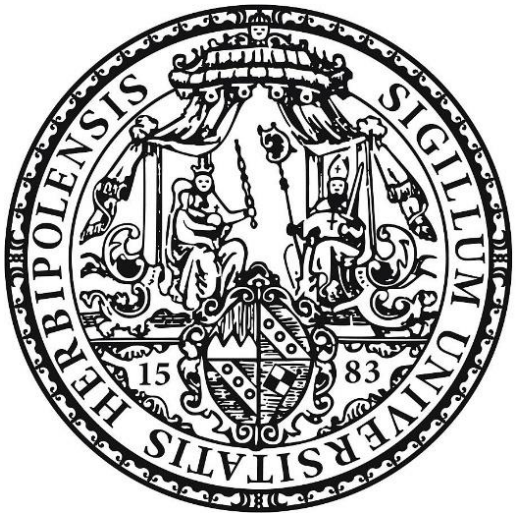
The role of *Rgs2* in animal models of affective disorders

Über die Bedeutung von *Rgs2* in Tiermodellen affektiver Störungen



Dissertation for a doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg
(Section Biomedicine)

submitted by

Annette Raab

from Breitengüßbach

Würzburg, April 2017

Submitted on: ……

Members of the *Promotionskomitee*:

Chairperson: Prof. Dr. Paul Pauli

Primary Supervisor: Prof. Dr. Jürgen Deckert

Supervisor (Second): Prof. Dr. Dr. Martin Heisenberg

Supervisor (Third): Prof. Dr. Martin Lohse

Supervisor (Fourth): Dr. Dr. Leif Hommers

Date of Public Defence: …….

Date of Receipt of Certificates: ……..

Table of contents

1 Introduction 1
1.1 Affective disorders 1
1.1.1 Anxiety disorders 2
1.1.2 Depressive disorders 4
1.2 Animal models of psychiatric disease 6
1.2.1 What is an animal model? 6
1.2.2 Validity of animal models 6
1.2.3 Evaluating fear and anxiety-related behavior 7
1.2.3.1 Innate anxiety 8
1.2.3.2 Learned fear 9
1.2.4 Evaluating depression-like behavior 10
1.3 G protein-coupled signaling 11
1.3.1 G protein-coupled receptor regulation 12
1.3.2 Regulators of G protein signaling 12
1.3.3 Regulator of G protein signaling 2 15
1.3.3.1 *RGS2* in the brain and its contribution to psychiatric disease 17
1.3.3.1.1 Human findings 17
1.3.3.1.2 Mouse model 17
1.4 MicroRNAs 20
1.4.1 Discovery 20
1.4.2 Biogenesis and function 21
1.4.3 MicroRNA in psychiatric disorders 22
2 Aim of the study 23
3 Materials 24
3.1 Chemicals and reagents 24
3.2 Technical equipment 25
3.3 Consumable supplies 26
3.4 DNA- and protein ladders 26
3.5 Commercial kits 26
3.6 Cell Lines 26
3.7 Cell culture medium 26
3.8 Plasmids 27
3.9 Solutions and buffers 27
3.10 Software 27

4 Methods 29
4.1 Animals 29
4.1.1 Genotyping of mice 29
4.1.1.1 Agarose gel electrophoresis 30
4.1.2 Analysis of body composition 31
4.1.3 Blood pressure measurements 31
4.1.4 Behavioral tests 31
4.1.4.1 Contextual and Cued Fear Conditioning 33
4.1.4.2 Elevated Plus Maze 34
4.1.4.3 Dark-Light Exploration 34
4.1.4.4 Open Field Locomotion 34
4.1.4.5 Barnes Maze 35
4.1.4.6 Intellicage 35
4.1.4.6.1 Apparatus 35
4.1.4.6.2 Place preference 36
4.1.4.7 Unpredictable Chronic Mild Stress 36
4.1.4.8 Sucrose Preference Measurements 37
4.1.4.9 Crawley´s Sociability and Preference for Social Novelty 37
4.1.4.10 Forced Swim Test 38
4.2 Cell culture techniques 38
4.2.1 Freezing cells 38
4.2.2 Thawing cells 38
4.2.3 MicroRNA mediated expression repression 38
4.2.3.1 Computational methods 38
4.2.3.2 Luciferase reporter assay 39
4.2.3.3 DNA-Transfection 39
4.2.3.4 Quantification of luciferase activity 39
4.3 MicroRNA Sequencing 40
4.4 High pressure liquid chromatography 40
4.5 Quantitative gene expression analysis 41
4.6 Statistical analysis 42
5 Results 43
5.1 General health 43
5.1.1 Body Weight, Food Intake and Body Composition 44
5.1.2 Blood pressure and heart rate 45
5.2 Memory and Learning 47

5.2.1 Aversive learning and memory 47
5.2.1.1 Short-term fear memory 48
5.2.1.2 Long-term fear memory and extinction learning 50
5.2.1.3 Gene expression analysis 51
5.2.2 Spatial learning 52
5.2.3 Reward learning and memory 56
5.3 Acute stress and its impact on innate anxiety 58
5.3.1 Elevated Plus Maze 58
5.3.2 Dark-Light Exploration 60
5.3.3 Open Field Locomotion 62
5.4 Chronic stress and its impact on anxiety and depressive behavior 65
5.4.1 Sucrose Preference and Food Consumption 65
5.4.2 Dark-Light Exploration 68
5.4.3 Social Interaction 71
5.4.4 Forced Swim Test 74
5.4.5 Gene expression analysis 75
5.5 Cell biological analysis 77
5.5.1 Neurotransmitter levels 77
5.5.2 G protein-coupled receptor expression 78
5.5.3 Regulator of G protein signaling protein expression 80
5.5.4 MicroRNA expression analysis 81
5.5.4.1.1 Luciferase Reporter Assay 81
5.5.4.1.2 MicroRNA Sequencing 82
6 Discussion 83
6.1 *Rgs2* deletion increases learning and memory 83
6.1.1 Behavioral testing 83
6.1.2 Gene expression and neurotransmitter level changes 84
6.1.2.1 Serotonergic system 85
6.1.2.2 Dopaminergic system 86
6.1.2.3 Intermediate early genes 87
6.2 *Rgs2* deletion provokes sex specific stress coping behavior 89
6.2.1 Behavioral testing 89
6.2.2 Gene expression changes 90
6.2.2.1 Adrenergic system 90
6.2.2.2 Neuropeptide Y system 91
6.2.2.3 microRNA Expression changes 91

6.3 *Rgs2* deletion increases innate anxiety 93
6.3.1 Behavioral testing 93
6.3.2 Gene expression changes 94
6.3.2.1 Serotonergic system 94
6.3.2.2 Adrenergic system 95
6.3.2.3 Neuropeptide Y system 95
6.4 *Rgs2* deletion increases depressive behavior 97
6.4.1 Behavioral testing 97
6.4.2 Gene expression analysis and neurotransmitter levels 97
6.4.2.1 Adrenergic system 97
6.4.2.2 Neurotransmitter level 98
6.5 Behavioral phenotyping issues 99
6.5.1 Health issues 99
6.5.2 Learning and memory testing 100
6.5.3 Anhedonia 100
6.5.4 Blood pressure measurements 101
7 Summary 103
8 Abbreviations 106
9 References 109
10 Appendix 127
10.1 Curriculum vitae 127
10.2 Publication list and conference contributions 129
10.3 Affidavit 131
10.4 Acknowledgments 132

Figures and Tables

Figure 1: Top 25 causes of global years lived with disability in 1990 and 2013 1
Figure 2: Total cost of all disorders of the brain in Europe by type of cost 2
Figure 3: Expression of fear, Charles Darwin, 1872 3
Figure 4: Behavioral tasks assessing innate anxiety in mice 9
Figure 5: Fear conditioning paradigm 10
Figure 6: Fine tuning of GPCR signaling by RGS proteins 13
Figure 7: The RGS fold of RGS4 14
Figure 8: RGS Proteins 14
Figure 9: Structural illustration of RGS2 in complex with $G\alpha_q$ 16
Figure 10: RGS2 regulation of synaptic signaling 19
Figure 11: General microRNA pathway 21
Figure 12: Experimental schedule of fear conditioning, short-term and long-term fear memory and extinction as well as acute stress susceptibility testing 32
Figure 13: Experimental schedule of chronic stress susceptibility testing 33
Figure 14: Time course of IntelliCage experiments 36
Figure 15: General health assessment 44
Figure 16: Home cage activity 45
Figure 17: Blood pressure and heart rate measurements 46
Figure 18: Short term fear learning and memory 48
Figure 19: Long term fear memory and extinction learning 50
Figure 20: *Rgs2* mRNA expression levels upon fear conditioning 51
Figure 21: Spatial learning in female mice 53
Figure 22: Spatial learning in male mice 55
Figure 23: Place preference learning 57
Figure 24: Elevated Plus Maze upon acute stress 59
Figure 25: Dark-Light Exploration upon acute stress 61
Figure 26: Open Field Locomotion upon acute stress 63
Figure 27: Sucrose Preference measurements 66
Figure 28: Food Intake measurements 67
Figure 29: Dark-Light Exploration upon Chronic Mild Stress 69
Figure 30: Social Interaction Test upon Chronic Mild Stress 72
Figure 31: Forced Swim Test upon Chronic Mild Stress 75
Figure 32: *Rgs2* mRNA expression levels upon Chronic Mild Stress 76
Figure 33: RGS protein mRNA expression in heart and hippocampus 80

Figure 34: Luciferase reporter Assay of 4 microRNAs regulating the expression of RGS2 by binding to the 3'UTR of *Rgs2* 81

Table 1: ANOVA and T-Test results for short-term fear learning and memory 49
Table 2: ANOVA results for long-term fear memory and extinction learning 51
Table 3: ANOVA results for spatial learning 54
Table 4: ANOVA results for place preference learning 57
Table 5: ANOVA results for Elevated Plus Maze upon acute stress 60
Table 6: ANOVA results for Dark-Light Exploration upon acute stress 62
Table 7: ANOVA results for Open Field Locomotion upon acute stress 64
Table 8: ANOVA results for Sucrose Preference 66
Table 9: ANOVA results for food intake 67
Table 10: ANOVA results for Dark-Light Exploration upon CMS 70
Table 11: ANOVA and T-test results for Social Interaction Test upon CMS 74
Table 12: ANOVA results for Forced Swim Test upon CMS 75
Table 13: Effect of *Rgs2* deletion on neurotransmitter levels in frontal cortex and hippocampus 78
Table 14: Effect of *Rgs2* deletion on GPCR mRNA expression in frontal cortex, hippocampus, atria and left ventricle 79
Table 15: Effect of *Rgs2* deletion on microRNA expression in the hippocampus 82

1 Introduction

1.1 Affective disorders

The term affective disorders encompasses all types and clinical pictures of both, anxiety and depressive disorders. The lifetime prevalence of anxiety disorders was estimated to be 28.8% in the U.S. (Kessler, Berglund et al. 2005). The global prevalence of anxiety disorders was estimated to be 7.3%, meaning that one in 14 people suffers from the illness at any given time (Baxter, Scott et al. 2013). Anxiety disorders lead to a severe reduction of quality of life and are the 9th leading cause of disability. They are associated with a substantial economic burden, estimated to be $46.6 billion in the 1990s in the US and 74€ billion by 2010 in Europe (Greenberg, Sisitsky et al. 1999, Mendlowicz and Stein 2000, Hoffman, Dukes et al. 2008, Gustavsson, Svensson et al. 2011, Baxter, Vos et al. 2014, Vos, Barber et al. 2015).

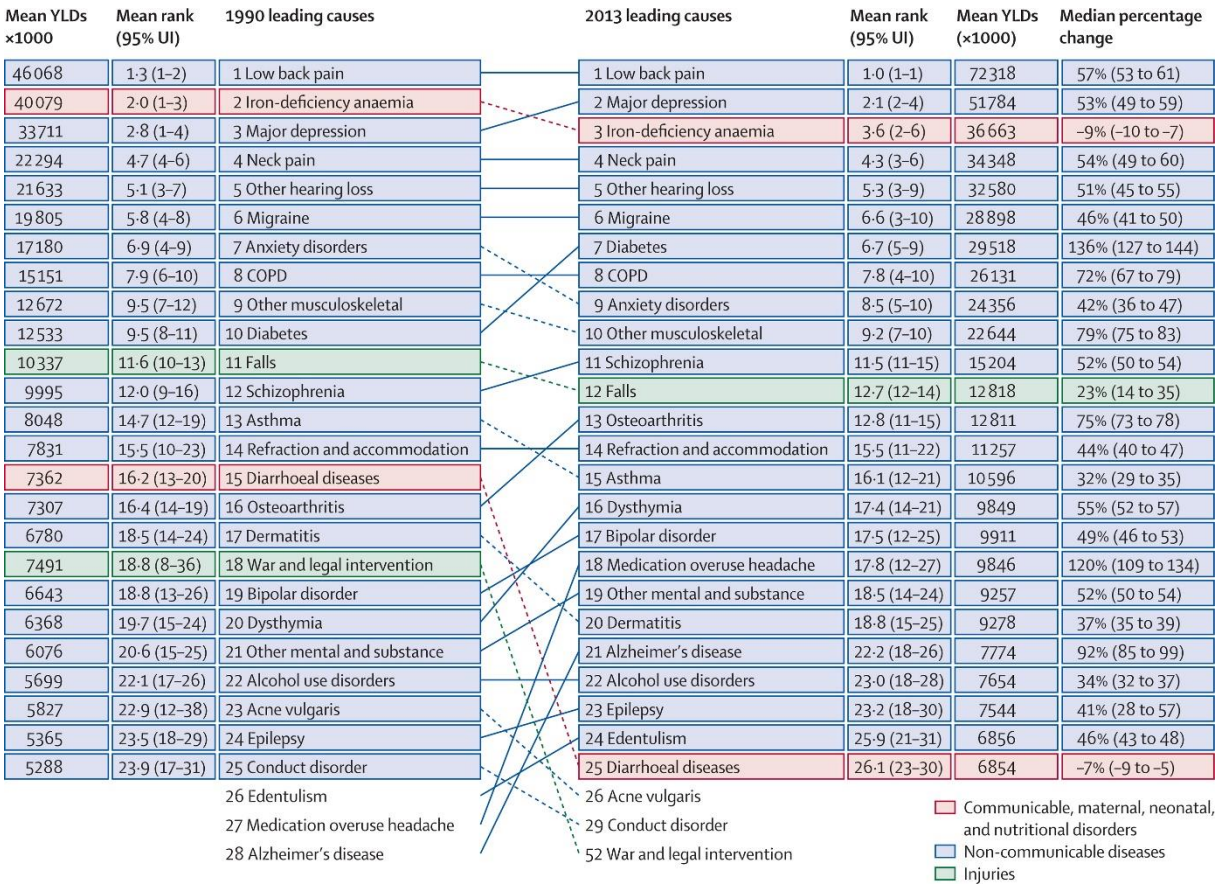


Figure 1: Top 25 causes of global years lived with disability in 1990 and 2013

YLD = years lived with disability. UI = uncertainty interval. COPD= chronic obstructive pulmonary disease (Vos, Barber et al. 2015)

Depressive disorders present with a lifetime prevalence of about 15-20%, and about 15% of the patients commit suicide (Nemeroff 1998, Fava and Kendler 2000, Kessler, Berglund et al. 2005). Major depression ranks 2nd in causes for years lived with disability in the Global Burden

of Disease Study 2013 (Vos, Barber et al. 2015). In 1992, the economic burden of depression was estimated at 43$ billion in the US and by 2010 113€ billion in Europe (Nemeroff 1998, Gustavsson, Svensson et al. 2011). Depression is a frequent comorbidity not only with anxiety disorders, but also with other illnesses such as cardiac disease, cerebrovascular disease and diabetes (Evans, Charney et al. 2005), often resulting in a negative impact on the outcome (Konstam, Moser et al. 2005, Faller, Stork et al. 2007, Serafini, Pompili et al. 2010).

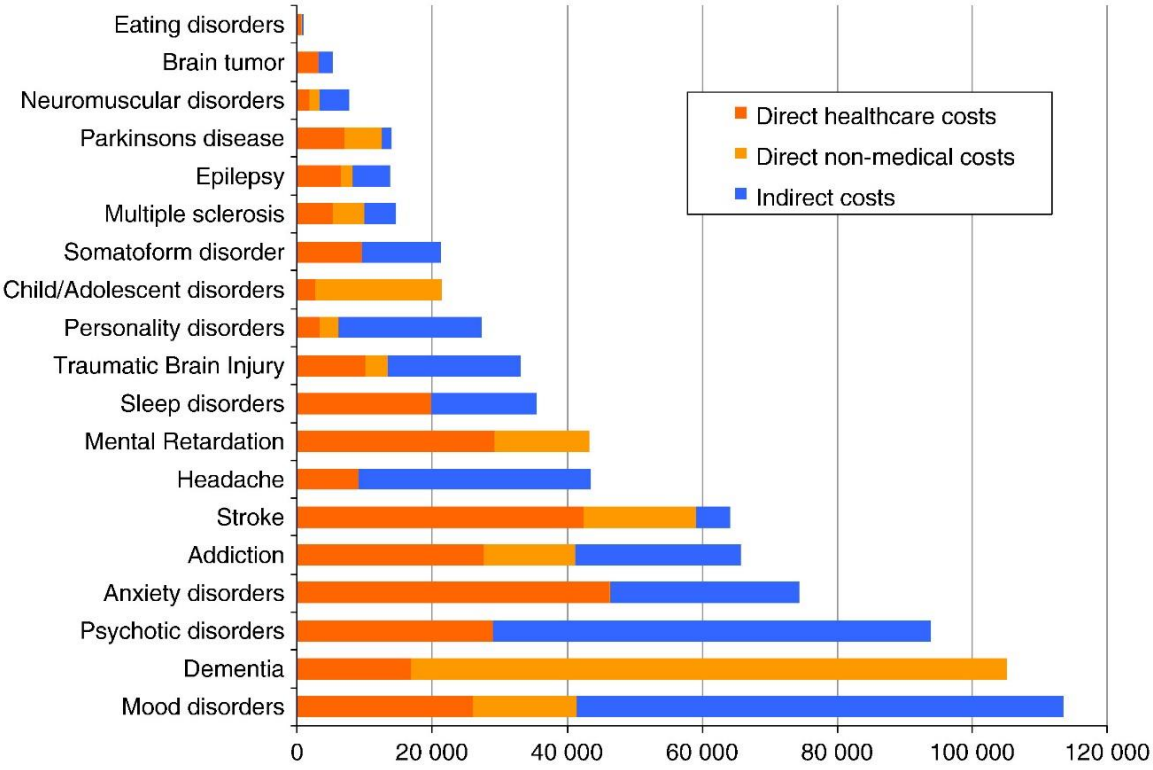


Figure 2: Total cost of all disorders of the brain in Europe by type of cost

(Gustavsson, Svensson et al. 2011)

Due to this substantial contribution of affective disorders to the global burden of disease, it is imperative to investigate possible causes and new treatment options.

1.1.1 Anxiety disorders

Anxiety disorders are defined by shared features of excessive fear and anxiety according to the *Diagnostic and Statistical Manual of Mental Disorders* in its 5th edition (DSM-V, APA, 2013).

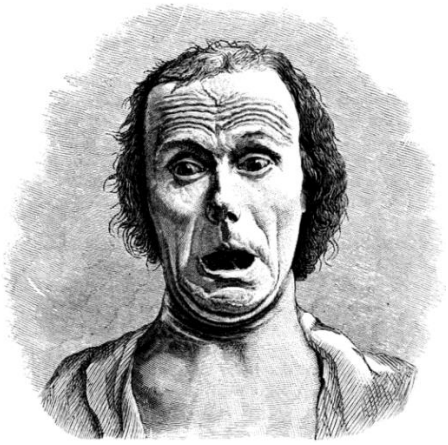


Figure 3: Expression of fear, Charles Darwin, 1872

Fear is a reaction to a real or potential immediate threat, which serves several physiological functions. The resulting physiological responses include heavier breathing, increased heart rate and hyper-vigilance in preparation of a fight or flight reaction (Shariff and Tracy 2011). The facial expression leads to widened eyes which increase the visual field and the speed of eye movement (Susskind and Anderson 2008). **Anxiety** is the anticipation of a perceived future threat and is often accompanied by increased muscle tension, heightened cautiousness and avoidance behavior. Fear and anxiety can be appropriate, but when experienced excessively or persistently over a longer period of time, the individual may suffer from an anxiety disorder (DSM-V, APA, 2013).

The etiology of anxiety disorders results from gene-environment interactions between candidate genes and stressful life events (Domschke and Deckert 2012). It involves biological and psychological vulnerability in addition to faulty learn processes. Anxiety disorders show a heritability between 32-67%, depending on the type of anxiety disorder (Domschke and Deckert 2007). Anxiety disorders are classified by age of onset and the type of objects or situations that elicit fear, anxiety or avoidance (DSM-V, APA, 2013):

- separation anxiety – fear or anxiety about separation from attachment figures
- selective mutism – inability to speak in social settings
- specific phobias – fear of specific objects or environments, e.g. arachnophobia
- social anxiety disorder – fear, anxiety or avoidance of social interactions
- panic disorder – recurrent unexpected panic attacks and the permanent worry to have more panic attacks
- agoraphobia – fear or anxiety towards being in open spaces, enclosed spaces, being in a crowd, using public transportation or being outside of one's home alone
- generalized anxiety disorder – persistent anxiety and concern about various parts of life, which the individual finds difficult to control.

Another subgroup of anxiety disorders are trauma and stress-related disorders including post-traumatic stress disorder (PTSD) and acute stress disorder. They result most likely from a slightly different etiology. Specific traumatic events must precede the onset of PTSD and acute stress disorder. Fear, anxiety or avoidance are especially, but not exclusively, related to situations reminding of the trauma in these disorders.

Anxiety disorders are treated with psychotherapy and supportive pharmacological interventions. According to the *S3-Leitlinie Behandlung von Angststörungen (2014)* pharmacological interventions such as selective serotonin reuptake inhibitors (paroxetine), serotonin-norepinephrine reuptake inhibitors (venlafaxine), tricyclic antidepressants (clomipramine), tricyclic anxiolytics (opipramol), $5HT_{1A}$ –agonists (buspirone), Benzodiazepines (Diazepam), calcium channel blocker (pregabaline) and reversible inhibitors of monoamine oxidase A (moclobemide) are suitable.

The most common acute pharmacological intervention manipulates the GABAergic system using benzodiazepines. Interestingly, five out of eight pharmacological interventions lead to an increase in available serotonin or noradrenaline in the synaptic cleft, indicating a disruption of the serotonergic (5-HT) and/ or noradrenergic (NA) system in anxiety disorders. Serotonin and noradrenaline take their effect on corresponding receptors which, almost all, are part of the G protein-coupled receptor family (see 1.2).

Anxiety disorders are frequently comorbid with major depressive disorder. This comorbidity is associated with greater symptom severity and higher incidence of suicidality (Kaufman and Charney 2000, Brown, Campbell et al. 2001). This might be due to particular genes contributing to the development of both disorders (Nemeroff 2002) and partially overlapping etiologies. Seligman 1975 proposed the theory of learned helplessness stating both humans and animals may develop a clinical depression upon exposition to inescapable aversive stimuli because after perceiving the aversive stimulus is inescapable both humans and animals cease all attempts to escape them.

1.1.2 Depressive disorders

Depressive disorders present with “sad, empty, depressed or irritable mood” together with somatic and cognitive changes that inhibit the individual to function normally (DSM-V, APA, 2013).

One important hypothesis of the etiology of depression is the stress-diathesis model. It postulates that genetic factors contribute to biological vulnerability towards stressful life events (e.g. physical disease, hormonal change or psychosocial factors), which initiate biochemical changes in the brain leading to the development of depressive disorders

(Nemeroff 1998, Caspi, Sugden et al. 2003). Recent reports postulate that the pathophysiology of depression rests on gene x environment interactions leading to alterations in three major monoamine systems: the serotonergic, noradrenergic and dopaminergic system (Dudley, Li et al. 2011, Saveanu and Nemeroff 2012). Depressive disorders show heritability, with major depressive disorder ranging from 37-42% (Ebmeier, Donaghey et al. 2006, Flint and Kendler 2014).

Depressive disorders can be classified according to duration, onset or presumed etiology (DSM-V, APA, 2013):

- disruptive mood dysregulation – persistent irritability and intolerance of frustration with extreme behavioral dyscontrol (children up to 12 years old)
- major depressive disorder – mood disturbance of at least 2 weeks involving changes in affect, cognition and neuro-vegetative functions
- persistent depressive disorder (dysthymia) – mood disturbance for over 2 years
- premenstrual dysphoric disorder

The main goals of intervention in depressive disorders are symptomatic relief, thereby reducing the likelihood of suicide, to reestablish social relations and economic productivity and to prevent relapse. Depression treatment can be divided into three phases: acute, maintenance and relapse prevention, all including psychotherapeutical and psychopharmacological interventions, depending on the severity of depression. According to the *S3-Leitlinie Unipolare Depression* pharmacological interventions such as tricyclic antidepressants (amitriptyline), selective serotonin reuptake inhibitors (citalopram), reversible inhibitor of monoamine oxidase A (moclobemide), serotonin-norepinephrine reuptake inhibitors (venlafaxine), alpha2-antagonists (mirtazapine), selective noradrenalin-dopamine reuptake inhibitors (bupropione), melatonin receptor agonists (agomelatine), Lithium and phytopharmaceuticals (St.-John's-wort) are suitable.

Most pharmacological interventions interfere with serotonin, norepinephrine or dopamine concentrations in the synaptic cleft or interact with serotoninergic or adrenergic G protein-coupled receptors. This fact gives rise to the importance of G protein-coupled receptors in affective disorders.

1.2 Animal models of psychiatric disease

One way to assess the relevance of specific candidate genes in psychiatric disorders is using mouse models. Genetically altered mice, either transgenic mice, overexpressing a candidate gene or knockout mice, with lowered or absent expression of a candidate gene can be generated are evaluated in animal models of psychiatric disorders.

1.2.1 What is an animal model?

"Animal models represent experimental preparations developed in one species for the purpose of studying phenomena in another species" – this simple definition of William McKinney in 1984 still holds true, but requires more than initially obvious.

The basic problem with animal models of psychiatric disorders is the inability of animals to unequivocally express their feelings, thus knowing whether a mouse is feeling afraid, anxious or depressed is not possible for a human investigator. Additionally, major mental illnesses may involve neuronal circuits unique to humans. It is however possible to observe rodent behavior and physiological responses upon certain stimuli. Furthermore, brain anatomy, physiology, and neurochemistry of i.e. mice are comparable to humans in many respects for example in both species the striatum and prefrontal cortex are involved in spatial learning (Chrousos 1998, Woolley, Laeremans et al. 2013).

The reproduction of an entire human neuropsychiatric disorder in mice is therefore not possible, but individual symptoms, causes and treatment responses can be modeled. So called *endophenotypes*, quantifiable behavioral, anatomical, biochemical or neurophysiological markers, make it possible to model multifactorial human behavior in animals to elucidate gene-to-behavior pathways of human neuropsychiatric disorders (Gould and Gottesman 2006). Therefore, animal models try to mirror one or more components or endophenotypes of the disorder, not the disorder in its entirety.

Animal models therefore serve two major purposes: (I) to verify hypothesis about the mechanism of a disease and (II) to predict treatment outcome in humans.

1.2.2 Validity of animal models

Animal models have to fulfil certain requirements to be considered valid and reliable. Three types of validity are especially important: *face*, *construct* and *predictive validity* (Willner 1984). An animal model of psychiatric disorders has to replicate one or more symptoms of the human disorder in order to display *face validity* (McKinney 1984). For example one mouse model of depression, the Chronic Mild Stress model (CMS), allows the investigation of behavioral and

physiological effects of chronic stress, using a parameter common with and resembling human depression: decreased feeding and associated weight loss (Willner 1997). The more similarities a model and a disorder share, the stronger the face validity (Willner and Mitchell 2002). *Construct validity* requires a hypothesized process or etiology underlying the human disorder. Knowledge of human neuronal circuits can therefore strengthen construct validity if the same neuronal structures are proven to be of importance in i.e. mice. Concerning anxiety and fear research, it is hypothesized that antipredator behavior and its neuronal basis is evolutionary conserved across species. The fight-flight-freeze system as well as the behavioral inhibition system can be investigated across species and always elicits an approach-avoidance conflict (Maximino, de Brito et al. 2010, Walz, Mühlberger et al. 2016). The third type of validity, *predictive validity*, centers on the response to treatments effective in humans. If a model shows similar results of an intervention as in a patient population, it supports its *predictive validity* (Willner 1984). The Forced Swim Test, evaluating depression-like behavior, displays strong predictive pharmacological validity. Drugs used to treat depression in humans reliably increase time spend swimming in the Forced Swim Test (Lucki 1997). It is desirable that a model shows a "true positive effect", i.e. responding to an intervention effective in humans and a "true negative effect", i.e. not responding to interventions ineffective in humans (Willner and Mitchell 2002). Additionally, animal models should mirror multiple components of the human disorder i.e. behavioral symptoms, neurochemical and neuroanatomical abnormalities and be robust, simple and of course reproducible.

It is imperative to recognize the limitations of an animal model mimicking only a part of a complex disorder, rather than its entirety (Crawley 2007).

1.2.3 Evaluating fear and anxiety-related behavior

Current tests of anxiety-like behavior in rodents either asses learned fear or innate anxiety behavior (Millan 2003). Since the mechanisms of inherent fear and anxiety are most likely well conserved across species, construct validity is achieved.

Human symptoms of anxiety are worrying about potential threats in the future and avoidance of places or situations that make a potential threat more likely to occur. In humans, anxious behavior may for example arise in a dark alley, provoking worry about a possible criminal event taking place. Subsequently this alley is avoided when possible or cautiously approached when passage is necessary. While worrying is not quantifiable in mice, approach-avoidance behavior is. Mice are given a choice between two rooms in an apparatus, one dark and small, the other open, very bright and spacious. Mice show innate anxiety towards brightly lit spaces, possibly due to a perceived increased visibility to potential predators, but also explore the environment in search of resources, such as food, despite potential predation.

If confronted with an unmistakable immediate threat, such as a criminal pointing a gun, humans react with fearful behavior and may freeze on the spot. When presented with an immediate threat such as a predator, animals react comparable with freezing behavior. The freezing response is typically evoked by presenting the mouse with stimuli such as an electroshock or a model of a predator. Face validity for anxiety and fear response is therefore present (Adhikari, Topiwala et al. 2010).

1.2.3.1 Innate anxiety

Anxiety-like behavior, the response to a potential threat, is typically investigated in the context of innate responses to non-learned stimuli. Innate anxiety is mainly modeled by tests based on approach-avoidance conflicts in mice. To elicit this conflict, the model environment consists of sections that are "safer" opposed to sections that are more "dangerous".

The Open Field, Dark-Light Exploration and Elevated Plus Maze Tests all provoke the approach-avoidance conflict between exploration and avoidance of a novel environment. The "anxiogenic" or "dangerous" areas in these tests are the exposed brightly lit center of the arena in the Open Field, the brightly lit half of the dark-light arena and the open elevated arms of the Elevated Plus Maze apparatus. Anxiety-like behavior is quantified by measuring the amount of time exploring the aversive areas in the testing arenas to the total exploration time. Increased defecation and urination are also indicative of increased anxious behavior (Hall 1934, Crawley and Goodwin 1980, Pellow and File 1986).

These tasks hold high construct validity, and various publications support a high pharmacological predictive validity. Anxiety measures are reduced by the acute administration of benzodiazepines in all three tests (Belzung, Misslin et al. 1987, Lister 1987), anxiety measures in the Open Field Test are also sensitive to chronic treatment with SSRIs (Borsini, Podhorna et al. 2002). The Social Interaction Test has even face validity for certain types of social anxiety disorder (File 1980).

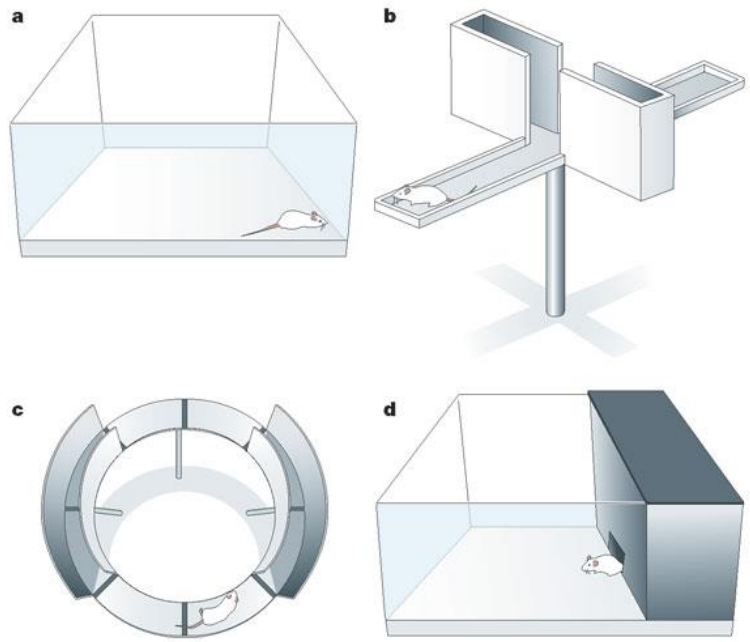


Figure 4: Behavioral tasks assessing innate anxiety in mice

Tests based on approach avoidance behavior, the Open Field (a), the Elevated Plus Maze (b), the Circular Maze (c) and the Dark Light Exploration Test (d) (Cryan and Holmes 2005)

1.2.3.2 Learned fear

Fear-like behavior to an immediate threat is typically investigated in the context of learning, by training animals to perceive a particular stimulus as an immediate threat.

Since animals fear predators and injuries, exposure to predator odor or painful foot shocks are used to model fear-like behavior.

Learned fear is most commonly assessed using contextual and cued fear conditioning as developed by Pavlov in 1927. This paradigm of associative learning has been successfully adapted for many species (Fanselow and Poulos 2005, Kim and Jung 2006). Fear conditioning consists of three basic features: a neutral stimulus (tone, conditioned stimulus, CS), an actively threatening aversive stimulus (foot shock, unconditioned stimulus, US) and a behavioral consequence of the fear response (freezing, complete immobility except breathing). During fear conditioning, the CS is paired to the US to facilitate associative learning in a distinct conditioning context (conditioning chamber). Fear-like behavior is quantified by evaluating freezing time during re-exposure to the conditioning chamber and the CS in a second, different chamber.

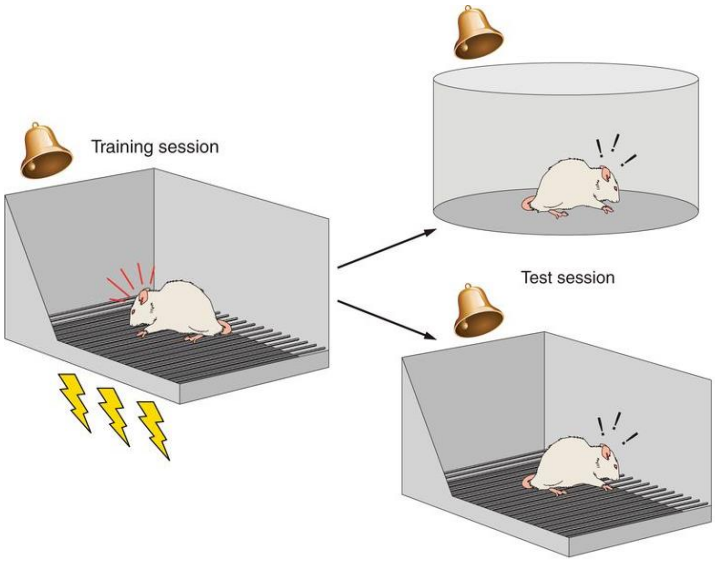


Figure 5: Fear conditioning paradigm

Schematic representation of contextual and cued fear conditioning. Mice are placed in a fear conditioning chamber where after an exploration phase a conditioned stimulus (tone) is paired with an unconditioned stimulus (foot shock). The animals learn to freeze to the context (the fear conditioning chamber) and the conditioned stimulus in an altered surrounding (Izquierdo, Furini et al. 2016).

1.2.4 Evaluating depression-like behavior

As mice cannot self-report depressed mood, only certain endophenotypes of depression can be assessed to evaluate depression-like behavior in rodents (Hasler, Drevets et al. 2004, Cryan and Holmes 2005). *Anhedonia*, the loss of interest in pleasurable or rewarding behavior, is a core symptom of depression and may be quantified using Sucrose Preference in mice. Mice normally display distinct preference for a sucrose solution opposed to pure water (Strekalova, Spanagel et al. 2004). Changes in appetite or disturbances of body weight may easily be quantified by regularly weighing mice and their food. In the behavioral despair test or Forced Swim Test rodents are exposed to the stressful threat of drowning. The time spent swimming and climbing opposed to the time spent immobile is quantified. Immobility time decreases with the administration of various antidepressants (Porsolt, Le Pichon et al. 1977). The *Chronic Mild Stress* paradigm assesses the ability to cope with uncontrollable stressors, triggering long-term behavioral and neuronal changes comparable to those in depressed patients. Effects caused by the CMS paradigm may be reversed by chronic antidepressant treatment (Willner, Towell et al. 1987, Willner 1997).

1.3 G protein-coupled signaling

G protein-coupled receptors (GPCR) are the largest family of membrane receptors transducing extracellular signals into the intracellular compartment. Their versatile members include sensory receptors for light, taste and smell as well as receptors for neurotransmitters, hormones, amino acids, chemokines and ions. Thereby, GPCRs control various physiological processes and drugs targeting these receptors represent 40-50% of drugs currently on the market (Dixon, Kobilka et al. 1986, Pierce, Premont et al. 2002, Lundstrom 2006, Lagerstrom and Schioth 2008).

GPCRs consist of seven transmembrane domains and couple to heterotrimeric G proteins (composed of an α-, a β- and a γ-subunit), which elicit intracellular downstream signaling upon GPCR activation. Agonist binding leads to conformational changes of the intracellular loops of the GPCR, promoting the exchange of GTP for GDP on the Gα-subunit, followed by the dissociation of GTP-Gα and Gβγ. Both GTP-Gα and Gβγ activate or inhibit various downstream effectors and second messenger pathways. GTP hydrolysis through intrinsic GTPase activity of the Gα subunit and subsequent re-association with Gβγ and the receptor terminates G protein signaling. Therefore, the rate of GTP hydrolysis at least partly determines the duration of GPCR signaling (Gilman 1987, Patel 2004).

The first GPCR structure solved was bovine rhodopsin with a 2.8 Å resolution (Palczewski, Kumasaka et al. 2000), followed by the human β_2-adrenergic receptor bound to an inverse agonist (Rasmussen, Choi et al. 2007, Rosenbaum, Cherezov et al. 2007). GPCR crystallography holds several challenges, GPCRs exhibit poor thermodynamic and proteolytic stability as well as problematic solubility (Rosenbaum, Rasmussen et al. 2009). In recent years, a total of 127 GPCR structures have been solved giving further insight into ligand binding modes, GPCR activation, dimerization and allosteric modulation (Katritch, Cherezov et al. 2013, Stevens, Cherezov et al. 2013, Zhang, Zhao et al. 2015).

G proteins can be classified into four subfamilies by their Gα subunits indicating their predominant intracellular signaling cascade. $G\alpha_s$ proteins canonically stimulate the adenylyl cyclases, which in turn leads to increased production of cyclic AMP, $G\alpha_i$ proteins inhibit adenylyl cyclases and activate G-protein-coupled inward rectifying potassium (GIRK) cannels, modulating neuronal excitability in the central nervous system (Lüscher and Slesinger 2010). Furthermore, after presynaptic $G\alpha_{i/o}$ activation, the Gβγ subunit acts as an effector and inhibits presynaptic N-type and P/Q-type Ca^{2+} channels, preventing Ca^{2+} influx and neurotransmitter release (Atwood, Lovinger et al. 2014). $G\alpha_q$ proteins activate phospholipase Cβ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacyl glycerol (DAG) and inositol trisphosphate (IP_3). DAG in turn activates protein kinase C (PKC) while IP_3 increases

cytosolic Ca^{2+} concentrations by activating IP_3 receptors in the endoplasmatic reticulum. $G\alpha_{12/13}$ activates Rho guanine-nucleotide exchange factors (GEFs) (Gilman 1987).

1.3.1 G protein-coupled receptor regulation

GPCR regulation is activated upon receptor stimulation. Second messenger kinases like protein kinase A (PKA) or protein kinase C (PKC) phosphorylate G protein-coupled receptors, thereby inhibiting G protein signaling after agonist binding. G protein-coupled receptor kinases (GRKs) phosphorylate active GPCRs promoting binding of β-arrestin to the phosphorylated receptor and resulting in either clathrin-mediated endocytosis for either degradation or dephosphorylation and resensitization (Pierce, Premont et al. 2002). Furthermore, internalized GPCRs can initiate G protein independent GPCR signaling (Calebiro, Nikolaev et al. 2009). GPCR signaling can also be modulated on the level of G proteins. Regulator of G protein signaling (RGS) proteins accelerate the hydrolysis of GTP bound to the Gα subunit, thereby leading to earlier termination of signaling (Magalhaes, Dunn et al. 2012).

1.3.2 Regulators of G protein signaling

The protein family Regulator of G protein signaling (RGS) negatively regulates GPCR signaling by acting as GTPase accelerating proteins (GAPs) towards $G\alpha_i$ and $G\alpha_q$ subunits. RGS proteins bind to Gα and substantially increase its intrinsic GTPase activity promoting the re-association of Gα and Gβγ and the termination of downstream GPCR signaling. The GAP mechanism of RGS proteins is to stabilize the transition state conformation, lowering the free energy necessary for the activation of GTP hydrolysis (Berman, Wilkie et al. 1996, Tesmer, Berman et al. 1997).

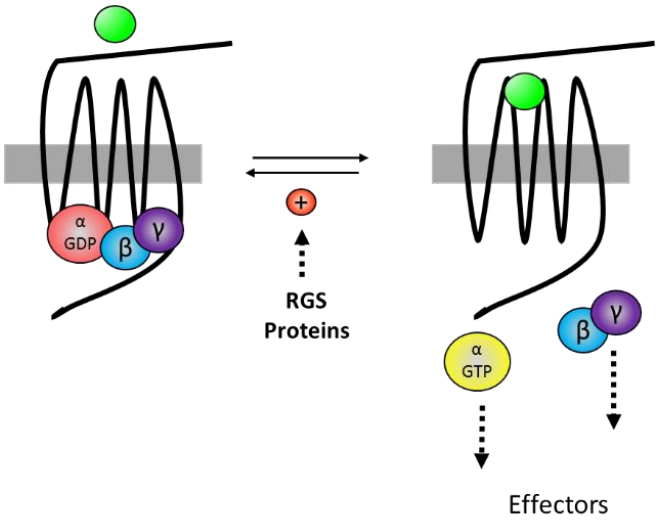


Figure 6: Fine tuning of GPCR signaling by RGS proteins

Schematic representation of the fine tuning of GPCR signaling by RGS proteins adapted from (Siderovski and Willard 2005). Upon binding of an activating ligand, the GPCR releases GDP and binds GTP acting as a GEF. This exchange results in the dissociation of $G_{\beta\gamma}$ and G_{α}-GTP allowing the activation of downstream signaling. Downstream effectors are activated until the GTP is hydrolyzed by the intrinsic GTP hydrolysis activity of the G_{α} subunit. Upon hydrolysis G_{α}-GDP rebinds $G_{\beta\gamma}$ returning the system to the inactive state. The rate of GTP hydrolysis can be significantly enhanced by RGS proteins, which act as GAPs for G_{α} subunits.

The discovery of the RGS protein family goes back to Sst2, a gene in *Saccharomyces cerevisiae*, responsible for desensitization of yeast mating pheromones (Chan and Otte 1982). During the 1990s several laboratories identified a conserved RGS homology domain (RH domain, ~120 amino acids long) responsible for binding to the Gα subunit and mediating the GAP function (De Vries, Mousli et al. 1995, Dohlman, Apaniesk et al. 1995, Druey, Blumer et al. 1996). The RH domain consists of nine α helices forming an oblong bundle. Upon interaction with the three switch regions of Gα, the transition state-like conformation of GTP hydrolysis is stabilized, thereby reducing the energy necessary for GTP-hydrolysis (Tesmer, Berman et al. 1997, Tesmer 2009).

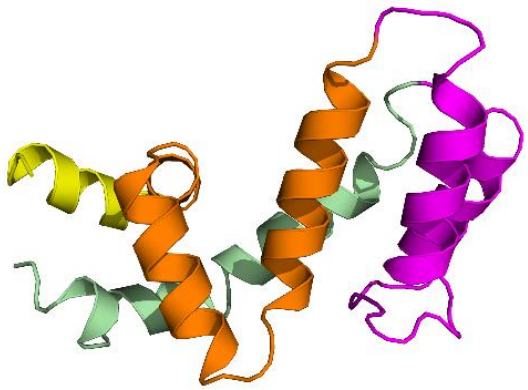


Figure 7: The RGS fold of RGS4

Ribbon diagram illustrating the tertiary structure of RGS4. The RGS4 box consists of nine helices: α1 (yellow), α2-4 (orange), α 5-6 (pink) and α 7-9 (sage) adapted from (Tesmer, Berman et al. 1997). The majority of residues interacting with G_α are on the bottom of the shown bundle.

Presently, 20 canonical RGS proteins acting as GAPs and additional 17 proteins containing a nonfunctional RGS domain are known. They are divided into 8 subfamilies according to their sequence homology and/ or non-RGS domains. RGS proteins are expressed in every cell type, tissue or organ in humans and vertebrates.

