Role of mir-483-5p in stress and anxiety

Submitted by Jaison Blesson Kolenchery, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Medical Studies.

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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Abstract

Stress and anxiety disorders are often severely debilitating to the individual and contribute immensely to the health care costs. Surveys have estimated that 33.7% of the population is affected by these disorders at least once in their lifetime. Financial burden of anxiety disorders within the European Union is over 41 billion Euros in 2004. A comprehensive understanding of the underlying neuronal and synaptic plasticity mechanisms that contribute to the adaptation and maladaptation to stress and anxiety is essential to offer therapeutic interventions to stress and anxiety disorders. microRNAs (miRs) have been identified to be involved in the orchestration of cellular mechanisms facilitating neuronal and synaptic plasticity. Animal models have been used to understand these mechanisms since they offer flexibility in studying several aspects of these conditions that are unobtainable from human studies due to technical and ethical issues. Here, we have used mice model to study the role of miRs in the amygdala, a brain region implicated in stress, fear and anxiety. We have identified several miRs, one of which is mir-483-5p, that is upregulated in the amygdala following 6hr restraint stress (6hRS). Further investigation revealed that mir-483-5p is enriched and upregulated in synaptosomes of the amygdalar neurons following stress. Moreover, overexpression of mir-483-5p was sufficient to induce changes in dendritic arborisation and spine proportions. Twelve genes were identified as potential targets of mir-483-5p that are expressed in the amygdala and respond to stress using miR target prediction algorithms and gene ontology lists. Out of these twelve *in silico* predicted targets, three genes (*Pgap2*, *Gpx3* and *Macf1*) showed down-regulation by mir-483-5p in N2a cells. Furthermore, using luciferase assay, we established a direct interaction of mir-483-5p at the predicted 3'UTR of these three target mRNAs. *In vivo* experiments showed that these targets are down-regulated in the amygdala following 6hRS where *Pgap2* also showed a reduction in synaptosomal compartments. Finally, we assayed the behaviour of mice after bilaterally overexpressing mir-48-5p or knocking down *Pgap2* in mice amygdala. Both of these cohorts of animals, relative to their controls, exhibited anxiolytic behaviour. Hitherto, our data suggest that mir-483-5p, through the downregulation of *Pgap2* in the amygdala, contribute to the mediation of anxiolytic behaviour in mice. Understanding the mechanisms and deciphering the molecular partners of mir-483-5p/*Pgap2* interaction could advance our understanding of the mechanisms involved in neuronal and synaptic plasticity leading to anxiolytic behaviour and adaptive response to stress.

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Abbreviations

6hr	6 hours
6hRS	6 hours restraint stress
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
ADORA2A	Adenosine A2a Receptor
AGO2	Argonaute RISC Catalytic Component 2
ALAD	Aminolevulinate dehydratase
ALP	Alkaline phosphatase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
APC	Adenomatous polyposis coli protein
APT1	Acyl-protein thioesterases 1
BBB	Blood brain barrier
Bcr	Breakpoint Cluster Region
BDNF	Brain-derived Neurotrophic Factor
BLA	Baso-Lateral Amygdala
CA	Central Amygdala
Ca2+	Calcium ions
CAMKII	Ca2+/calmodulin-dependent protein kinase II
CCKBR	Cholecystokinin B Receptor
CD1 mice	CD-1 ICR outbred mice strain
Cdk7	Cyclin Dependent Kinase 7
cDNA	Complementary DNA
CFR1	Cysteine-rich fibroblast growth factor receptor 1
CORT	Corticosterone
CREB	cAMP Response Element-Binding
CRF	Corticotrophin releasing factor
CRFR1	Corticotropin-releasing hormone receptor 1
CRH	Corticotrophin releasing hormone
CRHR2	Corticotropin-releasing hormone receptor 2
Ctnnb1	Catenin Beta 1
Ctnnbip1	Catenin beta interacting protein 1
Cul4a	Cullin 4A
DICER	Double-Stranded RNA-Specific Endoribonuclease
DNA	Deoxyribonucleic-Acid
E	Epinephrine
ECM	Extra-cellular matrix
eIFC2	Argonaute
EPM	Elevated Plus Maze

ER	Endoplasmic Reticulum
FKBP51	FKBP Prolyl Isomerase 5
FMR1	Fragile X mental retardation 1 gene
FMRP	Fragile X mental retardation protein
GABA	Gamma-Aminobutyric Acid
GABRA6	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha6
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GluRA	Glutamate receptor-A
GluRB	Glutamate receptor-B
GPCs	Glypicans
GPI	Glycosylphosphatidylinositol
GPI-Ap	Glycosylphosphatidylinositol anchored protein
GPx	Glutathione Peroxidase
Gpx3	Glutathione Peroxidase 3
GRM7	Glutamate metabotropic receptor 7
GSK3B	Glycogen synthase kinase-3 beta
HPA	Hypothalamic Pituitary Adrenal
HPC	Hippocampus
HPMRS	Hyperphosphatasia with mental retardation syndrome
HTR2C	5-Hydroxytryptamine Receptor 2C
Igf2	Insulin like growth factor 2
LC	Locus Coeruleus
LIMK1	LIM Domain Kinase 1
LTD	Long term depression
LTP	Long term polarisation
Macf1	Microtubule cross linking factor 1
MAO-A	Monoamine oxidase A
MCS	Multiple cloning site
MeCP2	Methyl CpG binding protein 2
MEF2	Myocyte enhancer factor 2
Mg2+	Magnesium ion
miR/miRNA	Micro RNA
mPFC	Medial Prefrontal Cortex
mPVN	Medial Paraventricular nuclei
NAc	Nucleus accumbens
NCK	Non-Catalytic Region Of Tyrosine Kinase
NE	Norepinephrine
NMDAr	N-methyl-D-aspartate receptor
NTRK3	Neurotrophic Receptor Tyrosine Kinase 3
nts	Nucleotides
o/n	Over night
p	P-value

Pak	p21 activated kinase
PASHA	DiGeorge Syndrome Critical Region Gene 8
PD	Panic Disorder
PFC	Prefrontal Cortex
Pgap2	Post-GPI attachment protein 2
PKA	Protein Kinase A
PLD	Phospholipase D
PND	Postnatal days
POMC	Pro-opiomelanocortin
PSD	Post-synaptic density
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Rbpj	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
RE1	Restrictive element-1
REST	Restrictive element-1 silencing transcription factor
RGS2	Regulator of G Protein Signalling 2
RISC	RNA induce silencing complex
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
Saal1	Serum Amyloid A Like 1
SAD	Social Anxiety Disorder
SC35	Serine/arginine-rich splicing factor SC35
SD rats	Sprague Dawley rat
SDE3	Silencing defective protein 3
SE	Status Epilepticus
SEM	Standard error of mean
SERT	Serotonin transporter
Shh	Sonic Hedgehog
SLC6A2	Solute Carrier Family 6 Member 2
SNPs	Single nucleotide polymorphisms
Sop2	Sensory rhodopsin-2
SPS	Single prolonged stress
SSRIs	Selective serotonin reuptake inhibitors
Tbkbp1	TANK-Binding Kinase 1-Binding Protein 1
TGN	Trans-golgi network
Tnik	TRAF2 And NCK Interacting Kinase
Traf2	TNF Receptor Associated Factor 2
TRBP	Transactivation response element RNA-binding protein
UTR	Untranslated region
VPA	Valproic acid
VTA	Ventral tegmental area

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CHAPTER 1 Introduction

1.1 Stress, fear and anxiety

Survival of organisms depends on the effectiveness of detecting and responding to potentially harmful stimuli while being able to seek and utilise beneficial stimuli (Belzung & Philippot, 2007). Simple organisms like bacteria have mechanisms that enable them to move away from potentially harmful substances and move closer to nutritional sources. Multicellular organisms have to avoid environmental dangers and inter-/intra- species danger. Some animals have developed complex mechanisms to detect and respond to intra- and inter-species threats. Stress, fear and anxiety thus enable organisms to not only to detect such perceived dangers but also respond to it by careful allocation of biological and psychological resources. Activation of flight or fight response is one of the mechanisms that facilitate this process (Box 1, p. 19 & Figure 1.1, p. 59). Stress, fear and anxiety are evolved mechanisms that can be seen as an extension of this danger detection mechanism. However, failure in the successful orchestration of these mechanisms could be pathological and can result in stress, fear and anxiety related disorders.

Robert Hook first used the word ‘stress’ in mechanics in 1658 to describe the tension and strain of elastic objects. The meaning of stress in this context is the ‘force applied to a unit area’. The use of the word ‘stress’ in a biological context is credited to Dr Hans Selye. Selye in his letter to the editor of Nature in 1936 titled “A syndrome produced by diverse nocuous agents” outlined his observations from the experiments he had conducted (Selye, 1936/1998). The experiments involved injecting animals with various agents or introducing surgical injuries. He called the observed reactions “generalised adaptation syndrome” (Selye, 1950) as he considered these changes to be generalised attempts by the

animal to adapt to the new conditions. Changes he observed included thymicolymphatic involution, gastric ulcers, lipid discharge from the adrenal glands and loss of chromaffinity in the medulla. Following further studies and observations, he published a monograph titled "The physiology and pathology of exposure to stress, a treatise based on the concepts of the general adaptation-syndrome and the diseases of adaptation" (Selye, 1950). This is the first time he used the word “stress” to describe the phenotype after the experimental treatment of animals. In his work he used the word stress to name the reaction to innocuous agents. Since the introduction of the word stress in this context, its usage spread into the general vernacular to describe anything unpleasant. As time went on its ambiguous usage introduced confusion to its exact meaning. Paul Rosch, who was the president of American Institute of Stress and co-authored several works with Selye, described stress the following way: “Stress, in addition to being itself, was also the cause of itself, and the result of itself” (Humphrey, 2005, p. VIII). This is evident of the diversity of semantics associated with the word stress. In neuroscience literature and this thesis, the word stress is generally used to describe stimuli that cause the physiological and psychological response, associated with the stress response. Due to the ambiguities associated with the word stress and adaptation to stress, several researchers have suggested the use of ‘allostasis’, ‘allostatic load’ and ‘allostatic overload’ to describe adaptive measures taken by the stress response system to achieve homeostasis, cumulative effects of stressors in normal physiological and pathological conditions, respectively (McEwen, 2005; McEwen, Gray, & Nasca, 2015). However, other researchers have pointed out that this mere change in semantics is unlikely to introduce a significant difference in the ways stress and the response

to stress is scientifically investigated (Godoy, Rossignoli, Delfino-Pereira, Garcia-Cairasco, & de Lima Umeoka, 2018). While acknowledging that semantic changes could help in focusing the scientific enquiry, in this thesis, we adhere to the traditional use of the word stress and stress response.

Fear and anxiety, on the other hand, have relatively better clarification within the scientific community. One pragmatic definition of fear is “phasic and abrupt flight-or-fight response accompanied by intense arousal in response to an immediate and identifiable threat” (Duval, Javanbakht, & Liberzon, 2015). “Anxiety is defined as the prolonged state of tension, worry, and apprehension regarding uncertain and potentially negative future events” (Duval et al., 2015). Fear and anxiety are evolutionary adaptations that play an essential function in the survival of organisms. While fear is useful to deal with immediate danger, anxiety is useful to increase the alertness and vigilance, which increases the chances of identifying a potential danger. The critical distinction to note is that in anxiety, unlike in fear, the flight or fight response is triggered with varying level of intensity in the absence of any threatening stimulus. Studies in rodent models have revealed that despite having some overlap in the neural mechanisms, fear and anxiety are different functions with different neural pathways (Tovote, Fadok, & Lüthi, 2015). Lesions induced to a particular region can selectively attenuate either fear or anxiety (Duval et al., 2015). Also, depending on the disorder and the individuals, the intensity and duration of the response can be different (Jakovcevski, Schachner, & Morellini, 2008). The following sections and chapter focus more on anxiety than fear.

Box 1: Stress response

Stress and the response to stress are considered to be among the most important phenomenon under neuroscientific investigation (Godoy et al., 2018; McEwen et al., 2015). Stress response system has evolved over the millennia to enable organisms to respond to stressful stimuli and adapt effectively. However, maladaptation in this system is, directly and indirectly, responsible for several mental disorders. Response to stress starts from the recognition of a perceived stressor. Depending on the physiological or psychological nature of stress, the stress response and the brain regions responsible for this response can be different. However, there is a significant overlap in response to stress and the brain areas responsible for it. This section attempts to describe the response to psychological stressors. Several brain regions, hormones, neurotransmitters, parasympathetic and sympathetic nervous system, chemokines and cytokines respond in a coordinated manner to fine-tune the stress response (Godoy et al., 2018; McEwen, 2004; McEwen et al., 2015). These brain regions include Pre-frontal cortex (PFC), Amygdala, Hippocampus, Paraventricular nucleus (PVN), Ventral tegmental area (VTA) and Nucleus accumbens (NAc). The stress response is facilitated via two main components of the stress system, Sympathetic Adreno-Medullar (SAM) and Hypothalamus-Pituitary Axis (HPA), Figure 1.1, p. 59. SAM axis is involved in the secretion of catecholamines like epinephrine (E) and norepinephrine (NEs), which is mainly responsible for mediating the visceral response to stress via the ANS (Autonomic Nervous System). This response involves changes in blood vessels, glands, organs and muscles that are associated with the fight or flight response (Tank & Wong, 2015). These changes are brought forward by the increase in plasma E and NE. The behavioural and physiological consequences of these changes are hyper-alertness, glycogen catabolism, gluconeogenesis, lipolysis, increased oxygen demand, thermoregulation and hyperventilation (Godoy et al., 2018). Moreover, SAM triggers several neurochemical changes and activation of neurocircuitry as preparative and adaptive measures against stress. These activities are mainly facilitated through the activation of adrenoreceptors that are distributed throughout peripheral tissues and several neuronal nuclei in the CNS (Central Nervous System). One such activation is the stimulation of neuronal projections

from LC (Locus Coeruleus) to mPVN (medial PVN) of the hypothalamus that facilitates the modulation of the HPA (Armario et al., 2012). Following the activation, PVN secretes oxytocin, vasopressin and corticotrophin-releasing hormone (CRH). CRH then stimulates to the production of corticotrophs that results in the release of adrenocorticotrophic hormone (ACTH). The release of ACTH stimulates the cortex of adrenal gland that secretes glucocorticoids (GCs) to the blood plasma (Vale, Rivier, Yang, Minick, & Guillemin, 1978; Vale, Spiess, Rivier, & Rivier, 1981). Cortisol is the human GCs and corticosterones being the rodent equivalent. Once released to the blood plasma, GCs can act on several peripheral organs and several brain regions as it can pass the blood-brain barrier (BBB). In the brain, GCs can affect two main receptor types glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) (Godoy et al., 2018). The affinity of MRs to GCs is ten times higher than that of GRs. Therefore, the relative availability of GCs in the blood plasma, distribution of both of these receptors in the CNS and the MRs to GRs ratio in a given population of cells can determine the effects of GRs at a given time. GRs are ubiquitously expressed in the brain while MRs are restricted to specific neuronal populations. However, the amygdala is one of the few regions where both GRs and MRs are co-localised. Adrenoreceptors are also present in the amygdala, and this allows the control of these regions by both SAM and HPA. The protein complexes that are formed after the activation of GRs and MRs can bind to several promoter regions know as glucocorticoid responsive elements (GREs). Receptor activation by GCs can also hinder the function of several transcription factors. These coordinated responses result in massive changes in gene regulation. Thus, GCs can have both short and long-term effects in physiology and behaviours. Through the coordinated actions of SAM axis and HPA, several adaptive measures are triggered in response to a stressor that enable organisms to adapt to the current and future situations.

1.2 Anxiety disorders and their prevalence

Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM5) describes anxiety disorders, also called ‘stress and anxiety disorder', as an excessive fear response or worry that interferes with functioning or causing significant distress (American Psychiatric Association, 2013). Anxiety disorders are the most prevalent of mental disorders (Duval et al., 2015). The median age of onset is around 11 years and has a lifetime prevalence of 28% (Kessler, Chiu, Demler, & Walters, 2005). A 1996 study estimated $47 billion as the direct and indirect cost of anxiety disorders in the USA alone. This is almost 32% of the total expenditure on all the mental disorders (DuPont et al., 1996). In the UK, National Mental Health in the fact-file three presented that the total direct and indirect cost of mental health is £77 billion per year. Some researchers suggest that this is likely to be an underestimation, probably due to the complex interactions of these disorders and other conditions (Prince et al., 2007). The damaging effects of these disorders are overlooked in society and the media. Moreover, the sufferers of these disorders generally retract from themselves and from society, which reduces their chance of getting proper diagnosis and treatment. A survey showed that only around 12% of the people who suffer from these disorders receive minimally adequate treatment (P. S. Wang et al., 2005). Also, there is a high degree of co-morbidity between anxiety disorders and major depressive disorders, especially at older ages (Coffey & Coffey, 2016). Some researchers have proposed that the likely scenario is that anxiety disorders predispose individuals to depression (Coffey & Coffey, 2016; Wetherell, Gatz, & Pedersen, 2001). Contrastingly, a meta-analysis has shown that having anxiety does not predict the prognosis of second generation treatment by antidepressants to

patients suffering from major depression (Nelson, Delucchi, & Schneider, 2009). However, it is important to note that this observation does not necessarily exclude anxiety being one of the potential causes of depression. Furthermore, anxious individuals are at a higher risk of suicide ideation, attempts and deaths (Bentley et al., 2016; Bolton et al., 2008; Nepon, Belik, Bolton, & Sareen, 2010). A survey found that over 70% of individuals who reported a lifetime prevalence of suicide attempts also suffered from at least one anxiety disorder (Nepon et al., 2010). A better understanding of the disorders and the treatment options could lead to more people being treated. The major anxiety disorders include Panic Disorder (PD), Specific Phobia (SP), Post Traumatic Stress Disorder (PTSD), Obsessive Compulsive Disorder (OCD), Social Anxiety Disorder (SAD) and Generalised Anxiety Disorder (GAD) (Barton, Karner, Salih, Baldwin, & Edwards, 2014; Durbano, 2015). While it is difficult to present a clear definition of each disorder, several different criteria are used to diagnose these conditions (American Psychiatric Association, 2013; Barton et al., 2014; Ströhle, Gensichen, & Domschke, 2018). Next section briefly outlines important aspects of these anxiety disorders. See DSM-5 (American Psychiatric Association, 2013) or the ICD-11 (World Health Organisation, 2018) for a detailed guidelines for diagnosing anxiety disorders.

The most distinguishable feature of PD is the repeated and unexpected ‘panic attacks’ (Barton et al., 2014; Ströhle et al., 2018). A panic attack involves a sudden increase in heartbeats, shortness of breath, tightness in chest, tingling sensation, gastrointestinal distress, sweating, hot/cold flashes, fear of dying and fear of losing control. PD patients can also suffer from agoraphobia, and they tend to avoid places and situations that are perceived to be the triggers of panic

attacks (Barton et al., 2014; Ströhle et al., 2018). The unexpected nature of the panic attacks often leaves the patients worried about the next panic attack which then increases their basal anxiety level(Barton et al., 2014; Ströhle et al., 2018). On the other hand, SP is characterised by excessive fear of specific objects, animals or situations/places (Barton et al., 2014). Uncontrollable fear induced due to the object or situation can result in panic attacks, intense distress and avoidance of the situations that are perceived to be the triggers of phobia. Another important condition that is considered an anxiety disorder is OCD. OCD is characterised by the compulsion to engage in activities repeatedly without any apparent reduction in the sensation of compulsion (Barton et al., 2014; Ströhle et al., 2018). Due to this lack of reduction in the feeling of compulsion, patients often find themselves engaging in these activities to a pathological level, often resulting in injury or debilitation. PTSD is another anxiety disorder that develops in certain individuals after exposure to a physiological or psychological trauma or being a witness to the trauma of someone else. PTSD is characterised by the 'persistent re-experience' of the trauma, nightmares and flashbacks of the traumatic event (Barton et al., 2014, p. 46). These can be triggered by a memory of the traumatic event, any other object that triggers the memory of the traumatic event or changes in mood. Another anxiety disorder is SAD. Individuals who suffer from SAD feel severe and persistent fear of situations that require social performance (Barton et al., 2014; Ströhle et al., 2018). Excessive self-monitoring and cognitive distortions are frequent in these individuals. These individuals tend to avoid social situations at any cost. In a related condition called GAD, the fear is felt towards general situations. Patients suffering from GAD experience prolonged, unfounded and severe anxiety, worry and apprehensive expectations from

general and unrelated situations (Barton et al., 2014; Hayes, 2011; Ströhle et al., 2018). Unlike other anxiety disorders, GAD does not have to be triggered by any specific stimulus. However, GAD can manifest itself following an episode of other disorders. GAD can also be a result of excessive general worry about other conditions (Hayes, 2011). For this reason, several patients suffer from more than one anxiety disorder at the same time. Also, anxiety disorders have comorbidity with other mental disorders like major depressive disorders, schizophrenia, delusions and mood disorders and with other chronic conditions such as cancer and cystic fibrosis (Duff, 2015; Watts et al., 2014). A study in war veterans has shown that GAD sufferers are 1.36 times more likely to suffer from metabolic syndrome (Dohrenwend, 2006; Phillips., 2011). Interestingly, a longitudinal study in 425 women over seven years identified that psychological distress elevated the risk of metabolic disorders (Räikkönen, Matthews, & Kuller, 2002).

As seen in the above sections anxiety disorder can have a diverse array of symptoms and triggers (Marwaha, Parsons, Flanagan, & Broome, 2013; Stansfeld et al., 2016). However, from these observations and other studies, it is likely that anxiety disorders and major depressive disorders could have similar underlying mechanisms. Furthermore, the observation that the patients suffering from different anxiety disorders can benefit from similar treatments strengthens this view. Moreover, it is often the case that one disorder can exacerbate the other. Additionally, some common brain regions are active in different anxiety disorders. One such common anatomical feature shared among these mental disorders is the involvement of the limbic system, especially the amygdala.

1.3 Organisation and function of the limbic system

The history behind the origin and popularisation of the limbic system is also the history of various advancements in neuroscience, especially affective neuroscience that attempts to understand emotions in human and in other species. Since ancient history, humans were interested in emotions and its control centres. Aristotle (384-322BCE) thought that the centre of intelligence and emotions was the heart (Carlino, 1999). Since then, many scientists, philosophers and physicians have tried to understand human emotions and attempted to draw similarities between human and animals' emotions. The study of emotions also started with the realisation that the brain is the centre of our thoughts and cognition. One of the earliest observations that brain structures are correlated with some sensory physiology was probably by Da Vinci (1432-1519) (Pevsner, 2002, 2019; Roxo, Franceschini, Zubaran, Kleber, & Sander, 2011). For example, Da Vinci has conducted extensive research in the brain anatomy (Pevsner, 2019). He was the first to pith a frog and to use a solidifying medium to study internal structures of the brain by injecting wax into ox brain (Pevsner, 2002, 2019). In the persistent attempts to pursue the understanding of human emotions, several animal experiments, human experiments and case studies were instrumental. The staining by Ramon y Cajal (Tessman & Suarez, 2002) and several case studies were instrumental. These include some of the well-known case studies of patents such as Phineas Gage by Harlow Vermont's (O'Driscoll & Leach, 1998; Rippon, 2001), Louis Victor Leborgne by Paul Broca (Broca, 1861; Thiebaut De Schotten et al., 2015), Henry Molaison by Scoviille and Milner (Scoviille & Milner, 1957) and others (Thiebaut De Schotten et al., 2015).

contributed to the understanding of human emotions and the idea that particular regions in the brain can have a more significant role to play in certain behaviours.

The word 'limbic' means 'border' in Latin. The use of the term 'limbic' in a biological context is credited to Paul Broca in 1860 (Greenblatt, 1984; Roxo et al., 2011). The identification of the Broca's area in human cortex, which has an important role in language processing, as the name indicates, is also credited to Broca. He was interested in the identifying brain structures comparatively in all the species he could find. Broca described "The great limbic lobe" to characterise a region in the brain that is housed distinctively from the other regions (Greenblatt, 1984). Several landmark studies and theories by various scientists like Papez, Cannon-Bard, Kluver and Bucy and many others showed that the limbic system has an important role to play in the regulation of emotions. It was MacLean who reintroduced the term limbic system to the neuroscientific community (Lautin, 2001; MacLean, 1949; Pessoa & Hof, 2015). MacLean identified the limbic system as necessary for emotional processing. Even though the limbic system was not conceptualised initially to be an area that deals with emotional processing, through earlier experiments involving electrical stimulation and dissection of brain regions that helped to correlate specific brain damages to emotional dysregulation, it came to light that the structures within the limbic system have a critical role in emotional processing (Klüver & Bucy, 1937; Peper & Markowitsch, 2001; Roxo et al., 2011). Later it became clear that the limbic system is not only involved in emotional processing but also other diverse cognitive functions such as memory (J. E. LeDoux, 1991; Rolls, 2015; Roxo et al., 2011). There are ongoing debates in identifying the regions belong to the limbic system and its role in emotional processing. For examples, arguments

were presented that not all structures within the limbic system contribute towards emotional process and these structures do not function as a single system (Rolls, 2015). Furthermore, the connections between certain structures within the limbic system like the hippocampus and non-limbic areas are so extensive, it is difficult to establish a clear border (Roxo et al., 2011). However, the commonly agreed structures in the limbic system include; amygdala, hippocampus, fornix, mammillary body, thalamus, hypothalamus, cingulate gyrus and prefrontal cortex. It is beyond the scope of this thesis to explain all the limbic system structures in detail. The section below attempts to further explain the amygdala since the experiments in this thesis focus on the amygdala, and it is one of the most studied brain regions regulating stress, fear and anxiety.

1.3.1 The Amygdala

Amygdala, meaning almond in Greek, is a structure deep in the brain in the medial temporal lobe and forms an essential structure in the limbic system. The first person to have used the term amygdala to describe distinct cellular clusters in the grey matter close to the limbic areas was Burdach in 1819 (Pabba, 2013). However, the first anatomical description of the amygdala was first given by Meynert in 1867 (Pabba, 2013). The first *in vivo* demonstration of the role of amygdala region in emotional processing is given by the experiments conducted by Brown and Schaffer in rhesus monkeys in 1888 (Brown & Schafer, 1888). They were studying the effects of surgical removal of a specific brain region to correlate any behavioural changes that followed. After the bilateral removal of the temporal lobe in a rhesus monkey, authors observed significant changes in the behaviour of the animal regarding its aggression, response to potential danger and memory. Before the surgery, the animal was “wild, fierce and assaulting” to

the handlers (Brown & Schafer, 1888, p. 306). However, after the surgery, the animal became friendly and allowed the researchers to handle, tease and even slap it. After the surgery, the animal did not make any attempts to escape or retaliate. Authors also saw the subject trying to feel, taste and smell both familiar and unfamiliar, animate and inanimate objects including his cage mates. He also approached another unfamiliar newly introduced cage mate of “wild and savage” nature (Brown & Schafer, 1888, p. 311). Persistent attempts to investigate the stranger without any ‘fear or suspicion’ was seen even after suffering injuries from an attack by the stranger cage mate. This experiment showed an essential role of the regions in the temporal lobe for fear and aggressive behaviours.

