimuli, and with that Ca^{2+}-influx. This might provide an extra level of control, in addition to the expression of the *p2rx4* gene.

P2X4R activation within the PVN leads to anxiolysis

ATP is the endogenous ligand of all P2XR. CTP and ATP contain similar functional groups within their base ring structure, but CTP has a weaker effect on receptor activity than ATP (Soto et al., 1996). The smaller cytidine base cannot fill the agonist binding site of the P2XR completely, which diminishes the extent to which CTP can bind to and activate the receptor (Hattori and Gouaux, 2012). However, CTP is able to activate P2X4R (Soto et al., 1996), although its EC_{50} is 20 times higher than that of ATP (Coddou et al., 2011). Thus, in attempt to activate the P2X4R as specifically as possible, I decided to use CTP for the behavioural experiments. Since it was not used *in vivo* before, I tested two different doses of CTP based on the comparison of the EC_{50}s of ATP and CTP (Coddou et al., 2011). While a tendency towards an anxiolytic effect was observed with the lower dose (4 nmol/0.5 µl), the higher dose (40 nmol/0.5 µl) had the predicted anxiolytic effect on both the EPM and the LDB 10 min after administration. The 10 min time point was chosen based on former studies, which showed that the anxiolytic activity of OT manifests itself 10 min following local infusion in the PVN (Blume et al., 2008; Jurek et al., 2012).

The observed anxiolytic effect of P2X4R activation correlates with the results of studies using the positive allosteric modulator of P2X4R, IVM, which showed decreased anxiety-like behaviour when administered systemically (Bortolato et al., 2013). However, IVM potentiates $GABA_A$ receptors as well (Krusek and Zemkova, 1994), and given the well-known role of GABA in anxiety regulation, IVM is likely to exert its anxiolytic properties via this mechanism. It is therefore not specific enough for the elucidation of P2X4R's role in anxiety. Indeed, the effects of IVM on anxiety-like behaviour in P2X4R KO mice were similar to those observed in wild type mice (Bortolato et al., 2013).

CTP had similar effects on anxiety-related behaviour in males as well as in virgin female rats, reflecting the findings regarding basal P2X4R expression in HAB and LAB rats, which were also not sex-dependent. The underlying cellular mechanisms that lead to P2X4R-induced anxiolysis are likely to be identical in males and females, just as it was observed for OT-induced anxiolysis, which is mediated via MAPK-activation in male (Blume et al., 2008) as well as in female rats (Jurek et al., 2012).

Administration of ATP, given in a concentration 20-times less than CTP, based on the EC_{50}s for P2X4R, exerted no effect on anxiety-related behaviour. Here, all existing functional ATP-receptors in the PVN that are responsive to the administered ATP-concentration were activated. Although the occupancy of P2X4R by ATP is likely to be lower due to the binding to other receptors, the absence of any consequences for anxiety-related behaviour indicates that other ATP-receptors in the PVN play a role in anxiety regulation as well. It seems, however, that those tend to reverse P2X4R's anxiolytic effect, highlighting the specific role of the P2X4R subunit in anxiolysis.

Since CTP lacks complete specificity for the P2X4R, I confirmed the anxiolytic effect of P2X4R by inhibiting it with the specific antagonist 5-BDBD prior to CTP administration. Indeed, 5-BDBD prevented the anxiolytic effect normally observed following CTP infusion, indicating an exclusive role of P2X4R within the PVN in the control of anxiety-related behaviour of rats. However, in addition to this pharmacological verification, adeno-associated virus (AAV)-mediated knock down of P2X4R within the PVN would greatly strengthen the hypothesis. Therefore, ongoing studies utilizing local AAV administration are taking place to confirm the pharmacological data.

Apart from studies mentioned above relating to P2X4R and anxiety, P2XRs have been substantially implicated in depression, a common co-morbidity to anxiety. In this context, P2X7R has received most of the attention, as it was shown that SNPs within the human P2X7R gene are associated with major depressive disorder (Lucae et al., 2006; Soronen et al., 2011). Furthermore, the absence of P2X7R leads to an antidepressant phenotype and increased food intake without changes in basal locomotor activity or anxiety in mice (Basso et al., 2009). It is not clear yet how P2X7R leads to the alterations in behaviour, but it was found by means of deletion experiments that the responsible P2X7R-expressing cells are most likely neurons or astrocytes rather than other cell types (Csolle et al., 2013; Sperlagh et al., 2012), giving a further example for the connection of central purinergic signalling and mood.

In contrast to P2X7R, activation of P2X2R results in an anti-depressant phenotype. The specific response is initiated by astrocyte-derived ATP in the medial prefrontal cortex (Cao et al., 2013), as shown in mice using the Forced-Swim-Test (FST). Intriguingly, preliminary experiments showed depressive-like effects of CTP in male Wistar rats, indicated by a higher

% time spent immobile in the FST (Martinetz, unpublished), which fits with previous literature (Bortolato et al., 2013). Additionally, the findings for P2X7R and P2X2R demonstrate that different P2XR subunits can have opposite regulatory effects on the same behaviour.

P2X4R modulation influences inborn anxiety levels

Local administration of CTP into the PVN of HAB rats showed that CTP is an efficient anxiolytic even in rats with a strong genetic predisposition to high anxiety. In contrast, P2X4R inhibition by 5-BDBD in LAB rats leads to a more anxious phenotype. Interestingly, 5-BDBD has no effect on anxiety-related behaviour in Wistar rats. This phenomenon is probably justified by the abnormally high P2X4R expression in the LAB PVN. A down-regulation of this highly active P2X4R system, which is normally responsible for the maintenance of the low anxiety-levels, results in a normal anxious phenotype. In Wistar rats, on the other hand, the P2X4R system might not be permanently active and its inhibition has little to no effects on anxiety. This again, underlines the outstanding importance of P2X4R in the regulation of anxiety-related behaviour by confirming CTP's behavioural effect in a psychopathological animal model.