RGS Proteins	A/RZ	GAIP, RGS19, RET-RGS1, RGSZ1/RGS20, RGSZ2/RGS17
	B/R4	RGS3
		RGS1, 2, 4, 5, 8, 13, 16, 18, 21
	C/ R7	RGS6, 7, 9, 11
	D/R12	RGS10
		RGS12
		RGS14
	E/RA	Axin, Conductin
	F/GEF	P115-RhoGEF
		PDZ-RhoGEF, LARG
	G/GRK	GRK2, GRK3
		GRK1, 4, 5, 6, 7
	H/SNX	RGS-PX1, SNX14, SNX25
	I/D-AKAP2	D-AKAP2
		RGS22

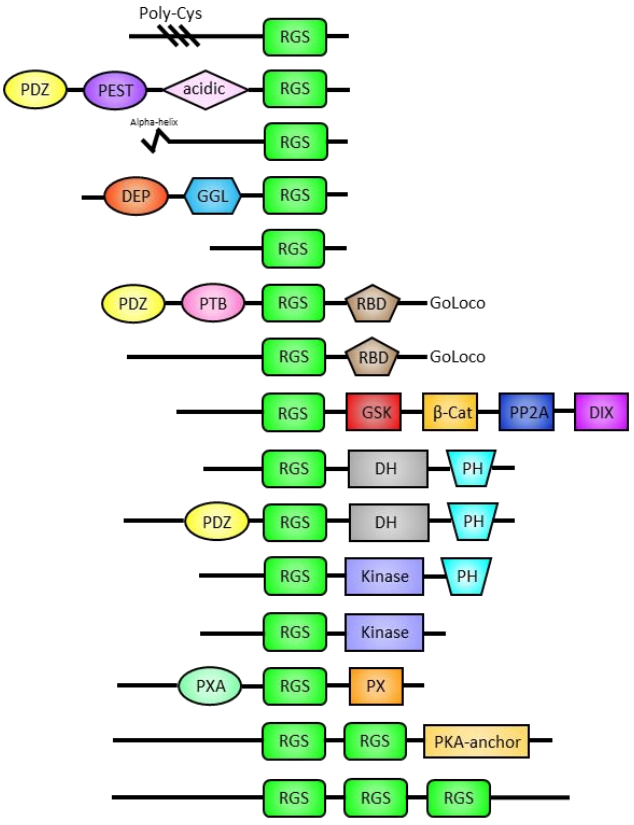


Figure 8: RGS Proteins

Classification of mammalian RGS protein members into subfamilies and their protein structures showing identified motifs and domains, adapted from (Bansal, Druey et al. 2007)

The various domains and motifs in the structurally diverse RGS protein family point towards the fact, that acting as a GAP for Gα is not the predominant function of these proteins (Burchett 2000, Sethakorn, Yau et al. 2010). For instance, the canonical function of G protein-coupled receptor kinases (GRKs) is to phosphorylate the intracellular domains of activated G protein-coupled receptors (Premont and Gainetdinov 2007), while A-kinase anchor proteins (AKAPs) are scaffolding proteins determining the subcellular location of protein kinase A (Greenwald and Saucerman 2011).

The R4 subfamily encompasses the smallest RGS proteins, containing only short peptide sequences next to the RGS homology domain, except RGS3 which also contains a PDZ, a PEST and an acidic domain. Despite being the smallest RGS proteins, their physiological functions are numerous. RGS1 and RGS13 are important in processes related to B-lymphocyte homeostasis and adaptive immune response, RGS4 regulates pain sensitivity and RGS18 modulates osteoclastogenesis (Bansal, Druey et al. 2007).

RGS2, the protein of interest in this thesis, is also a member of the R4 subfamily.

1.3.3 Regulator of G protein signaling 2

In mice and humans, the RGS2 locus is located on chromosome 1 and contains five exons. Encoded is a 212-amino acid long, ~24 kDa protein containing one RGS domain of approximately 120 amino acids flanked by an ~80-residue N-terminal domain and a short C-terminal tail. The N-terminal domain of RGS2 has membrane targeting function as well as proposed importance in associating RGS2 with other components of the G protein signaling complex. This structure characterizes RGS2 as a class B/R4 RGS protein (Siderovski, Heximer et al. 1994, Siderovski, Hessel et al. 1996). The structure of RGS2 in complex with AlF_4^+ activated $G\alpha_q$ was solved in 2013, giving insight into the $G\alpha_q$ selectivity of RGS2 (Nance, Kreutz et al. 2013).

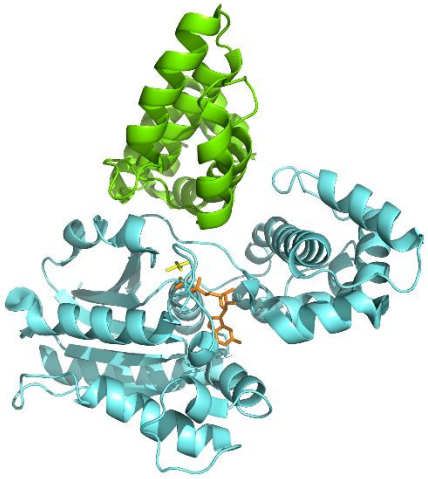


Figure 9: Structural illustration of RGS2 in complex with $G\alpha_q$

RGS2 in green, $G\alpha_q$ in blue, GDP in orange, AlF_4^+ in yellow adapted from (Nance, Kreutz et al. 2013)

RGS2 shows GAP selectivity towards $G\alpha_q$ in vitro, due to a unique tilt of RGS2 when bound to $G\alpha_q$ and a strong interaction between RGS2 and the long alpha helical domain of $G\alpha_q$ (Heximer, Watson et al. 1997, Nance, Kreutz et al. 2013). However, some studies report an interaction of RGS2 with $G\alpha_{i/o}$. In dopaminergic neurons RGS2 reduces the coupling efficiency of $GABA_B$ receptors and associated GIRK channels, thereby mediating the inhibitory postsynaptic effects of $G\alpha_{i/o}$ coupled receptors (Labouebe, Lomazzi et al. 2007). This could indicate that the interaction of RGS2 with specific GPCRs can shift the GAP activity of RGS2 to $G\alpha_{i/o}$ coupled signaling processes (Ingi, Krumins et al. 1998, Heximer, Srinivasa et al. 1999, Han, Mark et al. 2006, Labouebe, Lomazzi et al. 2007). Additionally, RGS2 has been reported to impair $G\alpha_s$ function by directly inhibiting adenylyl cyclase isoforms III, V and VI (Sinnarajah, Dessauer et al. 2001) and preventing signaling via phospholipase Cβ by sterically hindering its access to $G\alpha_q$ (Anger, Zhang et al. 2004).

RGS2/Rgs2 is ubiquitously expressed in human and rodent tissues (Kehrl and Sinnarajah 2002) and has various cellular functions. Osteoblast proliferation under stress conditions i.e. after fracture is hypothesized to be RGS2 dependent (Roy, Nunn et al. 2006) and immune response is impaired upon *RGS2* deletion (Oliveira-Dos-Santos, Matsumoto et al. 2000). Several studies in humans and mice have also reported a role of *RGS2/Rgs2* in cardiac remodeling, arrhythmia and blood pressure regulation (Riddle, Schwartzman et al. 2005, Wieland, Lutz et al. 2007, Gu, Cifelli et al. 2009, Tsang, Woo et al. 2010, Zhang and Mende 2014).

1.3.3.1 *RGS2* in the brain and its contribution to psychiatric disease

1.3.3.1.1 Human findings

In genetic association studies polymorphisms in and flanking the RGS2 gene were associated with higher incidence of several neuropsychiatric disorders. Lower RGS2 expression was associated with higher incidence of the respective disorder (Semplicini, Lenzini et al. 2006). Reports were made for increased symptoms of post-traumatic stress disorder (Amstadter, Koenen et al. 2009), increased suicidal ideation after a traumatic event (Amstadter, Koenen et al. 2009) and increased number of suicides (Cui, Nishiguchi et al. 2008). Furthermore, RGS2 was reported to be associated with a higher incidence of panic disorder, generalized anxiety disorder and social anxiety disorder (Leygraf, Hohoff et al. 2006, Smoller, Paulus et al. 2008, Koenen, Amstadter et al. 2009, Otowa, Shimada et al. 2011, Stein, Keshaviah et al. 2014, Hohoff, Weber et al. 2015). Even reduced treatment response to sertraline of patients suffering of social anxiety disorder is associated with RGS2 polymorphisms (Stein, Keshaviah et al. 2014). However, results unable to replicate these findings were also reported (Mouri, Hishimoto et al. 2010, Strug, Suresh et al. 2010, Hettema, Sun et al. 2013), suggesting RGS2 to be one among several genes contributing to human anxiety.

Two polymorphisms tagging the gene of microRNA hsa-miR-22 were nominally associated with panic disorder. Subsequently hsa-miR-22 was shown to regulate the expression of several candidate genes of panic disorder including *RGS2* (Muinos-Gimeno, Espinosa-Parrilla et al. 2011). A polymorphism upstream of the gene of a microRNA (hsa-miR-4717-5p) regulating *RGS2* was also mildly associated with higher incidence of panic disorder (Hommers, Raab et al. 2015). microRNA

Conversely, there are also publications reporting no significant association (Mouri, Hishimoto et al. 2010, Hettema, Sun et al. 2013), suggesting, that *RGS2* to be one among many factors involved and thereby only account for part of the effect.

1.3.3.1.2 Mouse model

In 2000 Oliveira-Dos-Santos and coworkers could delete exons 4 and 5 of the *Rgs2* mouse genome thereby *Rgs2* heterozygous and homozygous knockout mice were created. Homozygous *Rgs2*$^{-/-}$ mice were viable, however *Rgs2* deletion could be linked to increased anxious behavior (Oliveira-Dos-Santos, Matsumoto et al. 2000, Lifschytz, Broner et al. 2012). *Rgs2* was furthermore identified as part of a quantitative trait locus for anxiety-related behavior (Yalcin, Willis-Owen et al. 2004), and increased RGS2 expression was observed upon

treatment with oxytocin resulting in anxiolysis (Okimoto, Bosch et al. 2012). Additionally, homozygous and heterozygous deletion of *Rgs2* triggered depression-like behavior in mice (Lifschytz, Broner et al. 2012).

RGS2 is expressed throughout all areas of the brain. Prominent expression has been reported in the hippocampus, cortex, striatum, ventral tegmental area and the amygdala (Grafstein-Dunn, Young et al. 2001, Ingi and Aoki 2002, Taymans, Wintmolders et al. 2002). Intermediate early genes have been linked to activity-dependent plasticity in the brain (French, O'Connor et al. 2001, Minatohara, Akiyoshi et al. 2015). Upon stimuli evoking intermediate early gene response and/ or synaptic plasticity, *RGS2* expression was reported to be rapidly upregulated in cortex, striatum and hippocampus (Ingi, Krumins et al. 1998). Amphetamine administration and treatment with haloperidol as well as risperidone lead to an increase of *RGS2* expression in the rat striatum (Burchett, Volk et al. 1998, Robinet, Geurts et al. 2001, Taymans, Wintmolders et al. 2002, Taymans, Leysen et al. 2003).

In the hippocampus, RGS2 affects short-term synaptic plasticity. With increasing RGS2 expression, paired pulse depression (PPD) is triggered and subsequent neurotransmitter release is possible. Consequently, low RGS2 levels lead to paired pulse facilitation (PPF) and a lower probability of neurotransmitter release. Since pertussis toxin prevents PPF in neurons of *Rgs2*$^{-/-}$ mice, the effect is most likely mediated by modulation of presynaptic $G\alpha_{i/o}$ signaling (Han, Mark et al. 2006). After presynaptic $G\alpha_{i/o}$ activation, the Gβγ subunit acts as an effector and inhibits presynaptic N-type Ca^{2+} channels (Figure 10). Thereby, calcium influx and associated neurotransmitter release is prevented (Ikeda 1996, Jarvis and Zamponi 2001, Kajikawa, Saitoh et al. 2001). RGS2 is also able to decrease P/Q-type Ca^{2+} channel inhibition via $G\alpha_{i/o}$ in vitro, supporting the hypothesized mechanism (Mark, Wittemann et al. 2000). In conclusion, RGS2 modulates synaptic strength.

The amount of spines in neurons is an established marker for the total number of synapses and subsequently for synaptic plasticity (Moser 1999). In hippocampal CA1 neurons of *Rgs2*$^{-/-}$ mice, less apical and basilar spines of dendrites were detected compared to *Rgs2*$^{-/+}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000). However, these findings were not confirmed comparing hippocampal neurons from *Rgs2*$^{-/-}$ and WT mice (Han, Mark et al. 2006). Furthermore, after excitation with Schaeffer collaterals, *Rgs2*$^{-/-}$ CA1 neurons showed a reduced collective basal electrical activity (Oliveira-Dos-Santos, Matsumoto et al. 2000), suggesting an importance of *Rgs2* in synaptic development and neuronal activity.

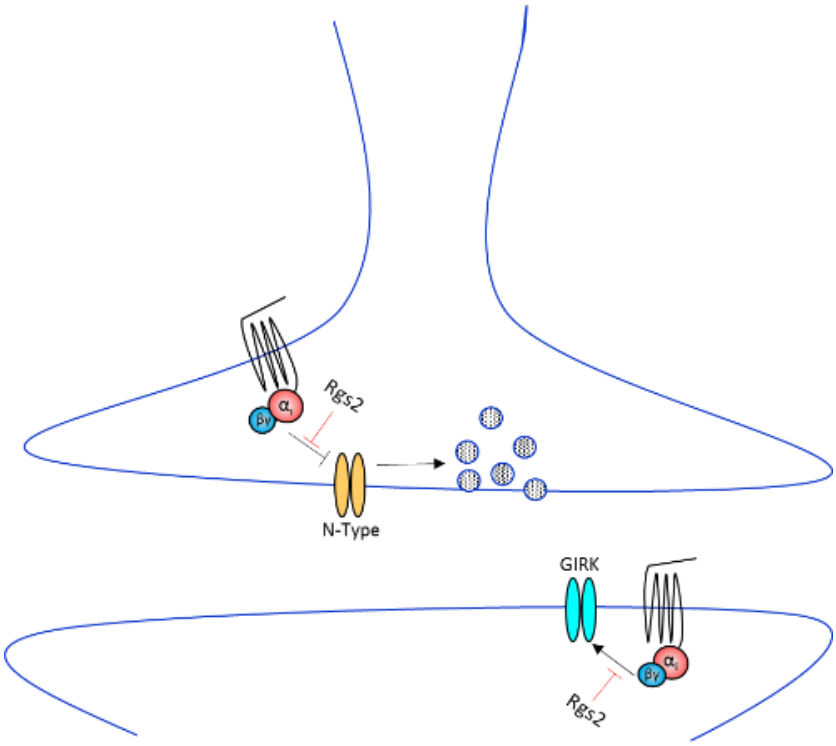


Figure 10: RGS2 regulation of synaptic signaling

Activation of presynaptic GPCRs releases $G_{\beta\gamma}$ to inhibit N-Type Ca^{2+} channels suppressing neurotransmitter release. Upregulation of Rgs2 expression blocks $G_{\beta\gamma}$ inhibition of N-Type Ca^{2+} channels thereby facilitating neurotransmitter release. Rgs2 inhibits postsynaptic $GABA_B$ receptor activated GIRK currents by promoting $G_{\beta\gamma}$ deactivation. Adapted from (Gerber, Squires et al. 2016)

Opposing results regarding the impact of *Rgs2* on canonical long-term potentiation (LTP) in the hippocampus have been reported. While (Oliveira-Dos-Santos, Matsumoto et al. 2000) observed no effect of *Rgs2* on hippocampal LTP by comparing *Rgs2*$^{-/-}$ and *Rgs2*$^{-/+}$ mice, Hutchison and coworkers reported augmented LTP comparing hippocampal neurons *of Rgs2*$^{-/-}$ and WT mice (Hutchison, Chidiac et al. 2009). Increased hippocampal LTP is linked to improved learning and memory and reduced LTP to impaired learning and memory (Cercato, Colettis et al. 2014, Stuchlik 2014, Gruart, Leal-Campanario et al. 2015). However, spatial learning and memory as tested with the Morris Water Maze, however, was comparable among *Rgs2*$^{-/-}$ and *Rgs2*$^{-/+}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000).

In the ventral tegmental area (VTA), RGS2 decreases the ability of $GABA_B$ receptors to activate GIRK channels at the post-synaptic membrane (Figure 10). $G\alpha_{i/o}$ mediated activation of GIRK channels leads to postsynaptic inhibition, by triggering an inhibitory postsynaptic potential. It was suggested, that regulating RGS2 expression patterns due to stimuli could be part of a tolerance mechanism relevant in addiction (Labouebe, Lomazzi et al. 2007).

1.4 MicroRNAs

Epidemiological studies have suggested that environmental factors such as psychological or physiological stress contribute to psychiatric morbidity (see 1.1.1 and 1.1.2). Environmental factors may affect gene expression levels by epigenetic mechanisms including histone modifications, DNA methylation and post-transcriptional regulation by microRNAs. The relevance of microRNAs in psychiatric disorders is investigated using *in vitro* and *in vivo* methods in patients and animal models. (Issler and Chen 2015). MicroRNA hsa-miR-4717-5p, regulating the expression of RGS2, in an *in vitro* luciferase assay, was mildly associated with panic disorder with comorbid agoraphobia in a human patient case control sample (Hommers, Raab et al. 2015).

1.4.1 Discovery

Nucleic acids were first discovered in the 1900s by Friedrich Miescher (Dahm 2005). Subsequently the mechanism of RNA mediated translation, from DNA via mRNA to protein, was identified (Crick 1958). However, this “canonical” function of RNA is not its only one. In recent years, it was discovered that about 97% of RNA genes transcribe to non-coding RNA (Eddy 2001, Mattick 2001, Mattick 2003, Mattick and Makunin 2006). The first non-coding RNA (ncRNA), described in 1965, was alanine transfer RNA (tRNA) recovered from baker`s yeast (Holley, Apgar et al. 1965). Subsequently other classes of ncRNAs where identified such as tRNAs, ribosomal RNAs (rRNA), small interfering RNAs (siRNA) and microRNAs (miRNA). NcRNAs have various functions, rRNA is part of the ribosome facilitating protein synthesis, tRNA enables translation of mRNA to protein, siRNA and microRNA regulate post-transcriptional gene expression.

MicroRNAs are small single stranded endogenous RNA molecules, about 22 nucleotides long. They play an important role in translational regulation (Ambros 2004). The first microRNA described was the 22 nt RNA *lin-4* in C. elegans. *Lin-4* drives the postembryonic development of C. elegans by temporarily decreasing the level of LIN-14. The *lin-4* gene encodes for two small RNAs of about 22 and 61 nt in length (Lee, Feinbaum et al. 1993). The 61 nt RNA species was assumed to fold into a stem loop and suggested to be the precursor of the 22 nt RNA species. Both *lin-4* RNAs showed antisense complementarity to several sites in the 3`UTR of the *lin-14* gene. The proposed model of post transcriptional regulation was a pairing of *lin-4* RNAs to the 3`UTR of *lin-14* mRNA and the subsequent repression of *lin-14* translation (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993).

With the discovery of *let-7*, regulating *lin-41*, in C. elegans and the identification of gene homologs of *let-7* in human and other animals it was proven that the regulatory ability of *lin-4* is not species specific nor unique (Pasquinelli, Reinhart et al. 2000, Reinhart, Slack et al.

2000). In recent years the number of annotated microRNAs in the database mirBase continuously increased, in 2016, 2588 human mature microRNAs were described.

1.4.2 Biogenesis and function

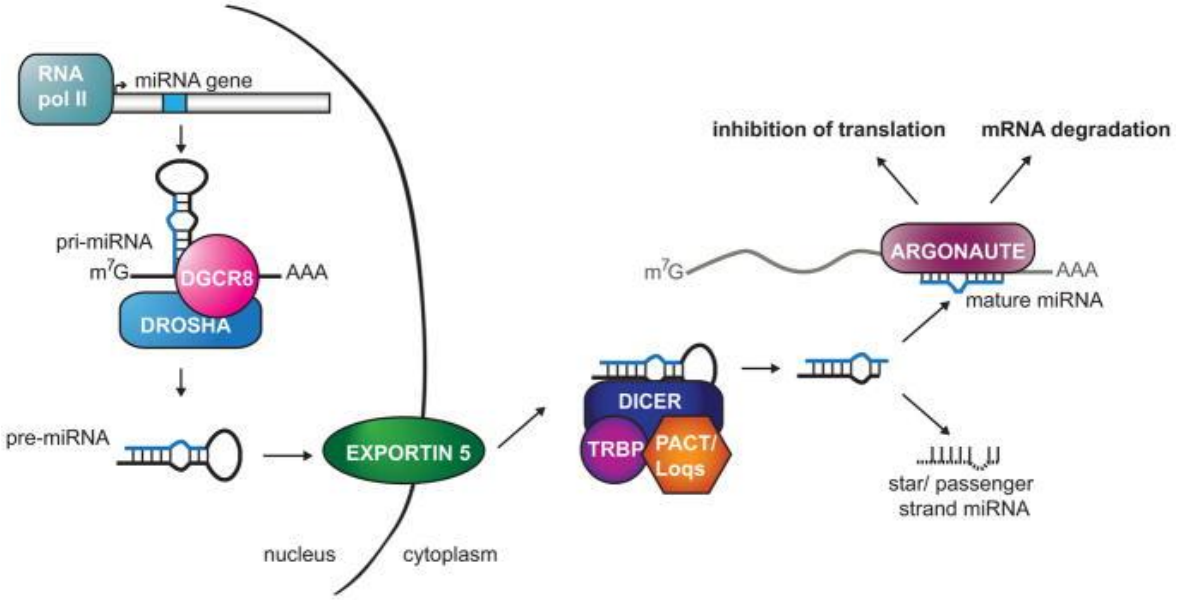


Figure 11: General microRNA pathway

MicroRNAs are predominantly transcribed by RNA polymerase II resulting primary microRNA transcripts (pri-miRNA). The pri-miRNA is cleaved by a microprocessor including Drosha and DGCR8. This process produces the precursor microRNA hairpin (pre-miRNA). Exportin 5 exports the pre-miRNA out of the nucleus where the pre-miRNA is processed by the Dicer complex. Dicer cleaves the hairpin loop and one strand of microRNA is loaded onto Argonaute forming the microRNA Induced Silencing Complex (miRISC). miRISC is then able to regulate the gene expression through mRNA degradation or translation inhibition (Finnegan and Pasquinelli 2013).

MicroRNA genes are predominantly transcribed by RNA polymerase II. The resulting long primary microRNA (pri-microRNA) contains the mature microRNA sequence (Kim, Han et al. 2009, Winter, Jung et al. 2009) and is processed by a type-III endonuclease Drosha and its cofactor DGCR8, generating 60-70 nt long hairpin precursor microRNAs (pre-microRNA). Exportin 5 transports the pre-microRNA from the nucleus to the cytoplasm, where the 21-24 nt long duplex microRNA is cleaved by Dicer, another type-III endonuclease. The microRNA is then incorporated into the RNA induced silencing complex (RISC) by loading the mature microRNA sequence onto Argonaut 2. This miRISC complex is guided to specific mRNAs by imperfect base pairings between mature microRNA and mRNA, provoking mRNA destabilization and degradation or mRNA translational repression through steric hindrance, finally leading to down-regulation of protein expression (Huntzinger and Izaurralde 2011, Pasquinelli 2012). Due to the ability of microRNAs to target mRNAs through imperfect base pairings, every microRNA has several possible targets, putatively leading to regulation of more than half of the human genome (Bartel 2009, Friedman, Farh et al. 2009).

1.4.3 MicroRNA in psychiatric disorders

Post-mortem studies of patients with major depressive disorder, suggest an important role for two microRNAs in the brain. Hsa-miR-1202 was reduced in prefrontal tissues of patients suffering from major depressive disorder (MDD) and blood levels of hsa-miR-1202 increased upon antidepressant treatment only in responding patients, possibility allowing to use hsa-miR-1202 as a biomarker. Bioinformatic analysis and in vitro studies revealed *GRM4* (metabotropic glutamate receptor 4) as a target gene of hsa-miR-1202 (Lopez, Lim et al. 2014). In raphe nuclei of suicide victims with MDD hsa-miR-135 was markedly reduced. In subsequent experiments, using mouse models and *in vitro* studies mmu-miR-135 was identified to be essential for chronic stress resilience and antidepressant efficacy. *Sert* and *Htr1a* genes were identified as target genes via luciferase assay (Issler, Haramati et al. 2014).

Brain specific miR-128b was shown to regulate fear extinction in a mouse model using lentiviral overexpression and sponge knockdown of miR-128b in the prefrontal cortex (Lin, Wei et al. 2011). The Notch pathway and miR-34a were identified to regulate fear memory consolidation. Confirmed by virus-induced overexpression in the amygdala, miR-34a was able to inhibit stress induced anxiety via target gene *Crfr1* (Dias, Goodman et al. 2014).

Presently, while a large number of microRNAs were implicated in psychiatric disorders by animal models or human studies, most microRNAs require further experimental validation and mechanistic evaluation to interpret their pathological relevance for psychiatric disorders (Hommers, Domschke et al. 2015).

2 Aim of the study

Anxiety and depressive disorders present with increasing prevalence in the last decade, yet their etiology is still poorly understood. Numerous candidate genes have been identified, however, few are sufficiently validated. One candidate gene, *RGS2/Rgs2*, was previously implicated in human and rodent anxiety as well as rodent depression-like behavior. Furthermore, *Rgs2* was implicated in molecular processes of learning and memory, however opposing reports leave the role of *Rgs2* in learning and memory unclear.

The aim of the present study is to further elucidate behavioral alterations in RGS2 knockout mice, in order to strengthen the base of human studies and indicate possible therapeutic developments.

Four main questions were addressed in this thesis:

1. Is emotional learning altered in *Rgs2*$^{-/-}$ mice? Does *Rgs2* affect learning and memory in non-aversive paradigms?
2. Does *Rgs2* play a role in acute and chronic stress coping?
3. Does *Rgs2* play a role in anxiety and depression-like behavior?
4. Which underlying molecular mechanism could be responsible for observed behavioral changes upon *Rgs2* deletion?

3 Materials

3.1 Chemicals and reagents

chelex-100	Bio-Rad Inc., Hercules, USA
dimethylsufoxide (DMSO)	AppliChem GmbH, Darmstadt, Germany
dNTP Set 100 mM	Thermo Fischer Scientific, Waltham, USA
Dulbecco's Modified Eagle Medium	PAN Biothech, Aidenbach, Germany
ethidium bromid solution (1%)	AppliChem GmbH, Darmstadt, Germany
fetal calf serum	PAN Biothech, Aidenbach, Germany
L-glutamine	PAN Biothech, Aidenbach, Germany
methanol	AppliChem GmbH, Darmstadt, Germany
mirvana MicroRNA mimics	Thermo Fischer Scientific, Waltham, USA
N-lauroylsarcosin sodium salt	Sigma Aldrich, St. Louis, USA
para formaldehyde	Merck KGaA, Darmstadt, Germany
peqGOLD universal agarose	PeqLab, Erlangen, Germany
Proteinase-K	Sigma Aldrich, St. Louis, USA
RNasin	Promega, Madison, USA
sodium chloride (NaCl)	AppliChem GmbH, Darmstadt, Germany
sucrose	Sigma Aldrich, St. Louis, USA
Taq DNA Polymerase, recombinant	Thermo Fischer Scientific, Waltham, USA
TaqMan® gene expression assays	Thermo Fischer Scientific, Waltham, USA
Gene	Assay ID
Adra2a	Mm00845383_s1
Adra2b	Mm00477390_s1
Adra2c	Mm00431686_s1
Adrb1	Mm00431701_s1
Adrb2	Mm02524224_s1
Cck	Mm00446170_m1
Cckar	Mm00438060_m1
Cckbr	Mm00432329_m1
Crhr1	Mm00432670_m1
Drd2	Mm00438545_m1
Drd3	Mm00432887_m1
Drd4	Mm00432893_m1
Gabbr1	Mm00444578_m1
Gabbr2	Mm01352554_m1
Gapdh	Mm99999915_g1
Htr1a	Mm00434106_s1

Htr1b	Mm00439377_s1
Htr2a	Mm00555764_m1
Htr2c	Mm00434127_m1
Nps	Mm03990645_m1
Npsr1	Mm00558817_m1
Npy	Mm01410146_m1
Npy1r	Mm00650798_g1
Npy2r	Mm01956783_s1
Npy5r	Mm02620267_s1
Rgs2	Mm00501385_m1
TaqMan® Universal Master Mix II, no UNG	Thermo Fischer Scientific, Waltham, USA
trypsin EDTA	PAN Biothech, Aidenbach, Germany
penicillin/streptomycin	PAN Biothech, Aidenbach, Germany

3.2 Technical equipment

1100 HPLC system	Agilent, Santa Clara, USA
5000mL beaker	Hartenstein, Würzburg, Germany
5415D centrifuge	Eppendorf, Hamburg, Germany
BP-2000 Blood pressure analysis system	Visitech Systems, Ape, USA
C1000TM thermal cycler	Bio-Rad Inc., Hercules, USA
CFX384 Real-Time PCR detection system	Bio-Rad Inc., Hercules, USA
cryo box	Hartenstein, Würzburg, Germany
Dark-Light Exploration apparatus	TSE Systems, Bad Homburg, Germany
electrochemical detector	Macherey-Nagel, Düren, Germany
Elevated Plus Maze	TSE Systems, Bad Homburg, Germany
EnVision 2104 Multilabel Reader	PerkinElmer, Waltham, USA
Fear Conditioning Chamber	TSE Systems, Bad Homburg, Germany
Intellicage apparatus	New Behavior AG, Zürich, Switzerland
minispec LF50 mq 7.5 NMR analyzer	Bruker BioSpin GmbH, Billerica, USA
NanoDrop 2000c Spectrophometer	PeqLab, Erlangen, Germany
Next Seq 500 system	Illumina Inc., San Diego, USA
Open Field	TSE Systems, Bad Homburg, Germany
Radiofrequency Identification Transponders	Planet ID GmbH, Essen, Germany
reversed-phase column 100-3C18 nucleosil	Macherey-Nagel, Düren, Germany
Ultra-Turrax Homogenizer (IKA T 10 basic)	IKA, Staufen im Breisgau, Germany
universal 320 R centrifuge	Hettich, Tuttlingen, Germany

3.3 Consumable supplies

384-well plate	Bio-Rad Inc., Hercules, USA
96-well plate	Sarstedt, Nümbrecht, Germany
96-well plate, white	Thermo Fischer Scientific, Waltham, USA
cryo vials	Thermo Fischer Scientific, Waltham, USA
culture dishes	Sarstedt, Nümbrecht, Germany
culture flasks	Sarstedt, Nümbrecht, Germany
falcon tubes (15 and 50 ml)	Corning Inc. Reynosa, Mexico
PCR tubes	Hartenstein, Würzburg, Germany
pipette tips RNAse free	Biozym GmbH, Oldendorf, Germany
pipette tips	Eppendorf, Hamburg, Germany
reaction tubes (1.5 and 2 ml)	Eppendorf, Hamburg, Germany
surgical disposable scalpel	Feather Safety, Okasa, Japan

3.4 DNA- and protein ladders

100bp DNA ladder	NEB, Frankfurt am Main, Germany

3.5 Commercial kits

Luc-Pair™ Duo-Luciferase Assay Kit 2.0	GeneCopoeia, Rockville, USA
NucleoSpin® miRNA kit	Macherey-Nagel, Düren, Germany
SuperScript® II reverse transcriptase kit	Thermo Fischer Scientific, Waltham, USA

3.6 Cell Lines

Hek293AD Cells	Thermo Fischer Scientific, Waltham, USA

3.7 Cell culture medium

Unless otherwise indicated, chemicals are given in (%) designated volume per volume (v/v).

Complete Dulbecco's Modified Eagle Medium (DMEM):

DMEM supplemented with:	4.5 g/L	glucose
	2 mM	L-glutamine
	10 %	fetal calf serum
	100 U/mL	penicillin
	100 µg/mL	streptomycin

pure DMEM:

DMEM supplemented with:	4.5 g/L	glucose

	2 mM	L-glutamine

Freezing medium:

DMEM supplemented with:	4.5 g/L	glucose
	2 mM	L-glutamine
	40 %	fetal calf serum
	10 %	DMSO

3.8 Plasmids

RGS2 3´UTR (MmiT054664-MT06)	GeneCopoeia, Rockville, USA
Control 3´UTR (CmiT000001-MT06)	GeneCopoeia, Rockville, USA

3.9 Solutions and buffers

Unless otherwise indicated, chemicals are given in (%) designated volume per volume (v/v)

TAE Buffer:	40 mM	Tris
	20 mM	CH_3CO_2H
	1 mM	EDTA
	pH 8.5	
Transmitter buffer:	150 mM	H_3PO_4
	500 µM	DTPA
HPLC mobile phase	90% 0.65mM	octanesulfonic acid
	10 %	methanol
	0.5 mM	trimethylamine
	0.1 mM	EDTA
	0.1 M	NaH_2PO_4
Digestion Buffer:	2.5 ml	Na-laurylsarcosin
	1 ml	NaCl 5M
	2.5 g	Chelex 100
	ad 50 ml	H_20
Proteinase K solution	10 mg/ml	in H_2O

3.10 Software

CFX Manager™ Software	Bio-Rad Inc., Hercules, USA
FCS software	TSE Systems, Bad Homburg, Germany
GraphPad Prism 7	GraphPad Software Inc., La Jolla, USA
Intellicage (Designer, Controller and Analyzer)	New Behavior AG, Zürich, Switzerland
minispec analysis, minispec plus 4.1.5	Bruker BioSpin GmbH, Billerica, USA

Pymol	Schrödinger, Cambridge, USA
VideoMot 2	TSE Systems, Bad Homburg, Germany

4 Methods

4.1 Animals

All animals were kept at the Center for Experimental Molecular Medicine (ZEMM) at the University of Würzburg on a regular 12 h light/ 12 h dark cycle in a temperature (21 ± 0.5°C) and humidity (50 ± 5%) controlled environment with food and water *ad libitum*. All experiments were performed during the light phase between 9.00 am and 3.00 pm. Male and female mice, wildtype C57BL/6J and *Rgs2* knockout on C57BL/6J background, generously provided by J. Penninger (Oliveira-Dos-Santos, Matsumoto et al. 2000), aged 8-12 weeks were used for all experiments. Mice were housed in same-genotype groups of 2-3 animals per cage, except for IntelliCage and Barnes Maze tests, for which mice were held in mixed genotype groups of ten mice per cage. All were offspring of homozygous wildtype and homozygous *Rgs2*$^{-/-}$ matings.

All animal protocols were in line with the provisions of the Animal Protection Law according to the Directive of the European Communities Council of 1986 (86/609/EEC), and have been reviewed as well as approved by the District Government of Lower Franconia and the University of Wuerzburg.

4.1.1 Genotyping of mice

Genotyping was performed using a polymerase chain reaction (PCR) on ear-punch biopsies of *Rgs2*$^{-/-}$, *Rgs2*$^{-/+}$ or *Rgs2*$^{+/+}$ (wildtype) mice. Ear punch biopsies were lysed in 50 µl digestion buffer and 3 µl proteinase K solution while shaking at 55 °C for 3 h. Lysates were vortexed, centrifuged for 1 min at 15700 x g and then boiled at 100 °C for 8 min. To remove insoluble material, lysates were again centrifuged at 15700 x g for 8 min. The supernatant was diluted 1:5 for PCR analysis. PCR was performed using *Taq* DNA polymerase. Primers detect the *Rgs2* wildtype allele at 583 bp and the *Rgs2* mutant allele at 693 bp (Oliveira-Dos-Santos, Matsumoto et al. 2000).

Primers:

Wildtype allele:	FW-CCG AGT TCT GTG AAG AAA ACA TTG
	RW-GGG ACT CCT GGT CTC ATG TAG CAT
Rgs2$^{-/-}$ mutant allele	FW-GCT AAA GCG CAT GCT CCA GAC
	RW-GGC CCA CAT TTA CAC GAA CC

For the polymerase chain reaction 4 µl diluted lysed ear-punch biopsy (template) were added to the following PCR reaction mix.
The PCR reaction mix contained:

1 µl 10µM forward primer
1 µl 10µM reverse primer
2 µl 2mM dNTP
2 µl *Taq* buffer
1 µl 50mM $MgCl_2$
0.2 µl 5U/µl *Taq* DNA polymerase

The compete mixture was then processed in the PCR thermal cycler as follows.
PCR Protocol:

(1) initialization step: 94°C – 2 min
(2) denaturation step: 94°C – 30 sec
(3) annealing step: 57°C – 30 sec
(4) elongation step: 72°C – 40 sec
(5) final elongation step: 72°C – 7 min
(6) final hold: 16°C - ∞
(2) to (4) were repeated for 40 reaction cycles

In a polymerase chain reaction, a specific part of a DNA molecule is amplified repeatedly. This method was originally developed to amplify coding sequences of interest. In a PCR tube the template DNA is amplified using sequence complementary forward and reverse primer, deoxynucleoside triphosphates and a thermostable polymerase. The reaction is initiated by the initialization and denaturation step, during these steps the hot start polymerase is activated and the hydrogen bonds between the double strand DNA helix are dissolved. Now the single strand DNA is accessible so that, in the annealing step, the forward and reverse primer can attach to the sequence complementary single strand DNA. In the elongation step the polymerase attaches the complementary deoxynucleoside triphosphates to re-complete the double strand DNA molecule. These cycles are repeated 30-40 times to repeatedly amplify the selected DNA sequence.

4.1.1.1 Agarose gel electrophoresis

In order to separate DNA fragments after PCR, agarose gel electrophoresis was used. 2.5 % agarose (w/v) was melted in TAE buffer, after cooling the mixture to about 50 °C 0.005 % ethidium bromide was added. The gel was then poured into a gel tray with comb in place. A 100 bp DNA ladder was used as standard. DNA was separated according to molecular size using an electrophorese chamber at a constant voltage of 120 V with TAE as running buffer. DNA fragments were visualized under ultraviolet light (300 - 360 nm) via fluorescence of intercalated ethidium bromide.

4.1.2 Analysis of body composition

Non-invasive nuclear magnetic resonance (NMR) analysis of living and awake mice were carried out using a Bruker Minispec LF50/mq7.5 analyzer (Trujillo Viera, El-Merahbi et al. 2016). Prior to analysis, each mouse was weighted to allow correction of lean, fat and free fluid mass measurements to total body weight. Mice were directed into an animal restrainer and put into the minispec probe. Mass of lean, fat as well as free fluid were determined according to (Kunnecke, Verry et al. 2004). Data were the mean of three repeated measurements for each mouse.

4.1.3 Blood pressure measurements

Tail blood pressure was determined using a non-invasive blood pressure analyzer for mice (Krege, Hodgin et al. 1995). The tail blood pressure and heart rate were determined using transmission photoplethysmography, meaning the light transmitted through the tail is analyzed. This is possible due to changed light scatter corresponding to changed vessel size upon pressure waves triggered by each heartbeat. Animals were trained for four consecutive days to habituate to the measurement process, measurements on the fifth day were then evaluated. Each measurement process consisted of 15 individual blood pressure measurements per mouse. The first 5 measurements were discarded due to habituation to the restraint during the measurement process. Blood pressure and heart rate were then averaged of 3-6 selected valid measurements of the remaining 10 measurements per animal.

4.1.4 Behavioral tests

Behavioral tests were divided in three experimental subgroups.

Experiment 1:

Mice were randomly assigned to a non-stressed control group (CTR male: 18 *Rgs2*$^{-/-}$ and 18 WT, female: 18 *Rgs2*$^{-/-}$ and 18 WT) or an acute stress group (FC male: 25 *Rgs2*$^{-/-}$ and 25 WT, female: 18 *Rgs2*$^{-/-}$ and 18 WT). FC mice were subjected to contextual and cued fear conditioning and underwent short-term fear memory tests 24h after conditioning. Subsequently, 18 CTR and 18 FC mice per genotype and sex were tested for innate anxiety using three different tests based on approach-avoidance conflict to evaluate the impact of acute stress on innate anxiety: elevated plus maze (EPM), dark/light box (DLB) and open field (OF), while 7 male mice per genotype were subjected to fear conditioning and fear memory tests at 24h, 7d and 14d to assess both short- and long-term fear memory as well as fear extinction. The experimental design is depicted in Figure 12.

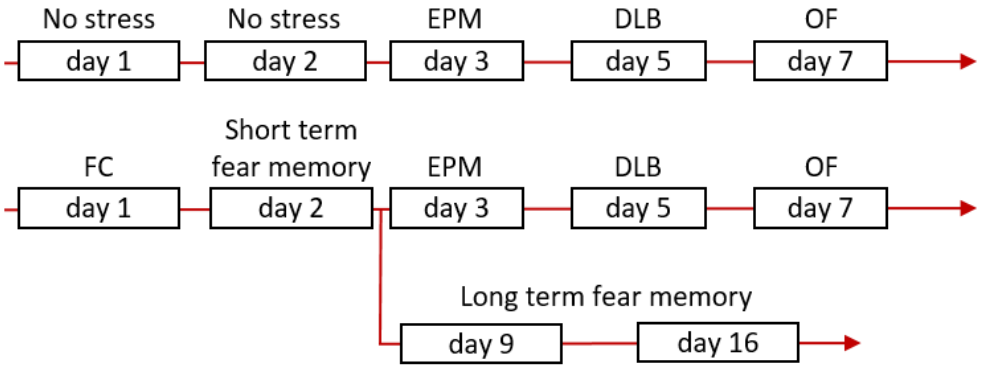


Figure 12: Experimental schedule of fear conditioning, short-term and long-term fear memory and extinction as well as acute stress susceptibility testing

Time course of the test battery applied to elucidate the impact of acute stress on innate anxiety. FC: Fear Conditioning; EPM: Elevated Plus Maze; DLB: Dark-Light Exploration; OF: Open Field Locomotion

Experiment 2:

Male (10 *Rgs2*$^{-/-}$ and 10 WT) and female (10 *Rgs2*$^{-/-}$ and 10 WT) mice were tested for visuo-spatial learning and memory in the Barnes maze, which takes advantage of mildly aversive stimuli (i.e. bright light) to provide motivation to locate an escape chamber. An additional independent cohort of 10 male *Rgs2*$^{-/-}$ and 10 male WT mice was subjected to a place preference paradigm in the IntelliCage to assess reward motivated spatial learning and re-learning (reversal) in a non-aversive home cage setting.

Experiment 3:

Mice were randomly assigned to a non-stressed control group (CTR: 10 mice per genotype and sex) or a chronic stress group (CMS: 10 mice per genotype and sex). The CMS group was subjected to 3 weeks of chronic unpredictable mild stress as depicted in Figure 13, while the control group was kept undisturbed in their home cage. Subsequently, CTR and CMS groups were tested in the DLB, SI and FST to investigate the impact of chronic mild stress on innate anxiety, social behavior and depression-like behavior as depicted in Figure 13.

Day	Stressor	Duration
Monday	Exposure to an empty water bottle	1 hour
Tuesday	Change of cage mates	2 hours
Wednesday	Illumination	Overnight
Thursday	Tilted cage (45°)	Overnight
Friday	Food deprivation	Overnight
Saturday	Removal of nesting material	Overnight
Sunday	Water deprivation	Overnight

+ every 3-4 days 15 min restraint
+ every 10 days soiled cage overnight

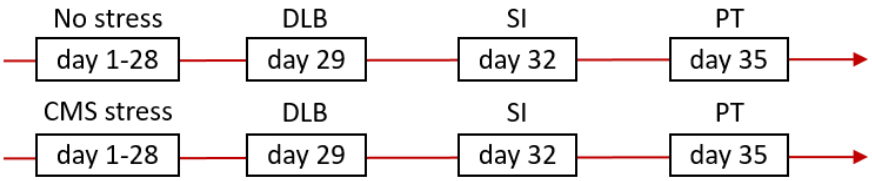


Figure 13: Experimental schedule of chronic stress susceptibility testing

Time course of the test battery to elucidate the impact of Chronic Mild Stress on innate anxiety, social behavior and depression-like behavior. CMS: Chronic Mild Stress; DLB: Dark-Light Exploration; SI: Social Interaction; FST: Forced Swim Test

4.1.4.1 Contextual and Cued Fear Conditioning

An automated fear conditioning chamber was used to investigate associative fear learning and memory (Fischer, Radulovic et al. 2007). During conditioning (day 0), mice were subjected to a 2-min habituation phase followed by the presentation of an auditory cue (4 kHz tone; 80 dB, conditioned stimulus, CS) for 30 s, co-terminating with a 0.6 mA scrambled foot-shock (unconditioned stimulus, US) during the last 2 s. Mice received two CS-US pairings with an inter-trial interval of 90 s. The second CS-US pairing was followed by a 30 s delay phase before mice were returned to their home cage. On day 1, mice were tested for contextual fear memory by exposition to the original conditioning chamber for 5 min. Two hours later, cued fear memory was evaluated in a modified environment. Mice were habituated to the modified environment for 2 min and then presented with the auditory cue 4 times for 30 s with an inter-trial interval of 5 s. During the entire experiment, behavioral responses were automatically recorded via infrared light barriers and a webcam. The infrared light barriers are comprised of a light emitting and a light receiving point. They are mounted into the apparatus at ground level to allow the tracking of the animal position and horizontal movement. Additionally, a second set of light barriers is mounted at an adjustable height above the cage floor to detect vertical activity. Once a mouse is placed into the test chamber some light barriers are blocked, and the position and movement of the mouse are tracked. The distance traveled, rearing (vertical activity), activity (the duration of movement above a speed threshold of 2 cm/s),

maximum speed and time freezing (complete immobility for a duration of > 2s) were quantified.

4.1.4.2 Elevated Plus Maze

The Elevated Plus Maze Test was performed as described previously. (Pellow and File 1986, Hogg 1996, Carobrez and Bertoglio 2005, Komada, Takao et al. 2008). In short, the Elevated Plus Maze consisted of two open arms (30 x 5 cm, 50 lx illumination) and two closed arms (30 x 5 x 15 cm, 5 lx illumination) extending from a common central area (5 x 5cm), elevated 60 cm above ground level. The maze was and semipermeable to infrared light to allow the visualization of black mice on a non-aversive black apparatus (Post, Weyers et al. 2011). Mice were individually placed in the central area facing an open arm and allowed to explore the maze for 10 min. The 10 min session was recorded using a CCD camera mounted to the ceiling and behavior was automatically tracked using VideoMot2 Software. Time spent in the open arms, number of open arm entries as measures of anxiety-like behavior, number of open and closed arm entries combined and total distance traveled to measure general exploratory behavior were quantified.

4.1.4.3 Dark-Light Exploration

Dark-Light Exploration Test was conducted as previously described (Crawley and Goodwin 1980, Bourin and Hascoet 2003). The apparatus consisted of an opaque white box (50 x 50 x 40 cm) with a black insert comprising of one third of the total box size with a rectangular opening (7 x 5 cm) at floor level. The insert was semipermeable to infrared light to allow the visualization of black mice in a dark surrounding. Illumination of the dark compartment was between 0-5 lx whereas the light compartment was illuminated at about 100 lx. Mice were individually placed in the dark compartment and allowed to explore freely for 10 min. The session was recorded using a CCD camera mounted to the ceiling and behavior was analyzed using automated tracking software VideoMot2. The number of transitions between the two compartments, the time needed to first enter the lit compartment (latency) as well as time spent in the lit compartment were quantified to evaluate anxiety-like behavior. Additionally, the total distance traveled was monitored in order to assess general exploratory behavior.

4.1.4.4 Open Field Locomotion

The Open Field Test assesses general exploratory behavior and anxiety-like behavior in rodents. The Open Field Test was conducted as previously described (Hall 1934, Prut and Belzung 2003, Seibenhener and Wooten 2015). The Open Field consisted of an opaque square box (50 x 50 x 40 cm) semipermeable to infrared light with an illumination of 100 lx in the

center of the Open Field to 50 lx at the walls of the apparatus (Post, Weyers et al. 2011). The black semipermeable material made it possible to visualize black mice on non-aversive black surrounding using infrared light not visual to mice. Mice were individually placed in one corner of the Open Field. The movement was automatically tracked and analyzed using a CCD camera positioned above the center of the box and VideoMot2 Software. The software was used to evaluate the distance traveled as a measure of general locomotor activity, as well as the time spent in the central zone of the arena as a measure of anxiety-like behavior.