Almost five decades later another group of researchers, Kluver and Bucy, performed similar experiments in rhesus monkeys where they removed temporal lobe bilaterally (Klüver & Bucy, 1937). The authors observed behaviour changes similar to the 1888 Brown and Schafer experiments. They called the effects they saw ‘Psychic Blindness’, now known as Kluver-Busy syndrome, which involved an absence of fear and anger associated behaviours, hyper-orality (feeling both animate and inanimate objects by mouth) and hypersexuality (Klüver & Bucy, 1937). In both of the experiments by Brown and Schafer and Kluver and Bucy, the animals did not suffer from any other illness, or behavioural traits and their sensory and motor systems were functional as before. These experiments showed that the amygdala region is vital in the detection of fear.

It is during this time that Ivan Pavlov published his finding of conditioned reflexes in 1927 (Pavlov, 1927/2010). While studying the salivation in dogs, he came across a method to teach the animals to make an association between neutral stimuli, such as a bell ring, and a physiological outcome, such as

salivation. This idea was later translated to study fear learning using a technique called fear conditioning. This method allowed scientists to teach animals or humans to associate a neutral stimulus (CS or conditioned stimulus), like light or tone, to an aversive stimulus like an electric shock (UCS or unconditioned stimulus). This is done by presenting both stimuli in succession with little or no gap, or by presenting at the same time. After a few learning sessions, the presentation of the hitherto neutral stimuli is sufficient to trigger behavioural outcomes such as freezing and flinching in animals or galvanic skin response in humans.

The 1956 study by Weiskrantz also studied bilateral lesions in monkeys, which showed similar results as previous studies. However, using the fear-conditioning paradigm, he also identified impairment in learned fear in monkeys with bilateral amygdala lesions. More studies in amygdala lesions and stimulations in rodents (Blanchard & Blanchard, 1972; LeDoux, Ciocchetti, Xagoraris, & Romanski, 1990; Weiskrantz, 1956) also corroborated the previous findings. These experiments point towards the vital role of the amygdala in fear associated behaviours, especially in learned fear. The function of the amygdala is essential for defensive and threat behaviours were not only seen in animal studies but also human studies. In the 1978 study (Halgren, Walter, Cherlow, & Crandall, 1978) the author electrically stimulated amygdala and the hippocampal area in epileptic patients. The patients reported hallucinations and other sensations along with the feeling of being scared and fearful. Similar effects were seen in a relatively recent study where amygdala was stimulated in epilepsy patients (Lanteaume et al., 2007). In this case, patients reported a feeling of fear and anger. Interestingly, the authors also observed a functional asymmetry with

the reported emotions depending on which amygdala was stimulated, suggesting the possibility of lateral bias in amygdala function in humans.

In 1994 a case study was published about a patient referred to as S.M. or SM-046 with bilateral amygdala lesion caused by a rare genetic condition called Urbach-Wiethe-disease (Adolphs, Tranel, Damasio, & Damasio, 1994). This was a 30-year-old woman with average IQ, and all the senses functionally intact. However, she suffered from a lack of fear from early childhood. Upon the presentation of human facial expressions, SM-046 rated the expression of fear, anger and surprise as significantly less intense when compared to the brain-damaged controls. Moreover, she suffered from severe impairment in recognising faces with fear expressions when compared to healthy controls. Other studies have shown that damage in amygdala results in impairment of fear conditioning in humans (Bechara et al., 1995). Also, fear conditioning increases the activity in the amygdala when measured by fMRI (Büchel, Morris, Dolan, & Friston, 1998; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998). These effects also seem to occur not only in response to the conscious stimuli but also to the stimuli presented subliminally (Morris, Ohman, & Dolan, 1998). These findings in humans, monkeys and rodents demonstrate the evolutionarily conserved role of the amygdala in the fear response. More studies revealed that the circuitry and functions of the amygdala are conserved across species (Mcdonald, 1998). Reptiles, birds and fish also have ‘amygdala like’ regions in their brain which carry out the similar functions as in mammals (Jarvis et al., 2005; Johnston, 1923; Lanuza et al., 1998). Even though several behaviours are influenced by amygdala (Gupta, Koscik, Bechara, & Tranel, 2011), the cells in the amygdala are mostly valence selective. This means that the cells that respond to fear cues

do not necessarily respond to reward cues (Belova, Paton, Morrison, & Salzman, 2007; Paton, Belova, Morrison, & Salzman, 2006; Sangha, Chadick, & Janak, 2013; Schoenbaum, Chiba, & Gallagher, 1999; Shabel & Janak, 2009; Shabel, Schairer, Donahue, Powell, & Janak, 2011; Uwano, Nishijo, Ono, & Tamura, 1995). This suggests that there are different cell types with inter- and intra-projections from the amygdala (Etkin, 2010). These cell types are clustered together to form separate nuclei within the amygdala. The next section focuses on the different nuclei of the amygdala.

1.3.2 The neurological architecture of Amygdala

Amygdala often called amygdaloid complex is subdivided into approximately 13 nuclei. This division is based on cytoarchitecture, histochemistry and connections (Sah, Faber, Armentia, & Power, 2008). The commonly recognised nuclei in the amygdala are divided into Basolateral amygdala (BLA), central amygdala (CeA), medial amygdala and cortical amygdala (Figure 1.2, p. 61). BLA is again divided into Lateral amygdala (LA), Basal Amygdala (BA) and Basomedial Amygdala (BM). CeA encompass the centrolateral (CeL) and centromedial (CeM) nuclei. Other regions around amygdala raise ambiguity on whether to be included as part of the amygdala or not. These regions include the amygdaloid part of the bed nucleus of stria terminalis, anterior amygdala area, the amygdalo-hippocampal area and the intercalated nuclei (Sah et al., 2008). The amygdala nuclei receive and send a plethora of efferent and afferent connections that mediate many functions related to emotional regulation. Most of these studies have used anterograde or retrograde tracers to study these connections (Sah et al., 2008). However, the recent invention of optogenetic methods has helped us to dissect the role of these connections with much better resolution. Since each amygdala

nuclei have a different proportion of cell types, optogenetic methods can be used for selective manipulation of particular types of neurons thereby understanding its functions more clearly (Tye et al., 2011).

BLA has approximately 90% of glutamatergic neurons (Carlsen, 1988; Y. Smith & Parè, 1994) while CeA has about 95% GABAergic medium spiny neurons (McDonald, 1982). BLA is the primary input site of the amygdala (Etkin, 2010) while CeM is the primary output region (Krettek & Price, 1978a, 1978b). BLA receives various inputs from somatosensory cortex, PFC, thalamus, hippocampus and other regions while CeM primarily receives input from BLA and CeL while it outputs to hypothalamus and brainstem. The consequence of these biases in nuclei is that each nucleus might have a relatively more or less important role on various aspects of the amygdala functions. It is known that the amygdala does not have be wholly intact for fear conditioning to take place. For example, experiments have shown that the dorsal region of lateral amygdala and CeA are more important for fear conditioning than the other regions (Joseph LeDoux, 2003). Even though the major circuitry of the amygdala is conserved, some species differences are observed. The brains of reptiles do not possess PFC, and therefore these animals do not have a connection between amygdala and PFC. Interestingly, the size of BLA relative to CeA is larger in primates when compared with rodent BLA. One possible explanation given to this species difference in size of BLA is correlated to the relatively larger cortex in primates, which consequently results in an increased number of connections from the cortex to the BLA in primates (Chareyron, Banta Lavenex, Amaral, & Lavenex, 2011).

1.4 **Role of Amygdala in anxiety**

Even though the amygdala has been studied for several phenomena like fear, anxiety, feeding and reward and its role in decision making (Gupta et al., 2011) this thesis focuses on amygdala and its role in anxiety. Many of the studies in rodents relied on lesions, electrical stimulation, pharmacological manipulation or optogenetic dissection of the amygdalar region (Adhikari et al., 2015; Allsop, Vander Weele, Wichmann, & Tye, 2014; LaLumiere, 2014). However, some other studies looked at the changes in amygdala after inducing anxiety in animals by exposure to stressful stimuli like restraint stress, social defeat, maternal separation and environmental manipulations. Some other studies used fMRI scans to image the activity in the amygdala in human subjects. Through all of these studies, several interesting discoveries came to light. Figure 1.3 (p. 63) shows the current model of anxiety circuitry involving the amygdala.

Meta-analyses of human fMRI studies have confirmed that patients suffering from anxiety disorder have a heightened amygdala activity when compared against the controls (Etkin & Wager, 2007). The activation of amygdala can occur consciously or unconsciously when presented with a threat-stimulus (Bishop, 2004). When presented unconsciously, fear-expressing facial features resulted in elevated activity in BLA in anxious subjects versus controls (Etkin et al., 2004). Heightened amygdala activation increases the likelihood of a subject finding threat-stimuli such as fearful faces. The consequence of elevated amygdala activity in anxiety patients is that they experience not only hyper-vigilance but also hyperarousal of fear response when presented with such stimuli (Etkin et al., 2004). This is one of the defining characteristics of anxiety disorders. Many anxiety disorders are associated with intolerance of uncertainty

or ambiguity (Boelen & Reijntjes, 2009; Grillon et al., 2008; Holaway, Heimberg, & Coles, 2006) and negative interpretations of ambiguous material (Bishop, 2007; Eysenck, Mogg, May, Richards, & et al, 1991).

Studies have found that simple unpredictability can result in activation of the amygdala and behaviours in both humans and mice (Herry et al., 2007). This shows that specific characteristics of anxiety disorders could also be the result of amygdala activation. Furthermore, PTSD patients have more basal activity in the amygdala when compared against the controls (VanElzakker, Kathryn Dahlgren, Caroline Davis, Dubois, & Shin, 2014). These findings tell a story where simple unpredictable events could lead to the activation of the amygdala in certain individuals consciously or unconsciously. Such an elevated amygdala activity can increase a basal anxiety level. Heightened basal anxiety can then predispose individuals to perceive neutral stimuli as aversive or overestimate the intensity from otherwise mildly threatening situations. Activation of the amygdala and environmental cues could then form a positive feedback loop. Experiments have found that reappraising negative emotions can reduce anxiety while engaging with an anxiogenic task (Hofmann, Heering, Sawyer, & Asnaani, 2009). The importance of threat appraisal in the development of anxiety (Britton, Lissek, Grillon, Norcross, & Pine, 2011) and the role of emotional regulation in anxiety disorders (Cisler & Olatunji, 2012) support the above notion. Potential circuitry from PFC to amygdala is proposed to be important in threat appraisal and emotional regulation (Banks, Eddy, Angstadt, Nathan, & Luan Phan, 2007; Cisler & Olatunji, 2012; Cisler, Olatunji, Feldner, & Forsyth, 2010; Goldin, McRae, Ramel, & Gross, 2008; Urry, 2006).

Even though the above human studies help us understand anxiety and the role of the amygdala, one fundamental limitation is insufficient resolution in studying the amygdala circuitry. Since amygdala consists of several nuclei with diverse functions, it is essential to understand the circuitry and its nuclei better to elucidate the mechanisms properly. This is necessary for any useful potential pharmacological manipulations. Animal models of anxiety are instrumental in studying the anxiety circuitry in much more detail.

1.5 Animal models to study anxiety-related disorders

Animal models play an important role in all of the biomedical research. In order to study the psychopathology of anxiety in animal models, it is important to use a valid model of anxiety. The three main types of validity applied to a good animal models are; face validity, construct validity and predictive validity (Davis, Charney, & Coyle, 2002). Face validity refers to the outward similarities between the model and the illness while the construct validity means the internal mechanism. Predictive validity refers to the ability to make therapeutic predictions based on the model. An ideal model organism should be able to have at least both construct and predictive validity (Davis et al., 2002). Like in other areas of biomedical research, in the scientific study of emotions, animal models are a useful choice. This is not surprising given the evolutionary conservation of certain emotional behaviours and brain structures in humans and other animals (DeFelipe, 2011). The first person who probably drew a direct line to show the similarities in human and animal emotions is Charles Darwin (Darwin, 1872). The fact that most animals display clear defensive behaviours when presented with threat stimuli is a useful endophenotype to study in the area of affective neuroscience. Even though there are some species-specific differences in these

behaviours, the underlying mechanisms are likely to be conserved and therefore similar. The examples of such behaviour in defensive behaviours include vocalisations, freezing, fleeing and aggression.

The hormonal response to threat stimuli involving CRH and HPA axis also seems to be similar in mammals. In anxiety research, animal models are used not only to understand the neurobiology of these disorders (Bourin, Petit-Demoulière, Dhonnchadha, & Hascöet, 2007), but also to test any potential drug with anxiolytic effect before it reaches the clinical trials (Haller & Alicki, 2012). The similarities in the defensive behaviours, evolutionary conservations of underlying mechanisms and the practicality of designing and testing pharmacological and therapy-based interventions in animal models fulfil all the three criteria mentioned above of useful animal models to study stress, anxiety and fear.

Several studies have been used to study the various aspects of anxiety or fear including non-human primates, rodents, birds and sometimes even insects like Drosophila and bumble bees. One of the most commonly used groups of animals is rodents. The earliest evolutionary common ancestry between humans and rodents were 75 million years ago (Sartori, Landgraf, & Singewald, 2011). For most human genes, it is likely to find homologous mouse genes (Carver & Stubbs, 1997; Nilsson et al., 2001). Moreover, the structuring of the human brain is similar to the mouse brain (Jones, 2009). There is a phylogenetic relationship between the anxiety process and the regions involved in humans and mice (Belzung & Philippot, 2007; Canteras, Resstel, Bertoglio, de Pádua Carobrez, & Guimarães, 2010). Among rodents, rats (*Rattus norvegicus*) used to be the primary choice for decades (Sartori et al., 2011). This is mainly due to the

practicality of tissue and fluid sampling and the superiority of rat in cognitive and operant performance in behavioural neuropharmacology over other rodents. Partially due to the arrival of gene targeting methods and other advancements in molecular biology, mice (*Mus musculus*) model of anxiety is now preferred by many researchers (Cryan & Holmes, 2005). These advancements help researchers to introduce genetic changes to study their effect on anxiety (Hirst, 1994) and test previously identified genetic associations of anxiety (Hovatta et al., 2005). Other properties such as relatively smaller size and requirement of space and food provide direct and indirect practical and economic advantages that make mice a pre-eminent model organism to study anxiety.

The similarities between humans and rodents are apparent in the hitherto described observations and experiments. Anxiety features like the fight/flight responses, avoidance, freezing, urination/defecation, attention/vigilance, autonomic hyperarousal or muscular tension can be seen in both humans and mice (Sartori et al., 2011). A simple tone-shock conditioning in rodents results in significant changes in rodent amygdala (Quirk, Armony, & LeDoux, 1997; Quirk, Repa, & LeDoux, 1995). Similar effects on amygdala were also found in human studies using fMRI scans and simple conditioning paradigm (LaBar et al., 1998; Morris et al., 1998). Moreover, the pathway from vmPFC to amygdala exists both in humans (Phelps, Delgado, Nearing, & Ledoux, 2004) and rodents (Amano, Unal, & Paré, 2010; Morris et al., 1998). Experiments have shown that these pathways mediate the extinction of fear memories acquired through conditioning in humans as well as in rodents. Moreover, there are also studies suggesting that memory consolidation and post-retrieval reconsolidation mechanisms might be similar in rodents and humans.

Researchers were able to affect the fear memory by inhibiting protein synthesis (Nader, Schafe, & Le Doux, 2000) or by the presentation of neutral stimuli during the retrieval of fear memory (Monfils, Cowansage, Klann, & LeDoux, 2009). Interestingly, a similar method was also used in humans to manipulate fear memory non-invasively (Quirk et al., 2010; Schiller et al., 2010). The similarities of human and rodent amygdala function in fear learning also extend to anxiety-like behaviours. Researchers observed that in GAD patients BLA-CeA pathway was abnormal. When compared to the control group, GAD patients had a significantly less distinct connection between BLA and CeA (Etkin, Prater, Schatzberg, Menon, & Greicius, 2009). Following from this observation researchers made optogenetic manipulation of this circuitry in rodents. They were able to either inhibit or stimulate the BLA-CeA pathway in rodents which changed the anxiety-like behaviours (Tye et al., 2011). This is a good demonstration of how observations from human studies can be further explored to infer a causal relationship using animal models. Furthermore, optogenetic activation of BLA cell body decreases time spend in the open arm of the elevated plus maze (EPM) (Tye et al., 2011), suggestive of anxiety like behaviour. Moreover, an SNP (single nucleotide polymorphism) in the *Oprl1* gene (opioid receptor-like 1) was associated with self-reported history of childhood trauma and PTSD symptoms. Altered expression of this gene was found in the amygdala of mice with impaired fear learning. Additionally, activating these receptors using a selective agonist impaired fear memory consolidation (Andero et al., 2013).

1.6 **Neural circuitry of anxiety in rodents**

The invention of optogenetics and the concomitant ability to dissect the circuit optogenetically enabled us to discover the role of amygdala nuclei in anxiety with

much better resolution. As mentioned before different, nuclei within amygdala possess different properties due to their cellular architecture. Other brain areas such as BNST, hypothalamus, mPFC, locus coeruleus, periaqueductal grey, parabrachial nucleus, raphe nucleus, lateral septum also have important roles to play in anxiety-like behaviours. Due to the central role of the amygdala, this section focuses on neurocircuitry of this structure. Several neuronal circuits have been implicated in anxiety. The heterogeneity of amygdala nuclei results in facilitation of both anxiogenic and anxiolytic behaviours upon their stimulation. While the somatic activation of BLA projection neurons increases anxiety, selective excitation of BLA axonal projections terminates at CeL, reducing anxious phenotype (Tye et al., 2011). This anxiolytic effect could be due to the activation of CeM cells by the CeL cells or due to the activation of BNST neurons from the CeL projections to BNST (Tye et al., 2011). It is known that the chemical or electrical stimulation of CeM activates defensive behaviours through brain stem (Tye et al., 2011).

Further investigations are required to understand the exact circuitry that mediates the anxiolytic effect through CeM cells. One possibility is that the CeL connections to CeM might be activating a different subpopulation of cells compared to the chemical or electrical activation of the CeM. Alternatively, the observed reduction in anxiety might be due to the function of BNST. Stimulation of BLA inputs to adBNST has been shown to have an anxiolytic effect (Kim et al., 2013). Moreover, the activation of BLA-CeA projection neurons also reduces anxiety in murine models (Felix-Ortiz et al., 2013). Furthermore, stimulation of BLA-vHC projection not only has an anxiogenic effect (Felix-Ortiz et al., 2013) but also decreases social interaction (Felix-Ortiz & Tye, 2014). Some of the

circuitry that has been implicated in anxiety is also implicated in fear behaviour or extinction of fear learning. Projections to BLA from mPFC have been shown to be necessary for fear extinction. The rhythmic excitation of these projections are shown to have an anxiolytic effect and might be involved in signalling safety (Likhtik, Stujenske, Topiwala, Harris, & Gordon, 2013). These findings demonstrate the complexity of amygdala and the circuitry it facilitates.

Teaching patients suffering from anxiety to appraise and deal with fearful stimuli might facilitate the reduction in severity of anxiety mediated through the activation of mPFC-BLA pathway. By comparing the differences in molecular architecture and basal anxiety levels in different strains, new mechanisms or pathways affecting anxiety-like behaviours can be identified. Strains such as BALB/c have five-fold lower levels of benzodiazepine binding sites in the amygdala when compared to C57BL/6 mice. This was given as a plausible explanation for the increased trait anxiety (see Box 5, p. 239) in BALB/c mice relative to C57BL/6 mice.

1.7 Aetiology and treatment of anxiety disorders

Like so many other conditions, anxiety disorders also stem from a complex interaction of environmental variables and genetic heterogeneity. Twin studies have shown that this is to be the likely case (Eley, 2007). Genetic variation in regulatory gene regions might play a disproportionally larger part in the development and prognosis of anxiety disorders when compared to the variation within protein-coding genes (Knight, 2005; Rockman & Wray, 2002). This is unsurprising when considering that 98% of the human genome does not code for proteins, but regulatory regions (Elgar & Vavouri, 2008). A longitudinal study in more than 34,000 adults showed that experiencing early childhood trauma

increased the likelihood of developing psychiatric disorders, including anxiety disorders and in heterotypic continuity (the progression from one disorder to another) (Walsh, McLaughlin, Hamilton, & Keyes, 2017). Rodent models using rats have also shown that exposure to early life stress is linked to anxiety-like behaviours later in life (Liu, Atrooz, Salvi, & Salim, 2017). Epidemiological studies have shown that exposure to moderate-severe early childhood traumatic brain injury is associated with vulnerability of developing anxiety disorders even 13 years after the event (Albicini & McKinlay, 2017).

There are several approaches to treating anxiety disorders that exploit various dimensions of the underlying mechanisms. Cognitive behavioural therapy (CBT) with or without the aid of pharmacological agents is usually the first approach to the treatment. However, exercise, meditation and a healthy diet are all likely to contribute positively towards the prognosis of anxiety disorders. Even though tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) used to be the first choice for treatment, due to their side effects that resulted in noncompliance in many of the patients, it is no longer the primary choice. Selective serotonin reuptake inhibitors (SSRIs) and selective norepinephrine inhibitors (SNRIs) are now the preferred choices of treatments of anxiety disorders (Stein, Ipser, Seedat, Sager, & Amos, 2006). These drugs increase the availability of serotonin or norepinephrine at the synapses by blocking their re-uptake. The resulting increase in the potent neurotransmission and downstream effects on other neurotransmitters are the mechanisms that contribute to the drug efficacy (Smith, Kuczenski, George-Friedman, Malley, & Foote, 2000). Benzodiazepines are another class of drugs that are extremely useful, especially for situations that require a fast-acting pharmacological agent

(Kent, Coplan, & Gorman, 1998). Benzodiazepines are thought to be involved in enhancing the inhibitory effects of γ–aminobutyric acid (GABA) through its action on $GABA_A$ receptors (Martin, 1987). However, the users of these drugs can form high dependence. Moreover, rebound anxiety is also common in patients who discontinue their use. Anticonvulsants like Pregabilin and GABApentin started to become increasingly useful due to the reduced potential for dependence relative to benzodiazepines. Other drugs which involved the modulation of NMDA receptors and neurotransmitters are also being used for treating anxiety disorders (Ledgerwood, Richardson, & Cranney, 2003, 2005; Yamamoto et al., 2010).

While psychological, electrophysiological, neurological and behavioural methods are all valid ways to understand anxiety, in order to achieve a comprehensive understanding, it is crucial to incorporate molecular underpinnings of these mechanisms. One of the molecular mechanisms that can regulate anxiety and behaviour is through precise control of gene expressions. microRNAs (miRs) form an important and interesting part of the puzzle in the orchestration of gene expression and therefore the neural activity and the resulting behavioural outcome. The next section introduces miRs and their role in anxiety-like behaviours.

1.8 What are microRNAs?

MicroRNAs, henceforth referred to as miRs, belong to the category of non-coding RNAs. Figure 1.4 (p. 65) shows the classification of all RNAs in the genome. miRs are approximately 22 nucleotides in length and have an important role in the regulation of almost all of the biological processes studied so far. Their primary function is the precise control of the gene regulation mainly by mRNA degradation or translational repression by binding the mRNA sequences at 3' or the 5’ UTRs.

However, recent evidence has implicated miRs in increasing the protein expression by aiding RNA translation (Gokhale & Gadgil, 2012; Rusk, 2008; Saraiya, Li, & Wang, 2013).

The discovery of miRs was a relatively recent event which happened in 1993. The first miR discovered was *lin-4* in *C.elegans* (Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993). The events leading to the discovery started with Victor Ambrose and Gary Ruvkon while they were the students at Robert Hortvitzs' lab. They were studying the heterochronic genes, genes involved in the temporal developmental patterns in *C.elegans* by studying null mutations. One such gene was *Lin-4*. *Lin-4* loss of function mutation (lin-4^{e912}) showed phenotypes like the inability to lay eggs, not because eggs were not formed, but due to missing vulva and other structures required for laying eggs (Almeida, Reis, & Calin, 2011; R. Lee, Feinbaum, & Ambros, 2004). The eggs often ended up hatching inside the mother. Later it became clear that the *Lin-4* is required for the transition from larval stage L1 to L2 (Chalfie, Horvitz, & Sulston, 1981; Lee et al., 2004). Interestingly, they noticed that a suppressor mutation in *Lin-14* was able to revert the phenotype of lin-4^{e912} (Ferguson, 1987). Moreover, null mutations in *Lin-14* resulted in an opposite phenotype of *Lin-4* null mutations (Ferguson, 1987; Lee et al., 2004). From these finding, they inferred that the *Lin-4* could negatively influence *Lin-14*. When the 700bp sequence that has the *Lin-4* gene was obtained they realised that it did not have ORF with a start and a stop codon, which was thought to be one of the characterising features of a protein-coding gene. However, they persevered in studying this region. Later they identified another two mutants that showed the same phenotype as the lin-4^{e912}. These mutations (n355 and n536) were on 3' UTR region of the *Lin-14* mRNA

and were involved in the post-transcriptional down-regulation of the *Lin-14* mRNA (Ruvkun, Wightman, Bürglin, & Arasu, 1991; Wightman et al., 1993). The realisation that *Lin-4* could not encode for a protein-coding gene, evolutionary conservation of *Lin-14* 3' UTR, the necessity of intact *Lin-14* 3' UTR for the observed down-regulation and previous research showing the presence of antisense sequences in eukaryotes (Hildebrandt & Nellen, 1992) pointed towards the possibility of the down-regulation of *Lin-14* by *Lin-4* through its action at the 3'UTR. Moreover, when sequencing the *Lin-4* and the 3' UTR region of the *Lin-14*, Ruvkun and Ambrose independently noticed the complementarity in these sequences (R. C. Lee et al., 1993; Wightman et al., 1993). By doing other experiments and more careful analysis, two manuscripts were published in Cell in 1993 which was the first paper describing the role of *Lin-4* short RNA sequence and posttranscriptional down-regulation of *Lin-14* mRNA through its 3' UTR region (R. C. Lee et al., 1993; Wightman et al., 1993). Thus, the study of miRs was born. However, even after identifying these mechanisms Ambrose and Ruvkon were not sure if this was a global phenomenon or a species-specific mechanism. Seven years later when *Let-7* was identified, it became clear that this could be a conserved mechanism in many species. One-year later, discovery of miRs in HeLa* cells, Drosophila and other organisms also invited much

* HeLa cells are named after Henrietta Lacks, the source of this first human cell line to be established, thanks to their ability to continually divide indefinitely. HeLa cell line have been instrumental not only in cancer studies and other cellular and molecular studies, but also in stimulating and directing fruitful discussion and debate in bioethics and implementation of informed consent. Debate around the HeLa cells helped put an end to the practice of using patients' specimens without their consent (Beskow, 2016).

attention to this newly identified mechanism (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001). Later in 2002 miRs were implicated in cancer and then in 2004 miRs were also identified in plants (Lindow & Kauppinen, 2012). In 2011, miRBase, the largest depository of miR sequences reported over 25,000 mature miRs in 193 species (Kozomara & Griffiths-Jones, 2011). Hitherto, miRBase reported nearly two thousand miRs in humans alone. It is reported that at least 30-80% of all human protein-coding genes are regulated by miRs (Filipowicz, Bhattacharyya, & Sonenberg, 2008; Friedman, Farh, Burge, & Bartel, 2009; Lu & Clark, 2012). While miRs comprise of 1% of all genes (Bartel, 2004), a single miR can target more than 100 genes (Lu & Clark, 2012) which makes them the most abundant class of gene regulators (Muiños-Gimeno et al., 2011). Some have called the miRs the ‘meta-controllers' of the evolution of the brain (Serafini et al., 2012). Given their importance and ubiquitous nature, some researchers have even suggested seeing miRs as ‘genes' (Yang & Zhou, 2013). The next section introduces the mechanism of miR actions.