P2X4R-activated signalling cascades in hypothalamic cells

The observed differences in MAP-kinase activity between male and female rats are not in line with the finding that CTP decreases anxiety in both genders and are therefore almost certainly not involved in the mediation of the anxiolytic P2X4R effect. It is thus very likely

that the anxiolytic effect of P2X4R is not mediated by activating the OT-system, as discussed above. In contrast, eEF2, a key mediator of protein synthesis, is activated in male and female rats alike, 10 min after CTP administration and would thus serve as an interesting candidate for further examination. As I demonstrated in part II of my results, protein synthesis is an important factor of OT-mediated anxiolysis, and might therefore be involved in the acute effect of P2X4R activation as well.

In summary, the present findings highlight, for the first time, the role of the hypothalamic purinergic system in the regulation of anxiety. In this regard, specifically the P2XR subunit P2X4R exerts strong anxiolytic effects shortly after local activation in the PVN. The relevance of P2X4R is greatly underlined by the finding that even inborn, extreme, anxiety levels can be reversed by activation or inhibition of the P2X4R. Altogether, the study provides new insights into the engaged processes of anxiety-regulation in the PVN and suggests P2X4R as a novel target for possible pharmacological interventions for treatment of anxiety disorders.

Part II: Oxytocin activates protein synthesis in the rat hypothalamus

Former studies revealed that acute OT-induced anxiolysis is at least partly mediated via the MEK1/2-pathway (Blume et al., 2008; Jurek et al., 2012). Additional pathways downstream of the OTR in the PVN that might mediate OT's long-term anxiolytic effects were unclear. Therefore, in the second aim of my thesis, I investigated this behavioural phenomenon in depth and hypothesised that OT would lead to protein synthesis and modulation of microRNAs and thus the previously described long-term anxiolysis (Waldherr and Neumann, 2007).

OT activates eEF2 in a PKC-dependent manner

My observation that OT activates eEF2 in hypothalamic cells as well as in the PVN indicates that OT promotes protein synthesis. Interestingly, other intracellular factors that stimulate protein synthesis, mTOR and p90RSK, were not activated, demonstrating that OT recruits only a specific component of the protein translation machinery. Dephosphorylated eEF2 promotes the translocation step of peptide chain elongation and is therefore a key regulator of protein synthesis. When phosphorylated at residue Thr56 in its GTP-binding domain, its binding to the ribosomes and, hence its activity, is impaired (Carlberg et al., 1990). The calcium/calmodulin (CaM)-dependent protein kinase eEF2k is responsible for the phosphorylation of eEF2 (Ryazanov et al., 1997). eEF2k can be regulated by phosphorylation at several sites and the kinases shown to be involved are mTOR, RSK, and AMP-activated protein kinase (AMPK) (Browne et al., 2004; Wang and Proud, 2006).

My experiments revealed that the OT-stimulated dephosphorylation of eEF2 in the PVN is blocked when the cells were pre-incubated with a PKC-inhibitor. I chose to study, as OT's anxiolytic effect was previously shown to be Ca^{2+}-dependent (Van den Burg et al., unpublished), and as PKC is one of the targets of the $G_{q/11}$-protein-coupled OTR. This result was in line with a previous finding in OT-treated myometrial cells (Devost et al., 2008), although the exact pathway is not elucidated yet. On the basis of the present findings and current literature, the following signalling pathway can be suggested: As a $G_{q/11}$-protein-coupled receptor, the OTR activates PLCβ, which in turn generates IP3 and DAG and triggers the release of Ca^{2+} from intracellular stores. Consequently, PKC is activated and might phosphorylate AMPK at Ser485/491, a phosphorylation site that inhibits AMPK (Woods et al., 2003). AMPK usually directly phosphorylates and thus activates eEF2k at Ser398 (Browne

et al., 2004). This activation is now blocked and with that the subsequent phosphorylation at Thr56 and thus inactivation of eEF2 by eEF2k as well. In this way, OT could control the regulation of *de novo* protein synthesis in a Ca^{2+}-dependent manner in the PVN and might contribute to OT's anxiolytic effect.

Protein synthesis in hypothalamic cells is enhanced by OT

In order to demonstrate that the identified pathway leads indeed to protein synthesis, I stimulated hypothalamic cells with OT in a medium where methionine was replaced by the synthetic amino acid L-AHA. The detection of the incorporation rate of L-AHA by means of Click-chemistry revealed that new proteins are indeed generated in response to an OT stimulus. Protein synthesis is already visible 30 min after stimulation and is greatly enhanced after 3 h of incubation. Without OT, *de novo* protein synthesis is hardly detectable, illustrating the significance of OT on this intracellular process. Enhanced protein synthesis is generally considered as a marker for cell activity and thus, the present finding demonstrates that OT has not only acute effects on the cell like the elevation of intracellular Ca^{2+} levels and increased MAPK-activity (Blume et al., 2008; Jurek et al., 2012), but also provokes long-term intracellular changes that might serve as a substrate for long-term effects like modulation of structural plasticity in the PVN (Theodosis, 2002) and stress response (Neumann et al., 2000).