4.1.4.5 Barnes Maze

The Barnes Maze test evaluated spatial learning (Barnes 1979, Rosenfeld and Ferguson 2014). The maze was a dark gray PVC disk with a diameter of 122 cm, elevated 80 cm above ground (TSE, Bad Homburg, Germany). 40 evenly spaced round openings with 50 mm in diameter were located at the outer margin of the disk. At the base of one hole an escape chamber was mounted. The spatial learning task was to locate and enter the escape chamber. Mice were given fifteen 2 min trials to locate the escape chamber. Upon entering the escape chamber, the trial ended. If the mouse was unable to locate the escape chamber in the 2-min period, it was gently guided by the experimenter to facilitate the learning process. After these fifteen trials reversal learning was tested. Therefore, the escape chamber was moved to the opposite hole on the maze. Mice were given five 2 min trials to learn the new position of the escape chamber. Each trial was recorded using a CCD camera mounted to the ceiling. All trials were carried out on 6 consecutive days, three to four trials on each day with an inter trial interval of approximately 30 min. Parameters considered were the time needed to locate the hole with the escape chamber (target latency), the number of wrong holes searched to before reaching hole with the escape chamber (primary errors), the time needed to enter the escape chamber (escape latency). Additional parameters considered were distance traveled until reaching the hole with the escape chamber (distance) and percent time spent in the correct target quadrant of total time (time in target quadrant). All parameters were evaluated in each trial using automated tracking software VideoMot2 by TSE Systems.

4.1.4.6 Intellicage

4.1.4.6.1 Apparatus

The IntelliCage apparatus was used to assess place preference learning. Male C57BL/6J and *Rgs2*$^{-/-}$ mice (*n* = 10 per genotype) were housed in mixed genotype groups of 10 mice per IntelliCage. During testing, animals had free access to shelters and standard mouse food. The IntelliCage provided access to water in each of the four conditioning chambers, fitted into the cage corners, accessible by one mouse at a time. In every corner two drinking bottles were available via two round openings (13 mm in diameter) outfitted with motorized doors. A

circular radiofrequency identification (RFID) antenna identified each mouse at the entrance to the conditioning corner. The duration of the corner visit was monitored by a temperature sensor. During a corner visit, number and duration of nosepokes at each door were quantified using infrared-light-beam sensors. Drinking behavior was evaluated by quantifying the duration of licking episode, the number of licks and total contact time with the bottle caps. All parameters were monitored and controlled using a central PC running IntelliCage software (Designer, Controller and Analyzer version 2.17.0.0, New Behavior AG). The Designer software was used to program the place preference learning task.

4.1.4.6.2 Place preference

Five days prior to testing, radiofrequency identification transponders were implanted subcutaneously in the dorso-cervical region of each mouse under isoflurane anesthesia. Prior to the place preference paradigm mice were habituated to the IntelliCage. Habituation started with a four-day free adaptation phase. During this time all doors were open and allowed free access to all eight drinking bottles. This was followed by a four-day nosepoke adaptation phase. During this phase all doors were closed and drinking was only possible when mice performed a nosepoke. This nosepoke opened the door for a 7 second drinking period once per corner visit.

After IntelliCage adaptation phase the place preference, a reward motivated spatial learning paradigm, started. Mice were randomly assigned to one corner where drinking, via nosepoke, was possible. In the other three corners, doors always remained closed. After 6 days of the place preference learning, the corner in which the water reward was previously given was switched to the opposite corner, termed place preference reversal. Re-learning of the newly assigned corner was tested for an additional 3 days (adapted from (Albuquerque, Haussler et al. 2013).

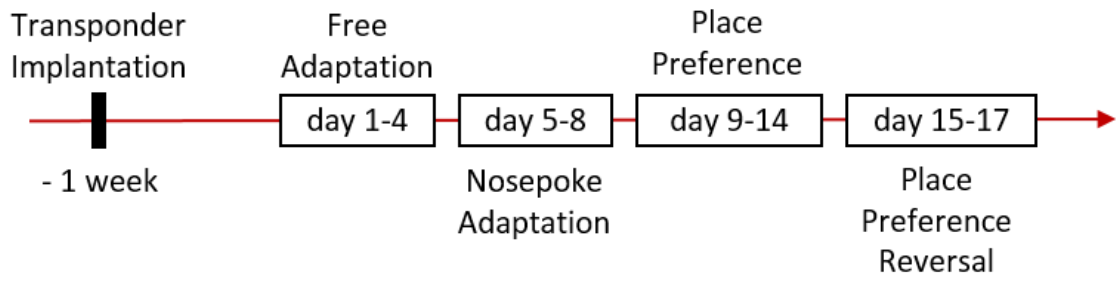


Figure 14: Time course of IntelliCage experiments

4.1.4.7 Unpredictable Chronic Mild Stress

The Chronic Mild Stress paradigm was performed as previously described (Katz 1981, Willner, Towell et al. 1987, Monleon, D'Aquila et al. 1995). The used stressors were adapted according

to options available in the animal facility (Zhu, Wang et al. 2014). The following stressors were used in a fixed weekly schedule; tilted cage (45 °), removal of nesting material, overnight food deprivation, overnight water deprivation followed by 1-hour exposure to an empty bottle, change of cage mate for 2 hours, overnight light, 15 min restraint and soiled cage overnight. Animals housed in groups of two mice per cage were subjected to the Chronic Mild Stress paradigm for 27 consecutive days (3 weeks). Behavior was evaluated after 3 weeks of stress.

4.1.4.8 Sucrose Preference Measurements

Sucrose Preference was conducted as previously described (Monleon, D'Aquila et al. 1995). In short, a two bottle approach giving mice the free choice to drink either plain water or a 1 % sucrose solution was used. The fluid intakes were evaluated over 48 h, after 24 h the two bottles were switched to avoid a place preference bias. Sucrose Preference was then calculated as % sucrose intake of total fluid intake.

4.1.4.9 Crawley´s Sociability and Preference for Social Novelty

The Sociability and Preference for Social Novelty Test (Social Interaction Test) was conducted as previously described (Moy, Nadler et al. 2004, Kaidanovich-Beilin, Lipina et al. 2011). The testing apparatus was made from clear Plexiglas and consisted of a rectangular three-chamber box (20 x 40 cm each) with dividing walls containing small doors (5 x 3cm) allowing free access to each chamber. Two identical plastic cup-like containers, perforated to allow nose contact but prevent fighting, were placed inside each side chamber.

During phase one (adaptation phase) both doors were closed and a subject mouse was placed in the middle chamber to habituate for 5 min. During phase two (sociability test) a mouse (stranger 1) having had no prior contact with the subject mouse, was placed inside a plastic cup in one side chamber while the other chamber contained an empty plastic cup. Both doors were then opened and the subject mouse was allowed to explore freely for 10 min. The location of stranger 1 was alternated between animals. In phase 3 (social novelty test), a second mouse (stranger 2) was placed in the previously empty plastic cup. The subject mouse was again allowed to explore freely for 10 min.

Each phase was recorded using a CCD camera mounted to the ceiling and recordings were quantified using tracking software VideoMot2. The total time spent in each compartment, the distance traveled in each compartment and the total distance to control for general locomotion were quantified for each phase of the test.

4.1.4.10 Forced Swim Test

The mouse Forced Swim Test was adapted and used to assess behavioral despair as an indicator of depression-like behavior (Porsolt, Le Pichon et al. 1977, Can, Dao et al. 2012). Mice were placed in a 5000 ml glass beaker filled with 3000 ml water at 25-27 °C. Behavior was recorded for 6 min using a webcam. The time spent immobile or floating during the last 4 min of the test, as well as the time passed to first float (latency) was evaluated.

4.2 Cell culture techniques

HEK293AD cells were used for all experiments (Shein and Enders 1962).

4.2.1 Freezing cells

A 250 ml flask with confluent cells was washed with DPBS, cells were trypsinized and re-suspended in complete DMEM. After centrifugation at 390 x g for 7 min at 4 °C, the supernatant was removed and the cell pellet was re-suspended in 5 ml freezing medium. The cell suspension was immediately aliquoted into five 2 ml cryo vials. The vials were stored in a cryo box overnight at -80 °C. On the next day, the vials were transferred into a storage box in the -80 °C freezer.

4.2.2 Thawing cells

Frozen cells were thawed at 37 °C in a water-bath. Once only a small ice crystal was left in the cryo vial, the cell suspension was transferred into 5 ml pre-warmed complete DMEM. After centrifugation at 390 x g for 7 min at 4 °C, the cell pellet was re-suspended in 10 ml of complete DMEM and transferred into a 250 ml flask at incubated at 37 °C, and 7 % $C0_2$.

4.2.3 MicroRNA mediated expression repression

4.2.3.1 Computational methods

Three web-based microRNA target prediction tools were used to predict microRNA regulation of *RGS2* gene expression through binding at its the 3'UTR: TargetScanHuman 6.2 (Grimson, Farh et al. 2007), DIANA microT-CDS (Paraskevopoulou, Georgakilas et al. 2013) and miRanda (Betel, Koppal et al. 2010). Annotation and mature microRNA sequences were acquired from miRBase release 21 (Kozomara and Griffiths-Jones 2014) and miRNAConverter of miRSystem was employed to convert names between different miRBase versions (Lu, Lee et al. 2012).

4.2.3.2 Luciferase reporter assay

Target gene expression regulation by microRNAs was assessed using a dual *firefly/renilla* luciferase assay. "Luciferase vectors" contained the 3´UTR of RGS2 fused to the cDNA of the *firefly* luciferase (RGS2 vector). No 3`UTR fused to the *firefly* luciferase (control vector) was used as a control. MirVana microRNA mimics, small chemically modified double stranded RNAs that mimic endogenous microRNAs and allow functional microRNA analysis, were co-transfected with either RGS2 or control vectors into HEK293AD cells. Thereby the microRNA interaction with the 3´UTR can repress *firefly* luciferase expression.

4.2.3.3 DNA-Transfection

HEK293AD cells were seeded in complete DMEM into a 96-well plate 4 hours prior transfection to reach approximately 60 % confluency. Each well was transfected with 40 ng of RGS2 or control vector plasmid and 3 pmol of a mirVana microRNA mimic. For the transfection the vector plasmid and the mirVana microRNA mimic were mixed with 8.25 µl pure DMEM and 0.15 µl Attractene. After a 10 min incubation period to allow formation of transfection complexes 10 µl of vector/mimic/Attractene/DMEM mix was added to each well.

To eliminate plate to plate variations, each plate contained measurements for RGS2- and control-vector for the same microRNA and a micro-RNA untransfected control to allow normalization. Additionally, each plate contained a negative control using a plant specific microRNA, ath-159a.

4.2.3.4 Quantification of luciferase activity

Cells were incubated for 40-48 h post transfection. Luciferase activity was quantified by an EnVision 2104 Multilabel Reader using the LucPair™ Duo-Luciferase Assay Kit according to the manufacturer's protocol. In short, culture medium was removed and cells were washed once with PBS. Then 14 µl of lysis buffer were added to each well. After 15 min incubation on an orbital shaker at room temperature, 70 µl of FLuc Assay Working solution were added. Following a 5 min incubation period at room temperature *firefly* luminescence was determined in the EnVision Reader. After completing *firefly* luciferase measurement, each well was spiked with 70 µl RLuc Working Solution and incubated for 5 min at room temperature. Then *renilla* luminescence was quantified by the EnVision Reader.

Luciferase expression suppression for each microRNA was calculated as follows. Luciferase activity was normalized to renilla activity for each well to correct for cell density and transfection efficiency in each well, yielding relative luciferase activity and technical triplicates were averaged. Relative luciferase activity of each microRNA was normalized to the relative luciferase activity of un-transfected (H_2O) control (maximal activity). To calculate normalized

luciferase activity, the maximal activity of each microRNA co-transfected with the RGS2 vector was normalized to the activity of that microRNA co-transfected with the control vector containing no 3`UTR to correct for unspecific microRNA-vector interaction.

4.3 MicroRNA Sequencing

Animals were sacrificed using cervical dislocation, the brain was surgically dissected and the hippocampus was frozen using liquid nitrogen and stored at -80°C. Total RNA was extracted using NucleoSpin® miRNA kit according to manufacturer's protocol. In the extraction step lysis buffer amount was adapted according to the amount of tissue. Total RNA concentrations were determined using UV-VIS spectrophotometry (NanoDrop®).

The library (adapter ligated microRNAs) for next generation sequencing was prepared using NEB Next Small RNA Library Prep for Illumina (Set 1 and 2) according to manufacturer's protocol. Size selection was performed using a 6% Novex® TBE PAGE gel with SYBR® Gold Nucleic Acid Gel Stain, the 140bp band corresponded to the Adapter-ligated microRNA constructs and was isolated. The sequencing was performed in a Next Seq 500 system using a Nest Seq 500 Kit v1 which includes a Paired End 75 Mid output flow cell.

The resulting reads were mapped an a microRNA expression profile was generated using the miRExpress algorithm (Wang, Lin et al. 2009).

4.4 High pressure liquid chromatography

2-month-old *Rgs2*$^{-/-}$ and wildtype mice were sacrificed using isofluran inhalation and perfused for 10 min using PBS. The hippocampus and the prefrontal cortex were dissected, immediately frozen using liquid nitrogen and stored at -80 °C until further analysis. Dissected frozen brain regions were homogenized in transmitter buffer on dry ice using an Ultra-Turrax Homogenizer in a CO_2 atmosphere, and centrifuged at 20879 x g for 12 min, the supernatant was transferred into Eppendorf-caps and stored at -20 °C until analysis. For HPLC analysis the supernatant was diluted 1:10 in transmitter buffer and 50 µl were injected into the HPLC system. Monoamine neurotransmitters Serotonin (5HT), Dopamine (DA), Norepinephrine (NE) and their metabolites 3-Methoxy-4-hydroxyphenylglycol (MHPG), 3,4-Dihydroxyphenylacetic acid (DOPAC), 5 Hydroxyindoleacetic acid (5HIAA), Homovanillic acid (HVA) were quantified using an Agilent 1100 HPLC system consisting of a reversed-phase column 100-3C18 nucleosil and an electrochemical detector at 0.75 V as previously described (Riederer & Burger, 2009). The amount of neurotransmitters and corresponding metabolites were normalized to the amount of brain tissue. The amount of the three neurotransmitters (DA, 5HT, NA), as well as their respective metabolic turnover ratios ((HVA + DOPAC) / DA; 5HIAA / 5HT; MHPG / NA)) were evaluated (Okada, Tachibana et al. 2013).

4.5 Quantitative gene expression analysis

Animals were sacrificed using cervical dislocation, their brains and/or their hearts were surgically dissected. Whole hearts, or dissected hearts (atria and ventricle), prefrontal cortices and hippocampus were frozen using liquid nitrogen and stored at -80°C until further analysis. Total RNA was extracted using NucleoSpin® miRNA kit according to manufacturer's protocol. In the extraction step lysis buffer amount was adapted according to the size of the respective tissue.

Total RNA concentrations were determined using UV-VIS spectrophotometry (NanoDrop). Reverse transcription of RNA to cDNA was performed using SuperScript® II reverse transcriptase kit.

For reverse transcription the following components were necessary for each sample:

1 µg	RNA in 9 µl RNase free water
2 µl	oligo dt
1 µl	10 mM dNTP

This mixture was heated at 70°C for 10 min to allow denaturation of RNA and oligo dt and then cooled on ice for at least one minute. Each sample was then spiked with the enzyme solution containing:

4 µl	5x first strand buffer
2 µl	100mM DDT
0.9 µl	RNase free water
0.1 µl	RNasin
1 µl	superscript II reverse transcriptase

Samples were incubated at 42°C for 60 min for reverse transcription. The superscript II reverse transcriptase was then inactivated by heating at 70°C for 10 min.

100 ng cDNA were used for each quantitative real time PCR. Quantitative real time PCR was performed in a CFX384 Real-Time PCR detection system using TaqMan® Universal Master Mix II and appropriate qPCR primers. TaqMan®-probes are hydrolysis probes consist of a specific oligonucleotide sequence fused to fluorescent tag and a quencher. During polymerization the exonuclease activity of the *Taq* polymerase degrades the probe, thereby separating the fluorophore from the quencher allowing quantification of the amount of DNA template via fluorescence.

The PCR reaction mix contained:

5 µl	20ng/µl cDNA

1 µl 20x TaqMan gene expression assay
10 µl 2x TaqMan universal master mix
4 µl nuclease free water

PCR Protocol:

(1) initialization step: 95°C – 10 min
(2) denaturation step: 95°C – 15 sec
(3) annealing step: 60°C – 60 sec
(4) final hold: 16°C - ∞
(2) to (3) 40 reaction cycles

4.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.01. Students-t- tests, regular Two-Way ANOVA or repeated measures ANOVA were performed as needed. Bonferroni's multiple comparisons test was used if group effects or interactions were significant. If not indicated otherwise, a p-value below 0.05 was considered to be statistically significant. Data are shown as mean ± standard error of the mean.

5 Results

Male and female mice were evaluated separately in all tests due to the variability of behavior in female mice on account of their estrous cycle (Palanza 2001) and due to the differential stress vulnerability of male and female mice (Adamec, Head et al. 2006, Weinstock 2007).

5.1 General health

Alterations in general health of laboratory mice might interfere with behavioral testing. Oliveira-Dos-Santos and coworkers examined vibrissae, eyes, rearing/standing, muscle tone, righting reflex, balance, ear reflex, hearing, response to light and olfaction of *Rgs2*$^{-/-}$ and *Rgs2*$^{+/-}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000). Additionally, motor coordination using the Rotarod test and exploratory behavior using circadian and Open Field activities were examined. Male and female *Rgs2*$^{-/-}$ mice showed no abnormalities in all tests. To corroborate these findings in the present study and ensure *Rgs2*$^{-/-}$ mice had no physical impairments confounding behavioral output, body weight, food intake and body composition as well as home cage activity were evaluated. In line with the previous findings *Rgs2*$^{-/-}$ mice were expected to show unaltered general health.

5.1.1 Body Weight, Food Intake and Body Composition

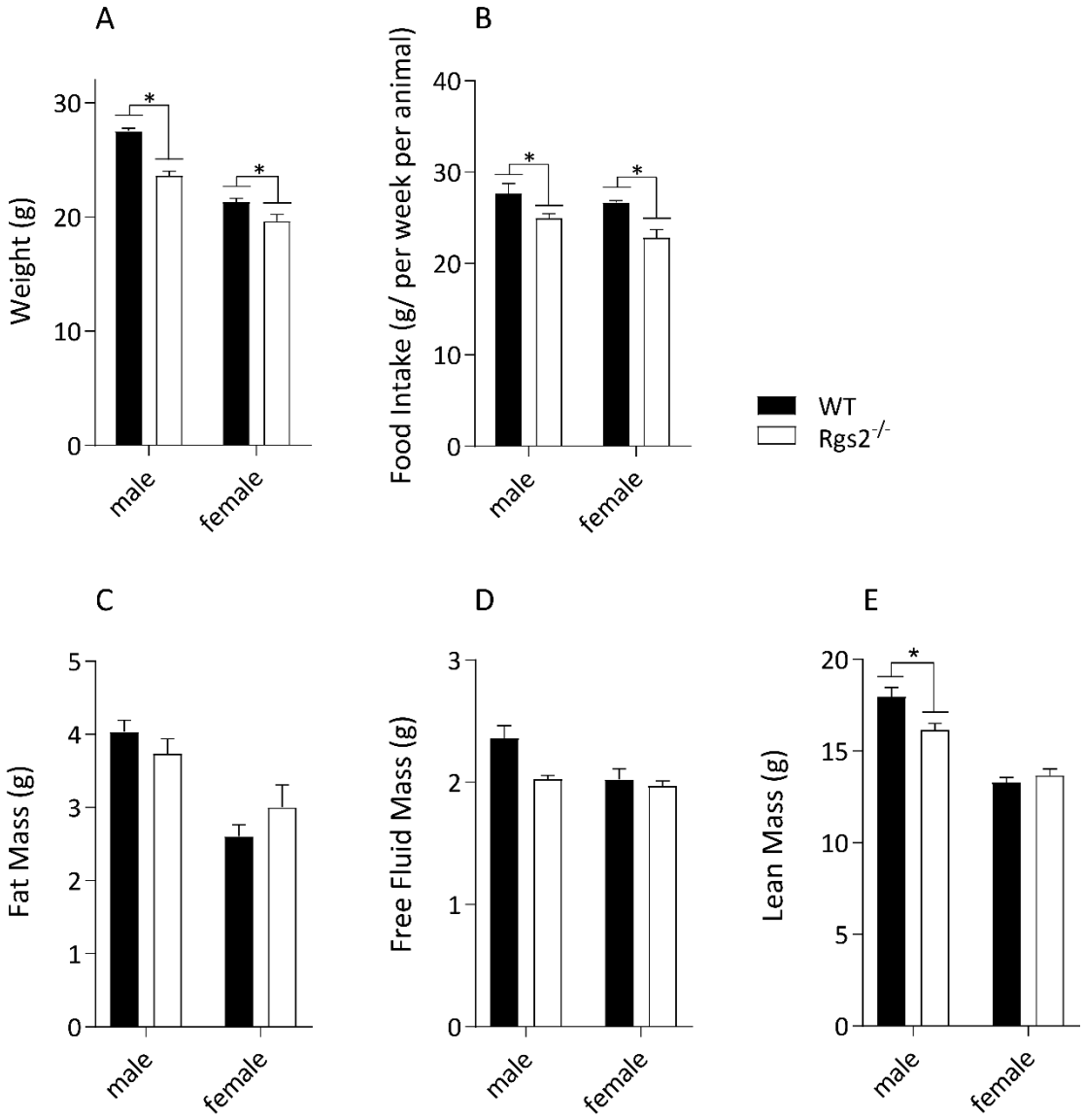


Figure 15: General health assessment

General health was determined by quantification of (A) body weight (B) food intake and (C-E) body composition. Body composition was determined using NMR analysis and yielded data for (C) fat mass, (D) free fluid mass and (E) lean mass. Data are mean ± SEM, n=17-21/genotype and sex for body weight measurements, n=10-11 cages/genotype and sex for food intake measurements and n= 6-9/genotype for body composition measurements. WT are depicted in black bars, *Rgs2*$^{-/-}$ are depicted in white bars. * indicates $p<0.05$ in t-tests.

2-month-old (+/- 5 days) *Rgs2*$^{-/-}$ mice showed reduced body weight compared to same sex WT mice (Figure 15A). Reduced body weight might be due to decreased food intake. As shown in Figure 15B, *Rgs2*$^{-/-}$ mice consumed less food during one week compared to same sex WT. Body composition as assessed by nuclear magnetic resonance imaging revealed reduced lean tissue in male but not female *Rgs2*$^{-/-}$ mice (Figure 15C-E).

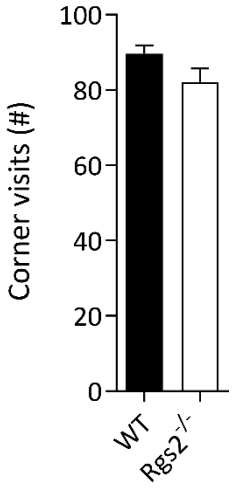


Figure 16: Home cage activity

Male mice were housed in the IntelliCage for a 4-day period. Evaluated were the mean number of corner visits as an indicator of home cage activity. Data are mean ± SEM, n=10-12/genotype, WT are depicted in black circles, *Rgs2*$^{-/-}$ are depicted in white circles.

Home cage activity was assessed using the IntelliCage by counting the number of corner visits of each mouse over a four-night period, revealing comparable activity in male *Rgs2*$^{-/-}$ and WT mice (Figure 16). Female mice were not evaluated in the IntelliCage.

Results regarding weight, food intake and body composition did not corroborate previous findings and were not in line with the expected results. However, home cage activity was unaltered, as expected. Since movement and activity of *Rgs2*$^{-/-}$ mice were not impaired, observed changes in weight, food intake and body composition were expected not to alter behavioral measures.

5.1.2 Blood pressure and heart rate

Several publications reported a hypertensive phenotype of *Rgs2*$^{-/-}$ mice (Heximer, Knutsen et al. 2003, Tang, Wang et al. 2003). Systolic and diastolic blood pressure, as well as heart rate were therefore evaluated using the non-invasive tail-cuff method. The hypothesis was that systolic and diastolic blood pressure are elevated in *Rgs2*$^{-/-}$ mice.

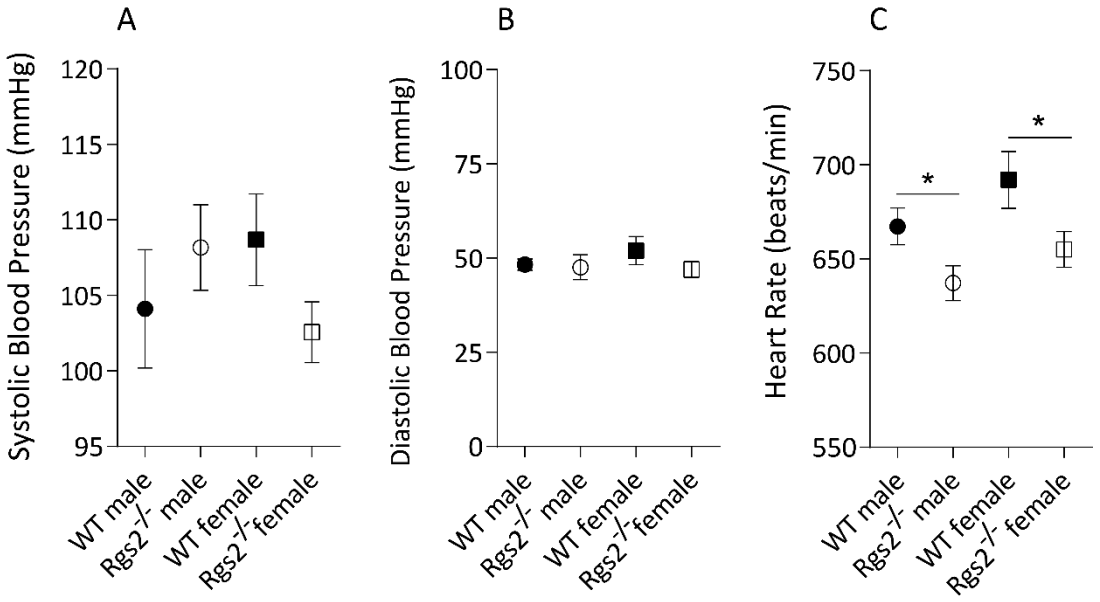


Figure 17: Blood pressure and heart rate measurements

Awake mice were tested in a non-invasive tail cuff system to evaluate blood pressure and heart rate. Illustrated are (A) systolic and (B) diastolic blood pressures, as well as (C) heart rate. Data are mean ± SEM, n=23-25/genotype and sex WT are depicted in black bars, *Rgs2*$^{-/-}$ are depicted in white bars. * indicates $p<0.05$ in t-tests.

Systolic and diastolic blood pressure were comparable for male and female *Rgs2*$^{-/-}$ mice compared to same sex WT (Figure 17A-B). However, the heart rate was decreased in *Rgs2*$^{-/-}$ mice compared to WT for both sexes (Figure 17C).

The hypertensive phenotype of *Rgs2*$^{-/-}$ mice was not confirmed using the tail-cuff method. However, a bradycardic heart rate was observed.

5.2 Memory and Learning

A previous publication reported comparable spatial and conditional learning in Water Maze and passive avoidance experiments of homozygous *Rgs2*$^{-/-}$ mice compared to heterozygous *Rgs2*$^{-/+}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000). Additionally, several publications suggest increased innate anxiety in *Rgs2*$^{-/-}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000, Lifschytz, Broner et al. 2012). The etiology of anxiety disorders involves interactions between candidate genes and stressful life events (see 1.1.1). Whether *Rgs2* is a candidate gene of fear learning and memory was assessed using the Pavlovian contextual and cued fear conditioning paradigm. This paradigm tests short term fear memory, long term fear memory and fear memory extinction. It was hypothesized that aversive learning, specifically fear learning including short and long-term fear memory, are increased in *Rgs2*$^{-/-}$ mice.

5.2.1 Aversive learning and memory

Contextual and cued Pavlovian fear conditioning is a task assessing the ability to associate an aversive, fear inducing, experience (an electric foot shock) with a distinct environmental cue (distinct tone). The main measures are (I) how fast mice create (learning) and (II) how long mice retain the aversive association (memory). How fast mice create the association was tested in the conditioning session. How long mice can retain this association was evaluated (A) 24h after the conditioning session, thereby assessing short term fear memory in context and cue tests and (B) one and two weeks after the conditioning session, thereby assessing long term fear memory and fear extinction learning in context and cue tests. The evaluated parameter was the relative freezing time (percent freezing time of total time). Freezing is defined as complete immobility except breathing. Relative freezing time was expected to be increased among *Rgs2*$^{-/-}$ mice in each phase of the test indicating increased fear learning and memory of *Rgs2*$^{-/-}$ mice. 25 male mice per genotype and 18 female mice per genotype were tested for fear learning and short term fear memory. 7 male mice per genotype were tested for long term fear memory and fear extinction learning.

5.2.1.1 Short-term fear memory

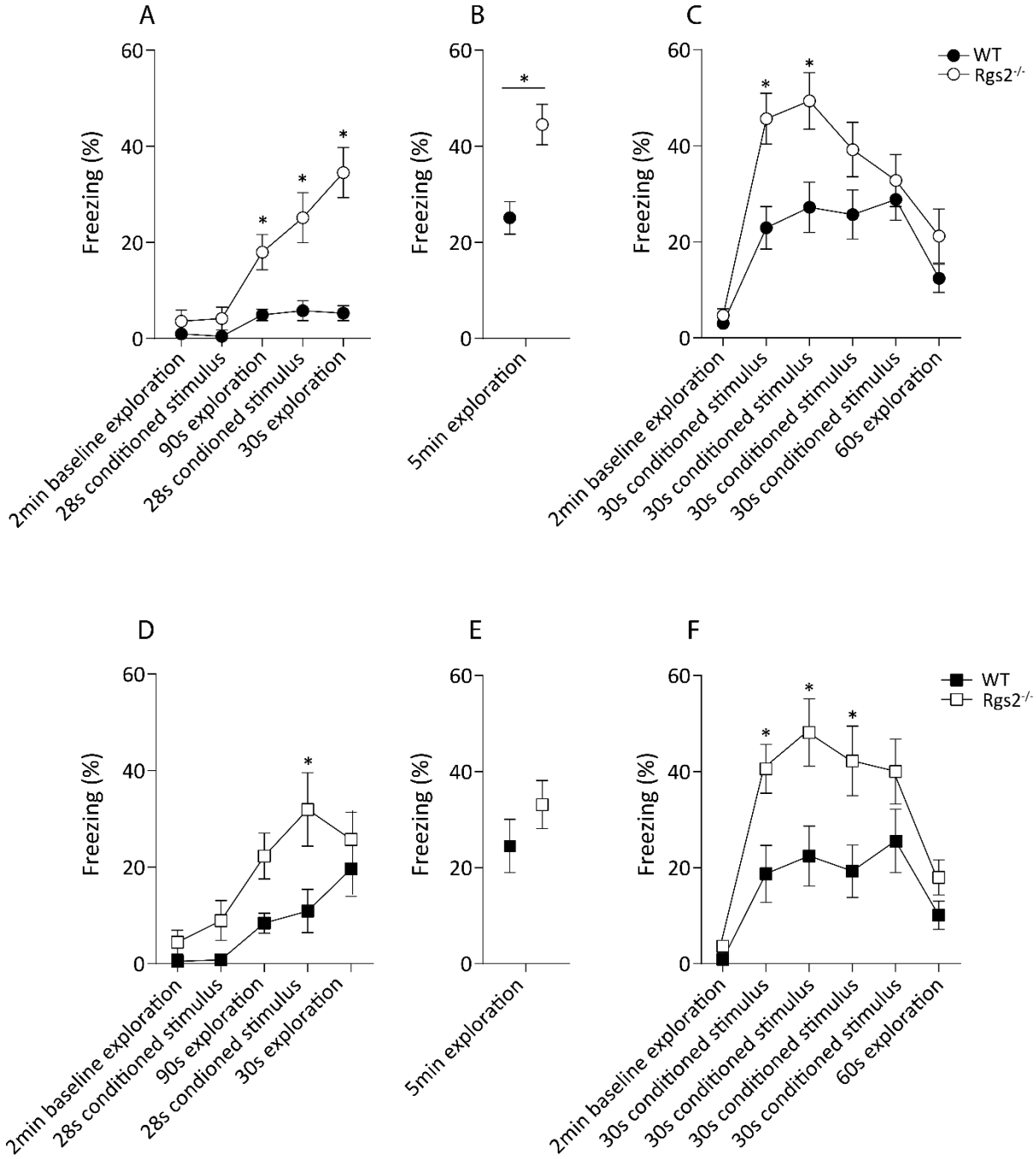


Figure 18: Short term fear learning and memory

Mice were subjected to a fear conditioning paradigm. (A/D) Time course of conditioning phase with two tone-shock pairings, (B/E) context memory test 24h after conditioning, (C/F) time course of cue memory test 26h after conditioning. Figures A-C illustrate results of male mice, D-F of female mice. Data are mean ± SEM, n=19-25/genotype and sex, WT male are depicted in black circles, *Rgs2*$^{-/-}$ male are depicted in white circles. WT female are depicted in black squares, *Rgs2*$^{-/-}$ female are depicted in white squares * indicates $p<0.05$ in ANOVA main effects, $^{+}$ indicates $p<0.05$ in Bonferroni's post hoc test.

Relative freezing times did not differ between genotypes during the 2min baseline exploration phase and the first CS presentation in male mice. However, male *Rgs2*$^{-/-}$ mice showed higher

levels of relative freezing time compared to WT after the first presentation of a foot shock (US) in the conditioning session (Figure 18A). Likewise, female *Rgs2*$^{-/-}$ mice displayed almost absent and similar relative freezing time compared to WT mice during the baseline exploration phase, as well as the first presentation of the CS in the conditioning session (Figure 18D). After the first foot shock (US), female *Rgs2*$^{-/-}$ mice showed increased relative freezing time compared to WT during the second presentation of the CS. These results indicate faster fear learning in male and female *Rgs2*$^{-/-}$ mice exposed to aversive stimuli in the conditioning session as expected.

In the contextual fear memory test 1 day later, male *Rgs2*$^{-/-}$ mice showed higher relative freezing time compared to WT (Figure 18B). However, female *Rgs2*$^{-/-}$ mice showed comparable relative freezing time in the context memory test compared to WT (Figure 18E), indicating a sex specific enhanced contextual fear memory in male *Rgs2*$^{-/-}$ mice.

In the cue memory test, both male (Figure 18C) and female (Figure 18F) *Rgs2*$^{-/-}$ mice displayed increased relative freezing time upon presentation of the conditioned tone (CS) in an altered surrounding. For both sexes, relative freezing did not differ between genotypes during the 2min baseline exploration phase. However, relative freezing time was increased in male *Rgs2*$^{-/-}$ mice compared to WT controls during the 1st and 2nd CS presentation, but remained comparable during the 3rd and 4th CS. Likewise, female *Rgs2*$^{-/-}$ mice displayed elevated freezing time upon presentation of the 1st, 2nd and 3rd CS. These data, as expected, suggest augmented short-term cued fear learning and memory in male and female *Rgs2*$^{-/-}$ mice.

Taken together, these results suggest deletion of *Rgs2* to promote faster fear learning and increased short term fear memory.

Table 1: ANOVA and T-Test results for short-term fear learning and memory

		male		female	
	effect	**$F_{(4;188)/(1;47)}$**	**significance**	**$F_{(4;204)/(1.34)}$**	**significance**
Conditioning	Genotype x time	9.382	**p < 0.0001**	1.87	p = 0.1192
	Genotype	24.69	**p < 0.0001**	6.224	**p < 0.05**
	Time	18.76	**p < 0.0001**	13.68	**p < 0.0001**
Cue	Genotype x time	3.272	**p < 0.01**	2.556	**p < 0.05**
	Genotype	6.383	**p < 0.05**	8.55	**p < 0.01**
	Time	27.92	**P < 0.0001**	20.68	**p < 0.0001**
Context	Genotype	$t_{(47)}$ = 3.578	**p < 0.001**	$t_{(34)}$ =1.150	p = 0.2584

5.2.1.2 Long-term fear memory and extinction learning

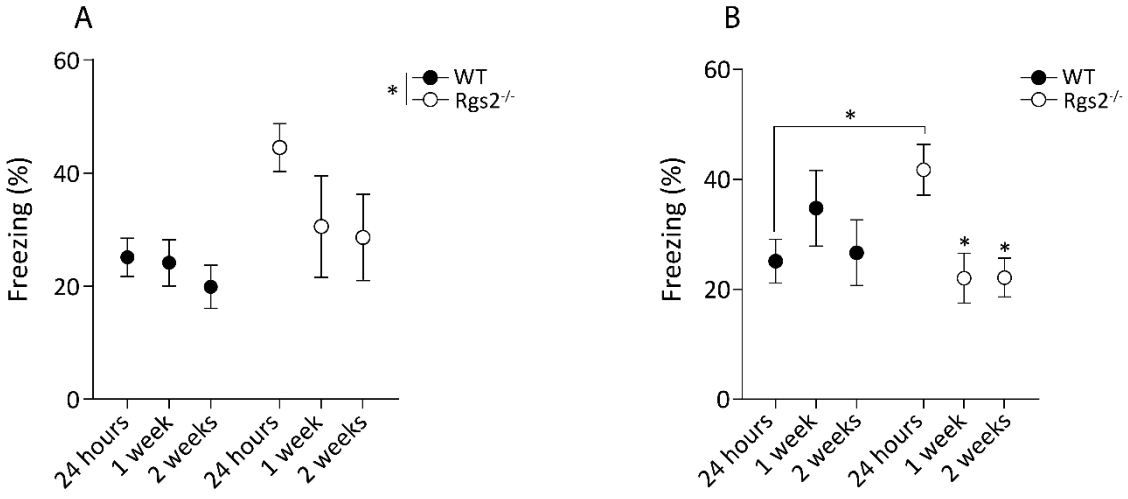


Figure 19: Long term fear memory and extinction learning

Male mice were tested in context and cue tests 24h, 1 week and 2 weeks after conditioning. (A) time course of relative freezing time in context tests and (B) time course of relative freezing time in cue tests. Data are mean ± SEM, n=7/genotype, WT are depicted in black circles, *Rgs2*$^{-/-}$ are depicted in white circles. * indicates p<0.05 in ANOVA main effects, $^{+}$ indicates p<0.05 in Bonferroni's post hoc test.

Male *Rgs2*$^{-/-}$ mice tested for short and long term fear memory, showed higher relative freezing time in context memory tests 24h, one and two weeks after conditioning compared to WT (Figure 19A).

In cue memory tests (Figure 19B), an increase of cue freezing time 24h after conditioning ($p<0.05$) was observed in *Rgs2*$^{-/-}$ mice compared to WT, while one and two weeks later there was no difference. *Rgs2*$^{-/-}$ mice showed a reduction of relative freezing time 1 week ($p<0.05$) and 2 weeks ($p<0.05$) after conditioning compared to 24h after conditioning, indicating extinction of cued fear memory over time. WT mice did not show a reduction of relative freezing time over 2 weeks, arguing for long term memory of cued fear in WT mice.

Taken together, these results confirm the hypothesis of increased long term contextual fear memory in *Rgs2*$^{-/-}$ mice. However, they indicate faster cued fear extinction or extinction learning and no increase in long term cue memory of male *Rgs2*$^{-/-}$ mice compared to WT. These results suggest deletion of *Rgs2*$^{-/-}$ to promote faster cued fear extinction learning and increased long term contextual fear memory.

Table 2: ANOVA results for long-term fear memory and extinction learning

	effect	$F_{(2;71)/(1;71)}$	significance
Context memory	Genotype x time	0.9342	p = 0.3977
	Genotype	5.387	**p < 0.05**
	Time	2.232	p = 0.1148
Cue memory	Genotype x time	3.970	**p < 0.05**
	Genotype	0.001	p = 0.9679
	Time	1.331	p = 0.2706

5.2.1.3 Gene expression analysis

Various stimuli triggering neuronal plasticity modulate the mRNA expression level of *Rgs2* in several brain regions, rendering *Rgs2* to be an intermediate early gene (Burchett, Volk et al. 1998, Ingi, Krumins et al. 1998). Whether *Rgs2* mRNA expression is altered by fear conditioning was assessed using quantitative real time PCR. The hippocampus and frontal cortices of WT mice were dissected one and six hours after the conditioning phase of contextual and cued fear conditioning and *Rgs2* mRNA expression was analyzed. The hypothesis was, that FC triggers an increase in the mRNA expression level of *Rgs2*.

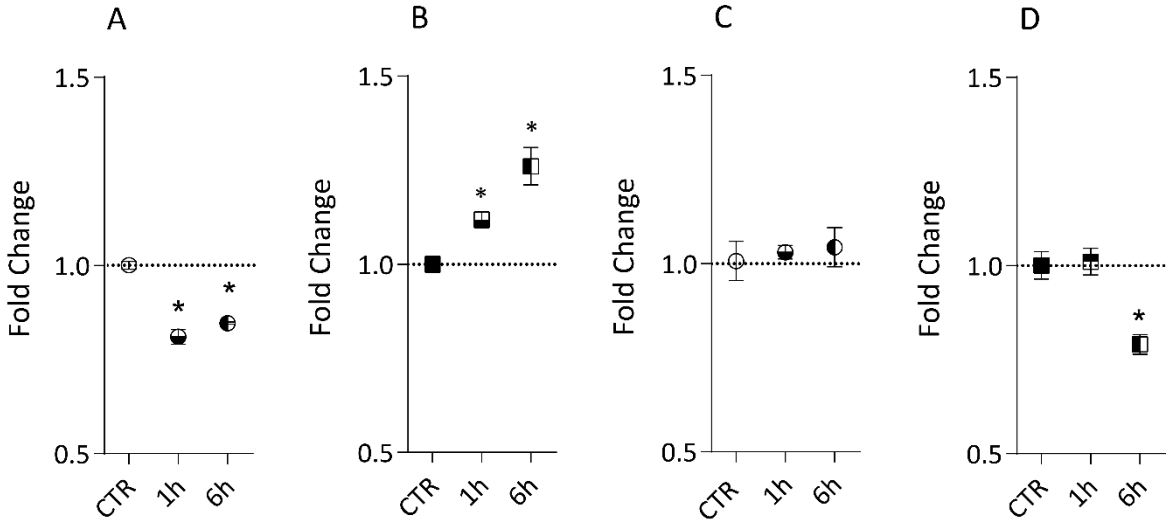


Figure 20: *Rgs2* mRNA expression levels upon fear conditioning

Time course of relative mRNA expression changes evaluated by quantitative real time PCR upon fear conditioning. (A/C) Time course of hippocampal mRNA expression, (B/D) time course of prefrontal cortex mRNA expression. Data are mean ± SEM, n=6/genotype, males are depicted in circles, female are depicted in squares. * indicates p<0.05 in t-tests.

In male mice, fear conditioning mildly reduced *Rgs2* mRNA expression levels in the hippocampus 1h and 6h after the conditioning phase (Figure 20A). In frontal cortices (Figure 20B), *Rgs2* expression levels were increased 1h after conditioning and further increased at 6h. Female mice showed no change of *Rgs2* mRNA expression levels in hippocampal preparations

1h or 6h after conditioning (Figure 20C), however 6h after conditioning *Rgs2* expression levels were reduced in frontal cortices (Figure 20D).

These results suggest FC stress to be sufficient to elicit *Rgs2* mRNA expression change, however *Rgs2* mRNA levels were not only increased but also decreased after FC stress. Deletion of *Rgs2* may thus alter dynamic regulation of GPCR signaling upon stressful stimuli in a sex-specific manner.

5.2.2 Spatial learning

Previous results indicated comparable spatial learning in the Water Maze for *Rgs2*$^{-/-}$ compared to *Rgs2*$^{-/+}$ mice (Oliveira-Dos-Santos, Matsumoto et al. 2000). However, this test is considered very stressful and stress has been shown to affect learning, especially in mice with increased innate anxiety (Harrison, Hosseini et al. 2009). Both fear learning and cue extinction learning were increased in Pavlovian contextual and cued fear conditioning of *Rgs2*$^{-/-}$ mice (see 5.2.1). It was hypothesized, that *Rgs2*$^{-/-}$ mice show increased aversive emotional learning with unaltered learning in other non-aversive paradigms. The Barnes Maze was conducted to evaluate spatial learning and a place preference paradigm in the IntelliCage was used to investigate reward motivated spatial learning. Since the Barnes Maze is less stressful than the Water Maze (Paul, Magda et al. 2009, Sharma, Rakoczy et al. 2010), the confounding effect of stress is reduced. Ten mice per genotype and sex, housed in mixed genotype groups since weaning, were tested.

The Barnes Maze consists of a circular platform with 40 evenly spaced holes at the outer margin. Mice are trained to locate one target hole and remember its location. Underneath the target hole and escape chamber in mounted. The mouse is trained to enter this escape chamber. Each trial lasts for 2 min. If the mouse fails to locate the escape chamber within this time, it is guided to the escape chamber by the experimenter. Each mouse was given 15 trials to locate and enter the escape chamber (acquisition phase), subsequently the escape chamber is moved to the opposite hole on the Barnes Maze and mice were tested to relearn the new location (reversal phase). In the acquisition phase spatial learning is assessed, in the reversal phase cognitive flexibility.

The following parameters were considered: (I) the time it takes to locate the target hole (target latency), (II) the number of wrong holes searched before locating the target hole (primary errors), (III) the time needed to enter the escape chamber (escape latency), (IV) the distance traveled until reaching the target hole (distance) and (V) the relative time spent in the correct target quadrant (time in target quadrant). Faster learning would be indicated by reduced target latency, escape latency, primary errors and distance as well as by increased time in target quadrant.

