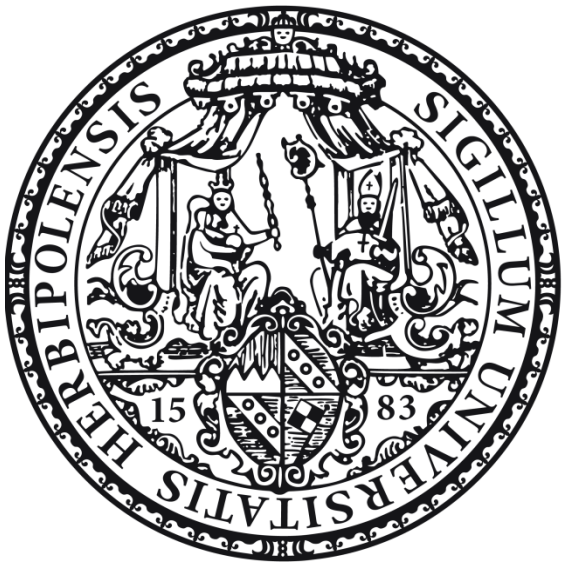

The regulation of *corticotropin releasing hormone receptor 1* gene expression and its role in panic disorder

Die Regulation der *Corticotropin Releasing Hormon Rezeptor 1* Genexpression und ihre Rolle bei der Panikstörung

Doctoral thesis for a doctoral degree
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“The pleasure lies not in discovering truth, but in searching for it.”

— Leo Tolstoy, Anna Karenina

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Abstract

Panic Disorder (PD) is characterized by unexpected, recurrent panic attacks, which are not restricted to certain situations, medication or stimuli. Like other anxiety disorders, PD is a multifactorial disorder and develops through the interaction of genetic and environmental risk factors. Despite an estimated heritability of up to 48%, no distinct genetic mechanism could be revealed yet. A dysregulation of the stress response has been shown in patients with PD and several studies could find an association of components of the corticotropin-releasing factor (CRF) system with PD. The corticotropin releasing hormone receptor 1 (CRHR1) is the main receptor of CRF in the brain and thus a crucial regulator of cerebral CRF signaling. Recent genetic studies found an association of certain *CRHR1* single nucleotide polymorphisms (SNPs) with PD and other anxiety disorders. Among the associated *CRHR1* SNPs, rs17689918 showed further evidence in a multilevel study regulating *CRHR1* gene expression in panic-relevant brain regions and affecting brain activation in fMRI experiments, as well as flight behavior in a behavioral avoidance task (Weber *et al*, 2015). Here, we aimed to investigate the underlying neurogenetic and neurobiological mechanisms, by which the rs17689918 risk allele affects *CRHR1* gene expression and receptor function, and its putative function in the pathophysiology of PD.

Due to its intronic position and the predicted change of splicing regulatory elements by the risk allele of rs17689918, the expression of alternative spliced *CRHR1* isoforms was investigated using quantitative real-time PCR (qPCR) in a human post-mortem brain tissue sample. Of eight known *CRHR1* isoforms, expression of three *CRHR1* isoforms and the *CRHR1-IT1-CRHR1* readthrough transcript variant 5 – all expressing the seven transmembrane domains needed for functional receptors – was analyzed. Subsequently, electrophysiological assays were developed to measure the receptor activity of differentially expressed CRHR1 isoforms via co-expressed Kir2.3 potassium channels *in vitro*. In a second approach, possible epigenetic regulation of *CRHR1* expression by rs17689918 was investigated by analyses of DNA methylation patterns of a CpG Island within the *CRHR1*

promoter region, firstly in a case-control sample for PD and secondly in a healthy control sample, separated in high and low anxious individuals. To investigate a possible gene × epigene × environment interaction, the impact of early life stress by means of childhood trauma was evaluated via the childhood trauma questionnaire (CTQ). Finally, consequences of differential DNA methylation of the *CRHR1* promoter region on gene expression were investigated by luciferase-based reporter gene assays *in vitro*.

The expression of *CRHR1β* was significantly decreased in amygdalae and midbrains of risk allele carriers. The expression of *CRHR1-IT1-CRHR1* readthrough transcript variant 5 was significantly increased in forebrains and midbrains of risk allele carriers. All other analyzed isoforms showed no differences in expression between non-risk and risk allele carriers of rs17689918. The electrophysiological recordings of membrane potential showed an activation of Kir2.3 channels by CRHR1β in contrast to an inconsistent mix of activation and inhibition of Kir2.3 by the main isoform CRHR1α. DNA methylation of the *CRHR1* promoter region was significantly reduced in panic disorder patients, as well as in high anxious individuals of an independent healthy control sample, but no direct relation to the rs17689918 risk allele could be discerned. However, the combination of carrying the risk allele, low DNA methylation and high CTQ scores lead to increased sum scores in the Beck Anxiety Inventory (BAI) in healthy individuals. Functional analyses revealed an activation of gene expression by decreased DNA methylation of the promoter region *in vitro*.

Our results revealed that rs17689918 regulates CRHR1 function by increasing the expression of alternative transcript variants with altered function. Our analyses of DNA methylation revealed decreased methylation as a new risk factor for panic disorder and high anxious behavior, which in combination with other risk factors like childhood trauma and the rs17689918 risk allele might further increase cognitive and somatic anxiety symptoms. This supports the role of *CRHR1* as a plasticity gene of anxiety behavior, i.e. a gene that is highly regulated by epigenetic or post-transcriptional mechanisms in response to environmental stressors. By its role in CRF signaling, the dysregulation of CRHR1 might extensively affect the stress response and contribute to the pathophysiology of stress-related disorders like PD.

The understanding of the underlying mechanisms, especially the genetic and epigenetic regulation, would however enhance CRHR1 as a target of improved future therapeutics for PD and other anxiety disorders.

Zusammenfassung

Die Panikstörung manifestiert sich durch unerwartete, wiederkehrende Panikattacken, welche sich nicht auf bestimmte Situationen, Medikationen oder Stimuli zurückführen lassen. Bei der Panikstörung handelt es sich, wie bei allen psychischen Erkrankungen, um eine sogenannte multifaktorielle Erkrankung, d.h. sie entwickelt sich aus einem Zusammenspiel von genetischen Faktoren und Umweltfaktoren. Trotz einer geschätzten Heritabilität von bis zu 48% ist bisher kein eindeutiger genetischer Mechanismus bekannt, der zur Entwicklung einer Panikstörung führt. Mehrere Studien konnten eine Fehlregulation der Stressantwort bei Patienten mit Panikstörung feststellen. Dabei konnten mehrfach Polymorphismen in Genen des Corticotropin-Releasing Faktor (CRF) Systems mit Panikstörung assoziiert werden. Insbesondere der Hauptrezeptor von CRF im Gehirn, der Corticotropin Releasing Hormon Rezeptor 1 (CRHR1), konnte in mehreren Studien mit Panikstörung und anderen Angsterkrankungen assoziiert werden. In einer kürzlich erschienenen Studie wurde gezeigt, dass der *CRHR1* Einzelnukleotid-Polymorphismus rs17689918 die Genexpression von *CRHR1* in Gehirnregionen reguliert, die auch in Angsterkrankungen eine Schlüsselrolle spielen. Zusätzlich zeigten Risikoallelträger eine veränderte Gehirnaktivierung in fMRT Experimenten und ein verändertes Fluchtverhalten in einem Verhaltenstest (Weber *et al*, 2015). In der vorliegenden Studie wurden die zugrundeliegenden neurogenetischen und neurobiologischen Mechanismen untersucht, anhand derer das Risikoallel von rs17689918 die *CRHR1* Genexpression und Rezeptorfunktion beeinflusst, und welche Rolle diese in der Pathophysiologie der Panikstörung spielen.

Aufgrund der Position von rs17689918 im Intron von *CRHR1* und der *in silico* berechneten Änderung der Erkennungssequenz für Spleißregulatoren durch das Risikoallel von rs17689918 wurde die Expression alternativer Spleißformen von *CRHR1* mittels quantitativer real-time PCR in humanen *post-mortem* Gehirnproben analysiert. Insgesamt wurde die Expression von vier CRHR1 Isoformen und die Expression der *CRHR1-IT1-CRHR1* readthrough Transkriptvariante 5 analysiert. Für funktionelle Analysen wurden elektrophysiologische Assays entwickelt, um durch die Messung der Aktivität von ko-

exprimierten Kir2.3 Kaliumkanälen die Rezeptoraktivität der CRHR1 Isoformen *in vitro* bestimmen zu können. Zusätzlich wurde eine mögliche epigenetische Regulation der *CRHR1* Genexpression durch rs17689918 untersucht. Hierfür wurden DNA Methylierungsmuster eines Abschnittes einer CpG-Insel im Promoterbereich des *CRHR1* Gens in einer Fall-Kontroll-Stichprobe für Panikstörung und einer weiteren gesunden Kontrollstichprobe – unterteilt in eine hoch-ängstliche und eine niedrig-ängstliche Gruppe – analysiert. Um mögliche Gen-Epigen-Umweltinteraktionen zu untersuchen, wurde zusätzlich der Einfluss belastender Lebensereignisse in frühen Lebensabschnitten mittels dem *Childhood Trauma Questionnaire (CTQ)* erfasst. Die Funktionalität von differenziell methylierten Abschnitten der *CRHR1* Promoterregion wurde mittels Luziferase-basierten Reportergenassays *in vitro* untersucht.

Die Ergebnisse der Expressionsanalyse zeigen eine signifikant verminderte Expression der Isoform *CRHR1β* in Amygdala- und Mittelhirnproben von Risikoallelträgern. Gleichzeitig war *CRHR1-IT1-CRHR1* Transkriptvariante 5 in Vorderhirn- und Mittelhirnproben von Risikoallelträgern signifikant höher exprimiert. Alle anderen Isoformen zeigten keinen Expressionsunterschied zwischen Risiko- und Nicht-Risikoallelträgern von rs17689918. Elektrophysiologische Messungen des Membranpotentials fanden eine Aktivierung der ko-exprimierten Kir2.3 Kanäle durch Isoform CRHR1β, im Gegensatz zu einer inkonsistenten Regulation aus Aktivierung und Inhibition der Kanäle durch die Hauptvariante CRHR1α. Patienten mit Panikstörung zeigten eine geringere DNA-Methylierung der *CRHR1* Promoterregion im Vergleich zu gesunden Kontrollen. Gleichzeitig haben hoch-ängstliche gesunde Probanden eine geringere DNA Methylierung der *CRHR1* Promoterregion als weniger ängstlichen Probanden. Allerdings konnte kein Einfluss von rs17689918 auf die DNA-Methylierung gefunden werden. Probanden mit einer Akkumulation von Risikofaktoren wie dem Risikoallel von rs17689918, geringer DNA-Methylierung und hohen CTQ-Werten erreichen außerdem höhere Summenwerte im BAI. Die funktionellen Analysen zeigten eine Aktivierung der Genexpression infolge einer geringen DNA-Methylierung der differenziell methylierten CpG-Stellen.

Die Ergebnisse zeigen, dass rs17689918 die Funktion von CRHR1 durch eine Verschiebung der Expression zu alternativen, weniger oder nicht-funktionellen Spleißvarianten steuert. Zusätzlich zeigen die Analysen, dass eine verringerte DNA-Methylierung der *CRHR1* Promoterregion ein Risikofaktor für Panikstörung und erhöhtes Angstverhalten ist, welcher in Kombination mit weiteren Risikofaktoren wie Kindheitstraumata oder dem rs17689918 Risikoallel ängstliche Verhaltenszüge begünstigt. Dies unterstützt die Hypothese, dass *CRHR1* die Funktion eines sogenannten „Plastizitätsgens“ für ängstliches Verhalten und Angsterkrankungen hat, d.h. ein Gen dessen Expression durch epigenetische und posttranskriptionale Modulation in Reaktion auf Umwelteinflüsse reguliert wird. Durch seine wichtige Funktion im CRF-Signalweg könnte eine Fehlregulation von CRHR1 einen weitreichenden Einfluss auf die humane Stressantwort haben und somit auch zur Pathophysiologie von stress-bedingten Erkrankungen wie Panikstörung beitragen. Das Verständnis der zugrundeliegenden Mechanismen, besonders der genetischen und epigenetischen Regulation, würde dazu beitragen CRHR1 als möglichen Ansatzpunkt zukünftiger Therapien für Panikstörung und anderen Angsterkrankungen zu erschließen.

1. Introduction

1.1. Panic disorder

Prevalence and symptoms

It is estimated that every year about 27% of all adults of the European population are affected by mental disorders, causing estimated costs of several hundred million euros (Wittchen *et al*, 2011). The most frequent mental disorders are depressive disorders, like major depression, substance use disorders, like alcoholism, and anxiety disorders, like panic disorder (Wittchen *et al*, 2011). Panic disorder (PD) has a lifetime prevalence of about 5.2 % (Kessler *et al*, 2012) and a 12-month prevalence of about 1.8 % (Wittchen *et al*, 2011). With an average onset at the age of 23 years (Kessler *et al*, 2012) the DALY (disability adjusted life years) of panic disorder ranks at 10 in total samples and 8 in females among all neuropsychiatric disorders (Wittchen *et al*, 2011).

Panic disorder emerges as unexpected panic attacks which are recurrent and not restricted to certain situations or stimuli, like substances or medication (ICD-10, DSM-V; WHO, 1992; Kogan et al., 2016). These attacks are defined as episodes of intense fear and are often accompanied by characteristic autonomic symptoms like sweating, trembling, hot flushes or the fear of losing control (Kogan *et al*, 2016). Patients suffering from panic attacks often have a persistent fear or worry about future attacks and try to avoid situations where they experienced panic attacks. Therefore, they often suffer from impairment of their familial, social and economic life (Kogan *et al*, 2016). Panic disorder often occurs together with other comorbid psychiatric disorders (Schumacher *et al*, 2011).

Etiology of Panic Disorder

Like other psychiatric disorders, PD is a multifactorial disorder. By so called gene-environment interactions (G × E), several genetic risk factors together with environmental risk factors form a more vulnerable or more resilient intermediate phenotype that, under persistent influence of further environmental and genetic factors, eventually lead to the pathophysiology

of a disorder. These interactions are possibly mediated by epigenetic mechanism, like DNA methylation or histone acetylation (details in the epigenetic section below; Meaney and Szyf, 2005).

For panic disorder, family and twin studies revealed a heritability of up to 48 % (Hettema *et al*, 2001; Schumacher *et al*, 2011), leaving the remaining 52 % to environmental factors. The accumulation of life events is thought to impact on the etiology of panic disorder (Klauke *et al*, 2010), but no distinct risk factor could be elucidated yet. Also life events in critical time periods might further modulate the risk for panic disorder in concert with genetic factors, as it was shown for an interaction of a serotonin transporter polymorphism and childhood trauma leading to increased dimensional anxiety traits (Klauke *et al*, 2011). Moreover, perturbed conditioned or learned fear to a situation where previous panic attacks occurred, accompanied by a overgeneralization of fear might also contribute to the development of panic disorder (Lissek *et al*, 2010). Deficits in the fear regulation might thereby be again modulated by variants of known risk genes in the stress response and the serotonergic neurotransmitter system (Heitland *et al*, 2013).

Among the genetic risk factors are several candidate genes coding for important components of neurotransmitter systems, e.g. the monoamine oxidase A (*MAOA*) gene (Domschke *et al*, 2012; Reif *et al*, 2014), adenosine A2a receptor (*ADORA2A*) gene (Deckert *et al*, 1998; Hohoff *et al*, 2010) or neuropeptide S receptor 1 (*NPSR1*) gene (Domschke *et al*, 2011), as well as for important regulatory systems like the stress response (Keck *et al*, 2008; Weber *et al*, 2015). Like in other stress-related disorders, e.g. depression or posttraumatic stress disorder (PTSD), a dysregulation of the stress response is discussed as possible underlying mechanism in the pathophysiology of PD. Indeed, several studies showed an association of variations of candidate genes involved in the regulation of the stress response with anxiety disorders and depression (Ising *et al*, 2012; Keck *et al*, 2008; Shin and Liberzon, 2010). Further, the stress response in patients of depression and anxiety shows a highly dysregulated stress adaption (Gold, 2015; Shin and Liberzon, 2010). Moreover, a dysregulation of the HPA axis in patients suffering from panic disorder was also described

repeatedly, but replication of these findings are inconsistent, mostly due to difficulties in study design and measurements of HPA axis dysregulation in those patients (Abelson *et al*, 2007; Dieleman *et al*, 2015; Graeff, 2007; Graeff *et al*, 2005; Schreiber *et al*, 1996).

1.2. The human stress response

Since the first definition of the stress reaction by Hans Selyle, the “general alarm reaction” and “general adaption reaction” were extensively studied (Selye, 1936). Today, stress is defined as state of real or perceived threat to homeostasis of the body (Smith and Vale, 2006). The appropriate response to these threatening factors and the restoration of homeostasis is crucial for survival. The right amount of stress to the body and brain is considered as stimulatory and thus beneficial. Our stress perception thereby follows the concept of an inverted U, meaning the understimulation and overstimulation with stressors are less beneficial than the optimal level of stress in between (Sapolsky, 2015). The excess of stress in a chronic way, i.e. over a longer time period, is an overstimulation of the stress response which we perceive as stressful and harmful (Sapolsky, 2015). This perception of stressful stimuli depends on our individual stress response, which is regulated by the expression and abundance of certain mediators and receptors.

The chronic persistence of certain stressors is, according to the allostatic load hypothesis, a risk factor for many psychiatric disorders (McEwen, 2000), especially for so-called stress-related disorders like major depression and anxiety disorders. The stress response is disturbed either by chronic novel stressors that constantly activate the stress response or by a dysregulated secretion of stress hormones leading to a constant stress state (McEwen, 1998). Also, the stage of development at which stress is experienced has an impact on the stress response, and by this on the development of a dysregulation or disorder. Early life stress, like childhood maltreatment, has been shown to be a risk factor for psychiatric pathology (Cicchetti and Rogosch, 2001; Gunnar and Quevedo, 2007; Heim *et al*, 2001). Several studies could show an impact of prenatal stress and prenatal anxiety on stress response in later life (O’Connor *et al*, 2005, 2013). For instance O´Connor *et al.* could show

disturbances of cortisol levels in 10 year old children that were exposed to prenatal stress (O'Connor *et al*, 2005).

1.2.1. Stress pathways

SAM system

Two pathways – the sympathetic-adrenal-medullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis – regulate the human stress response. The SAM system regulates a fast response via adrenaline/noradrenaline release in response to a threat culminating in the typical "fight or flight" reaction. As a part of the sympathetic nervous system, preganglionic cells from the spinal cord form direct cholinergic synapses with chromaffin cells in the adrenal medulla. These cells secrete catecholamines like adrenaline and noradrenaline (Gunnar and Quevedo, 2007). Through binding to receptors throughout the body, catecholamines activate the "fight or flight" reaction by for instance increasing the heart rate, vasodilation to activate muscles, and increasing glucose levels in the blood to ensure availability of sufficient energy reserves for the brain and body (Gunnar and Quevedo, 2007). In the brain noradrenaline is also secreted by the locus coeruleus to ensure increased vigilance and attention for possible threats (Gunnar and Quevedo, 2007).

HPA axis

The slower stress response, and therefore more important for chronic stress experience, is mediated by the HPA axis (Figure 1). The stress response is initiated by the secretion of corticotropin releasing hormone or corticotropin-releasing factor (CRH or CRF) from parvocellular neurons of the paraventricular nuclei of the hypothalamus (PVH). The CRF-secreting neurons of the PVH receive various inputs, e.g. solitary-hypothalamic projections from the nucleus of the solitary tract, the periaqueductal grey or the locus coeruleus via noradrenergic projections. Also input from limbic structures, like hippocampus or amygdala, as well as from suprachiasmatic structures, important for instance for circadian rhythm, are known for PVH neurons. Thereby, the CRF signaling of PVH is highly regulated by local interneurons.

CRF signaling from PVH to the median eminence of the hypothalamus induces the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH in turn is transported to the adrenal cortex and stimulates the secretion of the corticosteroids, like cortisol in humans. Cortisol is synthesized in mitochondria of the adrenal gland from cholesterol under enzymatic catalysis of several enzymes like P450scc, cytochrome P450 and steroidogenic acute regulatory (StAR) protein. Cortisol has wide-ranging metabolic effects in the body, for instance induction of gluconeogenesis and activation of the lipometabolism to recruit the body's energy reserves. Additionally, it interacts with several transcription factors of the immune system (Barnes and Adcock, 1993; Igarashi *et al*, 2005; Saatcioglu *et al*, 1994). In general, i.e. under basal, non-stressful conditions, cortisol secretion follows a circadian rhythm with peak levels in the early morning and lowest levels in the evening together with an additional pulsatile secretion (Walker *et al*, 2012). The HPA axis is regulated by several positive and negative feedback mechanisms, like the regulation of StAR protein abundance by ACTH levels in the adrenal cortex.

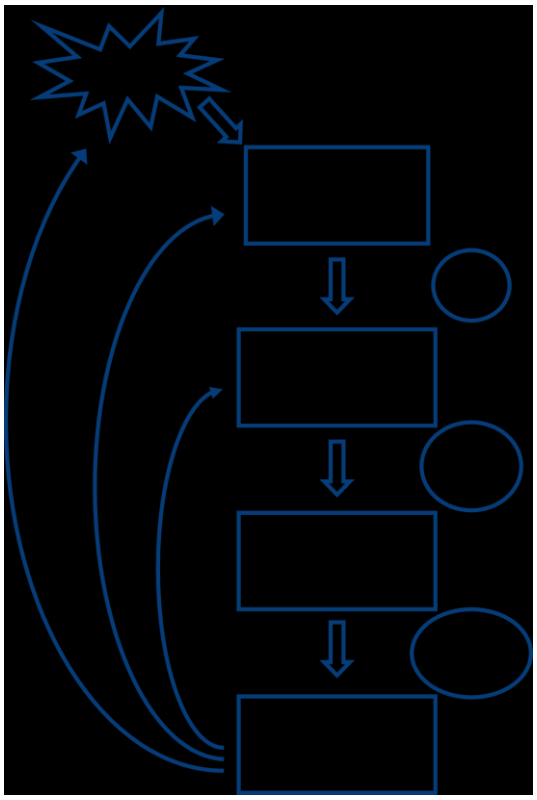

Figure 1: Schematic illustration of the stress response via the HPA axis. The HPA axis mediates the slow and chronic stress response via secretion of CRF from the paraventricular neurons of the hypothalamus, leading to ACTH release from the anterior pituitary gland. ACTH in turn activates cortisol release from the adrenal cortex, which initiates the stress response of the body by several metabolic effects. Positive and negative feedback loops, mostly mediated by action of glucocorticoid and mineralglucocorticoid receptors, regulate the different components of the HPA axis.

The effects of cortisol are delayed compared to those of the fast stress response due to its mode of action. While noradrenaline acts via receptor binding, cortisol works via regulation of gene expression. Hence, cortisol binds to glucocorticoid receptor (GR) or mineralocorticoid receptors (MR) in the cytoplasm of target cells and activates them. These activated receptors are then translocated to the nucleus, where they regulate the expression of downstream target genes (De Kloet *et al*, 1991). Under basal activity over ¾ of the MR are occupied while GRs are only activated during stress reaction or at peak levels (de Kloet *et al*, 1999; De Kloet *et al*, 1991). GR and MR activity can thereby have opposing effects, for instance increase or decrease the responsiveness to excitatory stimuli (De Kloet *et al*, 1991). It is thought that these opposing effects might mediate the salutary versus deleterious effects of stress (Sapolsky, 2015).

1.2.2. The CRF system

The signaling pathways of corticotropin-releasing factor (CRF) play an important role in the regulation of the stress response in general, and therefore are of special interest in several psychiatric disorders including PD. As described above, CRF is released from hypothalamus upon external stressors and initiates the activation of the HPA axis by signaling from the hypothalamus to the pituitary gland, which results in the release of ACTH and finally of cortisol from the adrenal gland (Figure 2; Vale *et al*, 1981; Rivier and Vale, 1983; Hauger *et al*, 2006; Binder and Nemeroff, 2010). By activation of noradrenergic signaling pathways in the locus coeruleus, CRF additionally influences the fast stress response of the SAM system in the limbic system and also directly triggers the release of corticosteroids from the adrenal gland (Binder and Nemeroff, 2010; Hauger *et al*, 2006). Those wide-reaching regulatory functions make CRF to a central regulator of stress response and stress-related signaling in the brain. Thereby, the CRF system is regulated via several different mechanisms including negative and positive feedback loops (Figure 2).