NPY5R as an example for OT-induced de novo *synthesis*

I discovered that the NPY receptor NPY5R is one of the proteins that is newly synthesised in the PVN in response to an OT-stimulus. The receptor belongs to the group of proteins whose expression level rises in the early 30 min phase and stays elevated, even if slightly dampened, for 3 h. NPY5R is one of the four known NPY receptors in the brain (Y1, Y2, Y4, Y5) through which the highly abundant neuropeptide NPY exerts its effects (Redrobe et al., 1999). The involvement of NPY in several biological functions, including feeding, epilepsy, depression, and anxiety has long been known (Heilig, 2004; Meurs et al., 2007; Stanley and Leibowitz, 1985). Central administration of NPY results in anxiolysis in rats (Heilig et al., 1989) and interestingly, the effect lasts up to 3 days (Heilig and Murison, 1987). All NPY receptor subtypes are expressed in the PVN (Kask et al., 2002).

The involvement of NPY5R in the anxiolytic effect of NPY lacks complete elucidation. It is believed that both NPY1R and the NPY5R are crucial for the required signalling for anxiolysis (Sorensen et al., 2004). Central as well as intra-amygdalar administration of the specific NPY5R agonist [cPP$^{1–7}$,NPY$^{19\ –23}$,Ala^{31},Aib^{32},Gln^{34}]hPP ([cPP]hPP) (Cabrele et al., 2000) decrease anxiety-like behaviour in rats (Sajdyk et al., 2002; Sorensen et al., 2004) and the effect of NPY can be blocked by pre-treatment with a Y5 antagonist (Sajdyk et al., 2002).

An OT-induced increase in hypothalamic NPY5R expression therefore elevates the cell's susceptibility for NPY and might contribute to an enhanced anxiolytic NPY response amplifying the OT effect. NPY is synthesised in neurons of the hypothalamic arcuate nucleus, which project, amongst others, to the PVN where NPY is released (Chronwall et al., 1985; Morris, 1989). However, so far, the NPY system in the PVN is better known for its regulatory effect on feeding behaviour (Stanley and Leibowitz, 1985). OT is involved in the regulation of

food intake as well (Arletti et al., 1989), providing another possibility for an interaction of the two neuropeptidergic systems. If so, OT and NPY would antagonize each other's function rather than enhance it, since OT reduces food intake (Arletti et al., 1989), whereas NPY increases it (Stanley and Leibowitz, 1985). One possibility is, for instance, that the anorexic effect of OT is counteracted by NPY and NPY5R, to compensate for the presence of exogenous OT.

The interplay of the OT- and NPY-system and their putative shared role in anxiety in the PVN requires follow-up studies. These could involve a local inhibition of NPY5R in the PVN and assessment of the effects of a subsequent OT infusion. Also, the cellular localisation of the NPY5R in the PVN should be clarified; especially its co-localisation with OTR is of relevance in the context of the regulation of anxiety-like behaviour.

Protein synthesis is needed for the anxiolytic effect of OT

The absence of OT-induced anxiolysis following pre-treatment with the general protein synthesis inhibitor anisomycin shows that OT-induced anxiolysis depends on protein synthesis within the PVN at the 30 min time-point. Anisomycin blocks translational elongation (Vazquez et al., 1969), which is, according to my results, activated by OT via the PKC/eEF2 pathway. Amongst the proteins that are newly synthesised 30 min after the OT stimulus, must be candidates that contribute to anxiolysis, since anxiolysis does not appear when protein synthesis is blocked. One of those candidates could be NPY5R, whose increased expression leads to an enhanced NPY response of the cells. It would also be possible that OT induces increased expression of ion channels, facilitating neuronal

excitability, or enhances the cell's configuration of neurotransmitter receptors. Fast protein synthesis, as observed in my study, occurs usually in synapses where the required mRNA is already stored, ready for immediate translation (Meister, 2011). Such local protein synthesis is important for certain forms of synaptic plasticity, including the endocytosis of AMPA receptors via *de novo* synthesis of cytoskeletal microtubule-associated protein 1B (Davidkova and Carroll, 2007; Heise et al., 2014). Synaptic protein synthesis furthermore involves the synthesis of intracellular signalling molecules, such as CaMKIIα, which may occur within minutes of receptor activation (Kanhema et al., 2006; Scheetz et al., 2000). An additional protein involved in anxiolysis would be the regulator of G-protein signalling 2 (RGS2) (Leygraf et al., 2006; Okimoto et al., 2012). RGS2 has been shown to mediate anxiety in mice and humans (Oliveira-Dos-Santos et al., 2000; Smoller et al., 2008), and its expression is, interestingly enough, induced after OT-treatment. Higher RGS2 levels in the CeA of female mice were shown to be linked with anxiolysis (Okimoto et al., 2012). The detailed mechanisms underlying the connection of RGS2 and anxiety are still unknown, but it was shown that RGS2 increases synaptic vesicle release by down-regulating the G_i-mediated pre-synaptic Ca^{2+} channel inhibition (Han et al., 2006).

In contrast to the 30 min time-point, inhibition of protein synthesis within the PVN had no effect on the long-term (3 h) OT-induced anxiolysis. A possible scenario would be that the long-term anxiolytic effect of OT is not generated in the PVN, but by OT that is released in another brain region involved in anxiety regulation. Such a region can be the CeA, a structure where axons of hypothalamic OT-neurons have been found to activate local GABAergic circuits to decrease freezing responses in fear-conditioned rats (Knobloch et al., 2012; Viviani et al., 2011). Other regions that have been shown to be highly innervated by hypothalamic

OT-neurons and play a role in anxiety regulation are the lateral septum, the BNST, the vHPC, as well as the mPFC (Anthony et al., 2014; Kim et al., 2013; Knobloch et al., 2012; Sabihi et al., 2014).