1.8.1 How do miRs work?

50% of all miRs are encoded on the non-protein coding regions of the genome while the other 50% are on the introns of protein-coding regions (Saini, Griffiths-Jones, & Enright, 2007). Some of the miRs like *Let-7b*, mir-17-5p and mir-21 are ubiquitously expressed in all cells (Tang et al., 2007) while others exhibit cell specificity (Brown & Naldini, 2009) and expressed in a heterochronological manner. mir-134-1 is expressed solely in the brain and spinal cord (Tang et al., 2007) while mir-409-3p is expressed during the brain development in mice (Krichevsky, King, Donahue, Khrapko, & Kosik, 2003). Figure 1.5 (p. 67) shows biogenesis and mechanisms of miR function. The complex processes of

producing mature miRs are initialised by the transcription of miR regions performed by ribonuclease III (DROSHA) enzymes and the DiGeorge syndrome critical region eight protein (DGCR8). This results in the production of pre-miR, which is a 70-100nts long precursor of mature miR. Pre-miR is transported to the cytoplasm by EXPORTIN-5 and then cleaved by DICER, another RNase III enzyme, and trans-activation-responsive RNA binding protein (TRBP). This process generates the double-stranded miR of 22nt length. Following this, helicase enzyme unwinds the double strand, and one of the strands is degraded while the other, known as the guide strand, functions as the mature miR. miRs also undergo post-transcriptional modification which involves the insertion or deletion of nucleotides through a process called RNA editing. RNA editing is tissue-specific which increases the complexity of miR-mRNA interactions (Erson & Petty, 2008). Mature miR then forms the miRISC complex, miR-induced silencing complex, with other proteins. miRISC binds mostly to 3' UTR of mRNA before the mRNA is degraded or its translation is repressed (Zhang et al., 2007). However, miRs can also target the mRNA at the 5’ UTRs or on the coding regions (Duursma, Kedde, Schrier, le Sage, & Agami, 2008; Kloosterman, Wienholds, Ketting, & Plasterk, 2004).

The region of the miR that binds to the target region is called the seed region which is the most critical region of a mature miR (Brennecke, Stark, Russell, & Cohen, 2005). If the guide strand is 100% complementary with the region in the mRNA, the mRNA will be degraded. In case of less than 100% complementarity, the target mRNA is deadenylated. Deadenylation can lead to decapping which makes the mRNA prone to exonucleolytic digestion. Additionally, deadenylation can also result in translational repression, and the

exact mechanisms employed for repression depends on the stage of mRNA translation (de Kloet, Fitzsimons, Datson, Meijer, & Vreugdenhil, 2009; Kuss & Chen, 2008). This mechanistic network can get complex when considering that miRs can indirectly regulate other genes in the networks which can, in turn, control the transcription of miRs. Thus, miRs can provide precise temporal and spatial control of gene expression in a tissue-specific manner. Moreover, mutations in the seed region of mRNA or in the miR itself could either exclude canonical targets from being repressed and degraded or generate novel targets. This is one of the overlooked factors when analysing SNPs in the genome. Many of the silence mutations might not be silent since they might be downregulated by a miR through its novel seed site. miR identification should be followed by the prediction of their target genes. Many computational methods exist for this purpose, and they have a false positive rate of 24-70% (Baek et al., 2008; Easow, Teleman, & Cohen, 2007; Selbach et al., 2008). This is one of the reason why targets have to be experimentally validated (Thomson, Bracken, & Goodall, 2011), see Chapter 4 for more details.

1.9 **miRs in neurons**

miRs play important roles in various stages of the development of CNS and neuronal processes. They are involved in stress, learning and memory process, synaptic plasticity, synaptogenesis, neurogenesis and neurite outgrowth (Fiore & Schratt, 2007; He et al., 2007; Rogaev, 2005; Zhou et al., 2009). More specifically, mir-9 and mir-124a involved in neural lineage differentiation in embryonic stem (ES) cells (Krichevsky, Sonntag, Isacson, & Kosik, 2006) and the overexpression of this miR in non-neuronal cells changed the gene expression pattern of the cell to a pattern reminiscent of neurons (Lim et al., 2005;

Makeyev, Zhang, Carrasco, & Maniatis, 2007). Another miR, mir-132, when overexpressed in cortical neurons, increased the neurite outgrowth while inhibition reduced the outgrowth (Nakazawa, 2003; Okabe et al., 2003; Vo et al., 2005). This is achieved by the down-regulation of p250GAP, a GTPase activating protein. Another miR, mir-134 has been shown to reduce dendritic spine in the hippocampus and is also involved in synaptic plasticity (Fiore & Schratt, 2007; Hansen et al., 2007; Schratt et al., 2006). mir-134 targets the LIMK1 gene that regulates proteins like cofilin and actin which are involved in dendritic spine dynamics and postsynaptic density (Meng et al., 2002; Schratt et al., 2006). Given the role of miRs in various biological processes and many neuronal functions, it is unsurprising to learn that miRs also play an essential role in pathologies responsible for many psychiatric disorders including anxiety disorders. This is evident from many human studies and animal models of anxiety disorders. See Table 1.1 for selected examples of miRs implicated in human psychiatric conditions and rodent models. For a detailed review see (J. Donner et al., 2008; Malan-Müller, Joanna Hemmings, & Seedat, 2013)

1.9.1 miRs in human anxiety-related conditions

Stress, glucocorticoids and treatments involving the administration of mood stabilisers can affect miR expression pattern (Hunsberger, Austin, Chen, & Manji, 2009; Zhou et al., 2009). This shows that miRs are important in the pathology of the psychiatric conditions and the success of the pharmacological interventions. This is also evident from several studies that have implicated miRs in psychiatric conditions like PTSD (Giridharan et al., 2016), stress-related substance addiction (Doura & Unterwald, 2016), autism spectrum disorders (Abu-Elneel et al., 2008; Talebizadeh, Butler, & Theodoro, 2008), Rett syndrome (Klein et al., 2007) and

also in substance abuse disorders (Chandrasekar & Dreyer, 2009). Some of these studies analysed post-mortem brain samples from human donors in order to identify the miRs that are potentially involved in these conditions (Beveridge, Gardiner, Carroll, Tooney, & Cairns, 2010; Beveridge et al., 2008; Perkins et al., 2007). miRs are also thought to be epigenetic modulators in psychiatric disorders (Hunsberger et al., 2009; Knight, 2005). Polymorphisms in regulatory regions in the genome are considered to be risk factors for developing several complex disorders including psychiatric disorders (Knight, 2005). SNPs in the target region of the NTRK3 gene are associated with the hoarding phenotype of OCD (Muiños-Gimeno et al., 2009). This site also happened to be targeted by mir-485-3p. A study that looked into 712 SNPs in 325 mRNA in a Spanish population of PD patients shows several associations between SNPs and PD (Muiños-Gimeno et al., 2011). Three such SNPs were rs6502892, rs11763020 and rs73531. Rs6502892 was seen in mir-22 which is a predicted repressor of BDNF, HTR2C, MAO-A and RGS2. Furthermore, mir-339, a miR that targets ADORA2A, BDNF, CRHR2 and SLC6A2, also had an SNP, rs11763020, associated with PD. All of these genes are implicated in stress and anxiety pathways and disorders. SNPs in other miRs like mir-138-2, mir-148a, mir-488 and mir-491 were also associated with different phenotypes of PD. These miRs repress genes like GABRA6, CCKBR and POMC. SNP in mir-148a was shown to be associated with the age of onset for PD (Muiños-Gimeno et al., 2011). These studies also revealed that the density of SNPs is much lower in miR regions when compared to other genomic areas. Analysis of 117 miRs in four different human reference populations also corroborated this finding (Quach et al., 2009). Interestingly, none of the SNPs was in the mature regions of the miRs (Quach et al., 2009). These

findings show that mutations in the mature regions are naturally selected against and could have deleterious effects with severe complications. These studies underline the importance of incorporating miRs into our current biological model of psychiatric disorders.

Name of miR/s	Name of the condition	Referneces	Organism
mir-134, miR-219, several others	Schizophrenia	Stark et al 2008, Kocerha et al 2009, Hunsberger 2009	Humans, Rodents
mir-134	William's syndrome	Hoogenraad, Akhmanova, Galjart, & De Zeeuw, 2004	Humans
mir-132	Rett syndrome	Klein et al 2007	Humans
mir-148a	Phobia disorder (PD)	Muiños-Gimeno et al 2011	Humans
mir-570, mir-219-1-3p, several others	PTSD	Giridharan et al 2016	Humans, Rodents
mir-181a, several other	Cocaine addiction	Doura & Unterwald 2016	Rodents
mir-22	Panic disorder	Muinos-Gimeno et al 2011	Humans
mir-485-3p	OCD	Muinos-Gimeno et al 2009	Humans
mir-221	Nicotine Sensitivity	Gomez et al 2016	Rodents
mir-229, several others	Autism Spectrum Disorder	Abu-Elneel 2008	Humans
mir-211, mir-204	Social phobia	Donner 2008	Humans
mir-34, several others	Maternal separation stress	Uchida 2010	Rodents

Table 1.1 Examples of miRs implicated in human psychiatric conditions and rodent models

1.9.2 miRs in animal models of anxiety

Rodent models of anxiety disorders facilitate scientific investigations to understand the role of miRs in anxiety and to explore new horizons. For example, human association studies have linked an SNP (rs817782) in the 3'UTR region of ALAD to SP (Donner et al., 2008). This SNP made the 3' UTR region target of mir-211 and mir-204. ALAD was up-regulated in hippocampus and PAG of six different inbred mice strains that also exhibited anxiety. Even though a functional relationship between these miRs and anxiety is yet to be experimentally validated, these studies furthered our understating into the complex changes within the genome could predispose us to anxiety disorders or other conditions (Hovatta et al., 2005).

It is known that the malfunctioning of the HPA axis plays a crucial role in stress and anxiety disorders (McEwen, 2002). The fact that there are several miRs in the CNS with tissue-specific function implies the likelihood of miR mediated regulatory mechanisms in the CNS (Kuss & Chen, 2008; Sun & Tsao, 2008). An example of this is shown in the experiments that involve two different strains of rats that have differential stress responsiveness to repeated restraint stress (RRS). F344 rats are hyper-responsive to RRS when compared to SD rats (Uchida et al., 2008). Coincidentally, F344 rats also have relatively increased expression of mir-18 in the PVN region. These rats also have reduced Glucocorticoid receptors (GRs) in the same region. Studies in neuronal cultures have shown that mir-18 can inhibit the GR mRNA. Moreover, *in vitro* studies have shown that GRs can be down-regulated by mir-18 and mir-124a (Vreugdenhil et al., 2009). These pieces of evidence suggest that GRs could be down-regulated by mir-18 and mir-124a in the PVN of F344 rats which also correlates with their

increased susceptibility to RRS. This is an example of how miRs can regulate the responsiveness of an organism to stress.

There are other lines of evidence pointing towards the role of miRs in the rodent models of maternal separation (MS) stress models. Early childhood trauma and maternal separation are shown to be an important risk factor for developing psychiatric disorders, especially anxiety (Volk et al., 2016). Experiments have shown that some miRs and mRNA increase in expression following maternal separation. Among the miRs that are up-regulated after MS include mir-132, mir-124-1, mir-9-1, mir-9-3p, mir-212 and mir-29a. All of these genes possess a repressor element 1 (RE-1) site within 50kb of their promoter regions (Otto et al., 2007). It is also shown that repressor element 1 silencing transcription factor 4 (REST4) is a neuronal-specific transcriptional repressor which increases in its abundance after MS or chronic stress. Taken together these experiments suggests the tissue-specific role of certain transcriptional factors that contribute to the regulation of mRNAs involved in stress response is mediated via the orchestrated functioning of miRs (Conaco, Otto, Han, & Mandel, 2006; Kosik, 2006; Otto et al., 2007; Vo et al., 2005). Interestingly, another miR, mir-15a, was shown to be significantly higher in healthy human subjects treated with dexamethasone and in adults who experienced childhood trauma (Volk et al., 2016). The researchers were able to quantify these changes in the miR using peripheral blood samples, suggesting that this could potentially be used as a biomarker. Moreover, the mir-15a was also shown to be up-regulated in the amygdala of mice after exposure to chronic stress induced by social defeat paradigm. Levels of *Fkbp51*, a gene involved in psychiatric disorders and a predicted target for mir-15a, was down-regulated in amygdala after chronic

stress. Even though overexpression of mir-15a in BLA did not result in anxiety-like behaviours, it predisposed the animals to experience higher levels of anxiety after exposure to chronic stress (Volk et al., 2016).

miRs are also implicated in the trans-generational effects of anxiety in mice (Short et al., 2016). Analysis of sperm samples from male mice treated with CORT revealed up-regulation of several miRs. These miRs are predicted to target *Bdnf* and *Igf2*. The male, not the female, offspring of these mice also exhibited higher anxiety-like behaviours (Short et al., 2016). Previous studies have shown sex differences in anxiety disorders (Donner & Lowry, 2013) and gender bias in the role of miRs in neuropsychiatric disorders (Pak, Rao, Prins, & Mott, 2013). Experiments by Short et al (2016) suggest a potential sexually dimorphic role of several miRs in rodents. Meerson et al (2010) showed that acute and chronic stress results in the differential miR expression pattern in the CeA and hippocampus. While acute stress increased the expression of mir-134 and mir-183, chronic stress down-regulated mir-134 not only in CeA but also in hippocampus. These miRs are shown to down-regulate SC35, a protein involved in the alternative splicing of AChE. SC35 aid the alternative splicing of the synapse-associated AChE-*S* isoform to its rare protein soluble form of AChE-*R*. This alternative splicing is suggested to be a potential mechanism that is involved in the temporal and local regulation of neurotransmission of cholinergic neurons, which is vital in the stress response. Moreover, acute stress resulted in the increase of mir-34c (Haramati et al., 2011; Issler et al., 2014). When mir-34c was overexpressed in CeA of WT mice, subjects exhibited anxiolytic behaviour. Further analysis revealed that mir-34c target *Crfr1* mRNA at an evolutionarily conserved 3' UTR.

Acute and repeated stress can produce short-term and long-term changes in the brain areas involved. miRs could play an important role in the regulation of these changes. Studies have shown that short-term changes in neural transmission and gene regulation (Alfonso et al., 2006; Gao et al., 2006; H. Xu, He, Richardson, & Li, 2004) and long-term structural modifications were seen in the animals after restraint stress (Cook & Wellman, 2004; Donohue et al., 2006; Magarin˜os & McEwen, 1995). On a similar pattern, acute stress is also shown to result in rapid and transient modulation of miRs in the brain (Mannironi et al., 2017; Rinaldi et al., 2010). Northern blot analysis in CD1 mice after acute stress have shown an increase of *Let-7a*, mir-9, mir-26a and mir-26b specifically in PFC, but not in the hippocampus (Rinaldi et al., 2010). This is an example of tissue-specific temporal regulation of miRs by stress. A group of miRs called mir-17-92 cluster was previously implicated in brain development (Bian et al., 2013) and tumorigenesis (Olive, Jiang, & He, 2010). Recent experiments have shown that this miR cluster has a vital role to play in the pathogenesis of anxiety and depression-like behaviours in mice (Jin et al., 2016). There were able to show that the deletion of this cluster significantly affected the neurogenesis in hippocampus. Moreover, knocking out mir-17-92 cluster results in anxiety and depression-like behaviours in mice, while its overexpression results in the anxiolytic effect. In order to understand the global role of miRs, Giraldez et al. (2005) knocked down DICER in zebrafish. This knock down resulted in complications in embryonic neural plate and transformation to neural tube (Giraldez, 2005). Another group of researchers studied the effect of knockdown of DICER in CeA (Haramati et al., 2011). These animals showed a sudden spike in anxiety-like behaviours. These studies show the importance of miRs in

regulating the precise control of amygdala, not only in the developmental stage but also in the functional reactivity to stimuli.

Given the role of miRs, it is crucial that miRs are accounted for when considering potential drug targets or modulators. It might be even possible that miRs could be used as a predictor of prognosis. Given the importance of miRs in many psychiatric disorders, several reviews have acknowledged the nascent area of miRs being the targets of drugs in the fight against these conditions (O'Connor, Dinan, & Cryan, 2012; Scott et al., 2015). For example, lithium and Valproate (VPA) are used to treat bipolar disorders. VPA is used for the treatment of refractory anxiety disorder, an anxiety disorder that is comorbid with bipolar disorder. It is also used to enhance the efficacy of exposure-based cognitive behavioural therapy for anxiety and PTSD (Kuriyama, Honma, Koyama, & Kim, 2011). Chronic treatment using VPA and lithium in mice showed fluctuation in several miRs, eight of which were confirmed in hippocampus. The targets of these miRs are involved in many vital functions that can influence anxiety (Zhou et al., 2009; Zhou & Yang, 2012). One such miR was mir-34a that was shown to decrease in its expression when neuronal cultures were treated with VPA and lithium. Moreover, a decrease in mir-34-a is correlated with an increase in GRM7, which is an essential protein involved in the role of decreasing symptoms of patients with bipolar disorders. GRM7 is predicted to have a mir-34a target site. Additionally, treating the cell with the precursor or mir-34a decreases the expression of GRM7 while treating with an inhibitor of mir-34a result in an increase in GRM7 (Zhou et al., 2009). These experiments show that the effect of VPA and lithium could be mediated via the regulation of GRM7 by mir-34a. Another example is the role of a common SSRIs, Prozac, in regulating Serotonin

Transporter (SERT). SERT plays a crucial role in neuronal function and therefore in anxiety disorders, depression and suicidal behaviour (Van Praag, 1996). SSRIs are one of the effective pharmacological interventions that are currently employed for the treatment of anxiety disorders. As mentioned above, they work by increasing the availability of serotonin in the synapse by inhibiting the reuptake. This success of SSRIs depend on its ability to target SERT (Torres, Gainetdinov, & Caron, 2003). Chronic treatment of mice with Prozac results in the up-regulation of mir-16. mir-16 targets SERT and results in the down-regulation of SERT in the raphe nuclei (Baudry, Mouillet-Richard, Schneider, Launay, & Kellermann, 2010). This suggests that the function of Prozac could be partially dependent on the action of mir-16. Moreover, another group of researchers were able to demonstrate a strong interaction between mir-135 and SERT and serotonin receptor 1a mRNA (Issler et al., 2014). By genetically controlling the expression of mir-135 researchers were able to change anxiety-like behaviours, serotonin level and response to pharmacological interventions in rodent models. Reduced levels of mir-135 in the blood samples and brain of depressed individuals not only corroborate the role of mir-135 in psychiatric disorders like anxiety but also demonstrate the potentially powerful utility of miRs in diagnosis and prognosis of these disorders (Issler et al., 2014). These findings show the importance of incorporating these methods and understanding into the current ways of understanding and diagnosing mental disorders (Malan-Müller et al., 2013; Mannironi et al., 2013; O'Connor et al., 2012; Scott et al., 2015). So far, all the studies point towards the necessary role of miRs in the orchestration of precise cellular mechanisms in neuronal function. By understanding more about miRs and the specific gene networks they influence, we will inevitably contribute

to the success of future attempts to form an in-depth understanding of these mechanisms.

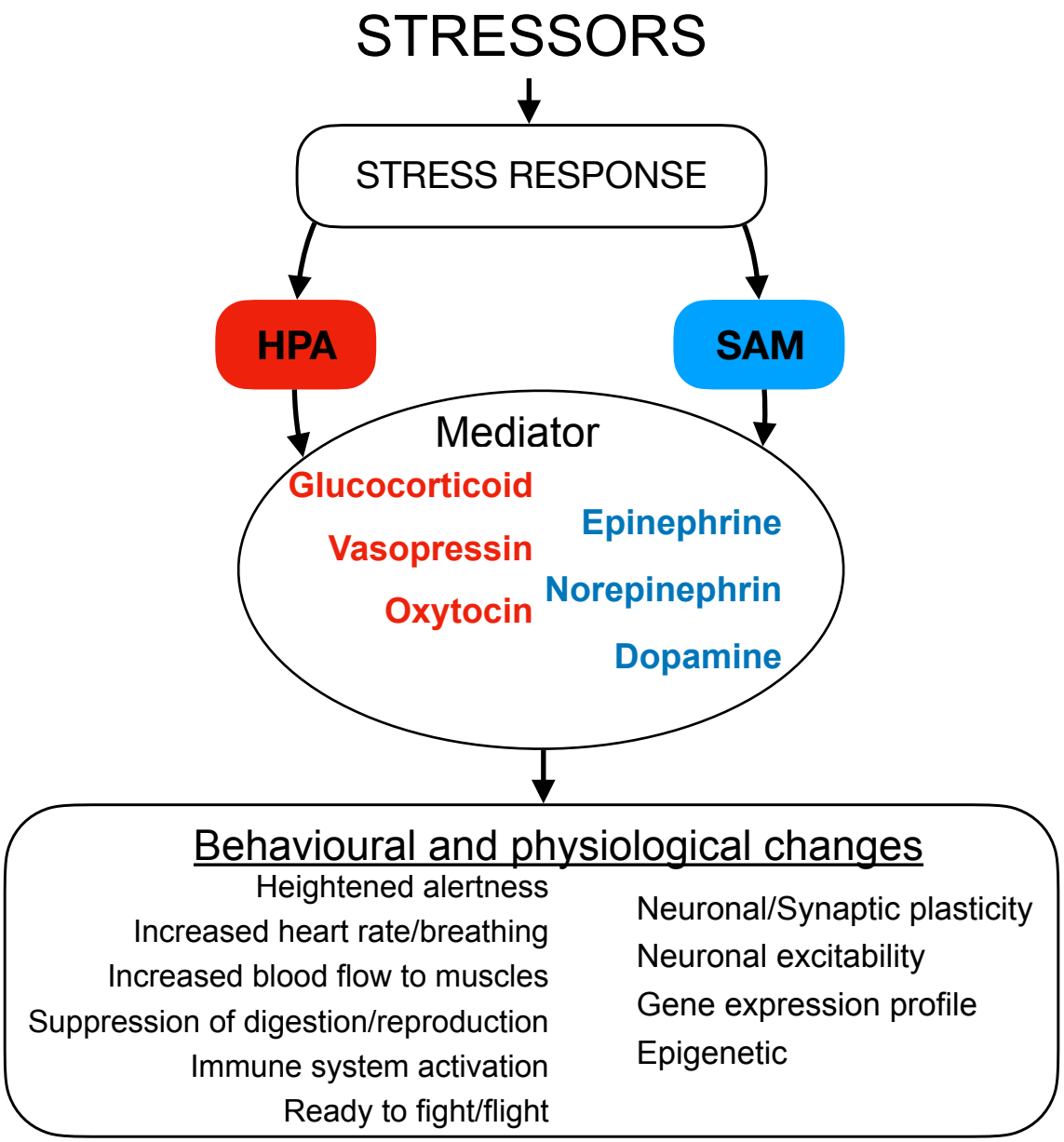

Figure 1.1 The stress system
In mammals, the perception of a stressor elicits the stress response. This includes the activation of two main components of the stress system, HPA (Hypothalamus-Pituitary-Axis) and SAM (Sympathetic-Adreno-Medullar) axis. While HPA involves the secretion of glucocorticoids, SAM axis involves the release of epinephrine and norepinephrine. Collectively, these two systems facilitate behavioural and physiological changes in preparation for and the execution of the flight or fight response. Ultimately, these changes lead to the adaptation to stressors by restoring homeostasis. See Box 1 for more details.

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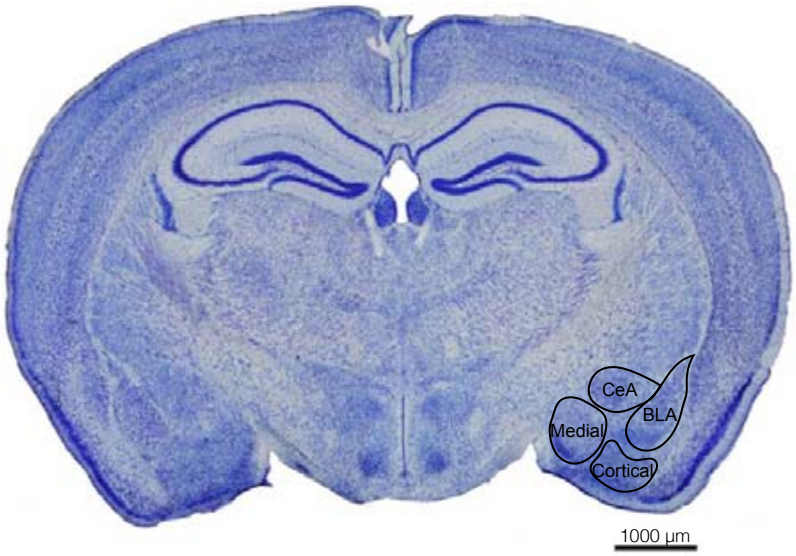

Figure 1.2 The amygdala
Photomicrograph from Nissl-stained coronal sections of a mouse brain showing the Amygdaloid complex. Among several other behaviours, the amygdala is important for the detection of threat stimuli and the adaptation to those stimuli. Damage to these regions induces malfunctions in threat detection and other behaviours. See section 1.3.1 for more details. BLA: Basolateral Amygdala, CeA: Central Amygdala, CeM: Medial Amygdala, Cortical Amygdala. Reproduced from DeFelipe et al, 2011 with permission.

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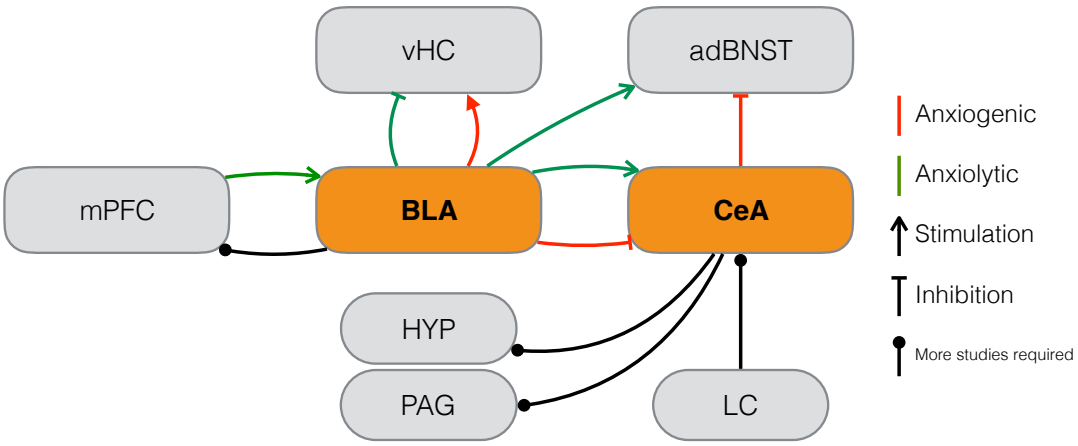

Figure 1.3 Anxiety circuits involving the amygdala
The colour and type of connections between regions show the effect on anxiety when stimulated or inhibited. Red lines show anxiogenic effects while the green line shows anxiolytic effects upon either stimulation (pointed arrowheads) or inhibition (flat line heads). Some of the connections require further studies to verify their exact role (round line heads). There are several other projections from other brain regions to the amygdala. For simplicity, only those projections that are known to affect anxiety is represented in this image. BLA: Basolateral amygdala, CeA:Central Amygdala, mPFC:Medical Prefrontal Cortex, vHC: ventral hippocampus, adBNST: anterodorsal Bed Nucleus of the Stria Terminalis, HYP:Hypothalamus, PAG: periaqueductal gray, LC: locus coeruleus. See section 1.6 for more details.