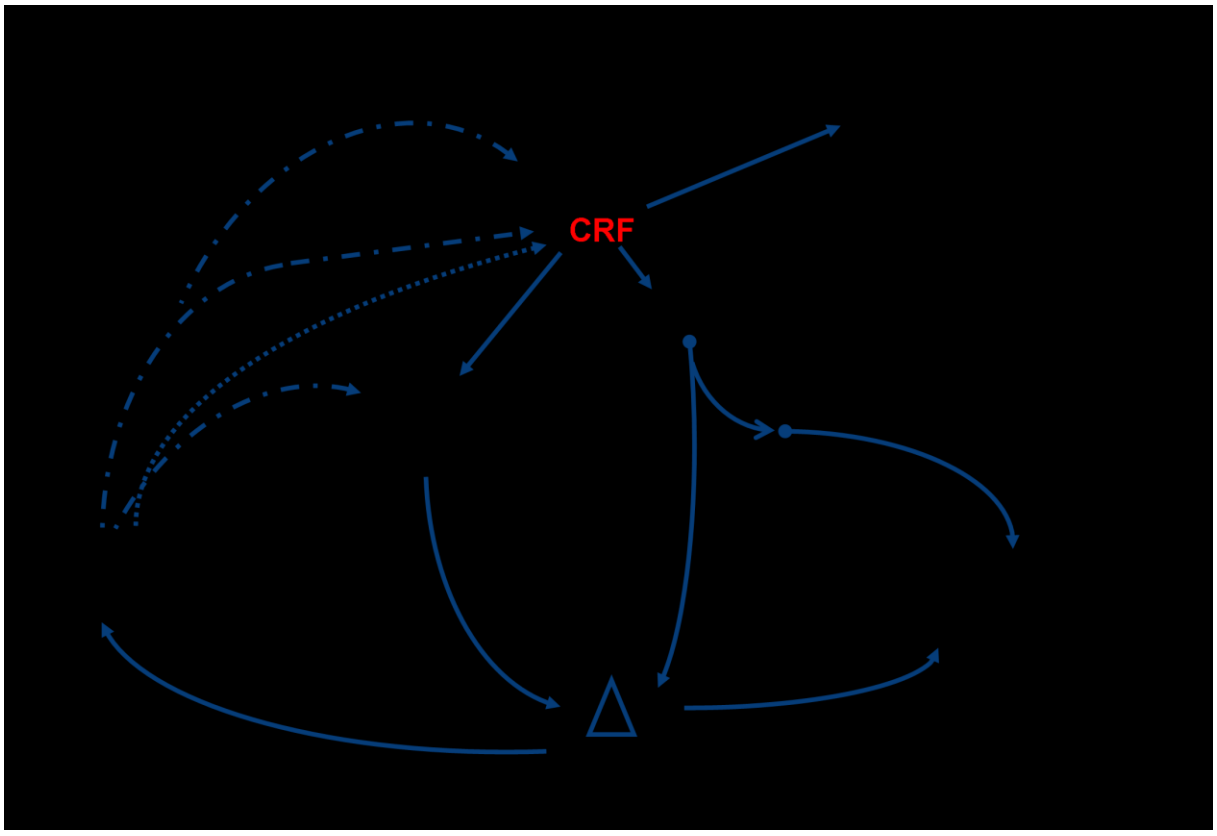

Figure 2: The CRF system regulates the stress response on various levels. By release from the hypothalamus, CRF mediates the ACTH release from the pituitary gland, which eventually enables corticosteroid release from the adrenal gland. Additionally, CRF activates cortisol and catecholamine synthesis directly in the adrenal gland. CRF innervates noradrenergic neurons through direct links to the locus coeruleus (LC) and by this affects the fast stress response via the SAM system. Centrally active CRF signaling in other brain regions like amygdala and hippocampus regulates several behavioral effects, for instance anxiety-related behavior, locomotion, learning and sleep. Glucocorticoids in turn regulate CRF release and function, as well as HPA axis activity via positive feedback (dashed line) and negative feedback (intermitted line) loops. Figure adapted from Binder and Nemeroff (Binder and Nemeroff, 2010).

Two distinct receptors exist for CRF – CRF receptor 1, also called corticotropin releasing hormone receptor 1 (CRHR1) and CRF receptor 2, also called corticotropin releasing hormone receptor 2 (CRHR2). Beside binding CRF, both receptors bind ligands of the urocortin protein family with different affinity, which is also thought of as a regulatory mechanism of CRF signaling (Binder and Nemeroff, 2010; Hauger *et al*, 2006). In the brain, CRHR1 shows highest affinity for CRF and is the major receptor in the brain, while CRHR2 is destined a more moderating role and is expressed in both the brain and the periphery (Binder and Nemeroff, 2010; Hauger *et al*, 2006). CRF binds with high affinity to CRHR1 (Janssen and Kozicz, 2013; Pioszak *et al*, 2008). Urocortin 1-3 but not CRF bind to CRHR2 underlining the assumption of a more moderating role of the second CRF receptor (Binder and Nemeroff, 2010; Janssen and

Kozicz, 2013; Pioszak *et al*, 2008). The abundance of all four ligands – CRF and Urocortin 1-3 – are regulated by the CRH binding protein (CRHBP), which regulates activation of the receptors by binding the ligands. CRHBP circulates in the blood stream and is co-expressed with CRF and CRHR1 in most relevant pathways indicating an important regulatory role for CRF signaling (Behan *et al*, 1995; Potter *et al*, 1992). By binding to CRHBP, CRF dimerizes and is inactivated. Thereby, activation of CRF signaling is regulated (Janssen and Kozicz, 2013). CRHR2 seems to have a more regulatory role, especially regarding the stress response, because its lacking of affinity for CRF, and also by its expression in the periphery, where it is for instance involved in the regulation of cerebral blood flow (Janssen and Kozicz, 2013). However, CRHR2 knockout mice show impairments of HPA axis maintenance and hypersensitivity to stress accompanied by anxiety-related behavior, which points to a more substantial role mediating sensitivity of the stress response via the HPA axis (Janssen and Kozicz, 2013). Anyway, the major receptor of CRF signaling regarding stress response in the brain is CRHR1.

1.3. Corticotropin releasing hormone receptor 1 – CRHR1

The corticotropin releasing hormone receptor 1 (*CRHR1*) gene spans over 51.5 kb on chromosome 17 in humans (Genomic location: 17q21.31), chromosome 10 in mice and chromosome 11 in rats. It codes for the eponym 415 amino acid (aa) long receptor CRHR1, whereas several alternative transcript variants are known (see section below, Pisarchik *et al*, 2001; Binder and Nemeroff, 2010). Corticotropin releasing hormone receptor 1 is a class B G-protein coupled receptor with seven characteristic transmembrane domains (Pioszak *et al*, 2008). Receptors bind ligands by a two-domain model, i.e. a N-terminal extracellular domain interacts with a C-terminal domain of the ligand and thereby activates the receptor (Hoare, 2005; Pioszak *et al*, 2008). The main signaling pathway via the activation of adenylyl cyclase increases intracellular cyclic adenosine monophosphate (cAMP) levels, whereas also alternative signaling via phospholipase C is known for the receptor (Seymour *et al*, 2003). *CRHR1* is expressed abundantly throughout the brain with highest expression in brainstem, medial and basolateral amygdala and cerebellum (Binder and Nemeroff, 2010; Potter *et al*, 1994). It represents the main regulator of CRF-action in the brain by binding CRF with highest affinity (Pioszak *et al*, 2008; Seymour *et al*, 2003).

CRHR1 variations and gene expression are frequently associated with anxiety and stress-related disorders. In recent years, *CRHR1* emerged to a candidate gene for anxiety and stress-related disorders. Along these lines, global *Crhr1* knockout mice have been reported to display decreased anxiety-like behavior along with blunted ACTH and cortisol levels (Timpl *et al*, 1998). Accordingly, conditional anterior forebrain (Wang *et al*, 2012) and limbic brain structure *Crhr1* knockout (Müller *et al*, 2003) as well as knockdown of *Crhr1* mRNA expression in the basolateral amygdala (Sztainberg *et al*, 2010) induced a significant decrease in anxiety levels. In a juvenile rhesus macaque model anxious temperament (AT) – analogous to a human childhood anxiety-risk phenotype – and related brain metabolic activity were influenced by *CRHR1* variation (Rogers *et al*, 2013).

In humans, deficits in fear acquisition were shown to be driven by risk allele of *CRHR1* SNP rs878886 (Heitland *et al*, 2013, 2015). The association of several *CRHR1* polymorphisms

(rs12944712, rs110402, rs12938031, rs4792887, rs242924) with stress- or anxiety-related phenotypes has been extended to a clinical context, i.e. PTSD (Amstadter *et al*, 2011; Boscarino *et al*, 2012; White *et al*, 2013) and PD (Ishitobi *et al*, 2012; Keck *et al*, 2008). In a recently published study from our department we could show an association of *CRHR1* SNP rs17689918 risk allele with panic disorder (Weber *et al*, 2015). Interestingly risk allele carriers of rs17688918 also scored higher on dimensional phenotypes for anxiety and agoraphobia, and showed decreased gene expression in amygdala and forebrain samples of an independent post-mortem brain tissue sample (Weber *et al*, 2015). Further, rs17689918 seems also to affect the functional and behavioral level as fMRI data showed differential activation of dorsolateral prefrontal cortices of risk allele carriers in a fear-conditioning task and increased activation of the amygdala in a safety-learning task. Confronted with stressful situation in an exposure test (cf. Richter *et al*, 2012), panic disorder patients carrying the rs17689918 risk allele could persevere better, accompanied by smaller increase in heart rates but rather increased anxious apprehension (Weber *et al*, 2015). This was interpreted as a mechanism in particular relevant for chronic anxious apprehension as opposed to acute panic and its strong autonomic arousal (cf. Richter *et al*, 2012; Hamm *et al*, 2014). *CRHR1* rs17689918 thereby regulates CRHR1 function presumably by shifting expression patterns and by this, protein abundance in relevant brain regions for panic disorder and anxious behavior.

It could not be clarified how rs17689918 risk allele regulates CRHR1 on multiple levels. *In silico* analyses of the sequence resulted in a predicted alteration of a regulatory sequence for splicing factors dependent on risk and non-risk alleles. So a regulation of the expression of alternative splicing transcripts of *CRHR1* dependent on rs17689918 genotype would be one possible underlying mechanism (Weber *et al*, 2015). As a SNP can influence gene expression and protein function in various ways, also other ways of regulation of gene expression are conceivable despite its intronic position. Weber *et al.* revealed 90 perfect proxy SNPs, i.e. SNPs in perfect linkage disequilibrium with rs17689918 (Weber *et al*, 2015). Indeed, none of them lies in the promoter region, but for instance an epigenetic regulation of *CRHR1* gene

expression dependent on genotypes of rs17689918 or one of its proxy SNPs might be another possible underlying mechanism.

1.3.1. Alternative Splicing of CRHR1

Splicing is a crucial mechanism to facilitate the diversity of proteins from the human genome. After transcription RNA contains all gene information including introns and exons. Already in the nucleus pre-mature RNA undergoes the process of RNA splicing where introns are removed by the spliceosome, which is built by small non-coding RNAs (snRNAs) and proteins. Correct recognition of the splice site is ensured by distinct splicing regulatory elements (SRE). Subsequently the 5' and 3' UTRs are modified and the resulting mRNA is transported to the cytoplasm and eventually translated into proteins. The mRNA thereby can be spliced in different ways and hence, produce different proteins. It is estimated that this alternative splicing of pre-mRNA accounts for one third of the protein diversity in humans and thereby potentiates the number of possible proteins that are expressed from the genome (Black *et al*, 2000). The splicing of alternative transcripts can be regulated depending on different environmental factors, like stress, as well as factors like sex, age or genotype (Black *et al*, 2000).

For *CRHR1,* eight alternative spliced transcript variants exist in humans (Table 1). Additionally, two more variants exist as readthrough of the upstream *CRHR1-Intron 1* (*CRHR1-IT1*) gene and *CRHR1*. The *CRHR1* gene contains 14 exons. The main variant CRHR1α contains all seven transmembrane proteins and the crucial regions of exon 3 that are necessary for efficient CRF binding (Pisarchik *et al*, 2001; Pisarchik and Slominski, 2004). CRHR1α is the most efficient isoform of CRHR1 showing the best signal transduction (Pisarchik *et al*, 2001).

Table 1: Overview of the CRHR1 isoforms. Eight isoforms are directly transcribed from *CRHR1* (α-h) and two are readthrough variants of *CRHR1* and the upstream *CRHR1-IT1*. Information on length and exons were derived from the NCBI website and Grammatopoulos and Chrousos (Grammatopoulos and Chrousos, 2002). aa = amino acids; UTR = untranslated region

Isoform	Protein length	missing Exons
CRHR1α	415 aa	6
CRHR1β	444 aa	–
CRHR1c	375 aa	3 and 6
CRHR1d	401 aa	6 and 12
CRHR1e	194 aa/ 240 aa	3, 4 and 6; two reading frames
CRHR1f	370 aa	6 and 11
CRHR1g	341 aa	6, 10 and partly 9 and 11
CRHR1h	145 aa	cryptic exon between 4 and 5
CRHR1-IT1-CRHR1 transcript variant 5	240 aa	readthrough variant with alternate 5' UTR
CRHR1-IT1-CRHR1 transcript variant 6	314 aa	readthrough variant with alternate 5' UTR

The longest isoform *CRHR1β* showed an impaired cAMP production which is 100 times reduced compared to *CRHR1α* (Nabhan *et al*, 1995; Pisarchik *et al*, 2001). *CRHR1c* lacks exon 3, which codes for the binding domain for CRF and therefore might have a poor CRF affinity. *CRHR1e-g* all contain frameshifts causing either translation stop signals or deletion of functional transmembrane domains. It is assumed that alternative splice variants of *CRHR1* that contain the CRF binding domain but not the transmembrane domains might act in a regulatory way analogue to the function of CRHBP (Pisarchik *et al*, 2001). Interestingly, *in vitro* experiments with cell culture showed a shift of expression between the alternative transcripts in response to environmental stress. Pisarchik *et al.* showed that cells treated with UV radiation increase expression of both *CRHR1α* and *CRHR1g* over other transcript variants (Pisarchik *et al*, 2001). This effect is considered a permanent shift as it persists in the following generations of cells. However, the study did not show if *CRHR1g* expression is increased in response to the stressor or in a more regulatory way upon increased CRF or *CRHR1α* expression.

As discussed above, CRHR1 is regularly associated with anxiety and stress-related disorders, but so far no studies could show a link between alternative splicing of *CRHR1* and psychiatric disorders, or other components of the CRF system (Binder and Nemeroff, 2010). Nevertheless, genetic variations in *CRHR1*, which regulate the expression of alternative

spliced transcript variants, might have a large impact on CRHR1 receptor function and thereby on activity of the HPA axis.

1.3.2. Epigenetic regulation of CRHR1

Epigenetic mechanisms are defined as alterations of a phenotype without permanent changes of the genotype and were first characterized by Waddington (Waddington, 1942). Today an epigenetic trait is defined as a "*stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"* (Berger *et al*, 2009). Thereby three aspects are important. First, an epigenetic mechanism has to be independent from its initial signal and self-sustaining without it. Second, it does not modify the sequence of the DNA itself and third, it has to be heritable from mother to daughter cell either via mitosis or meiosis (Berger *et al*, 2009; Dulac, 2010). The best-investigated epigenetic mechanisms are DNA methylation, histone modifications and action of non-coding RNAs (ncRNA) like micro RNA (miRNA; Figure 3).

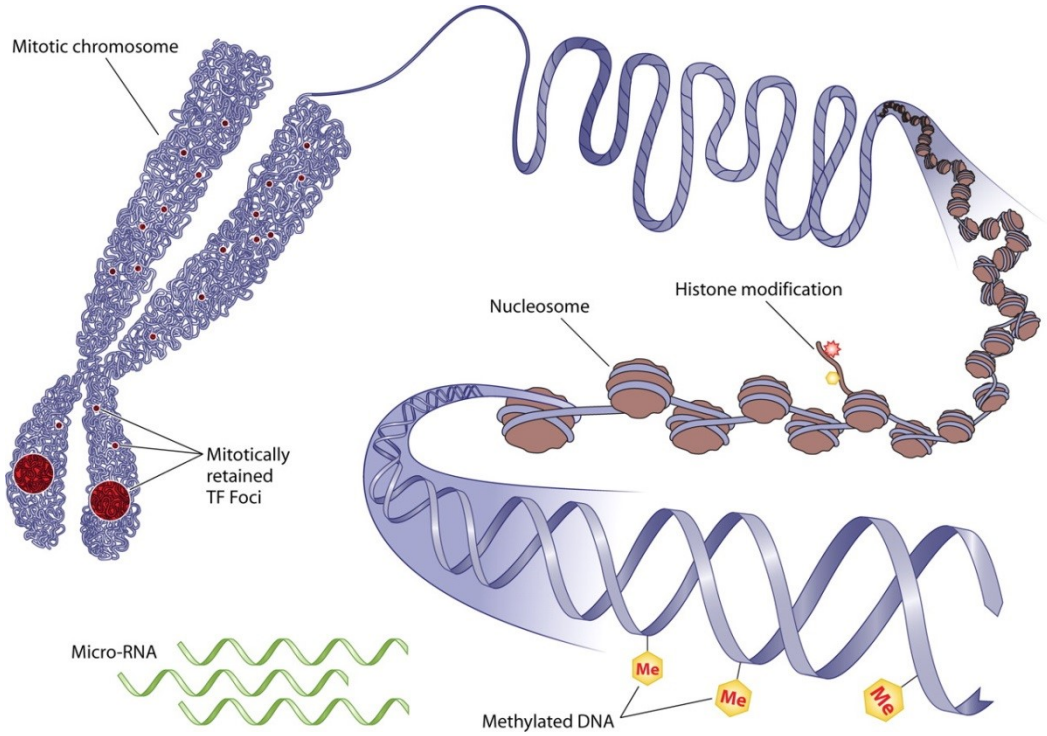

Figure 3: Epigenetic mechanisms regulate gene expression in multiple ways. Histone modifications (red and yellow stars) like acetylation or methylation of histone proteins regulate density of the chromatin. The denser the packing of the DNA around the nucleosomes, the less possible is the transcription of genes from DNA. DNA methylation activates or de-activates gene expression by chemical modification of certain bases (Jaenisch and Bird, 2003). Especially DNA methylation of gene promoter regions is considered to silence gene expression. Post-transcriptional mechanisms include miRNAs. About 20 bp long, miRNAs recognize corresponding mRNA sequences, bind to them and activate the protection mechanism of the cell, called RNA interference, which leads to degradation of the bound mRNA. Figure adapted from Zaidi et al. (Zaidi *et al*, 2010) Copyright © 2010, American Society for Microbiology.

Histone modification

In order to store DNA in the nucleus of the cell it has to be condensed 1,000 times and reduced to one third of its length. Therefore, the human DNA is packed into nucleosomes consisting of four histone proteins. With the aid of nucleosomes, DNA is packed into more condensed structures of chromatin and eventually chromosomes. Depending on the density of DNA packaging the expression or repression of genes in the respective area is regulated. Two distinct forms of chromatin exist – an open and a dense form. The open form, describing loose packed DNA ready for gene transcription, is called euchromatin. A more protected and dense form of chromatin that does not allow gene transcription is known as heterochromatin. Packaging of the chromatin into its different forms is accomplished with the aid of chromatin remodeling complexes that in turn are dependent on modifications of N-terminal tails of the

histone proteins. These modifications differ between the single histone proteins and can be phosphorylation, acetylation or methylation. These epigenetic histone modifications regulate density of chromatin and by this gene expression. This can be a short-term inactivation or even passed on to daughter cells. In terms of transcriptional regulation, for instance transcription activators recruit histone acetyltransferases, which transfer acetyl residues to the N-terminal ends of histones, and thereby make the underlying genes more accessible for the cell's transcriptional machinery. In turn, histone deacetylases attracted for instance by transcription repressors remove histone residues, making chromatin structure denser and thus, prevent gene expression. It is estimated that about half of the whole genome is regulated by epigenetically modified histones (Telese *et al*, 2013).

MicroRNA

A second form of epigenetic regulation of gene expression is the post-transcriptional regulation of mRNA translation by miRNA. Estimations from current sequencing analyses showed that about three-quarters of the human genome is transcribed but only one third of this is actually within genomic regions, letting the remaining two thirds to short and long ncRNA. An important role within the group of ncRNAs play miRNAs (Telese *et al*, 2013). MiRNAs form RNA-induced silencing complexes (RISC) together with proteins that recognize corresponding mRNA sequences and bind to them. Nucleases of RISC together with endonucleases like Dicer then lead to degradation of the mRNA without translation into protein. The process, called RNA interference, is also a defense mechanism to protect the cell from external mRNA introduced for instance by viruses or other pathogens. MiRNAs mostly bind common sequences in the untranslated regions of target mRNA and thus, can target more than on type of mRNA at once.

Expression and regulation of miRNA was recently associated with several psychiatric disorders, like bipolar disorder and schizophrenia (Hommers *et al*, 2014; Ripke *et al*, 2011; Whalley *et al*, 2012). Regarding anxiety disorders, miRNA 4717-5p could be associated with anxiety-related traits possibly by regulation the expression of candidate genes like *RGS2* and

CNR1 (Hommers *et al*, 2014, 2015). Our recently associated *CRHR1* SNP rs17689918 showed no differentially binding miRNA as did none of its proxy SNPs (Weber *et al*, 2015). Thus, no further miRNA analyses regarding regulation of *CRHR1* gene expression were performed within this study.

DNA methylation

The best investigated epigenetic mechanism up to date is the process of DNA methylation. DNA methylation describes the covalently binding of a methyl group to the cytosine base of the DNA. This chemical modification in most cases occurs on a so-called CpG site, i.e. a cytosine (C) base is followed by a guanine (G) base with a phosphate (p) residue in between. CpG sites are not randomly distributed in the genome, but several cluster with high density of CpG sites exist, so called CpG islands (Bird, 1980; Jaenisch and Bird, 2003). In general, CpG islands are unmethylated but can be methylated as a regulatory mechanism, for instance during cell or tissue development (Jaenisch and Bird, 2003). In human somatic cells 70-80 % of all CpG sites are methylated, while during embryogenesis only about 30 % are methylated (Bird, 2002). After implantation CpG sites are *de novo* methylated by DNA methyltransferases DNMT3a and DNMT3b. The methyltransferase DNMT1 maintains the methylation pattern in somatic cells by a semiconservative mechanism (Bird, 2002). CpG sites are palindromic sequences and the parental strand still carries the methylation. Hence, DNMT1 enzymes recognize the parental methylation and add methyl residues to the newly synthesized complementary DNA strand at the respective site. The importance of DNA methylation for the cell development and maintenance was shown by gene expression knockdown experiments of methyltransferases *DNMT1* or *DNMT3*. Complete knockout of *DNMT1* and *DNMT3* expression lead to lethal mice at embryonic state. Conditional knockout of *DNMT1* and *DNMT3* in forebrains of mice lead to reduced synaptic plasticity and thereby to deficits in learning and memory due to dysregulated maintenance of DNA methylation in the region (Feng *et al*, 2010). Further, DNMT3 has been shown to be an important regulator of emotional behavior by mediating synaptic plasticity in the nucleus accumbens (LaPlant *et al*, 2010).

Demethylation of CpG sites can occur by several mechanisms including removing the methyl group by deaminases and subsequent base excision repair, or the conversion of methylated cytosine (5-mC) to hydroxymethylated cytosine (5-hmC) by catalysis of ten eleven translocation enzymes (Tet). 5-hmCs are then further deaminated to 5-hydroxyuracils and again excised by DNA repair mechanisms (Telese *et al*, 2013). Despite being seen as a more stable modification in adult cells, DNA methylation could be shown to be highly plastic and activity-dependent in studies in rodents (Guo *et al*, 2011).

The impact of DNA methylation is highly dependent on the genetic region. While methylation of CpG sites and islands in the gene body is generally associated with active genes, DNA methylation on gene promoters is linked with repression of gene expression due to possible blocking of recognition elements of the transcriptional machinery (Suzuki and Bird, 2008). However, more recent studies showed that also non-promoter methylation impacts gene expression (Wu *et al*, 2010), leading to the assumption of a more diverse function of DNA methylation in the regulation of gene expression.

Moreover, DNA methylation has been shown to be important for the regulation of gene expression in response to environmental factors. Several studies investigated the change of epigenetic regulators, especially DNA methylation on genes of the HPA axis in response to environmental stressors. In 2007, a study in rats could show that depending on the amount of maternal care, pups show differential histone acetylation and DNA methylation followed by differential gene expression of glucocorticoid receptors in the hippocampus (Weaver *et al*, 2007). This differential expression was enabled due to increased binding of certain transcription factors known to regulate GR expression (Weaver *et al*, 2007). Mice exposed to prenatal stress showed increased ACTH and CRF levels in amygdalae accompanied by decreased DNA methylation of the GR gene *Nr3c1* and increased *Nr3c1* expression in hippocampus (Mueller and Bale, 2008). Also in humans it was found that suicides with a history of child maltreatment have decreased *NR3C1* expression and increased gene methylation (McGowan *et al*, 2009).

In psychiatric disorders, epigenetic mechanisms and especially DNA methylation might underlie the long investigated “hidden heritability” – the link between genetic and environmental factors. First studies with respect to anxiety disorders showed differential DNA methylation patterns in the monoamine oxidase A (*MAOA*) and glutamate decarboxylase 1 (*GAD1*) genes as well as in the oxytocin receptor (*OXTR*) gene to be associated with panic disorder (Domschke *et al*, 2012, 2013; Ziegler *et al*, 2016) and social anxiety disorder (Ziegler *et al*, 2015), respectively. It was shown recently that DNA methylation in the *MAOA* gene was decreased in panic disorder patients but could be increased with cognitive behavioral therapy (Ziegler *et al*, 2016). This reversibility was only observed in successfully treated panic disorder patients but not in therapy non-responder (Ziegler *et al*, 2016).

To date, no data are available on the role of *CRHR1* methylation in regard to anxiety disorders despite evidence from a rodent study for *Crhr1* promoter demethylation induced by gestational hypoxia to be associated with anxiety-like behavior (Wang *et al*, 2013). Another study by Sotnikov *et al*. showed bidirectional alterations of *Crhr1* gene expression in the amygdala of high anxious (HAB) and low anxious (LAB) mouse strains (Sotnikov *et al*, 2014). HAB and LAB mice thereby underwent a treatment of chronic mild stress or enriched environment and were analyzed for anxiety-related behavior as well as differences in genetic and epigenetic markers. Expression of *Crhr1* decreased in the amygdala of LAB and increased in the amygdala of HAB. Interestingly, these alterations in *Crhr1* expression were accompanied by an increased methylation of *Crhr1* promoter region. Finally, it could be shown that certain transcription factors regulate *Crhr1* expression in a methylation dependent manner (Sotnikov *et al*, 2014). However, so far no study investigated DNA methylation pattern of *CRHR1* promoter region in humans, which might add further evidence to an epigenetically regulated gene expression of *CRHR1* in response to environmental stimuli.