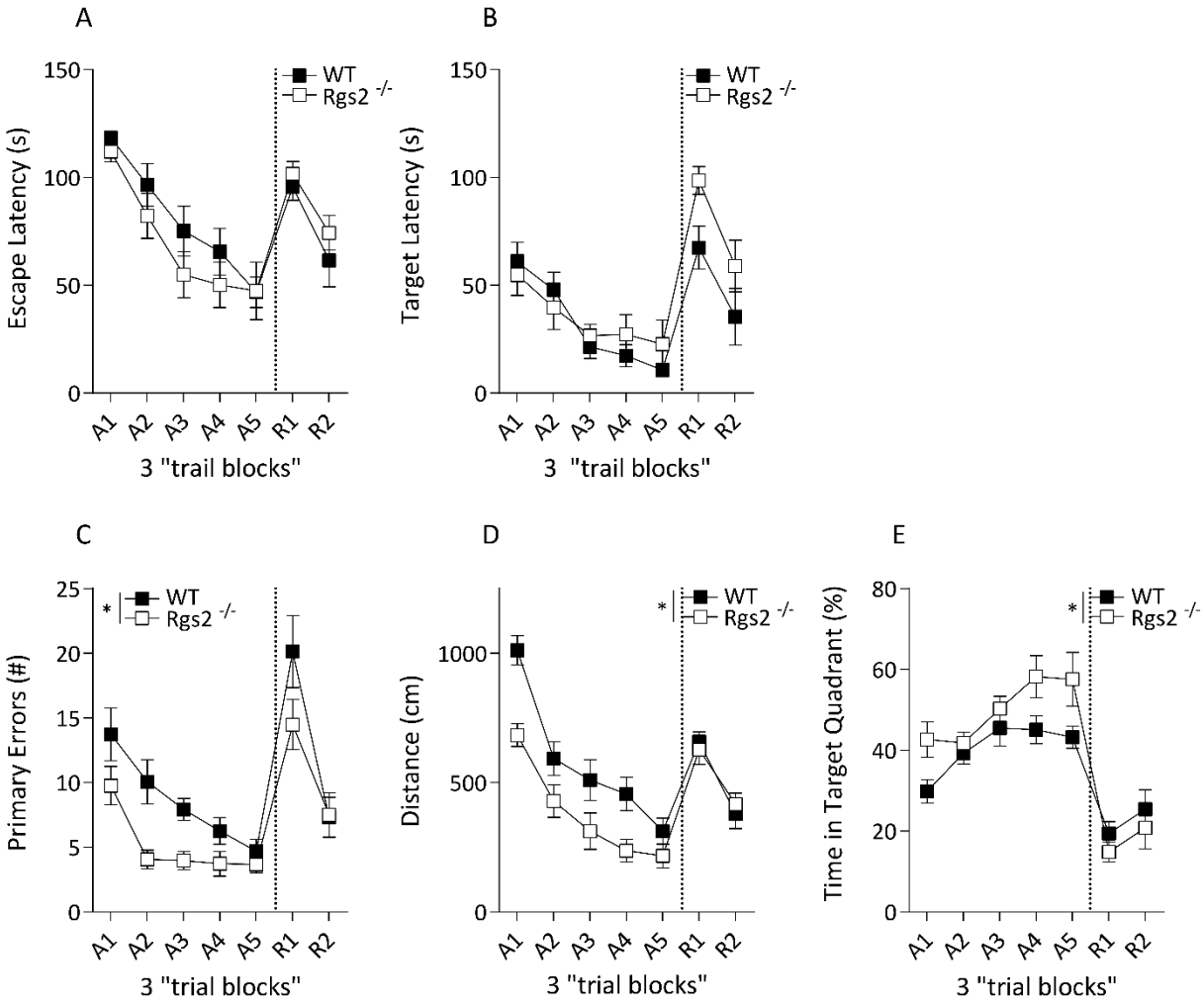


Figure 21: Spatial learning in female mice

Mice were tested in the Barnes Maze for spatial learning. (A) Time course of escape latency, (B) time course of target latency, (C) time course of number of primary errors, (D) time course of distance travelled and (E) time course of relative time spent in the target quadrant. A1-A5 illustrate the time course of the acquisition phase in 3 trial blocks, R1 and R2 the reversal phase. Data are mean ± SEM, n=10/genotype, WT are depicted in black squares, *Rgs2*$^{-/-}$ are depicted in white squares. * indicates $p<0.05$ in ANOVA main effects, $^{+}$ indicates $p<0.05$ in Bonferroni's post hoc test.

During the acquisition phase (A1-A5), female mice of both genotypes acquired the spatial learning task as shown by a time-dependent reduction of escape latency, target latency, primary errors and distance traveled (Figure 21A-D), as well as, increased time in the correct target quadrant (Figure 21E). However, female *Rgs2*$^{-/-}$ mice reached the target hole with significantly less primary errors (Figure 21C). Additionally, female *Rgs2*$^{-/-}$ mice traveled significantly shorter distances on the maze until escaping into the target hole (Figure 21D) and spent more time in the correct target quadrant (Figure 21E). These results indicated increased spatial memory of female *Rgs2*$^{-/-}$ mice.

Upon switching the target hole to the opposite hole of the maze (reversal phase, R1-R2), the escape and target latencies, number of primary errors and distance of both genotypes

transiently increased. Both genotypes relearned the new location of the correct hole. In the reversal phase, cognitive flexibility was comparable between female *Rgs2*$^{-/-}$ and WT mice.

Table 3: ANOVA results for spatial learning

		male		female	
	effect	**$F_{(4;76)/(1;19)}$**	**significance**	**$F_{(4;72)/(1.18)}$**	**significance**
Escape latency (s)	Genotype x time	3.305	**p < 0.05**	0.4867	p = 0.7454
	Genotype	8.094	**p < 0.05**	1.637	p = 0.217
	Time	6.575	**p < 0.0001**	21.50	**p < 0.0001**
Target latency (s)	Genotype x time	0.6455	p = 0.6317	1.043	p = 0.3912
	Genotype	0.1434	p = 0.7091	0.105	p = 0.7496
	Time	2.673	**p < 0.05**	14.75	**p < 0.0001**
Primary errors (#)	Genotype x time	1.056	p = 0.3840	1.303	p = 0.2772
	Genotype	2.681	p = 0.1180	16.37	**p < 0.001**
	Time	9.923	**p < 0.0001**	13.45	**p < 0.001**
Distance (cm)	Genotype x time	5.744	**p < 0.001**	1.29	P = 0.282
	Genotype	0.4343	p = 0.5178	15.79	**p < 0.001**
	Time	27.83	**p < 0.0001**	36.26	**p < 0.0001**
Time in target quadrant (%)	Genotype x time	0.8127	p = 0.5209	1.317	P = 0.2718
	Genotype	0.1483	p = 0.7044	6.344	**p < 0.05**
	Time	8.784	**p < 0.0001**	7.872	**p < 0.0001**

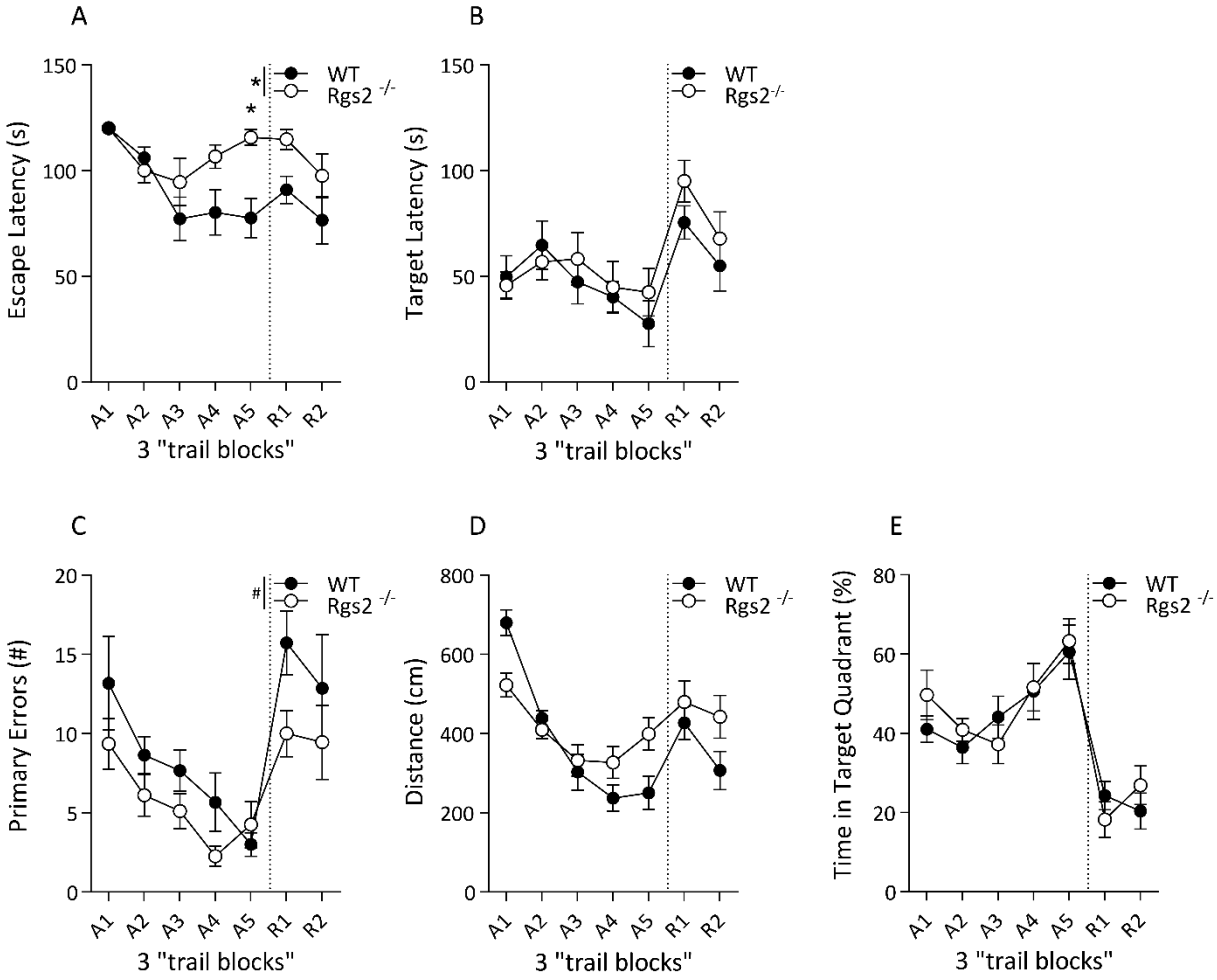


Figure 22: Spatial learning in male mice

Mice were tested in the Barnes Maze for spatial learning. (A) Time course of the escape latency, (B) time course of target latency, (C) time course of number of primary errors, (D) time course of distance travelled and (E) time course of relative time spent in the target quadrant. A1-A5 illustrate the time course of the acquisition phase in 3 trial blocks, R1 and R2 the reversal phase. Data are mean ± SEM, n=10/genotype, WT are depicted in black circles, *Rgs2*$^{-/-}$ are depicted in white circles. * indicates $p<0.05$ in ANOVA main effects, $^{+}$ indicates $p<0.05$ in Bonferroni's post hoc test.

Male mice acquired the spatial learning task, as indicated by a time dependent decrease of target latency, primary errors, distance and escape latency (Figure 22A-D) and an increase of time spent in the correct target quadrant (Figure 22E). While the escape latencies were comparable between genotypes at the acquisition trial blocks A1-A4, WT mice required less time to escape into the target hole at A5, compared to *Rgs2*$^{-/-}$ mice (Figure 22A). Conversely, *Rgs2*$^{-/-}$ mice showed a trend for less primary errors compared to WT mice (Figure 22C). Target latencies and time in the correct target quadrant were similar between genotypes (Figure 22B and E). These results suggest mildly increased spatial memory in male *Rgs2*$^{-/-}$ mice.

During the reversal phase, target latencies, number of primary errors and distance of both genotypes were transiently increased and both genotypes learned to locate the new position of the escape chamber. However, there was no difference in learning behavior between male

WT and *Rgs2*$^{-/-}$ mice in the reversal phase. This indicates comparable cognitive flexibility of male *Rgs2*$^{-/-}$ and WT mice.

Taken together, *Rgs2*$^{-/-}$ mice exhibit increased spatial learning, rendering increased learning not specific for aversive emotional learning. This effect is pronounced in female *Rgs2*$^{-/-}$ mice, whereas in male *Rgs2*$^{-/-}$ mice the effect is mild. Cognitive flexibility was not altered in *Rgs2*$^{-/-}$ mice.

5.2.3 Reward learning and memory

To further test the hypothesis, that increased learning in *Rgs2*$^{-/-}$ mice is specific for aversive emotional fear related learning, a reward motivated spatial learning task, a place preference paradigm in the IntelliCage was used. Since increased learning in the Barnes Maze was mild and not as clear in male *Rgs2*$^{-/-}$ mice, only male mice were tested in the IntelliCage apparatus. Ten male mice per genotype, housed in mixed genotype groups since weaning, were tested in two IntelliCages. Since the IntelliCage apparatus tests learning in a homecage environment, stress due to handling and novel environments was minimized.

The place preference paradigm assesses the ability to associate a rewarding experience (access to a water bottle) with a spatial location at one of four corners in the IntelliCage (assigned corner). A door at each corner prevents free access to water bottles. To open these doors, mice have to perform a “nosepoke” registered by a light beam sensor to open the door, resulting in a 7s drinking period after which the door closes again. The main measure is the relative number of “incorrect nosepokes” to gain access to a water bottle in the three corners not assigned. The hypothesis was that *Rgs2*$^{-/-}$ mice to show comparable reward motivated spatial learning, making an equivalent number of “incorrect nosepokes”.

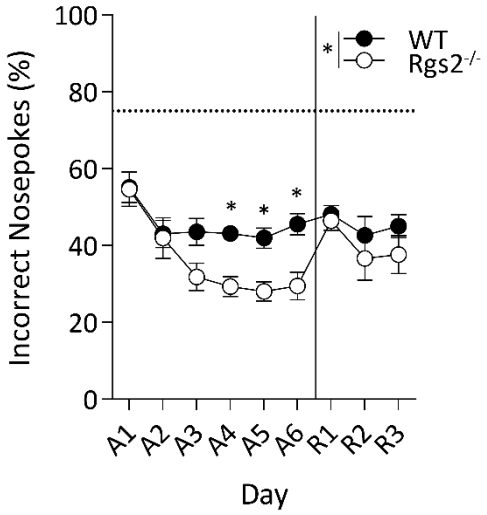


Figure 23: Place preference learning

Male mice were tested for reward motivated spatial learning using an IntelliCage apparatus. A1-A6 show the time course of relative incorrect nosepokes during of the 6-day place preference phase, R1-R3 show the time course of relative incorrect nosepokes during the 3-day reversal phase. The dotted line indicates the 75% random level. Data are mean ± SEM, n=10/genotype, WT are depicted in black circles, *Rgs2*$^{-/-}$ are depicted in white circles. * indicates p<0.05 in ANOVA main effects, $^{+}$ indicates p<0.05 in Bonferroni's post hoc test.

Both genotypes achieved nosepoke error rates below the 75% random level and showed improved error rates over the 6-day testing period, indicating successful acquisition of the learning task. While incorrect nosepokes did not differ between genotypes on days 1-3, *Rgs2*$^{-/-}$ mice made less incorrect nosepokes at days 4-6 compared to WT (Figure 23A), indicating increased learning of male *Rgs2*$^{-/-}$ mice. Upon switching the reward corner to the opposite side (place preference reversal), both genotypes again acquired the learning task over the 3-day testing period again with error rates comparable between genotypes (Figure 23B), indicating similar cognitive flexibility.

Taken together, results did not confirm the hypothesis that increased learning upon deletion of *Rgs2* is specific for emotional aversive paradigms, as it was also increased in the tested reward learning paradigm. Consistent with results of the Barnes Maze, cognitive flexibility was not enhanced.

Table 4: ANOVA results for place preference learning

	effect	**$F_{(5;85)/(1;17)}$**	**significance**
Incorrect nosepokes (#) A1-A6	Genotype x time	2.169	p = 0.065
	Genotype	14.15	**p < 0.01**
	Time	10.01	**p < 0.0001**
Incorrect nosepokes (#) R1-R3	Genotype x time	0.4899	p = 0.617
	Genotype	1.109	p = 0.3071
	Time	3.586	**p < 0.05**

5.3 Acute stress and its impact on innate anxiety

Acute stress can potentiate anxious behavior and induce fear generalization or exacerbate anxiety-like behavior (Grillon, Duncko et al. 2007, Greenwood, Thompson et al. 2014, Vanderheyden, George et al. 2015). Previous reports suggest RGS2 to modulate innate anxiety in humans and mice (Oliveira-Dos-Santos, Matsumoto et al. 2000, Leygraf, Hohoff et al. 2006, Lifschytz, Broner et al. 2012, Stein, Keshaviah et al. 2014). It was therefore investigated, whether deleting *Rgs2* impacts anxiety-like behavior and whether stress elicited by fear conditioning (FC) potentiates anxiety-like behavior in *Rgs2*$^{-/-}$ and WT mice. The role of *Rgs2* in innate anxiety was assessed using three tests based on the approach-avoidance conflict between exploring a novel environment and the aversive properties of a novel surrounding (EPM, DLB and OF). The impact of FC stress on innate anxiety was assessed using the same three tests, after mice had been subjected to the FC paradigm. The first part of the hypotheses was, that deletion of *Rgs2* increases anxiety-like behavior, and the second hypothesis was, that FC stress potentiates anxiety-like behavior more strongly in *Rgs2*$^{-/-}$ mice than in WT mice due to higher stress susceptibility.

5.3.1 Elevated Plus Maze

The Elevated Plus Maze Test elicits an approach avoidance conflict between the open and closed arms of the maze. The closed arms are surrounded by high black walls, have low illumination and represent the saver less aversive part of the maze. The open arms are brightly illuminated and reveal the elevation of the maze above ground, thereby representing the more aversive part of the maze. The assessed parameters were (I) the relative time spent on the open arms, (II) the number of times the mouse enters the open arms (open arm entries) and (III) the total distance traveled on the maze. Decreased relative time spent on the open arms and decreased open arm entries indicate increased anxiety-like behavior. Decreased total distance traveled on the maze indicates novelty-induced locomotion reflecting anxiety-like behavior. *Rgs2*$^{-/-}$ mice were expected to show increased innate anxiety as well as a stronger reaction to FC-induced stress further increasing anxiety-like behavior.

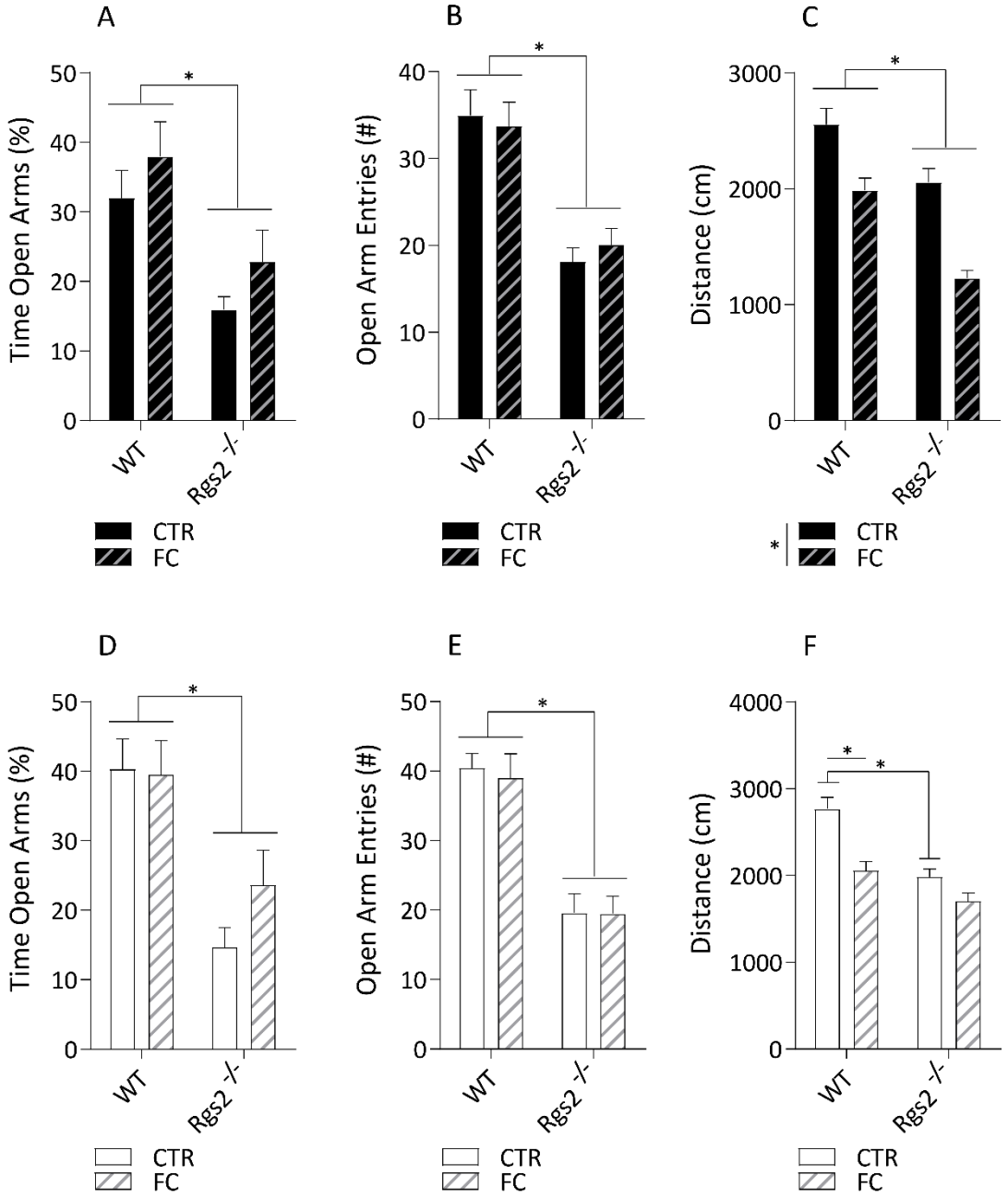


Figure 24: Elevated Plus Maze upon acute stress

Mice were tested in the Elevated Plus Maze 24h after exposure to acute fear conditioning stress (FC) or after being kept in their home cage (CTR). (A/D) Illustrated are relative time spent on open arms, (B/E) number of open arm entries and (C/F) total distance traveled. Data are mean ± SEM, n= 16-19/genotype and sex, CTR groups are depicted in plain bars, FC groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects, + indicates $p<0.05$ in Bonferroni's post hoc test.

Male *Rgs2*$^{-/-}$ mice spent less time on the open arms (Figure 24A) and entered the open arms less frequently (Figure 24B). Furthermore, male *Rgs2*$^{-/-}$ mice traveled less distance during the 10-min testing period compared to WT mice. Acute stress upon FC led to a reduced distance traveled in both genotypes (Figure 24C).

Comparable results were observed for female mice. Open arm exploration time was reduced in female *Rgs2*$^{-/-}$ mice (Figure 24D) as well as the number of open arm entries compared to WT (Figure 24E). The total distance traveled was reduced in female *Rgs2*$^{-/-}$ compared to WT. Upon FC stress the total distance traveled was reduced in WT mice compared to WT controls, but not in *Rgs2*$^{-/-}$ mice compared to *Rgs2*$^{-/-}$ controls (Figure 24F).

The reduction of relative time spent on the open arms, number of open arm entries and reduced locomotion suggested increased innate anxiety of male and female *Rgs2*$^{-/-}$ mice. Upon FC stress locomotor activity was reduced indicating heightened cautious behavior suggesting expected fear generalization in male *Rgs2*$^{-/-}$ and WT mice as well as in female WT mice. However, in female *Rgs2*$^{-/-}$ no fear generalization was observed.

Deletion of *Rgs2* increased innate anxiety in the Elevated Plus Maze. FC stress did not affect *Rgs2*$^{-/-}$ mice more strongly than WT mice, on the contrary, deletion of *Rgs2* appeared to prevent fear generalization in female mice.

Table 5: ANOVA results for Elevated Plus Maze upon acute stress

		male		female	
	effect	**$F_{(1;65)}$**	**significance**	**$F_{(1;62)}$**	**significance**
Time open arms (%)	Genotype x FC stress	0,01233	p = 0,9119	1,256	p = 0,2667
	Genotype	16,23	**p = 0,0001**	22,81	**p < 0,0001**
	FC stress	2,759	p = 0,1015	0,8878	p = 0,3497
Open arm entries (#)	Genotype x FC stress	0,4747	p = 0,4933	0,06030	p = 0,8068
	Genotype	43,60	**p < 0,0001**	55,87	**p < 0,0001**
	FC stress	0,02489	p = 0,8751	0,08359	p = 0,7735
Distance (cm)	Genotype x FC stress	1,286	p = 0,2610	3,923	p = 0,0521
	Genotype	30,79	**p < 0,0001**	27,31	**p < 0,0001**
	FC stress	38,23	**p < 0,0001**	20,69	**p < 0,0001**

5.3.2 Dark-Light Exploration

The Dark-Light Exploration Test elicits the approach avoidance conflict between a dark and a lit compartment. The dark compartment is surrounded by high black walls, has low illumination and represents the saver and less aversive part of the Dark-Light Exploration apparatus. The light compartment is brightly illuminated and represents the more aversive part of the Dark-Light Exploration apparatus. The assessed parameters were (I) the time needed to first enter the light compartment (latency time), (II) the relative time spent in the light compartment and (II) the total distance traveled in the Dark-Light Exploration apparatus. Increased latency, decreased relative time spent in the light compartment as well as decreased total distance traveled in the Dark-Light Exploration apparatus indicate increased anxiety-like behavior. *Rgs2*$^{-/-}$ mice were expected to show increased innate anxiety as well as a stronger reaction upon FC stress further increasing anxiety-like behavior.

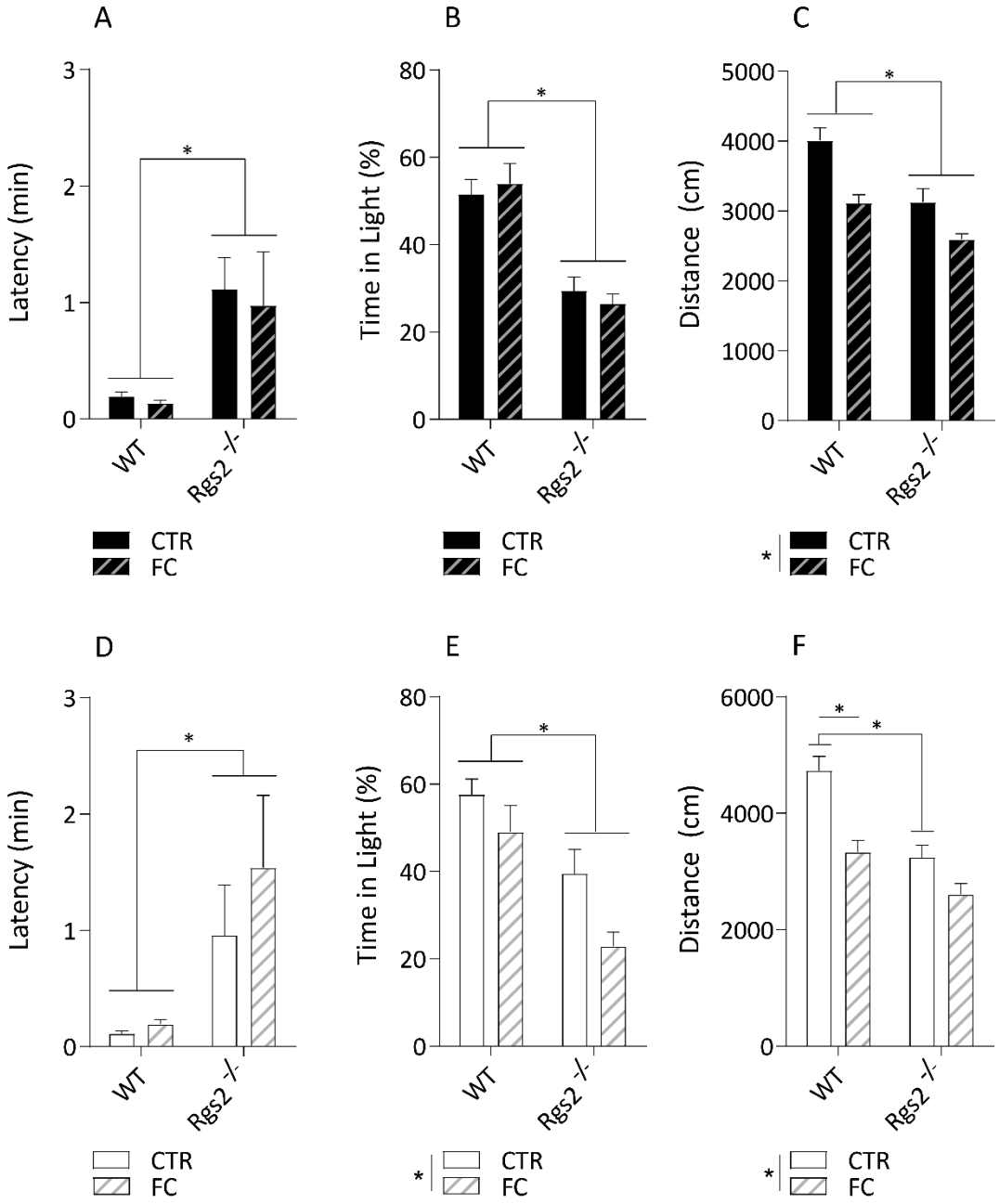


Figure 25: Dark-Light Exploration upon acute stress

Mice were tested in the Dark-Light Exploration Test 3 days after exposure to acute fear conditioning stress (FC) or after being kept in their home cage (CTR). (A, B, C) show data of male mice, (D, E, F) of female mice. (A/D) Illustrated are latency time, (B/E) relative time spent in the light compartment and (C/F) total distance traveled. Data are mean ± SEM, n= 16-19/genotype and sex, CTR groups are depicted in plain bars, FC groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects, + indicates $p<0.05$ in Bonferroni's post hoc test.

Male *Rgs2*$^{-/-}$ mice showed an increased latency time (Figure 25A) and spent less time in the lit compartment compared to WT (Figure 25B). Male *Rgs2*$^{-/-}$ mice traveled less distance during the 10-min testing period, additionally the total distance traveled was reduced for both genotypes upon FC stress (Figure 25C).

Female *Rgs2*$^{-/-}$ mice exhibited an increased latency time and spent less time in the lit compartment was observed compared to WT (Figure 25D-E). Additionally, female mice of both genotypes spent less time in the lit compartment upon FC stress (Figure 25E). The total distance traveled was reduced among female *Rgs2*$^{-/-}$ mice compared to WT. Moreover, FC stress led to an additional reduction of the distance traveled in WT, but not *Rgs2*$^{-/-}$ mice (Figure 25F).

Results confirm increased innate anxiety in *Rgs2*$^{-/-}$ mice regardless of sex as shown by increased latency times and reduced time spent in the lit compartment. FC stress reduced locomotor activity suggesting fear generalization in male *Rgs2*$^{-/-}$ and WT mice as well as in female WT mice as expected, comparable to results obtained for the Elevated Plus Maze. However, this effect was not observed in female *Rgs2*$^{-/-}$ mice.

Taken together, deletion of *Rgs2* increased innate anxiety in the Dark-Light Exploration Test. FC stress did not show a stronger effect on *Rgs2*$^{-/-}$ mice than WT mice. Contrary, *Rgs2* deletion may rather prevent fear generalization in female mice.

Table 6: ANOVA results for Dark-Light Exploration upon acute stress

		male		female	
	effect	**$F_{(1;66)}$**	**significance**	**$F_{(1;65)}$**	**significance**
Latency (min)	Genotype x FC stress	0,02248	p = 0,8813	0,4423	p = 0,5083
	Genotype	10,95	**p = 0,0015**	8,567	**p = 0,0047**
	FC stress	0,1437	p = 0,7059	0,7834	p = 0,3794
Time in light (min)	Genotype x FC stress	0,5974	p = 0,4423	0,7540	p = 0,3885
	Genotype	49,73	**p < 0,0001**	22,89	**p < 0,0001**
	FC stress	0,005946	p = 0,9388	7,401	**p = 0,0084**
Distance (cm)	Genotype x FC stress	1,440	p = 0,2344	3,194	p = 0,0786
	Genotype	21,16	**p < 0,0001**	27,00	**p < 0,0001**
	FC stress	22,25	**p < 0,0001**	22,95	**p < 0,0001**

5.3.3 Open Field Locomotion

The Open Field Test elicits the approach avoidance conflict between the brightly lit center of the Open Field and thigmotaxis in the corners and walls of the Open Field. Thigmotactic movement and wall proximity represents the less aversive option, while movement in the brightly illuminated center of the Open Field is more aversive. The assessed parameters are (I) the relative time spent in the center and (II) the total distance traveled in the Open Field. Decreased relative time spent in the center as well as decreased total distance traveled in the Open Field indicate increased anxiety-like behavior. *Rgs2*$^{-/-}$ mice were expected to show increased innate anxiety as well as a stronger reaction upon FC stress further increasing anxiety-like behavior.

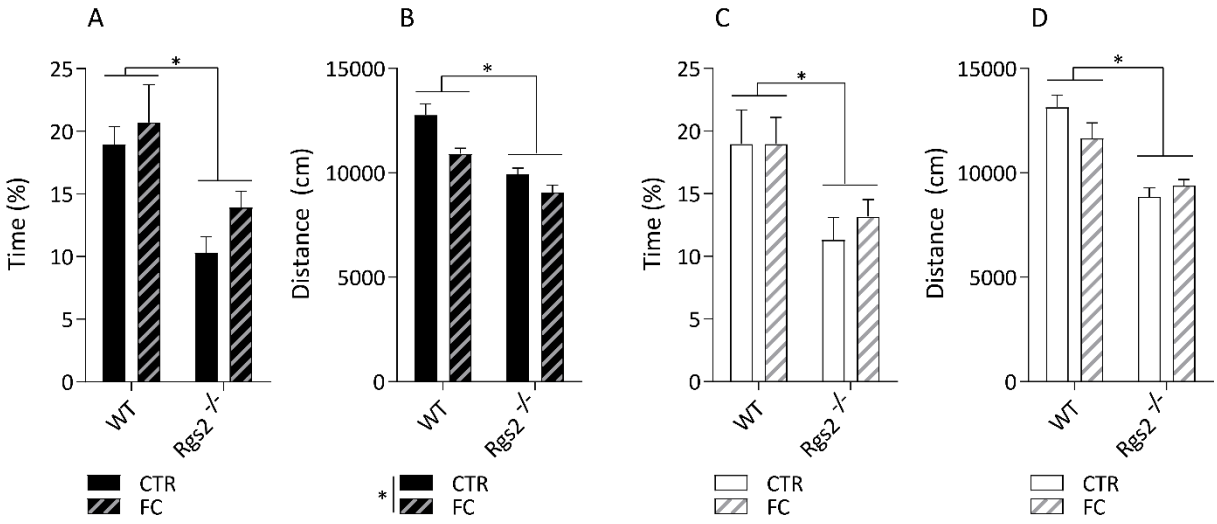


Figure 26: Open Field Locomotion upon acute stress

Mice were tested in the Open Field Locomotion Test 5 days after exposure to acute fear conditioning stress (FC) or after being kept in their home cage (CTR). (A, B) show data of male mice, (C, D) of female mice. (A/C) Illustrated are relative time spent in the center of the Open Field, (B/D) total distance traveled. Data are mean ± SEM, n= 16-19/genotype and sex, CTR groups are depicted in plain bars, FC groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects.

Male *Rgs2*$^{-/-}$ mice spent less time in the center of the Open Field (Figure 26A) and the total distance traveled was reduced compared to WT controls (Figure 26B) Upon FC stress, total distance traveled of both genotypes was further reduced (Figure 26B).

Female *Rgs2*$^{-/-}$ mice spent less time in the center of the Open Field (Figure 26C) and traveled less total distance compared to WT controls (Figure 26D). FC stress had no effect on female mice of both genotypes.

Open Field Test results confirmed increased innate anxiety in *Rgs2*$^{-/-}$ mice regardless of as sex shown by a decreased center time and decreased activity. Reduced locomotor activity upon FC confirmed fear generalization in male *Rgs2*$^{-/-}$ and WT mice. However, this effect was not observed in female *Rgs2*$^{-/-}$ and WT mice.

Rgs2 deletion thus increases innate anxiety in the Open Field Test. FC stress did not affect *Rgs2*$^{-/-}$ mice more strongly than WT mice, as *Rgs2* deletion may rather prevent fear generalization in female mice.

Table 7: ANOVA results for Open Field Locomotion upon acute stress

		male		female	
	effect	**$F_{(1;63)}$**	**significance**	**$F_{(1;67)}$**	**significance**
Center time (%)	Genotype x FC stress	0,2813	p = 0,5977	0,2086	p = 0,6493
	Genotype	19,29	**p < 0,0001**	10,89	**p = 0,0015**
	FC stress	2,307	p = 0,1338	0,2093	p = 0,6488
Distance (cm)	Genotype x FC stress	1,588	p = 0,2123	3,453	p = 0,0675
	Genotype	36,78	**p < 0,0001**	36,48	**p < 0,0001**
	FC stress	12,48	**p = 0,0008**	0,7629	p = 0,3855

5.4 Chronic stress and its impact on anxiety and depressive behavior

Anxiety disorders and depressive disorders are common comorbidities (Judd, Kessler et al. 1998, Brown, Campbell et al. 2001, Kessler, Berglund et al. 2003). Previous reports suggested *Rgs2* to modulate depression-like behavior in mice (Lifschytz, Broner et al. 2012). Whether deletion of *Rgs2* increases the susceptibility to stress-induced depression-like behavior in rodents has not been tested yet. Therefore, mice were subjected to the chronic mild stress (CMS) paradigm to provoke depression-like behavior (Katz 1981, Willner, Towell et al. 1987, Monleon, D'Aquila et al. 1995, Willner 2005). The severity of depression-like symptoms in mice such as reward behavior (anhedonic behavior) (Griffiths, Shanks et al. 1992, Harkin, Houlihan et al. 2002, Ducottet and Belzung 2004, Pothion, Bizot et al. 2004), disturbances in social behavior and behavioral despair was evaluated after 3 weeks of CMS (Czeh, Fuchs et al. 2016). Additionally, anxiety-like behavior was assessed to quantify a potential induction of a comorbidity of depression (Czeh, Fuchs et al. 2016).

Sucrose Preference and food intake were used to assess anhedonic behavior. The Forced Swim Test was used to evaluate behavioral despair, the Social Interaction Test to investigate social behavior and the Dark-Light Exploration Test to assess anxiety-like behavior.

5.4.1 Sucrose Preference and Food Consumption

The core symptom of human depression - depressed mood and loss of interest in pleasurable activities - are modeled in mice using the behavioral endophenotype of anhedonic behavior (Czeh, Fuchs et al. 2016). Anhedonic behavior was assessed using Sucrose Preference and food intake one week prior to and in the 3rd week of CMS. Reduced Sucrose Preference and reduced food intake indicate increased anhedonic behavior. The hypothesis was, that upon CMS, anhedonic-like behavior increases more strongly in *Rgs2*$^{-/-}$ mice compared to WT due to a possibly increased susceptibility to chronic stress.

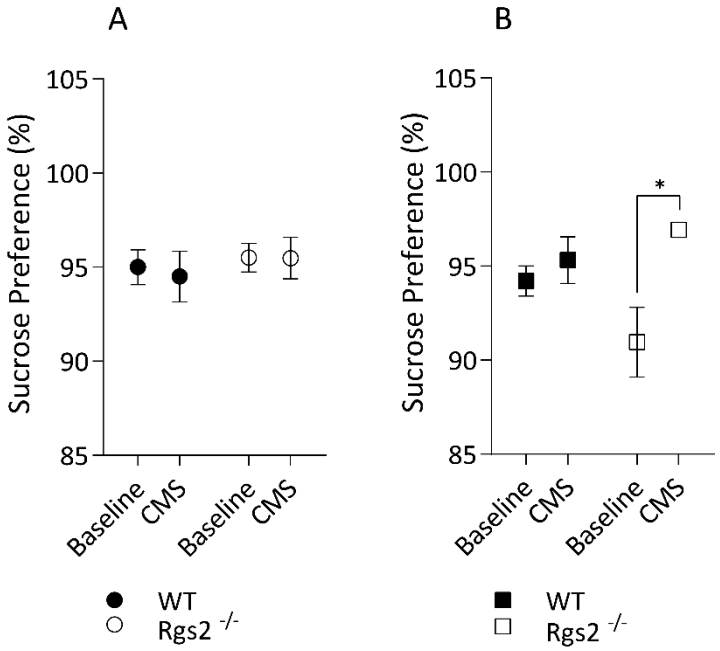


Figure 27: Sucrose Preference measurements

Time course of relative sucrose intake, illustrated are baseline levels and levels after three weeks of CMS. Data are mean ± SEM, n=5-6 cages/genotype and sex, WT male are depicted in black circles, *Rgs2*$^{-/-}$ male are depicted in white circles. WT female are depicted in black squares, *Rgs2*$^{-/-}$ female are depicted in white squares * indicates p<0.05 in ANOVA main effects, $^{+}$ indicates p<0.05 in Bonferroni's post hoc test.

Sucrose preference in male mice was comparable for both genotypes as well as CMS groups and control groups (Figure 27A). However, female *Rgs2*$^{-/-}$ mice showed increased sucrose preference upon CMS, whereas female WT mice exhibited no alteration in sucrose preference upon CMS (Figure 27B).

Table 8: ANOVA results for Sucrose Preference

		male		female	
	effect	**$F_{(1;10)}$**	**significance**	**$F_{(1;10)}$**	**significance**
Sucrose preference (%)	Genotype x CMS	0.05	p = 0.8280	6.85	**p < 0.05**
	Genotype	0.4533	p = 0.5177	0.3389	p = 0.5734
	CMS	0.05875	p = 0.8139	14.57	**p < 0.05**

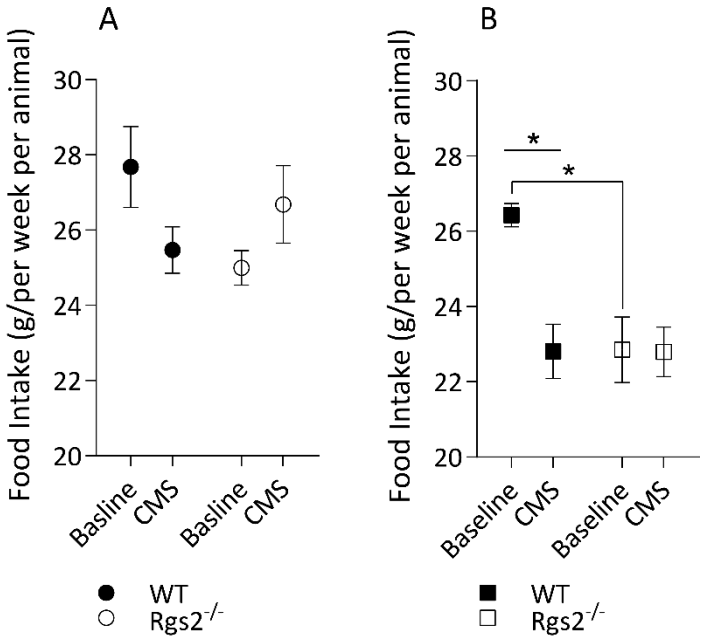


Figure 28: Food Intake measurements

Time course of food intake, illustrated are baseline levels and levels upon three weeks of CMS. Data are mean ± SEM, n=5-6 cages/genotype and sex, WT male are depicted in black circles, *Rgs2*$^{-/-}$ male are depicted in white circles. WT female are depicted in black squares, *Rgs2*$^{-/-}$ female are depicted in white squares * indicates p<0.05 in ANOVA main effects, $^{+}$ indicates p<0.05 in Bonferroni's post hoc test.

Upon CMS, food intake was mildly reduced in male WT mice but increased in male *Rgs2*$^{-/-}$ mice (Figure 28A). Female WT mice ate less food after 3 weeks of CMS. CMS had no impact on food consumption of female *Rgs2*$^{-/-}$ mice (Figure 28B).

Contrary than expected, CMS provoked anhedonic behavior in WT mice but not in *Rgs2*$^{-/-}$ mice as shown by reduced food intake. Furthermore, food intake measurements prior to CMS indicate anhedonic behavior in *Rgs2*$^{-/-}$ mice at baseline. Interestingly, this behavior was not intensified by CMS but reduced after 3 weeks of CMS. Taken together, anhedonic behavior is triggered by *Rgs2* deletion, but not intensified by chronic stress upon deletion of *Rgs2*.

Table 9: ANOVA results for food intake

		male		female	
	effect	**$F_{(1;10)}$**	**significance**	**$F_{(1;10)}$**	**significance**
Food intake	Genotype x CMS	5.329	**p < 0.05**	7.448	**p < 0.01**
	Genotype	0.7693	p = 0.386	7.537	**p < 0.01**
	CMS	0.09652	p = 0.7577	7.9	**p < 0.01**

5.4.2 Dark-Light Exploration

Increased anxiety-like behavior is a common comorbidity of depression (Czeh, Fuchs et al. 2016). *Rgs2*$^{-/-}$ mice show increased innate anxiety (see 5.3) and CMS has been reported to robustly cause anxiety-like behavior in rodents using various tests including the Dark-Light Exploration Test (Ma, Jiang et al. 2011, Jung, Hong et al. 2014, Zhu, Wang et al. 2014). To evaluate whether anxiety-like behavior is provoked in WT mice and further intensified in *Rgs2*$^{-/-}$ mice upon CMS, in line with the hypothesis of increased stress susceptibility of *Rgs2*$^{-/-}$ mice, mice were tested in the Dark-Light Exploration Test after 3 weeks of chronic mild stress exposure (see 5.3.2).