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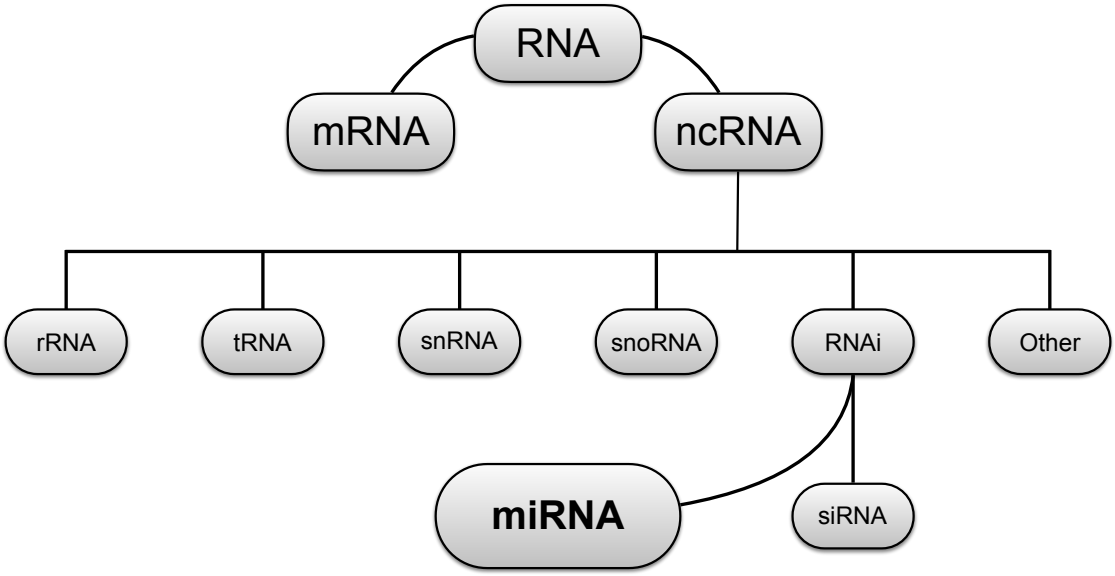

Figure 1.4 Classification of RNAs in the genome
The vast majority of the genome is non-coding RNAs. One of the important classes of non-coding RNAs are the microRNAs (miRs/miRNAs). Nc: non-coding RNAs, rRNA: ribosomal RNA is involved in protein translation, tRNA: transfer RNA facilitates translation by forming an RNA-amino acid interface, snRNA: small nuclear RNA forms part of splicesome, snoRNA: small RNAs in the nucleolus and involved in rRNA modification, RNAi: RNA interference is the mechanism by which the gene expression is regulated via RNA. Others include RNAs involved in chromatin structure modification and imprinting. RNA interference mechanisms are facilitated by short interference RNA (siRNA) and miRNAs.

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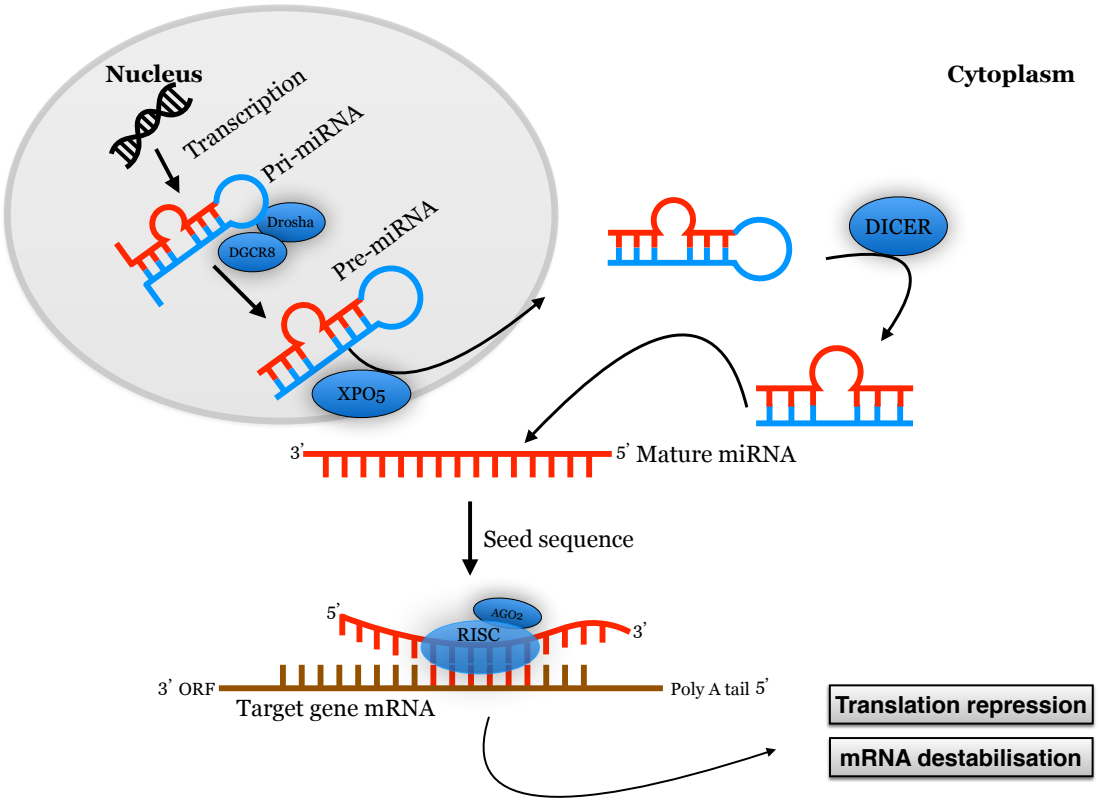

Figure 1.5 Biogenesis and function of miRs
RNA polymerase enzyme II transcribes the pri-miRNA from the DNA. Pri-miRNA is then processed by DROSHA and DGCR8 to form pre-miRNA. Following this, pre-miRNAs are transported into the cytoplasm. DICER further processes pre-miRNAs into its mature form. Mature miRNA then form complexes with RISC and AGO2 to bind to the target mRNA to engage in translational repression or degradation. See section 1.8 for more details. Figure adapted from Davis et al., 2015.

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CHAPTER 2 Materials and Methods

2.1 Mice

Male wild-type mice (C57BL/6J) of 8-12 weeks of age were used throughout the experiments listed. Three to five mice were housed in each cage with *ad libitum* access to food pellets and water. Cages were provided with bedding, commercial chow and housing. The cages were kept in moisture and temperature-controlled rooms with 12-hour light/dark cycles. All of the experiments were performed during the light half of the cycle. Approvals of the University of Exeter ethical committee, Home Office (UK, Project Licence#: 40/3502) and the Bioethics Commission (Cracow, Poland Project Licence#: 1152/2015) were obtained before conducting the experiments.

2.2 Behavioural analysis

Animals were left undisturbed in their home cages for one week before the start of the experiment. Experiments were performed during the light period of the 12:12 hour light-dark cycles. Restraint stress was performed within the home cages either using a metal mesh or perforated 50ml falcon tubes. Animals were left undisturbed for at least 12 hours (o/n) after restraint stress and behavioural tests were performed the following day. Before the behavioural test started, animals were habituated to the new conditions by leaving the animals in their home cages in the behavioural experimental room for one hour prior to the test.

EPM apparatus was constructed of four non-transparent white Plexiglas arms (50 x 10 x 30cm) that bisected in the middle to form a plus sign (Figure 2.1, p. 85). The maze was 50cm above the floor, and the arms were dimly illuminated with the closed arm having less light relative to the open arms. Mice were placed one at a time at the centre of the maze facing the open arm and allowed to explore the maze for five minutes. An overhead camera was used to record the activity.

The videos were analysed semi-automatically using AnyMaze software (Stoelting Europe). After each mouse, the maze was cleaned with 70% ethanol and left to dry for two minutes before introducing the next animal to the maze. While testing, the experimenter was blind to the experimental conditions of the animals in order to avoid potential confounders and bias.

2.3 Cell culture experiments

Neuro-2A (N2a) or HEK293T (HEK) cells were incubated at 37^{o}C at 5% CO_2 in DMEM culture media with 1% Penicillin-streptomycin, 1% non-essential amino acids (N2a cells only) and 5% v/v foetal bovine serum (FBS) (10% v/v FBS for HEK cells). Experiments were performed when the cell cultures reached 70-80% confluency. In order to make new cultures, cells were washed with sterile PBS after removing the media. 1ml of Trypsin (Sigma) was used to detach the cells from the growth surface. Contents were transferred into falcon tubes and centrifuged for 10 minutes. The supernatant was disposed and the pellet was suspended in fresh media. An appropriate amount of the re-suspended cells were added to new plates and fresh media were added.

2.4 Primary culture preparation

Amygdale from P01 C57BL6/6J WT mice was dissected and placed on a dish containing 9.1 mM glucose, 25 mM Hepes, 5 mM KCl and 120 mM NaCl. Tissue was homogenised using the surgical blade and digested in buffer containing 5 mg of pronase E and 5 mg of thermolysin (Sigma) at RT for 30 minutes. Following trituration of the tissue, it was plated on poly-D-lysine - coated (Sigma) coverslips placed in 6 wells plate. Plates were incubated at 37^{o}C and 5% CO_2 for 24 hours following which 5 µM cytosine β–D-arabinofuranoside (Ara-C; Sigma) was added

for 48 h. Neurons were allowed to maturate for 17-21 days after in neurobasal medium. Lipofectamine was used to transfect the neurons at day 7-9 with a plasmid overexpressing mir-483-5p under the CMV promoter.

Primary cultures were made with the help from Dr Anna Skrzypiec and Ms Valentina Brambilla.

2.5 **Spine analysis**

Dendritic spine morphology was analysed semi-automatically. Spine parameters like length, head width and scale-free parameter (the length to width ratio) were determined to analyse the spine shape. The bottom part of the spine is considered to be 1/3rd of the spine length adjacent to the dendrite and was excluded from analysis. The head width of the spine was chosen to be the diameter of the largest spine head. In order to avoid any systematic spatial bias due to the difference in morphology of spines on dendrites, only spines that are distinguishable in the transverse direction and spines on the secondary dendrites were selected for analysis. The limitation of the image resolution was accounted for by discarding any spines that are smaller than 0.2μm. The parameters used to identify spines were described elsewhere (Bourgin, Murai, Richter, & Pasquale, 2007; Mariusz Mucha et al., 2011). Details of this are described in the Table 2.1 below. Spine densities were estimated along 650–1015 μm of secondary/tertiary dendritic branches per group. A total of 113 sections were analysed, 59 of which were scrambled conditions and 54 mir-483-5p conditions.

Spine analysis was performed in collaboration with Dr Anna Skrzypiec.

2.6 **Sholl analysis**

Sholl analysis was performed using Imaris software, where concentric circles with a 10µm gap between each circle was drawn starting from the soma following digital tracing of the dendritic tree. The number of intersections of a circle and dendritic tree was then measured. Primary axons were identified and excluded so that the quantification is an accurate reflection of dendritic complexity. A total of 55 neurons were analysed for Sholl analysis, 21 neurons for scramble conditions and 34 for mir-483-5p conditions.

Sholl analysis was performed in collaboration with Dr Anna Skrzypiec.

Spine type	Length	Width	Defining feature
Mushroom	<2 μm	>0.5 μm	Connected to the dendritic shaft by a narrower segment (neck)
Stubby	<2 μm	>0.5 μm	Do not possess a defined neck
Thin	<2 μm	<0.5 μm	Have a neck
Filopodia	>2 μm	<0.5 μm	Do not have a distinctive spine head
Irregular	variable	variable	More than one heads or necks

Table 2.1 Criteria for differentiating spine types

2.7 Synaptosomes preparation

Synaptosome preparations were performed using Syn-PER™ synaptosome preparation reagent (Thermo Scientific, Cat# 87793) following the manufacturer instructions. Briefly, mice were terminally anaesthetised with overdose of pentobarbital and then perfused transcardially with RNase free ice-cold PBS. Amygdale was dissected and weight was measured. Tissue samples were placed inside a pre-chilled Dounce tissue grinder. ~ 10 slow stroked were performed on the tissue samples after adding ten volumes of the reagent. Homogenate was centrifuged at 1,200 x *g* for 10 minutes at 4°C. The pellet was discarded and the supernatant was centrifuged for 15,000 x *g* for 20 minutes at 4°C. The resulting pellet was used for RNA extraction.

2.8 qRT-PCRs for miRs and mRNAs

Mice were terminally anaesthetised with overdose of pentobarbital and then perfused transcardially with RNase free ice-cold PBS. Amygdalae were dissected from a coronal slice (-0.58 to -2.3mm relative to Bregma) and submerged in RNase later solution to prevent RNA degradation. Before the extraction bench and pipettes were wiped using RNase-Zap (Sigma) and RNase-free pipette tips were used. Total RNA was extracted using the RNeasy Lipid tissue mini kit (Qiagen, cat# 74804) by following the manufacturer protocol. Tissue samples were placed in RNase free Eppendorf tube containing QIAzol and homogenised using a motorised pestle. After incubating the mix at RT for 5 minutes, 200µlof chloroform was added and vigorously mixed. Tubes were then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The aqueous upper layer was transferred into a new tube containing 70% ethanol and mixed thoroughly. The solution was then transferred to a spin column. After several washing steps and DNase digestion,

RNA was eluted from the spin column using elution buffer. Concentration and quality of RNA were verified using NanoDrop and electrophoresis. A total of 1-2ug of total RNA was converted to cDNA using Superscript III (Invitrogen), oligo (dT) primers by following the manufacturer's instructions. The list of primers used for the qRT-PCR assays is shown in Table 2.2 below.

Total RNA from cell lines was extracted using the RNeasy Mini Kit (Qiagen, Cat# 74104). After removing the media from the wells and gently washing with PBS, QIAzol was added to lyse the cells. Lysed cells were then moved to an Eppendorf tube and 70% ethanol was added. Essentially the similar step used for RNA extraction from tissue was followed but using specialised buffers of RNA extraction from cell lines. 1-2 µg of total RNA was used for subsequent cDNA synthesis and 1µl for the cDNA was used for q-PCR reactions into a final volume of 20µl (QuantityNova, Qiagen, Cat# 208052).

miRNeasy Mini Kit (Qiagen, Cat# 217004) were used for the miR extraction. Tissue samples were submerged in QIAzol and homogenised using motorised pellets. When synaptosomes were used for RNA extraction, purified synaptosomes were added to QIAzol and similar steps were followed. 0.5-2ug of total RNA was used for cDNA synthesis. miScript II RT Kit (Qiagen, Cat# 218160) was used to convert mature miR into cDNA using miScript Reverse Transcriptase mix and nucleics mix. miScript SYBR® Green PCR Kit (Qiagen, Cat# 218073) was used to analyse the relative level of mir-483-5p using the custom synthesised primers for mir-483-5p using the miScript Primer Assay system (Qiagen, Cat#: 218300). mir-483-5p is normalised to RNU6-2 nuclear RNA (Qiagen).

Gene symbol	Forward primer (5' to 3')	Reverse primer (5' to 3')
Bcr	TGTCTGAGGCTACCATCGTG	TGTTCCAAACGAGGAATCTGC
Shh	AGGAAAACACGGGAGCAGAC	GGTCACTCGCAGCTTCACTC
Cdk7	CGGGTCTGGTTTCTCACTCG	CGTCGCAAACTGTCCCTCTC
Ctnnbip1	CGGCACCTTGCTATTTCTTCTC	AGTTCTGGGGACACCTGCTTC
Cul4a	GGACAGGGAGGTTCCAGAATAC	CTCCACACAGGCAATCAACG
Pgap2	CATCCCTCGGTTACATGCTCC	GACTTGCGATCTGTGTGCTTC
Gpx3	GGGGGCTTTGTGCCTAATTTC	TGGGAGGGCAGGAGTTCTTC
Macf1	ATGACCTTTGTGATGGTTCTGC	TCTGCAACCCTTTTGTTCCG
Rbpj	AGAGTTTGTGGAAGATGGCG	CACTGTTTGATCCCCTCGTTC
Saal1	TGGAGGGGCTGGATGTTTAC	TATCAGGACACTGCACGATGG
Tbkbp1	TTCATTCTGTGCGGGCTTCC	CACCGTCTCCATCAGTAGGTTG
Tnik	CCAAGGTGAAACCAGAAGAATCC	AATGCCGTCAGATCCTCATC
ß-actin	TGCTCCTCCTGAGCGCAAGTACTC	CGGACTCATCGTACTCCTGCTTGC

Table 2.2 Primers used for PCR assays

2.9 Bioinformatics

We have used three different databases to predict potential targets of mir-483-5p. EIMMo (http://www.mirz.unibas.ch) database was used to search for targets that are expressed in the mouse amygdala that produced 215 possible transcripts. AmiGo (http://amigo.geneontology.org) database was used to predict the transcripts that are stress related. The results outputted 4,816 possible targets. Then we used TargetScan (www.targetscan.org) algorithm to predict the targets for mir-483-5p to obtain 525 possible targets. Twelve of the transcripts that were found to be common in all of the three separate lists were used for subsequent studies to verify if they were down-regulated by mir-483-5p.

Bioinformatical analysis were performed in collaboration with Dr Anna Skrzypiec.

2.10 Dexamethasone treatment of N2a cells

N2a cells were grown on six-well plates as mentioned above. When 70-80% confluency is reached, the cells were transfected with 1µg per well of either a control plasmid or mir-483-5p (pEGP-mir483-5p, Cell Biolabs). Cells were transfected using TurboFect™ (Thermo Fisher Scientific) by following the manufacturers' protocol. 24hrs post transfection cells were treated with dexamethasone for three consecutive days at a final concentration of 1µM. On the fourth day total RNA was extracted and qRT-PCR performed as described in section 2.8 (p. 75).

Data from dexamethasone treatment of N2a cells were obtained through collaboration with Dr Anna Skrzypiec.

2.11 Immunohistochemistry

Mice were injected intraperitoneally with an overdose of pentobarbital to euthanise and then perfused transcardially with ice-cold PBS followed by 4% PFA

in PBS. The brains were extracted and fixed in 4% PFA in PBS overnight. The following day, brains were washed with PBS and 70 µm thick coronal sections were cut using a vibratome (Campden Instruments, UK). Sections were washed in PBS 3 x 10 minutes. Washing steps were followed by incubation and gentle agitation of the sections in PBS + 0.1% Triton X-100 for 1 hour at RT. Slices were incubated with primary antibody overnight at 4^{o}C while being gently rotated. Antibodies used were: *Pgap2* (1:500, Abcam cat#: ab175493), GPX3 (1:1000, Abcam cat#: ab27325), MACF1 (1:1000, Santa Cruz Biotechnology cat#: SC-68430), NeuN (Abcam, cat#: 104224), GFAP (Abcam, cat#: ab7260). Sections were washed (3 x 15 minutes) in PBS + 0.1% Triton X-100, 10% FBS at RT for 1 hour. Then, sections were incubated with gentle mixing and protected from light in secondary antibodies conjugated with fluorescent tags. In cases where the primary antibody is conjugated with a fluorophore, the sections were processed without secondary antibody incubation, and everything else was the same. After incubation, slices were washed for 3 x 15 minutes with 1 x PBS + 0.1% Triton X-100. Slices were then mounted on glass slides using mounting reagent, left to dry at RT protected from light before undergoing imaging using a confocal microscope.

Immunohistochemistry was performed with help from Dr Anna Skrzypiec and Ruby McDonald.

2.12 **Dual-Glo luciferase activity**

Dual-Glo® Luciferase assay (Promega, cat#: E1910) system is used to perform luciferase assay to check for direct interaction between mir-483-5p and *Pgap2*, *Gpx3* and *Macf1* 3'UTR regions. This assay system utilises *Firefly* luciferase to quantify the signal due to miR interaction and *Renilla* luciferase to normalise for

global and specific effects such as viable cell number and transfection efficiency, Figure 2.2 (p. 87). 3' UTR region containing the seed sequence for *Pgap2, Gpx3* or *Macf1* was inserted to the MCS site at 3' of the Firefly luciferase gene (*luc2*) using SacI and XbaI restriction sites.

In order to induce mutations in the seed regions of 3'UTR's of the target genes, primers with substituted bases that span the seed regions (Sigma) induced mutations. For *Gpx3* and *Macf1* the plasmids are amplified using the primers while for *Pgap2*, mutated primers were designed to synthesise the 3'UTR regions which are later digested and ligated into the pmiRGLO Dual-Luciferase vector. The substitutions induced in the seed regions are shown in Figure 4.6 (p. 181). Mutated WT 3'UTR regions are ligated into the pmiRGLO-Dual Luciferase vector (Promega, Cat#E1330). The 3'UTR region is ligated downstream of the *Firefly* luciferase gene, and the luminescence from firefly luciferase is used as the primary reporter gene. The vectors also have gene encoding for *Renilla* luciferase (*hRluc-neo*) acting as a control reporter to normalise for transfection efficiency. N2a cells are co-transfected with pmiRGLO Dual-Luciferase miR Target Expression Vector and vector overexpressing mir-483-5p or scramble sequence. Cells were grown and transfected in 96 well plates. Twenty-four hours after the transfection the wells were washed with PBS and lysed for 10 minutes. Following the cell lysis luminescence from Firefly luciferase was measured using PHERAStar® Plus (BMG LABTECH). Following the initial reading, Dual-Glo® Stop & Glo® reagents were added to quench the *Firefly* luciferase and elicit luminescence from *Renilla* luciferase. The plate was incubated with gentle shaking at RT for 10 minutes. Following the incubation, *Renilla* luminescence was measured. The activity of mir-483-5p was calculated as a ratio of *Firefly*

luminescence to *Renilla* luminescence for scramble and mir-483-5p conditions. Data is represented in the graphs as percentage decrease in luminescence relative to scramble.

Luciferase assay was performed with the help from Dr Anna Skrzypiec.

2.13 **Stereotaxic surgery**

Mice aged 6-8 weeks were used for the surgery. Mice were introduced to the chamber with a constant supply of 5% isoflurane with 4 l/min of oxygen to induce anaesthesia. Following the induction, the animal was prepared for the surgery and placed on a mouse stereotaxic frame (Stoelting Europe, Figure 2.3, p. 89) while supplied with a constant rate of 2.5% isoflurane and 1 l/min oxygen through a mouse face mask (Stoelting Europe) to maintain the anaesthesia. Breathing of the animals was monitored visually, and core temperature was monitored using a rectal thermometer. The stereotaxic coordinates used to target the basolateral amygdala (BLA) were; −1.5 mm anteroposterior (AP), ±3.0 mm mediolateral (ML) and −4.2 & -4.7 mm dorsoventral (DV). First, the required amount of lenti particles were aspirated into a 10µl NanoFil© (World Precision Instruments) syringe. The needle was inserted to -4.7mm (DV) and the lenti particles were injected at a rate of 10nl per minute. After the injection, the needle is left undisturbed at the location for another 5 minutes after which it is retracted back by another 0.5mm to reach –4.2mm. After 5 minutes of resting period, the needle is completely withdrawn from the brain. Particles were injected bilaterally. Following the injection, the incision wound was closed using Vetbond, and the animals were left to recover for 3-4 weeks. Animals were examined daily for a week and regularly inspected until the behavioural experiments. After the experiments, animals were sacrificed by an overdose of anaesthesia and brain extracted to verify the injection sites.

Immunohistochemistry using fluorescently tagged anti-eGFP antibodies were used to (Santa Cruz Biotechnology Inc,; cat# sc-9996) visualise the accuracy of injection sites (Figure 5.4, p. 215 & Figure 5.7, p. 221).

2.13.1 Lentiviral gene transfer

mir-483-5p sequences or a control scramble sequence were inserted into the LV-pUltra plasmid empty backbone (Addgene, cat#: 24129). NheI (restriction site at the 5′ terminus) and the SacI (restriction site at the 3′ terminus) were used to ligate the insert. The insert is ligated downstream of an enhanced green fluorescence protein (EGFP), separated by a self-cleaving peptide P2A.

In order to produce the lentiviral particles, HEK293t cells were grown to reach 70-80% confluence. Next, cells were co- transfected with addressing plasmid LV-pUltra-miR and packaging plasmids (pCMV delta R8.2, Addgene cat#12263) and (pCMV-VSV-G, Addgene cat#8454). Forty-eight hours post transfection the virus particles were concentrated using ultracentrifugation. The particles were diluted in cell culture media, and virus titre was performed using qRT-PCR using standard curve method. The estimated concentration was at 9.9 x 10^7 particles/ml.

Lentiviral particles to knock down all of the known transcripts of *Pgap2* (NM_001291358.1 & NM_145583.3) were purchased from AMSBIO. A negative scramble control (GTCTCCACGCGCAGTACATTT) was also made. shRNA stem-loop structure was inserted into the vector that drives the expression through the H1 promoter. Lentiviral vector was sequence verified and was co-transfected with packaging plasmids (Cat# HT-pack) into a cell line (Cat# TLV-C). DMEM medium with 10% serum was used for viral packaging. Viruses were collected and filtered through 0.45μm filters. Particles were used to transduce

HT1080 cells, and GFP positive cells were counted after 72 hours to obtain infection-forming units (IFU/ml) per millilitre. The concentration was estimated to be 1.07 x 10^7 IFU/ml.

Lentiviral particles were prepared with the help from Dr Mariusz Mucha.

2.14 Statistics

Data are expressed as mean ± standard error of the mean (SEM). Student's t-test (when two groups were compared) or analysis of variance (ANOVA) followed by Tukey's HSD Multiple Comparison post-tests was used as appropriate. P values of less than .05 were considered significant. ANOVA P values are reported in the text. Asterisks indicate the P values that are lower than 0.05.

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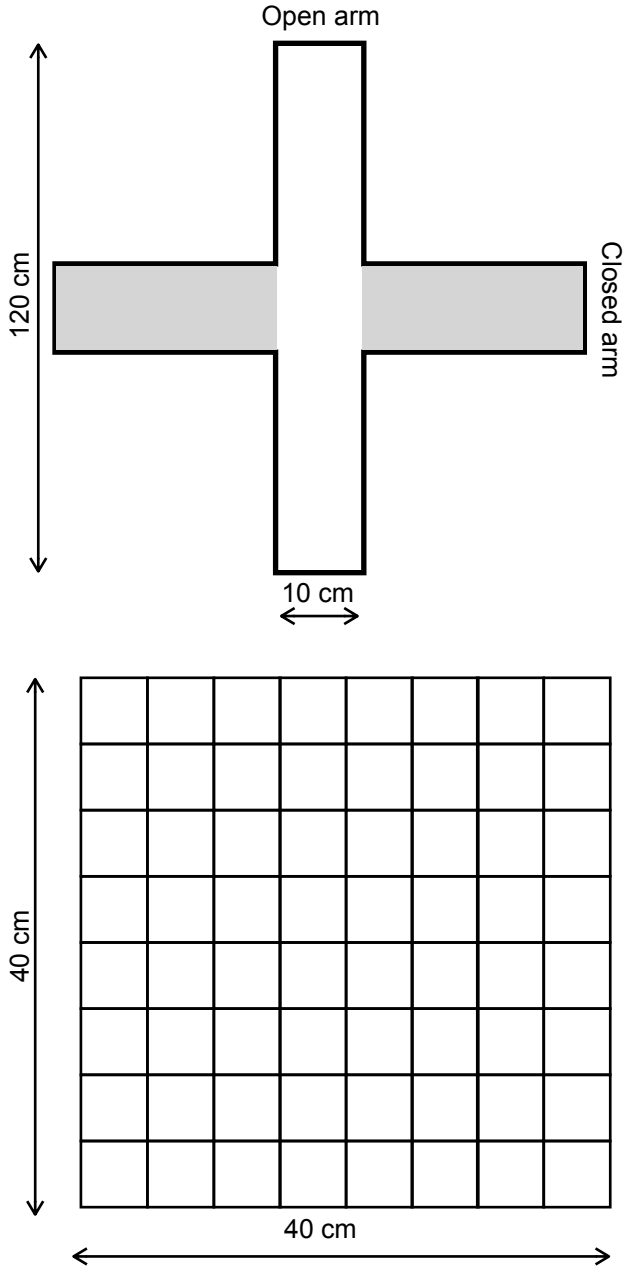

Figure 2.1 Dimensions of behavioural equipment
Diagrams above show the measurements of Elevated plus maze (EPM) (top) and open arena (bottom). Both apparatuses were placed 50 cm above the floor level on a stand. The subject was placed in the middle of the apparatus and the activity was recorded using a camera attached to the ceiling. Illumination is provided using two 60W lambs. See section 2.2 for more details.