1.4. Aim of the study

Based on the previous study showing the impact of *CRHR1* rs17689918 on several levels, from gene expression in distinct brain regions to behavioral and functional domains (Weber *et al*, 2015), we here aimed to characterize the functional basis underlying this association. As outlined above, due to its intronic position and the change of splice factor regulatory elements (SRE), rs17689918 might influence CRHR1 function by changing the expression of alternative spliced transcript variants. Hence, in a first approach we investigated expression of alternative splicing of *CRHR1* in human brain tissue samples. Further, to clarify functional consequences of differentially expressed transcript variants, respective alternative variants were characterized by electrophysiological measures in the heterologous expression system of *Xenopus laevis* oocytes.

Given the evidence for a genetically driven and in particular epigenetically modified function of *CRHR1* in anxiety disorders, we studied the epigenetic regulation of *CRHR1* gene expression for the first time in humans. To this end, human *CRHR1* gene sequence was analyzed *in silico* for CpG islands, followed up by analyses of DNA methylation regarding its role in panic disorder by applying a case-control approach. Additionally, we analyzed the association of *CRHR1* methylation with a dimensional phenotype for anxiety – the Beck Anxiety Inventory (BAI) in interaction with early life stress measured by means of the Childhood Trauma Questionnaire (CTQ). Finally, the functional relevance of altered *CRHR1* promoter methylation was investigated by means of luciferase-based reporter gene assays.

We hypothesized to discern an influence of rs17689918 on the expression of alternative splicing variants, leading to a shift to CRHR1 variants with differential function. Influence on DNA methylation pattern might be less strong due to the SNPs distal position. However, elucidating the role of DNA methylation of *CRHR1* might help to clarify the function and regulation of CRHR1 and the CRF system in anxiety disorders and particularly in panic disorder.

2. Materials and Methods

2.1. Materials

Plasmids, vectors

pcDNA3.1(+)-CRHR1beta	Creative Biogene, Shirley, USA
pCpGL-basic	RG Rehli, Regensburg, Germany
pCPGL-CMV/EF1α	RG Rehli, Regensburg, Germany
pCpGL_CMV/EF1α	own production
pCpGL_CRHR1	own production
pGL4.74	Promega, Mannheim, Germany
pSGEM	RG Wischmeyer, Würzburg, Germany
pSGEM-CRHR1α	own production
pSGEM-CRHR1β	own production

NCBI Refseq accession numbers

CRHR1α	NM_004382
CRHR1β	NM_001145146
CRHR1c	NM_001145147
CRHR1d	NM_001145148
CRHR1-IT1-CRHR1 readthrough, transcript variant 5	NM_001256299

Oligonucleotides

Name	Sense 5' → 3'	Antisense 5' → 3'
CMV/EF1alpha_amplification	AGATCTGGAGTCAATGGGAAAAACC	AAAGCTTGAATGTTCACAGAGACTA
CRHR1_expression	AATTACTCCGAGTGCCAGGAGA	AGATACAGTGGCCCAGGTAGT
CRHR1_isoform1 β	TGGTGCCCCATTTCAGGTTC	TAGTTGTAGGCGGCTGTCAC
CRHR1_isoform1 c	AGCAACATCTCAGACAATGGCTA	CACCAGGGAGATACAGTGGC
CRHR1_isoform1 d	GGAATCCTTCCAGGTCCGTT	GACTGCTGTGGACTGCTTGA
CRHR1_meth_P1	AGTTTTGTTTATTTGGAAGGGTTGG	ACCCTTTAAACCTAAAAACCCCAAA
CRHR1_rs17689918	AGCCACTGGGGGTCTTCATA	AAAGTTGGAAGCAAGCACCG
CRHR1_variant5	TCTAGGCTTGGTTGAACCGT	ACAATTCATGGGGACCCTGC
CRHR1alpha_mutagenesis	CCTTTGTCCTCTTTCTGCGGCTCAGGA GCATCCGGTGCCTGCGAAACATC	GATGTTTCGCAGGCACCGGATGCTCC TGAGCCGCAGAAAGAGGACAAAGG
GUSB	GCCGACTTCTCTGACAACCG	GGAACTGGCATGTCCACGG
HPRT1	TGCTTTCCTTGGTCAGGCAG	TTCGTGGGGTCCTTTTCACC
pCpGL_CMV_colony	TGTTTATGTGAGCAAACAGCAGA	GGGGCAGGGCCTTTCTTAAT
pCpGL_colony	TAAATTTTTTTGTTTAGTTTTTTGTTTTT A	CCATTAAAAAATCCCTATTAACTCAATA TA
pCpGL_seq_triplicate	TTGTTTATGTGAGCAAACAGCA	GGTTTTTCCCATTGACTCCA
pSGEM_CRHR1_ATG	GTGGCGGCCGCGCCACCATGGGAGG GCACCCGCAG	CTGCGGGTGCCCTCCCATGGTGGCG CGGCCGCCAC
pSGEM_seq rev		TTCGGGTGTTCTTGAGGCTGG
pSGEM_T7_seq	TAATACGACTCACTATAGGG	

SDHA	AACATCGGAACTGCGACTC	CTTCTTGCAACACGCTTCCC
TFRC	CGCGCTAGTGTTCTTCTGTG	TGACAATGGTTCTCCACCAAA
Triplicate insert	gTCGCCGCGCTGCAACGCAATGCGCG CCCGGCTTTTCGCCGCGCTGCAACGC AATGCGCGCCCGGCTTTTCGCCGCGC TGCAACGCAATGCGCGCCCGGCTTTg	acgtcAGCGGCGCGACGTTGCGTTACG CGCGGGCCGAAAAGCGGCGCGACGT TGCGTTACGCGCGGGCCGAAAAGCG GCGCGACGTTGCGTTACGCGCGGGC CGAAAcctag

Agonist, Antagonist

Corticotropin Releasing Factor, human	Sigma-Aldrich, Taufenkirchen, Germany
Corticotropin Releasing Factor, human	Tocris, Wiesbaden, Germany
CP-154526	Sigma-Aldrich, Taufenkirchen, Germany

Equipment

Device name	Manufacturer	Type
Amplifier	npi electronic, Tamm, Germany	Turbo Tec-10 C
Autoclave	Systec, Linden, Germany	V150
Cell counting device	Nexcelom, Lawrence, USA	Cellometer Auto T4
Cell culture safety hood	NuAire, Eurasburg, Germany	ClassII Biological Safety Cabin
Centrifuge	Hettich Zentrifugen, Tuttlingen, Germany	Rotofix 32A
Freezer (-20°C)	Liebherr, Biberach, Germany	
Freezer (-80°C)	Fisher Scientific, Schwerte, Germany	Forme -86°C
Gel chamber	BioRad Laboratories, Munich, Germany	B2
Heated shaker	EB Johanna Otto, Hechingen, Germany	TH15
Incubator (Cell lines)	Thermo Scientific, Munich, Germany	Hera Cell 150
Incubator (Oocytes)	Memmert, Schwabach, Germany	IPP 30
Luminometer	Promega, Mannheim, Germany	Glomax Multi Detection system
Micro pipette puller	Sutter Instrument, Novato, USA	P97
Micro scissor	Fine Science Tools, Heidelberg, Germany	
Microscope binocular	Wild Heerbrugg, Heerbrugg, Switzerland	204946
Microwave	Sharp, Hamburg, Germany	Express
Nano Injector	World Precision Instruments, Sarasota, USA	Nanoliter 2010
Perfusion system	ALA Scientific Instrument	12031041
Photometer	peqLab, Erlangen, Germany	NanoDrop ND1000
Photometer	Amersham Systems, Amersham, UK	GeneQuant pro
Pump	ALA Scientific Instruments, NY, USA	12031041
Scale	Mettler Toledo, Giessen, Germany	PM300

Thermocycler	Biometra, Göttingen, Germany	Biometra Thermocycler
Thermocycler (real-time)	BioRad, Munich, Germany	CFX384 real-time Thermocycler
Thermomixer	Eppendorf, Hamburg, Germany	Thermomixer comfort
UV-developer	BioRad, Munich, Germany	ChemiDoc UV Shield
Vacuum pump	Welch, Alton, Hampshire, UK	Standard Lab-Duty oil free pump
Voltage device	Consort, Turnhout, Belgium	E431
Vortex Mixer	Eppendorf, Hamburg, Germany	Microspin FV-2400
Water bath	Grant Instruments, Cambridgeshire, UK	JB Aqua 12
Water still	Thermo Scientific, Munich, Germany	Barnstead Nanopure Diamond

Buffers and stock solutions

Name	Recipe
Blue loading buffer	25 mg bromophenol blue-xylene cyanole 1.5 ml glycerol filled to 10 ml w/ ddH_2O
DEPC-treated ddH2O	0.1% v/v diethyl dicarbonate (DEPC) ddH_2O incubated for 1 h autoclaved for at least 15 min
High potassium (hK^+) buffer	2 mM NaCl 96 mM KCl 1.8 mM $CaCl_2$ 1 mM $MgCl_2$ 5 mM HEPES filled w/ ddH_2O; pH 7.4
High salt LB medium	5 g/l NaCl 5 g/l yeast extract 10 g/l tryptone pH 7.2 at 37°C; autoclaved
LB-Agar	15 g Select Agar filled to 1 l w/ LB medium; autoclaved
Low salt LB medium	0.5 g/l NaCl 5 g/l yeast extract 10 g/l tryptone pH 7.2 at 37°C; autoclaved
Lysis buffer	155 ml 1 M NH_4Cl 10 ml 1 M $KHCO_3$ 200 µl 0.5 M EDTA, ph 8.0 filled to 1 l w/ ddH_2O
MOPS buffer	0.1 M MOPS 40 mM NaAc 5 mM EDTA pH 8.0 filled w/ ddH_2O; pH 7,0
ND96	96 mM NaCl

	2 mM KCl 1.8 mM $CaCl_2$ 1 mM $MgCl_2$ 5 mM HEPES filled w/ ddH_2O; pH 7.4
$ND96^+$	2.5 mM Sodiumpyruvate 100 µg/ml gentamicin filled w/ ND96; pH 7.4
PCR buffer	500 µl 1 M KCl 100 µl 1 M tris-HCl pH 8.3 25 µl 10% Polysorbate 20 (Tween 20) 100 µl 0.1 M $MgCl_2$ 200 µl ddH_2O
RNA-loading buffer	6,5% Formaldehyde 50% Formamide 5% Glycerol 0.1 mM EDTA 0.025% Bromophenol blue filled with 1x MOPS buffer
SE buffer	75 mM NaCl 50 ml 0.5M EDTA ph 8.0 filled to 1 l w/ ddH_2O
TAE buffer	121 g/l tris 28.5 ml glacial acetic acid 50 ml EDTA 0.5 M, pH 8.0 filled to 1 l w/ ddH_2O
TE buffer	10 mM tris, pH 8 1 mM EDTA
Tricain 0.1%	1g Tricain 1 g $NaHCO_3$ filled to 1 l w/ ddH_2O

Kits

Description	Manufacturer, City, Country
Dual Luciferase Assay Kit	Promega, Mannheim, Germany
EpiTect 96 Bisulfite Kit	Qiagen, Hilden, Germany
FlexiGene DNA Kit	Qiagen, Hilden, Germany
Hotstar Taq Plus Polymerase Mastermix	Qiagen, Hilden, Germany
iScript™ cDNA Synthesis Kit	BioRad, Munich, Germany
NucleoSpin® Gel and PCR Clean-up	Machery-Nagel, Düren, Germany
PureYield™ Plasmid Midiprep System	Promega, Mannheim, Germany
QiaEx II Gel Extraction Kit	Qiagen, Hilden, Germany
QuikChange II Site-Directed Mutagenesis Kit	Agilent Technologies, Waldbronn, Germany
RNeasy Mini Plus Kit	Qiagen, Hilden, Germany
SYBR® Select Master Mix for CFX	Life Technologies, Darmstadt, Germany
TaqMan® Universal Master Mix	Life Technologies, Darmstadt, Germany

TransFast Transfection reagent	Promega, Mannheim, Germany
Wizard ® Plus SV Miniprep DNA Purification System	Promega, Mannheim, Germany

Chemicals

0.05 % trypsine- EDTA	Gibco, Karlsruhe, Germany
0.5 M EDTA pH 8.0	AppliChem, Darmstadt, Germany
1x TE Buffer pH 8.0	AppliChem, Darmstadt, Germany
2-Propanol	Sigma-Aldrich, Taufenkirchen, Germany
3-Morpholinopropane-1-sulfonic acid (MOPS)	Sigma-Aldrich, Taufenkirchen, Germany
Acetic acid, glacial	Sigma-Aldrich, Taufenkirchen, Germany
Agarose peqGold	peqLab, Erlangen, Germany
Ampicillin	Agilent Technologies, Waldbronn, Germany
Bromophenol blue	Affymetrix, Santa Clara, USA
$CaCl_2$	Sigma-Aldrich, Taufenkirchen, Germany
Chloroform	Sigma-Aldrich, Taufenkirchen, Germany
Deoxyribonucleotide triphosphate (dNTP)	Promega, Mannheim, Germany
Dulbeccos modified Eagles Medium (DMEM)/F12 1:1	Gibco, Karlsruhe, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethidiumbromide solution	Sigma-Aldrich, Taufenkirchen, Germany
Ethyl3-aminobenzoate methan-sulfonate (Tricain)	Sigma-Aldrich, Taufenkirchen, Germany
Fetal bovine serum (FBS), Hi	Gibco, Karlsruhe, Germany
Formaldehyde	Sigma-Aldrich, Taufenkirchen, Germany
Formamide	Sigma-Aldrich, Taufenkirchen, Germany
GeneRuler DNA-Ladder	Fermentas, St. Leon-Rot, Germany
Gentamicin sulfate salt	Sigma-Aldrich, Taufenkirchen, Germany
Glycerol	Sigma-Aldrich, Taufenkirchen, Germany
HEPES	Sigma-Aldrich, Taufenkirchen, Germany
KCl	Sigma-Aldrich, Taufenkirchen, Germany
$KHCO_3$	AppliChem, Darmstadt, Germany
m7G(5')ppp(5')G-cap	Roche, Mannheim, Germany
$MgCl_2$	Sigma-Aldrich, Taufenkirchen, Germany
Mineral oil	Sigma-Aldrich, Taufenkirchen, Germany
NaAc	Sigma-Aldrich, Taufenkirchen, Germany
NaCl	AppliChem, Darmstadt, Germany
$NaHCO_3$	Merck, Darmstadt, Germany
NH_4Cl	AppliChem, Darmstadt, Germany
Phosphate buffered saline (PBS)	Gibco, Karlsruhe, Germany
Protector RNase inhibitor	Roche, Mannheim, Germany
Ribonucleoside triphosphate set	Roche, Mannheim, Germany
Roti Phenol/Chloroform/Isoamylalkohol	Carl Roth, Karlsruhe, Germany
SDS	AppliChem, Darmstadt, Germany
SOC medium	Life Technologies, Darmstadt, Germany
Sodium pyruvate	Sigma-Aldrich, Taufenkirchen, Germany

ssRNA ladder	New England Biolabs, Frankfurt/Main, Germany
Tris	Sigma-Aldrich, Taufenkirchen, Germany
Tryptone	Sigma-Aldrich, Taufenkirchen, Germany
Turbo-ampicillin	Agilent Technologies, Waldbronn, Germany
Yeast extract	Sigma-Aldrich, Taufenkirchen, Germany
Zeocin	Life Technologies, Darmstadt, Germany

Animals, cell lines and bacteria

HEK293 cell line	ECACC, Salisbury, UK
One Shot® PIR1 chemically competent *E. coli*	Life Technologies, Darmstadt, Germany
Xenopus laevis	Xenopus Express Inc., Vernassal, France
XL1-Blue supercompetent Cells	Agilent Technologies, Waldbronn, Germany

Enzymes

Antarctic phosphatase	Fermentas, St.Leon-Rot, Germany
BamHI	New England Biolabs, Frankfurt/Main, Germany
BglII	New England Biolabs, Frankfurt/Main, Germany
Collagenase type 2	Cell Systems Biotechnology, Troisdorf, Germany
CpG methyltransferase (M.SssI)	New England Biolabs, Frankfurt/Main, Germany
Dnase	Roche, Mannheim, Germany
DpnI	Agilent Technologies, Waldbronn, Germany
HindIII	New England Biolabs, Frankfurt/Main, Germany
NheI	New England Biolabs, Frankfurt/Main, Germany
Pfu turbo DNA polymerase	Agilent Technologies, Waldbronn, Germany
Pronase E	AppliChem, Darmstadt, Germany
PstI	New England Biolabs, Frankfurt/Main, Germany
Reverse transcriptase	BioRad, Munich, Germany
T4 DNA ligase	Invitrogen, Karlsruhe, Germany
T4 polynucleotide kinase	New England Biolabs, Frankfurt/Main, Germany
T7- RNA polymerase	Roche, Mannheim, Germany
Taq polymerase	Eurogentec, Cologne, Germany
XbaI	New England Biolabs, Frankfurt/Main, Germany
XhoI	New England Biolabs, Frankfurt/Main, Germany

Consumables

24-well/96-well cell culture plate	Sarstedt, Nümbrecht, Germany
384-well qRT-PCR plate	4titude® Ltd, Berlin, Germany
Beaker (20-100 ml)	Schott-Duran, Wertheim, Germany
Borosilicate glass with filament GB150TF-10	Science Products, Hofheim, Germany
Cell culture flask T75/T150	Sarstedt, Nümbrecht, Germany
Cell scraper	Sarstedt, Nümbrecht, Germany
Cellometer counting chamber	Nexcelom, Lawrence, USA
Cryo sample tubes 1.5 ml/2 ml	Sarstedt, Nümbrecht, Germany
Eppendorf combitips	Eppendorf, Hamburg, Germany
Eppendorf reaction tube 1.5 ml/2 ml	Eppendorf, Hamburg, Germany
Erlenmeyer flask 100-1000 ml	Schott-Duran, Wertheim, Germany
Falcon sample tube 15 ml/50 ml	Greiner Bio-One, Frickenhausen, Germany
Glass pipette	Brand, Wertheim, Germany
Glass replacement 3.5 nl	World Precision Instruments, Sarasota, USA
Luminometer Plates	Promega, Mannheim, Germany
PCR reaction tube 300 µl	Sarstedt, Nümbrecht, Germany
Pipette (sterile) 2 ml/5 ml/10 ml/25 ml/50 ml	Sarstedt, Nümbrecht, Germany
Pipette tips (sterile) 10 µl/100 µl/1000 µl	Sarstedt, Nümbrecht, Germany
S-Monovette® 9 ml K3E	Sarstedt, Nümbrecht, Germany
Suture	Resorba, Nuremberg, Germany

Software

CFX Manager 3.0	BioRad, Munich Germany
ESME	Human Epigenome Project
Excel 2016	Microsoft Corporation, Seattle, USA
GraphPad	GraphPad Software, San Diego, USA
LinReg	linregpcr.hfrc.nl/
Pules/Pulsefit	HEKA Elektronik, Dr. Schulze GmbH, Lambrecht, Deutschland
SPSS	IBM Corp. Released 2014. IBM SPSS Statistics for Windows

2.2. Methods

2.2.1. Samples

Panic Disorder Sample

The panic disorder sample was recruited at the Department of Psychiatry and Psychotherapy of the University of Muenster by Prof. Dr. Dr. Domschke and colleagues. The sample consisted of 131 German patients with PD (f: N=85, age: 36.76±10.91 years±SD, m: N=46, age: 34.13±10.78 years±SD, min-max 17-63 years) as ascertained by experienced psychiatrists on the basis of medical records and a structured clinical interview according to the criteria of DSM-IV (SCID-I; Wittchen *et al*, 1997). Exclusion criteria comprised mental retardation, neurological or neurodegenerative disorders impairing psychiatric evaluation. Medication was recorded. 53 patients received SSRIs; 3 patients received venlafaxine and in each case one patient received trimipramine, agomelatine, opipramol, mirtazapine, valproate, quetiapine, and fluspirilene.

The control group consisted of 131 healthy subjects matched to the patient group by age (35.84±10.9 years±SD, min-max: 18-64 years; t=0.006, p=0.996) and gender (f=85, m=46; $\chi^2(1)=0.0$, p=1.0). Absence of current and lifetime mental axis I disorders was ascertained by experienced psychologists based on a structured clinical interview according to the criteria of DSM-IV (Mini International Neuropsychiatric Interview, M.I.N.I; Sheehan *et al*, 1998).

The study was approved by the ethics committee of the University of Muenster, Germany. All participants gave written informed consent and the study was conducted according to the ethical principles of the Helsinki Declaration.

Independent healthy control sample

An independent sample of healthy probands comprised 255 subjects (f: N=181, m: N=74, age: 25.29±6.02 years±SD) of Caucasian descent recruited at the University of Wuerzburg, Germany, within the Collaborative Research Center (CRC) TRR58, subproject Z02. Absence of current and lifetime mental axis I disorders was established by experienced psychologists on the basis of the M.I.N.I according to DSM-IV criteria (Sheehan *et al*, 1998).

Further exclusion criteria were severe medical conditions, intake of centrally active medication and excessive consumption of alcohol (>15 units/ week), nicotine consumption (>15 cigarettes/ day) or caffeine (>4 cups/ day). None of the recruited participants consumed nicotine. Dimensional anxiety was evaluated using the Beck Anxiety Inventory (BAI; Steer and Beck, 1990). Maltreatment and abuse in childhood was evaluated using Childhood Traumata Questionnaire (CTQ; Bernstein *et al*, 2003; Wingenfeld *et al*, 2010). Recruitment, blood sampling and psychophysiological characterization was conducted by Dipl.-Psych. Miriam Schiele. The ethics committee of the University of Wuerzburg approved the study. Written informed consent was obtained from all participating subjects. The study was conducted according to the ethical principles of the Helsinki Declaration.

Post-mortem brain sample

A human post-mortem sample of 76 deceased individuals (f: N=18, m: N=58, age: 48.6±12.8 years±SD, min-max: 16-74 years±SD) by the Medical Research Council (MRC) sudden death brain and tissue bank (Edinburgh, UK) was used for expression analyses of *CRHR1* isoforms in human tissue. RNA samples were isolated from three brain regions (amygdala, forebrain and midbrain) using MELT Total Nucleic Acid Isolation System (Applied Biosystem, AM Foster City, 1983) and stored at -80°C until usage. DNA extraction and RNA isolation were performed by the MRC Edinburgh, UK. Post-mortem intervals (PMI) ranged from 28 h to 111 h (mean: 54.84±16.68 h). DNA was available for all samples, while RNA was not available from all three brain regions for all participants. RNA integrity values (RIN) ranged from 2.4 to 8.1 of 10 across all samples and brain regions. Details on sample sizes for each brain region are given in results section and table 2.

2.2.2. Molecular biological methods

DNA purification from blood samples

Blood samples were collected from patients or probands using standardized EDTA-tubes by experienced psychiatrist or psychologist. DNA from blood samples was stored at

-20°C to preserve samples from degradation. DNA extractions were accomplished using either FlexiGen DNA extraction Kit or a standardized salting out procedure (Miller *et al*, 1988).

FlexiGen extraction was carried out according to the manufacturer's recommendations. In brief, 300 µl EDTA blood sample were mixed with 750 µl lysis buffer for cell lysis. After short centrifugation cell debris was denaturized with denaturation buffer, thoroughly mixed and incubated at 65°C for 5 min. For precipitation of DNA 150 µl 2-propanol were added and inverted until DNA became visible as a clump or a thread, and pelleted by centrifugation. After washing with 70 % EtOH and air-drying the DNA pellet, DNA was dissolved in FG3 Buffer.

Standardized blood extraction was performed according to Miller *et al*. (Miller *et al*, 1988). Cell lysis of erythrocytes was accomplished by gentle mix of approximately 9 ml EDTA-blood with 30 ml 4°C cold lyses buffer. After 30 min, incubation on ice the lysis mix was inverted four times and centrifuged for 15 min at 4°C. The supernatant was decanted and the pellet of leucocytes was resolved with 5 ml lysis buffer. After another centrifugation, lysis buffer was removed completely and the pellet was resuspended with 5 ml SE buffer, followed by adding 20 mg/ml pronase E and 0.5 % SDS to prevent protein contamination. The solution was incubated at 37°C water bath overnight. On the next day, 2.5 ml SE buffer were added and cell debris precipitated by mixing 6 M NaCl for 15 s and a centrifugation for 20 min at 4,000 rpm. Supernatant was mixed with 7.5 ml 2-propanol and centrifuged for 5 min at 3,000 rpm to precipitate the DNA. After washing the DNA pellet two times with 70% ethanol, DNA was dried at RT and resolved in TE-buffer by overnight incubation at 4°C. DNA was stored at -20°C until further use.

Photometric determination of nucleic acid concentration

DNA and RNA concentrations were quantified with the photometer NanoDrop ND100. For quantification 1.2 µl of DNA or RNA, respectively, were applied to the measuring field of the device. Then, extinction was determined in the wavelength range between 220 nm and 750 nm. The DNA or RNA concentration was subsequently determined based on the extinction at 260 nm. DNA and RNA purity was determined by extinction at 230 nm and 280 nm.

Bisulfite conversion of DNA

Bisulfite conversion of DNA for analyses of DNA methylation was performed using EpiTect ® Bisulfite Kit according to the standard protocol. To avoid batch effects case and control samples were bisulfite converted in the same run. In brief, for sodium bisulfite conversion of unmethylated cytosines, up to 2 µg per 20 µl DNA were mixed with 85 µl bisulfite mix, previously prepared with RNase-free water. After adding 35 µl DNA protect buffer, the mix was filled to 140 µl reaction volume with RNase-free water. Bisulfite reactions were performed in a thermal cycler with 5 min denaturation at 95°C, followed by 25 min incubation time at 60°C. This was repeated twice with prolonged reaction times of 85 min and 175 min each at 60°C. The reaction was terminated by incubation at 20°C up to 12 h (overnight). For cleanup the reaction plate was briefly centrifuged and mixed with 560 µl BL buffer in an EpiTect 96 Plate. The liquid was purged through the membrane of the EpiTect 96 plate, and the membranes were washed with 500 µl BW buffer per well. Removing the liquid by vacuum, 250 µl BD buffer were added per well, covered and incubated for 15 min at RT. Thereafter, samples were washed twice with 500 µl BW buffer and once with 150 µl 100 % EtOH followed by drying the membranes for 10 min. DNA was eluted by vacuum into fresh 96-well plates using 70 µl EB buffer topped with 10 µl Top Elute Fluid. Bisulfite converted DNA was stored at -20°C until further use.