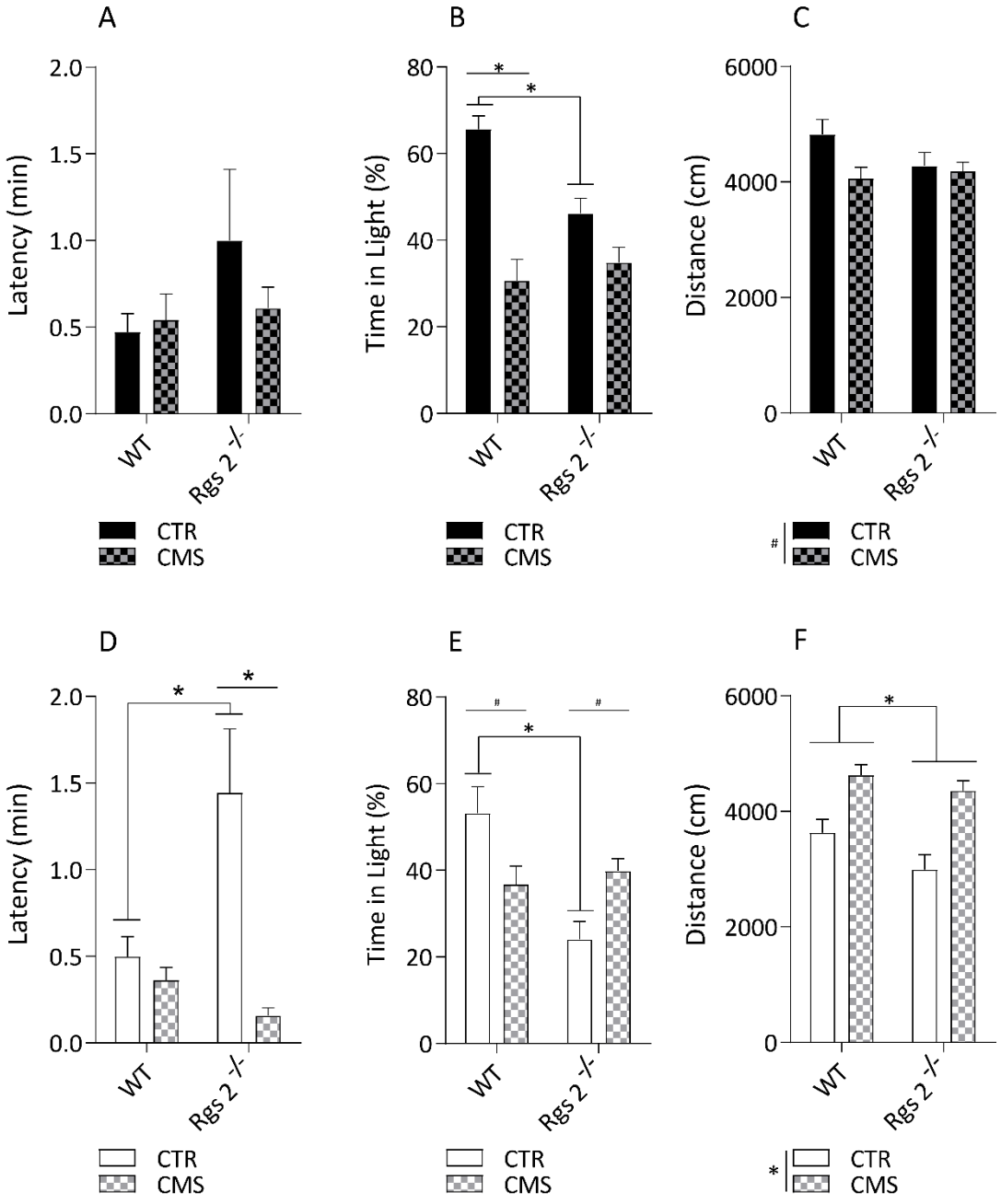


Figure 29: Dark-Light Exploration upon Chronic Mild Stress

Mice were tested in the Dark-Light Exploration Test 24 hours after exposure to Chronic Mild Stress (CMS) or after being kept in their home cage (CTR). (A/D) Illustrate are latency time, (B/E) relative time spent in the light compartment and (C/F) total distance traveled. Data are mean ± SEM, n= 10/genotype and sex, CTR groups are depicted in plain bars, CMS groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects, + indicates $p<0.05$ in Bonferroni's post hoc test.

Latency time was comparable between genotype and stress groups in male mice (Figure 29A). But, as illustrated in Figure 29B male control *Rgs2*$^{-/-}$ mice spent less time in the lit compartment compared to control WT mice, while there was no such difference after CMS. CMS led to a reduction of time spent in the lit compartment in WT mice. The total distance traveled of both genotypes was marginally reduced after CMS (Figure 29C).

Female control *Rgs2*$^{-/-}$ mice showed increased latency time compared to control WT mice, while there was no such difference after CMS. CMS led to a reduction of latency time in *Rgs2*$^{-/-}$ mice (Figure 29D). Control *Rgs2*$^{-/-}$ mice spent less time in the lit compartment compared to WT mice, while there was also no such difference after CMS (Figure 29E). While WT mice spent less time in the lit compartment upon CMS, *Rgs2*$^{-/-}$ spent more time in the lit compartment. As illustrated in Figure 29F, *Rgs2*$^{-/-}$ mice traveled less distance compared to WT mice during the 10-min testing period. CMS led to an increase of the total distance traveled for both genotypes.

CMS induced anxiety-like behavior in male and female WT mice as shown by increased latency times and less time spent in the lit compartment upon CMS. In *Rgs2*$^{-/-}$mice CMS had a sex-specific effect. While in male *Rgs2*$^{-/-}$ mice anxiety-like behavior was unaltered upon CMS, female *Rgs2*$^{-/-}$ mice experienced an anxiolytic effect reducing latency time and increasing time in the lit compartment upon CMS.

Results corroborate promotion of anxiety-like behavior by CMS, however this effect was only present in WT mice. CMS did not affect *Rgs2*$^{-/-}$ mice more strongly than WT mice; contrary male *Rgs2*$^{-/-}$ mice were unaffected while female *Rgs2*$^{-/-}$ mice rather showed anxiolysis upon CMS.

Table 10: ANOVA results for Dark-Light Exploration upon CMS

		male		female	
	effect	**$F_{(1;40)}$**	**significance**	**$F_{(1;40)}$**	**significance**
Latency (min)	Genotype x CMS	0.733	p = 0.397	9.999	**p < 0.01**
	Genotype	1.277	p = 0.2653	4.15	**p < 0.05**
	CMS	0.4821	p = 0.4915	15.40	**p < 0.001**
Time in light (min)	Genotype x CMS	8.557	**p < 0.01**	13.55	**p < 0.001**
	Genotype	3.557	p = 0.0664	8.883	**p < 0.01**
	CMS	32.79	**p < 0.0001**	0.005	p = 0.9429
Distance (cm)	Genotype x CMS	2.365	p = 0.1318	0.7986	p = 0.3769
	Genotype	0.943	p = 0.3372	4.725	**p < 0.05**
	CMS	3.847	p = 0.0567	31.6	**p < 0.0001**

5.4.3 Social Interaction

Dysfunctional social behavior is a symptom of depression in humans and can be modeled in mice using the Social Interaction Test (Czeh, Fuchs et al. 2016), thereby evaluating social anxiety, social motivation and affiliation as well as social memory (Kaidanovich-Beilin, Lipina et al. 2011). It has been shown, that CMS can impact social behavior (Otsuka, Shiuchi et al. 2015, Gross and Pinhasov 2016) and reported, that *Rgs2*$^{-/-}$ mice show disrupted social behavior (see 1.3.3.1.2). To evaluate whether social behavior is disrupted upon CMS due to increased stress susceptibility of *Rgs2*$^{-/-}$ mice, mice were tested in Crawley`s three chamber sociability and preference for social novelty test. This test assesses social behavior of mice in two phases. In phase I, sociability as an indicator for social affiliation and motivation is evaluated. “Sociability” is defined as the inclination to spent time with another mouse as opposed to staying alone in an empty chamber. In phase II, preference for social novelty indicative of intact social memory and novelty seeking is assessed. “Preference for social novelty” is defined as the inclination to spent time with a novel mouse as opposed to a familiar mouse. Mice with functional social behavior show sociability and preference for social novelty.

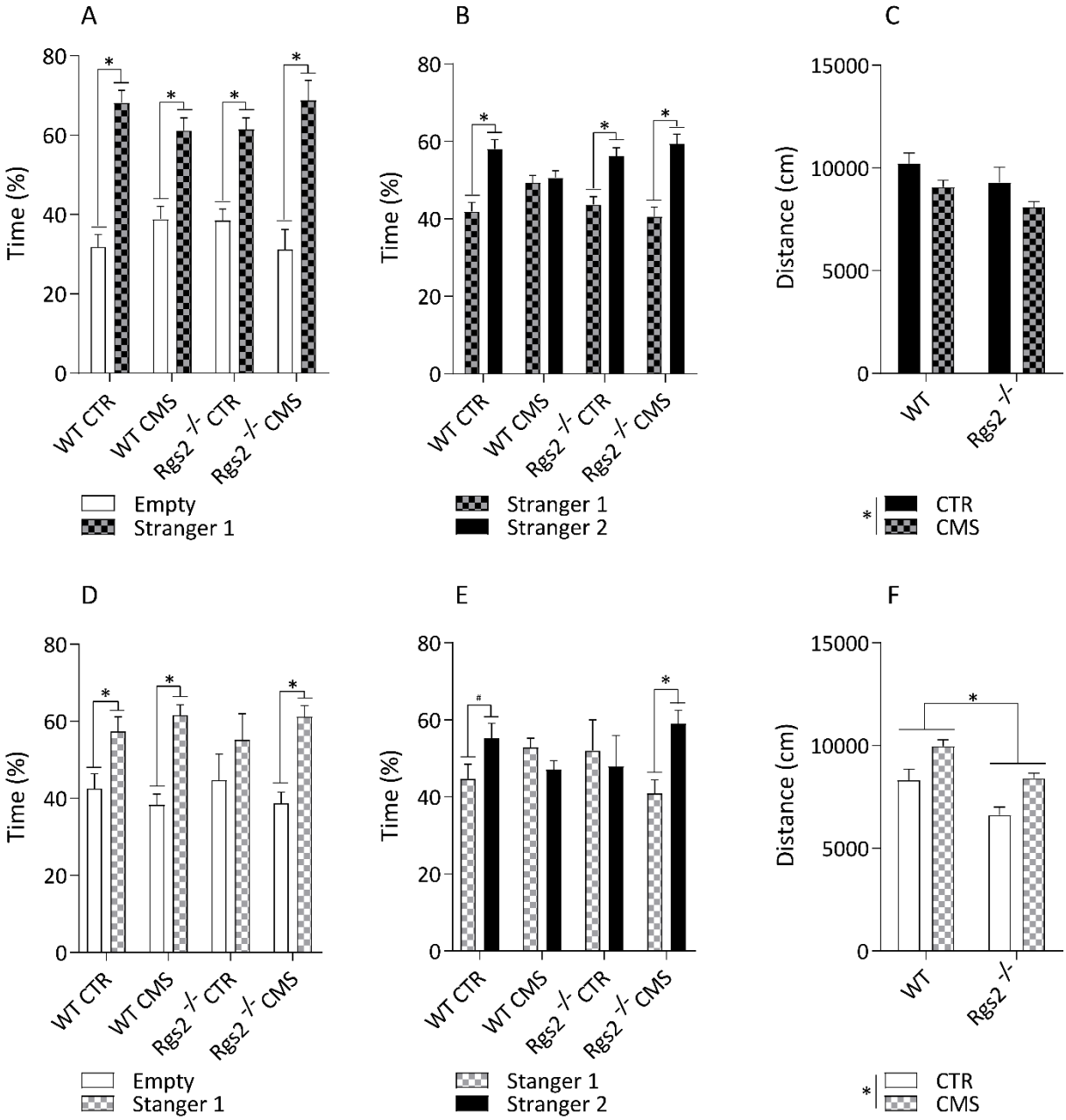


Figure 30: Social Interaction Test upon Chronic Mild Stress

Mice were tested in the Social Interaction Test 4 days after exposure to Chronic Mild Stress (CMS) or after being kept in their home cage (CTR). (A/D) Illustrated are sociability results relative time spent with a stranger mouse as opposed to an empty compartment, (B/E) social novelty results relative time spent with a novel stranger as opposed to a familiar mouse (C/F) total distance traveled. Data are mean ± SEM, n= 10/genotype and sex, CTR groups are depicted in plain bars, CMS groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects, + indicates $p<0.05$ in Bonferroni's post hoc test.

During phase I of the Social Interaction Test, male mice consistently preferred the compartment with stranger 1 mouse present, regardless of genotype or CMS (Figure 30A). During phase II of the SI test, WT control mice stayed longer in the compartment with a second new stranger 2 mouse. WT CMS mice exhibited no preference for either stranger 1 or 2. *Rgs2*$^{-/-}$ control and CMS mice showed preference for stranger 2 (Figure 30B). Consistent with

the findings in DLB, CMS led to a reduction of distance traveled by male mice of both genotypes (Figure 30C).

Female WT mice spent increased time with stranger 1 compared to the empty compartment in Phase I, regardless of CMS. *Rgs2*$^{-/-}$ control mice did not exhibit this preference, however, *Rgs2*$^{-/-}$ CMS mice did (Figure 30D). In phase II of the test, WT control mice showed preference for stranger 2, while WT CMS mice did not. *Rgs2*$^{-/-}$ control mice showed no preference for stranger 2, while *Rgs2*$^{-/-}$ CMS mice did (Figure 30E). In female mice of both genotypes CMS lead to an increase in activity indicated by increased total distance traveled. *Rgs2*$^{-/-}$ mice, irrespective of CMS and in line with findings in DLB, show novelty induced hypo-locomotion compared to WT mice (Figure 30E).

Taken together, these results indicate normal sociability in male and female WT mice, regardless of CMS. Male *Rgs2*$^{-/-}$ mice showed comparable behavior, while sociability behavior was disturbed in female *Rgs2*$^{-/-}$ mice, which was restored upon CMS to the level of functional social behavior. Preference for social novelty was present in male and female WT mice, however, CMS disturbed this preference for social novelty in both sexes. Male *Rgs2*$^{-/-}$ mice showed functional preference for social novelty, regardless of CMS. Preference for social novelty was disturbed in female *Rgs2*$^{-/-}$ mice, however, CMS restored functional behavior.

Results confirmed that CMS elicits dysfunctional social behavior in WT mice, however male *Rgs2*$^{-/-}$ mice again prove not affected by CMS whereas in female *Rgs2*$^{-/-}$ mice showed disturbed social behavior, which was restored upon CMS.

Table 11: ANOVA and T-test results for Social Interaction Test upon CMS

		male		female	
	Group	**T-test**	**significance**	**T-test**	**significance**
Empty vs Stanger 1	WT CTR	$t_{(18)}$=8.216	**p < 0.0001**	$t_{(18)}$=2.789	**p < 0.05**
	WT CMS	$t_{(24)}$=4.985	**p < 0.0001**	$t_{(22)}$=6.017	**p < 0.0001**
	Rgs2$^{-/-}$ CTR	$t_{(22)}$=5.591	**p < 0.0001**	$t_{(18)}$=1.105	ns.
	Rgs2$^{-/-}$ CMS	$t_{(18)}$=5.282	**p < 0.0001**	$t_{(20)}$=5.555	**p < 0.0001**
Stranger 1 vs Stranger 2	WT CTR	$t_{(18)}$=4.76	**p < 0.001**	$t_{(18)}$=1.985	ns.
	WT CMS	$t_{(24)}$=0.4688	ns.	$t_{(22)}$=1.7609	ns.
	Rgs2$^{-/-}$ CTR	$t_{(22)}$=4.307	**p < 0.001**	$t_{(18)}$=0.3555	ns.
	Rgs2$^{-/-}$ CMS	$t_{(18)}$=5.433	**p < 0.0001**	$t_{(20)}$=3.702	**p < 0.01**
		male		**female**	
	effect	**$F_{(1;40)}$**	**significance**	**$F_{(1;40)}$**	**significance**
Distance (cm)	Genotype x CMS	0.0021	p = 0.9632	0.0292	p = 0.8652
	Genotype	3.377	p = 0.0734	19.17	**p < 0.0001**
	CMS	5.182	**p < 0.05**	21.37	**p < 0.0001**

5.4.4 Forced Swim Test

The Forced Swim Test is used to model fatigue or loss of energy using the phenotype of behavioral despair as an indicator of depressive behavior in mice (Czeh, Fuchs et al. 2016). A depressive phenotype has been reported for *Rgs2*$^{-/-}$ mice (see 1.3.3.1.2). To evaluate, whether depressive behavior is provoked in WT and further intensified in *Rgs2*$^{-/-}$ mice upon CMS due to increased stress susceptibility, mice were tested in the Forced Swim Test. The Forced Swim Test exposes mice to an inescapable situation in a glass beaker filled with water. It evaluates the tendency to struggle, get free or escape opposed to the tendency to give up and resign. Two parameters were assessed: (I) the cumulative time spent floating or immobile in the last 4 minutes of the 6-minute testing phase (immobility time) and (II) the time spent struggling until the first floating occurs (latency to float). Increased floating time and decreased latency to float indicate behavioral despair or a depressed phenotype.

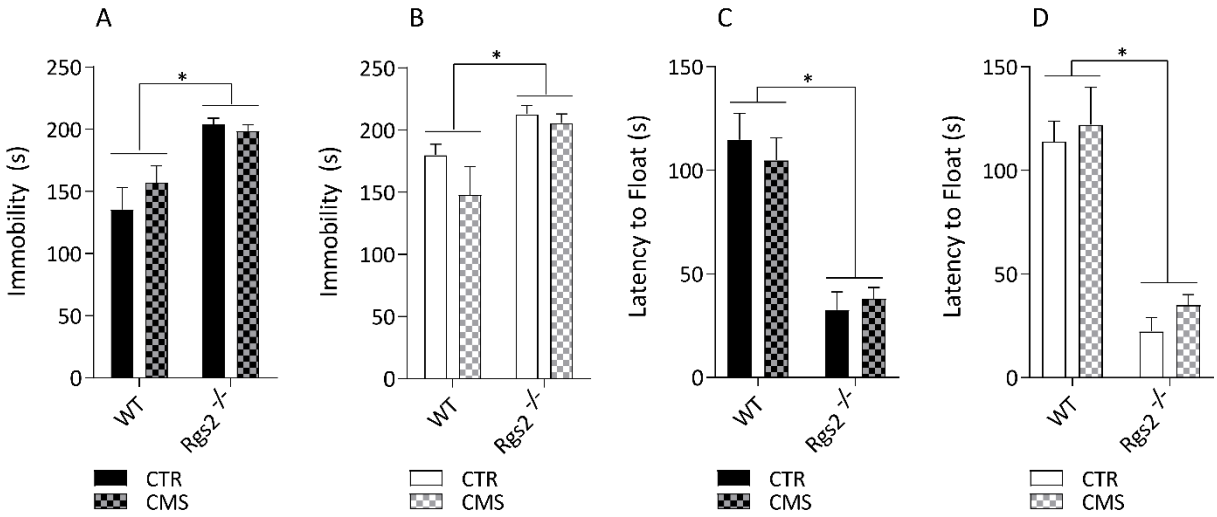


Figure 31: Forced Swim Test upon Chronic Mild Stress

Mice were tested in the Forced Swim Test days after exposure to Chronic Mild Stress (CMS) or after being kept in their home cage (CTR). (A/B) Illustrated are immobility time (cumulative time spent floating or immobile) and (C/D) latency to float (time spent struggling until the first floating). Data are mean ± SEM, n= 10/genotype and sex, CTR groups are depicted in plain bars, CMS groups are depicted in hatched bars, male mice are depicted in black, female mice are depicted in white. * indicates $p<0.05$ in ANOVA main effects.

Male and female *Rgs2*$^{-/-}$ mice showed increased immobility times compared to WT in the Forced Swim Test. (Figure 31A and B). Latencies to float were also reduced for male and female *Rgs2*$^{-/-}$ mice compared to WT (Figure 31C and D). Immobility time and latency to float were independent of CMS for both genotypes and sexes.

These results confirm depression-like behavior in male and female *Rgs2*$^{-/-}$ compared to WT. However, contrary to expectations, CMS had no effect on behavioral despair in either genotype or sex.

Table 12: ANOVA results for Forced Swim Test upon CMS

		male		female	
	effect	**$F_{(1;40)}$**	**significance**	**$F_{(1;40)}$**	**significance**
Immobility (s)	Genotype x CMS	1.331	p = 0.2553	0.7805	p = 0.3832
	Genotype	22.26	**p < 0.0001**	10.55	**p < 0.01**
	CMS	0.4975	p = 0.4846	1.944	p = 0.1709
Latency to float (s)	Genotype x CMS	0.6043	p = 0.4415	0.040	p = 0.8419
	Genotype	57.28	**p < 0.0001**	58.58	**p < 0.0001**
	CMS	0.055	p = 0.8151	0.8017	p = 0.3759

5.4.5 Gene expression analysis

Stimuli triggering neuronal plasticity modulate the mRNA expression level of *Rgs2* (see 5.2.1.3) Whether *Rgs2* mRNA expression is altered by CMS was assessed using quantitative real time PCR. Hippocampal and frontal cortices from WT mice were dissected 5-7 days after the last

behavioral test following CMS paradigme and *Rgs2* mRNA expression was quantified. The hypothesis was that CMS triggers an increase in the mRNA expression level of *Rgs2*.

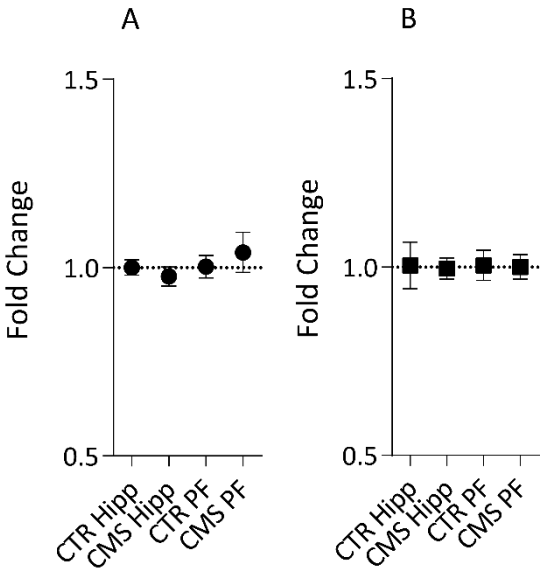


Figure 32: *Rgs2* mRNA expression levels upon Chronic Mild Stress

mRNA expression changes evaluated by quantitative real time PCR in hippocampus and prefrontal cortex upon 3 weeks of Chronic Mild Stress (CMS) compared to control conditions (CTR). (A) *Rgs2* mRNA expression in male mice and(B) *Rgs2* mRNA expression in female mice. Data are mean ± SEM, n=4/genotype, males are depicted in circles, females are depicted in squares. * indicates $p<0.05$ in t-test.

There were no changes of *Rgs2* mRNA expression in hippocampal and prefrontal cortices after CMS (Figure 32A and B) in male or female mice. Results suggest that CMS does not elicit a change in *Rgs2* mRNA expression.

5.5 Cell biological analysis

5.5.1 Neurotransmitter levels

Anxiety disorders and depression are associated with disturbed neurotransmitter systems and have therefore been classified as secondary neurotransmitter disorders (Kurian, Gissen et al. 2011, Ng, Papandreou et al. 2015). These disturbances are most likely due to alterations in pre- and postsynaptic signal transmission and are reflected by corresponding behavioral changes and are therapeutically treated with drugs modulating monoaminergic neurotransmitter systems and (Cassano, Baldini Rossi et al. 2002, Dell'Osso, Buoli et al. 2010, Blier 2013). To investigate the effects of *Rgs2* deletion monoaminergic neurotransmitter system in the brain, neurotransmitter levels in two brain regions implicated in depression, anxiety and fear (frontal cortices and hippocampi) were determined by means of high-performance liquid chromatography (HPLC). In line with observed behavioral changes, reduced monoaminergic neurotransmitter levels were expected. Table 13 summarizes the effects of *Rgs2* deletion on dopamine, serotonin, norepinephrine and their corresponding metabolite quotients in frontal cortex and hippocampus.

Table 13: Effect of *Rgs2* deletion on neurotransmitter levels in frontal cortex and hippocampus
Data are mean neurotransmitter level in ng/g tissue ± SEM, n=6 male mice/genotype.
* indicates p<0.05 in one sample t-tests, p values were not adjusted for multiple testing.

	Neurotransmitter	WT (ng/g)	*Rgs2*$^{-/-}$ (ng/g)	significance
Hippocampus	Dopamine	65.85 ± 5.86	48.08 ± 5.86	$t_{(10)}$ =5.25 **p< 0.001**
	(HVA + DOPAC) / DA	13.16 ± 1.98	16.02 ± 3.01	$t_{(10)}$ =1.94 ns
	Serotonin	480.50 ± 108.90	378.10 ± 43.92	$t_{(10)}$ =2.13 p= 0.0586
	5-HIAA / 5-HT	0.80 ± 0.08	0.98 ± 0.13	$t_{(10)}$ =2.90 **p< 0.05**
	Norepinephrine	223.30 ± 24.96	134.90 ± 15.91	$t_{(10)}$ =7.32 **p< 0.0001**
	MHPG / NA	3.41 ± 0.48	4.78 ± 0.31	$t_{(10)}$ =5.93 **p< 0.001**
Frontal cortex	Dopamine	63.66 ± 16.80	52.89 ± 10.21	$t_{(10)}$ =1.34 ns
	(HVA + DOPAC) / DA	9.71 ± 1.46	9.06 ± 1.65	$t_{(10)}$ =0.71 ns
	Serotonin	267.00 ± 24.53	171.50 ± 9.33	$t_{(10)}$ =8.91 **p< 0.0001**
	5-HIAA / 5-HT	0.56 ± 0.06	0.65 ± 0.06	$t_{(10)}$ =2.77 **p< 0.05**
	Norepinephrine	152.60 ± 5.45	90.28 ± 6.59	$t_{(10)}$ =17.85 **p< 0.0001**
	MHPG / NA	3.24 ± 0.55	4.46 ± 0.33	$t_{(10)}$ =4.66 **p< 0.001**

Dopamine levels were reduced in hippocampal preparations of *Rgs2*$^{-/-}$ mice, but not in the frontal cortex. The ratio between dopamine and its metabolites HVA and DOPAC was comparable for WT and *Rgs2*$^{-/-}$ mice. Concerning serotonin, levels were reduced in the frontal cortex, but not in the hippocampus. However, the ratio of serotonin and its metabolite 5-HIAA was significantly increased in both regions of *Rgs2*$^{-/-}$ mice. Norepinephrine concentrations were significantly decreased in *Rgs2*$^{-/-}$ mice in both regions, whereas ratio of norepinephrine and its metabolite MHPG was significantly increased.

As expected, *Rgs2* deletion leads to changes in neurotransmitter concentrations in both hippocampus and frontal cortex at might contribute to observed behavioral changes.

5.5.2 G protein-coupled receptor expression

RGS proteins regulate the duration of G protein-coupled signaling by accelerating signaling termination (see 1.3.2). The deletion of *Rgs2* may therefore result in altered expression levels of various GPCRs due to regulatory processes such as internalization and degradation, thereby

counteracting prolonged signaling upon deletion of *Rgs2*. Due to the role of *Rgs2* in fear learning, analysis of GPCR expression was focused on the hippocampus and the prefrontal cortex in male mice. Moreover, expression levels were also evaluated in atria and left ventricle of the heart separately, due to reported alterations in the blood pressure control and cardiac hypertrophy in *Rgs2*$^{-/-}$ mice (see 1.3.3.1.2). Therefore, expression changes in GPCRs possibly implicated in anxiety and depression, but also in cardiovascular dysregulation were investigated. The results are illustrated in Table 14.

Table 14: Effect of *Rgs2* deletion on GPCR mRNA expression in frontal cortex, hippocampus, atria and left ventricle

Data are mean fold change ± SEM, n=4-6 male mice/genotype. Grey background indicates p<0.05 in one sample t-tests, p values were not adjusted for multiple testing.

	Brain		**Heart**	
G protein-coupled receptor	Hippocampus	Frontal cortex	Atria	Left ventricle
ADRA2A	0.68 ± 0.11	1.14 ± 0.25	n.a.	n.a.
ADRAB1	n.a.	n.a.	1.08 ± 0.31	0.77 ± 0.14
ADRAB2	n.a.	n.a.	1.05 ± 0.36	0.87 ± 0.10
CCK	1.06 ± 0.26	0.92 ± 0.13	n.a.	n.a.
CCKAR	n.a.	n.a.	n.a.	n.a.
CCKBR	0.98 ± 0.16	0.92 ± 0.16	n.a.	n.a.
DRD2	0.82 ± 0.16	1.17 ± 0.16	n.a.	n.a.
DRD3	n.a.	n.a.	n.a.	n.a.
DRD4	n.a.	n.a.	n.a.	n.a.
GABAB1	0.87 ± 0.12	0.97 ± 0.08	n.a.	n.a.
GABAB2	0.97 ± 0.19	1.13 ± 0.08	n.a.	n.a.
HTR1A	0.96 ± 0.06	1.05 ± 0.13	n.a.	n.a.
HTR1B	0.87 ± 0.23	1.03 ± 0.17	n.a.	n.a.
HTR2A	1.49 ± 0.33	1.02 ± 0.12	n.a.	n.a.
HTR2C	0.94 ± 0.25	0.84 ± 0.15	n.a.	n.a.
NPSR1	n.a	0.95 ± 0.16	n.a.	n.a.
NPY	0.95 ± 0.13	0.97 ± 0.12	n.a.	n.a.
NPY1R	0.88 ± 0.11	0.95 ± 0.06	0.37 ± 0.04	0.92 ± 0.24
NPY2R	1.01 ± 0.09	1.03 ± 0.17	n.a.	n.a.
NPY5R	0.72 ± 0.11	1.34 ± 0.39	n.a.	n.a.

In hippocampal preparations of male *Rgs2*$^{-/-}$ mice, mRNA expression levels of the adrenergic receptor α_{2A}, the dopaminergic receptor D_2, the neuropeptide Y_1 receptor and neuropeptide Y_5 receptor are reduced, whereas the serotonin receptor 5-HT_{2A} is significantly increased. In frontal cortices, the mRNA expression of the dopamine receptor D_2 and GABAergic receptor B_2 is increased, while expression levels of the serotonin receptor 5-HT_{2C} are decreased.

In pooled atria, mRNA expression of the neuropeptide Y_1 receptor is reduced in *Rgs2*$^{-/-}$ mice. In the left ventricle, mRNA expression of both beta-adrenergic receptors, β_1 and β_2, are reduced upon *Rgs2* deletion.

Taken together, *Rgs2* deletion disrupts GPCR homeostasis and is associated with dysregulation of mRNA expression of several GPCRs.

5.5.3 Regulator of G protein signaling protein expression

Several members of the RGS protein family show a high sequence similarity, especially within the R4 family (see 1.3.2). A loss of *Rgs2* may therefore be compensated by an increased expression of other RGS proteins, partly taking over the physiological function of *Rgs2*. Han and coworkers previously reported unchanged expression of RGS5, RGS7 RGS8 in neuronal cultures (Han, Mark et al. 2006). Therefore, expression levels of all RGS protein family members were determined on the mRNA level by quantitative real time PCR analysis in hippocampus and whole heart preparations of male *Rgs2*$^{-/-}$ compared to WT mice.

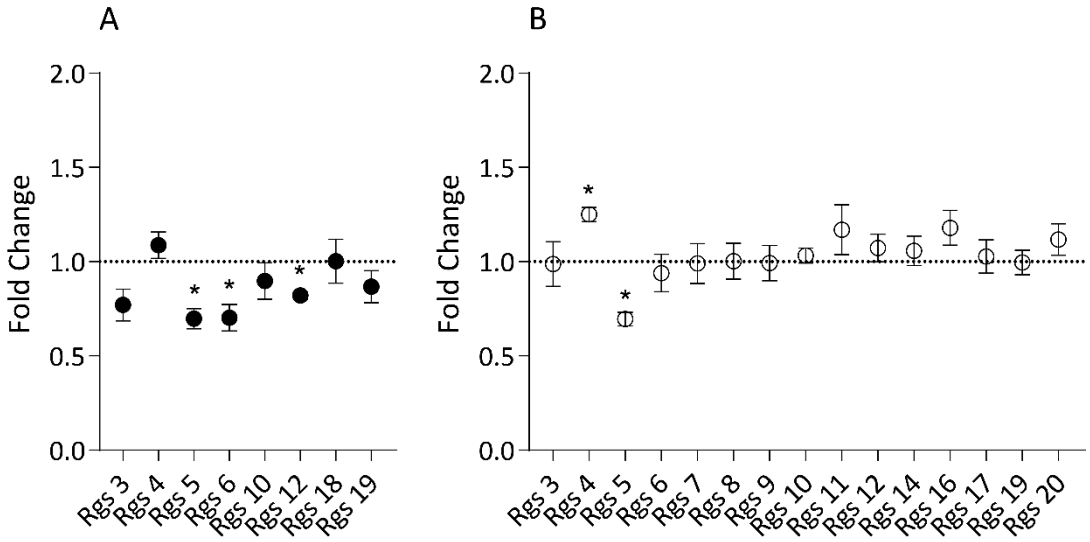


Figure 33: RGS protein mRNA expression in heart and hippocampus

mRNA expression changes evaluated by quantitative real time PCR upon *Rgs2* deletion. Depicted are fold changes (*Rgs2*$^{-/-}$ vs WT) of RGS proteins above the limit of detection. (A) RGS protein mRNA expression changes in hippocampus upon *Rgs2* deletion (B) RGS protein mRNA expression changes in whole heart upon *Rgs2* deletion. Data are mean ± SEM, n=4/genotype. * indicates $p<0.05$ in t-tests, p values were not adjusted for multiple testing.

Expression levels of RGS5, RGS6 and RGS12 were reduced in whole heart preparations in *Rgs2*$^{-/-}$ mice (Figure 33A). In the hippocampus, mRNA levels of RGS4, the closest relative to RGS2, was increased, whereas expression of RGS5 – another member of the R4 family – was reduced (Figure 33B).

Taken together, results suggest a compensatory increase of RGS4 the closest relative of RGS2 in the heart. However, contrary to expectations, RGS5, RGS6 and RGS12 expression levels were reduced upon *Rgs2* deletion.

5.5.4 MicroRNA expression analysis

MicroRNAs are involved in neuronal differentiation and synaptic plasticity and have been implicated in various neuronal processes including learning, memory formation, and psychiatric disorders (Schratt, Tuebing et al. 2006, Smalheiser and Lugli 2009, Issler and Chen 2015). Therefore, microRNAs potentially regulating *Rgs2* expression were identified using three web-based microRNA target prediction tools and 94 microRNAs putatively regulating *Rgs2* expression were subsequently investigated in a luciferase reporter assay. Furthermore, microRNAs potentially deregulated upon *Rgs2* deletion were assessed in the hippocampus of *Rgs2*$^{-/-}$ and WT mice, using microRNA sequencing. The hypothesis was, that *Rgs2* deletion alters microRNA expressions of microRNAs implicated in learning and memory as well as affective disorders and stress resilience.

5.5.4.1.1 Luciferase Reporter Assay

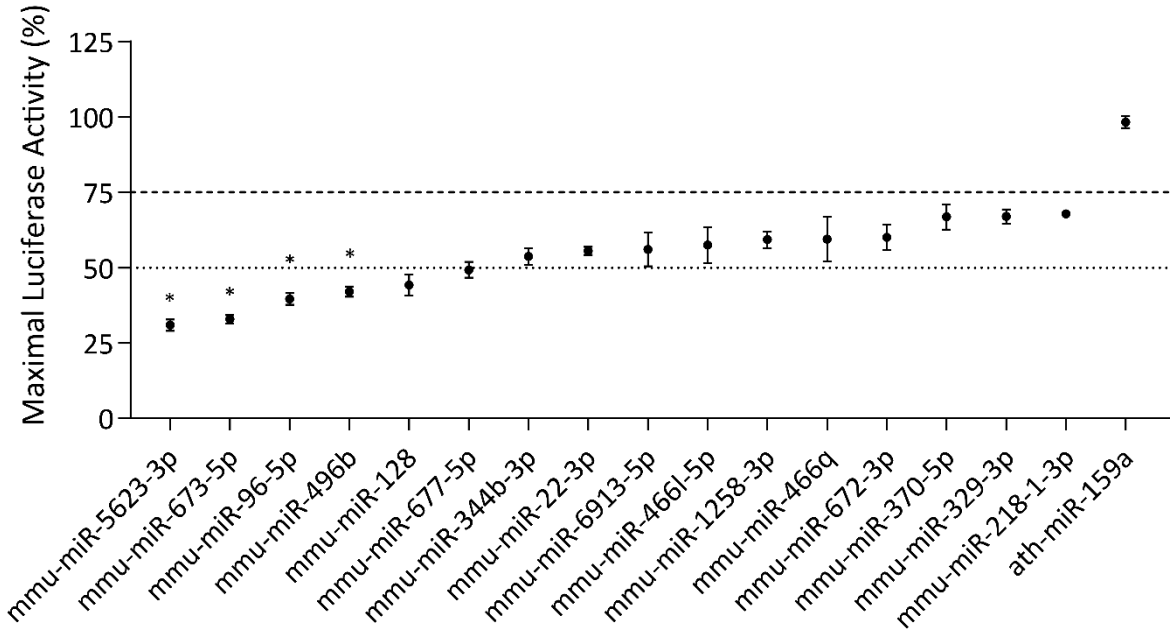


Figure 34: Luciferase reporter Assay of 4 microRNAs regulating the expression of RGS2 by binding to the 3'UTR of *Rgs2*

Predicted microRNAs were tested in a luciferase reporter assay using the 3'UTR of Rgs2 fused to a firefly luciferase. Illustrated are the luciferase activity repression of the co-expressed microRNAs below 50% maximal expression. The dotted line indicated 50% repression of luciferase activity, the dashed line 75% repression of luciferase activity. Ath-miR-159a serves as a negative control indicating maximal luciferase activity. Data are mean ± SEM of 3-5 trials per microRNA. *, $p<0.05$ below 50% of maximal luciferase activity, p values were not adjusted for multiple testing.

Four microRNAs repressed the luciferase activity below 50%, mmu-miR-5623-3p, mmu-miR-673-5p, mmu-miR-96-5p and mmu-miR-496b. Results suggest theses microRNAs to be able to post transcriptionally down-regulate RGS2 expression.

5.5.4.1.2 MicroRNA Sequencing

Hippocampal RNA preparations of six *Rgs2*$^{-/-}$ and six WT mice were investigated for microRNA expression using a sequencing approach. 346 unique microRNAs were mapped to the sequencing results; 42 microRNAs were significantly dysregulated and 8 microRNAs were dysregulated with a log fold change of at least 0.5 upon *Rgs2* deletion. Out of these eight microRNAs seven were up-regulated and one microRNA was down-regulated as depicted in Table 15. These microRNAs may be involved in regulating learning and memory processes, anxiety-like and depression-like behaviors as well as stress susceptibility.

Table 15: Effect of *Rgs2* deletion on microRNA expression in the hippocampus

Illustrated are microRNAs with raw microRNA expression counts of at least 30, a corresponding log2 fold change of no less than 0.5 and an adjusted p value ≤ 0.05 (Benjamini & Hochberg correction). Data are mean ± SD, n= 6 male mice/genotype.

mature microRNA	raw miREx *Rgs2*$^{-/-}$ (counts ± SD)	rlog miREx *Rgs2*$^{-/-}$ ± SD	raw miREx WT (counts ± SD)	rlog miREx WT ± SD	log2 Fold Change	adjusted p value
mmu-miR-1264-5p	95.83 ± 41.6	6.12 ± 0.42	33.17 ± 21.74	5.34 ± 0.32	0.83	0.02
mmu-miR-135a-5p	1450 ± 352.72	10.4 ± 0.3	847.5 ± 396.72	9.93 ± 0.4	0.55	0.04
mmu-miR-204-3p	88.33 ± 35.52	6.15 ± 0.37	43.83 ± 18.47	5.66 ± 0.22	0.67	0.03
mmu-miR-204-5p	4203.33 ± 1654.83	11.73 ± 0.44	1759.33 ± 983.48	10.95 ± 0.34	0.79	0.01
mmu-miR-34a-5p	254.17 ± 25.7	7.9 ± 0.11	156.33 ± 42.73	7.52 ± 0.22	0.53	0.01
mmu-miR-376b-5p	93 ± 14	6.47 ± 0.16	57.5 ± 16.49	6.14 ± 0.24	0.5	0.02
mmu-miR-450a-5p	129.83 ± 31.52	6.88 ± 0.23	76 ± 33.44	6.46 ± 0.37	0.53	0.05
mmu-miR-490-3p	166.33 ± 29.91	7.42 ± 0.17	241 ± 36.94	7.85 ± 0.09	-0.61	< 0.001

6 Discussion

Regulator of G protein signaling 2 is a protein widely expressed. It regulates several G protein-coupled pathways and is thereby involved in numerous physiological processes. This present study focuses on neurophysiological aspects such as learning and memory, anxiety-like behavior, depression-like behavior, stress coping and its underlying molecular causes using a mouse model with deleted *Rgs2* expression.

6.1 *Rgs2* deletion increases learning and memory

6.1.1 Behavioral testing

To date, conflicting reports of *Rgs2*-related effects on memory and learning have been published. Behavioral tests comparing homozygous and heterozygous knockout mice on C57BL/6J background revealed comparable spatial and conditional learning in Water Maze and step down avoidance tests (Oliveira-Dos-Santos, Matsumoto et al. 2000). In 2012, Lifschytz and coworkers, conducted further studies using a different mouse model exhibiting reduced RGS2 gene expression via promoter exchange on a background involving 129P2/OlaHsd and C57BL/6J mice. They observed no genotype effect comparing WT, heterozygous and homozygous *Rgs2* knockout mice in a novelty object recognition task. According to the Hebbian learning model, increasing synaptic strength provides a biological basis of learning and memory (Hebb, 1949). Strengthening of synapses can be tested by measuring long-term potentiation (LTP) and increased LTP has been associated with increased learning (Bliss and Collingridge 1993). Hippocampal LTP was comparable between *Rgs2*$^{-/-}$ and *Rgs2*$^{-/+}$ mice in hippocampal slices (Oliveira-Dos-Santos, Matsumoto et al. 2000), however, *in vivo* readings of LTP were increased in *Rgs2*$^{-/-}$ compared to WT mice (Hutchison, Chidiac et al. 2009).

Data of the present study showed *Rgs2* deletion to enhance learning and memory in three independent tasks: (I) an emotional aversive-associative learning paradigm, (II) a spatial learning and (III) a reward motivated spatial learning task. These paradigms employ varying stimuli, reinforcements, motivators and stress levels. Pavlovian fear conditioning revealed increased immediate learning, short term fear memory and extinction learning (the latter tested in male mice only) as shown by augmented “relative freezing time” (see Figure 18 and Figure 19). Reduced “primary errors” in the Barnes Maze test indicated increased spatial learning (see Figure 21 and Figure 22) and reduced “incorrect nosepokes” in a place preference paradigm demonstrated increased reward motivated spatial learning (see Figure 23).

Data in this study compared 2-month old WT and *Rgs2*$^{-/-}$ mice. LTP was reported to be increased when comparing WT and homozygous mice but not when comparing WT and *Rg2*$^{-/+}$. In line with LTP readings, possibly increased learning of *Rgs2*$^{-/-}$ mice may have been occluded in the study of Oliveira-Dos-Santos and coworkers by comparing heterozygous and homozygous mice (Oliveira-Dos-Santos, Matsumoto et al. 2000). Furthermore, inbred strain background may severely confound behavioral measures and may therefore also occlude effects (Crawley, Belknap et al. 1997, Bailey, Rustay et al. 2006). Mice on a 129P2/OlaHsd background showed reduced learning in a habituation experiment as well as in Barnes Maze testing compared to mice on a C57Bl/6J background. In line with these results, LTP readings were also reduced in 129P2/OlaHsd mice (Nguyen, Abel et al. 2000, Bolivar 2009). Memory testing by Lifschytz and coworkers could have therefore been confounded by the mixed background strain of 129P2/OlaHsd and C57BL/6. Moreover, learning behavior may be age dependent (Foster 1999). In the present study 2-month old mice were used for memory testing, as opposed to 4-5 month old mice used by Oliveira-Dos-Santos and coworkers, possibly additionally affecting the results.

Reduced expression of *RGS2* in human patients was associated with a higher incidence of anxiety disorders in several studies (see 1.3.3.1.1). The etiology of anxiety disorders involves faulty learn processes (see 1.1.1). Psychotherapeutic treatment approaches such as systematic desensitization and exposure therapy utilize pavlovian counter conditioning or fear extinction in order to attenuate a patients' pathological associations.

Cue fear extinction is tested by repeated exposure to the cue in a changed surrounding to remove the aversive association of the cue in the mouse Pavlovian fear conditioning paradigm. This parallels closely fear extinction of exposure therapy in humans. In case of arachnophobia, the patient is repeatedly exposed to the spider in a safe environment to remove the negative association of the spider.

Rgs2$^{-/-}$ mice exhibited enhanced learning in conditioning paradigms including enhanced cue extinction learning. These results suggest, that treatment response to behavioral therapy in human patients may correlate with polymorphisms associated with reduced *Rgs2* expression.

6.1.2 Gene expression and neurotransmitter level changes

RGS2 acts as a GTPase activating protein in numerous $G_{\alpha i}$ and $G_{\alpha q}$ GPCR pathways (Bansal, Druey et al. 2007). *Rgs2* deletion may therefore result in prolonged GPCR signaling, which may be accompanied by compensatory changes in GPCR expression.

6.1.2.1 Serotonergic system

The 5-HT_{2A} receptor is a postsynaptic receptor of the serotonergic system expressed in excitatory as well as inhibitory cells localized in cortex, hippocampus, ventral striatum and the amygdala (Pompeiano, Palacios et al. 1994, Cornea-Hebert, Riad et al. 1999, Lopez-Gimenez, Vilaro et al. 2001). Several results suggest an important role of this receptor in learning and memory. A global deletion of 5-HT_{2A} receptors resulted in impaired memory performance in recognition and working memory tasks (Morici, Ciccia et al. 2015). The medial prefrontal cortex was suggested to be responsible for this effect, however, a contribution of other brain structures including the hippocampus could not be excluded (Morici, Ciccia et al. 2015). Additionally, pharmacological activation of the 5-HT_{2A} receptor was reported to enhance consolidation and extinction of fear memory, which are hippocampal and amygdala dependent memory processes (Zhang, Ásgeirsdóttir et al. 2013). The upregulation of 5-HT_{2A} expression in the hippocampus of *Rgs2*$^{-/-}$ mice (see Table 14) may therefore be reflected in enhanced hippocampus dependent learning of *Rgs2*$^{-/-}$ mice.

The constitutive activity of 5-HT_{2C} receptors contributes to the serotonergic inhibition of the mesolimbic-mesocortical dopamine pathway (Alex and Pehek 2007). 5-HT_{2C} receptors are expressed in the bed nucleus of the stria terminalis, amygdala, prefrontal cortex, hippocampus, striatum and ventral tegmental area (Pompeiano, Palacios et al. 1994, Basura and Walker 2000). Antagonism or inverse agonism of 5-HT_{2C} receptors increases dopamine efflux in the nucleus accumbens and prefrontal cortex via the mesolimbic and mesocortical pathway. Furthermore, 5-HT_{2C} antagonism increases the firing rate of dopaminergic neurons in the ventral tegmental area. (Gobert, Rivet et al. 2000, Hutson, Barton et al. 2000, Alex and Pehek 2007, Di Matteo, Di Giovanni et al. 2008). Consequently, modulation of 5-HT_{2C} receptors may impact the response towards rewarding stimuli via the mesolimbic pathway as well as cognitive processes such as attention and memory via the mesocortical pathway. Global deletion of 5-HT_{2C} receptors was reported to lead to increased affective responses i.e. freezing and ultrasonic vocalizations towards foot shocks in a startle response paradigm in mice. Disinhibition of the mesolimbic dopamine system was suggested to be the primary mechanism of this result (Bonasera, Schenk et al. 2015). Furthermore, global deletion of 5-HT_{2C} receptors lead to an enhanced cocaine-induced dopamine efflux in the nucleus accumbens. Mice showed preference for self-administration of cocaine suggesting enhanced rewarding properties of cocaine (Rocha, Goulding et al. 2002). 5-HT_{2C} mRNA expression was reduced in the frontal cortices of *Rgs2*$^{-/-}$ mice and accompanied by reduced 5-HT neurotransmitter levels in the present study. Reduced 5-HT_{2C} signaling in *Rgs2*$^{-/-}$ mice may therefore lead to decreased serotonergic inhibition of the mesolimbic and mesocortical dopamine pathway. Consequently, rewarding stimuli may be perceived with more attention, promoting increased reward learning.