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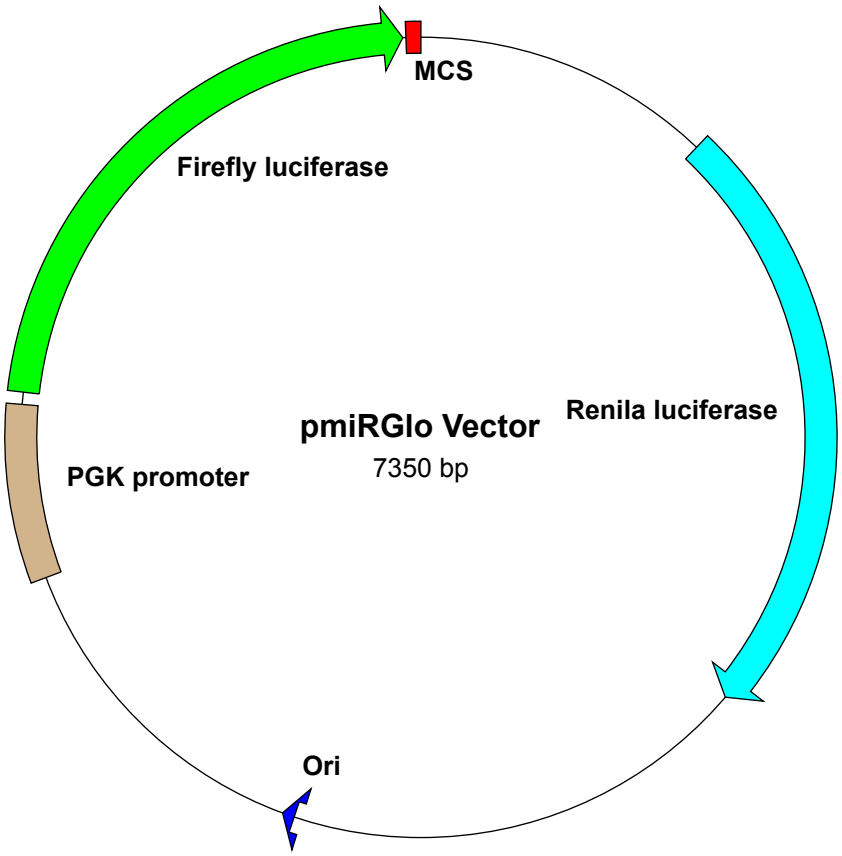

Figure 2.2 pmiRGlo Dual-luciferase vector
Firefly luciferase gene is expressed under the ubiquitous phosphoglycerate kinase (PGK) promoter. The UTR of the gene of interest is cloned to the MCS (Multiple cloning site) downstream of the Firefly luciferase gene. The luminescence emitted following the translation of the Firefly luciferase can be quantified to determine the efficiency of translational inhibition by miR of interest. If miR does target the predicted UTR, then less *Firefly* gene will be translated and concomitantly results in relatively reduced luminescence compared to the controls. The *Renilla* luciferase also expresses another gene, translation of which also results in luminescence. At the end of the assay, luminescence from the *Firefly* luciferase is normalised to the luminescence from *Renilla* luciferase to quantify the level of translational inhibition by miR.

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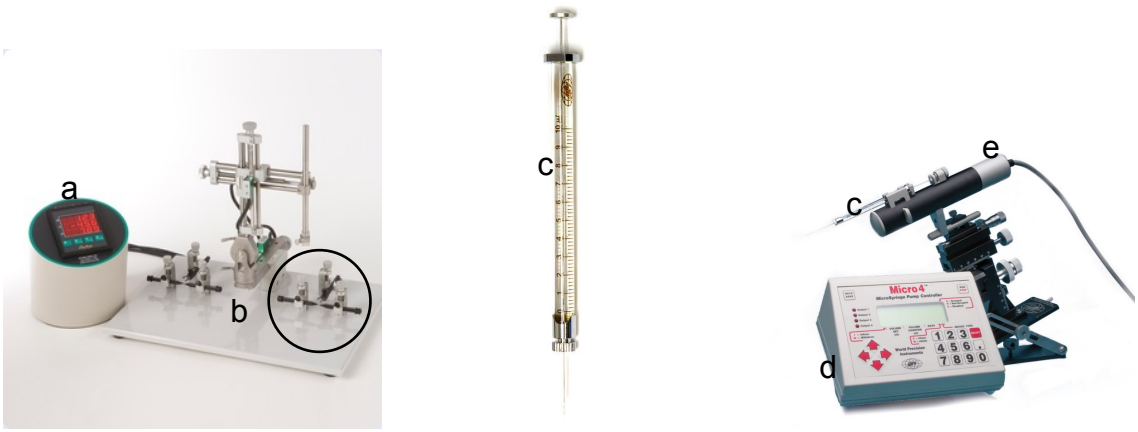

Figure 2.3 Stereotaxic surgery equipment
Surgery was performed using the above pieces of equipment. Following Anaesthesia mouse head was fixed on the frame (on the area shown in the circle). See section 2.13 for more details. a: Digital reader to establish the coordinates, b: Stereotaxic frame, c: Nanofil syringe with needle, d: Digital controller for the micro-injector, e: Micro-injector used to inject virus particles to the amygdala.

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CHAPTER 3 Role of mir-483-5p in neuronal plasticity

3.1 Introduction to neuronal plasticity

Survivability and reproductive success of organisms depend on its adaptability, ability to learn, repair injuries and respond to threats and rewards. In complex organisms, the brain or nervous system enables these activities by guiding the behaviour through the activation of neuronal networks that encodes external environmental representations. The term neuronal plasticity encompasses the structural and functional changes in neurons and synapses that allow the nervous system to adapt to new conditions through learning, memory and repair. Regulation of neuronal plasticity involves modifications of structural and functional characteristics of neurons. Structural plasticity includes changes in dendritic arborisation, dendritic spine morphology, axonal sprouting and synaptogenesis, while functional plasticity involves long-term potentiation (LTP), long-term depression (LTD) and homeostatic plasticity (Ye, Xu, Su, & He, 2016). The use of terms structural and functional can be sometimes misleading because functional changes in neurons can lead to concomitant structural changes and vice versa. The primary determinant of plasticity is neuronal activity and intra- and inter- neuronal biochemical milieu. Several signalling cascades underlie the mechanisms by which plasticity-related changes are manifested. Irrespective of these changes, the basis of these mechanisms involves protein synthesis, protein degradation, regulation of receptor density, receptor membrane trafficking, protein transport and post-translational modifications like phosphorylation, palmitoylation and ubiquitination. These biochemical processes underlie the machinery that enables neuronal plasticity.

A breakdown in these mechanisms can lead to several pathologies that can be manifested in various forms of neurological and cognitive disorders.

These include Alzheimer's disease, Fragile X Syndrome, epilepsy, schizophrenia, autism, cognitive impairments, Major Depressive Disorders (MDD), PTSD, Phobias, OCD and anxiety disorders (Bechtel et al., 2014; Chang, Zhang, Xu, Jing, & Zhan, 2014; Chiu, Alqadah, & Chang, 2014; Huang, Guo, Wang, & Chen, 2014; Pittenger, 2013; X.-L. Xu et al., 2011; Ye et al., 2016). Aetiology of these disorders is heterogeneous; however, it can include age-related decline, maladaptation to stress, brain injury, severe psychological trauma and genetic mutations. A combination of these factors or one of the factors alone can result in severe malfunctioning of plasticity-related mechanisms. Therefore, a comprehensive understanding of these mechanisms is vital for the effective management of these conditions. Several studies have unveiled the importance of mechanisms involved in synaptic plasticity and tried to elucidate the complex orchestrated molecular changes that facilitate these mechanisms. Protein synthesis and degradation are one of the mechanisms that lie at the heart of these orchestrated changes. As translational repressors, miRs have essential roles to play, both directly and indirectly, in staging the right conditions and fine-tuning the molecular pathway necessary for optimal neuronal plasticity to take place. This is unsurprising given more than half of the detectable miRs are identified within the nervous system (Holt & Schuman, 2013; Jobe, McQuate, & Zhao, 2012; Lenkala, Gamazon, Lacroix, Im, & Huang, 2015) suggesting its role in a plethora of mechanisms including synaptic plasticity. Indeed, several miRs are shown to be involved in the precise control of mechanisms at pre- and post-synapses that leads to synaptic plasticity by regulating one or more of its aspects (Ye et al. 2016). We have known that LTP causes an increase in spine density (Maletic-Savatic, Malinow, & Svoboda, 1999;

Shi et al., 1999) and enlargement (Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004), while LTD causes spine shrinkage in neurons (Zhou, Homma, & Poo, 2004). Studies have also shown that several miRs are up-regulated or down-regulated in a tissue-specific and spatiotemporal manner upon the induction of LTP or LTD (Park & Tang, 2009). These studies strengthen the observations that miRs are crucial regulators of neuronal plasticity changes in the nervous system (Davis, Haas, & Pocock, 2015; Rajman & Schratt, 2017). Following sections will look at the properties of miRs that make these little molecules effective neuronal plasticity modulators and mechanisms through which these changes are facilitated.

3.2 miRs – efficient neuronal plasticity regulators

Neuronal plasticity is a complex process that requires effective orchestration of various biochemical pathways and synchronised molecular functioning. This section looks at some of the evidence exposing the role of miRs to function as active players in facilitating these mechanisms through their intrinsic properties. These properties enable miRs to target several mRNAs collectively, exhibit compartmentalised expression patterns and responded to neuronal activation. One such important characteristic that makes miRs ideal candidates for the fine-tuning of protein synthesis required during synapse formation is the ability to repress or degrade several mRNA targets simultaneously (Bartel, 2009; Lim et al., 2005). Additionally, miRs can be transcribed in clusters which increases the range of its activity by targeting several proteins that might have diverse functions (Clokie, Lau, Hee, Coon, & Klein, 2012). Furthermore, new pieces of evidence showing reversible suppression of mRNA by miRs are updating its functional abilities and add more flexibility to their modus operandi than previously

suspected (Bhattacharyya, Habermacher, Martine, Closs, & Filipowicz, 2006; Vasudevan, Tong, & Steitz, 2007). These characteristics enable miRs to changes the availability of several protein products by functioning as molecular brakes. This is important for maintaining homeostasis and for responding promptly to the needs of the cell or cellular compartments where effective response depends on the ability to regulate the available molecular machinery by quick temporal responsiveness.

Another important characteristic of miRs is their ability to respond to neuronal activation. Since synaptic plasticity is a function of neuronal activation and miRs are transcribed following neuronal activation, miR induced translational repression forms an important mechanism by which synaptic plasticity is facilitated. Researchers have identified several miRs that respond to neuronal activation (Schratt, 2009b, 2009a). One of the ways to signal the transcription of miRs following neuronal activation is via transcription factors that target miR genes. Studies have revealed that several transcription factors that are triggered by the neuronal activity can initiate the transcription of miR genes (Davis et al., 2015). For example, Fiore and colleagues in their experiment in rat primary neuronal cultures observed that depolarisation of neuronal cells resulted in increased expression of mir-379-410, a cluster of at least three different miRs (Fiore et al., 2009). Subsequently, they discovered that the up-regulation of this cluster is a result of binding of the transcription factor MEF2 (myocyte enhancer factor 2), a transcription factor released during neuronal activation. Moreover, studies have shown that MEF2 expression has an inverse relationship on synapse number (Shalizi & Bonni, 2005). One of the miR from the cluster, mir-134, can be affected by BDNF expression (Schratt et al., 2006). BDNF is an

important transcription factor involved in neuronal plasticity and is up-regulated following neuronal activity. Additionally, BDNF is involved in synaptic strength and implicated in several CNS disorders (Autry & Monteggia, 2012; Pezet & Malcangio, 2004). Additionally, mir-134 is involved in the regulation of dendritic outgrowth. This is one example of how neuronal activity-dependent release of transcription factors can, in turn, up-regulate several miRs through which protein translation could be repressed to modify specific aspects of neuronal plasticity. Additionally, another miR, mir-132, has been shown to be up-regulated in neurons following its activation and it is involved in the regulation of spine formation through the activation of *Rac1-Pak* actin remodelling pathway (Impey et al., 2010). Further evidence for this phenomenon comes from the studies using hippocampal slices and synaptosomes.

Synaptosomes are small vesicles made from the brain tissue homogenate and are enriched with dendritic spines. Several proteins and ncRNAs, especially miRs, and post-synaptic densities (PSDs) were identified from synaptosomes (Lugli, Torvik, Larson, & Smalheiser, 2008). Synaptosomes have also been used to study functional aspects like neurotransmitter release, proteomic studies, RNA studies, calcium reuptake assays among other aspects of synaptic physiology (Evans, 2015). These properties of synaptosomes make these attractive tools to study several aspects of neuronal plasticity. Stimulating hippocampal slices or intracellular activation of synaptosomes release an active form of DICER and eIF2c, both of which are crucial to form active complexes required for miR mediated translational repression (Lugli, Larson, Martone, Jones, & Smalheiser, 2005). Furthermore, studies in synaptosomes have shown that Calpain, a Ca^{2+} dependent cysteine protease (duVerle, Ono, Sorimachi, Mamitsuka, & Muller,

2011), can cleave and thus activate DICER, a mechanism that might be responsible for the elevated expression of miRs in active neurons and synapses (Lugli et al., 2005). These experiments and several other studies have shown miRs can be up-regulated or transcribed following neuronal activity in response to the environmental stimuli, and several of these miRs are involved in the modification of various aspects of neuronal plasticity (Hollins & Cairns, 2016).

Another important feature of a neuronal plasticity regulator is its ability to change protein turnover in localised compartments. Anisotropic property of neurons separates synapses, up to 100s of microns, from its soma. Recent evidence suggests that there might be compartmentalisation and sub-compartmentalisation within spines that enable it to respond effectively to accommodate the need for optimal synaptic plasticity to occur (Chen & Sabatini, 2012; Colgan & Yasuda, 2014; Newpher & Ehlers, 2009). Electron micrographs have shown that endoplasmic reticulum is present within spines and postsynaptic regions. This strengthens the idea that there is localised translation and therefore post-translational modifications within these regions.

Additionally, several studies have suggested that the protein turnover at synaptic compartments are independently managed from other intra-cellular areas (Alvarez-Castelao & Schuman, 2015; Bai & Witzmann, 2007). Unique structural characteristics of spines enable them to maintain different biochemical milieu compared to the soma. The presence of such localised machinery is essential in order to respond effectively during high demand circumstances like the processes leading to synaptic plasticity (Alvarez-Castelao & Schuman, 2015; Sambandan et al., 2017). Overall, the available data suggest that separate protein translation and translational repression takes place at juxta-synaptic

regions and the local control of the synaptic process is essential for a synapse to function (Holt & Schuman, 2013; Sutton & Schuman, 2005).

miRs are ideal candidates for the regulation of processes that have a compartmental bias. Experiments have shown that miRs indeed have such spatial bias and involved in mechanisms locally, independent from the protein transcription and translation close to the soma (Cohen, Lee, Chen, Li, & Fields, 2011; Fiore et al., 2009; Roberto Fiore, Siegel, & Schratt, 2008; Kye et al., 2007; Sambandan et al., 2017; Schratt, 2009a). Some experiments used multiplex reverse transcription and laser-capture microdissection to investigate the spatial expression pattern of miRs. They have found that 187 miRs in rat hippocampal neurons exhibit compartmentalisation (Kye et al., 2007). Several other studies have used synaptosomal fractions to screen for miRs with spatial bias and identified several miRs that are expressed preferentially in the dendrites when compared to somatic fractions (Lugli et al., 2008; Siegel, Saba, & Schratt, 2011; Smalheiser & Lugli, 2009). In a further study when researchers used Pilocarpine to induce status epilepticus in rat hippocampal neurons, they identified differential regulatory patterns of miRs in nuclear fractions compared to synaptosomal fractions (Risbud & Porter, 2013). Proteins that are typically associated with miR processing, DICER, AGO2, FMRP and various P-body components are distributed within the dendrites of mature neurons (Barbee et al., 2006; Lugli et al., 2005) showing that the miR induced translational regulation takes place at these sites. Moreover, it has been shown that P-bodies containing miRs have been responding to neuronal activation in dendrites (Cougot et al., 2008). P-bodies are cytoplasmic structures that contain miRs, mRNAs and protein complexes required for mRNA decay like deadenylases and exonucleases.

Hitherto available studies indicate that miRs are preferentially expressed in juxta-synaptic regions and are involved in the local regulation of protein translation thereby functioning as effective plasticity regulators. Incorporating actions of miRs to the understanding of regulatory gene networks is essential to comprehend and possibly manipulate the mechanisms that underlie neuronal plasticity. The following section will look at some of the evidence showing how miRs can function as effective plasticity regulators.

3.3 **Putative mechanisms of miR-mediated neuronal plasticity changes**

Neuronal plasticity is a product of several mechanisms working in unison. These include dendritic arborisation, spine formation, modification and protein synthesis. It has been known that large pools of miRs are transcribed during synaptic development in post-mitotic neurons; most of these are associated with translational regulator complexes (Kim et al., 2004). These data and many others show the miRs are important players for optimal neurogenesis and maturation (Follert, Cremer, & Béclin, 2014; Rajman & Schratt, 2017). As outlined in the previous section, it has been the scientific consensus that miRs play a crucial role in various aspects of neuronal plasticity, owing to their unique properties. The following section will look at the role of miRs in some of the aspects of neuronal morphology.

Induction of LTP and LTD is used to study the neuronal connection between circuits. Protein synthesis and degradation are necessary for LTP and LTD to take place. Studies have shown that several miRs are up-regulated and down-regulated when LTP or LTD is induced, suggesting the role of miRs in these phenomena (Park & Tang, 2009). Furthermore, experiments in *C.elegans* have shown that mir-1 regulates the availability of *ACh* (Acetylcholine) receptor post-

synaptically. Interestingly, the same miR has been shown to affect transcription factor MEF2 and control the presynaptic neurotransmitter releases. Previously, it has been demonstrated that MEF2 can activate the transcription of miR clusters that are important in synaptic plasticity (Fiore et al., 2009). This shows the dynamic potential of miRs in fine-tuning receptor signal interaction across pre- and post-synapse. Moreover, in *Drosophila melanogaster*, it has been shown that mir-284 regulates the availability of GluRA and GluRB subunits of fly AMPA type glutamate receptors in neuromuscular junction of glutamatergic synapses (Karr et al., 2009). The availability of these subunits has been shown to be a marker for postsynaptic strength (Shi et al., 1999). These subunits are orthologous of mammalian GluR1 and GluR2 subunits in hippocampal dendrites (Ju et al., 2004). These findings suggest that miRs can affect synaptic plasticity by regulating receptor dynamics across the synapse and can be involved potentially in feedback / feed-forward mechanisms.

Another mechanism of fine-tuning synaptic plasticity is by affecting the dendritic arborisation or dendritic spine morphology. Understanding mechanisms involved in dendrite arborisation and spine dynamics are critical for the understanding of synaptic plasticity. Dendrites are the first points where an action potential from neighbouring neurons contacts postsynaptic neurons. Research has shown that protein synthesis is essential for dendrite growth (Jaworski, Spangler, Seeburg, Hoogenraad, & Sheng, 2005). The role of miRs in the dendritic arborisation is slowly starting to emerge. One piece of evidence to support the role of miRs in dendritic spine dynamics come from studies that show DICER null mouse exhibit significant changes in spine morphology, but not in spine number (Davis et al., 2008). These findings were reproduced in cultured

neurons where the authors showed that knockdown of DICER resulted in significant reduction of miRs and a concomitant reduction in dendritogenesis in post-mitotic neurons (Davis et al., 2008). Additionally, experiments involving knocking down of enzyme SDE3 helicase Armitage showed significant changes in miR induced translational repression, memory formation and olfactory learning in Drosophila (Ashraf, McLoon, Sclarsic, & Kunes, 2006). This is thought to be via the pathway that involves CAMKIIα, an important protein in synaptic plasticity regulation via LTP. Moreover, knockdown of enzymes required for miR biogenesis, DICER and PASHA, results in the failure of dendritic targeting in Drosophila neurons (Berdnik, Fan, Potter, & Luo, 2008). These pieces of evidence suggest that miRs are effective regulators of specific aspects of dendritic spine dynamics.

Another proposed mechanism that regulates dendritic arborisation by miR is through the indirect control of actin cytoskeleton. One of the first miR that was identified to have this role is mir-132 (Vo et al., 2005). mir-132 transcription is induced by CREB, a Ca^{2+} sensitive transcription factor that couples neuronal activation to neuronal plasticity (Sakamoto, Karelina, & Obrietan, 2011). Studies in neuronal cultures have shown that mir-132 is necessary and sufficient to stimulate neurite outgrowth (Vo et al., 2005). Moreover, mir-132 is shown to be involved in both basal and activity-dependent synaptic plasticity in neurons (Fiore et al., 2009; Wayman et al., 2008). Further studies have shown that mir-132 can bi-directionally regulate neuronal outgrowth by targeting the repression of GTPase-activating protein, p250GAP (Impey et al., 2010; Vo et al., 2005; Wayman et al., 2008). These GTPases are important regulators of the actin cytoskeleton in dendrites. The importance of these pathways in modulating

dendritic dynamics suggests that these mechanisms might be evolutionarily conserved. For example, mir-124, a miR previously identified to be involved in neuronal fate (Yu, Chung, Deo, Thompson, & Turner, 2008), has been shown to affect dendritic arborisation in *Drosophila melanogaster* (Xia-Lian Xu, Li, Wang, & Gao, 2008), regulate long-term synaptic plasticity in *Aplysia californica* (Rajasethupathy et al., 2009) and in rodents (Chandrasekar & Dreyer, 2009). This evidence points towards evolutionarily conserved miR regulated neuronal plasticity mechanisms underlying dendritic arborisation.

The function of miRs in regulating neuronal plasticity is not limited to functional plasticity or in dendritic arborisation. miRs have also been studied in the context of spine dynamics. One of the ways miRs can regulate spine dynamics is by down-regulating LIMK1. LIMK1 is a kinase that phosphorylates cofilin, an actin-depolarising factor, and thus increases spine growth (Schratt et al., 2006). It has been shown that mir-134 is localised to dendrites of hippocampal neurons where it down-regulates LIMK1 levels (Schratt et al., 2006). Interestingly, when BDNF is expressed, the effect of mir-134 induced LIMK1 reduction was attenuated. Because BDNF levels are dependent on neuronal activity, mir-134 could be potentially involved in the regulation of spine growth based on neuronal activation. Another proposed mechanism involves manipulation of post-translational modifications. mir-138 targets an enzyme, APT1, involved in de-palmitoylation of proteins involved in the synapse regulation (Siegel et al., 2009). So far, the evidence suggests that the miRs can suppress spine enlargement by controlling the palmitoylation of $G\alpha_{13}$ subunits of G proteins. Altogether, data suggest that miRs are effective dynamic regulators of spine morphology, regulate dendritic arborisation and functional plasticity. Research is slowly unveiling a

complex network of interactions and mechanisms employed by miRs to affect neuronal plasticity, some of these mechanisms might be evolutionarily conserved. Therefore, it is rather unsurprising to see miRs being involved in disorders involving synaptic plasticity. The next sections look at some of these miRs that are involved in the disorders of synaptic plasticity.

3.4 **miRs in the synaptic plasticity-related disorders**

Functional involvement of miRs in synaptic plasticity suggests that they may play important roles in disorders of synaptic plasticity. Several neurological disorders like schizophrenia, mental retardation and neurodegenerative disorders have faulty synapses, a pattern that is recognised as a central theme in many of the neurological conditions (Pietrzykowski et al., 2008). Several RNA binding proteins like FMR1 have been implicated in neurological disorders such as Fragile-X-syndrome (Jin et al., 2004) suggesting the role of non-coding RNAs in these disorders. Moreover, miRs are shown to be useful biomarkers for detecting Alzheimer's disease (Cogswell et al., 2008) and in status epilepticus (Raoof et al., 2017). Mouse models with a deletion the chromosomal region 22q11, a region that is correlated with schizophrenia in humans, have been developed. These mice show significant changes in the mature miR repertoire. One of the miRs that is significantly up-regulated is mir-134 (Stark et al., 2008) which was previously shown to be important in spine morphology (Schratt et al., 2006). As seen previously, mir-134 targets LIMK1, a kinase important to neuronal plasticity and shown to be relevant in William's syndrome (Hoogenraad, Akhmanova, Galjart, & De Zeeuw, 2004). Another example suggesting the role of miRs in these disorders is the role of mir-219 in schizophrenia (Kocerha et al., 2009). The proposed pathway of this phenomenon is by affecting the CaMKIIγ, which is a

downstream effector of the NMDA receptor (NMDAr). NMDAr plays important roles in synaptic plasticity by enabling calcium to influx into the post synapse and by triggering several signalling cascades that result in changes to AMPA receptor density and activation of several kinases. Another example of a plasticity-associated condition is Rett syndrome. It is a sex chromosome-linked disorder caused by mutations in the MeCP2 gene (Liyanage & Rastegar, 2014; Martínez de Paz & Ausió, 2017). Interestingly, mir-132, which was involved in activity-dependent neuronal plasticity (Fiore et al., 2009), is shown to be targeting MeCP2 and in the development of Rett syndrome (Klein et al., 2007). Interestingly, adenovirus-mediated knockdown of MeCP2 in BLA is shown to increase anxiety in mice (Adachi, Autry, Covington, & Monteggia, 2009), suggesting its potential role in anxiety-related disorders. Another mechanism important in the neuronal plasticity is axon guidance. miRs play an important role in axon guidance (Iyer, Bellon, & Baudet, 2014) which is an essential feature of the neurons during development and *de novo* synaptogenesis. Additionally, miRs are essential for neuronal placement and polarity (Kos et al., 2017). For example, while knock-down of mir-338 resulted in the loss of neuronal polarity and the number of neurons within the mouse cortical layer, the overexpression resulted in contrasting effects. Furthermore, several miRs, mir-124, let-7 and mir-181a are misregulated during cocaine-induced synaptic plasticity, suggesting its potential use in the intervention, prevention, treatment, diagnosis or prognosis of addictions (Chandrasekar & Dreyer, 2009). The role of miRs in neurological disorders is extended to the homeostatic mechanisms that allow neurons to maintain a stable firing rate during extreme circumstances, also known as neuronal homeostasis (Ramocki & Zoghbi, 2008; Turrigiano, 2007). The failure

of neuronal homeostasis is thought to be one of the important features leading to several neuropsychiatric disorders (Ramocki & Zoghbi, 2008). Table 1.1 shows examples of miRs that are shown to be involved in several psychiatric disorders and in animal models.