Oligonucleotide design

All oligonucleotide primers used for PCR and qPCR were designed using primer-BLAST online database (www.ncbi.com/blast; Altschul *et al*, 1990; Ye *et al*, 2012). Primer length varied between 20 and 25 bp, had between 40-60 % GCs and an optimal melting temperature around 60°C. To avoid the amplification of unintended amplicons in qPCR experiments, e.g. from gDNA residues in the RNA samples, all primers were designed as intron spanning, if applicable. Specificity of primers for each *CRHR1* isoform was evaluated by *in silico* analyses on BLAST database and by means of correct band sizes on a DNA gel.

Primers for bisulfite converted DNA were designed using MethPrimer database of the Peking Union Medical College Hospital, Chinese Academy of Medical Sciences (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi; Li and Dahiya, 2002). Position and sequences of *CRHR1* CpG island were obtained from UCSC genome browser (https://genome.ucsc.edu; Kent *et al*, 2002) and NCBI Refseq database (Pruitt *et al*, 2014). The CpG island was subdivided into target regions of about 300-500 bp depending on appropriate binding sites for primer pairs. Primers were designed accordingly. The MethPrimer online tool selects primer in the CpG island sequence considering possible masking effects of DNA methylation at all CpG sites in the sequence (Li and Dahiya, 2002). Hence, the obtained oligonucleotide primers bind to both methylated and non-methylated DNA. Oligonucleotide primers were validated using primer design and search tool BiSearch (http://bisearch.enzim.hu/?m=genompsearch; Tusnády *et al*, 2005). Primers were optimized using a gradient PCR with annealing temperatures between 57-67°C. Optimal results were obtained with an annealing temperature of 61.4°C. All oligonucleotides were ordered and purchased at Eurofinsgenomics (Ebersberg, Germany) or Sigma-Aldrich (Munich, Germany).

Genotyping CRHR1 SNP rs17689918

Genotyping of previously with panic disorder associated *CRHR1* SNP rs17689918 was performed using a restriction fragment length polymorphism (RFLP) assay. The target region of rs17689918 was amplified by PCR using 10 mM forward primer and reverse primer (CRHR1_rs17689918, see materials), 2.5 µl PCR buffer, 1 µl 10 mM dNTPs and 0.3 µl Taq-polymerase in 25 µl PCR mixes. PCR was initialized for 3 min at 95°C to detach both DNA and primers, followed by 35 cycles denaturation at 95°C, annealing at 60.5°C and elongation at 72°C each for 45 s. The PCR amplicon was then digested with restriction enzyme HpyCH4V for 4 h at 37°C and run on a 4% agarose gel. Genotype was analyzed based on length of DNA fragments.

Genotyping of the MRC samples was performed previously by Weber *et al.* using a custom TaqMan SNP genotyping assay (Weber *et al*, 2015). TaqMan assays were performed

in 10 µl reaction volume on a CFX384 real-time thermocycler, according to the recommendations by the manufacturer. Samples were run 2 min at 50°C followed 10 min at 95°C. Thereafter, 60 cycles with 15 s at 92°C followed by 1 min at 60°C and a plate read. Allelic distribution could be read out using CFX Manager software.

Agarose gel electrophoreses

The agarose gel electrophoresis was used to separate DNA or RNA fragments according to their size, for instance to define the length of amplified products of a PCR, cRNA or cDNA. The negatively charged nucleic acid fragments pass the sieve of the gel-matrix, shaped by the agarose, along an electric field to the anode. Thereby the fragments are separated according to their size. For the agarose gel, 1-4 % w/v peqGold agarose powder was heated to around 90°C in TAE buffer in a microwave and stirred until the agarose was completely dissolved. At a temperature of about 55°C 3-6 µl 0.025 % ethidium bromide were added and stirred. The gel was poured into a gel chamber with a 16-well comb. After about 30 min the gel was cured and ready to use or storable at 4°C. The samples were mixed with 20-30 % of their volume of blue loading buffer and filled in the comb-wells of the gel. As reference for the length of the analyzed DNA fragments served the GeneRuler DNA-ladder, which consists of distinctly sized DNA fragments with a size range between 100 to 1000 bp, defined by the manufacturer. The size of the analyzed DNA was approved with the respective ladder. Depending on the expected DNA size, the gels were run between 30 min and 1 h at 120 V in a TAE buffer filled electrophoresis chamber. The gel was analyzed and photographed with UV light at a wavelength of 302 nm in a ChemiDoc UV-chamber.

cDNA synthesis from RNA

The synthesis of the first-strand complementary DNA (cDNA) from extracted RNA was required for qPCR. The cDNA synthesis was accomplished using iScript cDNA synthesis kit. For the transcription of 300-1000 ng of RNA, the appropriate amount of diluted RNA was mixed with DEPC-treated ddH2O to a volume of 15 µl. This premix was filled up to 20 µl with 4 µl 5x

iScript reaction mix and 1 µl iScript reverse transcriptase. The entire reaction mix was incubated in a thermocycler for 5 min at 25°C, followed by an incubation of 30 min at 42°C for reverse transcription. The reaction was terminated by incubation at 85°C for 5 min. After the synthesis, the cDNA was diluted in 80 µl TE buffer. cDNA was stored at -20°C until further use.

Quantitative real time PCR

Gene expression analyses in post-mortem RNA samples were performed using quantitative real-time PCR (qPCR). QPCR was set up in 384-well plates in 10 µl volume, each containing 10 µM specific forward and reverse primer, 5 µl SYBR Select Mastermix, 1 µl cDNA and 4 µl nuclease free H_2O. The reaction was run in a CFX 384-well real-time PCR thermocycler. Starting with a denaturation at 95°C for 5 min, followed by 40 cycles of 10 s denaturation at 95°C, 30 s primer annealing and elongation in one step at 60°C. To measure the hitherto amplified amount of the transcript of interest, after each step the fluorescence of SYBR-green was quantified. As termination another 10 s at 95°C denaturation time were applied.

Due to limited amounts of RNA from post-mortem samples, adjustments to the standardized *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE) guidelines (Bustin *et al*, 2009; Vandenbroucke *et al*, 2001) had to be made. Instead of working in triplicates, expression was analyzed in duplicates. The data was analyzed and normalized using CFX software version 3.0 and LinReg software for PCR efficiencies (http://LinRegPCR.HFRC.nl; Ramakers *et al*, 2003; Ruijter *et al*, 2009). Relative expression was calculated with the two most stable out of four reference genes (*GUSB*, *HPRT1*, *SDHA*, *TFRC*). *GUSB* and *SDHA* were the two most stable reference genes with a M-value of 0.5383 and a coefficient variance of 0.1835 and 0.1709, respectively.

Mutagenesis PCR

Preparation of a pSGEM-CRHR1α vector was accomplished using site directed mutagenesis to eliminate excessive exon 6 of the *CRHR1* open-reading frame (ORF) in the

initial pSGEM-CRHR1β vector. Site-directed mutagenesis PCR was performed using the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer's protocol. Forward and reverse primer (CRHR1α_mutagenesis, see materials) were designed with 25 base pair overhang to each side of exon 5 and exon 7 of *CRHR1β* to eliminate the excessive exon 6 and create the *CRHR1α* ORF by amplification with *Pfu* polymerase. For mutagenesis PCR the following parameter were chosen: after an initial denaturation at 95°C for 30 s, 18 cycles of 30 s denaturation at 95°C, 60 s annealing at 55°C and 4 min elongation at 68°C were conducted. The reaction was terminated by cooling the reaction mix at 4°C. For better translation in oocytes, a consensus sequence of GCCACC (see materials) was inserted before the initial ATG of both *CRHR1* ORFs. In order to eliminate residual template DNA, amplification products were digested using the restriction enzyme DpnI for 1 h at 37°C. DpnI specifically digests methylated DNA and hence, only the methylated template DNA is digested. Correct mutagenesis was validated by Sanger sequencing (LGC Genomics, Berlin, Germany). PCR products were stored at -20°C until further use.

In vitro transcription

In vitro transcription of pSGEM vectors was conducted to obtain cRNA for expression of *CRHR1* variants α and β, as well as the inwardly-rectifying potassium channel Kir2.3 in oocytes of *Xenopus laevis*. For that, 15 µg of pSGEM DNA was linearized using NheI restriction enzyme, producing a 5' overhang required for T7 RNA polymerase, and cleaned up using phenol-chloroform extraction method. Therefore, 200 µl phenol/chloroform mix were added to the reaction mix and centrifuged for 5 min at 13,000 rpm and RT. The aqueous phase was transferred to a fresh sterile reaction tube and 100 µl DEPC-treated ddH_2O were added to the phenol/chloroform mix to repeat the extraction. After another 5 min centrifugation, the aqueous phase was again transferred to the reaction tube and mixed with the threefold volume of -20°C cooled 100% EtOH and $\frac{1}{10}$ volume of 3 M NaAc (pH 5.2). After overnight incubation at -20°C, the reaction mix was centrifuged for 30 min at 4°C and washed with 70% EtOH. DNA was dried

at 37°C and resuspended in 25 µl DEPC-treated ddH_2O, after another 10 min centrifugation at 4°C.

Concentration was measured with a photometer. Between 2-3 µg of template DNA were mixed with 5 µl 10 mM ATP, CTP, UTP and GTP + m^7G(5')ppp(5')G-cap each, 5 µl 10× transcription buffer, 2.5 µl RNase Inhibitor and 2.5 µl T7 RNA polymerase, and filled to 50 µl with DEPC-treated ddH_2O. The reaction mix was incubated at 37°C for 1 h, followed by adding 5 µl DNase to digest residual template DNA at 37°C for 15 min. Transcription was terminated by RNA purification using phenol-chloroform precipitation. CRNA was diluted in 10 µl DEPC-treated ddH_2O. Concentration of cRNA was estimated upon band signal on a 1.5 % RNA gel.

Bisulfite PCR

The target amplicon was analyzed with PCR using the HotStarTaq Plus Master Mix according to the standard protocol (Domschke *et al*, 2012). In brief, 2 µl bisulfite converted DNA were mixed with 10 µl HotStarTaq Plus Master Mix, 0.8 µl 10 µM forward and reverse primer (CRHR1_meth_P1, see materials), respectively, 1 µl 25 mM $MgCl_2$ and subsequently filled with water to a reaction volume of 20 µl. Optimized PCR conditions resulted in 35 cycles at 95°C denaturation followed by annealing at 61.4 °C and elongation at 72°C each for 45 s. The amplicon was purified and Sanger sequenced on ABI 3730XI sequencer platforms by LGC Genomics (Berlin, Germany). To avoid batch effects, case and control samples were sequenced in the same run. Sequencing data were analyzed quantitatively using the Epigenetic Sequencing Methylation (ESME) software as applied in previous studies by Domschke *et al*. and Ziegler *et al*. (Domschke *et al*, 2008, 2012, 2013, Ziegler *et al*, 2015, 2016). ESME software analyses the sequencing results and compares the bisulfite converted DNA sequence with the original sequence obtained from the NCBI reference sequence database (http://www.ncbi.nlm.nih.gov/refseq/; Pruitt *et al*, 2014). Thereby, the algorithm calculates the DNA methylation at each CpG in percent. To account for run variability, all samples were tested in duplicates, yielding a mean individual methylation score for each CpG site.

2.2.3. Molecular cloning

pSGEM vectors

For functional analyses of CRHR1 splice variants using electrophysiological recordings, single *CRHR1* isoforms, namely isoform α and β, were cloned in a pSGEM vector system by courtesy of Prof. Wischmeyer, University of Wuerzburg, to enable *in vitro* cRNA transcription (Figure 4).

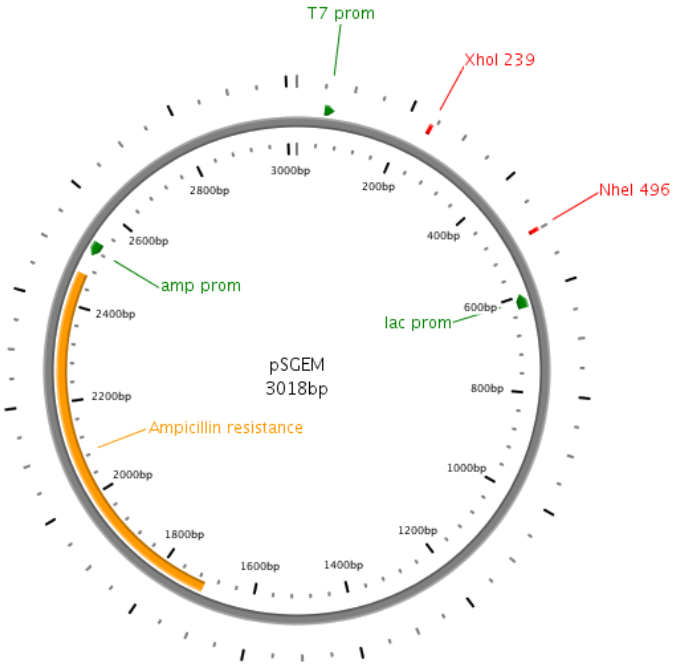

Figure 4: Vector map of the pSGEM vector used for *in vitro* transcription of cRNA. XhoI and NheI mark the restriction enzymes in the multiple cloning site of the vector used for linearization and ligation of the target ORF. T7 prom marks the T7 promoter used for *in vitro* transcription of cRNA. Ampicillin resistance was used for selection of successful transformed bacteria. Lac prom and amp prom mark two further promoter for ampicillin gene and SP6 promoter (not used in this experiment). bp = base pairs; prom = promoter. The vector map was created using PlasMapper version 2 online tool (http://wishart.biology.ualberta.ca/PlasMapper/; Dong *et al*, 2004).

A commercially available pcDNA3.1(+) vector containing the ORF of the longest *CRHR1* isoform β (NM_001145146.1) was purchased from Creative Biogene. The target *CRHR1β* ORF was extracted from vector with restriction enzymes NheI and XhoI, and purified from a 1% agarose gel using QiaEx II Gel Extraction Kit. Subsequently, *CRHR1β* ORF insert

was cloned into XbaI and NheI linearized pSGEM vector using T4 DNA ligase. NheI and XbaI restriction endonucleases produce identical restriction site overhangs, hence corresponding sticky ends of insert and vector. Before the ligation reaction, linearized vectors were dephosphorylated using Antarctic phosphatase to prevent religation of the vector. Nevertheless, reaction mixes without the insert were used as a control for self-ligation of the vector.

Ligated vectors were subsequently amplified by transformation into XL-1 blue *E.coli* bacteria. 50 µl of bacteria were thawed from -80°C on ice and mixed gently with 5 µl ligation mix. After 30 min incubation on ice, bacteria underwent heat-shock at 37°C for 45 s, followed by an instant incubation on ice for 2 min. After adding 150-250 µl of SOC medium, transformed bacteria were initially amplified by shaking for 1.5 h at 225 rpm and 37°C. For each sample, two LB-agar-ampicillin plates were used, one with 10 µl and the other with 100 µl of bacteria mix, and incubated overnight at 37°C. On next day, single colonies were picked with a sterile pipette tip or toothpick and incubated in Miniprep-cultures of 2 ml LB medium and 100 µM ampicillin between 10 and 16 h at 225 rpm and 37°C. For DNA clean up, the Wizard® Plus SV Miniprep DNA Purification System was used following the standard protocol. The obtained DNA was quantified using a NanoDrop Photometer. Successful ligation was validated by Sanger sequencing (LGC Genomics, Berlin, Germany). Vectors carrying the correct *CRHR1* insert were amplified using PureYield™ Plasmid Midiprep System according to the manufacturer's recommendations. To ensure correctness of the amplified vectors, each vector was again sequenced (LGC genomics, Berlin, Germany). Correct vectors pSGEM-CRHR1α and pSGEM-CRHR1β were used for *in vitro* transcription of *CRHR1* cRNA.

The purchased control vector pGL4.74 from Promega carries also an ampicillin resistance and was therefore amplified in the same way.

pCpGL vectors

For functional analyses of the target CpG region of *CRHR1*, triplicates of the target sequence were cloned in the multiple cloning site upstream of a CMV/EF1α promoter of a

pCpGL-basic vector by courtesy of Prof. Rehli, University of Regensburg, Germany (Klug and Rehli, 2014). Firstly, CMV/EF1α promoter region was amplified from a pCpGL-CMV/EF1α vector, producing new overhangs for restriction enzymes BglII and HindIII. After linearization of a pCpGL-basic vector with the same enzymes, the CMV/EF1α vector was ligated using T4 DNA ligase according to the manufacturer's instructions at the end of the multiple cloning site of pCpGL. The insert was obtained by annealing of synthesized 3x CpG9-15 forward and reverse strand (see materials) and ligated into the linearized new pCpGL_CMV/EF1α vector using T4 DNA Ligase according to the manufacturer's instructions (Figure 5). Successful ligation was validated by Sanger sequencing (LGC Genomics, Berlin, Germany).

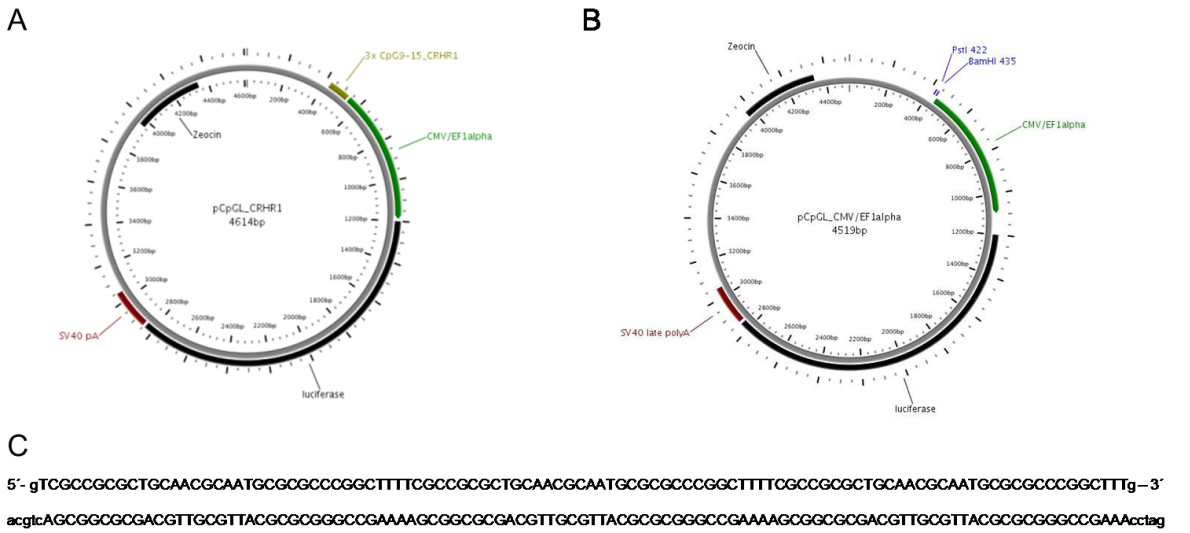

Figure 5: Vector maps of pCpGL vectors used in this study. A: pCpGL_CRHR1 vector containing a triple repeat of the sequence spanning *CRHR1* CpG sites 9-15 (C) inserted proximally to a CMV/EF1α promoter (green). Expression of luciferase reporter gene (black) was used to analyze the functionality of DNA methylation of the CpG sites. Zeocin (black) was needed to select positive transformed bacteria colonies. B: pCpGL_CMV/EF1α vector, obtained by cloning a CMV/EF1α promoter into pCpGL-basic vector using BglII and HindIII restriction sites, was used for control experiments in functional analyses. PstI and BamHI mark the restriction enzymes used for the insertion of the target sequence. C: Sequence of the triplicate of *CRHR1* CpG sites 9-15 (see also Figure 12 B, bold and lowercase). Lowercase letters indicate overhangs for restriction enzymes PstI and BamHI. bp = base pairs, The vector map was created using PlasMapper version 2 online tool (http://wishart.biology.ualberta.ca/PlasMapper/; Dong *et al*, 2004). Figure adapted from Schartner *et al*, 2017.

pCpGL_CMV/EF1α and pCpGL_CRHR1 vectors were transformed to PIR1 bacteria. Successfully transformed bacteria were selected by plating on low-salt-LB-agar-zeocin plates overnight at 37°C. Single colonies were picked with sterile pipet-tips and purified using

Wizard® Plus SV Miniprep DNA Purification System or PureYield™ Plasmid Midiprep System, respectively.

2.2.4. Cell biological methods

Cell culture

The human embryonic kidney cell line HEK293 was cultivated in DMEM high glucose/L-glutamine growth medium substituted with 10 % fetal bovine serum in T75 cell+ culture flasks at 37°C and 5 % v/v CO_2. For optimal growth conditions, cells were passaged regularly at about 80 % confluence.

For seeding, cells were trypsinized with 2-3 ml 0.05 % trypsin/EDTA solution and resuspended in 10 ml growth medium, counted on a cell counter and seeded to 24-well cell+ culture plates with a density of $5x10^4$ cells per well. After 24 h of incubation, cells were used for transfection experiments.

Cell transfection

Transfection of HEK293 cells was performed using TransFast transfection reagent according to the manufacturer. After optimization with several DNA amounts and ratios, 250 ng of total DNA and a 1:1 TransFast to DNA ratio were used. For transfection, 225 ng of pCpGL vector and 25 ng of pGL4.74 vector were mixed with 2.25 µl TransFast reagent in 200 µl serum free DMEM/F12 medium and incubated for 15 min at RT. Growth medium was removed from cells and replaced by 200 µl transfection mix. After 1 h incubation at 37°C and 5 % v/v CO_2, 800 µl growth medium were added per well. Cells were incubated between 40 and 48 h until further use.

Reporter gene assays

Reporter gene assays with pCpGL vectors were conducted using the Dual-Luciferase Reporter Assay Kit. In brief, cells were lysed using 100 µl passive lysis buffer by shaking 15 min at 400 rpm on a thermomixer. 20 µl cell lysate were transferred to white luminometer

plates. First, 100 µl luciferase assay reagent II were added per well and firefly luciferase activity was measured for 10 s after a 2 s reaction delay. Subsequently, 100 µl of Stop&Glo reagent were added to stop the reaction of the firefly luciferase, while starting the reaction of renilla luciferase expressed by the co-transfected pGL4.74 vector. Relative light units from luminometer were normalized to transfection efficiency by renilla luminescence and standardized to z-score to eliminate measurement-variations.

2.2.5. Electrophysiological methods

Laboratory animals and cell harvesting

Oocytes of female *Xenopus laevis* frogs were dissected with permission of the regional authority of lower Franconia under the standards of the German animal protection act. Wildtype, female *Xenopus laevis* (Xenopus Express Inc., Vernassal, France) were biannually used for oocyte preparation. For dissection, frogs were anaesthetized for 15-25 min in 0.1 % tricain solution until the animal was completely unconscious. The frog was placed on the back on ice and opened at the lower third of the belly for about 1 cm using a sterile scalpel. Directly beneath the fatty layer, oocytes were easily visible and could be extracted using a sterile tweezer. Each dissection gained several 100 oocytes, which were incubated in $ND96^{+}$ solution until further use. After dissection, frogs were sutured with reabsorbing suture and placed on wet tissues in a glass chamber under surveillance until fully recovered.

In order to separate the ovary and de-folliculize the oocytes, the ovary was dissected into small parts and incubated in collagenases solution (0.5 U/ml in $ND96^{Ca2+}$) for 2-3 h on a shaker with 50 rpm. After three washing steps in ND96 and two washing steps in $ND96^{+}$, separated oocytes were incubated at 19°C in $ND96^{+}$ solution until further use.

The electrophysiological characterization of *CRHR1* isoforms *in vitro* was approached by the injection of cRNA of *CRHR1* isoform α or β respectively, together with 50x diluted cRNA of ion channel *Kir2.3* in a ratio of 2:1. For injection, RNA of *Kir2.3* and *CRHR1* was filled in a glass injection capillary, dragged at 710°C in a micropipette puller. It was filled with mineral oil priorly, in order to provide an air-free system that warrants a correct and consistent injection

volume. 1-2 µl cRNA (150-250 pg/µl) were filled in the injection capillary and 41.4 nl cRNA were injected per oocyte with the injector. After injection with cRNA, oocytes were incubated per 5-10 cells per well of a 12-well plate for 48-72 h in $ND96^{+}$ medium at 19°C until further experiments. Meanwhile, dead or damaged oocytes were evaluated daily and discarded. $ND96^{+}$ solution was replaced if necessary.

Two electrode voltage clamp

The two electrode voltage clamp (TECV) analyses of *Xenopus laevis* oocytes were performed to evaluate differences in receptor activities of *CRHR1* transcript variants via measurements of ion currents and the conductivity of the cell membrane. To do so, two electrodes – one voltage and one current electrode – were dragged from a borosilicate glass capillary with filament in a micropipette puller at 710°C and chopped under a binocular with micro scissors to obtain a resistance between 1 and 3 MΩ. Prior to measurement, capillaries were filled with 3 M KCl and adjusted to the chlorinated silver-electrodes. Oocytes were placed in a small volume perfusion chamber, connected with a perfusion system, which allowed constant flow of different measuring solutions. Conductivity of the cell membrane was recorded using Pulse/Pulsefit software on a Macintosh computer and a TURBO TEC-10 C amplifier system.

Each measurement was started with a voltage ramp from -100 mV to +60 mV (Figure 6 A) to control for expression of the ion channels in the respective oocyte. For measurements of receptor activity, a many jumps (maju) protocol with voltage jumps to -40 V every 3 s was used (Figure 6 B). The receptor activity of CRHR1 in presence of different measuring solutions containing agonist (Corticotropin Releasing Factor, CRF), diluted in hK^{+}-medium, was recorded constantly via current changes. The oocyte was constantly monitored during measurement.