In summary, protein synthesis is important for OT-mediated anxiolysis in the PVN of rats, at least at the mid-term (30 min). This time period is in agreement with the rapid activation of eEF2. Activation of eEF2 is also transient, and eEF2 phosphorylation has returned to baseline within 60 min after OT application. However, at a later time point, OT seems to subsequently activate additional mechanisms leading to further enhanced protein synthesis, since I observed a great amount of newly synthesised proteins 3 h after OT-application. In contrast to the first pool of proteins, the second appears not to contribute to anxiolysis directly within the PVN. The long-term anxiolytic effect, although it should start in the PVN where OT was infused, could depend on PVN output to other brain regions involved in the control of anxiety-like behaviour.

Part III: Oxytocin has regulatory effects on the microRNAome of the hypothalamus

A potential pathway underlying the long-term effects of OT within the PVN is the modulation of microRNA levels. Deep Sequencing revealed dozens of microRNAs to be either up- or down-regulated 3 h after OT stimulation of hypothalamic cells. By means of qPCR, I was able to verify that OT activates some of the most distributed microRNAs in the brain, namely miR-124, miR-132, and miR-212, and increases their expression level by 1.8- to 2.4-fold. Moreover, OT up-regulates miR-34c expression, a microRNA that was already shown to exert anxiolytic actions in the CeA (Haramati et al., 2011). In the end, the two mechanisms protein

synthesis and microRNA regulation are not mutually exclusive, since the regulation of microRNA levels by OT would subsequently affect protein expression either directly (translation of target mRNAs) or indirectly (regulation of other mRNAs/proteins by the target mRNAs).

Regulation of protein synthesis can occur at several levels. One of these concerns the regulation at the post-transcriptional level, being altered microRNA expression. MicroRNAs are responsible for the fine-tuning of mRNA translation within the cell, and their regulation can thus impact the proteome. MicroRNA regulation by neuropeptides has not received much attention so far. In the research presented in this thesis, I investigated the influence of OT on microRNA expression.

OT increases microRNA expression in the hypothalamus

On the basis of Deep Sequencing, I succeeded to reveal an OT-induced increase in the expression of five microRNAs in primary hypothalamic cells 3 h after the onset of OT stimulation: miR-124, miR-132, miR-212, miR-34c, and miR-30a. These findings give completely new insights into the functioning of the neuropeptide. The expression of all of the validated microRNAs is increased by OT, so that the translation of their target mRNAs should be reduced. In addition, the Deep Sequencing analysis showed that OT down-regulates several microRNAs, demonstrating that OT influences the cellular post-transcriptional modification positively as well as negatively.

The highly abundant neural microRNA miR-124 is one of the positively regulated targets of OT. miR-124 is expressed in neurons but not astrocytes, and together with miR-9, miR-124

stimulates neuronal and represses glial differentiation (Makeyev et al., 2007). Moreover, acute stress negatively regulates miR-124 in the mouse amygdala, which is correlated with a simultaneous increase in mineralocorticoid receptor (MR) expression (Mannironi et al., 2013). Importantly, miR-124 is a regulator of glucocorticoid receptors (GR) as well and is thus involved in cortisol/corticosterone feedback (Vreugdenhil et al., 2009). In the hippocampus, OT indeed induces changes in the expression of MR and GR, although, here, GR was down- and MR was up-regulated (Petersson and Uvnas-Moberg, 2003). Both intracellular receptors bind glucocorticoids, with the MR having an approximately ten times higher affinity than the co-localized GR (Reul and de Kloet, 1985). Thus, down-regulation of both receptors in hypothalamic cells through up-regulation of miR-124 decreases the cell's sensitivity for glucocorticoids and could hence alter the stress response on the basis of feedback attenuation.

miR-132 and miR-212 are members of the same family, have similar mature sequences and share the same seed region (Wanet et al., 2012). They are encoded by the same intron of a small non-coding gene and their expression is induced by a variety of signals, including stimulation by brain-derived neurotrophic factor (BDNF) and synaptic activity. The expression of both microRNAs in neuronal cells is regulated by cAMP response element-binding protein (CREB) via MEK1/2 activation (Remenyi et al., 2010). This is of particular interest, because OT activates MEK1/2 to bring about acute anxiolysis (Blume et al., 2008; Jurek et al., 2012). miR-132/212 are therefore possible down-stream targets of OT-induced MEK1/2 activation, leading to increased long-term synaptic activity and cellular excitability (Cheng et al., 2007; Nudelman et al., 2010) in the hypothalamus, possibly by regulating the number of post-synaptic AMPA receptors (Remenyi et al., 2010).

The validated microRNA that showed the strongest regulation by OT in the hypothalamus is miR-30a. Currently, not much is known about the functions of this particular microRNA in the brain, but Deep Sequencing showed that it is one of the most abundant microRNAs in hypothalamic cells. One of the predicted and validated targets of miR-30a is BDNF (Mellios et al., 2008), but the involvement of BDNF in anxiety regulation is not fully elucidated yet (Martinowich et al., 2007). The prominent effect of OT on miR-30a expression is therefore worth further investigation.

Interestingly, four of the validated microRNAs, miR-30a, miR-132/212, and miR-124 share one predicted target, and this is MeCP2. MeCP2 binds specifically to methylated DNA and recruits co-repressors and chromatin remodelling proteins, functioning as a transcriptional repressor (Nan et al., 1998). OT could therefore even modulate the epigenetic machinery via microRNAs in the hypothalamus.