6.1.2.2 Dopaminergic system

Dopamine receptors are classified into D_1-like and D_2-like families. D_1-like receptors (D_1 and D_5) couple to $G_{\alpha s}$, D_2-like receptors (D_2, D_3 and D_4) to $G_{\alpha i}$ (Ilani, Ben-Shachar et al. 2001, Le Foll, Gallo et al. 2009). RGS2 increases predominantly the GTPase activity of $G_{\alpha i}$ and $G_{\alpha q}$. Therefore, expression of the D_2-like familiy was of most interest for the present study.

D_2 receptors are expressed in the striatum, olfactory tubercle, nucleus accumbes, striatum, hypothalamus, ventral tegmental area, prefrontal cingulate temporal and enthorinal cortex, amygdala and hippocampus (Missale, Nash et al. 1998). A global deletion of the D_2 receptor in mice showed severly impaired hippocampal-memory performance in Morris Water Maze as well as LTP induction. Pharmacological blockage of D_2 reseptors with sulpiride, a D_2/D_3 receptor antagonist, induced comparable impairments in Morris Water Maze and LTP. This effect was related to presynaptic D_2 receptors and associated with elevated hippocampal dopamine levels (Rocchetti, Isingrini et al. 2015). The role of D_2 signaling in learning was further confirmed in humans and non-human primates using fMRI and single neuron recordings respectively, showing prefrontal D_2 activation during spatial memory tasks (Wang, Vijayraghavan et al. 2004, Gelao, Fazio et al. 2014). Likewise, increased surface expression of D_2 receptors using a transgenic mouse model enhanced spatial memory acquisition and novel environment exploration (Saab, Georgiou et al. 2009). Consequently, increased D_2 mRNA expression in the frontal cortex of *Rgs2*$^{-/-}$ mice may contribute to increased spatial learning in all three tested learning paradigms. However, D_2 mRNA expression of *Rgs2*$^{-/-}$ mice was reduced in hippocampus and accompanied by reduced dopamine levels, which would be expected to result in impaired memory performance according to Rocchetti and coworkers (Rocchetti, Isingrini et al. 2015). The D_2 receptor has two isoforms, D_2 long and D_2 short. The D_2 long is primarily located at postsynaptic sites, while the D_2 short is considered to be the predominant presynaptic dopaminergic auto receptor. Presynaptic deletion of D_2 expression by targeting dopamine transporter positive cells using the Cre/loxP technique, as used by Rocchetti and coworkers, should result in a loss of presynaptic inhibition and lead to increased dopamine levels. However, studying D_2 mRNA expression in the hippocampus of *Rgs2*$^{-/-}$ mice includes both pre- and postsynaptic receptors. Reduced D_2 expression was accompanied by a reduction of dopamine levels, which may result from compensatory processes. While global and pre-synaptic loss of D_2 function affects learning, a compensatory adaption of D_2 expression and dopamine levels upon *Rgs2* deletion may have no impact on learning and memory.

Expression levels of D_3 and D_4 receptors in hippocampus and prefrontal cortex were below the limit of detection in the quantitative real time PCR analysis of the present study. Previous reports have suggested an expression of D_3 and D_4 receptors in the prefrontal cortex (Suzuki,

Hurd et al. 1998, Wedzony, Chocyk et al. 2000) along with a modulatory function concerning cognition, learning and memory (Furth, Mastwal et al. 2013, Nakajima, Gerretsen et al. 2013). A global deletion of D_3 receptors in mice improved aversive associative learning using passive avoidance testing (Micale, Cristino et al. 2010) as well as spatial learning using the Morris Water Maze (Xing, Kong et al. 2010, Xing, Meng et al. 2010). Pharmacological agonists and antagonists of D_3 and D_4 receptors have shown mixed effects on cognitive function in humans and rodents. D_3 receptor blockage was suggested to enhance cognitive function while D_3 receptor activation impair the same (Nakajima, Gerretsen et al. 2013). D_4 receptor agonists increase working memory and fear acquisition in rodents (Bernaerts and Tirelli 2003, Browman, Curzon et al. 2005), however a global deletion of D_4 receptors did not induce alterations in learning and memory (Falzone, Gelman et al. 2002). In line with the results of the present study, Rocchetti and coworkers were unable to detect D_3 and D_4 receptor expression in the hippocampus (Rocchetti, Isingrini et al. 2015). However, a contribution of these D_2-like receptors to the observed learning phenotype of *Rgs2*$^{-/-}$ mice cannot be excluded.

6.1.2.3 Intermediate early genes

Intermediate early gene (IEG) expression has been proposed as an important process involved in plastic changes of synapses representing the molecular underlying of long-term memory formation (Minatohara, Akiyoshi et al. 2015). Neuronal gene expression, in particular intermediate early genes such as c-fos, Arc and Rgs2 are rapidly and dynamically changed upon neuronal activity. Neuronal activity can be triggered using pharmacologically induced convulsive and sensory stimuli, as well as behavioral tasks. In hippocampal dependent memory tasks, such as the Morris Water Maze and contextual fear conditioning, rapid IEG changes were observed (Lonergan, Gafford et al. 2010, Minatohara, Akiyoshi et al. 2015). Stimuli promoting changes of synaptic plasticity or intermediate early gene expression induce a rapid upregulation of *Rgs2* expression in several brain regions (see 1.3.3.1.2). These stimuli include neuronal activation with maximal electroconvulsive seizures (Ingi, Krumins et al. 1998) pharmacological intervention with amphetamine, (Burchett, Volk et al. 1998), risperidone and haloperidol (Robinet, Geurts et al. 2001). However, a RGS2 IEG response upon a hippocampal dependent behavioral task is yet to be investigated.

In the present study, an intermediate early gene response of *Rgs2* mRNA was observed 1 and 6 hours after the acquisition phase of the fear conditioning paradigm. However, this response was minor and sex-specific. Hippocampal *Rgs2* mRNA levels were decreased in male mice while there was no change in female mice. In the prefrontal cortex, *Rgs2* mRNA levels were increased in male mice and decreased in female (see Figure 20).

These results may suggest a role of dynamic RGS2 regulation in hippocampal dependent aversive learning. Reduced *Rgs2* mRNA expression in male mice upon fear acquisition may prolong GPCR signaling in the hippocampus, thereby facilitating long term memory formation. This concept is supported by increased hippocampal dependent learning in the context test of the fear conditioning paradigm by male *Rgs2*$^{-/-}$ mice only. Female *Rgs2*$^{-/-}$ mice did not show enhanced hippocampal dependent learning in the context test of the fear conditioning paradigm concurrent with unaltered hippocampal *Rgs2* mRNA expression.

To further investigate the role of dynamic RGS2 expression in learning and memory, hippocampal IEG expression of RGS2 needs to be evaluated in other hippocampus dependent learning tasks, such as the Barnes Maze, Morris Water Maze and novel environment exposure. Additionally, dynamic IEG expression is not restricted to hippocampal dependent processes, and mRNA expression results in the prefrontal cortex suggest that dynamic expression of RGS2 may influence synaptic plasticity in several brain regions.

6.2 *Rgs2* deletion provokes sex specific stress coping behavior

6.2.1 Behavioral testing

Stress, stress coping or stress resilience are part of the etiology of several mental illnesses including anxiety disorders and depressive disorders (see 1.1). Behavioral phenotyping of *Rgs2*$^{-/-}$ mice revealed increased anxiety- and depression-like behavior (see 6.2 and 6.4). However, there is no data of *Rgs2* on stress coping or stress resilience giving insight into a potential role of *Rgs2* in the etiology of anxiety and depressive disorders. In the present study, the impact of two forms of stress, acute and chronic on *Rgs2*$^{-/-}$ mice were investigated.

As a model for acute short term stress the fear conditioning paradigm were used. Electric foot shocks serve as acute stressors in this paradigm (Campos, Fogaca et al. 2013). Innate anxiety measures in approach-avoidance tests (EPM, DLB and OF) were used as an indicator of possible fear generalization after acute stress.

Fear generalization was suggested by heightened cautious behavior in novel surroundings (novelty-induced hypo-locomotion or neophobia) as indicated by decreased “total distance traveled” in all three tests. After acute stress, fear generalization occurred in male mice of both genotypes. In female mice fear generalization occurred in both genotypes, however, this effect was pronounced in WT mice. This might be either due to a floor effect in female *Rgs2*$^{-/-}$ mice or due to altered stress coping indicating a potential role of *Rgs2* in the etiology of anxiety disorders.

Chronic stress was caused using the Chronic Mild Stress model. Chronic Mild Stress is an established animal model of depression, based on the stress-diathesis hypothesis. It reveals alterations in stress coping and stress resilience and may point towards genetic susceptibility genes for depression. CMS induces persisting changes in rodents mirroring depression-like symptoms in humans. Antidepressant treatment can reverse most effects of CMS strengthening its predictive validity (Mineur, Belzung et al. 2006, Campos, Fogaca et al. 2013). Various protocols have been reported and the protocol of Zhu and coworkers was adapted according to options available and feasible in the used animal facility. It included commonly used stressors such as overnight light, food and water deprivation and others (see 5.4 (Willner 1997, Willner 2005, Zhu, Wang et al. 2014). Stress coping was evaluated concerning anhedonia, behavioral despair, social behavior and anxiety like behavior

Upon CMS, anhedonic behavior was provoked in WT mice, while baseline anhedonic behavior of *Rgs2*$^{-/-}$ mice was normalized. Behavioral despair was unchanged by CMS among both genotypes and sexes. CMS had sex specific effects on social and anxiety-like behavior; WT mice (male and female) showed disturbed social memory upon CMS, while male *Rgs2*$^{-/-}$ mice

showed unaffected normal social behavior irrespective of CMS. Disturbed social behavior of female *Rgs2*$^{-/-}$ mice was normalized upon CMS. Anxiety-like behavior was increased in WT mice (male and female), while male *Rgs2*$^{-/-}$ mice were unaffected and female *Rgs2*$^{-/-}$ mice conversely display anxiolysis, upon CMS. A confounding effect of increased activity after CMS (File and Seth 2003) can be excluded since female mice of both genotypes are more active after CMS.

The present study indicates stress susceptibility, stress resilience and stress coping to be sex specifically altered in *Rgs2*$^{-/-}$ mice, mirroring a sex specific effect of stress in the etiology of both anxiety and depression. Furthermore, results suggest *Rgs2* deletion to alter basal stress level possibly promoting a depressive and anxious phenotype in mice.

6.2.2 Gene expression changes

6.2.2.1 Adrenergic system

The α_{2A} receptor system is important for the regulation of neuropsychological stress responses and stress coping behavior (Stamatakis, Pondiki et al. 2008). Stress induces noradrenaline release in frontal cortex, amygdala and hippocampus (Millan 2003). Upon acute and long term exposure to stress, α_{2A} receptor function in the hippocampus at pre-synaptic sides can be increased, thereby reducing the responsiveness of the hippocampus to noradrenergic innervation (Fulford and Marsden 1997). In *Rgs2*$^{-/-}$ mice, α_{2A} receptor mRNA expression was reduced in the hippocampus and accompanied by a reduction of noradrenaline levels and noradrenaline turnover in the hippocampus and prefrontal cortex. This disruption of the noradrenergic system may impair stress induced compensatory mechanisms, thereby altering behavioral stress coping and response.

Stress induced sympathetic activation stimulates the release of norepinephrine at sympathetic nerve endings and provokes norepinephrine and epinephrine secretion from the adrenal gland. Thereby a fight or flight response including increased heart rate, heart contractility and blood pressure is mediated (Mazzeo, Micalizzi et al. 2014, Tank and Lee Wong 2015). At the heart, these effects are predominantly mediated by β_1 and β_2 adrenergic receptors. It has been suggested that Rgs2 regulates β adrenergic signaling in the heart and may thereby influence blood pressure regulation and cardiac dysfunction (Nunn, Zou et al. 2010, Chakir, Zhu et al. 2011). Furthermore, Gross and coworkers suggested an increased sympathetic tone in *Rgs2* deficient mice accompanied by an increased behavioral stress reaction to novelty (Gross, Tank et al. 2005). In the present study, mRNA expression of the β adrenergic receptors β_1 and β_2 was reduced in the left ventricle of the heart upon *Rgs2* deletion. This may reflect altered reactivity to stress induced sympathetic nerve activation as well as a compensatory adaption to an increased sympathetic tone.

6.2.2.2 Neuropeptide Y system

Via the neuropeptide Y_1 receptor, NPY can activate the hypothalamic-pituitary-adrenal axis and the sympathetic adrenomedullary system which both play an important role in stress reactivity (Renshaw, Thomson et al. 2000, Kask, Harro et al. 2002, Heilig 2004, Dimitrov, DeJoseph et al. 2007). Furthermore, NPY is a co-transmitter of norepinephrine in the sympathetic nervous system (Waeber, Aubert et al. 1988). Upon stress, NPY release is provoked in the central nervous system and leads to anxiolytic effects, primarily mediated by the NPY_1 receptor (Thorsell, Carlsson et al. 1999, Karlsson, Choe et al. 2008). Acute restraint stress enhanced exploratory behavior and reduced anxiety-like behavior in the Elevated Plus Maze in male mice with a global deletion of the NPY_1 receptor (Karl, Burne et al. 2006). Painsipp and coworkers showed a similar increase in exploratory behavior upon stress, elicited by the Forced Swim Test in female mice with a global deletion of the NPY_1 receptor, concluding that NPY via the NPY_1 receptor controls stress coping behaviors (Painsipp, Sperk et al. 2010). In humans, the haplotype-driven *NPY* expression predicts brain responses to emotional stress challenges and inversely correlates with trait anxiety, suggesting *NPY* to regulate stress resilience (Zhou, Zhu et al. 2008). Furthermore, the NPY_1 receptor is suggested to be involved in stress mediated cardiovascular response (Klemfuss, Southerland et al. 1998, Tovote, Meyer et al. 2004, Costoli, Sgoifo et al. 2005). NPY acts as a vasoconstrictor either directly or indirectly by potentiating noradrenaline-induced vasoconstriction. In the cardiovascular system, the NPY_1 receptor is the predominant NPY receptor in both blood vessels and the heart (Prieto, Buus et al. 2000). In the present study, NPY_1 receptor mRNA expression was reduced in the hippocampus and atria upon *Rgs2* deletion. Therefore, NPY effects mediated by the NPY_1 receptor upon stress may be attenuated and subsequently contribute to the observed alterations of stress coping and stress resilience of *Rgs2*$^{-/-}$ mice.

6.2.2.3 microRNA Expression changes

Recent studies have implicated microRNAs in neuropsychiatric disorders as well as cognitive function and stress response (Bredy, Lin et al. 2011, Konopka, Schutz et al. 2011, Smalheiser, Lugli et al. 2012, Wang, Kwon et al. 2012, Fan, Sun et al. 2014, Wang, Zhang et al. 2014). Mice lacking *Rgs2* revealed increased cognitive function (see 6.1), increased anxiety-like (see 6.2) and depression-like behavior (see 6.4) as well as altered stress resilience or stress coping behavior (see 6.2). MicroRNA sequencing of hippocampi of *Rgs2*$^{-/-}$ and WT mice showed increased expression of seven microRNAs, including miR-34a-5p and miR135a-5p. While these microRNAs do not directly regulate *Rgs2* expression as indicated by unchanged repression of luciferase activity in the luciferase reporter assay, these microRNAs have been implicated in several neurobiological processes.

Hsa-miR-34a-5p was reported to be upregulated in cerebral spinal fluid of patients suffering from major depressive disorder and suggested to be a biomarker for depression (Wan, Liu et al. 2015). Furthermore, mmu-miR-34a-5p was upregulated in the ventral tegmental area of mice subjected to two weeks of chronic mild stress, (Zurawek, Kusmider et al. 2016) and mice lacking mmu-miR-34 expression proved resilient to stress-induced anxiety suggesting mmu-miR-34 to be critical for the regulating the behavioral and neurochemical response to acute stress (Andolina, Di Segni et al. 2016). In the present study mmu-miR-34a-5p was upregulated in *Rgs2*$^{-/-}$ mice compared to WT under control conditions suggesting *Rgs2*$^{-/-}$ mice to be in a stressed state under normal housing conditions. Furthermore, the increased baseline stress level might alter stress resilience and stress coping upon subjection to further acute or chronic stressors.

Mmu-mir-135 mediates anxiety and depression-like behavior in mice and its overexpression was associated with a reduction of anxiety-like and depression-like behavior in mice. Additionally, mmu-miR-135a overexpressing mice were resilient to social defeat stress indicating mmu-miR-135 to mediate stress resilience. Furthermore, hsa-miR-135 was downregulated in depressed patients and was suggested to mediate antidepressant response (Issler, Haramati et al. 2014). Furthermore, miR-135a-5p expression was reduced in the prefrontal cortex of mice upon 2 weeks' chronic mild stress corroborating its importance in stress resilience (Zurawek, Kusmider et al. 2016). In the present study mmu-miR-135a-5p was upregulated in *Rgs2*$^{-/-}$ mice under control conditions compared to WT. Conversely, *Rgs2*$^{-/-}$ mice showed an anxious and depressed phenotype despite the increased mmu-miR-135a-5p expression. This finding might be due to further gene expression changes in *Rgs2*$^{-/-}$ mice, masking the mmu-miR-135a-5p effect. However, increased mmu-miR-135a-5p may contribute to altered stress coping and stress resilience in *Rgs2*$^{-/-}$ mice.

6.3 *Rgs2* deletion increases innate anxiety

6.3.1 Behavioral testing

Several reports in humans and mice suggest reduced RGS2 expression to correlate with increased anxiety (see 1.2.3.1). Increased incidence of several sub-types of anxiety disorders including panic disorder, generalized anxiety disorder and social anxiety disorder was suggested to be associated with polymorphisms in and flanking the human *RGS2* gene (Leygraf, Hohoff et al. 2006, Smoller, Paulus et al. 2008, Koenen, Amstadter et al. 2009, Otowa, Shimada et al. 2011, Stein, Keshaviah et al. 2014, Hohoff, Weber et al. 2015).

In 2000, Oliveira-dos-Santos and coworkers reported increased innate anxiety of *Rgs2*$^{-/-}$ mice using the Dark-Light Exploration Test and Open Field defecation (Oliveira-Dos-Santos, Matsumoto et al. 2000). Subsequently, Yalcin and coworkers mapped the *Rgs2* gene into a quantitative trail locus influencing anxiety in mice (Yalcin, Willis-Owen et al. 2004). Lifschytz and coworkers, applied a different mouse model (see 6.1.1) and extended these findings by reporting increased innate anxiety in the Elevated Plus Maze (Lifschytz, Broner et al. 2012). To date, all experiments were conducted only in male *Rgs2*$^{-/-}$ mice even though the prevalence of anxiety disorders is almost twice as high in women (McLean, Asnaani et al. 2011).

Animal models are essential for the investigation of anxiety-related disorders, new pharmacological treatments and new pharmacological targets (Campos, Fogaca et al. 2013). Innate anxiety is defined as unconditioned anxiety and gives insight into an animals' acute anxious state. The animals´ conflict between the tendency to approach and explore novel environments as opposed to an avoidance of unprotected open spaces is used to extrapolate innate anxiety (see 1.2.3.1). The tests trigger this approach avoidance conflict between sections of the test apparatus perceived as "safer" as opposed to more "dangerous" (Lister 1990). In the present study, a well validated test battery was used to assess innate anxiety. All tests are based on the approach-avoidance conflict: the Elevated Plus Maze, the Dark-Light Exploration and the Open Field Tests (Cryan and Holmes 2005).

The present study confirmed previous reports of increased innate anxiety in the Elevated Plus Maze, Dark-Light Exploration and Open Field Tests. *Rgs2*$^{-/-}$ mice also showed novelty-induced hypo-locomotion suggesting heightened cautious behavior in a novel environment and the endophenotype neophobia in all three tests (Bortolato, Chen et al. 2008). Normal habituation behavior, evaluated by distances travelled during 10-min intervals in the Open Field, was normal in both *Rgs2*$^{-/-}$ and WT mice, further corroborating the concept of neophobia in *Rgs2*$^{-/-}$ mice. Home cage activity, measured in the IntelliCage setting by counting the corner visits of each mouse, was unchanged in *Rgs2*$^{-/-}$ compared to WT mice confirming hypo-locomotion to be specific for novel environments.

Novelty-induced hypo-locomotion or neophobia may translate into the clinical picture of agoraphobia in humans. Interestingly, reduced RGS2 expression in humans was associated with a higher incidence of panic disorder with agoraphobia (Leygraf, Hohoff et al. 2006). Consequently, RGS2 may therefore represent a novel pharmacological target for agoraphobia.

6.3.2 Gene expression changes

6.3.2.1 Serotonergic system

Lifschytz and coworkers suggested an involvement of the serotonergic system in the observed behavioral alterations of *Rgs2*$^{-/-}$ mice. However, Lifschytz and coworkers used a different mouse model with a reduced *Rgs2* expression for their studies (see 6.1.1). The lack of RGS2 mediated termination of downstream signaling may induce a stronger serotonergic inhibitory tone in *Rgs2*$^{-/-}$ mice. In the raphe nuclei, 5-HT_{1A} receptors function mainly as inhibitory somatodendritic autoreceptors while 5-HT_{1B} receptors act as autoreceptors on serotonergic axons inhibiting 5-HT release and synthesis (McDevitt and Neumaier 2011) Consequently, a compensatory downregulation of serotonergic receptors 5-HT_{1A} and 5-HT_{1B} upon *Rgs2* deletion occurs to facilitate increased serotonergic transmission (Lifschytz, Broner et al. 2012). These results were supported by lower reactivity of *Rgs2*$^{-/-}$ mice to 8-OH-DPAT induced hypothermia, suggesting reduced HT_{1A} receptor function. 8-OH-DPAT may reduce the body temperature by activating 5-HT_{1A} receptors, therefore 8-OH-DPAT induced hypothermia gives insight into in vivo 5-HT_{1A} autoreceptor function (Martin, Phillips et al. 1992). Lifschytz and coworkers suggested this serotonergic disruption to be partly responsible for the anxiety and depression-like phenotype in *Rgs2*$^{-/-}$ mice.

In addition to their importance for learning and memory, brain regions with prominent 5-HT_{2A} receptor expression are also involved in modulating the behavioral response to threats and novelty, representing the innate anxiety state of an organism. Global deletion of 5-HT_{2A} receptors in mice provoked an anxiolytic phenotype in behavioral tests evaluating innate anxiety, while depression-like behavior remained unchanged (Weisstaub, Zhou et al. 2006). Conflicting results regarding the pharmacological manipulation of the 5-HT_{2A} receptor have been reported. While agonists seemed ineffective towards the anxiety state, antagonists were shown to be both anxiogenic and anxiolytic (Millan 2003). Consequently, it is unclear whether increased 5-HT_{2A} receptor expression in the hippocampus of *Rgs2*$^{-/-}$ mice contributes to increased innate anxiety of *Rgs2*$^{-/-}$ mice.

5-HT_{2C} receptors are suggested to control anxious states (Millan 2003). A global deletion of 5-HT_{2C} receptors in mice induced an anxiolytic phenotype (Heisler, Zhou et al. 2007). Accordingly, 5-HT_{2C} agonists were reported to be anxiogenic while antagonists were suggested to be anxiolytic (Millan 2003). Interestingly, a reduced expression of 5-HT_{2C} accompanied by

reduced 5-HT neurotransmitter level were noted in the prefrontal cortex of *Rgs2*$^{-/-}$ mice while conversly mice display an anxious phenotype. This might suggest that even in the presence of reduced 5-HT neurotransmitter levels and reduced 5-HT_{2C} receptor expression, deletion of *Rgs2* may result in increased 5-HT_{2C} mediated signaling, due to its strong accelerating effect on the GTPase activity of the $G\alpha_q$ subunit (Ross and Wilkie 2000). Therefore, anxiolytic effects provoked by decreased 5-HT_{2C} expression may be reverted into anxiogenic effects, suggesting an important regulatory role of *Rgs2* on 5-HT_{2C} mediated signaling and associated anxiety.

6.3.2.2 Adrenergic system

The adrenergic system is activated upon anxiogenic or stressful stimuli, thereby controlling the anxiety response of an organsim. The α_{2A} receptor is broadly expressed in the central nervous system (CNS) and in peripheral tissues. In the CNS, high pre- and postsynaptic α_{2A} receptor mRNA expressions levels are found in the caudate putamen, nucleus accumbens, hippocampus and substantia nigra. Several results suggest an important role of the α_{2A} receptor in innate anxiety. A global deletion model of the α_{2A} receptor in mice revealed increased anxiety and reduced locomotor activity in a novel environment (Schramm, McDonald et al. 2001, Lahdesmaki, Sallinen et al. 2002). Furthermore, the noradrenaline turnover in cortex and hippocampus were increased, putatively due to the loss of presynaptic inhibition of noradrenaline release by the α_{2A} receptor (Lakhlani, MacMillan et al. 1997, Lahdesmaki, Sallinen et al. 2002). Upon *Rgs2* deletion, α_{2A} receptor mRNA expression was reduced in the hippocampus. Furthermore, norepinephrine levels and the norepinephrine turnover were reduced in hippocampus and prefrontal cortex, possibly indicating reduced presynaptic inhibition. Increased innate anxiety as well as novelty-induced hypo-locomotion or neophobia could therefore be partly explained by a disruption of presynaptic noradrenergic inhibition. The noradrenergic system is also of great importance in depression, stress coping and stress reactivity this is discussed in chapters 6.4and 6.2.

6.3.2.3 Neuropeptide Y system

Neuropeptide Y is highly expressed in the cerebral cortex. It co-localizes with noradrenaline receptors in the locus ceruleus and the sympathetic nervous system (Illes and Regenold 1990). NPY binds to 6 G protein-coupled receptors Y_1 to Y_6, all coupling to $G_{\alpha i}$. The NPY_1 receptor is the most abundant NPY receptor in the CNS and highly expressed in the hippocampus, periaqueductal grey, frontal cortex, hypothalamus and amygdala (Millan 2003). The NPY_1 receptor is suggested to modulate anxiety-like behavior. Global deletion of the NPY_1 receptor in mice results in anxious behavior dependent on the type of anxiety test and the time of testing in the circadian cycle (Karl, Burne et al. 2006). Furthermore, conditional deletion of hippocampal NPY_1 receptors (Bertocchi, Oberto et al. 2011) as well as conditional inactivation of NPY_1 receptors in NPY_5 receptor positive cells (Longo, Mele et al. 2014) increases anxiety-

like behavior in mice. In line with these findings, hippocampal overexpression of NPY_1 receptor leads to decreased anxiety in mice (Olesen, Christiansen et al. 2012). Pharmacological modulation of the NPY_1 receptor affects anxiety-like behavior in rodents. NPY_1 receptor agonists are anxiolytic while antagonists are anxiogenic (Kask, Harro et al. 2002, Heilig 2004, Lin, Boey et al. 2004, Primeaux, Wilson et al. 2005, Eva, Serra et al. 2006). In conclusion, reduced NPY_1 receptor mRNA expression in the hippocampus of *Rgs2*$^{-/-}$ mice may contribute to heighten innate anxiety. Furthermore, the neuropeptide Y system is important in stress coping and reactivity (see 6.2).

6.4 *Rgs2* deletion increases depressive behavior

6.4.1 Behavioral testing

Previous results suggest *Rgs2* deletion to promote depression-like behavior in mice. Lifschytz and coworkers reported increased behavioral despair in *Rgs2*$^{-/-}$ mice using the Forced Swim Test. Additionally, social behavior tested in the Social Interaction Test was disrupted in *Rgs2*$^{-/-}$ mice and dysfunctional social behavior represents one endophenotype of depression in mice (Lifschytz, Broner et al. 2012). However, as mentioned above, Lifschytz and coworkers used a different mouse model on a different inbred strain background. In humans, no association of *RGS2* polymorphisms with depression has been observed so far. However, an association of *RSG2* polymorphisms with suicide was reported, putatively linking *RGS2* with depression, as depression is the neuropsychiatric disorder most commonly associated with suicide. The importance of *RGS2* in suicide was further strengthened by findings of increased RGS2 expression in postmortem brains of suicide subjects in the same study (Cui, Nishiguchi et al. 2008). To date, all experiments were carried out using male *Rgs2*$^{-/-}$ mice, even though sex specific effects regarding depression are well known and the prevalence of depression is approximately twice as high in women compared to men (Accortt, Freeman et al. 2008).

The present study indicates depression-like behavior in *Rgs2*$^{-/-}$ mice to be similarly more distinctive in female mice mirroring a sex specific effect of depression. Increased behavioral despair of male *Rgs2*$^{-/-}$ mice was replicated using the Forced Swim Test as done by Lifschytz and coworkers. Importantly, this finding was confirmed for female *Rgs2*$^{-/-}$ mice. Anhedonia was increased as suggested by Sucrose Preference and food intake. This effect was pronounced and social behavior was only disrupted in female *Rgs2*$^{-/-}$ mice.

Taken together the present study corroborates findings of Lifschytz and coworkers and suggests *Rgs2* as a contributing factor for the sex specificity of depression.

6.4.2 Gene expression analysis and neurotransmitter levels

6.4.2.1 Adrenergic system

A global deletion of the α_{2A} receptor in mice was suggested to result in a depression-like phenotype. Behavioral despair was increased in a modified version of the Porsolt test. However, the tricyclic antidepressant imipramine was unable to rescue this phenotype (Schramm, McDonald et al. 2001). Furthermore, postmortem studies in humans reporting altered functionality of α_{2A} receptors in depressed patients strengthen the role of α_{2A} receptors in depression (Valdizan, Diez-Alarcia et al. 2010). Genetic association studies were inconsistent, suggesting both no association with depression (Martin-Guerrero, Callado et al.

2006) as well as a link between *ADRA2A* variants and treatment response to SNRIs (Wakeno, Kato et al. 2008). Since α_{2A} receptor mRNA expression was reduced in the hippocampus upon *Rgs2* deletion, the noradrenergic system may also contribute to a depression-like phenotype of *Rgs2*$^{-/-}$ mice, in line with the results of Schramm and coworkers.

6.4.2.2 Neurotransmitter level

The monoamine-deficiency hypothesis is the pharmacologically most relevant hypothesis of depression (Hasler 2010, Hamon and Blier 2013). This hypothesis arises from the fact that almost every drug inhibiting monoamine reuptake or degradation and leading to increased concentrations of monoamines in the synaptic cleft, proves to be an effective antidepressant (Belmaker and Agam 2008, Morrissette and Stahl 2014). The monoamine-deficiency hypothesis suggests a depletion of the neurotransmitters serotonin, norepinephrine and dopamine in the central nervous system to be an underlying cause of depression.

In the present study, monoamine neurotransmitter levels in prefrontal cortex and hippocampus were reduced upon *Rgs2* deletion (see Table 13). This effect of *Rgs2* deletion may contribute to the observed depression-like phenotype.

6.5 Behavioral phenotyping issues

6.5.1 Health issues

Abnormalities in general health interfere with behavioral testing and can severely confound results or lead to false interpretations making unaltered general health of *Rgs2*$^{-/-}$ mice imperative for behavioral testing (Crawley, 2007).

General health of *Rgs2*$^{-/-}$ mice was anticipated to be comparable to WT mice due to previous publications (Oliveira-Dos-Santos, Matsumoto et al. 2000, Lifschytz, Broner et al. 2012). However, male and female *Rgs2*$^{-/-}$ mice showed reduced body weight compared to age matched WT mice giving rise to a possible role of RGS2 in developmental or growth regulating processes. Conversely, in humans reduced RGS2 expression due to polymorphism rs4606 was associated with increased BMI in hypertensive patients (Sartori, Ceolotto et al. 2008). Reduced body mass was accompanied by lower lean mass in male *Rgs2*$^{-/-}$ mice (see Figure 15). Reduced exercise or movement may result in lower body weight and be accompanied by reduced lean mass. Reduced food intake can also lead to lower body weight in mice, and in fact *Rgs2*$^{-/-}$ mice showed a significant 9.7-14.3 % reduction (see Figure 15).

Furthermore, contrary to previous reports, *Rgs2*$^{-/-}$ mice were less active in the present study ("total distance traveled"), indicating reduced exercise or movement in all conducted tests involving a novel environment. Previous reports suggested unchanged activity of *Rgs2*$^{-/-}$ mice in circadian activity measurements in a home cage setting for 48 hours. Possible novelty induced alterations such as novelty induced hypo-locomotion may have been occluded (Oliveira-Dos-Santos, Matsumoto et al. 2000). Further analysis in this study revealed similar home cage activity i.e. movement measured by counting corner visits in the IntelliCage adaptation phase of male *Rgs2*$^{-/-}$ and WT mice (see Figure 16), strengthening the interpretation of altered activity in novel environments only. Therefore, lower body weight and lean mass are probably not caused by reduced exercise or movement.

Lower food intake in an *ad libitum* food and water access situation may indicate a lower metabolism or anhedonic behavior. Further tests indicate depression-like behavior of *Rgs2*$^{-/-}$ mice to be increased in Forced Swim Test (see Figure 31) and Sucrose Preference Test (see Figure 27). This further corroborates interpretation as anhedonic behavior as opposed to lowered metabolism.

Taken together, altered developmental or growth regulating processes as well as lowered metabolism upon *Rgs2* deletion cannot be conclusively excluded. Due to the similar home cage activity, it was concluded general health issues did not interfere with behavioral testing.

6.5.2 Learning and memory testing

Pavlovian fear conditioning is an established paradigme to investigate aversive emotional learning and memory (Rodrigues, Schafe et al. 2004, Izquierdo, Furini et al. 2016). Learning is reinforced by a negative aversive stimulus, an inescapable electric foot shock which is paired with distinct environmental cues i.e. a specific tone (US) and specific cage/context. Mice are trained to learn and remember the association between the aversive stimulus the tone and the context or cage they were trained in. The main parameter evaluated is "relative freezing time" and freezing time is scored automatically by the fear conditioning software. A reduction of exploratory behavior, as observed with *Rgs2*$^{-/-}$ mice in novel environments, can confound the automatic measurement. To prevent scoring low exploratory behavior as freezing, the threshold to count immobility as freezing was set to >2s. Using this threshold, lower activity is unlikely to falsely inflate freezing scores.

The Barnes Maze test was developed to evaluate spatial memory on dry-land reducing the procedural difficulties and the stress component (Barnes 1979) elicited by swimming during Water Maze procedures (Morris 1984, Rosenfeld and Ferguson 2014). This is of importance as stress may impair learning and memory, thereby possibly confounding results (Kim, Song et al. 2006).

To corroborate successful learning in the Barnes Maze test, a time dependent decrease of the parameters "target latency", "escape latency", "primary errors" and "distance" as well as a time dependent increase in "time in target quadrant" have to occur. However, male *Rgs2*$^{-/-}$ mice showed increasing "escape latency" and "distance" starting with trial block A3 after an initial decrease, while "target latencies", "primary errors" and "time in target quadrant" continued to change in line with successful learning. Male *Rgs2*$^{-/-}$ mice traveled less "distance" during trial block A1 compared to WT mice, but they traveled more distance compared to WT during trial block A5. Reduced distance during trail block A1 might be caused by novelty-induced hypo-locomotion of male *Rgs2*$^{-/-}$ mice. Due to repeated exposure to the Barnes Maze over the course of the test, this effect may have dissipated. Familiarity with the maze may have triggered exploratory behavior leading to increased "distance" during trail block A5. Subsequently, the increased exploration may trigger increased "escape latencies" in *Rgs2*$^{-/-}$ mice from A3 to A5. In conclusion, increasing "distance" and "escape latencies" indicated increased exploratory behavior rather than unsuccessful learning of the task, suggesting that results are valid.

6.5.3 Anhedonia

Mice were housed in groups of two mice per cage. The social contact between two mice can attenuate the effect of CMS, thereby confounding the results of the test. Single housing would

have been preferable, as it is a stressor by itself and increases the strength of chronic stress. Consequently, Sucrose Preference and food intake measurements were a mean of two mice per cage.

No change in sucrose preference of male *Rgs2*$^{-/-}$ and WT mice after 3 weeks of CMS was an unexpected result. Sucrose Preference measurements were obtained using a two-bottle test without any food or water restriction directly before testing for motivational purposes. The test was carried out for a 48h period. The long testing period without prior food and water restriction may confound small changes induced by CMS. An one-hour testing period after a four-hour food and water restriction has been reported to yield more reliable results (Willner, Towell et al. 1987). Additionally, the concentration of 1% sucrose could have been too high to allow the measurement of small changes (Monleon, D'Aquila et al. 1995).

6.5.4 Blood pressure measurements

Previous publications report a hypertensive phenotype of *Rgs2*$^{-/-}$ mice (Heximer, Knutsen et al. 2003, Tang, Wang et al. 2003). These findings were obtained either via echocardiography of anesthetized mice or by telemetric catheter implants in awake freely moving mice. In the present study blood pressure and heart rate were measured using a non-invasive tail cuff method and no replication of this hypertensive phenotype was observed. Both, systolic and diastolic blood pressure were similar to WT mice, while the heart rate was moderately reduced in *Rgs2*$^{-/-}$ mice.

Each method features strength and limitations. Echocardiographic measurements give insight into cardiovascular structures and cardiac functions additionally to estimated blood pressure and heart rate values. However, echocardiographic measurements require anesthesia or a lengthy training period to allow measurements in conscious mice. Anesthesia depresses contraction, heart rate and autonomic reflex control, thereby possibly confounding echocardiographic measurements (Gao, Ho et al. 2011). Telemetric measurements make it possible to obtain the arterial blood pressure directly over a long period in freely moving mice. But telemetric blood pressure measurements involve surgery inserting a catheter into the aortic arch via the left carotid artery and a telemeter into the subcutaneous space. This telemeter weighs approximately 4.3g and may have profound effects on behavior and cardiovascular parameters of smaller mice (Van Vliet, McGuire et al. 2006). Tail cuff measurements of awake mice, asused in the present study, require a training phase of at least five days of repeated measurements to minimize the excitement or stress induced by the restraint (Krege, Hodgin et al. 1995). Furthermore, the tail-cuff method yields peripheral arterial tail blood pressure opposed to central arterial pressure determined in telemetric settings (Zhao, Ho et al. 2011). Gross and coworkers previously reported an increase of ~

10mmHg mean arterial blood pressure via telemetry in *Rgs2*$^{-/-}$ mice, the tail cuff method may not resolve such a mild increase (Gross, Tank et al. 2005).

7 Summary

Anxiety and depressive disorders result from a complex interplay of genetic and environmental factors and are common mutual comorbidities. On the level of cellular signaling, regulator of G protein signaling 2 (Rgs2) has been implicated in human and rodent anxiety as well as rodent depression. Rgs2 negatively regulates G protein-coupled receptor (GPCR) signaling by acting as a GTPase accelerating protein towards the Gα subunit.

The present study investigates, whether mice with a homozygous Rgs2 deletion (*Rgs2*$^{-/-}$) show behavioral alterations as well as an increased susceptibility to stressful life events related to human anxiety and depressive disorders and tries to elucidate molecular underlying's of these changes.

To this end, *Rgs2*$^{-/-}$ mice were characterized in an aversive-associative learning paradigm to evaluate learned fear as a model for the etiology of human anxiety disorders. Spatial learning and reward motivated spatial learning were evaluated to control for learning in non-aversive paradigms. *Rgs2* deletion enhanced learning in all three paradigms, rendering increased learning upon deletion of Rgs2 not specific for aversive learning. These data support reports indicating increased long-term potentiation in *Rgs2*$^{-/-}$ mice and may predict treatment response to conditioning based behavior therapy in patients with polymorphisms associated with reduced RGS2 expression. Previous reports of increased innate anxiety were corroborated in three tests based on the approach-avoidance conflict. Interestingly, *Rgs2*$^{-/-}$ mice showed novelty-induced hypo-locomotion suggesting neophobia, which may translate to the clinical picture of agoraphobia in humans and reduced RGS2 expression in humans was associated with a higher incidence of panic disorder with agoraphobia. Depression-like behavior was more distinctive in female *Rgs2*$^{-/-}$ mice. Stress resilience, tested in an acute and a chronic stress paradigm, was also more distinctive in female *Rgs2*$^{-/-}$ mice, suggesting *Rgs2* to contribute to sex specific effects of anxiety disorders and depression.

Rgs2 deletion was associated with GPCR expression changes of the adrenergic, serotonergic, dopaminergic and neuropeptide Y systems in the brain and heart as well as reduced monoaminergic neurotransmitter levels. Furthermore, the expression of two stress-related microRNAs was increased upon R*gs2* deletion. The aversive-associative learning paradigm induced a dynamic *Rgs2* expression change. The observed molecular changes may contribute to the anxious and depressed phenotype as well as promote altered stress reactivity, while reflecting an alter basal stress level and a disrupted sympathetic tone. Dynamic *Rgs2* expression may mediate changes in GPCR signaling duration during memory formation.

Taken together, *Rgs2* deletion promotes increased anxiety-like and depression-like behavior, altered stress reactivity as well as increased cognitive function.

Zusammenfassung

Angststörungen sowie Depressionserkrankungen entstehen in der Regel aus der Interaktion genetischer Faktoren mit Umwelteinflüssen und sind häufig gegenseitige Begleiterkrankungen. Das Protein, Regulator of G protein signaling 2 (Rgs2), wurde mit dem vermehrten Auftreten von Angststörungen im Menschen, sowie mit angstähnlichem sowie depressionsähnlichem Verhalten im Mausmodell assoziiert. Rgs2 beeinflusst auf zellulärer Ebene G Protein gekoppelte Signalwege, indem es die GTPase Aktivität der G_{α} Untereinheit beschleunigt.

In der vorliegenden Arbeit wurden die Folgen einer homozygoten *Rgs2*-Defizienz im Mausmodell untersucht. In Anlehnung an die humanen Krankheitsbilder wurde angst- und depressions-ähnliches Verhalten, Stress Reaktivität und den phänotypischen Veränderungen zugrundeliegende molekulare Ursachen evaluiert.

Erlernte Furcht gilt als Model der Ätiologie humaner Angsterkrankungen. Aus diesem Grund, wurden *Rgs2*$^{-/-}$ Mäuse in einem aversiv-assoziativen Lernmodell, der sogenannten Furcht-Konditionierung, untersucht. Dabei zeigte sich erhöhtes Furchtlernen und Furchtgedächtnis in *Rgs2*$^{-/-}$ Mäusen. Um zu zeigen, dass die erhöhte kognitive Fähigkeit spezifisch für erlernte Furcht sei, wurde räumliches Lernen in zwei Modellen getestet. *Rgs2*-Defizienz verbesserte auch in diesen Modellen die Lernfähigkeit. Somit konnte gezeigt werden, dass verbesserte kognitive Fähigkeit nicht spezifisch für emotionales Lernen war. Diese Daten auf Verhaltensebene unterstützen bisherige Befunde von erhöhter Langzeit Potenzierung im Hippocampus von *Rgs2*$^{-/-}$ Mäusen. Im Menschen könnte eine durch Polymorphismen vermittelte reduzierte *Rgs2* Expression das Therapieansprechen auf konditionierungsbasierte Verhaltenstherapien verbessern. Bisherige Befunde von erhöhter, angeborener Angst in *Rgs2*$^{-/-}$ Mäusen konnten in drei Tests, basierend auf dem Annäherungs-Vermeidungs-Konflikt, bestätigt werden. Interessanterweise, zeigten *Rgs2*$^{-/-}$ Mäuse in allen Tests verminderte Lokomotion in neuen, ungewohnten Umgebungen. Dies könnte auf Neophobie und somit auf das Krankheitsbild der Agoraphobie im Menschen hindeuten. Tatsächlich wurden *RGS2* Polymorphismen bereits mit einer erhöhten Inzidenz von Panikstörung mit Agoraphobie assoziiert. *Rgs2*$^{-/-}$ Mäuse zeigten zudem depressionsähnliches Verhalten, welches in weiblichen Mäusen ausgeprägter war. Des Weiteren zeigten, insbesondere weibliche *Rgs2*$^{-/-}$ Mäuse, erhöhte Stress Resilienz nach akuter und chronischer Stressexposition. *Rgs2* könnte somit ein Faktor der Geschlechtsspezifität von Angst und Depressionserkrankungen sein.

Rgs2-Defizienz konnte mit Expressionsänderungen von G Protein gekoppelten Rezeptoren des adrenergen, serotonergen, dopaminergen und Neuropeptid Y Systems in Gehirn und Herz, sowie mit verminderten Spiegeln monoaminerger Neurotransmitter assoziiert werden. Diese

Veränderungen könnten zu dem beobachteten ängstlichen sowie depressiven Phänotyp und der veränderten Stress Reaktivität beitragen. Des Weiteren war die Expression zweier, in der Stressreaktion involvierten, microRNAs erhöht. Dies könnte auf einen veränderten basalen Stress Level hindeuten. Furcht-Konditionierung löste dynamische Expressionsänderungen der *Rgs2* mRNA aus. Somit könnte die GPCR Signaldauer während der Gedächtnisbildung durch *Rgs2* moduliert werden.

Zusammengefasst, führt *Rgs2*-Defizienz im Mausmodell zu erhöhtem angst- und depressionsähnlichem Verhalten, veränderter Stress Reaktivität sowie erhöhter kognitiver Leistung.