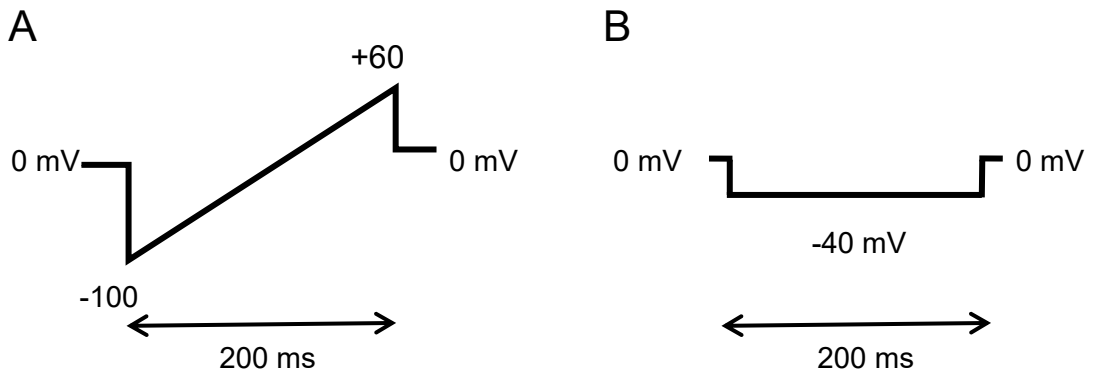

Figure 6: Different protocols, which were applied to the voltage clamped oocytes. A: Ramp from -100 mV to 60 mV over a time period of 200 ms. B: The many jumps protocol (maju) applied voltage jumps to -40 mV every 3 s. Currents were recorded. mV = milli Volt; ms = milli seconds

2.2.6. Statistical and *in silico* analyses

Statistical analyses

Hardy-Weinberg criteria were calculated using the DeFinetti online program (https://ihg.gsf.de/cgi-bin/hw/hwa2.pl; Wienker TF and Strom TM) and were fulfilled for *CRHR1* rs17689918 genotype distribution in all samples ($p>0.05$). For further analyses, genotypes were grouped according to a previous study (AA/AG genotype carriers = risk vs. GG genotype carriers = non-risk; Weber *et al*, 2015).

DNA methylation data were controlled for outliers and normal distribution prior to analyses. DNA methylation patterns were analyzed using mean scores for each sample. All CpG sites with DNA methylation < 5% were excluded, which left 7 CpG sites (CpG9-15) for further analyses (Table 4 and Table 5; Roberts *et al*, 2014). Associations of DNA methylation with age were evaluated using Pearson correlation for each CpG site. Effects of gender, age, drinking or smoking on DNA methylation were analyzed using independent sample t-tests. Age was used as covariate, where applicable. Impact of *CRHR1* rs17689918 genotype on phenotype and gender was assessed using χ^2-tests. Differences in DNA methylation between PD patients and controls, or between low and high anxious individuals of the independent healthy control group were tested using mixed linear model for repeated measures, with DNA methylation as within factor and group (e.g. patients vs. controls or BAI group) as between factor (Schuster *et al*, 2016; Unternaehrer *et al*, 2015; Ziegler *et al*, 2016). Follow-up analyses

for individual CpG sites were performed using univariate analysis of variance (ANOVA). Significance level was set at $p<0.05$. Bonferroni correction for multiple testing set the significance level to $p\leq0.007$ (p-value divided by multiple tests for seven analyzed CpG sites).

Hierarchical multiple regression was used to analyze the effect of *CRHR1* rs17689918 genotype, *CRHR1* promoter methylation and childhood trauma, as well as their interactions on anxiety scores of BAI. Due to the correlation of DNA methylation, age was included in the interaction analyses. Risk and non-risk allele carriers of rs17689918 were operationalized (risk = -0.5; non-risk = 0.5). Age, CTQ scores and DNA methylation were normalized to z-scores (mean = 0, SD = 1). Regression analyses were carried out in four steps with BAI sum scores as dependent variable. In the first step, the single variables, z-scores of CTQ, age and *CRHR1* promoter methylation as well as operationalized *CRHR1* genotypes were entered. In the second step, two-way interactions were included (e.g. genotype × z_CTQ; genotype × z_age, or z_CTQ × z_methylation). In the third step three-way interactions were entered (e.g. genotype × z_CTQ × z_methylation or genotype × z_age × z_CTQ). In the last step, a four-way interaction of all terms was entered (i.e. genotype × z_CTQ × z_age × z_methylation; Schiele *et al*, 2016).

Luciferase assay data were normalized to transfection efficiency by expression of renilla luciferase control gene and transformed to z-scores to eliminate day and measurement specific fluctuations. Differences in reporter gene expression were evaluated using independent sample two-sided t-tests. P-values <0.05 were considered significant.

Gene and isoform expression data from qPCR was obtained from relative light units and normalized using PCR efficiency for each individual gene with LinReg software (Ramakers *et al*, 2003; Ruijter *et al*, 2009). The expression of the two most stable out of four reference genes was used for normalization. Expression analyses of *CRHR1* isoforms were performed using CFX3.0 software (BioRad, Munich, Germany). Differences in gene expression between risk and non-risk allele were calculated using univariate analysis of variance (ANOVA) or Brown-Forsythe test, respectively.

Data from electrophysiological recordings was normalized to baseline activity in hK^+-medium. Differences in activity between the different receptor isoforms was calculated using two-sided t-tests or Mann-Whitney U-tests, respectively.

All statistical analyses were performed using SPSS 23 (IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY, USA). All graphical analyses were done using GraphPad Prism version 5 (GraphPad Software, San Diego CA USA, www.graphpad.com).

In silico analyses of transcription factor binding sites

In silico analyses to reveal potential transcription factor binding sites in the sequence spanning CpG sites 9-15 (see figure 12 B, bold and lowercase) were conducted using the online database JASPAR (http://jaspar.genereg.net/; Mathelier et al., 2015). All matrices available for *homo sapiens*, were selected.

3. Results

3.1. Alternative Splicing of CRHR1

3.1.1. Expression in post-mortem sample

The post-mortem MRC brain tissue sample consisted of 76 individuals (f: N=18, m: N=58, age: 48.6±12.8 years, min-max: 16-74 years). RNA was available for three brain regions – amygdala, midbrain and forebrain. Genotyping data for *CRHR1* rs17689918 was available for all 76 specimens (AA=3, AG=26, GG=47). Genotypes were grouped into risk (A) allele and non-risk (GG) allele carriers according to previous studies (Weber *et al*, 2015). No association of genotype with age or sex could be found ($p>0.05$). Due to previously published reduced expression of total *CRHR1* in risk allele carriers (Weber *et al*, 2015), total *CRHR1* gene expression was used as a covariate in expression analyses of all transcript variants. Relative gene expression of individual *CRHR1* isoforms between risk and non-risk allele carriers was analyzed separately for each brain region. Expression analyses were performed for three of eight known *CRHR1* isoforms (β, c and d) and one transcript variant 5, renamed into *CRHR1-IT1-CRHR1* readthrough transcript variant 5 throughout this study. Isoform *CRHR1β* and c express all seven transmembrane domains of CRHR1, isoform d lacks transmembrane domain 7. All other known *CRHR1* isoforms lack more than one transmembrane domain and were therefore excluded from analyses. The main *CRHR1* isoform *CRHR1α* could not be analyzed individually due to the lack of specific qPCR primers for this variation. Due to insufficient amplification or low efficiency, individual samples had to be excluded, so total numbers of samples for expression analyses were reduced (Table 2). Total numbers of samples, as well as sex and genotype distribution for each isoform and brain region are reported in detail in table 2.

Table 2: Expression data of all analyzed *CRHR1* isoforms including valid and invalid cases in relation to total available samples grouped by brain region. Age is indicated in years ± standard deviation. Genotyping data of rs17689918 is grouped in risk (A) allele carriers vs. non-risk (GG) allele carriers. Accession numbers of CRHR1 isoforms are according to NCBI website accessed on June 13th 2016.

							rs17689918	
Isoform; NCBI Accession Number	Brain region	Valid	Invalid	Total	Age	Female:Male	risk allele	non-risk allele
CRHR1β; NM_001145146	Amygdala	45	14	59	49.51±12.398	12:33	19	26
	Forebrain	60	6	66	47.87±12.705	14:46	25	35
	Midbrain	33	25	58	50.33±11.615	08:25	12	21
CRHR1c; NM_001145147	Amygdala	48	11	59	48.96±12.657	13:35	20	28
	Forebrain	64	2	66	48.97±13.162	15:49	25	39
	Midbrain	35	23	58	49.03±11.242	10:25	12	23
CRHR1d; NM_001145148	Amygdala	42	17	59	49.31±13.299	13:29	18	24
	Forebrain	59	7	66	47.90±12.811	14:45	24	35
	Midbrain	33	25	58	49.00±10.580	07:26	14	19
CRHR1-IT1-CRHR1 readthrough transcript variant 5; NM_001256299.2	Amygdala	54	5	59	49.65±12.358	14:40	23	31
	Forebrain	66	0	66	49.06±13.026	16:50	25	41
	Midbrain	53	5	58	50.25±12.542	15:38	17	36

Gene expression was analyzed based on the expression of the two most stable out of four reference genes (see methods) glucuronidase beta (*GUSB*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*). PCR efficiencies ranged between 65% for *CRHR1d* and 82.3% for *CRHR1β*, whereas mean efficiency was 78.54% over all primer pairs and samples.

mRNA expression of CRHR1 isoform β

In amygdala and midbrain samples risk allele carriers showed significant lower expression than non-risk allele carriers (Amygdala: $F_{1,38.775}$=10.374, p=0.003; Midbrain: $F_{1,19.925}$=5.723, p=0.027; Figure 7). In forebrain samples, no significant difference between risk and non-risk allele carriers could be detected ($F_{2,51}$=0.587, p=0.560).

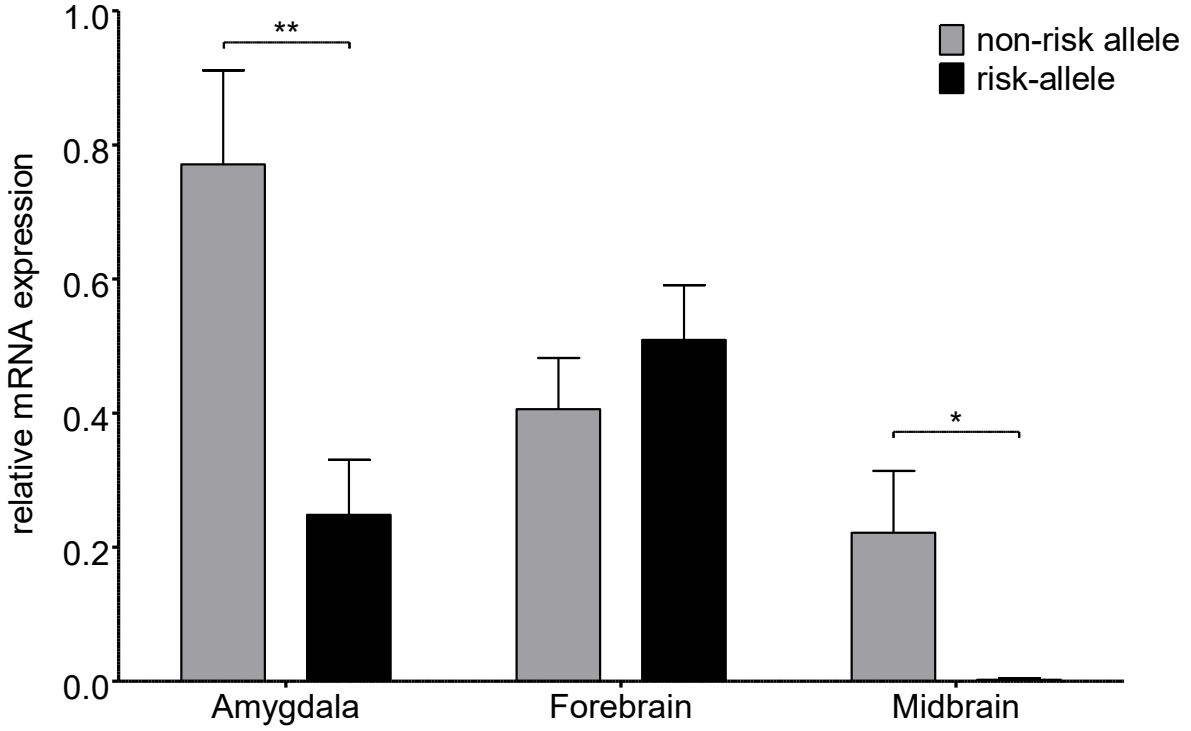

Figure 7: mRNA expression of isoform *CRHR1β* in risk allele (black columns) and non-risk allele (grey columns) carriers of *CRHR1* SNP rs17689918 in three different brain areas. Univariate analyses of variance indicated a significant reduction in expression of isoform β in amygdalae and midbrains of risk allele carriers; mRNA = messenger RNA; *: p ≤ 0.05; **: p ≤ 0.01

mRNA expression of CRHR1 isoform c and isoform d

Neither in amygdala, nor in forebrain or midbrain RNA samples, differences between risk and non-risk allele carriers regarding the expression of *CRHR1* isoforms c and d were detected (Table 3).

Table 3: mRNA expression of *CRHR1* isoform c and d showed no significant differences between risk and non-risk allele carriers of *CRHR1* rs17689918. ANOVA indicated univariate analyses of variances (F), p-value indicates significance when p≤0.05.

	Isoform *CRHR1c*		Isoform *CRHR1d*	
	ANOVA	p-value	ANOVA	p-value
Amygdala	$F_{2,44}$=1.610	0.211	$F_{1,25.8}$=3.544	0.071
Forebrain	$F_{2,55}$=0.877	0.422	$F_{2,50}$=0.037	0.964
Midbrain	$F_{2,24}$=0.556	0.581	$F_{2,21}$=0.519	0.602

mRNA expression of CRHR1-IT1-CRHR1 readthrough, transcript variant 5

In amygdala samples, no difference between risk and non-risk allele carriers was detected ($F_{2,50}$=2.292, p=0.112). In midbrains as well as in forebrains of risk allele carriers expression of transcript variant 5 was significantly increased compared to non-risk allele carriers (Midbrain: $F_{2,37}$=18.099, p<0.001; Forebrain: $F_{2,57}$=8.458, p=0.001; Figure 8).

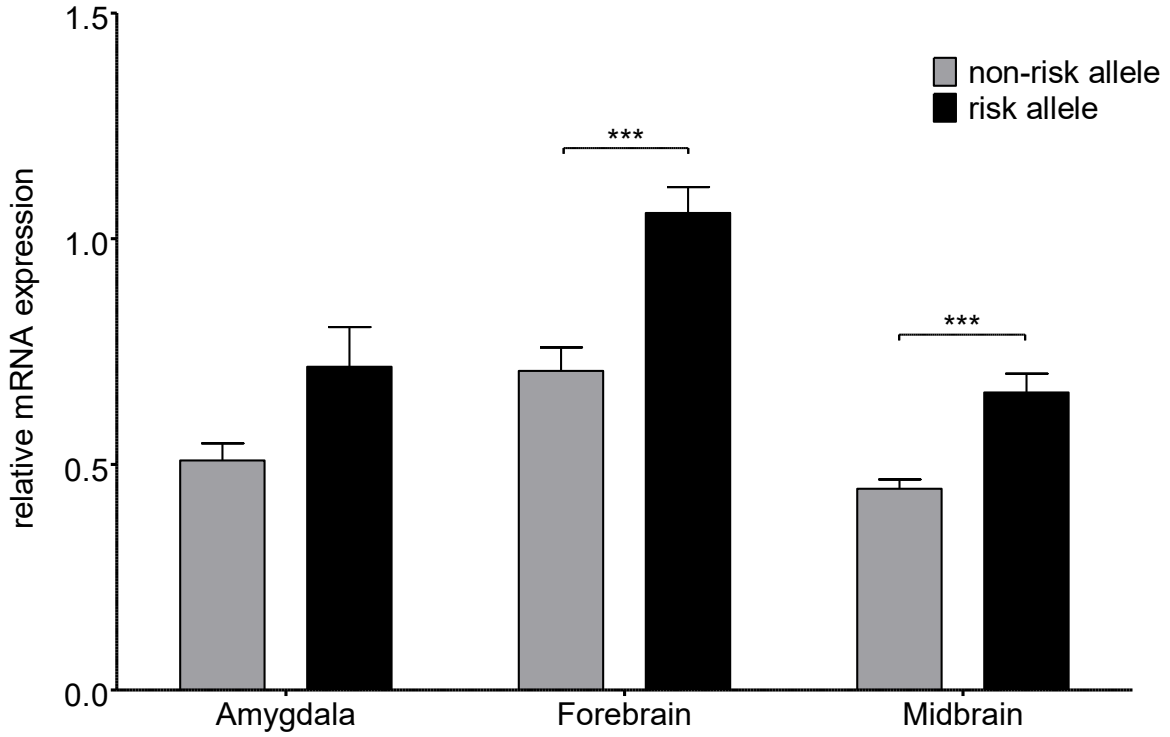

Figure 8: mRNA expression of *CRHR1-IT1-CRHR1* readthrough transcript variant 5 (NM_001256299.2) in risk allele (black columns) and non-risk allele (grey columns) carriers of *CRHR1* rs17689918 in three different brain areas. Univariate analyses of variance indicated a highly significant increase of transcript variant 5 mRNA expression in midbrains and forebrains of risk allele carriers; mRNA = messenger RNA; ***: $p \leq 0.001$

3.1.2. *In vitro* expression in oocytes

Activation and inhibition of Kir2.3 by CRHR1α isoform

In order to investigate differences in signaling between isoforms α and β of *CRHR1,* the activity of G-protein coupled Kir2.3 channels was recorded in oocytes. Both receptors were co-expressed in oocytes of *Xenopus laevis* and electrophysiologically analyzed using two-electrode voltage clamp (TEVC) measures. Therefore, expression and activation of Kir2.3 channels were first evaluated by voltage ramps from -100 mV to +60 mV during perfusion with different buffers. Baseline activity was recorded during ND96 buffer perfusion (Figure 9, light grey line). Kir2.3 channels were activated by perfusion in hK^+ buffer leading to a massive inward current of K^+ ions at hyperpolarized membrane potentials (Figure 9, dark grey lines). Similar current activation was recorded upon perfusion of the cells with 500 nM CRF in hK^+

buffer with different pattern between cells co-expressing CRHR1α (Figure 9 A, black line) or CRHR1β (Figure 9 B, black line).

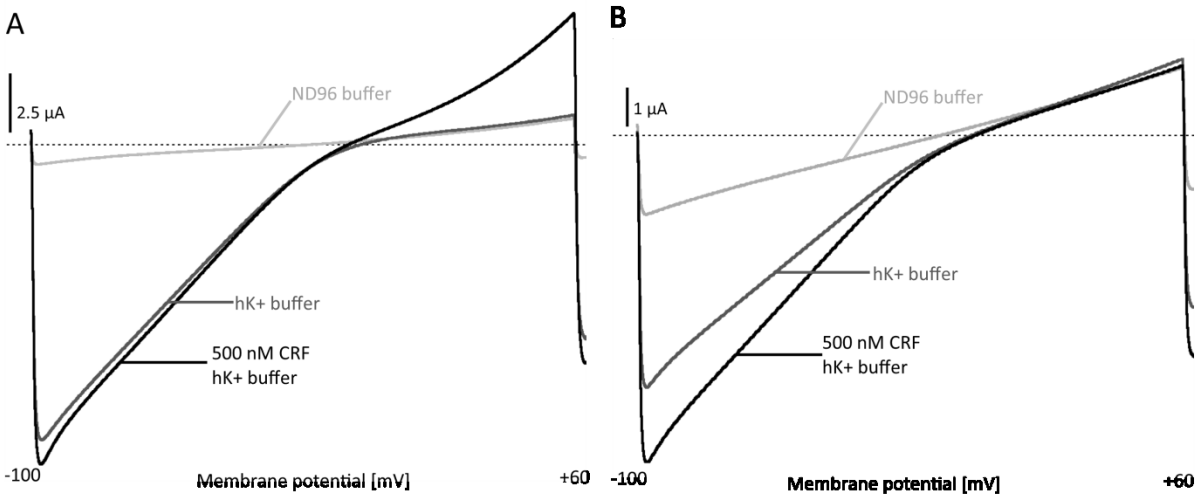

Figure 9: Current activations under voltage ramps from -100 mV to 60 mV proof expression of functional Kir2.3 channels and CRHR1 isoforms in oocytes. A: Ramp recordings from -100 mV to 60 mV evoke inwardly-rectifying currents by Kir2.3 channels activated by hK^+ buffer (dark grey line) and via CRHR1α by CRF application (black line). B: Ramp recordings from -100 mV to 60 mV evoke inwardly-rectifying currents by Kir2.3 channels activated by hK^+ buffer (dark grey line) and further activated by CRHR1β upon CRF application (black line).

Isoform *CRHR1α* showed inconsistent results. Using 500 nM CRF in hK^+ buffer, an inhibition of current amplitude was recorded in five cells, and a further activation of the Kir2.3 channels leading to a higher inward current was recorded in eight cells. Figure 10 A and B show a typical inhibition of inward current by CRF application of *CRHR1α*-expressing cells. CRHR1α transcript variant activated and inhibited Kir2.3 channels not significantly compared to baseline hK^+ buffer activation in the total sample of 13 cells ($T_{Total}(24)=1.025$, $p=0.326$; $N=13$). When analyzed separately, the inhibition of Kir2.3 channels reached significance ($U_{Inhibtion}=2.785$, $p=0.008$; $N=5$), while the activation did not, but showed a trend towards significance ($T_{Activation}(14)=2.319$, $p=0.053$, $N=8$).

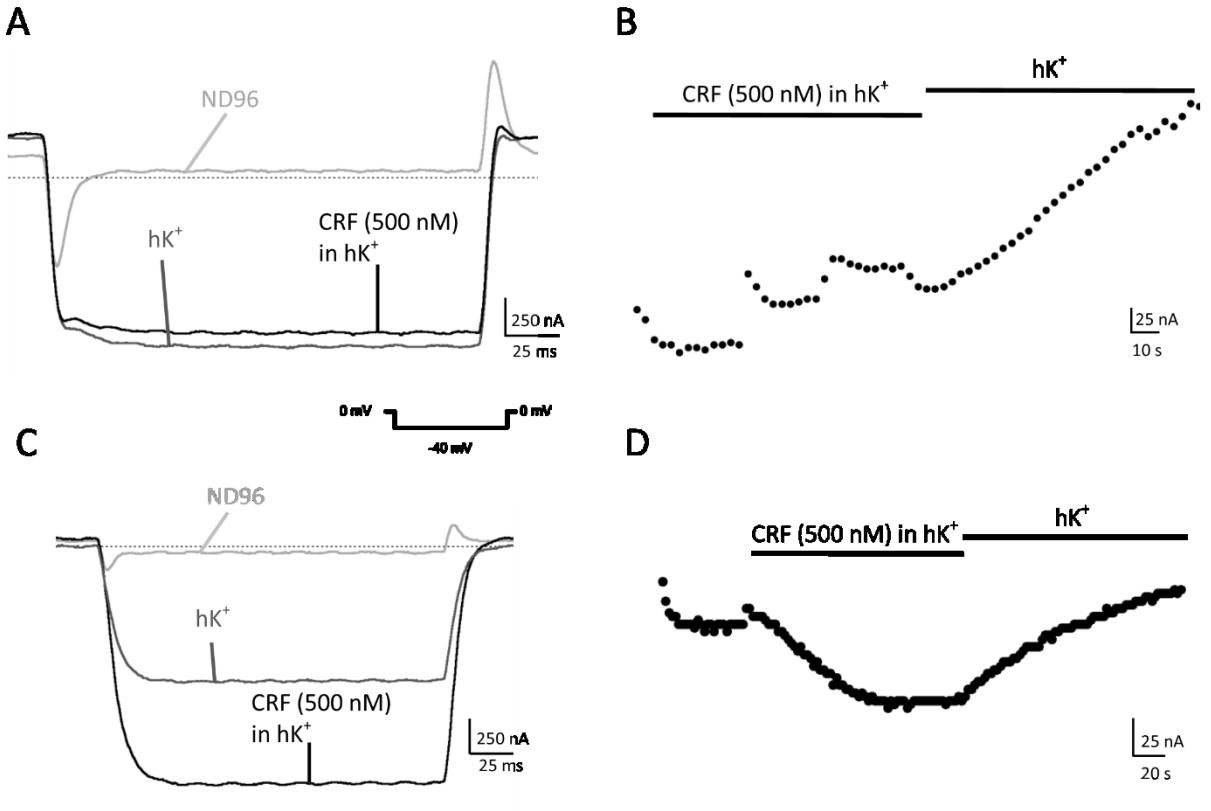

Figure 10: Typical activation and inhibition of Kir2.3 channels dependent on receptor activity of co-expressed CRHR1 isoforms α or β upon CRF perfusion. A: Membrane potential jumps to -40 mV demonstrate the inhibition of Kir2.3 channels by CRF perfusion. B: Recording the inward current during maju protocol shows inhibition of Kir2.3 activation after CRF application, which is not completely washed out by a switch to hK^+ buffer. C: Membrane potential jumps to -40 mV demonstrate the activation of Kir2.3 leading to a further activation of inward current after CRF application via co-expressed *CRHR1β* isoforms. D: Reversible activation of the inward current in CRHR1β expressing cells is elicited by CRF application and washed out by subsequent hK^+ buffer application. The inset shows the pulse protocol.

Isoform CRHR1β constantly activated co-expressed channels after CRF application (Figure 9 B and Figure 10 C, D). Activation of Kir2.3 channels was significantly higher than baseline Kir2.3 activation during hK^+ perfusion ($T_{CRHR1\beta}(13)=8.398$, $p<0.001$, $N=11$).