The microRNA that has a clear link with anxiety is miR-34c. When overexpressed in the CeA, miR-34c has anxiolytic properties (Haramati et al., 2011). Furthermore, CRFR1 was found to be one of the targets of miR-34c (Haramati et al., 2011), leading to a down-regulation of the anxiogenic CRFR1 in response to miR-34c activation (Muller et al., 2003). My observation that miR-34c expression is up-regulated in response to the OT-stimulus makes it possible that miR-34c exerts the same regulatory effects, *i.e.* via down-regulation of CRFR1, on anxiety-like behaviour in the PVN as in the CeA. Intriguingly, another target of miR-34c is eEF2k, the kinase responsible for eEF2 phosphorylation, and thus inactivation, of the key factor of protein synthesis that I showed to be activated by OT. Thus, by the up-regulation of miR-34c via OT, and subsequent inhibition of eEF2k mRNA translation, the production of the crucial factor leading to inhibition of OT-activated protein synthesis is prevented. This target

of miR-34c is particularly interesting since it is yet another example for the regulation of the eEF2k/eEF2-pathway within the hypothalamus, additionally to the already discussed dephosphorylation of eEF2 following OTR- and P2X4R-activation.

Another interesting microRNA that stood out in the Deep Sequencing results, although not validated, was miR-22. This particular microRNA belongs to the group of down-regulated transcripts, resulting in attenuated repression of its target mRNAs. One of those target mRNAs is RGS2, as revealed by overexpression of miR-22 in neuroblastoma cells and subsequent whole genome expression microarrays (Muiños-Gimeno et al., 2011). RGS2 reduces G-protein activity via its GTPase function (Watson et al., 1996). RGS2-KO mice show increased anxiety-like behaviour (Oliveira-Dos-Santos et al., 2000), and association studies in humans revealed four SNPs of the RGS2 gene associated with panic disorder (Leygraf et al., 2006). Moreover, OT induces the expression of RGS2 in the CaA of mice, and RGS2 levels are correlated with anxiolytic behaviour (Okimoto et al., 2012). Based on those findings, RGS2 might be one of the longer-term targets of OT, regulated indirectly by repressing the microRNA responsible for RGS2 regulation.

An additional example for an OT-induced decrease in microRNA expression was miR-339, which has previously been associated with anxiety as well: SNPs in the miR-339 gene were correlated with panic disorder in Spanish patients, and target prediction revealed, amongst others, CRFR2 (Muiños-Gimeno et al., 2011). CRFR2 activation in rodents has been reported to be anxiolytic, anxiogenic, or to have no effect (Bale et al., 2000; Cooper and Huhman, 2005; Coste et al., 2000), so its role is not yet clarified. However, CRFR2 immunoreactivity and mRNA were found in OT-neurons in the PVN (Dabrowska et al., 2011), indicating a direct connection between the two systems. One could therefore speculate that OT and CRF

regulate anxiety-like behaviour via CRFR2. Indeed, it has been proposed that CRFR2 mediates a feedback loop between CRF-containing neurons and OT-containing neurons (Dabrowska et al., 2011).

Just recently, miR-375 was shown to be up-regulated after unpredictable maternal stress in mice, and this regulation was even shown to be inheritable (Gapp et al., 2014). miR-375 is another microRNA that I found to be down-regulated by OT in the hypothalamus, possibly contributing to the stress-protective effect of OT (Neumann et al., 2000), giving an additional example for a potential regulatory mechanism of OT processed via microRNAs.

In summary, OT influences the expression of several microRNAs in hypothalamic cells, and thus has a regulatory effect on the post-transcriptional machinery of the cell. MicroRNAs repress the translation of their target mRNAs by binding to a so-called seed sequence on the mRNA and blocking protein synthesis. By up- or down-regulating microRNAs, OT affects protein synthesis via a mechanism in addition to the direct influence on eEF2 as shown above. Stress-related proteins like CRFR1, GR and MR are some of the targets of the OT-activated microRNAs, demonstrating a possible additional way of regulating the stress response. Other microRNAs, like miR-34c were already implicated in anxiety-regulation, although in other brain regions, and provide a new basis for an involvement of microRNAs in the regulatory effect of OT on anxiety-like behaviour. Deep Sequencing was performed in only one sample each (n = 1) and is currently being repeated in additional samples of OT-stimulated primary cells and *icv*-infused rats. These data will provide more insight into the regulation of microRNA expression by OT, but can unfortunately not be included in the thesis due to time constraints.

Conclusions and perspectives

The results presented in this study advance our understanding of the biology of anxiety. The use of rat strains bred for extremes in anxiety-like behaviour proved to be a fruitful approach to identify a previously unrecognised mediator of anxiety-like behaviour in the PVN of rats, P2X4R. These animals will likely continue to be of importance for the discovery of other factors that control anxiety. Of particular interest for P2X4R are the results of the recent flood of association studies, where genetic variations in the human genome, for example SNPs, are associated with diseases. Loss-of-function SNPs in the *p2rx4* gene, for instance, are linked to a higher risk for cardiovascular disease, a common co-morbidity to anxiety (Stokes et al., 2011). Furthermore, the chromosome, where *p2rx4* is located, was found to be an important genetic region for anxiety, bipolar, and unipolar disorders (Erhardt et al., 2007). The same applies to genetic studies on microRNAs as I described already above on the example of miR-339.