8 Abbreviations

3’UTR	three prime untranslated region
5HIAA	5 Hydroxyindoleacetic acid
5-HT	serotonin
Adra2a	alpha$_{2A}$ adrenergic receptor
Adra2b	alpha$_{2B}$ adrenergic receptor
Adra2c	alpha$_{2C}$ adrenergic receptor
Adrb1	beta$_1$ adrenergic receptor
Adrb2	beta$_2$ adrenergic receptor
AKAP	A-kinase anchor protein
AMP	adenosine monophosphate
CA1	Cornu Ammonis area 1
Cck	Cholecystokinin
Cckar	Cholecystokinin A receptor
Cckbr	Cholecystokinin B receptor
CMS	chronic mild stress
Crhr1	corticotropin-releasing hormone receptor 1
CS	conditioned stimulus
CTR	control
DA	dopamine
DAG	diacylglycerol
DLB	dark-Light exploration
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
Drd2	dopamine receptor D_2
Drd3	dopamine receptor D_3
Drd4	dopamine receptor D_4
EPM	elevated plus maze
FC	fear conditioning

FST	Forced swim test
Gabbr1	gamma-aminobutyric acid (GABA) B receptor 1
Gabbr2	gamma-aminobutyric acid (GABA) B receptor 2
GAP	GTPase activating protein
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCR	G protein coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
Htr1a	5-HT_{1A} receptor
Htr1b	5-HT_{1B} receptor
Htr2a	5-HT_{2A} receptor
Htr2c	5-HT_{2C} receptor
HVA	homovanillic acid
IP_3	inositol 1,4,5-trisphosphate
LTP	long term potentiation
MDD	major depressive disorder
MHPG	3-Methoxy-4-hydroxyphenylglycol
miRNA	microRNA
mRNA	messenger RNA
ncRNA	non-coding RNA
NE	norepinephrine
NMR	nuclear magnetic resonance
Nps	neuropeptide s
Npsr1	neuropeptide s receptor 1
Npy	neuropeptide Y
Npy1r	Neuropeptide Y receptor type 1
Npy2r	Neuropeptide Y receptor type 2
Npy5r	Neuropeptide Y receptor type 5

OF	open field locomotion
PCR	polymerase chain reaction
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PPD	paired pulse depression
PPF	paired pulse facilitation
pre-microRNA	precursor microRNAs
pri-microRNA	primary microRNA
PTSD	post-traumatic stress disorder
RGS	regulator of G protein signaling
Rgs2/RGS2	regulator of G Protein signaling
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SERT	serotonin transporter
SI	social interaction
siRNA	small interfering RNA
SSRI	selective serotonin reuptake inhibitor
tRNA	transfer RNA
US	unconditioned stimulus
VTA	ventral tegmental area
WT	wildtype

9 References

Accortt, E. E., M. P. Freeman and J. J. Allen (2008). "Women and major depressive disorder: clinical perspectives on causal pathways." J Womens Health (Larchmt) **17**(10): 1583-1590.

Adamec, R., D. Head, J. Blundell, P. Burton and O. Berton (2006). "Lasting anxiogenic effects of feline predator stress in mice: sex differences in vulnerability to stress and predicting severity of anxiogenic response from the stress experience." Physiol Behav **88**(1-2): 12-29.

Adhikari, A., M. A. Topiwala and J. A. Gordon (2010). "Synchronized activity between the ventral hippocampus and the medial prefrontal cortex during anxiety." Neuron **65**(2): 257-269.

Albuquerque, B., A. Haussler, E. Vannoni, D. P. Wolfer and I. Tegeder (2013). "Learning and memory with neuropathic pain: impact of old age and progranulin deficiency." Front Behav Neurosci **7**: 174.

Alex, K. D. and E. A. Pehek (2007). "Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission." Pharmacol Ther **113**(2): 296-320.

Ambros, V. (2004). "The functions of animal microRNAs." Nature **431**(7006): 350-355.

Amstadter, A. B., K. C. Koenen, K. J. Ruggiero, R. Acierno, S. Galea, D. G. Kilpatrick and J. Gelernter (2009). "Variation in RGS2 is associated with suicidal ideation in an epidemiological study of adults exposed to the 2004 Florida hurricanes." Arch Suicide Res **13**(4): 349-357.

Andolina, D., M. Di Segni, E. Bisicchia, F. D'Alessandro, V. Cestari, A. Ventura, C. Concepcion, S. Puglisi-Allegra and R. Ventura (2016). "Effects of lack of microRNA-34 on the neural circuitry underlying the stress response and anxiety." Neuropharmacology **107**: 305-316.

Anger, T., W. Zhang and U. Mende (2004). "Differential contribution of GTPase activation and effector antagonism to the inhibitory effect of RGS proteins on Gq-mediated signaling in vivo." J Biol Chem **279**(6): 3906-3915.

Atwood, B. K., D. M. Lovinger and B. N. Mathur (2014). "Presynaptic long-term depression mediated by Gi/o-coupled receptors." Trends Neurosci **37**(11): 663-673.

Bailey, K. R., N. R. Rustay and J. N. Crawley (2006). "Behavioral phenotyping of transgenic and knockout mice: practical concerns and potential pitfalls." Ilar j **47**(2): 124-131.

Bansal, G., K. M. Druey and Z. Xie (2007). "R4 RGS proteins: regulation of G-protein signaling and beyond." Pharmacol Ther **116**(3): 473-495.

Barnes, C. A. (1979). "Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat." J Comp Physiol Psychol **93**(1): 74-104.

Bartel, D. P. (2009). "MicroRNAs: target recognition and regulatory functions." Cell **136**(2): 215-233.

Basura, G. J. and P. D. Walker (2000). "Serotonin 2A and 2C receptor biosynthesis in the rodent striatum during postnatal development: mRNA expression and functional linkage to neuropeptide gene regulation." Synapse **38**(2): 216-225.

Baxter, A. J., K. M. Scott, T. Vos and H. A. Whiteford (2013). "Global prevalence of anxiety disorders: a systematic review and meta-regression." Psychol Med **43**(5): 897-910.

Baxter, A. J., T. Vos, K. M. Scott, A. J. Ferrari and H. A. Whiteford (2014). "The global burden of anxiety disorders in 2010." Psychol Med **44**(11): 2363-2374.

Belmaker, R. H. and G. Agam (2008). "Major depressive disorder." N Engl J Med **358**(1): 55-68.

Belzung, C., R. Misslin, E. Vogel, R. H. Dodd and G. Chapouthier (1987). "Anxiogenic effects of methyl-beta-carboline-3-carboxylate in a light/dark choice situation." Pharmacol Biochem Behav **28**(1): 29-33.

Berman, D. M., T. M. Wilkie and A. G. Gilman (1996). "GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits." Cell **86**(3): 445-452.

Bernaerts, P. and E. Tirelli (2003). "Facilitatory effect of the dopamine D4 receptor agonist PD168,077 on memory consolidation of an inhibitory avoidance learned response in C57BL/6J mice." Behav Brain Res **142**(1-2): 41-52.

Bertocchi, I., A. Oberto, A. Longo, P. Mele, M. Sabetta, A. Bartolomucci, P. Palanza, R. Sprengel and C. Eva (2011). "Regulatory functions of limbic Y1 receptors in body weight and anxiety uncovered by conditional knockout and maternal care." Proc Natl Acad Sci U S A **108**(48): 19395-19400.

Betel, D., A. Koppal, P. Agius, C. Sander and C. Leslie (2010). "Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites." Genome Biol **11**(8): R90.

Blier, P. (2013). "Neurotransmitter targeting in the treatment of depression." J Clin Psychiatry **74 Suppl 2**: 19-24.

Bliss, T. V. and G. L. Collingridge (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." Nature **361**(6407): 31-39.

Bolivar, V. J. (2009). "Intrasession and Intersession Habituation in Mice: From Inbred Strain Variability to Linkage Analysis." Neurobiology of learning and memory **92**(2): 206-214.

Bonasera, S. J., A. K. Schenk, E. J. Luxenberg, X. Wang, A. Basbaum and L. H. Tecott (2015). "Mice Lacking Serotonin 2C Receptors Have increased Affective Responses to Aversive Stimuli." PLoS One **10**(12): e0142906.

Borsini, F., J. Podhorna and D. Marazziti (2002). "Do animal models of anxiety predict anxiolytic-like effects of antidepressants?" Psychopharmacology (Berl) **163**(2): 121-141.

Bortolato, M., K. Chen and J. C. Shih (2008). "Monoamine oxidase inactivation: from pathophysiology to therapeutics." Adv Drug Deliv Rev **60**(13-14): 1527-1533.

Bourin, M. and M. Hascoet (2003). "The mouse light/dark box test." Eur J Pharmacol **463**(1-3): 55-65.

Bredy, T. W., Q. Lin, W. Wei, D. Baker-Andresen and J. S. Mattick (2011). "MicroRNA regulation of neural plasticity and memory." Neurobiol Learn Mem **96**(1): 89-94.

Browman, K. E., P. Curzon, J. B. Pan, A. L. Molesky, V. A. Komater, M. W. Decker, J. D. Brioni, R. B. Moreland and G. B. Fox (2005). "A-412997, a selective dopamine D4 agonist, improves cognitive performance in rats." Pharmacol Biochem Behav **82**(1): 148-155.

Brown, T. A., L. A. Campbell, C. L. Lehman, J. R. Grisham and R. B. Mancill (2001). "Current and lifetime comorbidity of the DSM-IV anxiety and mood disorders in a large clinical sample." Journal of Abnormal Psychology **110**(4): 585-599.

Burchett, S. A. (2000). "Regulators of G protein signaling: a bestiary of modular protein binding domains." J Neurochem **75**(4): 1335-1351.

Burchett, S. A., M. L. Volk, M. J. Bannon and J. G. Granneman (1998). "Regulators of G protein signaling: rapid changes in mRNA abundance in response to amphetamine." J Neurochem **70**(5): 2216-2219.

Calebiro, D., V. O. Nikolaev, M. C. Gagliani, T. de Filippis, C. Dees, C. Tacchetti, L. Persani and M. J. Lohse (2009). "Persistent cAMP-signals triggered by internalized G-protein-coupled receptors." PLoS Biol **7**(8): e1000172.

Campos, A. C., M. V. Fogaca, D. C. Aguiar and F. S. Guimaraes (2013). "Animal models of anxiety disorders and stress." Rev Bras Psiquiatr **35 Suppl 2**: S101-111.

Can, A., D. T. Dao, M. Arad, C. E. Terrillion, S. C. Piantadosi and T. D. Gould (2012). "The mouse forced swim test." J Vis Exp(59): e3638.

Carobrez, A. P. and L. J. Bertoglio (2005). "Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on." Neurosci Biobehav Rev **29**(8): 1193-1205.

Caspi, A., K. Sugden, T. E. Moffitt, A. Taylor, I. W. Craig, H. Harrington, J. McClay, J. Mill, J. Martin, A. Braithwaite and R. Poulton (2003). "Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene." Science **301**(5631): 386-389.

Cassano, G. B., N. Baldini Rossi and S. Pini (2002). "Psychopharmacology of anxiety disorders." Dialogues Clin Neurosci **4**(3): 271-285.

Cercato, M. C., N. Colettis, M. Snitcofsky, A. I. Aguirre, E. E. Kornisiuk, M. V. Baez and D. A. Jerusalinsky (2014). "Hippocampal NMDA receptors and the previous experience effect on memory." J Physiol Paris **108**(4-6): 263-269.

Chakir, K., W. Zhu, S. Tsang, A. Y. Woo, D. Yang, X. Wang, X. Zeng, M. H. Rhee, U. Mende, N. Koitabashi, E. Takimoto, K. J. Blumer, E. G. Lakatta, D. A. Kass and R. P. Xiao (2011). "RGS2 is a primary terminator of beta(2)-adrenergic receptor-mediated G(i) signaling." J Mol Cell Cardiol **50**(6): 1000-1007.

Chan, R. K. and C. A. Otte (1982). "Isolation and genetic analysis of Saccharomyces cerevisiae mutants supersensitive to G1 arrest by a factor and alpha factor pheromones." Mol Cell Biol **2**(1): 11-20.

Chrousos, G. P. (1998). "Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture." Ann N Y Acad Sci **851**: 311-335.

Cornea-Hebert, V., M. Riad, C. Wu, S. K. Singh and L. Descarries (1999). "Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat." J Comp Neurol **409**(2): 187-209.

Costoli, T., A. Sgoifo, D. Stilli, G. Flugge, W. Adriani, G. Laviola, E. Fuchs, T. Pedrazzini and E. Musso (2005). "Behavioural, neural and cardiovascular adaptations in mice lacking the NPY Y1 receptor." Neuroscience & Biobehavioral Reviews **29**(1): 113-123.

Crawley, J. and F. K. Goodwin (1980). "Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines." Pharmacol Biochem Behav **13**(2): 167-170.

Crawley, J. N., J. K. Belknap, A. Collins, J. C. Crabbe, W. Frankel, N. Henderson, R. J. Hitzemann, S. C. Maxson, L. L. Miner, A. J. Silva, J. M. Wehner, A. Wynshaw-Boris and R. Paylor (1997). "Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies." Psychopharmacology (Berl) **132**(2): 107-124.

Crick, F. H. (1958). "On protein synthesis." Symp Soc Exp Biol **12**: 138-163.

Cryan, J. F. and A. Holmes (2005). "The ascent of mouse: advances in modelling human depression and anxiety." Nat Rev Drug Discov **4**(9): 775-790.

Cui, H., N. Nishiguchi, E. Ivleva, M. Yanagi, M. Fukutake, H. Nushida, Y. Ueno, N. Kitamura, K. Maeda and O. Shirakawa (2008). "Association of RGS2 gene polymorphisms with suicide and increased RGS2 immunoreactivity in the postmortem brain of suicide victims." Neuropsychopharmacology **33**(7): 1537-1544.

Czeh, B., E. Fuchs, O. Wiborg and M. Simon (2016). "Animal models of major depression and their clinical implications." Prog Neuropsychopharmacol Biol Psychiatry **64**: 293-310.

Dahm, R. (2005). "Friedrich Miescher and the discovery of DNA." Dev Biol **278**(2): 274-288.

De Vries, L., M. Mousli, A. Wurmser and M. G. Farquhar (1995). "GAIP, a protein that specifically interacts with the trimeric G protein G alpha i3, is a member of a protein family with a highly conserved core domain." Proceedings of the National Academy of Sciences **92**(25): 11916-11920.

Dell'Osso, B., M. Buoli, D. S. Baldwin and A. C. Altamura (2010). "Serotonin norepinephrine reuptake inhibitors (SNRIs) in anxiety disorders: a comprehensive review of their clinical efficacy." Human Psychopharmacology: Clinical and Experimental **25**(1): 17-29.

Di Matteo, V., G. Di Giovanni, M. Pierucci and E. Esposito (2008). "Serotonin control of central dopaminergic function: focus on in vivo microdialysis studies." Prog Brain Res **172**: 7-44.

Dias, B. G., J. V. Goodman, R. Ahluwalia, A. E. Easton, R. Andero and K. J. Ressler (2014). "Amygdala-dependent fear memory consolidation via miR-34a and Notch signaling." Neuron **83**(4): 906-918.

Dimitrov, E. L., M. R. DeJoseph, M. S. Brownfield and J. H. Urban (2007). "Involvement of neuropeptide Y Y1 receptors in the regulation of neuroendocrine corticotropin-releasing hormone neuronal activity." Endocrinology **148**(8): 3666-3673.

Dixon, R. A., B. K. Kobilka, D. J. Strader, J. L. Benovic, H. G. Dohlman, T. Frielle, M. A. Bolanowski, C. D. Bennett, E. Rands, R. E. Diehl, R. A. Mumford, E. E. Slater, I. S. Sigal, M. G. Caron, R. J. Lefkowitz and C. D. Strader (1986). "Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin." Nature **321**(6065): 75-79.

Dohlman, H. G., D. Apaniesk, Y. Chen, J. Song and D. Nusskern (1995). "Inhibition of G-protein signaling by dominant gain-of-function mutations in Sst2p, a pheromone desensitization factor in Saccharomyces cerevisiae." Mol Cell Biol **15**(7): 3635-3643.

Domschke, K. and J. Deckert (2007). "[Genetics of anxiety disorders. Current clinical and molecular research]." Nervenarzt **78**(7): 825-833; quiz 834-825.

Domschke, K. and J. Deckert (2012). "Genetics of anxiety disorders - status quo and quo vadis." Curr Pharm Des **18**(35): 5691-5698.

Druey, K. M., K. J. Blumer, V. H. Kang and J. H. Kehrl (1996). "Inhibition of G-protein-mediated MAP kinase activation by a new mammalian gene family." Nature **379**(6567): 742-746.

Ducottet, C. and C. Belzung (2004). "Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice." Physiol Behav **81**(3): 417-426.

Dudley, K. J., X. Li, M. S. Kobor, T. E. Kippin and T. W. Bredy (2011). "Epigenetic mechanisms mediating vulnerability and resilience to psychiatric disorders." Neurosci Biobehav Rev **35**(7): 1544-1551.

Ebmeier, K. P., C. Donaghey and J. D. Steele (2006). "Recent developments and current controversies in depression." Lancet **367**(9505): 153-167.

Eddy, S. R. (2001). "Non-coding RNA genes and the modern RNA world." Nat Rev Genet **2**(12): 919-929.

Eva, C., M. Serra, P. Mele, G. Panzica and A. Oberto (2006). "Physiology and gene regulation of the brain NPY Y1 receptor." Front Neuroendocrinol **27**(3): 308-339.

Evans, D. L., D. S. Charney, L. Lewis, R. N. Golden, J. M. Gorman, K. R. Krishnan, C. B. Nemeroff, J. D. Bremner, R. M. Carney, J. C. Coyne, M. R. Delong, N. Frasure-Smith, A. H. Glassman, P. W. Gold, I. Grant, L. Gwyther, G. Ironson, R. L. Johnson, A. M. Kanner, W. J. Katon, P. G. Kaufmann, F. J. Keefe, T. Ketter, T. P. Laughren, J. Leserman, C. G. Lyketsos, W. M. McDonald, B. S. McEwen, A. H. Miller, D. Musselman, C. O'Connor, J. M. Petitto, B. G. Pollock, R. G. Robinson, S. P. Roose, J. Rowland, Y. Sheline, D. S. Sheps, G. Simon, D. Spiegel, A. Stunkard, T. Sunderland, P. Tibbits, Jr. and W. J. Valvo (2005). "Mood disorders in the medically ill: scientific review and recommendations." Biol Psychiatry **58**(3): 175-189.

Faller, H., S. Stork, M. Schowalter, T. Steinbuchel, V. Wollner, G. Ertl and C. E. Angermann (2007). "Depression and survival in chronic heart failure: does gender play a role?" Eur J Heart Fail **9**(10): 1018-1023.

Falzone, T. L., D. M. Gelman, J. I. Young, D. K. Grandy, M. J. Low and M. Rubinstein (2002). "Absence of dopamine D4 receptors results in enhanced reactivity to unconditioned, but not conditioned, fear." Eur J Neurosci **15**(1): 158-164.

Fan, H. M., X. Y. Sun, W. Guo, A. F. Zhong, W. Niu, L. Zhao, Y. H. Dai, Z. M. Guo, L. Y. Zhang and J. Lu (2014). "Differential expression of microRNA in peripheral blood mononuclear cells as specific biomarker for major depressive disorder patients." J Psychiatr Res **59**: 45-52.

Fanselow, M. S. and A. M. Poulos (2005). "The neuroscience of mammalian associative learning." Annu Rev Psychol **56**: 207-234.

Fava, M. and K. S. Kendler (2000). "Major depressive disorder." Neuron **28**(2): 335-341.

File, S. E. (1980). "The use of social interaction as a method for detecting anxiolytic activity of chlordiazepoxide-like drugs." J Neurosci Methods **2**(3): 219-238.

File, S. E. and P. Seth (2003). "A review of 25 years of the social interaction test." Eur J Pharmacol **463**(1-3): 35-53.

Finnegan, E. F. and A. E. Pasquinelli (2013). "MicroRNA biogenesis: regulating the regulators." Crit Rev Biochem Mol Biol **48**(1): 51-68.

Fischer, A., M. Radulovic, C. Schrick, F. Sananbenesi, J. Godovac-Zimmermann and J. Radulovic (2007). "Hippocampal Mek/Erk signaling mediates extinction of contextual freezing behavior." Neurobiol Learn Mem **87**(1): 149-158.

Flint, J. and K. S. Kendler (2014). "The genetics of major depression." Neuron **81**(3): 484-503.

Foster, T. C. (1999). "Involvement of hippocampal synaptic plasticity in age-related memory decline." Brain Res Brain Res Rev **30**(3): 236-249.

French, P. J., V. O'Connor, M. W. Jones, S. Davis, M. L. Errington, K. Voss, B. Truchet, C. Wotjak, T. Stean, V. Doyere, M. Maroun, S. Laroche and T. V. Bliss (2001). "Subfield-specific immediate early gene expression associated with hippocampal long-term potentiation in vivo." Eur J Neurosci **13**(5): 968-976.

Friedman, R. C., K. K. Farh, C. B. Burge and D. P. Bartel (2009). "Most mammalian mRNAs are conserved targets of microRNAs." Genome Res **19**(1): 92-105.

Fulford, A. J. and C. A. Marsden (1997). "Social isolation in the rat enhances alpha 2-autoreceptor function in the hippocampus in vivo." Neuroscience **77**(1): 57-64.

Furth, K. E., S. Mastwal, K. H. Wang, A. Buonanno and D. Vullhorst (2013). "Dopamine, cognitive function, and gamma oscillations: role of D4 receptors." Front Cell Neurosci **7**: 102.

Gao, S., D. Ho, D. E. Vatner and S. F. Vatner (2011). "Echocardiography in Mice." Current protocols in mouse biology **1**: 71-83.

Gelao, B., L. Fazio, P. Selvaggi, A. Di Giorgio, P. Taurisano, T. Quarto, R. Romano, A. Porcelli, M. Mancini, R. Masellis, G. Ursini, G. De Simeis, G. Caforio, L. Ferranti, L. Lo Bianco, A. Rampino, O. Todarello, T. Popolizio, G. Blasi and A. Bertolino (2014). "DRD2 genotype predicts prefrontal activity during working memory after stimulation of D2 receptors with bromocriptine." Psychopharmacology (Berl) **231**(11): 2361-2370.

Gerber, K. J., K. E. Squires and J. R. Hepler (2016). "Roles for Regulator of G Protein Signaling Proteins in Synaptic Signaling and Plasticity." Mol Pharmacol **89**(2): 273-286.

Gilman, A. G. (1987). "G proteins: transducers of receptor-generated signals." Annu Rev Biochem **56**: 615-649.

Gobert, A., J. M. Rivet, F. Lejeune, A. Newman-Tancredi, A. Adhumeau-Auclair, J. P. Nicolas, L. Cistarelli, C. Melon and M. J. Millan (2000). "Serotonin(2C) receptors tonically suppress the activity of mesocortical dopaminergic and adrenergic, but not serotonergic, pathways: a combined dialysis and electrophysiological analysis in the rat." Synapse **36**(3): 205-221.

Gould, T. D. and Gottesman, II (2006). "Psychiatric endophenotypes and the development of valid animal models." Genes Brain Behav **5**(2): 113-119.

Grafstein-Dunn, E., K. H. Young, M. I. Cockett and X. Z. Khawaja (2001). "Regional distribution of regulators of G-protein signaling (RGS) 1, 2, 13, 14, 16, and GAIP messenger ribonucleic acids by in situ hybridization in rat brain." Brain Res Mol Brain Res **88**(1-2): 113-123.

Greenberg, P. E., T. Sisitsky, R. C. Kessler, S. N. Finkelstein, E. R. Berndt, J. R. Davidson, J. C. Ballenger and A. J. Fyer (1999). "The economic burden of anxiety disorders in the 1990s." J Clin Psychiatry **60**(7): 427-435.

Greenwald, E. C. and J. J. Saucerman (2011). "Bigger, better, faster: principles and models of AKAP anchoring protein signaling." J Cardiovasc Pharmacol **58**(5): 462-469.

Greenwood, B. N., R. S. Thompson, M. R. Opp and M. Fleshner (2014). "Repeated Exposure to Conditioned Fear Stress Increases Anxiety and Delays Sleep Recovery Following Exposure to an Acute Traumatic Stressor." Frontiers in Psychiatry **5**: 146.

Griffiths, J., N. Shanks and H. Anisman (1992). "Strain-specific alterations in consumption of a palatable diet following repeated stressor exposure." Pharmacol Biochem Behav **42**(2): 219-227.

Grillon, C., R. Duncko, M. F. Covington, L. Kopperman and M. A. Kling (2007). "Acute stress potentiates anxiety in humans." Biol Psychiatry **62**(10): 1183-1186.

Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim and D. P. Bartel (2007). "MicroRNA targeting specificity in mammals: determinants beyond seed pairing." Mol Cell **27**(1): 91-105.

Gross, M. and A. Pinhasov (2016). "Chronic mild stress in submissive mice: Marked polydipsia and social avoidance without hedonic deficit in the sucrose preference test." Behavioural Brain Research **298, Part B**: 25-34.

Gross, V., J. Tank, M. Obst, R. Plehm, K. J. Blumer, A. Diedrich, J. Jordan and F. C. Luft (2005). "Autonomic nervous system and blood pressure regulation in RGS2-deficient mice." Am J Physiol Regul Integr Comp Physiol **288**(5): R1134-1142.

Gruart, A., R. Leal-Campanario, J. C. Lopez-Ramos and J. M. Delgado-Garcia (2015). "Functional basis of associative learning and its relationships with long-term potentiation evoked in the involved neural circuits: Lessons from studies in behaving mammals." Neurobiol Learn Mem **124**: 3-18.

Gu, S., C. Cifelli, S. Wang and S. P. Heximer (2009). "RGS proteins: identifying new GAPs in the understanding of blood pressure regulation and cardiovascular function." Clin Sci (Lond) **116**(5): 391-399.

Gustavsson, A., M. Svensson, F. Jacobi, C. Allgulander, J. Alonso, E. Beghi, R. Dodel, M. Ekman, C. Faravelli, L. Fratiglioni, B. Gannon, D. H. Jones, P. Jennum, A. Jordanova, L. Jonsson, K. Karampampa, M. Knapp, G. Kobelt, T. Kurth, R. Lieb, M. Linde, C. Ljungcrantz, A. Maercker, B. Melin, M. Moscarelli, A. Musayev, F. Norwood, M. Preisig, M. Pugliatti, J. Rehm, L. Salvador-Carulla, B. Schlehofer, R. Simon, H. C. Steinhausen, L. J. Stovner, J. M. Vallat, P. Van den Bergh, J. van Os, P. Vos, W. Xu, H. U. Wittchen, B. Jonsson and J. Olesen (2011). "Cost of disorders of the brain in Europe 2010." Eur Neuropsychopharmacol **21**(10): 718-779.

Hall, C. S. (1934). "Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality." Journal of Comparative Psychology **18**(3): 385-403.

Hamon, M. and P. Blier (2013). "Monoamine neurocircuitry in depression and strategies for new treatments." Prog Neuropsychopharmacol Biol Psychiatry **45**: 54-63.

Han, J., M. D. Mark, X. Li, M. Xie, S. Waka, J. Rettig and S. Herlitze (2006). "RGS2 determines short-term synaptic plasticity in hippocampal neurons by regulating Gi/o-mediated inhibition of presynaptic Ca2+ channels." Neuron **51**(5): 575-586.

Harkin, A., D. D. Houlihan and J. P. Kelly (2002). "Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine." J Psychopharmacol **16**(2): 115-123.

Harrison, F. E., A. H. Hosseini and M. P. McDonald (2009). "Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks." Behavioural brain research **198**(1): 247-251.

Hasler, G. (2010). "PATHOPHYSIOLOGY OF DEPRESSION: DO WE HAVE ANY SOLID EVIDENCE." World Psychiatry **9**(3): 155-161.

Hasler, G., W. C. Drevets, H. K. Manji and D. S. Charney (2004). "Discovering endophenotypes for major depression." Neuropsychopharmacology **29**(10): 1765-1781.

Heilig, M. (2004). "The NPY system in stress, anxiety and depression." Neuropeptides **38**(4): 213-224.

Heisler, L. K., L. Zhou, P. Bajwa, J. Hsu and L. H. Tecott (2007). "Serotonin 5-HT(2C) receptors regulate anxiety-like behavior." Genes Brain Behav **6**(5): 491-496.

Hettema, J. M., C. Sun, X. Chen and K. S. Kendler (2013). "Genetic association study between RGS2 and anxiety-related phenotypes." Psychiatr Genet **23**(2): 92.

Heximer, S. P., R. H. Knutsen, X. Sun, K. M. Kaltenbronn, M. H. Rhee, N. Peng, A. Oliveira-dos-Santos, J. M. Penninger, A. J. Muslin, T. H. Steinberg, J. M. Wyss, R. P. Mecham and K. J. Blumer (2003). "Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice." J Clin Invest **111**(4): 445-452.

Heximer, S. P., S. P. Srinivasa, L. S. Bernstein, J. L. Bernard, M. E. Linder, J. R. Hepler and K. J. Blumer (1999). "G protein selectivity is a determinant of RGS2 function." J Biol Chem **274**(48): 34253-34259.

Heximer, S. P., N. Watson, M. E. Linder, K. J. Blumer and J. R. Hepler (1997). "RGS2/G0S8 is a selective inhibitor of Gqalpha function." Proc Natl Acad Sci U S A **94**(26): 14389-14393.

Hoffman, D. L., E. M. Dukes and H. U. Wittchen (2008). "Human and economic burden of generalized anxiety disorder." Depress Anxiety **25**(1): 72-90.

Hogg, S. (1996). "A review of the validity and variability of the elevated plus-maze as an animal model of anxiety." Pharmacol Biochem Behav **54**(1): 21-30.

Hohoff, C., H. Weber, J. Richter, K. Domschke, P. M. Zwanzger, P. Ohrmann, J. Bauer, T. Suslow, H. Kugel, C. Baumann, B. Klauke, C. P. Jacob, J. Fritze, B. Bandelow, A. T. Gloster, A. L. Gerlach, T. Kircher, T. Lang, G. W. Alpers, A. Strohle, L. Fehm, H. U. Wittchen, V. Arolt, P. Pauli, A. Hamm, A. Reif and J. Deckert (2015). "RGS2 ggenetic variation: association analysis with panic disorder and dimensional as well as intermediate phenotypes of anxiety." Am J Med Genet B Neuropsychiatr Genet **168b**(3): 211-222.

Holley, R. W., J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick and A. Zamir (1965). "STRUCTURE OF A RIBONUCLEIC ACID." Science **147**(3664): 1462-1465.

Hommers, L., A. Raab, A. Bohl, H. Weber, C. J. Scholz, A. Erhardt, E. Binder, V. Arolt, A. Gerlach, A. Gloster, R. Kalisch, T. Kircher, T. Lonsdorf, A. Strohle, P. Zwanzger, M. Mattheisen, S. Cichon, K. P. Lesch, K. Domschke, A. Reif, M. J. Lohse and J. Deckert (2015). "MicroRNA hsa-miR-4717-5p regulates RGS2 and may be a risk factor for anxiety-related traits." Am J Med Genet B Neuropsychiatr Genet **168b**(4): 296-306.

Hommers, L. G., K. Domschke and J. Deckert (2015). "Heterogeneity and individuality: microRNAs in mental disorders." J Neural Transm (Vienna) **122**(1): 79-97.

Huntzinger, E. and E. Izaurralde (2011). "Gene silencing by microRNAs: contributions of translational repression and mRNA decay." Nat Rev Genet **12**(2): 99-110.

Hutchison, R. M., P. Chidiac and L. S. Leung (2009). "Hippocampal long-term potentiation is enhanced in urethane-anesthetized RGS2 knockout mice." Hippocampus **19**(8): 687-691.

Hutson, P. H., C. L. Barton, M. Jay, P. Blurton, F. Burkamp, R. Clarkson and L. J. Bristow (2000). "Activation of mesolimbic dopamine function by phencyclidine is enhanced by 5-HT(2C/2B) receptor antagonists: neurochemical and behavioural studies." Neuropharmacology **39**(12): 2318-2328.

Ikeda, S. R. (1996). "Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits." Nature **380**(6571): 255-258.

Ilani, T., D. Ben-Shachar, R. D. Strous, M. Mazor, A. Sheinkman, M. Kotler and S. Fuchs (2001). "A peripheral marker for schizophrenia: Increased levels of D3 dopamine receptor mRNA in blood lymphocytes." Proc Natl Acad Sci U S A **98**(2): 625-628.

Illes, P. and J. T. Regenold (1990). "Interaction between neuropeptide Y and noradrenaline on central catecholamine neurons." Nature **344**(6261): 62-63.

Ingi, T. and Y. Aoki (2002). "Expression of RGS2, RGS4 and RGS7 in the developing postnatal brain." Eur J Neurosci **15**(5): 929-936.

Ingi, T., A. M. Krumins, P. Chidiac, G. M. Brothers, S. Chung, B. E. Snow, C. A. Barnes, A. A. Lanahan, D. P. Siderovski, E. M. Ross, A. G. Gilman and P. F. Worley (1998). "Dynamic regulation of RGS2 suggests a novel mechanism in G-protein signaling and neuronal plasticity." J Neurosci **18**(18): 7178-7188.

Issler, O. and A. Chen (2015). "Determining the role of microRNAs in psychiatric disorders." Nat Rev Neurosci **16**(4): 201-212.

Issler, O., S. Haramati, E. D. Paul, H. Maeno, I. Navon, R. Zwang, S. Gil, H. S. Mayberg, B. W. Dunlop, A. Menke, R. Awatramani, E. B. Binder, E. S. Deneris, C. A. Lowry and A. Chen (2014). "MicroRNA 135 is essential for chronic stress resiliency, antidepressant efficacy, and intact serotonergic activity." Neuron **83**(2): 344-360.

Izquierdo, I., C. R. Furini and J. C. Myskiw (2016). "Fear Memory." Physiol Rev **96**(2): 695-750.

Jarvis, S. E. and G. W. Zamponi (2001). "Interactions between presynaptic Ca2+ channels, cytoplasmic messengers and proteins of the synaptic vesicle release complex." Trends Pharmacol Sci **22**(10): 519-525.

Judd, L. L., R. C. Kessler, M. P. Paulus, P. V. Zeller, H. U. Wittchen and J. L. Kunovac (1998). "Comorbidity as a fundamental feature of generalized anxiety disorders: results from the National Comorbidity Study (NCS)." Acta Psychiatr Scand Suppl **393**: 6-11.

Jung, Y. H., S. I. Hong, S. X. Ma, J. Y. Hwang, J. S. Kim, J. H. Lee, J. Y. Seo, S. Y. Lee and C. G. Jang (2014). "Strain differences in the chronic mild stress animal model of depression and anxiety in mice." Biomol Ther (Seoul) **22**(5): 453-459.

Kaidanovich-Beilin, O., T. Lipina, I. Vukobradovic, J. Roder and J. R. Woodgett (2011). "Assessment of social interaction behaviors." J Vis Exp(48).

Kajikawa, Y., N. Saitoh and T. Takahashi (2001). "GTP-binding protein beta gamma subunits mediate presynaptic calcium current inhibition by GABA(B) receptor." Proc Natl Acad Sci U S A **98**(14): 8054-8058.

Karl, T., T. H. Burne and H. Herzog (2006). "Effect of Y1 receptor deficiency on motor activity, exploration, and anxiety." Behav Brain Res **167**(1): 87-93.

Karlsson, R. M., J. S. Choe, H. A. Cameron, A. Thorsell, J. N. Crawley, A. Holmes and M. Heilig (2008). "The neuropeptide Y Y1 receptor subtype is necessary for the anxiolytic-like effects of neuropeptide Y, but not the antidepressant-like effects of fluoxetine, in mice." Psychopharmacology (Berl) **195**(4): 547-557.

Kask, A., J. Harro, S. von Horsten, J. P. Redrobe, Y. Dumont and R. Quirion (2002). "The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y." Neurosci Biobehav Rev **26**(3): 259-283.

Katritch, V., V. Cherezov and R. C. Stevens (2013). "Structure-Function of the G-protein-Coupled Receptor Superfamily." Annual review of pharmacology and toxicology **53**: 531-556.

Katz, R. J. (1981). "Animal models and human depressive disorders." Neurosci Biobehav Rev **5**(2): 231-246.

Kaufman, J. and D. Charney (2000). "Comorbidity of mood and anxiety disorders." Depression and Anxiety **12**(S1): 69-76.

Kehrl, J. H. and S. Sinnarajah (2002). "RGS2: a multifunctional regulator of G-protein signaling." Int J Biochem Cell Biol **34**(5): 432-438.

Kessler, R. C., P. Berglund, O. Demler, R. Jin, D. Koretz, K. R. Merikangas, A. J. Rush, E. E. Walters and P. S. Wang (2003). "The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R)." Jama **289**(23): 3095-3105.

Kessler, R. C., P. Berglund, O. Demler, R. Jin, K. R. Merikangas and E. E. Walters (2005). "Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication." Arch Gen Psychiatry **62**(6): 593-602.

Kim, J. J. and M. W. Jung (2006). "Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review." Neurosci Biobehav Rev **30**(2): 188-202.

Kim, J. J., E. Y. Song and T. A. Kosten (2006). "Stress effects in the hippocampus: synaptic plasticity and memory." Stress **9**(1): 1-11.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat Rev Mol Cell Biol **10**(2): 126-139.

Klemfuss, H., S. Southerland and K. T. Britton (1998). "Cardiovascular Actions of Neuropeptide Y and Social Stress." Peptides **19**(1): 85-92.

Koenen, K. C., A. B. Amstadter, K. J. Ruggiero, R. Acierno, S. Galea, D. G. Kilpatrick and J. Gelernter (2009). "RGS2 and generalized anxiety disorder in an epidemiologic sample of hurricane-exposed adults." Depress Anxiety **26**(4): 309-315.

Komada, M., K. Takao and T. Miyakawa (2008). "Elevated plus maze for mice." J Vis Exp(22).

Konopka, W., G. Schutz and L. Kaczmarek (2011). "The microRNA contribution to learning and memory." Neuroscientist **17**(5): 468-474.

Konstam, V., D. K. Moser and M. J. De Jong (2005). "Depression and anxiety in heart failure." J Card Fail **11**(6): 455-463.

Kozomara, A. and S. Griffiths-Jones (2014). "miRBase: annotating high confidence microRNAs using deep sequencing data." Nucleic Acids Res **42**(Database issue): D68-73.

Krege, J. H., J. B. Hodgin, J. R. Hagaman and O. Smithies (1995). "A noninvasive computerized tail-cuff system for measuring blood pressure in mice." Hypertension **25**(5): 1111-1115.

Kunnecke, B., P. Verry, A. Benardeau and M. von Kienlin (2004). "Quantitative body composition analysis in awake mice and rats by magnetic resonance relaxometry." Obes Res **12**(10): 1604-1615.

Kurian, M. A., P. Gissen, M. Smith, S. Heales, Jr. and P. T. Clayton (2011). "The monoamine neurotransmitter disorders: an expanding range of neurological syndromes." Lancet Neurol **10**(8): 721-733.

Labouebe, G., M. Lomazzi, H. G. Cruz, C. Creton, R. Lujan, M. Li, Y. Yanagawa, K. Obata, M. Watanabe, K. Wickman, S. B. Boyer, P. A. Slesinger and C. Luscher (2007). "RGS2 modulates coupling between GABAB receptors and GIRK channels in dopamine neurons of the ventral tegmental area." Nat Neurosci **10**(12): 1559-1568.

Lagerstrom, M. C. and H. B. Schioth (2008). "Structural diversity of G protein-coupled receptors and significance for drug discovery." Nat Rev Drug Discov **7**(4): 339-357.

Lahdesmaki, J., J. Sallinen, E. MacDonald, B. K. Kobilka, V. Fagerholm and M. Scheinin (2002). "Behavioral and neurochemical characterization of alpha(2A)-adrenergic receptor knockout mice." Neuroscience **113**(2): 289-299.

Lakhlani, P. P., L. B. MacMillan, T. Z. Guo, B. A. McCool, D. M. Lovinger, M. Maze and L. E. Limbird (1997). "Substitution of a mutant alpha2a-adrenergic receptor via "hit and run" gene targeting reveals the role of this subtype in sedative, analgesic, and anesthetic-sparing responses in vivo." Proc Natl Acad Sci U S A **94**(18): 9950-9955.

Le Foll, B., A. Gallo, Y. Le Strat, L. Lu and P. Gorwood (2009). "Genetics of dopamine receptors and drug addiction: a comprehensive review." Behav Pharmacol **20**(1): 1-17.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-854.

Leygraf, A., C. Hohoff, C. Freitag, S. A. Willis-Owen, P. Krakowitzky, J. Fritze, P. Franke, B. Bandelow, R. Fimmers, J. Flint and J. Deckert (2006). "Rgs 2 gene polymorphisms as modulators of anxiety in humans?" J Neural Transm (Vienna) **113**(12): 1921-1925.

Lifschytz, T., E. C. Broner, P. Zozulinsky, A. Slonimsky, R. Eitan, L. Greenbaum and B. Lerer (2012). "Relationship between Rgs2 gene expression level and anxiety and depression-like behaviour in a mutant mouse model: serotonergic involvement." Int J Neuropsychopharmacol **15**(9): 1307-1318.

Lin, Q., W. Wei, C. M. Coelho, X. Li, D. Baker-Andresen, K. Dudley, V. S. Ratnu, Z. Boskovic, M. S. Kobor, Y. E. Sun and T. W. Bredy (2011). "The brain-specific microRNA miR-128b regulates the formation of fear-extinction memory." Nat Neurosci **14**(9): 1115-1117.

Lin, S., D. Boey and H. Herzog (2004). "NPY and Y receptors: lessons from transgenic and knockout models." Neuropeptides **38**(4): 189-200.

Lister, R. G. (1987). "The use of a plus-maze to measure anxiety in the mouse." Psychopharmacology (Berl) **92**(2): 180-185.

Lister, R. G. (1990). "Ethologically-based animal models of anxiety disorders." Pharmacology & Therapeutics **46**(3): 321-340.

Lonergan, M. E., G. M. Gafford, T. J. Jarome and F. J. Helmstetter (2010). "Time-dependent expression of Arc and zif268 after acquisition of fear conditioning." Neural Plast **2010**: 139891.

Longo, A., P. Mele, I. Bertocchi, A. Oberto, A. Bachmann, A. Bartolomucci, P. Palanza, R. Sprengel and C. Eva (2014). "Conditional inactivation of neuropeptide Y Y1 receptors unravels the role of Y1 and Y5 receptors coexpressing neurons in anxiety." Biol Psychiatry **76**(11): 840-849.

Lopez-Gimenez, J. F., M. T. Vilaro, J. M. Palacios and G. Mengod (2001). "Mapping of 5-HT2A receptors and their mRNA in monkey brain: [3H]MDL100,907 autoradiography and in situ hybridization studies." J Comp Neurol **429**(4): 571-589.

Lopez, J. P., R. Lim, C. Cruceanu, L. Crapper, C. Fasano, B. Labonte, G. Maussion, J. P. Yang, V. Yerko, E. Vigneault, S. El Mestikawy, N. Mechawar, P. Pavlidis and G. Turecki (2014). "miR-1202 is a primate-specific and brain-enriched microRNA involved in major depression and antidepressant treatment." Nat Med **20**(7): 764-768.

Lu, T. P., C. Y. Lee, M. H. Tsai, Y. C. Chiu, C. K. Hsiao, L. C. Lai and E. Y. Chuang (2012). "miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets." PLoS One **7**(8): e42390.

Lucki, I. (1997). "The forced swimming test as a model for core and component behavioral effects of antidepressant drugs." Behav Pharmacol **8**(6-7): 523-532.

Lundstrom, K. (2006). "Latest development in drug discovery on G protein-coupled receptors." Curr Protein Pept Sci **7**(5): 465-470.

Lüscher, C. and P. A. Slesinger (2010). "Emerging concepts for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease." Nature reviews. Neuroscience **11**(5): 301-315.

Ma, X. C., D. Jiang, W. H. Jiang, F. Wang, M. Jia, J. Wu, K. Hashimoto, Y. H. Dang and C. G. Gao (2011). "Social isolation-induced aggression potentiates anxiety and depressive-like behavior in male mice subjected to unpredictable chronic mild stress." PLoS One **6**(6): e20955.

Magalhaes, A. C., H. Dunn and S. S. Ferguson (2012). "Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins." Br J Pharmacol **165**(6): 1717-1736.

Mark, M. D., S. Wittemann and S. Herlitze (2000). "G protein modulation of recombinant P/Q-type calcium channels by regulators of G protein signalling proteins." J Physiol **528 Pt 1**: 65-77.

Martin-Guerrero, I., L. F. Callado, K. Saitua, G. Rivero, A. Garcia-Orad and J. J. Meana (2006). "The N251K functional polymorphism in the alpha(2A)-adrenoceptor gene is not associated with depression: a study in suicide completers." Psychopharmacology (Berl) **184**(1): 82-86.

Martin, K. F., I. Phillips, M. Hearson, M. R. Prow and D. J. Heal (1992). "Characterization of 8-OH-DPAT-induced hypothermia in mice as a 5-HT1A autoreceptor response and its evaluation as a model to selectively identify antidepressants." British Journal of Pharmacology **107**(1): 15-21.

Mattick, J. S. (2001). "Non-coding RNAs: the architects of eukaryotic complexity." EMBO Rep **2**(11): 986-991.

Mattick, J. S. (2003). "Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms." Bioessays **25**(10): 930-939.

Mattick, J. S. and I. V. Makunin (2006). "Non-coding RNA." Hum Mol Genet **15 Spec No 1**: R17-29.

Maximino, C., T. M. de Brito and A. Gouveia Jr (2010). "Construct validity of behavioral models of anxiety: Where experimental psychopathology meets ecology and evolution." Psychology & Neuroscience **3**(1): 117-123.

Mazzeo, A. T., A. Micalizzi, L. Mascia, A. Scicolone, L. Siracusano and R. P. Mahajan (2014). "Brain–heart crosstalk: the many faces of stress-related cardiomyopathy syndromes in anaesthesia and intensive care." BJA: British Journal of Anaesthesia **112**(5): 803-815.

McDevitt, R. A. and J. F. Neumaier (2011). "Regulation of dorsal raphe nucleus function by serotonin autoreceptors: a behavioral perspective." Journal of chemical neuroanatomy **41**(4): 234-246.

McKinney, W. T. (1984). "Animal models of depression: an overview." Psychiatr Dev **2**(2): 77-96.

McLean, C. P., A. Asnaani, B. T. Litz and S. G. Hofmann (2011). "Gender Differences in Anxiety Disorders: Prevalence, Course of Illness, Comorbidity and Burden of Illness." Journal of psychiatric research **45**(8): 1027-1035.

Mendlowicz, M. V. and M. B. Stein (2000). "Quality of life in individuals with anxiety disorders." Am J Psychiatry **157**(5): 669-682.

Micale, V., L. Cristino, A. Tamburella, S. Petrosino, G. M. Leggio, V. Di Marzo and F. Drago (2010). "Enhanced cognitive performance of dopamine D3 receptor "knock-out" mice in the step-through passive-avoidance test: assessing the role of the endocannabinoid/endovanilloid systems." Pharmacol Res **61**(6): 531-536.

Millan, M. J. (2003). "The neurobiology and control of anxious states." Prog Neurobiol **70**(2): 83-244.

Minatohara, K., M. Akiyoshi and H. Okuno (2015). "Role of Immediate-Early Genes in Synaptic Plasticity and Neuronal Ensembles Underlying the Memory Trace." Front Mol Neurosci **8**: 78.

Minatohara, K., M. Akiyoshi and H. Okuno (2015). "Role of Immediate-Early Genes in Synaptic Plasticity and Neuronal Ensembles Underlying the Memory Trace." Frontiers in Molecular Neuroscience **8**: 78.

Mineur, Y. S., C. Belzung and W. E. Crusio (2006). "Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice." Behavioural Brain Research **175**(1): 43-50.

Missale, C., S. R. Nash, S. W. Robinson, M. Jaber and M. G. Caron (1998). "Dopamine receptors: from structure to function." Physiol Rev **78**(1): 189-225.