Additionally, CRHR1β activation was significantly higher than both, activation and inhibition of CRHR1α ($T_{Total}(25)=-5.621$, $p<0.001$, Figure 11). The same was true for differences between CRHR1β and CRHR1α inhibition or activation, when analyzed separately ($U_{Inhibtion}(10.801)=-3.240$, $p<0.001$; $T_{Activation}(20)=-3.887$, $p=0.001$; Figure 11).

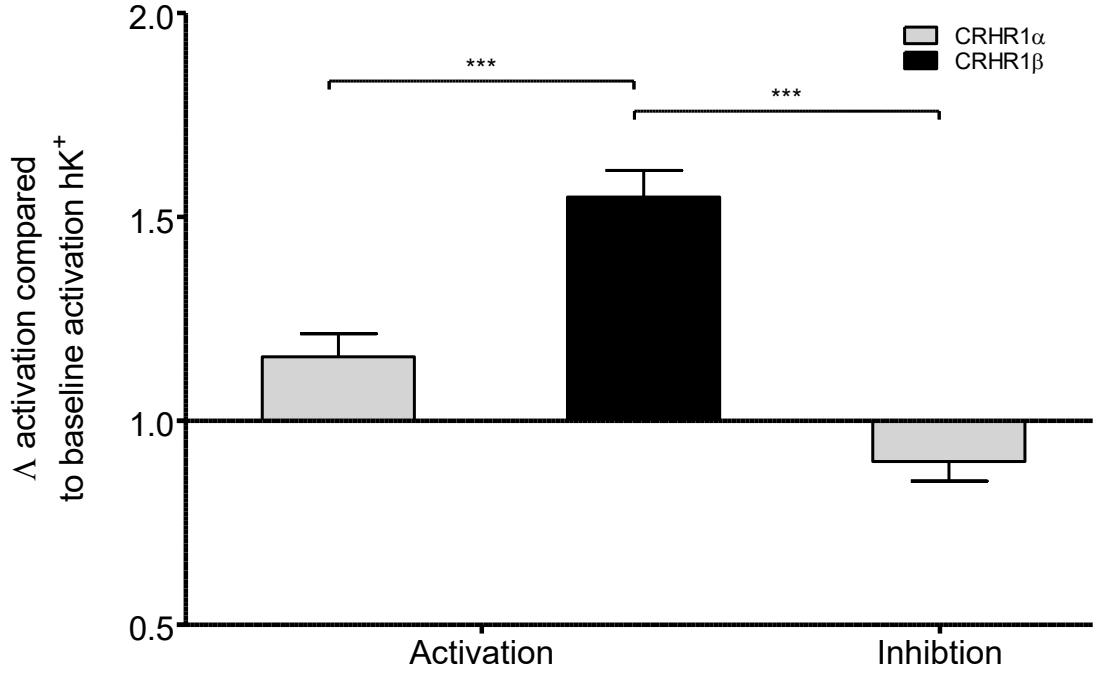

Figure 11: Relation of Kir2.3 activation and inhibition by co-expressed CRHR1α and β isoforms. CRHR1β lead to significantly stronger activation of Kir2.3 channels compared to inhibition as well as activation in presence of CRHR1α isoform.

3.2. Epigenetic regulation of CRHR1

3.2.1. DNA methylation of *CRHR1*

In search of regulatory regions relevant for DNA methylation in the *CRHR1* promoter region, the genome browser of the University of California in Santa Cruz (UCSC) was used (Kent *et al*, 2002). No CpG island could be detected in direct proximity to rs17689918 or one of its proxy SNPs, but further analyses lead to the discovery of a 2244 bp long CpG Island, spanning the transcription start *CRHR1* (chr17:45,783,319-45,785,562, UCSC Genome Browser (Kent *et al*, 2002) on Human Dec. 2013 [GRCh38/hg38] Assembly). In total, the CpG island contains 203 individual CpG sites. Assuming a regulatory effect of DNA methylation in the promoter region of *CRHR1*, an amplicon of 293 bp (chr17:45,783,249-45,783,542 based on UCSC Genome Browser (Kent *et al*, 2002) on Human Dec. 2013 [GRCh38/hg38] Assembly) covering the initial part of the CpG island approximately 1000 bp upstream of the transcription start was chosen for DNA methylation analyses (Figure 12 A). The amplicon contained 24 CpG sites; due to limitations of the bisulfite sequencing approach using commercially available Sanger sequencing, 15 of the 24 CpG sites were readable (Figure 12 B boxed).

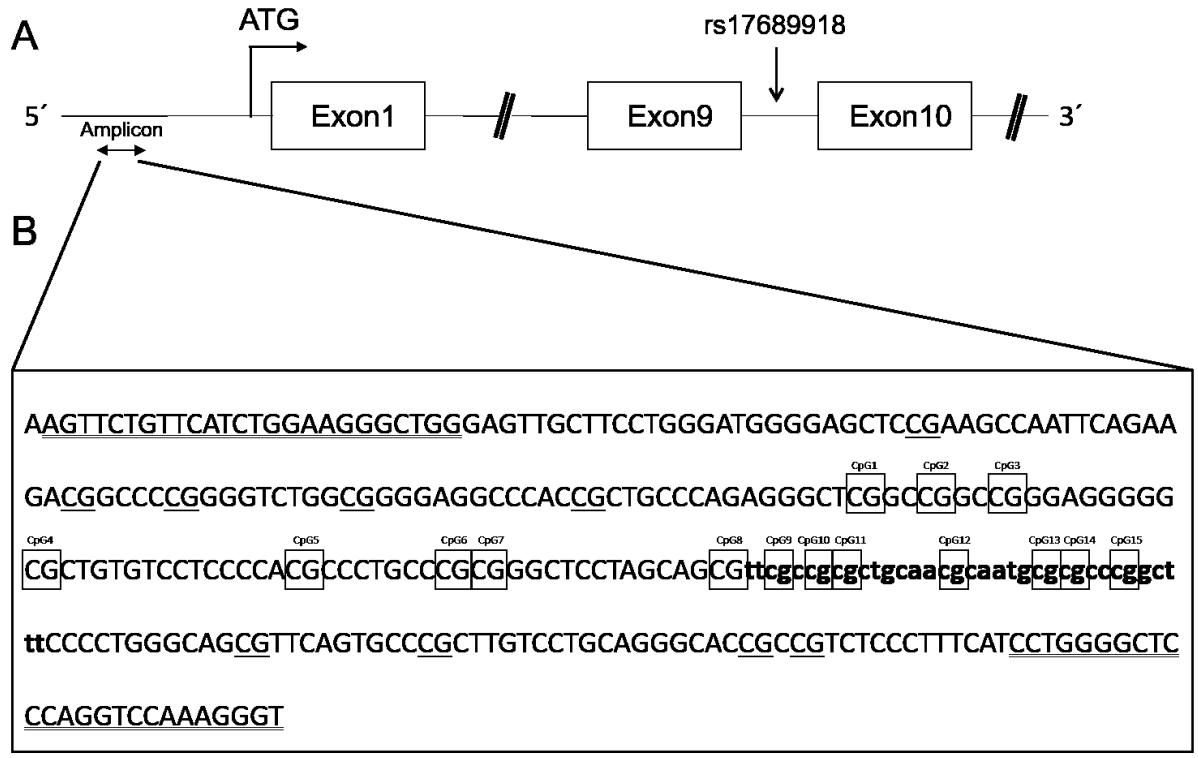

Figure 12: Schematic drawing of the *CRHR1* promoter region and investigated CpG island. A: The investigated amplicon is located approx. 1,000 bp upstream of the *CRHR1* transcription start (ATG; not to scale). B: Primer sequences used for PCR-amplification are double-underlined. Underlined CpG sites could not be analyzed due to technical issues (see methods). The 15 CpG sites robustly readable are boxed. Given insufficient methylation (<5 %) of CpG sites 1-8 (see Table 4), only CpG sites 9-15 were selected for further analyses (boxed bold lowercase, see methods). The sequence in bold and lowercase was used for *in silico* and *in vitro* analyses (see Figures 5 and 16 and Table 6); Figure adapted from Schartner *et al*, 2017.

Across the 15 readable CpG sites in the analyzed amplicon (see methods and Figure 12 B), only at CpG sites 9-15 a sufficient mean methylation of at least 5 % in one subsample was detected (see Table 4; Roberts *et al*, 2014). Thus, all subsequent epigenetic analyses were carried out for those seven CpG sites (CpG 9-15) only. No other CpG island across the *CRHR1* gene region could be detected.

3.2.2. Association of *CRHR1* methylation with panic disorder

Due to insufficient quality of sequencing data regarding DNA methylation, a reduced sample of 120 patients with panic disorder and 117 controls entered final epigenetic analyses. The patient sample consisted of 76 female patients and 44 male patients with an average age of 37.18±10.6 years±SD and 34.05±11.7 years±SD, respectively. The patient sample

consisted of 76 females and 41 males with an average age of 37.24±11.03 years±SD and 33.76 ±11.03 years±SD, respectively.

Individual average methylation of CpG sites 9-15 varied between 0.00 and 0.20 in patients and between 0.02 and 0.26 in controls (Table 4). Individual CpG sites correlated between 0.169 and 0.827 with p-values ranging from $p<0.001$ to $p=0.065$ (no significant correlation between CpG13 and CpG14) in patients and between 0.354 and 0.915 (all $p<0.001$) in controls. There was no association of methylation status (average methylation across CpG 9-15 and methylation at individual CpG sites) with age, medication or smoking (all $p>0.05$). While data on age were available for the complete sample, data on smoking behavior and medication intake were only available for the patient sample.

Table 4: *CRHR1* methylation in patients with panic disorder and matched healthy controls. Only CpGs 9-15 and average methylation across CpGs 9-15 (bold) entered analyses given a mean methylation <5 % at CpGs 1-8. [a)] = no homogeneity of variances required use of Brown-Forsythe tests; F = ANOVA; SD = standard deviation; p = p-value; * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant; [#] = p-values remaining significant after Bonferroni correction for multiple testing. Table adapted from Schartner *et al*, 2017.

	Patients N=120		**Controls N=117**		**Statistics**
	Mean methylation	SD	Mean methylation	SD	
CpG1	0.0133	0.02656	0.0075	0.01397	
CpG2	0.0209	0.04159	0.0152	0.01963	
CpG3	0.0096	0.02163	0.0155	0.02521	
CpG4	0.0084	0.01963	0.0177	0.03245	
CpG5	0.0037	0.01514	0.0012	0.00417	
CpG6	0.0051	0.01590	0.0048	0.01968	
CpG7	0.0221	0.02838	0.0242	0.03186	
CpG8	0.0090	0.02462	0.0093	0.02815	
CpG9	**0.0721**	**0.04418**	**0.0884**	**0.04689**	$F_{1,235}$=7.604, p=0.006**, #
CpG10	**0.0807**	**0.06104**	**0.1025**	**0.06124**	$F_{1,235}$=7.551, p=0.006**, #
CpG11[a)]	**0.1055**	**0.06957**	**0.1476**	**0.09189**	$F_{1,217.821}$=15.397, p<0.001***, #
CpG12	**0.0840**	**0.06334**	**0.1027**	**0.06723**	$F_{1,235}$=4.893, p=0.028*
CpG13[a)]	**0.1111**	**0.08093**	**0.1465**	**0.09106**	$F_{1,230.313}$=10.012, p=0.002**, #
CpG14	**0.0485**	**0.04721**	**0.0531**	**0.04477**	$F_{1,235}$=0.588, p=0.444, ns
CpG15[a)]	**0.0768**	**0.05572**	**0.1083**	**0.07206**	$F_{1,218.326}$=14.173, p<0.001***, #
Average CpGs 9-15	**0.0827**	**0.04355**	**0.1070**	**0.05528**	$F_{6,1410}$=4.450, p<0.001***

Neither an association of rs17689918 risk allele with panic disorder ($\chi^2(1)=0.252$; p=0.688), nor an significant effect of rs17689918 on average DNA methylation across CpG sites 9-15 in patients ($N_{risk}=80$; $N_{non\text{-}risk}=36$; $N_{missing}=4$; $F_{6,684}=1.294$, p=0.257) or controls ($N_{risk}=80$; $N_{non\text{-}risk}=37$; $F_{6,690}=0.873$, p=0.514) could be detected.

However, in PD patients average *CRHR1* methylation was significantly decreased compared to controls ($F_{6,1410}=4.450$, p<0.001). Analyses of individual CpG sites by means of univariate ANOVA revealed significantly decreased methylation at six CpG sites in patients as compared to controls (Table 4 and Figure 13). Results for CpG9, CpG10, CpG11, CpG13 and CpG15 survived Bonferroni correction for multiple testing (Table 4 and Figure 13).

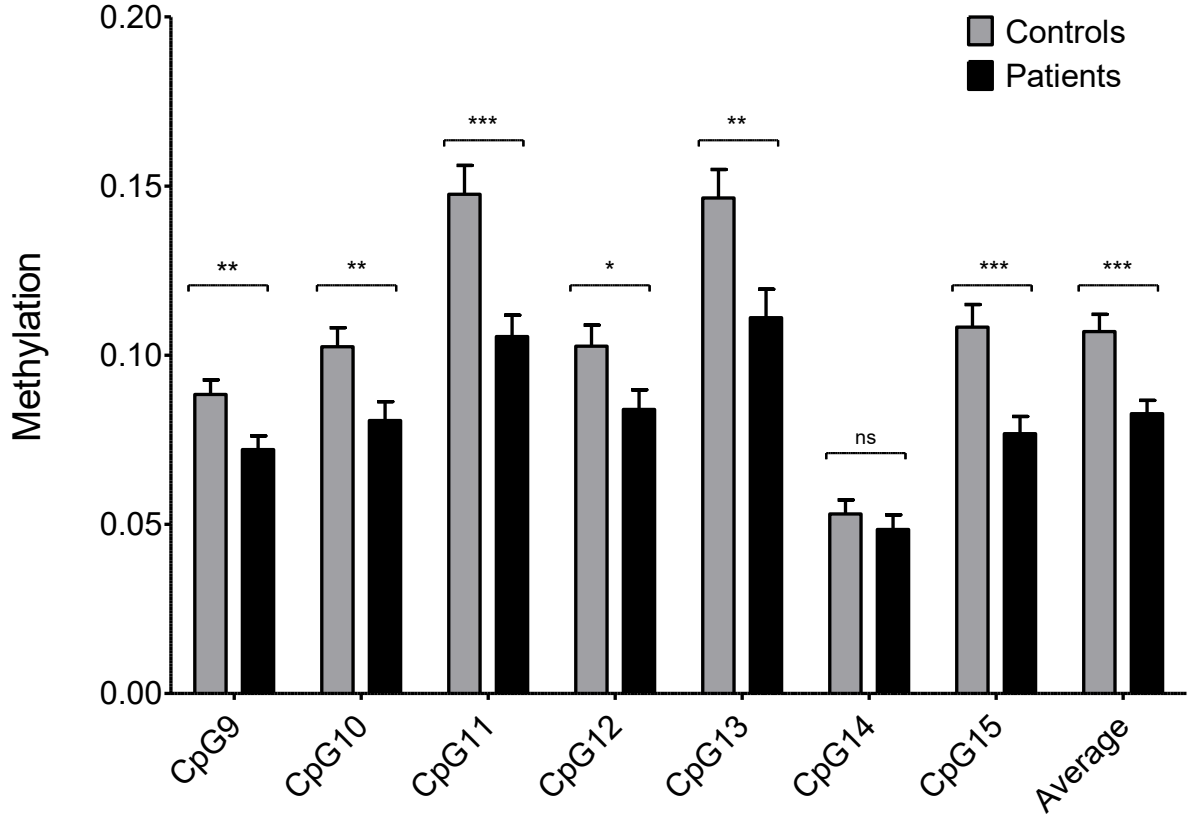

Figure 13: Mixed linear model for repeated measures revealed a significant group effect on average DNA methylation. Subsequent univariate analyses showed significantly decreased methylation at 6 out of 7 investigated CpG sites in patients (black columns) compared to healthy controls (grey columns). Results at CpG sites 9, 10, 11, 13 and 15 survived Bonferroni correction for multiple testing (see Table 4). Figure adapted from Schartner *et al*, 2017. * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant.

3.2.3. Association of *CRHR1* methylation with anxiety in controls

DNA methylation and BAI scores

Data on DNA methylation of *CRHR1* CpG site 9-15 were available for 255 samples. The independent sample of healthy participants was stratified into low- and high-anxious groups based on a median split of Beck Anxiety Inventory (BAI) sum scores. BAI data were available for all 255 subjects. BAI scores ranged from 0 to 35 with a variance of 7.39. Median, used for separation into “low-anxious” and “high-anxious” participants, was set at 6 points. Control values of BAI lie between 0 and 8, with panic disorder patients scoring ca. 14 and multiple anxiety disorders about 18 (Muntingh *et al*, 2011).

Individual mean methylation varied between 0.01 and 0.21 (individual min-max; Table 5). Correlations among the single CpG sites ranged from 0.253 to 0.904 (all p<0.001). Average

methylation across CpGs 9-15 correlated significantly with age (r=0.171, p=0.006) as did methylation at individual CpG sites 10, 11, 12 and 13 (CpG 10: r=0.152, p=0.015; CpG11: r=0.188, p=0.003; CpG12: r=126, p=0.044; CpG13: r=0.231, p<0.001). Thus, in further analyses, age was used as covariate.

Table 5: *CRHR1* methylation in high and low anxious healthy probands. Low anxious: probands with BAI scores <6; high anxious: probands with BAI scores ≥ 6 according to median split. Only CpGs 9-15 and average methylation across CpGs 9-15 (bold) entered analyses given a mean methylation <5 % at CpGs 1-8. BAI = Beck Anxiety Inventory, (Steer and Beck, 1990); SD = standard deviation; F = ANOVA; p = p-value; * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant; # = p-values remaining significant after Bonferroni correction for multiple testing. Table adapted from Schartner *et al*, 2017.

	High anxious (BAI ≥ 6), N=146		**Low anxious (BAI < 6), N=109**		**Statistics**
	Mean Methylation	SD	Mean Methylation	SD	
CpG1	0.0175	0.01964	0.0204	0.03916	
CpG2	0.0094	0.01514	0.0087	0.0122	
CpG3	0.0038	0.00772	0.0064	0.01529	
CpG4	0.0113	0.02272	0.0118	0.01884	
CpG5	0.0043	0.01317	0.0048	0.00948	
CpG6	0.0057	0.01732	0.0059	0.01267	
CpG7	0.0104	0.01562	0.0137	0.01829	
CpG8	0.0070	0.02059	0.0067	0.01657	
CpG9	**0.0751**	**0.04032**	**0.0793**	**0.04651**	$F_{2,252}$=0.334, p=0.717, ns
CpG10	**0.0732**	**0.03490**	**0.0836**	**0.04049**	$F_{2,252}$=5.282, p=0.006**, #
CpG11	**0.0782**	**0.04664**	**0.0942**	**0.05470**	$F_{2,252}$=7.699, p=0.001***, #
CpG12	**0.0586**	**0.03692**	**0.0701**	**0.04218**	$F_{2,252}$=4.631, p=0.011*
CpG13	**0.0828**	**0.06580**	**0.1055**	**0.07486**	$F_{2,252}$=10.405, p<0.001***, #
CpG14	**0.0371**	**0.03330**	**0.0529**	**0.03991**	$F_{2,252}$=6.016, p=0.003**, #
CpG15	**0.0396**	**0.03110**	**0.0442**	**0.03599**	$F_{2,252}$=1.075, p=0.343, ns
Average CpGs 9-15	**0.0635**	**0.03115**	**0.0757**	**0.03672**	$F_{6,1512}$=2.269, p=0.035*

No influence of rs17689918 genotype on average DNA methylation was detected ($F_{6,1434}$=0.386, p=0.888). High-anxious participants displayed significantly lower average *CRHR1* methylation than low-anxious probands, which held true for methylation at CpG sites

10 to 14 (Table 5 and Figure 14). Results for methylation at CpG10, CpG11, CpG13 and CpG14 survived Bonferroni correction for multiple testing.

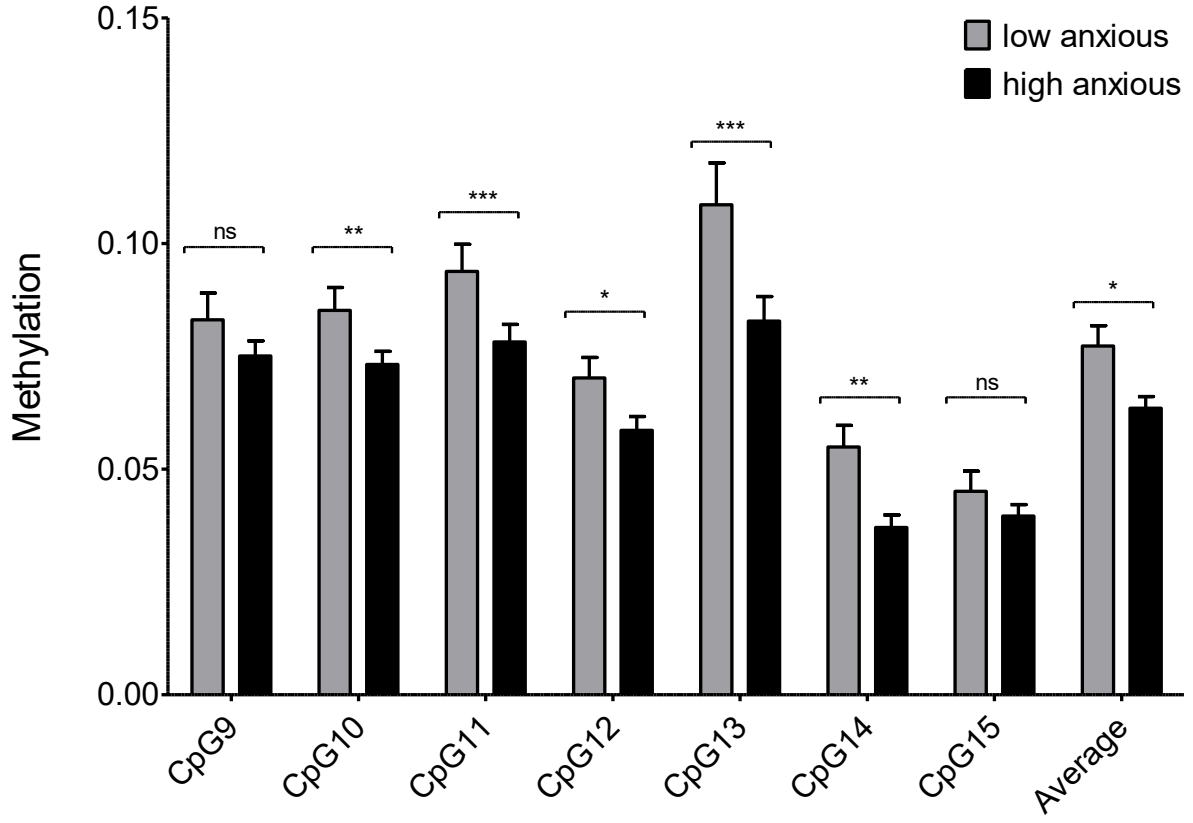

Figure 14: Differences in *CRHR1* methylation between low anxious and high anxious healthy subjects. Low anxious: probands with BAI scores <6; high anxious: probands with BAI scores ≥6 according to median split. BAI = Beck Anxiety Inventory (Steer and Beck, 1990). Mixed linear model for repeated measures with covariate age revealed a significant group effect on average DNA methylation. Subsequent univariate analyses showed significantly decreased methylation at 5 out of 7 CpG sites in high anxious probands; * = p<0.05; ** = p<0.01; *** = p<0.001; ns = not significant. Results at CpG sites 10, 11, 13 and 14 survived Bonferroni corrections for multiple testing (see Table 5). Figure adapted from Schartner *et al*, 2017.

DNA methylation – interaction with genotype and childhood trauma

In order to assess possible environmental factors interacting with the rs17689918 risk allele and *CRHR1* DNA methylation on anxious behavior, the Childhood Trauma Questionnaire (CTQ) was evaluated and set in relation to the genetic and phenotypic parameter with hierarchical multiple regression analyses. To correct for the influence of age on DNA methylation age was set as a fourth parameter of the interaction. Data on CTQ were available for all 255 subjects and ranged between 36 and 83 with a median of 42 and a variance of 34.

Regression analyses revealed an interaction of the risk allele of rs17689918 paired with low average DNA methylation across CpG9-15 and high scores of childhood trauma, mirrored by high CTQ sum scores, lead to significant higher BAI sum scores, i.e. an increased anxious phenotype ($F_{15,226}$=1.283, p=0.026; B=1.421; R^2=0.078; Figure 15).