OT has developed into a frequently applied drug in many fields of psychiatric research, since it was discovered that it can be easily administered with a nasal spray. Nevertheless, many factors involved in the functioning of OT are still to be uncovered, and for therapeutic use it should be handled with care. Some recent publications challenge the former pro-social view of OT (Kosfeld et al., 2005; Meyer-Lindenberg et al., 2011; Striepens et al., 2011) by findings showing that the effects of OT depend on situational and dispositional factors rather than improving pro-social behaviour per se (Bartz et al., 2011). OT even increases envy or defensiveness toward out-group members (De Dreu et al., 2011; Shamay-Tsoory et al., 2009). Furthermore, OT was associated with anxiogenic effects in the lateral septum (Guzman et al., 2013), and after chronic *icv* administration in mice (Peters et al., 2014). Thus,

a precise understanding of the OT-affected mechanisms in the brain is of great interest for further research regarding OT-mediated regulation of anxiety-related behaviour.

The discovery of two apparently different functional systems in the PVN that control anxiety-like behaviour, the purinergic and the OT systems, raises the question of whether activation of P2X4R and OTR leads to recruitment of shared intracellular factors that control the behavioural expression of anxiety. ATP, the natural ligand of P2X4R, is equally produced by glial cells and neurons and acts as a ubiquitous transmitter between neurons (Burnstock, 2006b). OT, on the other hand, is synthesised by a discrete population of neurons in the SON and the PVN. It is therefore important to identify the precise sources and targets of ATP and OT in the PVN to determine whether purinergic and oxytocinergic signalling converge to bring about the same effect: anxiolysis. Intracellularly, convergence might occur at the level of Ca^{2+}-dependent signalling, as both P2X4R- and OTR-activation lead to Ca^{2+}-influx (Egan and Khakh, 2004) (Van den Burg et al., unpublished). Another possible connection might be one or several of newly synthesised proteins. For OT I could show that it induces *de novo* protein synthesis through activation of eEF2 and additionally increases the expression of miR-34c, a regulator of the eEF2-inactivating eEF2k. Interestingly, P2X4R activation similarly promotes eEF2 activity. It is therefore important to characterize the newly synthesised proteins involved in the anxiolytic effect of P2X4R and OT. This could be achieved by separating these proteins on a gel, preferably in two dimensions, followed by mass spectrometry. The function of promising candidates, which I expect to be Ca^{2+}-dependent and/or to have a synaptic localisation, could then be assessed in tests for anxiety-like behaviour (EPM, LDB). Gain- and loss-of-function experiments of these proteins could be

realised by molecular (for instance viral and siRNA techniques) or pharmacological approaches (for instance by the use of agonists, antagonists, or ion channel blockers).

Furthermore, to dive deeper into the OT-induced microRNA regulation that I observed, and its importance for behavioural adaptations, selected microRNAs could be inhibited using locked nucleic acids (LNAs). Such synthetic oligonucleotides are used to knock down specific microRNAs (Elmen et al., 2008a; Elmen et al., 2008b; Orom et al., 2006), and could therefore be used *in vivo* to study the effect of eliminating one specific microRNA on predicted target protein synthesis and animal behaviour.

With the help of these approaches it should be possible to expand the findings presented in this thesis, and to come to an identification of factors that are crucial for the control of anxiety-like behaviour in the PVN. These factors could potentially serve as a basis for the development of novel pharmacological treatments of anxiety disorders.

Summary

Anxiety disorders are the most common psychiatric disorders and although there are a number of treatment options available, all have their limitations. Over the years, many studies have been conducted to discover the sites in the brain where anxiety-related behaviour is controlled, and the neural factors that mediate this behaviour. Despite these research efforts, our understanding of the biology of anxiety is far from complete, and the search for better understanding of the neural basis and effective treatments of anxiety disorders awaits significant advances in the field of the neurobiology of anxiety. The paraventricular nucleus of the hypothalamus (PVN) is one of the key brain regions involved in anxiety regulation. Therefore, the aims of my studies described in this thesis were therefore (1) to identify a new endogenous factor within the PVN that could be a new target for future pharmacological intervention of anxiety disorders, and (2) to determine the intracellular processes that sustain the anxiolytic effect following a single OT treatment within the PVN.

By making use of a microarray performed from PVN tissue of rats that had been selected for extreme low or high anxiety-related behaviour (the LAB and HAB rats), I discovered that the ATP-receptor P2X4R regulates anxiety-related behaviour within the PVN. P2X4R is a ligand-gated ion channel, highly permeable for Ca^{2+}, and thus increases intracellular Ca^{2+}-levels and downstream signalling when activated. Both mRNA and protein expression of P2X4R was strongly up-regulated in LAB rats when compared with the expression in HAB rats; independent of sex. CTP, a P2X4R agonist, decreased anxiety-like behaviour in Wistar rats as soon as 10 min after local infusion into the PVN. The anxiolytic effect of CTP was blocked by pre-treatment with the selective inhibitor of P2X4R, 5-BDBD, supporting the involvement of

P2X4R in anxiety regulation. Moreover, by infusion of CTP and 5-BDBD into the PVN of HAB and LAB rats, respectively, normalisation of the extreme phenotypes was achieved. Intracellular effectors of P2X4R stimulation included activation of eEF2, a regulator of peptide chain elongation, but whether *de novo* protein synthesis plays a role in P2X4R-mediated anxiolysis in the PVN is currently not known. However, the data clearly showed that activation of P2X4R within the PVN is a novel target for modulation of anxiety-related behaviour.