This chapter has presented the idea that miRs are important players in several aspects of neuronal plasticity. miRs are attractive agents through which particular aspects of neuronal plasticity can be manipulated. Here we report the identification and characterisation of mir-483-5p, a novel miR that is up-regulated locally in amygdala neurons after 6 hours of restraint stress (6hRS). Moreover, we show that mir-483-5p undergoes regional bias and overexpression of mir-483-5p is sufficient to produce significant differences in dendritic tree arborisation and spine maturation.

3.5 Results

3.5.1 Verification of targets identified by miR microarray assay

Neuroplastic changes triggered by environmental inputs consist the basis of mechanisms through which the functional properties of each brain domain is regulated. These properties form the part of network of interactions that predispose an organism to behavioural outcomes. Neuroplastic changes and network of neuronal interactions are facilitated by highly synchronised protein synthesise. Thus, the mechanisms regulating transcriptional and translational control of the protein synthesis form one of the basic processes of behavioural regulation. One such mechanism involves translational repression or mRNA degradation by miRs. Several studies have shown that miRs are up-regulated in the specific brain regions following restraint stress in rodents (Malan-Müller et al., 2013). It is important to understand the temporal and spatial regulation of miRs and their consequences within the brain to postulate a comprehensive understanding of mechanisms that underlie the basis of behaviour. One of the technological approaches allowing the identification of miRs is microarray analysis. The method is based on the detection and quantification of the change and magnitude of hundreds of different miR types within the same sample. In order to understand the changes in the miR milieu in rodent amygdala in response to restraint stress, C57/BL6 adult male mice were exposed to 6hr restraint stress (6hRS) and amygdalae were removed. Total RNA was extracted from the tissue, followed by miR microarray analysis, Figure 3.1a, p. 123. The numerical data representing change in the levels of various miR expression was subjected to post-hoc Bonferroni's correction resulting in the list of several miR expression levels being affected by restraint stress relative to levels found in the

stress naïve samples (Data not shown). Although we found five miRs to be significantly upregulated upon restraint stress (mir-483-5p, mir-1192, mir-1224, mir-1892 and mir-1894-3p), we decided to study the role of mir-483-5p in detail. The rationale behind this was that: (I) mir-483-5p is one of the highest of miRs upregulated upon stress and (II) the role of mir-483-5p has never been investigated in the context of stress and anxiety.

In order to verify the data obtained from miR microarray studies, we performed qRT-PCR assay using amygdala dissected either from stress naïve mice or from mice subjected to 6hRS. Relative mir-483-5p levels were quantified and normalised to the RNU6 RNA. The results, Figure 3.1b (p. 123), are shown in percentage change. We observed that upon stress, mir-483-5p level increased more than two-fold compared with stress-naïve animals (268% ±14%SEM, $t(2)=4.30$, $p=0.01$, $n = 3$). By using a sperate cohort of subjects, we have showed that the results of qRT-PCR is in agreement with the miR microarray findings.

3.5.2 Compartmentalisation of the mir-483-5p

Previous studies have demonstrated that protein translation can take place independently in distinct cellular compartments (Bai & Witzmann, 2007; Ziats & Rennert, 2014). One relevant example of this is the localised translation within dendritic spines that is separate from the global pool of translation taking place in the neuronal soma (Rochefort & Konnerth, 2012; Shi et al., 1999). Previous studies indicated miRs to play an active role in the translational repression of protein coding mRNAs within precisely defined cellular compartments. We reasoned that if mir-483-5p interfere with neuronal and synaptic functions, then it might be localised in specific neuronal compartment. Interestingly, others have demonstrated that restraint stress triggers morphological and long-lasting changes of neurons in mouse brain (Donohue et al., 2006; Mucha et al., 2011; Skrzypiec et al., 2013) triggered by miRs and can function independently in various cellular compartments (Davis et al., 2015; Fiore et al., 2008; Rajman & Schratt, 2017; Ziats & Rennert, 2014). If mir-483-5p expression triggers the synapse modification, then it's likely to be expressed within dendritic spines or synaptic boutons

In order to verify whether mir-483-5p expression follows a biased regulation in distinct cellular compartments, we extracted amygdalae from adult male C57/BL6J mice subjected to 6hRS. Next, we separated cytosolic and synaptosomal fractions and extracted RNA was from both 6hRS and stress-naïve mice. Relative mir-483-5p levels in cytosolic and synaptosomal fractions were measured and normalised to RNU6 RNA levels. ANOVA revealed a statistically significant difference among the groups ($F_{(3, 11)}$=23.11, p<0.001). Firstly, we saw an eight-fold increase of mir-483-5p in the synaptosomal fractions after stress

(TukeyHSD $p<0.001$) while no statistically significant increase was observed in the cytosolic fractions after stress (Figure 3.2, p. 125). Secondly, we observed a marked increase in the level of mir-483-5p in the synaptosomal fractions in comparison with cytosolic fractions only after stress (TukeyHSD $p<0.001$), but not under the control conditions. These data indicate that mir-483-5p is preferentially expressed in synaptosomes after stress demonstrating its potential involvement in the maintenance of biochemical milieu and in stress-induced functional changes in the juxta-synaptic regions. Finally, we found that the RNU6 RNA levels were similar in both compartments (data not shown) making it a good reference gene that can be used for normalisation when comparing cytosolic and synaptosomal fractions. While the previous experiment (Figure 3.1, p. 123) have shown that the level of mir-483-5p mRNA is elevated in the Amygdala following 6hRS, the current results provide better spatial resolution by providing us more insights into the compartments where the mir-483-5p levels are up-regulated. Moreover, our data point out that, compared to cytosolic fractions, mir-483-5p levels are enriched and selectively up-regulated in synaptosomes following 6hRS. Synaptosomal preparations contain the molecular machinery required for synaptic plasticity to occur. Therefore, it is likely that mir-483-5p is involved in these processes. Following from these findings, we next wanted to discover if mir-483-5p might be involved in morphological changes in neuronal cells.

3.5.3 mir-483 affects dendritic morphology

Our data showed that mir-483-5p is up-regulated in the amygdala following 6hRS and significant up-regulation was observed in the synaptosomal preparations compared to cytosolic fractions (Figure 3.2 p. 125). Moreover, previous research have demonstrated that restraint stress induce changes in neuronal morphology that can contribute to synaptic plasticity (Bourgognon et al., 2013; Mariusz Mucha et al., 2011; Roozendaal, McEwen, & Chattarji, 2009; Skrzypiec et al., 2013; Suvrathan et al., 2014; A Vyas, Jadhav, & Chattarji, 2006). These include changes in neuronal complexity and dendritic arborisation. These factors lead us to suspect that mir-483-5p might have discernible roles to play in morphological changes and its associated mechanisms. One of the ways to understand these morphological changes is by determining neuronal complexity. Sholl analysis is a commonly used and effective way to quantify neuronal tree complexity. It is performed by drawing concentric circles of incrementally increasing radial distance from the soma and then by counting the number of intersections of the circle and the dendrites, see methods sections for more details (Binley, Ng, Tribble, Song, & Morgan, 2014; Sholl, 1953). In order to understand whether mir-483-5p is sufficient to induce changes in neuronal morphology, primary neuronal cultures were made from the amygdalae of 1-day-old mice (P01). When the cultured neurons developed dendritic trees, they were double transfected with either mir-483-5p or scramble sequence expressing vector and plasmid expressing dTomato for visualisation of neuronal tree complexes. Next, neurons were imaged by confocal microscopy, and morphometric quantification was performed using Sholl analysis. Count of these intersections is then plotted against the distance (μm) from the soma, see Figure 3.3 (p. 127).

Our results showed that neuronal complexity is significantly reduced at the distal dendrites when transfected with the mir-483-5p compared to the control neurons. The observed reduction shows spatial selectiveness, up to 70% reduction only at 140-170 microns from the soma. Interestingly, the proximal dendrites, between 10-130 microns, seem to be unaffected by mir-483-5p overexpression. Figure 3.3b (p. 127) shows representative images of the neurons transfected with control or mir-483-5p constructs. This shows that mir-483-5p could affect dendritic arborisation selectively at distal ends of neurons. Moreover, neither the total length of the neurons nor the structural integrity was affected. This implies that the effect we observed is not because of the neurotoxic effect due to the ectopic expression of mir-483-5p, but due to specific mechanisms mediated by mir-483-5p to modify dendritic arbour dynamics. This led us to suspect that mir-483-5p is involved in mechanisms leading to synaptic plasticity by regulating the dendritic morphology. These discoveries prompted us to study the role of mir-483-5p in dendritic spine morphology and density.

3.5.4 mir-483-5p is sufficient to induce changes in spine dynamics

One of the morphological aspects that facilitate neuronal plasticity is through the regulation of dendritic spines. Dendritic spines are small protrusions from the dendritic shaft where a synapse could form (Rochefort & Konnerth, 2012). Several studies have shown that dendritic spines are affected by stress (Mucha et al., 2011; Pawlak et al., 2005). Our data suggested that mir-483-5p could have a specific role to play in these juxta-synaptic compartments where mir-483-5p is highly enriched and regulated after stress. To test this possibility, we investigated whether mir-483-5p affects the density or morphology of spines. Previous studies have shown that mushroom spines are more likely to form stable synapses, compared to more neuroplastic filopodia or thin spines (Bourne & Harris, 2007; Segal, 2017) and by studying the proportion of these spine-classes, the likelihood of new synapses being formed may be predicted. In order to study the potential role of mir-483-5p in spine formation and morphology we double transfected primary neuronal cultures with the mir-483-5p-pIRES-mCherry construct to overexpress mir-483-5p and AcGFP1-GFP to visualise dendritic spines. This construct has 20 amino acids fused to the N-terminal of Neuromodulin and AcGFP1. This construct overexpressed GFP1 in membranes and was used for spine visualisation. After transfection, cells were mounted on a slide and images were taken. The results are visualised in Figure 3.4, p. 129. We analysed 113 sections, 59 for scramble conditions and 54 for mir-483-5p conditions. Spines are differentiated by their morphology and size, as described in methods section. The different categories of spines analysed were: thin, stubby, branched, mushroom and filopodia-like spines. Figure 3.5 (p. 131) shows the representative images of the section for scrambled and mir-483-5p conditions. Firstly, we did not find any

statistical difference in the density of spines, Figure 3.5 (p. 131). Similarly, there was no statistically significant difference in the proportion of stubby, thin or branched spines. Interestingly, we found ~30% increase in mushroom spines ($t(111) = -4.08$, $p<0.001$) and ~60% decrease in filopodia-like spines ($p<0.001$). These results suggest that mir-483-5p overexpression is sufficient to facilitate the formation of mushroom-like dendritic spines and reduction on filopodia-like spines without affecting the overall density of spines. Interestingly, the effect observed in these neurons seems to be opposite to the effects produced by acute stress in amygdala neurons (Mucha et al., 2011; Skrzypiec et al., 2013), indicating that mir-483-5p might be involved in offsetting stress-induced changes in spine proportions.

3.6 Discussion

Neuronal plasticity underlies the processes by which learning and memory are encoded in the neural network. Optimal functioning of this network is essential for responding promptly and appropriately to environmental cues. However, severe or prolonged stress can result in neuronal plasticity related disorders or maladaptive outcomes. miRs constitute efficient players in the mechanisms involving neuronal plasticity and several miRs have been shown to be involved in neurological conditions related to anxiety and stress. In order to elucidate the roles of miRs in the regulation of anxiety-related behaviour we exploited the miR microarray technology to screen the changes in miR amygdala repertoire. We found several miRs to be up-regulated in the amygdala following 6hRS. One of the miRs that was identified to be significantly up-regulated was mir-483-5p. After the qRT-PCR confirmation of stress-induced changes in the expression of this miR (Figure 3.1, p. 123), we sought to understand if mir-483-5p shows spatial bias by looking at its relative expression in cytosolic vs. synaptosomal fractions before and after 6hRS. We found that the level of miR is preferentially up-regulated in the synaptosomal fractions as compared to the cytosolic fractions (Figure 3.2, p. 125). Further investigation revealed that mir-483-5p overexpression in primary neuronal culture is correlated with the reduction in the dendritic complexity in distal regions, as revealed by Sholl analysis (Figure 3.3, p. 127). Moreover, we have found that the overexpression of mir-483-5p resulted in the increase of mushroom spines and reduction in filopodia-like spines (Figure 3.4, p. 129) while maintaining the density of spines unchanged (Figure 3.5, p. 131). In our knowledge, this is the first time that mir-483-5p has been identified in the amygdala in relation to restraint stress. In this chapter, we are proposing

that mir-483-5p is a neuronal activity respondent of stress-related stimuli that undergoes compartmentalisation into the synaptic region. Moreover, our data show that mir-483-5p is involved in fine-tuning of specific aspects of dendritic arborisation and spine maturation.

3.6.1 Nesting gene independent transcription of mir-483-5p

Figure 3.6 (p. 133) shows the processing of the mir-483 stem-loop structure to form the mature mir-483-5p. mir-483-5p is transcribed from the intronic region of its nesting gene, Insulin like growth factor-2 (*Igf2)*. Studies have shown that mir-483-5p is co-expressed with *Igf2* suggesting co-transcription (Li et al., 2015; Ma et al., 2011). This poses the question if increase in the level of mir-483-5p is due to the transcription of its nesting gene or the transcription is independent. While our data show that mir-483-5p is overexpressed in the amygdala after stress, Figure 3.1 (p. 123), other researchers have found reduced *Igf2* levels in the amygdala (Jung et al., 2012). This indicates that mir-483-5p can be transcribed independently from *Ifg2* in the amygdala. *Igf2* independent transcription of mir-483-5p has also been identified in hepatocarcinoma (HCC) cells, and this is thought to be facilitated through ß-catenin/Wnt pathway (Veronese et al., 2011). Interestingly, ß-catenin/Wnt pathway is implicated in the amygdala functions (Teo, Soga, & Parhar, 2018) and memory consolidation (Kimberly A. Maguschak & Ressler, 2008). Further analysis of the ß-catenin/Wnt pathway's effect on mir-483-5p will be discussed in the final chapter. Taken together, it is likely the case that specific transcription of mir-483-5p can take place without the co-transcription of its nesting gene, suggesting the function of mir-483-5p is not limited to the mechanisms involving *Igf2*.

3.6.2 mir-483-5p responds to environmental stimuli

mir-483-5p had been identified in several conditions like cancers (Mullany et al., 2016), adipogenesis (Chen et al., 2015) and also suggested as potential biomarkers in nasopharyngeal carcinoma (Zheng et al., 2014), colorectal cancer (Cui et al., 2016), adrenocortical tumours (Patel et al., 2013; Patterson, Holloway, Weng, Fojo, & Kebebew, 2011), postoperative atrial fibrillation (POAF) (Harling et al., 2017) and in polycystic ovarian syndrome (Xiang et al., 2016). Scientific investigation of mir-483-5p is not limited to cancer and cell differentiation but also in CNS. In one study mir-483-5p was found to be one of the bio-markers for temporal lobe epilepsy and status epilepticus (Raoof et al., 2017). In another study researchers were analysing miR expression in PFC, Nucleus Accumbens (NAc) and dorsal striatum following nicotine admission; they found elevated levels of mir-483-5p in these regions (Gomez et al., 2015). Moreover, mir-483-5p has been demonstrated to inhibit melatonin synthesis in pinealocytes of rat brain by targeting the 3'UTR region of mRNA that codes for *Aanat*. *Aanat* codes for an enzyme that is the last modifier in the cascade of enzymatic interactions leading to the synthesis of functional melatonin (Clokie et al., 2012). Additionally, an epigenetic regulator gene *Mecp2*, an important for the healthy development of the human foetal brain, is shown to be regulated by the mir-483-5p 3' UTR interaction (Han et al., 2013). MeCP2 is previously implicated in synaptic plasticity (Klein et al., 2007; Smalheiser & Lugli, 2009), anxiety (Adachi et al., 2009) and dendrite formation (Smalheiser & Lugli, 2009; Smrt & Zhao, 2010). Moreover, a mutation in *Mecp2* is linked to the development of Rett Syndrome (Liyanage & Rastegar, 2014). These studies and our data show that mir-483-5p is active in CNS and might be important in the manifestation of several aspects

of stress, anxiety and neuronal plasticity. Indeed, bioinformatics analyses have revealed that mir-483-5p shares very high sequence homology with mice, humans and rat, Figure 3.7 (p. 135), suggesting potential functional conservation of mir-483-5p across species and validating our organism of choice to study this miR.

Our experiments revealed that mir-483-5p is up-regulated after stress in the amygdala. Even though previous research has shown that mir-483-5p is regulated in the CNS, to our knowledge this is the first time that mir-483-5p is shown to be responding to psychological stress. In light of previous research that has found mir-483-5p in CNS, it seems that it is transcribed in response to particular stimuli like repeated nicotine administration (Gomez et al., 2015) and SE (Risbud & Porter, 2013). These studies and our data suggest that mir-483-5p might be responding to powerful stimuli including acute stress. Another exciting discovery is that mir-483-5p is preferentially expressed and up-regulated in synaptosomes. Synaptosome preparations have been widely used to study aspects of synaptic physiology. We purified synaptosomes from amygdala tissue before and after stress and assayed the level of mir-483-5p in these structures. Corroborating other studies (Risbud & Porter, 2013), we found an increased expression of mir-483-5p in synaptosomes suggestive of its potential role in aspects of synaptic physiology. Previous studies have shown that independent protein synthesis and degradation happens in dendrites (Bai & Witzmann, 2007) and a subset of miRs observed in neurons are enriched in synaptosomes (Lugli et al., 2008). The Risbud and Porter study also corroborate this finding where they found mir-483-5p is preferentially expressed in the synaptosomes made from hippocampi in control conditions (Risbud & Porter, 2013). Interestingly the

same study also found that the compartmental bias of mir-483-5p ceased to exist 48hrs after the induction of SE, a pattern that was observed on other miRs that also exhibited compartmentalisation. The decrease of synaptosomal miRs prompted the authors to suggest that an episode of SE disrupts synaptoneurosomal milieu and therefore its normal function. Taken together our data and other studies show that biased regulation of mir-483-5p and other miRs is required for neuronal plasticity. This observation is further strengthened by the data from Sholl analysis and dendritic morphology analysis we performed on primary neuronal cultures overexpressing either mir-483-5p sequences or scrambled sequences, see below.

3.6.3 mir-483-5p potentially relives effects of stress

In order to understand if mir-483-5p is involved in neuronal plasticity related mechanisms, we made primary neuronal cultures from 1-day-old mice amygdalar tissue. After maturation, cultures were transfected with mir-483-5p or scrambled sequence. Neurons were imaged using confocal microscopy, and subsequent Sholl analysis and dendritic spine morphology analyses were performed. We found that mir-483-5p-transfected neurons exhibited reduced dendritic tree in the distal regions, Figure 3.4, p. 129. Moreover, we found an increase in mushroom spines and a concomitant decrease in filopodia-like spines. Additionally, no significant difference was observed in the density of spines. Dendritic tree architecture is important for effective communication between neurons. Several studies have found that stress could have differential effects on structural plasticity depending on specific brain region (Ajai Vyas, Bernal, & Chattarji, 2003; Ajai Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002), duration and intensity of stress response (Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; A

Vyas et al., 2006). Previous research in our lab has characterised structural changes in BLA following acute stress (Mucha et al., 2011; Skrzypiec et al., 2013). We have found that acute stress resulted in increased spine density and increased the percentage of stubby, irregular and filopodia-like spines (Skrzypiec et al., 2013). Interestingly, we also found a reduction in mature mushroom-like spines. An increase in spine density was also seen in the CA1 and CA3 regions of the hippocampus after acute restraint stress (Mucha et al., 2011). A similar increase in spine density was also seen in rat amygdala after acute restraint stress (Mitra et al., 2005). When the effect of stress on BLA-facilitated associative learning was investigated, researchers found that acute stress impairs extinction in rats by reducing the branch number and branch length in BLA neurons (Maroun et al., 2013). However, in one study researchers found acute stress correlated with changes in amygdala neuronal spine and anxiogenic behaviours only ten days after the stress. It is important to note that in this experiment researchers used a 2h immobilisation stress session. Overall, acute stress was shown to increase spine density, decrease mushroom-like mature spine and increase in another type of spines in the amygdalar neurons. Studies that looked at chronic stress also found interesting patterns. In male rats, CIS resulted in increased spine density in BLA which was correlated with anxiogenic behaviours (Mitra et al., 2005). Other studies that looked at chronic stress also found similar patterns (Ajai Vyas et al., 2002). In BLA chronic immobilisation stress (CIS) caused pyramidal neurons to increase in dendritic length and branching. Changes observed were most pronounced 60-160μm from the soma. A similar finding was also seen in stellate neurons where the most significant change was seen at 60μm from the soma. Taken together these studies suggest that stress can

induce functional changes in the amygdala neurons by increasing their spine density, reducing the mature mushroom spines while concomitantly increasing other types of spines. Studies have shown that stress also correlates with hyperexcitability of BLA neurons which then leads to anxiety-like behaviours or a predisposition to substance addiction (Rosenkranz, Venheim, & Padival, 2010; Sharp, 2017; Song et al., 2017).

In our experiments, when primary neuronal cultures were overexpressed with mir-483-5p, we found an effect that is opposite to stress-induced changes. We found that the spine density remained unchanged while the percentage of filopodia-like spines decreased with a concomitant increase in mature mushroom spines, Figure 3.4 (p. 129). Mushroom spines are termed mature spines or ‘memory' spines (Bourne & Harris, 2007) while filopodia-like spines are immature spines and are less likely to form synapses. Taken together, the changes in distal dendritic tree, decrease in filopodia-like spines and increase in mature mushroom spines might be a mechanism that offsets the potentially damaging effects of stress-induced structural changes and therefore stress-induced anxiety-like behaviours.

3.7 Conclusion

In this chapter, we have seen that miRs are effective regulators of structural and functional aspects of neuronal plasticity and constitute proficient fine-tuners to modulate behavioural outcomes. We have detected mir-483-5p up-regulation in the amygdala following acute stress, and the up-regulation is selective to juxta-synaptic regions where the molecular machinery is isolated from the rest of cell to respond effectively to local demands of protein synthesis and degradation. Moreover, we have found that overexpression of mir-483-5p in primary cultures affected dendritic arborisation and spine maturation in a pattern that is opposite to the changes induced by stress, suggesting that mir-483-5p might be involved in the offsetting of the maladaptive features induced by stress. Therefore, we suspect that mir-483-5p induced structural modifications in the amygdala are likely to produce an anxiolytic effect. Moreover, in the light of existing literature that links mir-483-5p to melatonin synthesis (Clokie et al., 2012), mediation of nicotine response (Gomez et al., 2015), temporal fluctuation and synaptosomal bias (Risbud & Porter, 2013) justifies the speculation that mir-483-5p could play important roles in other conditions that are comorbid with stress and anxiety, namely sleep disorders and substance abuse. This has a potential diagnostic relevance where stress and anxiety are shown to be comorbid with sleep disorders (Frank, 2012; Han, Kim, & Shim, 2012) and substance abuse (Gupta et al., 2011). Moreover, the finding by several studies that have used mir-483-5p as a biomarker might mean that mir-483-5p undergo active transport to the extracellular matrix and could be used as a biomarker for neuronal plasticity associated disorders. However, further studies are required in order to elucidate further the role of mir-483-5p in neuronal plasticity related to sleep disturbances,

substance abuse or its potential use as a biomarker in these disorders. The next chapter will look at the identification and characterisation of the potential targets of mir-483-5p in the amygdala.

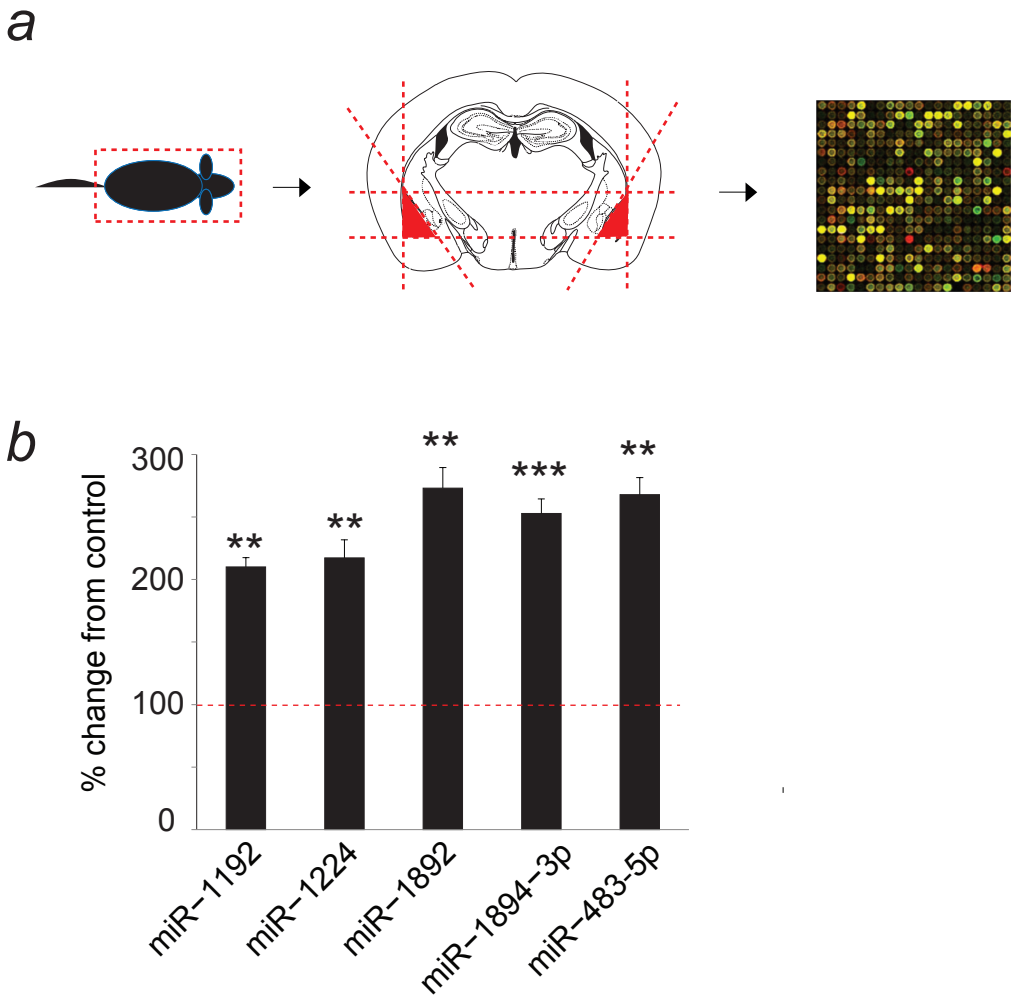

Figure 3.1 mir-483-5p is upregulated in the amygdala following stress
(a) Adult C57BL6J mice were either subjected to 6hr restraint stress (6hRS) or left undisturbed in their home cages. Animals were anaesthetised and their basolateral amygdalae were extracted. Samples were homogenised, miRs were extracted and hybridised to microarrays to analyse the level of miRs (data not shown). Following the miR microarray results, a new cohort of mice was subjected to stress in the same manner and samples analysed by qRT-PCR (b). qRT-PCR confirmed the results from the microarray study. mir-483-5p was one of the most prominently upregulated among all the miRs by showing a 2.7 fold increase when compared to the controls ($t(2)=4.30$, $p<0.01$, $n=3$ animals per group). Other miRs that were upregulated in the amygdala following stress are; mir-1192, mir-1224, mir-1892 and mir-1894-3p. Data are shown as Mean±SEM.