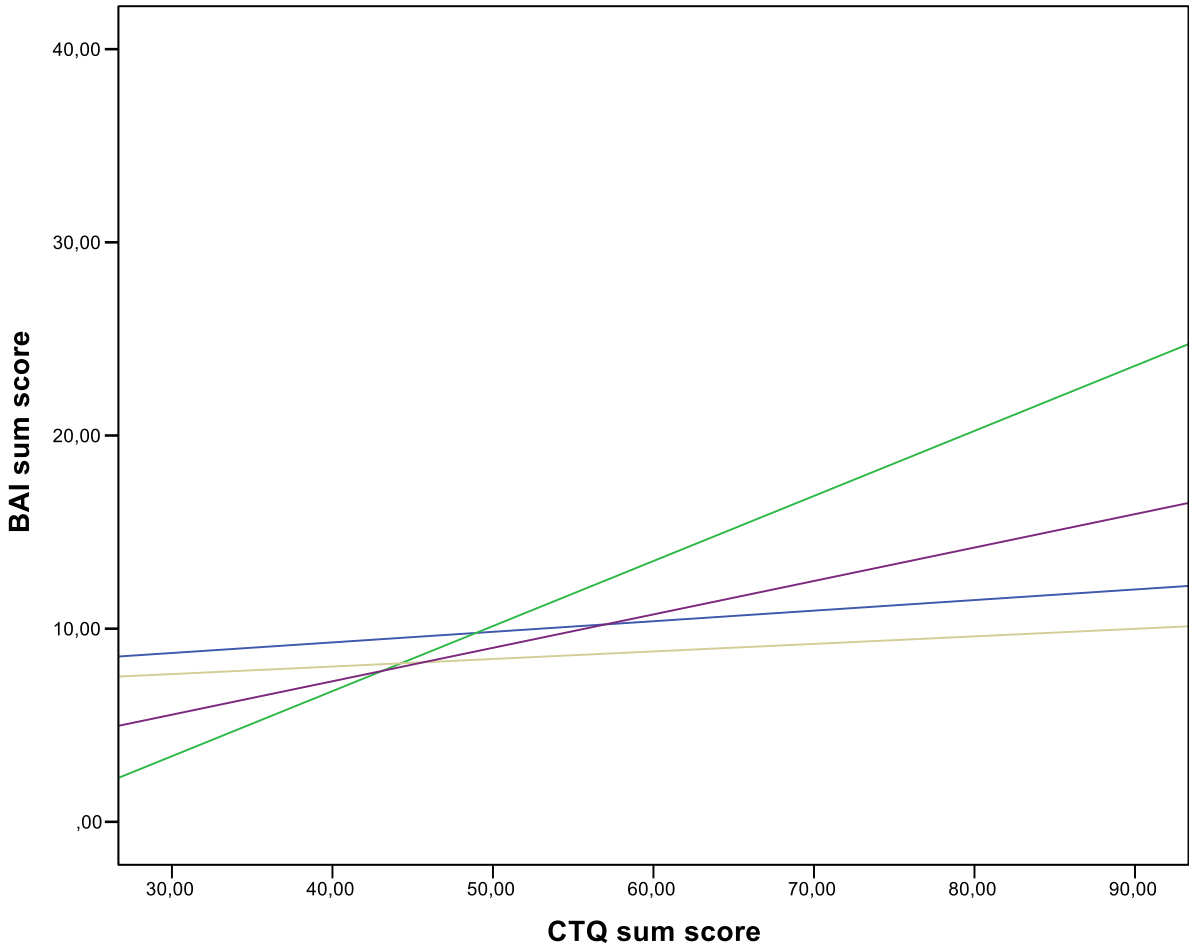

Figure 15: Interaction of rs17689918 genotype and DNA methylation with CTQ sum scores on BAI sum scores. Green line indicates risk (A) allele carriers and low methylated samples, blue line non-risk (GG) allele carries and low methylated samples, yellow line non-risk allele carries and high methylation, and purple line risk allele carriers and high methylation. The combination of risk allele and low DNA methylation together with high childhood trauma predicts higher scores on BAI with increasing age.

3.2.4. *In vitro* functional analyses

In order to validate a potentially functional effect of differential methylation at the investigated CpG sites on *CRHR1* gene expression, *in vitro* luciferase assays were performed. Methylation of pCpGL vectors was confirmed by methylation sensitive restriction enzyme

digest and visualized using gel-electrophoresis. Plasmid DNA amplified in bacteria is non-methylated, thus these DNA plasmids are cut by restriction enzymes, whereas *in vitro* CpG methylation blocks enzyme restriction. Only non-methylated vector controls were digested.

In HEK293 cells, unmethylated pCpGL_CRHR1 vectors showed a significant increase in luciferase gene expression as compared to methylated pCpGL_CRHR1 vectors ($t(16)=4.224$, $p<0.001$, Figure 16 A). PCpGL_CMV/EF1α vectors without insert showed no significant difference between the methylated and the unmethylated state ($t(16)=0.5271$, $p=0.605$, Figure 16 B).

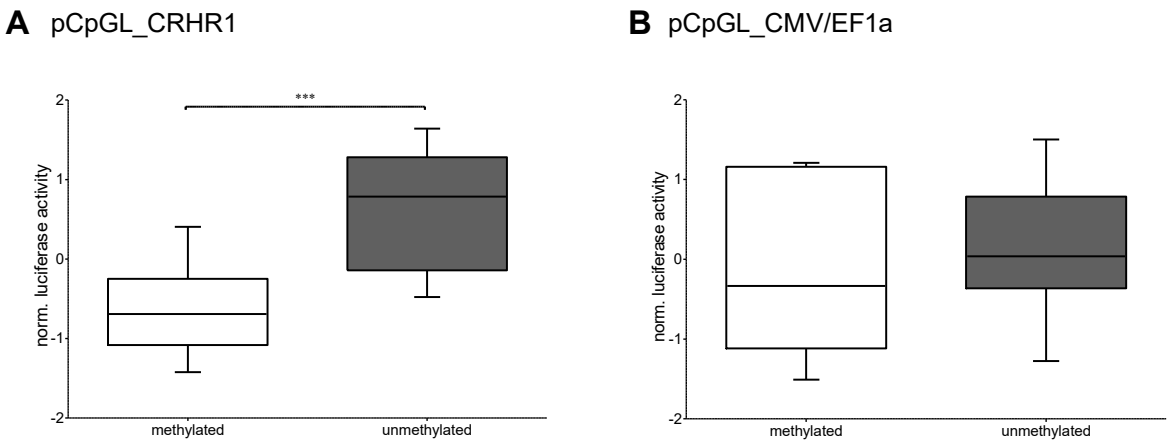

Figure 16: Functional analysis of *CRHR1* methylation using luciferase-based reporter gene assays. CpG-free luciferase-based reporter gene vectors pCpGL (Klug and Rehli, 2014) were *in vitro* methylated using MSss.I (white plots) or unmethylated (dark plots). A: Normalized luciferase gene expression was significantly decreased in presence of methylated pCpGL_CRHR1 vectors, containing triple repeats of the sequence spanning CpGs 9-15 (see Figure 12B), compared to unmethylated vectors. B: No significant difference in normalized luciferase gene expression was discerned between methylated or unmethylated pCpGL_CMV/EF1α control vectors lacking the insert of the sequence spanning CpGs 9-15. *** = $p<0.001$; Figure adapted from Schartner *et al*, 2017.

3.2.5. *In silico* functional prediction

Analysis of the sequence spanning CpG sites 9-15 (see Figure 12 B) revealed possibly masked transcription factor binding sites (Table 6).

Table 6: Predicted transcription factor binding sites in the *CRHR1* amplicon encompassing CpG sites 9-15. Prediction is based on results generated from JASPAR database (http://jaspar.genereg.net/; Mathelier *et al*, 2015). 'Start' and 'End' point indicate the respective bp in the investigated CpG island (see Figure 12 B); 'DNA-Strand' labels orientation on forward strand (1) or reverse strand (-1). Table adapted from Schartner *et al*, 2017.

Transcription factor	Gene name	Start	End	DNA-Strand	Predicted site sequence
HINFP	Histone H4 Transcription Factor	172	183	1	CAGCG[8]TTCG[9]CCG[10]
E2F4	E2F Transcription Factor 4	176	186	-1	[11]GC[10]GCG[9]GCGAAC
NFIX	Nuclear Factor I/X	185	193	-1	[12]CGTTGCAGC[11]
		199	207	1	CG[13]CG[14]CCCG[15]G
TEAD1	TEA Domain Family Member 1	191	200	-1	C[13]GCATT[12]GCGT
TEAD4	TEA Domain Family Member 4	191	200	-1	C[13]GCATT[12]GCGT
NOTO	Notochord Homeobox	192	201	-1	[14]GC[13]GCATT[12]GCG
TEAD3	TEA Domain Family Member 3	192	199	-1	[13]GCATT[12]GCG
NRF1	Nuclear Respiratory Factor 1	197	207	-1	C[15]CGGG[14]CG[13]CGCA
TFAP2A	Transcription Factor AP-2 Alpha	199	209	-1	AGC[15]CGGG[14]CG[13]CG
		199	209	1	CG[13]CG[14]CCCG[15]GCT
TFAP2B	Transcription Factor AP-2 Beta	199	209	1	CG[13]CG[14]CCCG[15]GCT
TFAP2C	Transcription Factor AP-2 Gamma	199	209	-1	AGC[15]CGGG[14]CG[13]CG
		199	209	1	CG[13]CG[14]CCCG[15]GCT
HIC2	Hypermethylated In Cancer 2	200	208	1	G[13]CG[14]CCCG[15]GC
THAP1	THAP Domain Containing, Apoptosis Associated Protein 1	200	208	1	G[13]CG[14]CCCG[15]GC
REL	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog	204	213	1	CCG[15]GCTTTCC
RELA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A	204	213	1	CCG[15]GCTTTCC

Literature research using the NCBI PubMed database revealed several transcription factors to be associated with anxiety disorders or related signaling pathways. However, for some candidates no according annotation could be found.

4. Discussion

4.1. Alternative splicing of *CRHR1* dependent on rs17689918 alleles

The present study investigated the regulation of *CRHR1* gene expression especially regarding its role in panic disorder. Based on a previous association of *CRHR1* rs17689918 showing impact on *CRHR1* gene expression and receptor function, the expression of alternative spliced transcript variants was investigated. Two transcript variants, *CRHR1β* and *CRHR1-IT1-CRHR1* transcript variant 5, were found to be differentially expressed in anxiety-relevant brain regions of a human post-mortem brain sample. While *CRHR1β* showed decreased expression in amygdalae and midbrains of rs17689918 risk allele carriers, expression of *CRHR1-IT1-CRHR1* transcript variant 5 was increased in forebrain and midbrain samples of risk allele carriers. Functional analyses of CRHR1β compared with the main variant CRHR1α by application of *in vitro* electrophysiological recordings revealed a significant activation of co-expressed G-protein-signaling-sensitive inwardly-rectifying K^+ channel Kir2.3 by transcript variant CRHR1β, while CRHR1α displayed inconsistent results of activation and inhibition.

Transcript variant CRHR1β deploys opposing signaling pathway

Signaling of G-protein coupled receptors (GPCR) in general is mediated by different pathways, which comprise from different G-protein subunits (G_s, $G_{i/o}$ and G_q). G_s and $G_{i/o}$ signaling is mediated via adenylyl cyclase and further activation or inhibition, respectively, of cAMP signaling. G_q-proteins activate phospholipase C (PLC), leading to hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2). PIP_2 then activates second messenger signaling pathways of inositol trisphosphate (IP_3) and diacylglycerol (DAG) resulting in either activation of intracellular Ca^{2+} channels (IP_3) or activation of protein kinases like protein kinase C (Figure 17).

CRHR1 employs all three G-protein coupled signaling pathways and thereby extends the signaling to other pathways like mitogen-activated protein kinase (MAPK) or NOS-I

dependent signaling (Bonfiglio *et al*, 2013; Grammatopoulos and Chrousos, 2002; Refojo *et al*, 2005). Our herein developed assay analyzed CRHR1 signaling via electrophysiological recordings of the G_q-protein coupled pathway, which might play an important role in the regulation of CRHR1 signaling by different transcript variants (Grammatopoulos and Chrousos, 2002).

CRF binding to CRHR1 leads to the activation of PLC, which hydrolyzes PIP_2. PIP_2 then inhibits the activated Kir2.3 channels, which can be recorded by voltage-clamp (Hatcher-Solis *et al*, 2014). Indeed, the inhibition of Kir2.3 channel activity could be shown in about half of the recordings in presence of CRHR1α. However, the other half of CRHR1α recordings and all measures of CRHR1β show the opposite effect – a further activation of Kir2.3 channels upon CRF perfusion. This activation and inhibition of co-expressed Kir2.3 channels represents the blend of different signaling pathways CRHR1 employs. While CRHR1α signaling mainly employs G_s-protein coupled signaling or a mixture of G_s and G_q coupled signaling pathways, possibly dependent on the predominant pathway expressed in each individual cell, CRHR1β couples G_q-protein signaling, but with an opposing effect. It thereby rather inhibits PLC activity, which leads to a further increase of PIP_2 and thus, further activation of Kir2.3 channels.

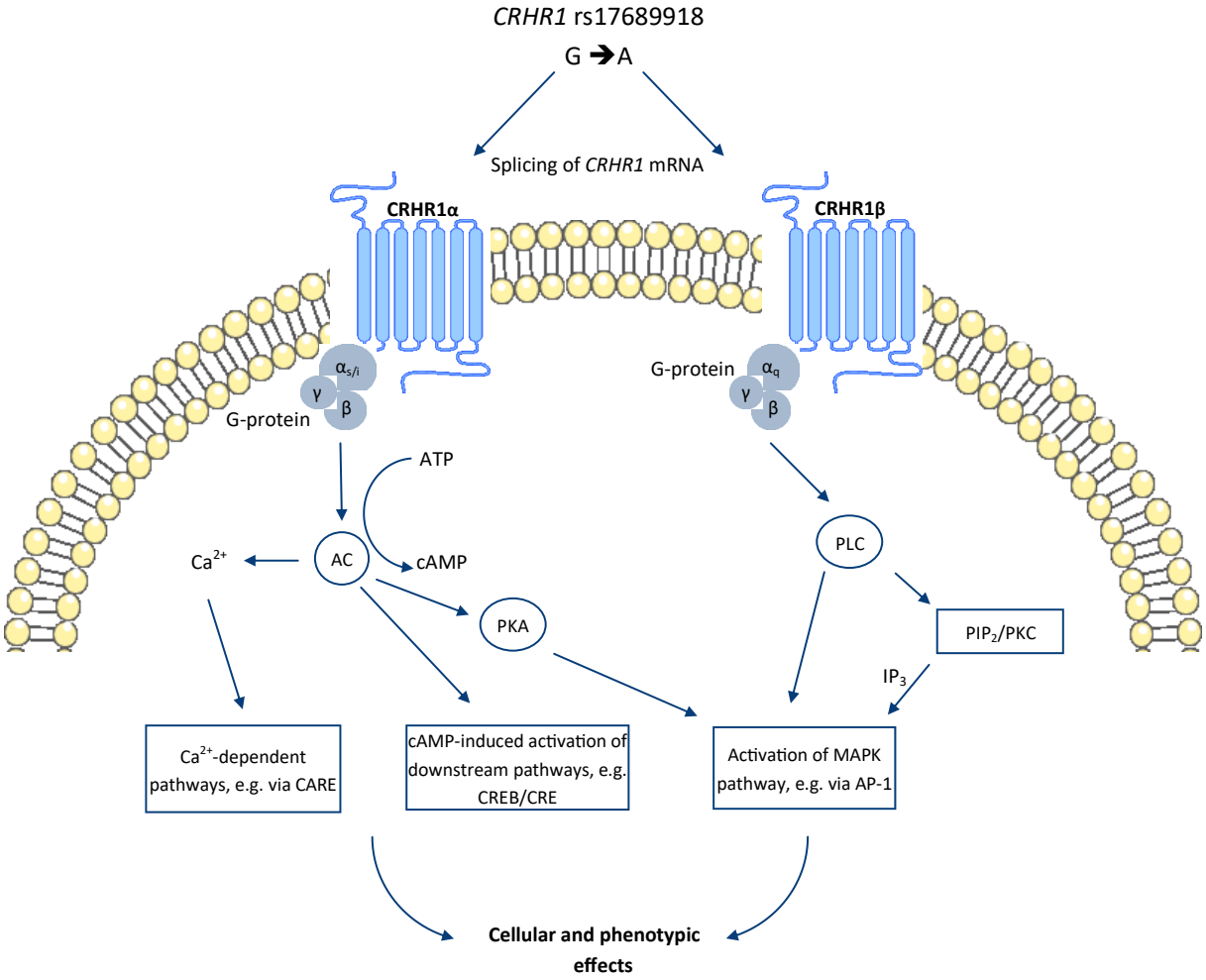

Figure 17: Schematic drawing of the differential signaling properties of CRHR1 isoforms. While CRHR1α signals mainly, but not solely, via G_s and G_i-protein coupled pathways, CRHR1β recruits the G_q-protein coupled pathway via phospholipase C inhibition. This might lead to differential regulation of the transcriptional activity and result in a different cellular response and eventually different phenotypic effects. α_s, α_i, α_q = G_α-protein subunits; AC = adenylyl cyclase; AP-1 = activator protein 1; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; Ca^{2+} = calcium ions; CARE = calcium-responsive element; CRE = cAMP-responsive element; IP_3 = Inositol trisphosphate; MAPK = mitogen-activated protein kinase; PIP_2 = phosphatidylinositol-4,5-bisphosphate; PLC = phospholipase C; PKA = protein kinase A; PKC = protein kinase C. Graphic was created with elements of ©Servier Medical Art elements by Servier (licensed under a Creative Commons Attribution 3.0 unported license; cf. Grammatopoulos and Chrousos, 2002; Zmijewski and Slominski, 2010).

In order to clarify the exact mechanism further studies are required, for instance with a selective inhibition of components of the pathways, like PIP_2, IP_3 or PLC (Hatcher-Solis *et al*, 2014). However, our results are in line with previous studies showing impaired G-protein coupling and CRF binding capability of CRHR1β compared to CRHR1α (Markovic *et al*, 2006; Pisarchik *et al*, 2001), as well as showing CRHR1β to be less functional on the G_s-protein coupled pathway, leading to less cAMP accumulation in the cell (Figure 17; Grammatopoulos

and Chrousos, 2002; Bonfiglio et al., 2011). Hence, CRHR1β might not only be less functional in sense of cAMP accumulation or CRF binding, but rather employ an opposing, potentially regulatory mechanism of CRHR1 signaling by possible inhibition of PLC via G_q-protein coupled signaling (Figure 17).

If CRHR1β also shows this contrary function compared to CRHR1α regarding other G-protein signaling pathways, namely G_s and $G_{i/O}$, has to be evaluated in future studies, for instance by co-expression of both variants with channels with different G-protein coupling. Additionally, one has to control for the variability of oocyte batches, the efficiency of RNA translation in oocytes and the isolated signaling of CRHR1 in oocytes, so the replication of the results and a translation into other, preferably human cell lines are needed to overcome the limitations of the current study. Importantly however, the increased expression of CRHR1β isoforms in risk allele carriers crucially changes CRHR1 receptor function and thereby signaling in risk allele carriers of rs17689918 (Figure 17).

Alternative splicing as important regulator of protein function

In general, alternative splicing of gene products is known to shift the function of an expressed gene to the opposite or even abolish the translation into protein (Glatt *et al*, 2011). Alternative spliced isoforms account for a high proportion of the diversity among proteins expressed from the human genome. During developmental processes alternative splicing regulates protein function and abundance, e.g. in synaptogenesis or cell fate determination (Glatt *et al*, 2011). In adult neurons alternative spliced transcripts regulate e.g. vehicle release (Sørensen *et al*, 2003), or are involved in signaling pathways like long-term potentiation (Glatt *et al*, 2011). Likewise for the biggest protein family of membrane receptors, the G-protein coupled receptors, which also include CRHR1, alternative splicing is known to regulate specific signaling pathways (Markovic, 2013).

Of the eight known alternative transcripts of *CRHR1* and *CRHR1-IT1-CRHR1* transcript variant 5, only three alternative transcripts code for all seven transmembrane domains (Zmijewski and Slominski, 2010), however little is known about the characteristics of *CRHR1-*

IT1-CRHR1 transcript variant 5. Alternative splicing of *CRHR1* has been shown to be shifted in response to environmental factors. Pisarchik *et al.* analyzed expression patterns of *CRHR1* isoforms in human skin cells before and after treating the cells with different stressors like UV light exposure (Pisarchik *et al*, 2001). All cells shifted the expression towards *CRHR1α* isoform and partially towards the expression of *CRHR1g* (Pisarchik *et al*, 2001). This shift in alternative transcripts persisted after repeated stress exposure, pointing to more permanent adaption of the cells to the stress by changing the receptor expression (Pisarchik *et al*, 2001). In the brain, the regulation of *CRHR1* alternative splicing could be modulated in a similar way, e.g. shifting the expression to less functional CRHR1 variants upon stress exposure. Indeed, *CRHR1β* was not found to be expressed in any of the investigated skin cells as it might be more restricted to the brain. The differences in expression of *CRHR1* splicing variants revealed in this study could be an adaption of risk allele carriers in response to either accumulated stressors or in response to a dysregulated stress response. Expression of certain isoforms with differential or regulatory function could therefore represent either a risk factor for, or a result of PD. Especially as this alteration in alternative splicing is probably accompanied by increased activation of the amygdala during safety learning, or decreased activation of prefrontal cortex areas in differential conditioning tasks in risk allele carriers as revealed previously by Weber *et al.* (Weber *et al*, 2015).

Dysregulated CRHR1 signaling in the fear network

Amygdala, forebrain and midbrain cover all brain regions of the fear network of panic disorder initially described and revised by Gorman et al. (Gorman *et al*, 1989, 2000). Since then, this fear network and the dysregulation of the same were investigated extensively in anxiety disorders and panic disorder using constantly evolving imaging and genetic techniques. However, the important regions of the fear network still comprise the prefrontal cortex, brainstem and the limbic system (Dresler *et al*, 2013). Expression of *CRHR1* was detected in all these regions underlining a role of CRHR1 signaling within the fear network (Kühne *et al*, 2012; Refojo *et al*, 2011).

A decreased expression of *CRHR1β* in risk allele carriers of rs17689918 might point to dysregulation of CRHR1 signaling in the amygdala and midbrain of risk allele carriers, which is in line with the decreased gene expression of overall *CRHR1* in amygdalae of risk allele carriers demonstrated earlier (Weber *et al*, 2015). The amygdala is one of the most important structures for emotional regulation and regularly associated with fear and anxiety (Davis and Whalen, 2001; Kim *et al*, 2012; Phelps *et al*, 2005). CRHR1 signaling in the amygdala was shown to mediate anxiety related behavior. Mice with a conditional gene knockout of *Crhr1* in the limbic system displayed reduced anxiety related behavior but normal HPA axis activity (Müller *et al*, 2003). Further, these mice showed also impaired adaption to stress by a putative dysregulation of downstream associated genes via corticosteroid signaling (Müller *et al*, 2003). Another study showed that rescue of extreme anxiety phenotypes of low anxious (LAB) and high anxious (HAB) mouse strains by stressful or enriched environment, respectively, resulted in bidirectional changes in *Crhr1* gene expression in the amygdala but not in other brain regions (Sotnikov *et al*, 2014). Additionally, the above described changes in anxious temperament in rhesus macaques were accompanied by changes in metabolic activity in amygdala and hippocampus (Rogers *et al*, 2013). Together with the aforementioned increased activation of the amygdala of risk allele carriers in a safety learning task (Weber *et al*, 2015), the hypothesis of a dysregulation of amygdala activity by aberrant CRHR1 signaling is further reinforced. Importantly, our data indicate that these disturbances are not only mediated by a decreased gene expression of total *CRHR1*, but also enhanced by a shift in expression to a less functional, or even contrarily functional, alternative spliced transcripts.

In forebrain and midbrain regions of risk allele carriers, the expression of *CRHR1-IT1-CRHR1* transcript variant 5 was significantly increased. This variant is a readthrough of upstream non-coding gene *CRHR1-Intronic transcript 1* and *CRHR1*. It encodes a protein with sequence identities with *CRHR1* but alternate 5’ structure including alternate exons and untranslated region (UTR), as well as an alternative translation start (Pruitt *et al*, 2014). So far, there are no functional studies available for *CRHR1-IT1-CRHR1* transcript variant 5. However,

as it shares important parts of the sequence with functional *CRHR1* transcript variants, transcript variant 5 might also be important for regulation of CRF signaling.

Moreover, midbrain and forebrain regions are important brain areas of the fear network and highly relevant for panic disorder. Several studies report differential brain activation in structures of midbrain and forebrain in panic disorder patients or risk allele carriers of candidate genes (Domschke *et al*, 2006, 2008; Dresler *et al*, 2013; Feldker *et al*, 2016). Imaging studies regularly associate structural alterations in brainstem and cortical areas with panic disorder (Dresler *et al*, 2013). Thereby, especially the interaction and signaling between these brain regions seems to play a crucial role in the pathophysiology of panic disorder (Dresler *et al*, 2013). Animal studies have also shown decreased anxiety-related behavior in prefrontal *Crhr1* knockout mice, which reduced also the impact of early life stress on anxiety-related behavior (Wang *et al*, 2012).

Interestingly, it has been shown that CRHR1 has quite an ambivalent role depending on the cell type and region of the brain where it is expressed. While *Crhr1* deletion in glutamatergic neurons of the forebrain had anxiolytic effects and impaired signaling with other brain areas like the amygdala, the deletion of *Crhr1* in midbrain dopaminergic neurons had an anxiogenic effect and impaired dopamine signaling of the prefrontal cortex (Refojo *et al*, 2011). An imbalance in the regulation of the signaling of midbrain and forebrain regions by CRHR1 might lead to the development of anxiety disorders (Refojo *et al*, 2011). This imbalance might not only be mediated by the depletion of CRHR1 in the respective brain area, but also be induced by the expression of less functional or regulatory alternative transcript variants like CRHR1β and CRHR1-IT1-CRHR1 transcript variant 5. However, to confirm that this effect is not only induced by a reduced expression of total CRHR1, but also by increased expression of alternative spliced isoforms like transcript variant 5, further studies for instance on the functional characterization of CRHR1 transcript variants are urgently needed.

Limitations

Beside the above discussed caveats, several limitations have to be considered in interpretation of our data. The post-mortem sample consisted of tissue samples of amygdala, forebrain and midbrain. Here no separation regarding sub-regions or cell types was possible. According to several studies showing signaling and expression differences between very distinct brain regions within the forebrain, midbrain and even the amygdala, our data gives a rather general overview and might not reflect the actual *CRHR1* expression in each brain sub-region. Replication of our findings in further independent samples is required as well as translation into animal models to get a greater resolution of regional and cell-type specific *CRHR1* expression and signaling.

Despite being tested with stable and good quality measures, the RNA from the post-mortem brains could be partially degraded as the post-mortem interval is between 28-111 h. Further, only limited data was available on the post-mortem brain sample, e.g. no data of medication or substance abuse. For instance substance abuse has been shown to influence or be influenced by alternative splicing of certain candidate genes, but an impact on alternative splicing of *CRHR1* or other G-protein coupled receptors is not known (Glatt *et al*, 2011).