Additionally, I examined the effects of exogenous intra-PVN OT and its effects in hypothalamic cells. This neuropeptide is synthesised in the PVN (among other regions) and is known for both its short- and long-term anxiolytic properties. I found that OT activates protein synthesis via a PKC-dependent dephosphorylation of eEF2, both *in vitro* in hypothalamic cells as well as *in vivo*, in the PVN of male Wistar rats. This activation occurs within 10 min and leads to a relatively fast increase of *de novo* protein synthesis within 30 min. Moreover, an even greater amount of newly synthesised proteins is detectable 3 h after OT-application in primary hypothalamic cells. Behavioural testing for anxiety-related behaviour of male Wistar rats, treated with the general protein synthesis inhibitor anisomycin, revealed that the mid-term (30 min) anxiolytic effect of locally administered OT depends on local protein synthesis. However, the long-term (3 h) anxiolytic effect was not abolished by local inhibition of protein synthesis within the PVN, suggesting an involvement of another brain region connected to the PVN controlling the OT-regulated anxiety-like behaviour.

The later time point (3 h) however, appeared to be of significance for OT-induced microRNA regulation. Deep Sequencing analysis revealed dozens of regulated microRNAs in primary

hypothalamic cells 3 h after OT-application. By means of qPCR, the up-regulation of essential neural microRNAs, including miR-124, miR-132, and miR-212, was validated. MicroRNA regulation by the neuropeptide OT is a completely novel finding and should contribute to our understanding of the long-term effects of OT.

Thus, by assessing the purinergic system as a novel regulator of anxiety in the PVN and by providing a greater understanding of the molecular underpinnings of the oxytocinergic system particularly within the PVN, I highlighted the role of this brain region in the mediation of anxiety-related behaviour. Taken together, the results presented in this thesis advance our knowledge about the biology of anxiety, and may help to develop new strategies for the treatment of anxiety disorders in the future.

Deutsche Zusammenfassung

Angsterkrankungen betreffen einen jährlich wachsenden Anteil der Weltbevölkerung. Dazu zählen Phobien, Panikattacken, Generalisierte Angststörung oder Zwangsstörung. Die Symptome und Beschwerden dieser Erkrankungen sind vielseitig und daher gestaltet es sich schwierig geeignete Behandlungsmöglichkeiten zu entwickeln. Ein großer Zweig der Neurobiologie beschäftigt sich aus diesem Grund mit der Aufklärung neuronaler Vorgänge, die zur Ausbildung von krankhaftem Angstverhalten führen. Trotz allem sind nach wie vor große Lücken zu füllen, um ein vollständiges Verständnis von Angst und Angsterkrankungen zu erlangen. Für die Entwicklung effektiver Behandlungsmöglichkeiten brauchen wir daher fundierte Kenntnisse über die molekularen Vorgänge und Mechanismen im Gehirn, die das Angstverhalten steuern.

Meine Studien waren daher zum einen darauf ausgerichtet einen endogenen Faktor zu finden, der Ziel einer zukünftigen Behandlungsmöglichkeit sein könnte und zum anderen intrazelluläre Prozesse zu bestimmen, die von einer schnellen Anxiolyse zu einem dauerhaft reduzierten Angstverhalten führen.

Die Vorgänge im paraventrikulären Nukleus des Hypothalamus (PVN), einer Kernregion der Angst-Regulation im Gehirn, standen dabei im Zentrum. Speziell gezüchtete Ratten, die sogenannten HAB und LAB Ratten, die sich auf extreme Art in ihrem Angstverhalten unterscheiden (sie weisen sehr hohes bzw. sehr niedriges Angstverhalten auf) bildeten dabei eines meiner Versuchsmodelle. Bei meinen Untersuchungen gelang es mir, den ATP-Rezeptor P2X4R als neuen Faktor der Angst-Regulation zu bestimmen. P2X4R ist ein Ionenkanal, der durch Bindung seines Liganden aktiviert, und somit geöffnet wird. Er ist besonders durchlässig für Ca^{2+}-Ionen und seine Aktivierung steigert daher den intrazellulären

Ca^{2+} Spiegel, was wiederum zur Aktivierung Ca^{2+}-sensibler Signalkaskaden führt. LAB Ratten zeichnen sich im Vergleich zu HAB Ratten durch eine erhöhte Expression jenes P2X4R aus. Eine Aktivierung des Rezeptors direkt im PVN, mit Hilfe des Agonisten CTP, führte nach 10 min zu einer Verringerung des Angstverhaltens von Ratten, die nicht auf einen spezifischen Phänotyp hin selektiert wurden (nachfolgend: Wistar Ratten). Dieser angstlösende Effekt von CTP konnte durch die Applikation des spezifischen P2X4R Antagonisten 5-BDBD verhindert werden, was darauf schließen lässt, dass P2X4R eine zentrale Rolle in der Regulation des Angstverhaltens spielt. Außerdem gelang es mir, durch eine lokale Infusion des Agonisten CTP bzw. des Antagonisten 5-BDBD in den PVN von HAB und LAB Ratten, deren extreme Phänotypen zu normalisieren. Intrazellulär konnte ich unter anderem den eukaryotischen Elongationsfaktor 2 (eEF2) als einen zentralen Faktor der P2X4R-Signalkaskade identifizieren. eEF2 ist während der Protein-Neusynthese entscheidend für die Bildung und Verlängerung der sich bildenden Peptidkette. Ob Protein-Neusynthese für den angstlösenden Effekt von P2X4R verantwortlich ist, ist noch nicht bekannt. Die gewonnenen Daten zeigen deutlich, dass eine Aktivierung des P2X4R im PVN zu vermindertem Angstverhalten führt und beschreiben P2X4R als ein neues pharmakologisches Ziel für die Regulierung des Angstverhaltens.