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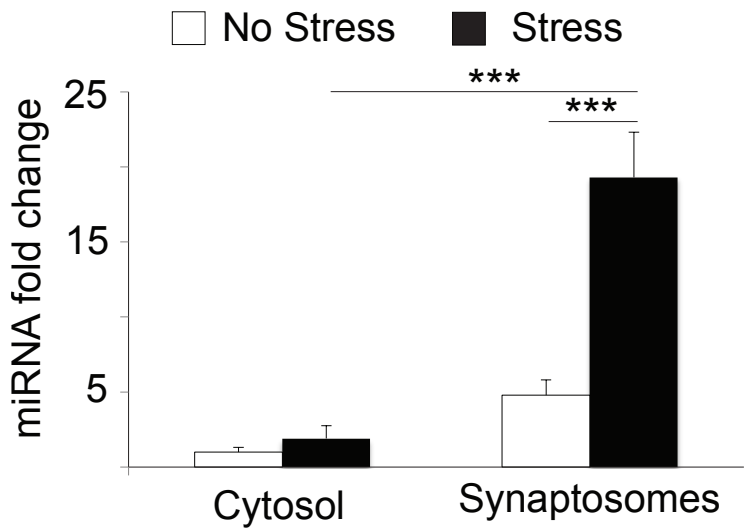

Figure 3.2 mir-483-5p exhibit compartmental bias

Mice were either left undisturbed in their home cages or subjected to 6hr restraint stress, and amygdalae were dissected after anaesthetising the animals. Tissue samples were homogenised, and synaptic fractions and cytosolic fractions were separated. RNA was extracted from these samples and subsequently analysed using qRT-PCR. Under control conditions (white bars) mir-483-5p is enriched in synaptosomes. After stress (black bars) mir-483-5p is preferentially upregulated in synaptosomes ($F(3, 11)=23.11$, $p<0.001$, n = 3-4 per group) while no significant differences were observed in cytosolic fractions.

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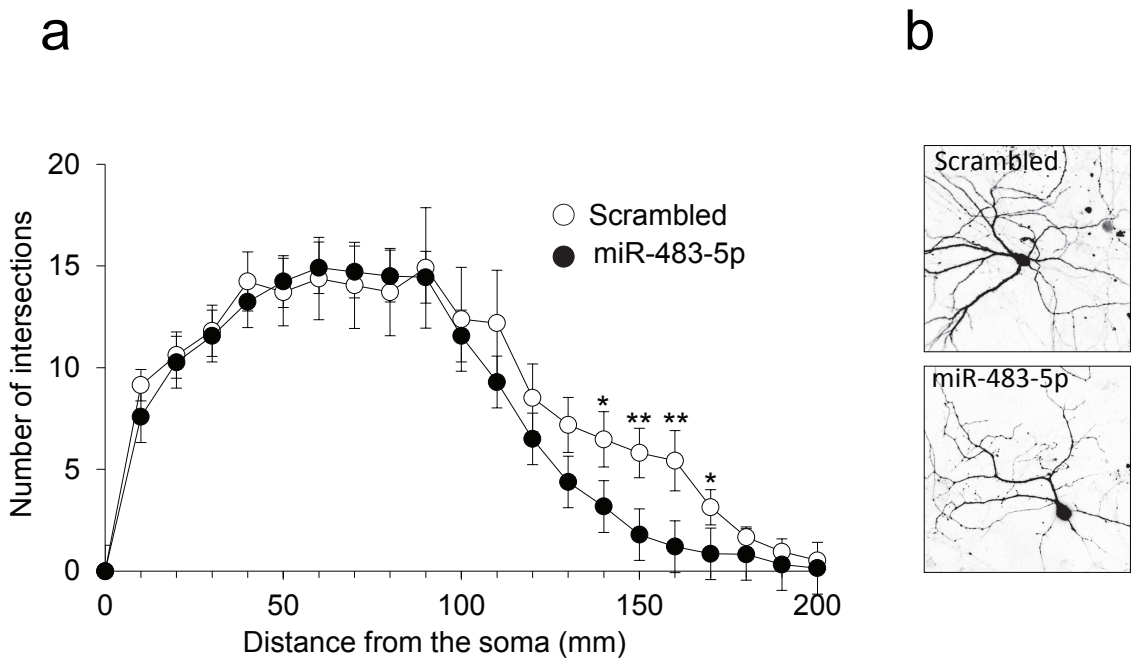

Figure 3.3 mir-483-5p affects dendritic arbour

Primary neuronal cultures were made from 1-day-old mice amygdala tissue and transfected with either a control scramble sequence or mir-483-5p. (a) Neurons were imaged using confocal microscopy and Sholl analysis performed. The analysis revealed that overexpression of mir-483-5p resulted in shrinkage only at the distal parts of the dendritic tree (140-170 microns from the soma; $0.05>p<0.001$; n = 21 and 34 for control and mir-483-5p respectively). (b) Representative images showing the reduced dendritic tree after mir-483-5p overexpression compared to the control.

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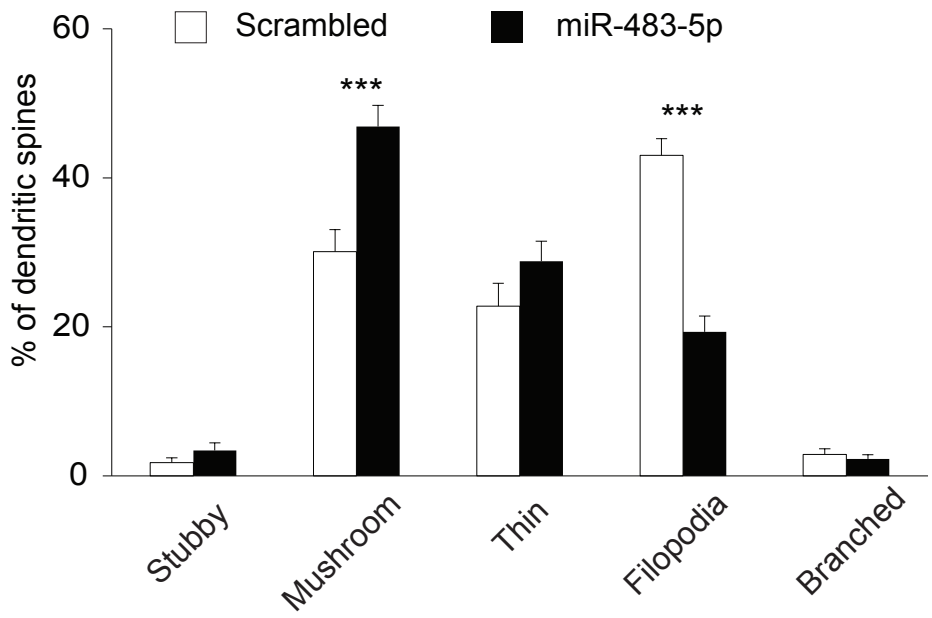

Figure 3.4 mir-483-5p affects dendritic spine proportions
Primary neuronal cultures were made from 1-day-old mice amygdalar tissue and transfected with plasmids (AcGFP1-GFP & miR-483-5p-pIRES-mCherry) to over-express either scramble sequence or mir-483-5p sequence. Dendrites were visualised using confocal microscopy and analysed to quantify dendritic spine morphology. Analysis revealed that the proportion of mushroom spines were increased by 30% ($p<0.001$) while a 60% ($p<0.001$) decrease in filopodia-like spines were detected ($n = 59$ segments from $N = 20$ neurons and $n = 54$, $N = 21$ for scrambled and mir-483-5p-transfected groups, respectively). Data are shown as mean±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

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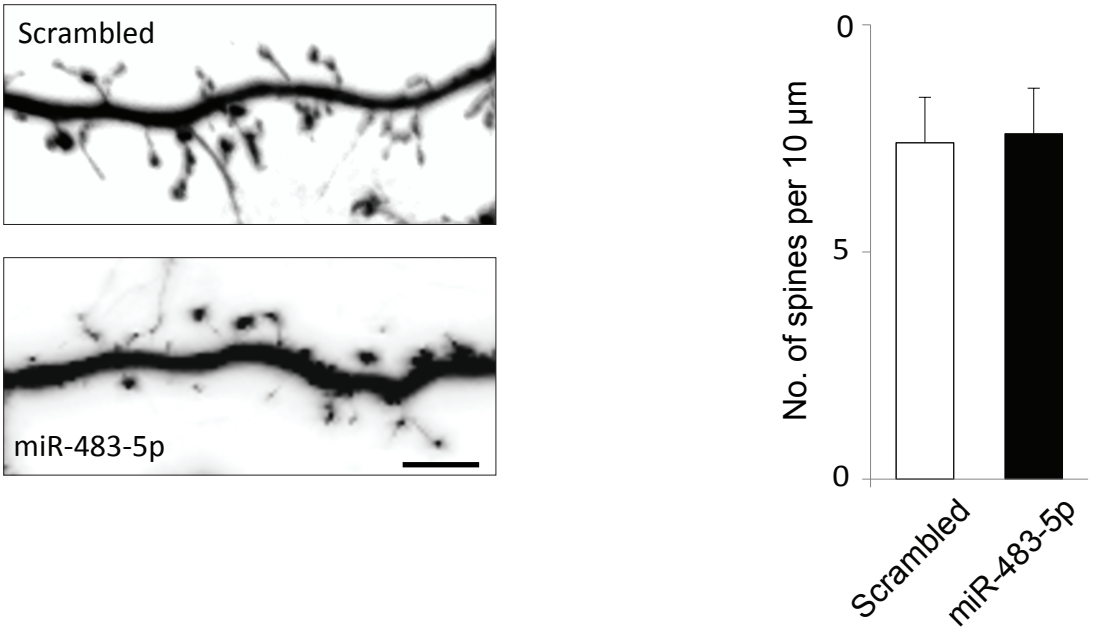

Figure 3.5 mir-483-5p does not affect dendritic spine density
Primary neuronal cultures were made from 1-day-old mice amygdalar tissue and transfected with plasmids (AcGFP1-GFP & miR-483-5p-pIRES-mCherry) to over-express either scramble sequence or mir-483-5p sequence. Dendrites were visualised using confocal microscopy and analysed to quantify dendritic spine morphology. (left) Shows representative images of dendritic spines transfected with scramble or mir-483-5p sequences. (right) Shows that the density of dendritic spines remained unaffected by the over-expression of mir-483-5p. Data are shown as mean±SEM. Scale bars c = 50 µm, e = 5 µm.

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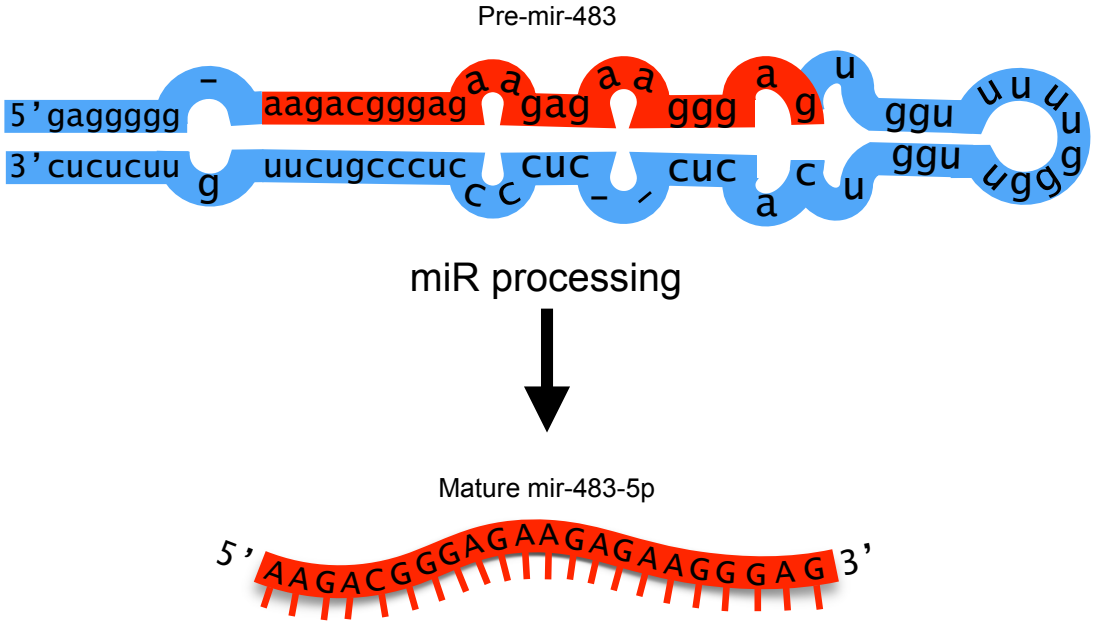

Figure 3.6 Pre- and mature mir-483-5p
The diagram above shows the Pre-mir-483 stem-loop structure, as predicted by miRBase, one of the most extensive archives for miRs. Pre-mir-483 stem-loop structure undergoes processing by DROSHA and DICER complex to form the mature functional miR-483-5p. '5p' at the end of the mir-483-5p signifies that this miR is made from the 5' end of the Pre-miR-483.

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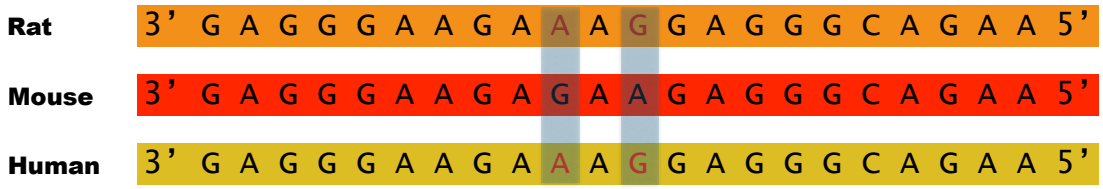

Figure 3.7 mir-483-5p homology with Rat and humans

The sequences above compare the Rat, mouse and human mir-483-5p. The highlighted region shows the bases that are different from the mouse sequence. Out of the 22 bases that form the mature miR, only two bases are different in mouse & rat and mouse & human mir-483-5p. However, rat & human mir-483 are identical to each other. This sequence homology of mir-483-5p in humans, rat and mice suggests potentially conserved functional homology.

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CHAPTER 4 Identifying the targets of mir-483-5p

4.1 Introduction

In the previous chapters, we have seen that stress induces multiple changes in the brain and effective adaptation to stress involves orchestration of various molecular pathways. Maladaptation to stress or breakdown in these pathways can result in several pathologies, such as anxiety disorders. miRs are important players in facilitating and fine-tuning these pathways. Recently, researchers have unveiled their importance in anxiety disorders and other neurological conditions. Characteristics of miRs - the ability to regulate several targets at once, transcription in clusters, compartmentalisation and neuronal activation responsiveness enable these little molecules to function as plasticity regulators. 2% of the human genome codes for proteins (Elgar & Vavouri, 2008), 80% of those proteins are estimated to be regulated by miRs (Filipowicz et al., 2008; Friedman et al., 2009; Lu & Clark, 2012). Their ability to control multiple targets makes them the most abundant gene regulators (Muiños-Gimeno et al., 2011). Unsurprisingly, miRs have been called the 'meta-controllers' of gene expression in synaptic activity and crucial players in the evolution of the brain (Ceman & Saugstad, 2011; W. Chen & Qin, 2015). To date, miRBase provides annotation for 2,654 mature human miRs and 1,978 mouse miRs. Even if we conservatively assume each miRs can target twenty-five mRNAs, the total number of potential miR targets exceeds the estimated number of total protein-coding genes. This crude estimation helps to comprehend the true capacity of miRs and their roles. However, understanding the roles of miRs and their utility in scientific study greatly depends on deciphering the dynamics of regulating their targets. Therefore, while studying miRs it is also necessary to consider their targets and the roles those target mRNAs.

4.2 Intro to target prediction algorithms

miR research is only ~25 years old, and we are still in the logarithmic phase of learning about their functions and *modus operandi*. Each miR has the potential to target 100s of mRNAs. This makes the identification and validation of miR targets a resource-consuming and error-prone endeavour. One of the efficient ways to increase the chances of identifying true targets of miRs is to use *in silico* prediction algorithms to get a crude list of putative mRNA targets and then experimentally verify each target. Several databases exist for this purpose, each with their respective pros and cons. The algorithms used by these databases are rule-based, data-based or a combination of these two approaches (Yue, Liu, & Huang, 2009). The initial studies that tried to understand the role of miRs and their interaction with mRNA showed that a perfect complementarity of mREs (mRNA regulatory elements) and the nucleotides between 2^{nd} and 8^{th} bases in the seed regions is crucial. This rule was among the first to be used in algorithms to predict miR:mRNA interactions (Lai, 2002). However, later studies revealed that rules regulating the miR:mRNA interaction are more complex than initially anticipated. Accounting for this new knowledge, the current algorithms use several rules to predict putative targets for miR:mRNA interaction. The following sections attempt to shed some light on the current understanding of miR:mRNA interaction while also briefly describing how an algorithm predict putative targets.

4.3 Importance of seed sequence

One of the most important features of miR:mRNA interaction is the seed sequence. Seed sequence is the 6-8 bases on the miR that forms perfect or nearly perfect Watson-Crick pairing at the corresponding site at 3' UTR, 5' UTR or coding regions of the target mRNA. This region on the target mRNA is termed

as seed site. It is also the best predictor of miR:mRNA interaction. Prediction algorithms use the seed sequence complementarity to provide a list of putative mRNA targets. The seed region starts from the second nucleotide (position two) and could extend to the eighth nucleotide (position eight) from the 5' end of the miR (Agarwal, Bell, Nam, & Bartel, 2015; Bartel, 2009; Saito & Saetrom, 2010). There are several types of miR:mRNA interaction at the seed sites. Their interaction could be perfect/stringent or imperfect pairing at the seed site. Seed sites with a perfect Watson-Crick pairing can be divided into four main types. These are 8mer site, 7mer-m8, 7mer-A1 and 6mer; see Figure 4.1, p. 171 (Agarwal et al., 2015; Bartel, 2009; Saito & Saetrom, 2010). These names are used based on the number of Watson-Crick pairings and the presence of Adenosine at position one. The 8mer site has an Adenosine present at the position one on the seed site and forms a perfect Watson-Crick pairing from position two to position eight. 7mer-m8 has no Adenosine present at position one and, like an 8mer, has perfect complementarity from position two to position eight at the seed site. In the 7mer-A1 site there is an Adenosine at position one and has perfect Watson-Crick pairing only until position seven. Unlike 8mer and 7mer-m8, 7mer-A1 type of interaction lacks pairing at the position eight. Finally, the 6mer site does not have Adenosine at position one and has perfect Watson-Crick pairing until position seven. The presence or absence of Adenosine is used in the naming of the site because it has been shown that adenosine at position one can increase the efficiency of target recognition (Baek et al., 2008; Lewis, Burge, & Bartel, 2005). Moreover, research has shown that the miR:mRNA interaction is not limited to a perfect seed sequence complementarity (Chi, Zang, Mele, & Darnell, 2009; Grimson et al., 2007; Ritchie & Rasko, 2014). These imperfect

type of pairings can have a wobble pairing (a non-canonical pairing of G:U), mismatch or a combination of the two (Saito & Saetrom, 2010). In one study where 27% of Ago-mRNAs detected in Argonaute and HITS-CLIP method showed no previously identified seed sequences (Chi et al., 2009). Similar pieces of evidence led the authors to attribute this finding to non-canonical binding such as wobble or bulge nucleotides (Didiano & Hobert, 2006). Furthermore, studies have shown that on top of seed pairing, additional pairing at the 3' end of miRs could enhance the efficacy of target prediction (Bartel, 2009; Grimson et al., 2007; Saito & Saetrom, 2010). The number of pairing that provides this advantage seems to depend on the type of seed pairing. 3-4 pairings seem to be enough for stringent seed pairing while 4-5 pairings are required for moderate pairings with the wobble of the bulge (Bartel, 2009). One recent study that looked into the relationship between the seed site type and mRNA expression in the samples from colorectal cancer patients showed that seed site type alone does not always predict mRNA expression, mRNA dysregulation or survival (Mullany et al., 2016). Some studies have suggested that the type of seed site could be a better indicator of the conservation of the seed site than the efficacy of repression (Ellwanger, Büttner, Mewes, & Stümpflen, 2011).

Seed sites and pairing at the 3' end of miR are not the only determinants of miR:mRNA interaction. Studies have shown that the location of the seed sequence is also important in determining their efficacy. Theoretically, a miR loaded RISC (RNA induced silencing complex) could bind to any region of an mRNA. However, studies have shown that RISC binding is biased towards the 3'UTR (Agarwal et al., 2015; Friedman et al., 2009; Guo, Ingolia, Weissman, & Bartel, 2010; Saito & Saetrom, 2010) and other studies have reported that the

length of 3'UTRs could be used as a predictor to determine if an mRNA is targeted by miR (Ellwanger et al., 2011; Stark, Brennecke, Bushati, Russell, & Cohen, 2005). An analysis has revealed that 3'UTR length and miR interaction is so strong that house-keeping genes have evolved to reduce their 3'UTR length to decrease their chances of being targeted by miRs. In another study that looked at protein output after miR transfection showed that, most of the proteins (24 out of 40) that are repressed by at least 50% had at least a seed site on the 3'UTRs compared to exons or 5' UTR (Baek et al., 2008). Among those that had seed sites on exons, these regions were less effective than the 3'UTRs (Baek et al., 2008; Grimson et al., 2007). These studies show that 3'UTR co-evolved with miRs in order to function as effective regulators and also to reduce off-target effects (Brennecke et al., 2005; A. Stark et al., 2005). However, some studies have noted that for some miRs the efficacy of down-regulation is similar irrespective of the seed site location on the target mRNAs (Lytle, Yario, & Steitz, 2007). An interesting question to ask then is why seed sites are not uniformly distributed across an mRNA instead of the dominating presence in 3'UTRs? One *ad hoc* explanation offered to this question posits that RISC has to compete with the translational machinery to engage in successful repression or degradation of mRNA. Since this translational machinery occupies 5' UTRs and exons, it is predominantly on 3'UTRs where miRs could occupy. Throughout evolution, these regions are naturally selected, and most of the miRs ended up having a seed site on 3'UTRs (Bartel, 2004; Saito & Saetrom, 2010). Interestingly some studies have suggested a new type of miR:mRNA interaction where a miR can simultaneously bind 3'UTR and 5'UTR of a target (Lee et al., 2009). In this model, on top of the canonical binding where the 5' end of miR binds to the 3'UTR of the

mRNA, the 3' end of miR also binds to the 5'UTR. The preliminary assay has found that this pattern of binding is present in the non-conserved miRs and also in the miR:mRNA interaction that resulted in the lowest protein output. However, more studies are required to understand if this is a true and generalisable mode of miR:mRNA interaction.

4.4 Conservation of the seed sequence

Another important consideration that algorithms use to predict the target sequences is the evolutionary conservation of seed sequences (Ritchie & Rasko, 2014). Studies have shown that miR seed sequences follow a high degree of conservation among distinct species unless the interaction is species specific. Evaluation of these conserved sites can significantly reduce the incidence of false positive predictions and therefore can be useful. Target Scan, one of the first and commonly used prediction algorithms, initially looks at the conservation among the seed sequences in distinct species and refines this list by looking at the target position at the 3' UTR and out-seed regions (Ritchie & Rasko, 2014). One of the drawbacks of this method is when identifying species-specific miR:mRNA interactions where these interactions are not evolutionarily conserved. Indeed, studies have shown that 30-40% of miRs are not evolutionarily conserved (Ellwanger et al., 2011; Saito & Saetrom, 2010). Another study that looked at humans miRs reported that many of the new miRs identified were found to be specific to humans (Berezikov et al., 2006) and many are not conserved beyond primates (Bentwich et al., 2005). These data suggest the species-specific roles of miRs in the evolutionary process. By restricting studies to miRs that are evolutionarily conserved, it is likely that the studies of endogenous pathways

responsible for phenomena like stress and anxiety in humans and other model organisms will be less comprehensive.

4.5 Other determinants of miR:mRNA interaction

There are other factors at play which determine the efficiency of miR:mRNA interaction, one of which is the seed site context or the adjacent regions where the seed site is located on an mRNA (Baek et al., 2008; Bartel, 2009; Broughton, Lovci, Huang, Yeo, & Pasquinelli, 2016; Grimson et al., 2007). An experiment using reporter assay showed that the efficacy of down-regulation of targets could be altered by varying the seed site context, but keeping the seed site at the 3'UTR constant (Saito & Saetrom, 2010). This shows that the seed site context can affect the efficacy of target repression. One such property of the 3'UTR context is the percentage of AU nucleotides in the upstream and downstream regions of the seed site. This effect might not be because of the presence of AU *per se,* but the biochemical consequences of having AU regions. An example of this is the chances of secondary structure formation. Presence of AU has been proposed to be a useful heuristic to compute the probability of secondary structure formation (Grimson et al., 2007; Saito & Saetrom, 2010). Additionally, formations of secondary structures are also linked to the thermodynamic stability of a miR. Moreover, secondary structures reduced the accessibility and binding of RISC during the silencing process. Prediction algorithms have started to use the probability of secondary RNA structure formation in a given target region to predict miR:mRNA interaction (Ritchie and Rasko, 2014). The algorithm scores different base pairs (C:G, A:U and G:U) to calculate ‘energy scores’ and uses it to calculate stability. One of the requirements is the relatively larger energy score towards the 5’ end of miR. Based on these energy scores and the probability of

having a duplex formed in an area, algorithms can increase their accuracy of finding a true miR:mRNA interaction at 3'UTR.

Another important, useful feature to determine if a gene is targeted by miR is to look for multiple seed sites in their 3'UTR. It has been shown that the presence of more than one seed site (seed sites of same miR or different miRs) greatly increases the efficacy of repression in an additive manner and, in some cases, synergistically (Sætrom et al., 2007; Saito & Saetrom, 2010). Moreover, presence or absence of binding sites for other regulatory proteins can also affect the likelihood of miR:mRNA interaction at that site. This could be due to the reduced accessibility of miRs to the seed sites. Other factors like the position of the seed sites away from the centre of miRs and supplementary paring at the 3' end of miR also seems to affect the efficacy of target repression (Grimson et al., 2007; Saito & Saetrom, 2010). The determinants of effective miR:mRNA interaction is a result of the interplay between several factors. These sophistications negatively add to the accuracy of current target prediction algorithms. Several other methods that circumvent these problems have been proposed. One method of identifying the targets is by using the expression data; by looking at experiments that used overexpression of certain miRs and their targets confirmed through experiments. However, this approach has an ouroboros relationship with *in silico* prediction and therefore limits the capacity of experiments to inform prediction data in order to produce results that are more likely to be true.