Due to the limited amount of post-mortem brain RNA some adjustments had to be made to the MIQE guidelines of qPCR analyses, e.g. measurements in duplicates instead of triplicates and use of four instead of five references genes (Hellemans *et al*, 2007; Ruijter *et al*, 2009; Vandenbroucke *et al*, 2001). Therefore, replication of the expression is urgently needed in a second independent post-mortem brain sample. In addition, the electrophysiological data has to be interpreted carefully. Firstly, only one possible signaling pathway was analyzed. Secondly, only two transcript variants were compared here, without controlling for impact of possible co-expressed compensatory isoforms that might be expressed in human cells as the highly regulated expression of alternative transcripts of *CRHR1* is known from different studies (Pisarchik *et al*, 2001; Pisarchik and Slominski, 2004). Thirdly, the electrophysiological measures were performed *in vitro* in an isolated system. CRHR1 is known to function differently in different cell environments, e.g. the opposite effects

on anxiety-related behavior between glutamatergic and dopaminergic neurons (Refojo *et al*, 2011), or the differential CRHR1 activation in presence of NMDA or AMPA receptors (Andres *et al*, 2013). Thus, the analyses of electrophysiological properties of CRHR1 transcript variants has to be extended to more variants including possible interactions or compensatory effects, and the characterization of variants in different cell types, e.g. human cell lines. Further, an over-expression and knockdown of single isoforms or a combination of different alternative transcript variants in human cell lines might improve possible translation of these findings and mirror actual CRHR1 signaling in human neurons. Besides the direct regulation of alternative splicing of *CRHR1*, rs17689918 could also affect downstream or upstream genes, especially as Weber *et al.* found 90 perfect proxies, which extend also to neighboring genes (Weber *et al*, 2015). This might also affect the regulation of *CRHR1* expression and splicing.

Outlook and future studies

Beside the already described replication in independent post-mortem samples, further experiments will help to clarify the role of CRHR1 in anxiety-related behavior and panic disorder. In this regard, post-mortem case/control samples for anxiety disorders and panic disorder might be of special interest. Also the investigation of epigenetic regulation of alternative splicing might add a new dimension to the regulation of CRHR1 signaling in the brain. An epigenetic regulation of NTRK2/TRKB isoforms in suicide completers was already shown by Ernst *et al.* (Ernst *et al*, 2009). Epigenetic analyses were not possible in our sample as only whole genome amplified DNA was available lacking the original methylation pattern.

Nevertheless, we could show that the previously associated SNP rs17689918 regulates alternative splicing of *CRHR1* in specific brain areas of stress response, fear and anxious behavior. Together with previous findings on molecular, functional and behavioral level, we find converging evidence that rs17689918 regulates efficiency of CRF signaling by shifting alternative transcription of its receptor. Future studies have to clarify the specific role of *CRHR1* alternative transcripts on cellular level and eventually in neuronal networks to understand the role of CRF signaling in PD and eventually other stress-related disorders.

4.2. Epigenetic regulation of CRHR1 promoter region

In the second approach of the present study, methylation patterns of a CpG island in the *CRHR1* promoter region were investigated for the first time regarding panic disorder and a related psychometric variable. Patients with panic disorder were found to display significantly decreased *CRHR1* methylation when compared to a sample of healthy controls. In line with this, an independent sample of healthy volunteers with an increased score on the Beck Anxiety Inventory (BAI), which has been proposed as particularly suitable to assess anxiety disorders with a high somatic component such as panic disorder (Cox *et al*, 1996; Creamer *et al*, 1995), showed decreased *CRHR1* methylation. Here, an accumulative impact of stressful experiences during childhood, risk allele of *CRHR1* SNP rs17689918 and *CRHR1* promoter methylation on sum scores of BAI could be discerned. The functional relevance of differential methylation of the investigated target sequence was confirmed with reporter gene assays, which revealed an increased *CRHR1* gene expression in non-methylated samples (Jaenisch and Bird, 2003; Suzuki and Bird, 2008).

CRHR1 expression regulates anxiety-related behavior

Decreased *CRHR1* promoter methylation, conferring increased *CRHR1* gene expression, as a possible marker of panic-related pathology provides a first translational support for a recent epigenetic study in rodents. *Crhr1* promoter demethylation leads to elevated *Crhr1* mRNA expression in offspring of prenatally stressed mice accompanied by anxiety-related behavior (Wang *et al*, 2013). Our results are in line with global *Crhr1* knockout mice displaying decreased anxiety-related behavior compared to wild types and concomitantly blunted ACTH and cortisol levels (Timpl *et al*, 1998). Furthermore, the aforementioned conditional knockdown of *Crhr1* in the anterior forebrain (Wang *et al*, 2012) as well as in limbic brain regions (Rogers *et al*, 2013; Sztainberg *et al*, 2010) lead to a hypersensitivity to stress along with decreased anxiety-related behavior. Together, these converging findings point to an epigenetically driven hyperactive stress axis with enhanced CRHR1 signaling in turn increasing anxiety risk.

However, as discussed above, several studies point to a more complex role of Crhr1 signaling in brain (Refojo *et al*, 2011) and also in humans, the directionality of CRHR1 signaling in the regulation of anxiety is not entirely clarified. While the recent association of the minor allele of rs17689918 with panic disorder accompanied with decreased mRNA expression in anxiety-related brain regions might appear as diverging, different aspects have to be taken into account. While the previous study focused rather on a separate aspect of anxious apprehension, showing decreased expression with an above discussed shift in alternative splicing of *CRHR1* in the respective anxiety-related brain regions, the second approach instead investigated phenotypes mirroring more acute panic states with increased strong autonomic arousal (Hamm *et al*, 2014; Weber *et al*, 2015). The underlying pathomechanism lies in an increased CRF transmission under mediation of an epigenetically controlled *CRHR1* expression. The underlying epigenetic processes might thereby serve as a biological attempt to antagonize genetic or environmental risk factors in a putative compensatory way (Figure 18).

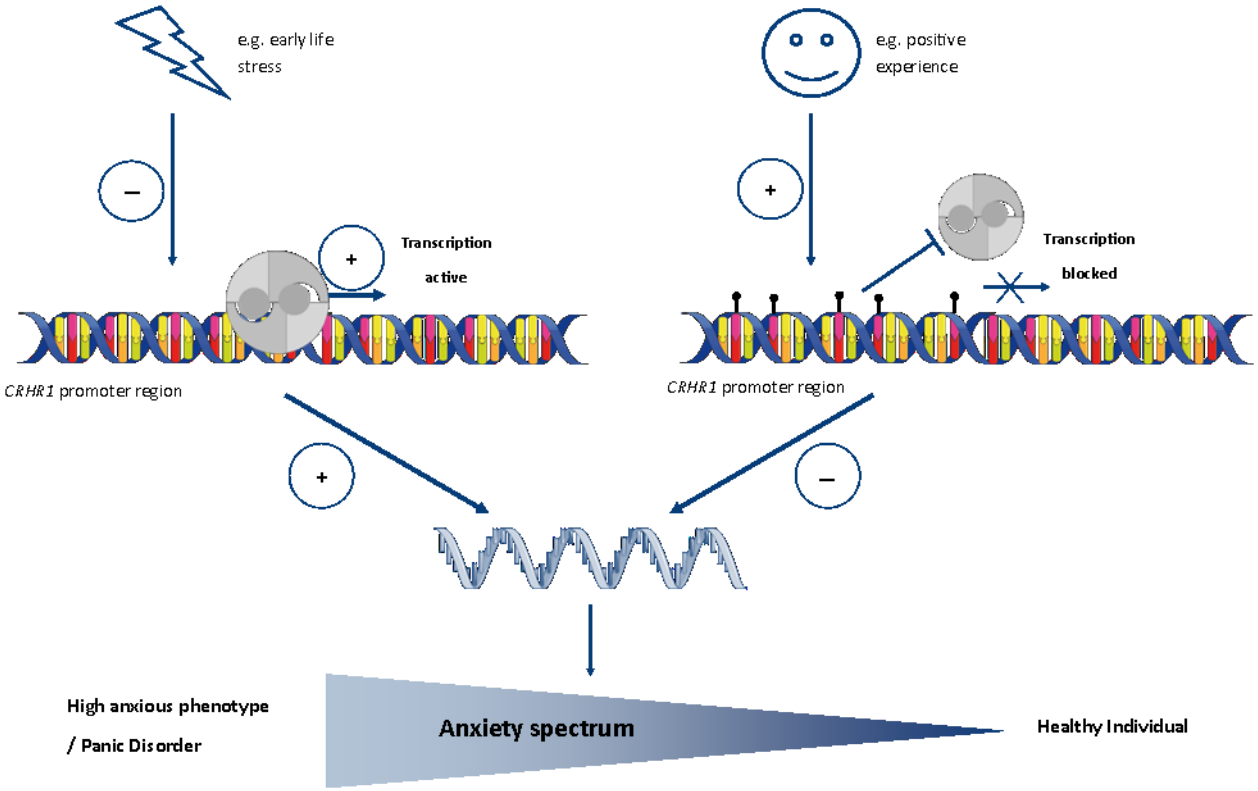

Figure 18: Hypothesized regulation of *CRHR1* expression by DNA methylation. *CRHR1* gene promoter methylation (black bubbles) is decreased in response to stressful life events or increased by positive life events, respectively. DNA methylation of the *CRHR1* promoter region leads to altered expression of *CRHR1* mRNA and eventually different amounts of receptors in the cell. This eventually leads to more or less anxious phenotype, which might turn into pathologic phenotypes like panic disorder. Graphic was created with elements of ©Servier Medical Art elements by Servier (licensed under a Creative Commons Attribution 3.0 unported license); cf. Sotnikov *et al*, 2014.

This is further reinforced by increased anxiety scores in healthy controls with accumulative risk factors of increased early life stress, low *CRHR1* promoter methylation and the minor allele of rs17689918. Despite not having a direct impact on DNA methylation, the risk allele of rs17689918 in concert with other risk factors clearly increases anxiety in general and hence, the risk of developing a related disorder. Interestingly, early life stress is associated with deficits in stress response and anxiety-like behavior conferred by impaired *Crhr1* expression (Sumner *et al*, 2014; Veenit *et al*, 2014; Wang *et al*, 2012), and also other components of the HPA axis were shown to be influenced by early life stress (Klengel *et al*, 2013). However, the effect of rs17689918 on *CRHR1* promoter methylation might rather be indirect as our results lack the direct association of rs17689918 with methylation, which either point to two independent underlying “risk” mechanisms – a genetic and an epigenetic mechanism, or an additive effect of risk factors lacking a conclusive connection. Both

possibilities are further endorsed by the small effect size of the interaction of epigenetic, genetic and environmental factors on BAI sum scores.

While the genetic mechanism is mediated by the SNPs impact on gene expression via the regulation of alternative splicing directly affecting the receptor functionality, the epigenetic regulation of *CRHR1* gene expression is an independent form of expression-regulation in response to environmental stressors, confirming *CRHR1* as a plasticity gene that regulates behavior along an anxiety continuum (Belsky *et al*, 2009; Sotnikov *et al*, 2014). However, given the limited samples size of the present sample this independence of genetic and epigenetic regulation could not be approved with certainty.

DNA methylation is masking important TFBS

The possible masking of transcription factor binding sites (TFBS) by DNA methylation at CpG sites 9-15 might constitute the underlying mechanism of the regulation of *CRHR1* gene expression. Among the predicted transcription factors, two candidates, namely TFAP2 and NFκB, have been identified to bind in the *CRHR1* promoter region in previous studies (Parham *et al*, 2004). Furthermore, *TFAP2*, coding for the Transcription factor Activating Enhancer Binding Protein 2 α/β, has already been shown to regulate serotonergic, dopaminergic, and noradrenergic signaling partly via regulation of CRF/CRHR1 pathways (Mitchell *et al*, 1991; Salim *et al*, 2007). Moreover, TFAP2A has been shown to be involved in CRF-dependent regulation of gene expression (Cheng and Handwerger, 2002; Damberg *et al*, 2000; Hubert *et al*, 2010). Certain genotypes of *TFAP2β* have also been associated with decreased anxiety-related personality traits in women (Damberg *et al*, 2003). Animal studies in rodents linked antidepressant treatment to *TFAP2* gene expression and DNA-binding activity (Damberg *et al*, 2000).

Other putative masked transcription factors like THAP1 (THAP Domain Containing 1) or REL/RELA (REL proto-oncogene, NF-kB subunit / RELA proto-oncogene, NF-kB subunit) have been shown to be involved in regulation of *CRF* and *CRHR1* expression in inflammatory processes and during pregnancy (e.g., REL / RELA; Markovic *et al*, 2013; Zocco *et al*, 2010)

as well as frustration behavior (THAP1; Sabariego *et al*, 2013). Further studies on DNA-protein interaction using methylated and unmethylated DNA are needed to clarify the exact mechanism involved in the regulation of gene expression and its relevance for stress- and particularly panic-related anxiety.

Whether these epigenetic processes are a compensatory biological attempt to antagonize a genetic predisposition and the impact of other environmental factors, rather than a relevant pathogenic mechanism by themselves, could not be evaluated conclusively in this study. Hence, further replication of the previous categorical association of *CRHR1* genetic polymorphisms with panic disorder together with epigenetic variation in longitudinal studies is clearly needed. Cohort studies will thereby allow to evaluate potential dynamics in *CRHR1* methylation dependent on environmental factors under careful consideration of clinical phenotype, comorbidities, duration of the disorder, medication and family history. These aspects are highly relevant given the crucial role of CRHR1 in the stress response dependent on environmental factors and the bidirectional dynamics of *Crhr1* gene expression under control of promoter DNA methylation upon differential environmental stimuli in the amygdala of extreme phenotypes in rodents (Holsboer, 1999; Risbrough and Stein, 2006; Schreiber *et al*, 1996; Sotnikov *et al*, 2014; Tyrka *et al*, 2009).

Limitations

Besides the above-mentioned caveats and suggestions for future studies, the following limitations have to be considered when interpreting the present results: Due to missing data on smoking behavior and psychotropic medication for parts of the sample, the influence of these factors on *CRHR1* promoter methylation could not be discerned. Future studies are needed to explore the effects of medication, smoking behavior and other potentially confounding factors on *CRHR1* methylation. Further, the sum scores of anxiety measured with BAI and trauma measured with CTQ in the independent healthy control samples suggest rather low anxiety and childhood trauma levels in the cohort. Here, future studies with a wider variation among the dimensional anxiety and stress levels are required to extend our findings

to the full continuum of anxiety. Additionally, the investigation of gene expression by luciferase assays encompassing the seven differentially expressed CpG sites cannot be directly extrapolated to *CRHR1* promoter regulation *in toto*. In this regard, no data on gene and protein expression were available leaving the extension of *CRHR1* methylation on gene expression level rather suggestive than conclusive. DNA methylation was measured in DNA from whole blood samples with several possible confounding factors like cell composition of the blood. Despite increasing evidence for blood samples being convenient proxies for methylation correlates in brain tissue, epigenetic patterns in peripheral biomaterial do not allow direct conclusions regarding central mechanisms (Davies *et al*, 2012; Ewald *et al*, 2014; Massart *et al*, 2016; Provençal *et al*, 2012; Ursini *et al*, 2011).

Future studies and outlook

In this respect, 'imaging epigenetic' studies using fMRI or PET correlating peripheral *CRHR1* methylation in leucocytes with brain activation patterns or central biochemical activity, respectively (cf. Demers et al., 2016; Shumay et al., 2012), will greatly advance our understanding of systemic epigenetic processes as sensors of alterations in the nervous system relevant for panic-related psychopathology. Also extending analyses of gene-environment interactions to more traumatized, anxious individuals as well as including data on stressful life events in later life should broaden the understanding of the impact on CRHR1 expression and signaling. Regarding DNA methylation patterns in brain tissue, future studies of DNA methylation paired with gene expression analyses in human post-mortem brain tissue would greatly enhance the understanding of the role of promoter methylation in *CRHR1* expression in the brain.

4.3. Conclusions

Taken together, in this study we investigated how *CRHR1* rs17689918 regulates gene expression and receptor function on multiple levels. In a first approach, we could reveal the differential expression of alternative splice products of *CRHR1* in brain areas relevant for anxiety and anxiety disorders dependent on rs17689918 genotype. Further, we developed an electrophysiological assay to analyze CRHR1 receptor function *in vitro* and revealed opposing signaling pathways employed by CRHR1α and β isoforms. Despite not showing a direct effect of rs17689918 alleles on DNA methylation of the *CRHR1* promoter region, we for the first time identified a potential role of *CRHR1* promoter hypomethylation, conferring increased *CRHR1* expression, in panic disorder and high anxious healthy individuals. The interactive impact of genetic risk, environmental risk and increased promoter methylation lead thereby to an increased anxious intermediate phenotype.

The interaction of genetic and environmental factors mediated by epigenetic factors might therefore exemplify the aforementioned multifactorial character of psychiatric disorders. The accumulation of several risk factors leads to an increased anxious intermediate phenotype even in a sample of young, in general low-anxious and low-traumatized individuals. This might mirror an increased allostatic stress load in these individuals leaving them more vulnerable for anxiety disorders. However, this view does not explain the healthiness of the tested individuals accounting for another hypothesis stating the mismatch between early and later environment to be critical for the development of the disorder (Bateson *et al*, 2004; Bodden *et al*, 2015). Both ways, there is a discrepancy between the results of decreased *CRHR1* mRNA expression regulated via alternative splicing on the one hand and the hypomethylation conferring increased *CRHR1* expression on the other hand.

Thus, CRHR1 might rather function as a plasticity gene, which is highly regulated upon environmental stressors, for instance by alternative splicing or epigenetic mechanisms. In contrast to classic risk genes or risk alleles, plasticity genes increase the individual vulnerability and susceptibility to environmental factors of all spectra and can therefore be regulated in a positive and negative manner (Belsky *et al*, 2009), as it was shown in the bidirectional

regulation of Crhr1 along an anxiety continuum in response to environmental stimuli (Sotnikov *et al*, 2014). By its central role in the regulation of the CRF system and HPA axis, CRHR1 dysregulation might greatly influence the stress response and by this favor pathomechanisms of panic disorder. In the light of a high regulation of the stress system by several positive and negative mechanisms, it is unlikely that the dysregulation of a single factor like CRHR1 expression or protein function might account for the development of a disorder. The understanding of the exact mechanisms of genetic and epigenetic regulation in anxiety and anxiety disorders would however enhance CRHR1 as a target for improved future therapeutics.

Affidavit

I hereby confirm that my thesis entitles “The Regulation of Corticotropin releasing hormone receptor 1 gene expression and its role in Panic Disorder” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, 07.03.2017

Eidestattliche Erklärung

Hiermit erkläre ich Eides statt, die Dissertation „Die Regulation der *Corticotropin Releasing Hormon Rezeptor 1* Genexpression und ihre Rolle bei der Panikstörung“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 07.03.2017

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Annex

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Abbreviations

#	5-hmC	5-Hydroxymethylcytosine
	5-mC	5-Methylcytosine
	%	percent
	α	alpha
	β	beta
	γ	gamma
	µ	micro
	µg	microgram
	µl	microliter
	µM	micro molar
	χ	chi
A	A	adenine
	A	Ampere
	aa	amino acid
	ACTH	adrenocorticotropic hormone
	ADORA2A	adenosine A2a receptor
	ANOVA	analysis of variance
	AT	anxious temperament
	ATP	adenosine triphosphate
B	BAI	Beck anxiety inventory
	BLAST	Basic Local Alignment Search Tool
	bp	base pairs
C	C	cytosine
	°C	degree Celsius
	©	copyright symbol
	CaCl2	calcium chloride
	cAMP	cyclic adenosine monophosphate
	cDNA	complementary DNA
	cf	confer
	chr	chromosome
	cm	centimeter
	CMV	cytomegalovirus
	CNR1	cannabinoid receptor 1
	CO_2	carbon dioxide
	CpG	cytosine phosphate guanine
	CRC	collaborative research center
	CRF	corticotropin-releasing factor
	CRH	corticotropin releasing hormone
	CRHBP	corticotropin releasing hormone binding protein
	CRHR1	corticotropin releasing hormone receptor 1
	CRHR1-IT	CRHR1-intronic variant 1
	CRHR2	corticotropin releasing hormone receptor 2
	cRNA	complementary RNA
	CTP	cytosine triphosphate

	CTQ	childhood trauma questionnaire
D	DAG	diacylglycerol
	DALY	disability adjusted life years
	ddH2O	double distilled H_2O
	DEPC	diethyl dicarbonate
	Dipl.-Psych	Diplom-Psychologe / Diplom-Psychologin
	DMEM	Dulbeccos modified Eagles Medium
	DNA	deoxyribonucleic acid
	DNMT1	DNA methyltransferase 1
	DNMT3a	DNA methyltransferase 3a
	DNMT3b	DNA methyltransferase 3b
	dNTP	deoxy nucleoside triphosphate
	Dr.	doctor
	DSM	Diagnostic and Statistical Manual of Mental Disorders
E	e.g.	exempli gratia
	EDTA	2,2',2",2"'-(Ethane-1,2-diyldinitrilo)tetraacetic acid
	EF1a	elongation factor 1 alpha
	ESME	epigenetic sequencing methylation
	et al.	*et alii*, Latin: and others
	EtOH	ethanol
F	f	female
	F	ANOVA test statistic
	FBS	fetal bovine serum
	fMRI	functional magnetic-resonance imaging
G	G	guanine
	g	gram
	GAD1	glutamate decarboxylase 1
	gDNA	genomic DNA
	GPCR	G-protein coupled receptors
	GR	glucocorticoid receptor
	GTP	guanine triphosphate
	GUSB	glucuronidase beta
	G × E	gene-environment interactions
H	h	hour
	HAB	high anxiety behavior
	HEK	human embryonic kidney
	HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
	hK+	high potassium
	HPA	hypothalamus-pituitary-adrenal
	HPRT1	hypoxanthine phosphoribosyltransferase 1
I	i.e.	*id est*; Latin: that is
	ICD	International Statistical Classification of Diseases and Related Health Problems
	IP3	intracellular Ca2+ channels
	IRK	inwardly-rectifying potassium channel
K	K	potassium
	KCl	potassium chloride

	KHCO3	potassium hydrogen carbonate
	Kir	Potassium inwardly-rectifying channel
L	l	liter
	LAB	low anxiety behavior
	LB	lysogeny broth
	LC	locus coeruleus
	Ltd	limited company
M	m	milli
	M	molar
	m	male
	M.I.N.I	mini international neuropsychiatric interview
	MaCl2	magnesium chloride
	maju	many jumps
	MAOA	monoamine oxidase A
	MAPK	mitogen-activated protein kinase
	max	maximal
	min	minute
	min	minimal
	MIQE	minimum information for publication of quantitative real-time PCR experiments
	miRNA	micro ribonucleic acid
	mM	milli molar
	MOPS	3-Morpholinopropane-1-sulfonic acid
	MR	mineralocorticoid receptor
	MRC	Medicl Research Council
	mRNA	messenger ribonucelic acid
	ms	millisecond
	mV	milli volt
	MΩ	mega ohm
N	n	nano
	Na	sodium
	NaAc	sodium acetate
	NaCl	sodium chloride
	$NaHCO_3$	sodium hydrogen carbonate
	NCBI	National Center for Biotechnology Information
	ncRNA	non-coding ribonucleic acid
	ng	nanogram
	NH4Cl	ammonium chloride
	nl	nanoliter
	nm	nanometer
	nM	nano molar
	NPSR1	neuropeptide S receptor 1
	NR3C1	nuclear receptor subfamily 3 group C member 1
	ns	not significant
	NTRK2	neurotrophic receptor tyrosine kinase 2
	NY	New York
O	ORF	open-reading frame

	OXTR	oxytocin receptor
P	p	p-value
	PBS	phosphate buffered saline
	PCR	polymerase chain reaction
	PD	panic disorder
	PET	positron emission tomography
	PIP2	phosphatidylinositol-4,5-bisphosphate
	PLC	phospholipase C
	Prof.	professor
	PTSD	posttraumatic stress disorder
	PUMCH	Peking Union Medical College Hospital
	PVH	paraventricular nuclei of the hypothalamus
Q	qPCR	quantitative real-time PCR
R	REL	REL proto-oncogene, NF-kB subunit
	RELA	RELA proto-oncogene, NF-kB subunit
	RFLP	restriction fragment length polymorphism
	RGS2	regulator of G-protein signaling 2
	RIN	RNA integrity value
	RISC	RNA-induced silencing complexes
	RNA	ribonucleic acid
	rpm	rounds per minute
	rs	reference SNP cluster number
	RT	room temperature
S	SAM	sympathetic-adrenal-medullary
	SCID-I	structured clinical interview for DSM IV Axis I disorders
	SD	standard deviation
	SDHA	succinate dehydrogenase complex flavoprotein subunit A
	SDS	Sodium lauryl sulfate
	SNP	single nucleotide polymorphism
	snRNA	small nuclear ribonucleic acid
	SOC	Super Optimal broth with Catabolite repression
	ss	single strand
	SSRI	selective serotonin re-uptake inhibitors
	StAR	steroidogenic acute regulatory
T	T	thymine
	t	t-test statistic
	TAE	tris acetate EDTA
	TE	tris EDTA
	TECV	two electrode voltage clamp
	Tet	ten eleven translocation
	TFAP2	Transcription factor Activating Enhancer Binding Protein 2 α/β
	TFBS	transcription factor binding site
	THAP1	THAP Domain Containing 1
	TM	trademark
	TFRC	transferrin receptor
	TRKB	Tropomyosin-Related Kinase B

	TRR58	Transregio 58
U	U/ml	units per millimeter
	UCSC	University of California Santa Cruz
	UK	United Kingdom
	USA	United States of America
	UTP	uracil triphosphate
	UTR	untranslated region
	UV	ultraviolet
V	V	volt
	v/v	volume per volume
W	w/	with
	w/v	weight per volume

Publication list

Schartner, C., Scholz, C.-J, Weber, H., Post, A., Freudenberg, F., Grünewald, L., Reif, A.; "The regulation of tetraspanin 8 gene expression - a potential new mechanism in the pathogenesis of bipolar disorder"; **under review**.

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