Monleon, S., P. D'Aquila, A. Parra, V. M. Simon, P. F. Brain and P. Willner (1995). "Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine." Psychopharmacology (Berl) **117**(4): 453-457.

Morici, J. F., L. Ciccia, G. Malleret, J. A. Gingrich, P. Bekinschtein and N. V. Weisstaub (2015). "Serotonin 2a Receptor and Serotonin 1a Receptor Interact Within the Medial Prefrontal Cortex During Recognition Memory in Mice." Front Pharmacol **6**: 298.

Morris, R. (1984). "Developments of a water-maze procedure for studying spatial learning in the rat." J Neurosci Methods **11**(1): 47-60.

Morrissette, D. A. and S. M. Stahl (2014). "Modulating the serotonin system in the treatment of major depressive disorder." CNS Spectrums **19**(S1): 54-68.

Moser, M. B. (1999). "Making more synapses: a way to store information?" Cell Mol Life Sci **55**(4): 593-600.

Mouri, K., A. Hishimoto, M. Fukutake, N. Nishiguchi, O. Shirakawa and K. Maeda (2010). "Association study of RGS2 gene polymorphisms with panic disorder in Japanese." Kobe J Med Sci **55**(5): E116-121.

Moy, S. S., J. J. Nadler, A. Perez, R. P. Barbaro, J. M. Johns, T. R. Magnuson, J. Piven and J. N. Crawley (2004). "Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice." Genes Brain Behav **3**(5): 287-302.

Muinos-Gimeno, M., Y. Espinosa-Parrilla, M. Guidi, B. Kagerbauer, T. Sipila, E. Maron, K. Pettai, L. Kananen, R. Navines, R. Martin-Santos, M. Gratacos, A. Metspalu, I. Hovatta and X. Estivill (2011). "Human microRNAs miR-22, miR-138-2, miR-148a, and miR-488 are associated with panic disorder and regulate several anxiety candidate genes and related pathways." Biol Psychiatry **69**(6): 526-533.

Nakajima, S., P. Gerretsen, H. Takeuchi, F. Caravaggio, T. Chow, B. Le Foll, B. Mulsant, B. Pollock and A. Graff-Guerrero (2013). "The potential role of dopamine D(3) receptor neurotransmission in cognition." European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology **23**(8): 799-813.

Nance, M. R., B. Kreutz, V. M. Tesmer, R. Sterne-Marr, T. Kozasa and J. J. Tesmer (2013). "Structural and functional analysis of the regulator of G protein signaling 2-galphaq complex." Structure **21**(3): 438-448.

Nemeroff, C. B. (1998). "The neurobiology of depression." Sci Am **278**(6): 42-49.

Nemeroff, C. B. (2002). "Comorbidity of Mood and Anxiety Disorders: The Rule, Not the Exception?" American Journal of Psychiatry **159**(1): 3-4.

Ng, J., A. Papandreou, S. J. Heales and M. A. Kurian (2015). "Monoamine neurotransmitter disorders--clinical advances and future perspectives." Nat Rev Neurol **11**(10): 567-584.

Nguyen, P. V., T. Abel, E. R. Kandel and R. Bourtchouladze (2000). "Strain-dependent Differences in LTP and Hippocampus-dependent Memory in Inbred Mice." Learning & Memory **7**(3): 170-179.

Nunn, C., M. X. Zou, A. J. Sobiesiak, A. A. Roy, L. A. Kirshenbaum and P. Chidiac (2010). "RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy." Cell Signal **22**(8): 1231-1239.

Okada, Y., K. Tachibana, S. Yanagita and K. Takeda (2013). "Prenatal exposure to zinc oxide particles alters monoaminergic neurotransmitter levels in the brain of mouse offspring." J Toxicol Sci **38**(3): 363-370.

Okimoto, N., O. J. Bosch, D. A. Slattery, K. Pflaum, H. Matsushita, F. Y. Wei, M. Ohmori, T. Nishiki, I. Ohmori, Y. Hiramatsu, H. Matsui, I. D. Neumann and K. Tomizawa (2012). "RGS2 mediates the anxiolytic effect of oxytocin." Brain Res **1453**: 26-33.

Olesen, M. V., S. H. Christiansen, C. R. Gotzsche, L. Nikitidou, M. Kokaia and D. P. Woldbye (2012). "Neuropeptide Y Y1 receptor hippocampal overexpression via viral vectors is associated with modest anxiolytic-like and proconvulsant effects in mice." J Neurosci Res **90**(2): 498-507.

Oliveira-Dos-Santos, A. J., G. Matsumoto, B. E. Snow, D. Bai, F. P. Houston, I. Q. Whishaw, S. Mariathasan, T. Sasaki, A. Wakeham, P. S. Ohashi, J. C. Roder, C. A. Barnes, D. P. Siderovski and J. M. Penninger (2000). "Regulation of T cell activation, anxiety, and male aggression by RGS2." Proc Natl Acad Sci U S A **97**(22): 12272-12277.

Otowa, T., T. Shimada, Y. Kawamura, N. Sugaya, E. Yoshida, K. Inoue, S. Yasuda, X. Liu, T. Minato, M. Tochigi, T. Umekage, K. Kasai, H. Tanii, Y. Okazaki, H. Kaiya and T. Sasaki (2011). "Association of RGS2 variants with panic disorder in a Japanese population." Am J Med Genet B Neuropsychiatr Genet **156b**(4): 430-434.

Otsuka, A., T. Shiuchi, S. Chikahisa, N. Shimizu and H. Séi (2015). "Voluntary exercise and increased food intake after mild chronic stress improve social avoidance behavior in mice." Physiology & Behavior **151**: 264-271.

Painsipp, E., G. Sperk, H. Herzog and P. Holzer (2010). "Delayed stress-induced differences in locomotor and depression-related behaviour in female neuropeptide-Y Y1 receptor knockout mice." J Psychopharmacol **24**(10): 1541-1549.

Palanza, P. (2001). "Animal models of anxiety and depression: how are females different?" Neurosci Biobehav Rev **25**(3): 219-233.

Palczewski, K., T. Kumasaka, T. Hori, C. A. Behnke, H. Motoshima, B. A. Fox, I. Le Trong, D. C. Teller, T. Okada, R. E. Stenkamp, M. Yamamoto and M. Miyano (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science **289**(5480): 739-745.

Paraskevopoulou, M. D., G. Georgakilas, N. Kostoulas, I. S. Vlachos, T. Vergoulis, M. Reczko, C. Filippidis, T. Dalamagas and A. G. Hatzigeorgiou (2013). "DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows." Nucleic Acids Res **41**(Web Server issue): W169-173.

Pasquinelli, A. E. (2012). "MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship." Nat Rev Genet **13**(4): 271-282.

Pasquinelli, A. E., B. J. Reinhart, F. Slack, M. Q. Martindale, M. I. Kuroda, B. Maller, D. C. Hayward, E. E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson and G. Ruvkun (2000). "Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA." Nature **408**(6808): 86-89.

Patel, T. B. (2004). "Single transmembrane spanning heterotrimeric g protein-coupled receptors and their signaling cascades." Pharmacol Rev **56**(3): 371-385.

Paul, C. M., G. Magda and S. Abel (2009). "Spatial memory: Theoretical basis and comparative review on experimental methods in rodents." Behav Brain Res **203**(2): 151-164.

Pellow, S. and S. E. File (1986). "Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat." Pharmacol Biochem Behav **24**(3): 525-529.

Pierce, K. L., R. T. Premont and R. J. Lefkowitz (2002). "Seven-transmembrane receptors." Nat Rev Mol Cell Biol **3**(9): 639-650.

Pompeiano, M., J. M. Palacios and G. Mengod (1994). "Distribution of the serotonin 5-HT2 receptor family mRNAs: comparison between 5-HT2A and 5-HT2C receptors." Brain Res Mol Brain Res **23**(1-2): 163-178.

Porsolt, R. D., M. Le Pichon and M. Jalfre (1977). "Depression: a new animal model sensitive to antidepressant treatments." Nature **266**(5604): 730-732.

Post, A. M., P. Weyers, P. Holzer, E. Painsipp, P. Pauli, T. Wultsch, A. Reif and K. P. Lesch (2011). "Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models." Behav Brain Res **218**(1): 99-105.

Pothion, S., J. C. Bizot, F. Trovero and C. Belzung (2004). "Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress." Behav Brain Res **155**(1): 135-146.

Premont, R. T. and R. R. Gainetdinov (2007). "Physiological roles of G protein-coupled receptor kinases and arrestins." Annu Rev Physiol **69**: 511-534.

Prieto, D., C. L. Buus, M. J. Mulvany and H. Nilsson (2000). "Neuropeptide Y regulates intracellular calcium through different signalling pathways linked to a Y(1)-receptor in rat mesenteric small arteries." British Journal of Pharmacology **129**(8): 1689-1699.

Primeaux, S. D., S. P. Wilson, M. C. Cusick, D. A. York and M. A. Wilson (2005). "Effects of altered amygdalar neuropeptide Y expression on anxiety-related behaviors." Neuropsychopharmacology **30**(9): 1589-1597.

Prut, L. and C. Belzung (2003). "The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review." Eur J Pharmacol **463**(1-3): 3-33.

Rasmussen, S. G., H. J. Choi, D. M. Rosenbaum, T. S. Kobilka, F. S. Thian, P. C. Edwards, M. Burghammer, V. R. Ratnala, R. Sanishvili, R. F. Fischetti, G. F. Schertler, W. I. Weis and B. K. Kobilka (2007). "Crystal structure of the human beta2 adrenergic G-protein-coupled receptor." Nature **450**(7168): 383-387.

Reinhart, B. J., F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun (2000). "The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans." Nature **403**(6772): 901-906.

Renshaw, D., L. M. Thomson, M. Carroll, S. Kapas and J. P. Hinson (2000). "Actions of neuropeptide Y on the rat adrenal cortex." Endocrinology **141**(1): 169-173.

Riddle, E. L., R. A. Schwartzman, M. Bond and P. A. Insel (2005). "Multi-tasking RGS proteins in the heart: the next therapeutic target?" Circ Res **96**(4): 401-411.

Robinet, E. A., M. Geurts, J. M. Maloteaux and P. J. Pauwels (2001). "Chronic treatment with certain antipsychotic drugs preserves upregulation of regulator of G-protein signalling 2 mRNA in rat striatum as opposed to c-fos mRNA." Neurosci Lett **307**(1): 45-48.

Rocchetti, J., E. Isingrini, G. Dal Bo, S. Sagheby, A. Menegaux, F. Tronche, D. Levesque, L. Moquin, A. Gratton, T. P. Wong, M. Rubinstein and B. Giros (2015). "Presynaptic D2 dopamine receptors control long-term depression expression and memory processes in the temporal hippocampus." Biol Psychiatry **77**(6): 513-525.

Rocha, B. A., E. H. Goulding, L. E. O'Dell, A. N. Mead, N. G. Coufal, L. H. Parsons and L. H. Tecott (2002). "Enhanced locomotor, reinforcing, and neurochemical effects of cocaine in serotonin 5-hydroxytryptamine 2C receptor mutant mice." J Neurosci **22**(22): 10039-10045.

Rodrigues, S. M., G. E. Schafe and J. E. LeDoux (2004). "Molecular mechanisms underlying emotional learning and memory in the lateral amygdala." Neuron **44**(1): 75-91.

Rosenbaum, D. M., V. Cherezov, M. A. Hanson, S. G. Rasmussen, F. S. Thian, T. S. Kobilka, H. J. Choi, X. J. Yao, W. I. Weis, R. C. Stevens and B. K. Kobilka (2007). "GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function." Science **318**(5854): 1266-1273.

Rosenbaum, D. M., S. G. Rasmussen and B. K. Kobilka (2009). "The structure and function of G-protein-coupled receptors." Nature **459**(7245): 356-363.

Rosenfeld, C. S. and S. A. Ferguson (2014). "Barnes maze testing strategies with small and large rodent models." J Vis Exp(84): e51194.

Ross, E. M. and T. M. Wilkie (2000). "GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins." Annu Rev Biochem **69**: 795-827.

Roy, A. A., C. Nunn, H. Ming, M. X. Zou, J. Penninger, L. A. Kirshenbaum, S. J. Dixon and P. Chidiac (2006). "Up-regulation of endogenous RGS2 mediates cross-desensitization between Gs and Gq signaling in osteoblasts." J Biol Chem **281**(43): 32684-32693.

Saab, B. J., J. Georgiou, A. Nath, F. J. S. Lee, M. Wang, A. Michalon, F. Liu, I. M. Mansuy and J. C. Roder (2009). "NCS-1 in the Dentate Gyrus Promotes Exploration, Synaptic Plasticity, and Rapid Acquisition of Spatial Memory." Neuron **63**(5): 643-656.

Sartori, M., G. Ceolotto, F. Dorigatti, L. Mos, M. Santonastaso, P. Bratti, I. Papparella, A. Semplicini and P. Palatini (2008). "RGS2 C1114G polymorphism and body weight gain in hypertensive patients." Metabolism **57**(3): 421-427.

Saveanu, R. V. and C. B. Nemeroff (2012). "Etiology of Depression: Genetic and Environmental Factors." Psychiatric Clinics of North America **35**(1): 51-71.

Schramm, N. L., M. P. McDonald and L. E. Limbird (2001). "The alpha(2a)-adrenergic receptor plays a protective role in mouse behavioral models of depression and anxiety." J Neurosci **21**(13): 4875-4882.

Schratt, G. M., F. Tuebing, E. A. Nigh, C. G. Kane, M. E. Sabatini, M. Kiebler and M. E. Greenberg (2006). "A brain-specific microRNA regulates dendritic spine development." Nature **439**(7074): 283-289.

Seibenhener, M. L. and M. C. Wooten (2015). "Use of the Open Field Maze to measure locomotor and anxiety-like behavior in mice." J Vis Exp(96): e52434.

Semplicini, A., L. Lenzini, M. Sartori, I. Papparella, L. A. Calo, E. Pagnin, G. Strapazzon, C. Benna, R. Costa, A. Avogaro, G. Ceolotto and A. C. Pessina (2006). "Reduced expression of regulator of G-protein signaling 2 (RGS2) in hypertensive patients increases calcium mobilization and ERK1/2 phosphorylation induced by angiotensin II." J Hypertens **24**(6): 1115-1124.

Serafini, G., M. Pompili, M. Innamorati, G. Iacorossi, I. Cuomo, M. Della Vista, D. Lester, L. De Biase, P. Girardi and R. Tatarelli (2010). "The impact of anxiety, depression, and suicidality on quality of life and functional status of patients with congestive heart failure and hypertension: an observational cross-sectional study." Prim Care Companion J Clin Psychiatry **12**(6).

Sethakorn, N., D. M. Yau and N. O. Dulin (2010). "Non-canonical functions of RGS proteins." Cell Signal **22**(9): 1274-1281.

Shariff, A. F. and J. L. Tracy (2011). "What Are Emotion Expressions For?" Current Directions in Psychological Science **20**(6): 395-399.

Sharma, S., S. Rakoczy and H. Brown-Borg (2010). "Assessment of spatial memory in mice." Life Sci **87**(17-18): 521-536.

Shein, H. M. and J. F. Enders (1962). "TRANSFORMATION INDUCED BY SIMIAN VIRUS 40 IN HUMAN RENAL CELL CULTURES, I. MORPHOLOGY AND GROWTH CHARACTERISTICS(*)." Proceedings of the National Academy of Sciences of the United States of America **48**(7): 1164-1172.

Siderovski, D. P., A. Hessel, S. Chung, T. W. Mak and M. Tyers (1996). "A new family of regulators of G-protein-coupled receptors?" Curr Biol **6**(2): 211-212.

Siderovski, D. P., S. P. Heximer and D. R. Forsdyke (1994). "A human gene encoding a putative basic helix-loop-helix phosphoprotein whose mRNA increases rapidly in cycloheximide-treated blood mononuclear cells." DNA Cell Biol **13**(2): 125-147.

Siderovski, D. P. and F. S. Willard (2005). "The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits." Int J Biol Sci **1**(2): 51-66.

Sinnarajah, S., C. W. Dessauer, D. Srikumar, J. Chen, J. Yuen, S. Yilma, J. C. Dennis, E. E. Morrison, V. Vodyanoy and J. H. Kehrl (2001). "RGS2 regulates signal transduction in olfactory neurons by attenuating activation of adenylyl cyclase III." Nature **409**(6823): 1051-1055.

Smalheiser, N. R. and G. Lugli (2009). "microRNA regulation of synaptic plasticity." Neuromolecular Med **11**(3): 133-140.

Smalheiser, N. R., G. Lugli, H. S. Rizavi, V. I. Torvik, G. Turecki and Y. Dwivedi (2012). "MicroRNA expression is down-regulated and reorganized in prefrontal cortex of depressed suicide subjects." PLoS One **7**(3): e33201.

Smoller, J. W., M. P. Paulus, J. A. Fagerness, S. Purcell, L. H. Yamaki, D. Hirshfeld-Becker, J. Biederman, J. F. Rosenbaum, J. Gelernter and M. B. Stein (2008). "Influence of RGS2 on anxiety-related temperament, personality, and brain function." Arch Gen Psychiatry **65**(3): 298-308.

Stamatakis, A., S. Pondiki, E. Kitraki, A. Diamantopoulou, T. Panagiotaropoulos, A. Raftogianni and F. Stylianopoulou (2008). "Effect of neonatal handling on adult rat spatial learning and memory following acute stress." Stress **11**(2): 148-159.

Stein, M. B., A. Keshaviah, S. A. Haddad, M. Van Ameringen, N. M. Simon, M. H. Pollack and J. W. Smoller (2014). "Influence of RGS2 on sertraline treatment for social anxiety disorder." Neuropsychopharmacology **39**(6): 1340-1346.

Stevens, R. C., V. Cherezov, V. Katritch, R. Abagyan, P. Kuhn, H. Rosen and K. Wuthrich (2013). "The GPCR Network: a large-scale collaboration to determine human GPCR structure and function." Nat Rev Drug Discov **12**(1): 25-34.

Strekalova, T., R. Spanagel, D. Bartsch, F. A. Henn and P. Gass (2004). "Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration." Neuropsychopharmacology **29**(11): 2007-2017.

Strug, L. J., R. Suresh, A. J. Fyer, A. Talati, P. B. Adams, W. Li, S. E. Hodge, T. C. Gilliam and M. M. Weissman (2010). "Panic disorder is associated with the serotonin transporter gene (SLC6A4) but not the promoter region (5-HTTLPR)." Mol Psychiatry **15**(2): 166-176.

Stuchlik, A. (2014). "Dynamic learning and memory, synaptic plasticity and neurogenesis: an update." Front Behav Neurosci **8**: 106.

Susskind, J. M. and A. K. Anderson (2008). "Facial expression form and function." Commun Integr Biol **1**(2): 148-149.

Suzuki, M., Y. L. Hurd, P. Sokoloff, J. C. Schwartz and G. Sedvall (1998). "D3 dopamine receptor mRNA is widely expressed in the human brain." Brain Res **779**(1-2): 58-74.

Tang, K. M., G. R. Wang, P. Lu, R. H. Karas, M. Aronovitz, S. P. Heximer, K. M. Kaltenbronn, K. J. Blumer, D. P. Siderovski, Y. Zhu and M. E. Mendelsohn (2003). "Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure." Nat Med **9**(12): 1506-1512.

Tank, A. W. and D. Lee Wong (2015). "Peripheral and central effects of circulating catecholamines." Compr Physiol **5**(1): 1-15.

Taymans, J. M., J. E. Leysen and X. Langlois (2003). "Striatal gene expression of RGS2 and RGS4 is specifically mediated by dopamine D1 and D2 receptors: clues for RGS2 and RGS4 functions." J Neurochem **84**(5): 1118-1127.

Taymans, J. M., C. Wintmolders, P. Te Riele, M. Jurzak, H. J. Groenewegen, J. E. Leysen and X. Langlois (2002). "Detailed localization of regulator of G protein signaling 2 messenger ribonucleic acid and protein in the rat brain." Neuroscience **114**(1): 39-53.

Tesmer, J. J. (2009). "Structure and function of regulator of G protein signaling homology domains." Prog Mol Biol Transl Sci **86**: 75-113.

Tesmer, J. J., D. M. Berman, A. G. Gilman and S. R. Sprang (1997). "Structure of RGS4 bound to AlF4--activated G(i alpha1): stabilization of the transition state for GTP hydrolysis." Cell **89**(2): 251-261.

Thorsell, A., K. Carlsson, R. Ekman and M. Heilig (1999). "Behavioral and endocrine adaptation, and up-regulation of NPY expression in rat amygdala following repeated restraint stress." Neuroreport **10**(14): 3003-3007.

Tovote, P., M. Meyer, A. G. Beck-Sickinger, S. von Hörsten, S. Ove Ögren, J. Spiess and O. Stiedl (2004). "Central NPY receptor-mediated alteration of heart rate dynamics in mice during expression of fear conditioned to an auditory cue." Regulatory Peptides **120**(1–3): 205-214.

Trujillo Viera, J., R. El-Merahbi, B. Nieswandt, D. Stegner and G. Sumara (2016). "Phospholipases D1 and D2 Suppress Appetite and Protect against Overweight." PLoS One **11**(6): e0157607.

Tsang, S., A. Y. Woo, W. Zhu and R. P. Xiao (2010). "Deregulation of RGS2 in cardiovascular diseases." Front Biosci (Schol Ed) **2**: 547-557.

Valdizan, E. M., R. Diez-Alarcia, J. Gonzalez-Maeso, F. Pilar-Cuellar, J. A. Garcia-Sevilla, J. J. Meana and A. Pazos (2010). "alpha(2)-Adrenoceptor functionality in postmortem frontal cortex of depressed suicide victims." Biol Psychiatry **68**(9): 869-872.

Van Vliet, B. N., J. McGuire, L. Chafe, A. Leonard, A. Joshi and J. P. Montani (2006). "Phenotyping the level of blood pressure by telemetry in mice." Clin Exp Pharmacol Physiol **33**(11): 1007-1015.

Vanderheyden, W. M., S. A. George, L. Urpa, M. Kehoe, I. Liberzon and G. R. Poe (2015). "Sleep Alterations Following Exposure to Stress Predict Fear-Associated Memory Impairments in a Rodent Model of PTSD." Experimental brain research **233**(8): 2335-2346.

Vos, T., R. M. Barber, B. Bell, A. Bertozzi-Villa, S. Biryukov, I. Bolliger, F. Charlson, A. Davis, L. Degenhardt, D. Dicker, L. Duan, H. Erskine, V. L. Feigin, A. J. Ferrari, C. Fitzmaurice, T. Fleming, N. Graetz, C. Guinovart, J. Haagsma, G. M. Hansen, S. W. Hanson, K. R. Heuton, H. Higashi, N. Kassebaum, H. Kyu, E. Laurie, X. Liang, K. Lofgren, R. Lozano, M. F. MacIntyre, M. Moradi-Lakeh, M. Naghavi, G. Nguyen, S. Odell, K. Ortblad, D. A. Roberts, G. A. Roth, L. Sandar, P. T. Serina, J. D. Stanaway, C. Steiner, B. Thomas, S. E. Vollset, H. Whiteford, T. M. Wolock, P. Ye, M. Zhou, M. A. Ãvila, G. M. Aasvang, C. Abbafati, A. A. Ozgoren, F. Abd-Allah, M. I. A. Aziz, S. F. Abera, V. Aboyans, J. P. Abraham, B. Abraham, I. Abubakar, L. J. Abu-Raddad, N. M. E. Abu-Rmeileh, T. C. Aburto, T. Achoki, I. N. Ackerman, A. Adelekan, Z. Ademi, A. K. Adou, J. C. Adsuar, J. Arnlov, E. E. Agardh, M. J. Al Khabouri, S. S. Alam, D. Alasfoor, M. I. Albittar, M. A. Alegretti, A. V. Aleman, Z. A. Alemu, R. Alfonso-Cristancho, S. Alhabib, R. Ali, F. Alla, P. Allebeck, P. J. Allen, M. A. AlMazroa, U. Alsharif, E. Alvarez, N. Alvis-Guzman, O. Ameli, H. Amini, W. Ammar, B. O. Anderson, H. R. Anderson, C. A. T. Antonio, P. Anwari, H. Apfel, V. S. A. Arsenijevic, A. Artaman, R. J. Asghar, R. Assadi, L. S. Atkins, C. Atkinson, A. Badawi, M. C. Bahit, T. Bakfalouni, K. Balakrishnan, S. Balalla, A. Banerjee, S. L. Barker-Collo, S. Barquera, L. Barregard, L. H. Barrero, S. Basu, A. Basu, A. Baxter, J. Beardsley, N. Bedi, E. Beghi, T. Bekele, M. L. Bell, C. Benjet, D. A. Bennett, I. M. Bensenor, H. Benzian, E. Bernabe, T. J. Beyene, N. Bhala, A. Bhalla, Z. Bhutta, K. Bienhoff, B. Bikbov, A. B. Abdulhak, J. D. Blore, F. M. Blyth, M. A. Bohensky, B. B. Basara, G. Borges, N. M. Bornstein, D. Bose, S. Boufous, R. R. Bourne, L. N. Boyers, M. Brainin, M. Brauer, C. E. G. Brayne, A. Brazinova, N. J. K. Breitborde, H. Brenner, A. D. M. Briggs, P. M. Brooks, J. Brown, T. S. Brugha, R. Buchbinder, G. C. Buckle, G. Bukhman, A. G. Bulloch, M. Burch, R. Burnett, R. Cardenas, N. L. Cabral, I. R. C. Nonato, J. C. Campuzano, J. R. Carapetis, D. O. Carpenter, V. Caso, C. A. Castaneda-Orjuela, F. Catala-Lopez, V. K. Chadha, J.-C. Chang, H. Chen, W. Chen, P. P. Chiang, O. Chimed-Ochir, R. Chowdhury, H. Christensen, C. A. Christophi, S. S. Chugh, M. Cirillo, M. Coggeshall, A. Cohen, V. Colistro, S. M. Colquhoun, A. G. Contreras, L. T. Cooper, C. Cooper, K. Cooperrider, J. Coresh, M. Cortinovis, M. H. Criqui, J. A. Crump, L. Cuevas-Nasu, R. Dandona, L. Dandona, E. Dansereau, H. G. Dantes, P. I. Dargan, G. Davey, D. V. Davitoiu, A. Dayama, V. De la Cruz-Gongora, S. F. de la Vega, D. De Leo, B. del Pozo-Cruz, R. P. Dellavalle, K. Deribe, S. Derrett, D. C. Des Jarlais, M. Dessalegn, G. A. deVeber, S. D. Dharmaratne, C. Diaz-Torne, E. L. Ding, K. Dokova, E. R. Dorsey, T. R. Driscoll, H. Duber, A. M. Durrani, K. M. Edmond, R. G. Ellenbogen, M. Endres, S. P. Ermakov, B. Eshrati, A. Esteghamati, K. Estep, S. Fahimi, F. Farzadfar, D. F. J. Fay, D. T. Felson, S.-M. Fereshtehnejad, J. G. Fernandes, C. P. Ferri, A. Flaxman, N. Foigt, K. J. Foreman, F. G. R. Fowkes, R. C. Franklin,

T. Furst, N. D. Futran, B. J. Gabbe, F. G. Gankpe, F. A. Garcia-Guerra, J. M. Geleijnse, B. D. Gessner, K. B. Gibney, R. F. Gillum, I. A. Ginawi, M. Giroud, G. Giussani, S. Goenka, K. Goginashvili, P. Gona, T. G. de Cosio, R. A. Gosselin, C. C. Gotay, A. Goto, H. N. Gouda, R. l. Guerrant, H. C. Gugnani, D. Gunnell, R. Gupta, R. Gupta, R. A. Gutierrez, N. Hafezi-Nejad, H. Hagan, Y. Halasa, R. R. Hamadeh, H. Hamavid, M. Hammami, G. J. Hankey, Y. Hao, H. L. Harb, J. M. Haro, R. Havmoeller, R. J. Hay, S. Hay, M. T. Hedayati, I. B. H. Pi, P. Heydarpour, M. Hijar, H. W. Hoek, H. J. Hoffman, J. C. Hornberger, H. D. Hosgood, M. Hossain, P. J. Hotez, D. G. Hoy, M. Hsairi, H. Hu, G. Hu, J. J. Huang, C. Huang, L. Huiart, A. Husseini, M. Iannarone, K. M. Iburg, K. Innos, M. Inoue, K. H. Jacobsen, S. K. Jassal, P. Jeemon, P. N. Jensen, V. Jha, G. Jiang, Y. Jiang, J. B. Jonas, J. Joseph, K. Juel, H. Kan, A. Karch, C. Karimkhani, G. Karthikeyan, R. Katz, A. Kaul, N. Kawakami, D. S. Kazi, A. H. Kemp, A. P. Kengne, Y. S. Khader, S. E. A. H. Khalifa, E. A. Khan, G. Khan, Y.-H. Khang, I. Khonelidze, C. Kieling, D. Kim, S. Kim, R. W. Kimokoti, Y. Kinfu, J. M. Kinge, B. M. Kissela, M. Kivipelto, L. Knibbs, A. K. Knudsen, Y. Kokubo, S. Kosen, A. Kramer, M. Kravchenko, R. V. Krishnamurthi, S. Krishnaswami, B. K. Defo, B. K. Bicer, E. J. Kuipers, V. S. Kulkarni, K. Kumar, G. A. Kumar, G. F. Kwan, T. Lai, R. Lalloo, H. Lam, Q. Lan, V. C. Lansingh, H. Larson, A. Larsson, A. E. B. Lawrynowicz, J. L. Leasher, J.-T. Lee, J. Leigh, R. Leung, M. Levi, B. Li, Y. Li, Y. Li, J. liang, S. Lim, H.-H. Lin, M. Lind, M. P. Lindsay, S. E. Lipshultz, S. Liu, B. K. Lloyd, S. L. Ohno, G. Logroscino, K. J. Looker, A. D. Lopez, N. Lopez-Olmedo, J. Lortet-Tieulent, P. A. Lotufo, N. Low, R. M. Lucas, R. Lunevicius, R. A. Lyons, J. Ma, S. Ma, M. T. Mackay, M. Majdan, R. Malekzadeh, C. C. Mapoma, W. Marcenes, L. M. March, C. Margono, G. B. Marks, M. B. Marzan, J. R. Masci, A. J. Mason-Jones, R. G. Matzopoulos, B. M. Mayosi, T. T. Mazorodze, N. W. McGill, J. J. McGrath, M. McKee, A. McLain, B. J. McMahon, P. A. Meaney, M. M. Mehndiratta, F. Mejia-Rodriguez, W. Mekonnen, Y. A. Melaku, M. Meltzer, Z. A. Memish, G. Mensah, A. Meretoja, F. A. Mhimbira, R. Micha, T. R. Miller, E. J. Mills, P. B. Mitchell, C. N. Mock, T. E. Moffitt, N. M. Ibrahim, K. A. Mohammad, A. H. Mokdad, G. L. Mola, L. Monasta, M. Montico, T. J. Montine, A. R. Moore, A. E. Moran, L. Morawska, R. Mori, J. Moschandreas, W. N. Moturi, M. Moyer, D. Mozaffarian, U. O. Mueller, M. Mukaigawara, M. E. Murdoch, J. Murray, K. S. Murthy, P. Naghavi, Z. Nahas, A. Naheed, K. S. Naidoo, L. Naldi, D. Nand, V. Nangia, K. M. V. Narayan, D. Nash, C. Nejjari, S. P. Neupane, L. M. Newman, C. R. Newton, M. Ng, F. N. Ngalesoni, N. T. Nhung, M. I. Nisar, S. Nolte, O. F. Norheim, R. E. Norman, B. Norrving, L. Nyakarahuka, I. H. Oh, T. Ohkubo, S. B. Omer, J. N. Opio, A. Ortiz, J. D. Pandian, C. I. A. Panelo, C. Papachristou, E.-K. Park, C. D. Parry, A. J. P. Caicedo, S. B. Patten, V. K. Paul, B. I. Pavlin, N. Pearce, L. S. Pedraza, C. A. Pellegrini, D. M. Pereira, F. P. Perez-Ruiz, N. Perico, A. Pervaiz, K. Pesudovs, C. B. Peterson, M. Petzold, M. R. Phillips, D. Phillips, B. Phillips, F. B. Piel, D. Plass, D. Poenaru, G. V. Polanczyk, S. Polinder, C. A. Pope, S. Popova, R. G. Poulton, F. Pourmalek, D. Prabhakaran, N. M. Prasad, D. Qato, D. A. Quistberg, A. Rafay, K. Rahimi, V. Rahimi-Movaghar, S. u. Rahman, M. Raju, I. Rakovac, S. M. Rana, H. Razavi, A. Refaat, J. Rehm, G. Remuzzi, S. Resnikoff, A. L. Ribeiro, P. M. Riccio, L. Richardson, J. H. Richardus, A. M. Riederer, M. Robinson, A. Roca, A. Rodriguez, D. Rojas-Rueda, L. Ronfani, D. Rothenbacher, N. Roy, G. M. Ruhago, N. Sabin, R. L. Sacco, K. Ksoreide, S. Saha, R. Sahathevan, M. A. Sahraian, U. Sampson, J. R. Sanabria, L. Sanchez-Riera, I. S. Santos, M. Satpathy, J. E. Saunders, M. Sawhney, M. I. Saylan, P. Scarborough, B. Schoettker, I. J. C. Schneider, D. C. Schwebel, J. G. Scott, S. Seedat, S. G. Sepanlou, B. Serdar, E. E. Servan-Mori, K. Shackelford, A. Shaheen, S. Shahraz, T. S. Levy, S. Shangguan, J. She, S. Sheikhbahaei, D. S. Shepard, P. Shi, K. Shibuya, Y. Shinohara, R. Shiri, K. Shishani, I. Shiue, M. G. Shrime, I. D. Sigfusdottir, D. H. Silberberg, E. P. Simard, S. Sindi, J. A. Singh, L. Singh, V. Skirbekk, K. Sliwa, M. Soljak, S. Soneji, S. S. Soshnikov, P. Speyer, L. A. Sposato, C. T. Sreeramareddy, H. Stoeckl, V. K. Stathopoulou, N. Steckling, M. B. Stein, D. J. Stein, T. J. Steiner, A. Stewart, E. Stork, L. J. Stovner, K. Stroumpoulis, L. Sturua, B. F. Sunguya, M. Swaroop, B. L. Sykes, K. M. Tabb, K. Takahashi, F. Tan, N. Tandon, D. Tanne, M. Tanner, M. Tavakkoli, H. R. Taylor, B. J. Te Ao, A. M. Temesgen, M. T. Have, E. Y. Tenkorang, A. S. Terkawi, A. M. Theadom, E. Thomas, A. L. Thorne-Lyman, A. G. Thrift, I. M. Tleyjeh, M. Tonelli, F. Topouzis, J. A. Towbin, H. Toyoshima, J. Traebert, B. X. Tran, L. Trasande, M. Trillini, T. Truelsen, U. Trujillo, M. Tsilimbaris, E. M. Tuzcu, K. N. Ukwaja, E. A. Undurraga, S. B. Uzun, W. H. van Brakel, S. van de Vijver, R. V. Dingenen, C. H. van Gool, Y. Y. Varakin, T. J. Vasankari, M. S. Vavilala, L. J. Veerman, G. Velasquez-Melendez, N. Venketasubramanian, L. Vijayakumar, S. Villalpando, F. S. Violante, V. V. Vlassov, S. Waller, M. T. Wallin, X. Wan, L. Wang, J. Wang, Y. Wang, T. S. Warouw, S. Weichenthal, E. Weiderpass, R. G. Weintraub, A. Werdecker, K. R. R. Wessells, R. Westerman, J. D. Wilkinson, H. C. Williams, T. N. Williams, S. M. Woldeyohannes, C. D. A. Wolfe, J. Q. Wong, H. Wong, A. D. Woolf, J. L. Wright, B. Wurtz, G. Xu, G. Yang, Y. Yano, M. A. Yenesew, G. K. Yentur, P. Yip, N. Yonemoto, S.-J. Yoon, M. Younis, C. Yu, K. Y. Kim, M. E. S. Zaki, Y. Zhang, Z. Zhao, Y. Zhao, J. Zhu, D. Zonies, J. R. Zunt, J. A. Salomon and C. J. L. Murray (2015). "Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013." The Lancet **386**(9995): 743-800.

Waeber, B., J. F. Aubert, R. Corder, D. Evequoz, J. Nussberger, R. Gaillard and H. R. Brunner (1988). "Cardiovascular effects of neuropeptide Y." Am J Hypertens **1**(2): 193-199.

Wakeno, M., M. Kato, G. Okugawa, T. Fukuda, Y. Hosoi, Y. Takekita, M. Yamashita, S. Nonen, J. Azuma and T. Kinoshita (2008). "The alpha 2A-adrenergic receptor gene polymorphism modifies antidepressant responses to milnacipran." J Clin Psychopharmacol **28**(5): 518-524.

Walz, N., A. Mühlberger and P. Pauli (2016). "A Human Open Field Test Reveals Thigmotaxis Related to Agoraphobic Fear." Biological Psychiatry **80**(5): 390-397.

Wan, Y., Y. Liu, X. Wang, J. Wu, K. Liu, J. Zhou, L. Liu and C. Zhang (2015). "Identification of differential microRNAs in cerebrospinal fluid and serum of patients with major depressive disorder." PLoS One **10**(3): e0121975.

Wang, M., S. Vijayraghavan and P. S. Goldman-Rakic (2004). "Selective D2 receptor actions on the functional circuitry of working memory." Science **303**(5659): 853-856.

Wang, W.-C., F.-M. Lin, W.-C. Chang, K.-Y. Lin, H.-D. Huang and N.-S. Lin (2009). "miRExpress: Analyzing high-throughput sequencing data for profiling microRNA expression." BMC Bioinformatics **10**(1): 328.

Wang, W., E. J. Kwon and L. H. Tsai (2012). "MicroRNAs in learning, memory, and neurological diseases." Learn Mem **19**(9): 359-368.

Wang, Z., C. Zhang, J. Huang, C. Yuan, W. Hong, J. Chen, S. Yu, L. Xu, K. Gao and Y. Fang (2014). "MiRNA-206 and BDNF genes interacted in bipolar I disorder." J Affect Disord **162**: 116-119.

Wedzony, K., A. Chocyk, M. Mackowiak, K. Fijal and A. Czyrak (2000). "Cortical localization of dopamine D4 receptors in the rat brain--immunocytochemical study." J Physiol Pharmacol **51**(2): 205-221.

Weinstock, M. (2007). "Gender differences in the effects of prenatal stress on brain development and behaviour." Neurochem Res **32**(10): 1730-1740.

Weisstaub, N. V., M. Zhou, A. Lira, E. Lambe, J. Gonzalez-Maeso, J. P. Hornung, E. Sibille, M. Underwood, S. Itohara, W. T. Dauer, M. S. Ansorge, E. Morelli, J. J. Mann, M. Toth, G. Aghajanian, S. C. Sealfon, R. Hen and J. A. Gingrich (2006). "Cortical 5-HT2A receptor signaling modulates anxiety-like behaviors in mice." Science **313**(5786): 536-540.

Wieland, T., S. Lutz and P. Chidiac (2007). "Regulators of G protein signalling: a spotlight on emerging functions in the cardiovascular system." Curr Opin Pharmacol **7**(2): 201-207.

Wightman, B., I. Ha and G. Ruvkun (1993). "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans." Cell **75**(5): 855-862.

Willner, P. (1984). "The validity of animal models of depression." Psychopharmacology (Berl) **83**(1): 1-16.

Willner, P. (1997). "Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation." Psychopharmacology (Berl) **134**(4): 319-329.

Willner, P. (2005). "Chronic Mild Stress (CMS) Revisited: Consistency and Behavioural-Neurobiological Concordance in the Effects of CMS." Neuropsychobiology **52**(2): 90-110.

Willner, P. and P. J. Mitchell (2002). "The validity of animal models of predisposition to depression." Behav Pharmacol **13**(3): 169-188.

Willner, P., A. Towell, D. Sampson, S. Sophokleous and R. Muscat (1987). "Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant." Psychopharmacology (Berl) **93**(3): 358-364.

Winter, J., S. Jung, S. Keller, R. I. Gregory and S. Diederichs (2009). "Many roads to maturity: microRNA biogenesis pathways and their regulation." Nat Cell Biol **11**(3): 228-234.

Woolley, D. G., A. Laeremans, I. Gantois, D. Mantini, B. Vermaercke, H. P. Op de Beeck, S. P. Swinnen, N. Wenderoth, L. Arckens and R. D'Hooge (2013). "Homologous involvement of striatum and prefrontal cortex in rodent and human water maze learning." Proc Natl Acad Sci U S A **110**(8): 3131-3136.

Xing, B., H. Kong, X. Meng, S. G. Wei, M. Xu and S. B. Li (2010). "Dopamine D1 but not D3 receptor is critical for spatial learning and related signaling in the hippocampus." Neuroscience **169**(4): 1511-1519.

Xing, B., X. Meng, S. Wei and S. Li (2010). "Influence of dopamine D3 receptor knockout on age-related decline of spatial memory." Neurosci Lett **481**(3): 149-153.

Yalcin, B., S. A. Willis-Owen, J. Fullerton, A. Meesaq, R. M. Deacon, J. N. Rawlins, R. R. Copley, A. P. Morris, J. Flint and R. Mott (2004). "Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice." Nat Genet **36**(11): 1197-1202.

Zhang, D., Q. Zhao and B. Wu (2015). "Structural Studies of G Protein-Coupled Receptors." Mol Cells **38**(10): 836-842.

Zhang, G., H. N. Ásgeirsdóttir, S. J. Cohen, A. H. Munchow, M. P. Barrera and R. W. Stackman Jr (2013). "Stimulation of serotonin 2A receptors facilitates consolidation and extinction of fear memory in C57BL/6J mice." Neuropharmacology **64**: 403-413.

Zhang, P. and U. Mende (2014). "Functional role, mechanisms of regulation, and therapeutic potential of regulator of G protein signaling 2 in the heart." Trends Cardiovasc Med **24**(2): 85-93.

Zhao, X., D. Ho, S. Gao, C. Hong, D. E. Vatner and S. F. Vatner (2011). "Arterial Pressure Monitoring in Mice." Current protocols in mouse biology **1**: 105-122.

Zhou, Z., G. Zhu, A. R. Hariri, M. A. Enoch, D. Scott, R. Sinha, M. Virkkunen, D. C. Mash, R. H. Lipsky, X. Z. Hu, C. A. Hodgkinson, K. Xu, B. Buzas, Q. Yuan, P. H. Shen, R. E. Ferrell, S. B. Manuck, S. M. Brown, R. L. Hauger, C. S. Stohler, J. K. Zubieta and D. Goldman (2008). "Genetic variation in human NPY expression affects stress response and emotion." Nature **452**(7190): 997-1001.

Zhu, S., J. Wang, Y. Zhang, V. Li, J. Kong, J. He and X. M. Li (2014). "Unpredictable chronic mild stress induces anxiety and depression-like behaviors and inactivates AMP-activated protein kinase in mice." Brain Res **1576**: 81-90.

Zurawek, D., M. Kusmider, A. Faron-Gorecka, P. Gruca, P. Pabian, J. Solich, M. Kolasa, M. Papp and M. Dziedzicka-Wasylewska (2016). "Reciprocal MicroRNA Expression in Mesocortical Circuit and Its Interplay with Serotonin Transporter Define Resilient Rats in the Chronic Mild Stress." Mol Neurobiol.

10 Appendix

10.1 Curriculum vitae

10.2 Publication list and conference contributions

Research articles

1. L. Hommers*, A. Raab*, A. Bohl, H. Weber, C. J. Scholz, A. Erhardt, E. Binder, V. Arolt, A. Gerlach, A. Gloster, R. Kalisch, T. Kircher, T. Lonsdorf, A. Strohle, P. Zwanzger, M. Mattheisen, S. Cichon, K. P. Lesch, K. Domschke, A. Reif, M. J. Lohse and J. Deckert (2015). "MicroRNA hsa-miR-4717-5p regulates RGS2 and may be a risk factor for anxiety-related traits." Am J Med Genet B Neuropsychiatr Genet 168b(4): 296-306.

* equal contribution

Oral presentations

Date	Organizer	Presentation Title
July 2013	Annual retreat of the Department of Pharmacology	RGS2 and its influence on G protein signaling processes
July 2014	Annual retreat of the Department of Pharmacology	Fear Conditioning and behavioral phenotyping
2015	Annual retreat of the Department of Pharmacology	Dynamic regulation of *RGS2* after Fear Conditioning
January 2016	Neurobiological Colloquium of the Department of Psychiatry Psychosomatics and Psychotherapy	Behavioral Phenotyping of RGS2$^{-/-}$ mice
September 2016	Annual meeting of the DGBP / Poster prize presentation	Deletion of RGS2 leads to enhanced learning and memory as well as differential stress resilience in mice

Poster presentations

Date	Organizer	Presentation Title
October 2014	Annual GSLS Conference, Würzburg	MicroRNAs regulating pharmacological target genes of Antidepressants
March 2015	Annual DGPT Conference, Kiel	MicroRNAs regulating RGS2 and SLC6A4 as novel targets for anxiety disorders
November 2015	DGPPN Berlin	Deletion of RGS2 leads to enhanced fear memory in contextual and cued fear conditioning in mice
June 2016	IZKF Retreat	Deletion of RGS2 leads to enhanced fear memory in contextual and cued fear conditioning in mice
September 2016	DGBP Würzburg	Deletion of RGS2 leads to enhanced learning and memory as well as differential stress resilience in mice

November 2016	SFN San Diego	Deletion of RGS2 leads to enhanced learning and memory as well as differential stress resilience in mice

10.3 Affidavit

I hereby declare that my thesis entitled

“The role of *Rgs2* in animal models of affective disorders”

is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or 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 similar form.

Würzburg,

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation

„Über die Bedeutung von *Rgs2* in Tiermodellen affektiver Störungen“

eigenständig, d.h. insbesondere selbständig 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 bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg,

10.4 Acknowledgments

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Dr. med. Leif Hommers for giving me the opportunity to pursue my Doctoral studies under his supervision. His continuous support, patience, motivation and knowledge and of immense help.

Secondly, I would also like to thank my thesis committee: Prof. Martin J. Lohse, Prof. Jürgen Deckert and Prof Martin Heisenberg, for their thought-provoking comments, encouragement and interpretative insights in my annual meetings.

I would like to thank Sandy for introducing me to the animal facilities, without her guidance and our lively discussions this thesis would not have been possible. Special thanks to Angelika for teaching me how to handle a mouse brain. I would like to thank Alex for the warm welcome into the research group, Brigit for all her support and excellent technical assistant. Most importantly, I would like to express my deepest gratitude to all the smart, scared and sometimes surprisingly stubborn mice.

Lastly, I would like to thank all the people of the Pharmacology department, especially Angela, Gabriela, Sandra and Evelyn, who made this time bearable, fun, happy and full of good memory's.