Zusätzlich habe ich die Auswirkungen von exogen appliziertem Oxytocin (OT) im PVN und in hypothalamen Zellen untersucht. Das im PVN produzierte Neuropeptid ist bekannt für seinen kurzfristig, wie auch langfristig angstlösenden Effekt. Meine Experimente zeigten *in vitro* (in hypothalamen Zellen), genauso wie *in vivo* (im PVN männlicher Wistar Ratten), dass OT die Protein-Neusynthese durch die Protein Kinase C (PKC)-abhängige Dephosphorylierung von eEF2 aktiviert. Dieser Effekt war bereits nach 10 min *in vitro* wie *in vivo* zu beobachten

und führte nach 30 min zu einem raschen Anstieg neu synthetisierter Proteine in primären hypothalamen Zellen. 3 h nach Stimulation jener Zellen mit OT konnte ich gegenüber den Vehikel-stimulierten Zellen eine 4-fach größere Menge neu synthetisierter Proteine erfassen. Der lokal in den PVN applizierte Proteinsynthese-Inhibitor Anisomycin blockierte den mittelfristigen (30 min) anxiolytischen Effekt von exogenem OT im PVN männlicher Wistar Ratten. Jedoch hatte Anisomycin keine Auswirkungen auf die OT-induzierte langfristige (3 h) Anxiolyse. Dies lässt darauf schließen, dass zu diesem Zeitpunkt weitere Gehirnareale, die mit dem PVN in Verbindung stehen (wie z.B. Amygdala oder präfrontaler Cortex), für die Ausbildung des angstlösenden Effektes von OT verantwortlich sind.

Der spätere Zeitpunkt (3 h) stellte sich jedoch als bedeutend für die Regulation der microRNA Expression durch OT heraus. Das Deep Sequencing des Transkriptoms OT-stimulierter (3 h) primärer hypothalamer Zellen, lieferte Dutzende regulierte microRNAs. Mit Hilfe von qPCR konnte die Hoch-Regulation bedeutender neuronaler microRNAs, darunter miR-124, miR-132 sowie miR-212 validiert werden. Die Fähigkeit, auf die Expression von microRNAs Einfluss zu nehmen, ist ein völlig neuer Aspekt des Neuropeptids OT und könnte zu unserem Verständnis langfristiger Effekte von OT entscheidend beitragen.

Zusammengenommen bereichern die Ergebnisse, die ich in dieser Arbeit präsentiere, unser Wissen über die Biologie der Angst und können von großer Bedeutung bei der Entwicklung neuer Strategien für die Behandlung von Angsterkrankungen in der Zukunft sein.

List of abbreviations

4E-BP	4E binding protein
5-BDBD	5-(3-Bromophenyl)-1,3-dihydro-2*H*-benzofuro[3,2-*e*]-1,4-diazepin-2-one
5-HT1A	serotonergic receptor 1a
AAV	adeno-associated virus
AMPK	AMP-activated protein kinase
ATP	adenosine-triphosphate
AVP	vasopressin
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
Ca^{2+}	calcium
CaM	calcium/calmodulin
CaMK	CaM kinase
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CeA	central amygdala
CNS	central nervous system
CREB	cAMP response element-binding protein
CRF	corticotropin-releasing factor
CRFR1	CRF receptor 1
CRFR2	CRF receptor 2
CTP	cytidine triphosphate

DAG	1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EDC	l-ethyl-3-(3-dimethylaminopropyl) carbodiimide
eEF	eukaryotic elongation factor
eEF2k	eukaryotic elongation factor-2 kinase
EGF	epidermal growth factor
eIF	eukaryotic initiation factor
EPM	Elevated Plusmaze
eTF	eukaryotic termination factor
FBS	fetal bovine serum
FST	Forced-Swim-Test
GABA	γ-aminobutyric acid
GAD	generalized anxiety disorder
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GR	glucocorticoid receptors
HAB	high-anxiety behaviour
HBSS	Hank's balanced salt solution
HPA axis	hypothalamic pituitary adrenal axis
HRP	horseradish peroxidase
IP3	inositol triphosphate
IVM	ivermectin
KO	kock-out
LAB	low-anxiety behaviour

L-AHA	L-azidohomoalanine
LDB	Light-Dark-box
LNA	locked nucleic acids
MAO	monoamine oxidase
MAP	mitogen-activated protein
MAPK	MAP kinase
MeCP2	methyl CpG binding protein 2
mPFC	medial pre-frontal cortex
MR	mineralocorticoid receptor
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NPS	neuropeptide S
NPY	neuropeptide Y
OCD	obsessive-compulsive disorder
OT	oxytocin
OTR	oxytocin receptor
P2X4R	P2X4 receptor
P2XR	P2X receptor
P2YR	P2Y receptor
PAA	polyacrylamide
PBS	phosphate buffered saline
PBST	PBS/0.3 % Triton X 100

PFA	paraformaldehyde
PKC	protein kinase C
PLCβ	phospholipase C-β
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
qPCR	quantitative PCR
RGS2	regulator of G-protein signalling 2
RNAi	RNA interference
Rpl	ribosomal protein L13a
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
SNRI	selective serotonin-norepinephrine reuptake inhibitor
SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
TBST	Tris-buffered saline with 0.001 % Tween-20
TCA	tricyclic antidepressants
TNP-ATP	2',3'-0-(2,4,6-trinitrophenyl) adenosine 5-triphosphate
tRNA	transfer RNA
TRPV2	transient receptor potential cation channel subfamily V member 2
URT-primer	Universal Reverse Transcription primer
UTR	untranslated region
Veh	vehicle

vHPC	ventral hippocampus
VIP	vasoactive intestinal peptide

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Stefanie Martinetz, David A. Slattery, Inga D. Neumann, Erwin H. van den Burg. Oxytocin mediates anxiolysis within the PVN via PKC-dependent dephosphorylation of eukaryotic elongation factor 2, in preparation

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