4.6 **Importance of experimentally confirming targets**

The scientific community has come a long way in understanding miRs and how they interact with their targets. However, much more research is needed to reach

a comprehensive understanding of these mechanisms and to predict miR:mRNA interaction with better specificity and sensitivity. Given the complexity of miR:mRNA interaction and our relative lack of understanding about the factors leading to a successful miR induced repression, it is unsurprising that the currently available algorithms suffer from reduced specificity and sensitivity in detecting true targets of miRs. Nonetheless, the availability of such platforms is fortuitous as it helps to filter down to a smaller collection of miR targets which then needs to be experimentally verified. However, using any single algorithm to get a list of putative targets is limited. This is because, more often than not, these algorithms produce more than 100s of targets for each gene. Using more than one algorithm to find overlapping genes can be beneficial to further decrease the number of putative targets to a manageable collection. Moreover, by using gene ontology databases, one can tease apart the targets that might be involved in particular pathways or present in specific tissues. In short, obtaining *in silico* prediction of putative targets is a complex process, and the current system can produce significant variability in the results. Studies have shown that the majority of experimentally confirmed miR:mRNA interactions were missed by the algorithms (Selbach et al., 2008). Additionally, algorithms have been seen to produce inconsistent and conflicting results. This is thought to be mainly due to different algorithms using different models to make the prediction. Using different databases for genes and 3'UTRs by different algorithms can also contributes to the conflicting results by each algorithm. Therefore, it is imperative to use experimental techniques to validate these results from prediction algorithms and also acknowledge that algorithms might miss certain targets. Using databases like TarBase and mirRecords, which lists only the experimentally validated

targets of miRs, can circumvent some of these problems. However, one glaring issue with this is the only miRs that have been adequately studied and experimentally validated will make it to this list and cannot be used to study novel targets of miRs. Albeit, it can be used to inform us about the principles of miR:mRNA interaction and thus revise the algorithm to produce better results.

In our research, miR microarray technology is exploited to catalogue miRs that showed significantly altered regulation after 6hRS. In the previous chapters, we studied one of those miRs, mir-483-5p in detail and established its role in regulating spine dynamics in primary cultures and showed that they exhibit biased regulation after stress *in vivo*. In this chapter, we sought to identify the targets of mir-483-5p and check if those targets are down-regulated in the amygdala after 6hRS. Here we report the identification and characterisation of the target genes. Firstly, using a bioinformatics/data-mining approach, we identified 12 potential targets of the mir-483-5p. We then assayed if these mRNAs are down-regulated in N2a cells following the ectopic overexpression of mir-483-5p. Out of the 12 miRs, three genes (*Pgap2*, *Gpx3* and *Macf1*) are identified as potential targets. Moreover, we used luciferase reporter assay to show a direct interaction of mir-483-5p at the predicted seed sites at the 3'UTR region of these target mRNAs. Furthermore, using multi-label immunostaining we saw that three of the targets of mir-483-5p is expressed in the BLA neurons. Finally, we analysed total and synaptosomal fractions made from amygdalae of mice before and after 6hRS to show that these targets are indeed down-regulated in the amygdala, as predicted by the putative interaction of mir-483-5p at the seed sites. However, only *Pgap2* showed a significant down-regulation in both total and synaptosomal fractions. Here we predict that *Pgap2* is the likely candidate for the mir-483-5p

induced effects on neurons and will form the primary candidate of interest in the following experiments.

4.7 Results

4.7.1 Putative targets of mir-483-5p

In previous chapters, we have identified mir-483-5p, a miR that is significantly up-regulated in the amygdala following 6hRS. Moreover, we reported compartmental bias of this miR and that its overexpression is sufficient to induce changes in dendritic arbour and spine proportion, a pattern reminiscent of adaptive measures against stress-induced neuronal changes. The next logical step to follow in order to understand the role of mir-483-5p is to predict and then validate the mRNA targets of mir-483-5p. Several online databases using various algorithms exist with an aim to predict putative miR targets. These algorithms use different methods, but the core of their predictive power utilises the seed site complementarity and thermodynamic stability of these sequences (Agarwal et al., 2015). Combinations of methods have been explored to predict the number of targets that have functional relevance in stress-induced synaptic plasticity. Firstly, we used the TargetScan (Agarwal et al., 2015) to run a target prediction for mir-48-5p which resulted in 525 putative targets, Figure 4.2 (p. 173). Then we used the EIMMo database to narrow down the number of potential targets expressed in the amygdala leading to 215 results. Then we compared 215 targets against the list of genes expressed in amygdala using AmiGo search algorithm to scan for stress-related genes. Crosschecking these three different lists resulted in an overlap of potential targets. These targets are *Bcr, Cdk7, Ctnnbip1, Cul4a, Gpx3, Macf1, Pgap2, Rbpj, Saal1, Shh, Tbkbp1* and *Tnik*. The systematic approach we

used increased the probability of identification of true mRNA targets of mir-483-5p that are likely to be regulated by stress in the amygdala. Next, we investigated whether these target genes undergo down-regulation by mir-483-5p in a biological system.

4.7.2 mir-483-5p down-regulates several predicted targets

Using bioinformatics and data mining approach, we identified 12 putative mRNA targets for mir-483-5p that are likely to be stress-regulated and expressed in the amygdala. However, given the complexity of the biological systems, *in silico* prediction is not a guaranteed method to obtain a list of true targets (Baek et al., 2008; Easow et al., 2007; Selbach et al., 2008). In order to verify whether mir-483-5p is capable of reducing the mRNA levels of the predicted targets, we overexpressed mir-483-5p in mouse neuroblastoma cells (N2a) treated with dexamethasone (Dex), an analogue of corticosterone and quantified the target mRNA levels. We transfected N2a cells with pEGP-mir-483-5p expressing plasmid or scramble control sequence expressing plasmid. 24hrs post-transfection, Dex was added to the culture to form a final concentration of 1µM. RNA was extracted on the 4th day, and levels of each target gene was measured and normalised to the mRNA levels of house-keeping gene, ß-actin (Figure 4.3, p. 175 & Figure 4.4, p. 177). Out of the 12 putative target genes, significant reductions in mRNA levels were seen in *Pgap2* (46% ±8%, $t(6)= 2.45$, $p<0.05$), *Gpx3* (51% ±3%, $t(6)= 2.45$, $p<0.001$) and *Macf1* (95% ±2%, $t(4)= 2.78$, $p<0.05$), Figure 4.3 (p. 175). Nine of the other target genes did not show statistically significant change in their mRNA levels, Figure 4.4 (p. 177). Using N2a cell-based overexpression system, we identified a reduction in the mRNA levels of these three targets upon increasing the level of mir-483-5p. These results prompted us

to consider *Pgap2*, *Gpx3* and *Macf1* for further analysis. Following from this experiment we decided to investigate whether these targets are downregulated through a direct interaction of mir-483-5p at the 3'UTR region of *Gpx3*, *Macf1* and *Pgap2*.

4.7.3 mir-483-5p downregulates targets through 3'UTR interaction.

In order to understand if mir-483-5p down-regulates *Pgap2*, *Gpx3* and *Macf1* we cloned the 3'UTR region of these genes into pmiRGLO Dual- Luciferase vector of Dual-Glo® Luciferase assay system from Promega (Figure 2.2, p. 87). N2a cells were double transfected with mir-483-5p or scramble sequence overexpressing plasmid and pmiRGLO Dual- Luciferase vector. 24hrs after transfection, luminescence from the primary reporter gene, firefly luciferase, is measured and normalised to the *Renilla* luciferase luminescence (control reporter gene). Figure 4.5 (p. 179) shows the results represented as the percentage change in luminescence. *Pgap2* (62%±5, $t_{9.58} = 5.77$, $p<0.001$), Gpx3 (62%±4, $t_{8.96} = 4.74$, $p<0.01$) and Macf1 (47%±9, $t_{6.61} = 3.61$, $p<0.01$) showed significant reduction in luciferase activity relative to the scramble controls demonstrating that mir-483-5p overexpression is sufficient to observe a reduction in luciferase due to the interaction of mir-483-5p and 3'UTR region of the target mRNA.

In order to further establish the observed down-regulation is due to the 3'UTR interaction at the predicted seed site, we introduced point mutations (up to three nucleotides) into the seed site at randomly chosen sites (Figure 4.6, p. 181) and repeated luciferase assay as mentioned above. Introduction of mutations at the seed site eradicated the reduced luminescence in these assays, suggesting a direct interaction of mir-483-5p at the seed site of predicted 3'UTRs, Figure 4.5 (p. 179). Our experiments, for the first time in the literature, have

demonstrated that mir-483-5p interact with the predicted 3'UTR seed site of *Pgap2*, *Gpx3* and *Macf1*.

4.7.4 PGAP2, GPX3 and MACF1 are detected in BLA neurons

Our experiments hitherto revealed that mir-483-5p binds to *Pgap2*, *Gpx3* and *Macf1* at their 3'UTR region to reduce their expression in cell cultures. We also saw that mir-483-5p is enriched in synaptic compartments and upregulated in those compartments after stress. Next, we sought to investigate if these target proteins are expressed in BLA neurons. Moreover, we wanted to identify the expression pattern of these proteins within the cell. These three target proteins could be expressed in a diffused manner in the cytoplasm or in the cell membrane. In order to check this, we performed immunostaining on brain slices of WT mice using antibodies against for PGAP2, GPX3 or MACF1. In this multi-label immunostaining, we have used NeuN (Neuronal Nuclei Antigen) antibody as the neuronal markers and GFAP (Glial Fibrillary Acidic Protein) antibody as a marker for glial cells. NeuN and GFAP staining are commonly used as markers to identify respective cell types. Figure 4.7 (p. 183) shows the immunostaining of BLA neurons using antibodies against one of the target genes, NeuN and GFAP. The results showed that all of the three genuine targets of mir-483-5p are expressed in BLA neurons. GPX3 showed significant signal also in GFAP positive cells indicating GPX3 is expressed in both neurons and glial cells. Furthermore, pattern of signal revealed that the target proteins are expressed in the cell membrane instead of showing a cytosolic diffused pattern like that of NeuN. Next, we wanted to investigate if these three targets are downregulated in synaptosomes and total cell lysates after 6hRS.

4.7.5 Pgap2, a target of mir-483-5p is down-regulated in synaptosomes

Our previous experiments showed that following 6hRS mir-483-5p is overexpressed in mouse amygdala, and the regulation of mir-483-5p is biased towards synaptosomes. Moreover, we also found that the overexpression of mir-483-5p is sufficient to offset stress-induced changes in dendritic arborisation and spine classes proportions in primary cultures. Furthermore, while reporter assay showed that mir-483-5p targets *Pagp2*, *Gpx3* and *Macf1* at the predicted 3'UTR seed site, multi-label immunostaining showed that the target gene products are present in BLA neurons. These results suggest the target mRNAs could be down-regulated in the amygdala by mir-483-5p. And, if the mir-483-5p induced morphological changes are facilitated via the predicted targets, then a reduced expression of these targets should be also found in the synaptosomes after 6hRS. In order to test these predictions, we extracted total and synaptosomal mRNA from the BLA of control and stressed animals, and performed qRT-PCR to assess the levels of the three target genes. As suggested by our data, all of the three targets were significantly reduced in the total amygdala homogenate RNA ('Total' in Figure 4.8, p. 185): *Pagp2* (52% ±7, $t_{3.87} = 3.72$, $p<0.05$), *Gpx3* (42% ±20, $t_{3.75} = 3.69$, $p<0.05$) and *Macf1* (40% ±10, $t_{2.44} = 5.22$, $p<0.05$). This suggests that the down-regulation of target genes in the total amygdala RNA pool is likely due to the mir-483-5p interaction. Furthermore, our data also showed that one of the target genes, *Pgap2* (53% ±3, $t_{3.34} = 3.19$, $p<0.05$) is significantly down-regulated in synaptosomal fractions. Interestingly, no significant changes in *Gpx3* and *Macf1* levels were found in synaptosomal fractions, compared to the total fractions. Only *Pgap2* showed significant down-regulation in both total fractions and synaptosomes. This suggests that mir-483-5p induced changes in juxta-

synaptic compartments might be facilitated through *Pgap2*, and not by *Gpx3* or *Macf1*.

4.8 Discussion

4.8.1 Multiple databases can be used to filter potential targets of mir-483-5p

Our research has identified a novel function of mir-483-5p in structural neuronal plasticity. We have shown that following 6hRS mir-483-5p is preferentially up-regulated in synaptosomal compartments (Chapter 3). Moreover, we present evidence showing that the overexpression of mir-483-5p is sufficient to induce changes in structural plasticity like dendritic arborisation and spine proportion in primary neuronal cultures. This pattern is reminiscent of adaptive measures against stress-induced changes in the amygdala neurons (Chapter 3). In this chapter, we have identified 12 potential targets of mir-483-5p from a list of over 5,000 genes by cross-comparing multiple databases (Figure 4.2, p. 173). Firstly, TargetScan database was used to obtain a list of putative mir-483-5p targets. A total of 525 targets were found using this database. The TargetScan algorithm was used due to its relatively superior specificity reported by previous studies. One study compared experimentally observed protein output in response to ectopic expression of a miR (mir-124) with the targets predicted by several databases. TargetScan was one of the databases that produced one of the best sensitivities (Baek et al., 2008). Authors associated this increased sensitivity of TargetScan to its algorithm that prioritises 8mer and 7mer-m8 sites to predict targets. This is because these type of seed sites results in more efficient repression of targets than other seed interactions. Moreover, TargetScan analyses the 3'UTR environment of predicted seed sequences to provide a

context score which helps to increase the specificity. Other research has also found that, relative to other prediction programs, TargetScan provides high precision and specificity (Alexiou, Maragkakis, Papadopoulos, Reczko, & Hatzigeorgiou, 2009). However, it is important to note that only 1/3rd of the targets predicted by the TargetScan showed any response in the studies by Baek and colleagues. This is one reason why it is crucial to validate the predictions outputted by these algorithms experimentally. Moreover, other databases can be used to tease apart the genes that are likely expressed in specific tissues and have been previously predicted or experimentally implicated in the phenomenon under study. In order to further filter the list of genes predicted by TargetScan and to study phenomena we are interested in, EIMMo and AmiGo databases were used to find the overlap between the predicted targets, genes that are expressed in amygdala and altered in response to stress. Figure 4.2 (p. 173) shows the number of hits for each search parameters. By cross-comparing these three lists, TargetScan, EIMMo and AmiGo, we found an overlap of 12 putative targets to investigate further. By doing so, we systematically filtered the total number of genes to a manageable number. Moreover, these 12 genes will likely contain targets of mir-483-5p that are implicated in stress and expressed in the amygdala. The list of these 12 genes is presented in Figure 4.2 (p. 173).

4.8.2 Targets of mir-483-5p in a biological system

In silico predictions do not necessarily predicate biological significance. In order to establish if these genes could be down-regulated by mir-483-5p, we used N2a overexpression system to quantify the mRNA levels of all 12 genes. When mir-483-5p is overexpressed in N2a cells in the presence of Dex, 3 out of 12 genes (*Pgap2*, *Gpx3* and *Macf1*) were significantly down-regulated. This also aligns with

the previous research that reported a 3rd of predicted genes by TargetScan are experimentally verified (Baek et al., 2008). Moreover, these results suggest that the nine genes that did not show down-regulation are unlikely to be the targets of mir-483-5p. However, there is a plausibility that some of these nine genes could be the targets and induced down-regulation of these targets by forced overexpression of mir-483-5p triggers an alternate feedback mechanism that increases their transcription and thereby compensates for their reduction. Nonetheless, these possible pathways could be N2a cell specific or endogenous to neurons. In either case, such a pathway would indicate that these nine genes are unlikely to play active roles in specific effects induced by mir-483-5p *in vivo*. Based on these results, only those genes that showed significant down-regulation in response to mir-483-5p overexpression have been exposed to further investigation.

It is important to note that we have reduced the list down to 12 from a potential 525 TargetScan search hits using gene ontology and tissue expression. As a result, the targets that are focused in this research will involve only those that satisfy these criteria. Therefore, it is likely that not all of the targets mir-483-5p are investigated. Indeed, mir-483-5p is likely to have other mRNA targets that are involved in other pathways and mechanisms. To study all of the targets of mir-483-5p comprehensively, it will require a much larger collaboration from researchers involved in different areas of interest including stress and anxiety. Currently, this is beyond the scope of this research and outside what is possible with our available resources. Nevertheless, the three genes identified in these experiments could be part of cognate mechanisms involved in stress and anxiety. Our discovery could open doors to previously unidentified mechanisms that

contribute to neuronal plasticity in this context and therefore are inherently interesting.

In this experiment, we have found a correlation between overexpression of mir-483-5p and down-regulation of three targets in N2a cells. However, this observation does not suffice to imply a causal relationship. Using DualGlo luciferase assay system and mutating different nucleotides in the seed site, we show that mir-483-5p targets these three genes at the predicted 3'UTR seed. Firstly, by cloning the 3'UTR region downstream of a reporter gene, we show that the luminescence from the reporter gene is reduced when cells are transfected with mir-483-5p. Secondly, we demonstrate that the reduction in luminescence is eliminated only when the nucleotides in the predicted seed site are mutated. These observations unequivocally demonstrate that the predicted seed site in their 3'UTR context is necessary for the repressive action of mir-483-5p suggesting that mir-483-5p form canonical Watson-Crick pairing at the seed sites of *Pgap2*, *Gpx3* and *Macf1*. Moreover, the predicted seed sites form the 7mer-m8 type of stringent pairings with all of the three target mRNAs. However, these experiments do not suggest that mir-483-5p can down-regulate these targets in the amygdala following 6hRS. In order to verify such a hypothesis, repression of these genes in the amygdala following 6hRS should be detected. Moreover, any genes that are involved in the mechanisms that require translation at juxta-synaptic compartments could also show a down-regulation in those compartments after 6hRS. Investigating these predictions were the purpose of the next experiment. In order to do this, we made total fractions and synaptosomal fractions from amygdala tissues obtained from animals before (controls) and after 6hRS. mRNA levels of *Pgap2, Gpx3* and *Macf1* in these

fractions were assayed using qRT-PCR. As predicted, a down-regulation of these three genes (*Pgap2*, *Gpx3* and *Macf1*) in the total fraction was observed, Figure 4.8 (p. 185). Our previous experiments showed that mir-483-5p is up-regulated in total fractions, but also in synaptosomal fractions. In agreement with our finding, all three genes showed significant down-regulation in total fractions suggesting that mir-483-5p regulates these three targets in amygdalae after 6hRS.

Interestingly, only *Pgap2* showed significant down-regulation in synaptosomes. One probable reason for this could be that, unlike *Gpx3* and *Macf1, Pgap2* is the only gene that undergoes localised translation in synaptosomes. This explanation is supported by our data that show significant enrichment of *Pgap2*, ~4.5 times, in synaptosomes relative to total fractions. In the case of *Gpx3* and *Macf1*, their synaptosomal mRNA levels were lower relative to total fractions, 0.7 and 0.6 times respectively. When considered along with that fact that mir-483-5p is also significantly enriched in synaptosomes following 6hRS, *Pgap2* is expected to show down-regulation in synaptosomal fractions. As we will see in the later sections, this also corroborates the functions of these genes. Additionally, this observation also suggests that *Pgap2* has crucial roles to play in juxta-synaptic compartments under normal conditions. Furthermore, immunostaining of amygdala neurons suggests that *Pgap2* and *Macf1* are present in neurons while *Gpx3* is present in both neurons and GFAP positive cells, mainly astrocytes, Figure 4.7 (p. 183). See box 2, 3 & 4 (pp. 160, 163 & 165) which describes the roles of *Gpx3, Macf1* and *Pgap2* in detail. Following sections attempt to suggest potential mechanisms of the action of the three targets in rodent stress and anxiety.

In our experiment, we show that *Pgap2* mRNA levels in total fractions and synaptosomal fractions after 6hRS (Figure 4.8, p. 185) is negatively correlated with an up-regulation of mir-483-5p in those fractions. Similarly, we were able to reproduce this negative relationship in N2a cells when mir-483-5p was overexpressed. Furthermore, using DualGlo luciferase reporter assay, we show a direct interaction of mir-483-5p at the 3'UTR of *Pgap2*. Taken together, our data suggest that *Pgap2* is targeted by mir-483-5p in the total and synaptosomal fractions in response to 6hRS. Our experiments also show that among the three verified targets of mir-483-5p, only *Pgap2* mRNA demonstrated significant down-regulation in both fractions. However, the implication of this down-regulation at a molecular level needs further study.

There are several possible mechanisms that could contribute to the *Pgap2* induced changes in neuronal plasticity. Reduction in the availability of *Pgap2* could lead to a global dysregulation in GPI anchoring, a local increase in alkaline phosphatase (ALP) levels, or relative increase in GPI-Aps cleavage. Since several signalling molecules are transported to the lipid raft in response to stress, reducing *Pgap2* levels could result in transient attenuation in the glypiation of several proteins and therefore the number of functional receptors in the lipid raft. By temporarily regulating these proteins, it might be possible to reduce the overall cellular signals received by the cell in response to stress. However, further studies are required to understand if down-regulation of *Pgap2* will result in a concomitant reduction in the number of glypiated proteins expressed in the lipid raft and if the reduction is sufficient to reduce the overall signal load received by a cell. Some evidence for this 'lipid raft gate' hypothesis comes from studies that showed the disruption of lipid rafts can interfere with signalling cascade involving

Protein kinase C, Src-dependent protein kinase D activation and phosphorylation of several proteins that links cytoskeleton and signalling molecules (Cabrera-Poch, Sánchez-Ruiloba, Rodríguez-Martínez, & Iglesias, 2004). Moreover, studies have also shown that lipid rafts can modulate GPCR signalling by manipulating the availability of signalling receptors (Czysz & Rasenick, 2013). Furthermore, studies have reported the importance of lipid rafts in neuronal growth, signalling and in neurodegenerative conditions (Head, Patel, & Insel, 2014; Um & Ko, 2017). However, the majority of studies focused on GPI-Aps already present in the lipid raft and how the structure of lipid raft affects these signalling pathways. How does the regulation of glypiation process *per se* affect the lipid rafts and the consequences of this process in the signal transduction through the lipid rafts proteins is still elusive and requires further investigation.

Box 2: *Gpx3*

One of the genes we identified as a target of mir-483-5p is *Gpx3* which is a selenocysteine-containing plasma protein that is a member of glutathione peroxidase (GPx) family. GPxs catalyse hydroperoxides, hydrogen peroxide (H_2O_2) and scavenge reactive oxygen species (ROs) in cells (Jin et al., 2011). This anti-oxidant enzyme, like the other GPx gene products, catabolises H_2O_2 to $2H_2O$ thus absorbing some of the adverse effects of oxidative stress. Moreover, GPx3 is the only one in the GPx family of proteins that are released to the extracellular matrix. Consistent with its role, induced expression of GPx3 reduces extra-cellular H_2O_2 levels (Chung et al., 2009). However, studies have shown that glutathione family of genes might have tissue-specific and cell-specific roles (Brigelius-Flohé, 1999). Moreover, GPx3 is proposed to be an active player in both intracellular and extra-cellular H_2O_2 reduction. These studies point towards the potential role of *Gpx3* in neurological conditions. Indeed, several researchers have investigated oxidative stress and the role of H_2O_2 in brain regions in response to stress. Some studies have shown that oxidative stress induced by ROs is one of the common mechanisms underlying bipolar disorder, depression and schizophrenia (Fullerton et al., 2010). Moreover, defects in these systems can exacerbate the progression of certain psychiatric diseases (Fullerton et al., 2010).

Interestingly, roles of H_2O_2 in these mechanisms are not that clear. Later studies have shown that H_2O_2 is essential for synaptic transmission and plasticity in the rodent brain (Armogida, Nisticò, & Mercuri, 2012). Moreover, H_2O_2 in the extracellular environment is also implicated in the reduction of metabotropic signal transduction, intracellular Ca^{2+} signalling and organelle function modulation in rat hippocampal neurons (Gerich, Funke, Hildebrandt, Faßhauer, & Müller, 2009). Furthermore, studies have shown that H_2O_2 is an important neuroprotective molecule that is essential in attenuating the ischemia-induced neuronal damage (Armogida et al., 2012). This neuroprotective effect of H_2O_2 is partially due to the careful regulation of glutathione peroxidase family of genes. Furthermore, studies using knockout or overexpression of antioxidant enzymes have shown a relationship between anxiety-like behaviour and oxidative stress (Krolow, Arcego, Noschang, Weis, & Dalmaz, 2014). These research findings

point toward the idea that by varying the release and expression of H_2O_2 and antioxidants like GPx3, it is possible to produce pleiotropic effects on cellular functions. In agreement with this, GPX3 deficient mice showed an increase of P-selectin and cyclic cGMP in the plasma, both of which are implicated in depression or anxiety disorders. Additionally, compared to the WTs, $GPX3^{-/-}$ mice showed an increased neurological score (Jin et al., 2011), suggesting it is one of the necessary components for learning and plasticity. This is further demonstrated through a study that showed an interaction between SOP2 (Sensory rhodopsin-2) and GPX3 which might contribute to the bipolar disorder (Fullerton et al., 2010). Despite these studies and being the only GPx family of proteins released to the ECM, *Gpx3* has never been probed in the context of stress or anxiety. In our experiments, we found that mir-483-5p directly targets GPx3 at a specific 3'UTR and the level of GPx3 mRNA is reduced in the amygdala following 6hRS. It is difficult not to speculate possible mechanisms where the *Gpx3* level reduces in the amygdala neurons thereby selectively reducing the relative peroxidase activity in the cell or the ECM. This reduction in the peroxidase activity can potentially modify H_2O_2 levels and thus steer several aspects of neuronal physiology. Interestingly, in our experiments, we failed to see a reduction in *Gpx3* mRNA in synaptosomes following stress. This could be because, unlike *Pgap2*, the relative expression of *Gpx3* is significantly lower in synaptosomes, compared to total fractions. However, an alternative explanation could be that *Gpx3* mRNA level is selectively repressed in the total fractions but not in the synaptosomes. This could be due to the presence of a significantly larger pool of *Pgap2* mRNAs in the synaptosomes competing for mir-483-5p RISC and concomitantly reducing the repression of *Gpx3* mRNA. This is supported by the observation unveiled by our data that showed the ratio of synaptosomal *Gpx3* mRNA to total mRNA levels has increased after 6hRS. Further experimental evidence is required to falsify this claim and to study if this biased regulation of *Gpx3* mRNA also reflects the GPX3 protein expression. Furthermore, empirical evidence is not available to show GPX3 levels can modulate H_2O_2 levels in amygdalar neurons. Nonetheless, extrapolation from our data will be the first to point out that relative H_2O_2 levels can be selectively increased in ECM environment with relatively shorter spatial proximity to areas

where synapses are being formed. This way GPX3 can manipulate the relative H_2O_2 levels outside the cell and control site-specific signals responding to H_2O_2. Taken together, *Gpx3* seems to have good potential to steer neuronal plasticity by regulating H_2O_2 levels in neuronal structures. Our data might form the first in the series of further studies that investigate *Gpx3* and its potential role in stress and anxiety disorders.

Box 3: *Macf1*

Another important gene that is targeted by mir-483-5p and down-regulated in amygdala homogenate is microtubule-actin cross-linking factor 1 (*Macf1*). It is the largest gene (the coding region is >22kb) targeted by mir-483-5p and is expressed ubiquitously in lungs and nervous tissues (Goryunov, He, Lin, Leung, & Liem, 2010). The function of *Macf1* is to cross-link microtubules, actin and intermediary filaments and thus bridge different elements of the cytoskeleton to form functional networks at cellular junctions and the cell periphery. Microtubules, actins and intermediate filaments together form cellular cytoskeleton. It is important for maintaining the structural integrity of cells, cellular movement and transportation of proteins within each cell. Previous studies have implicated *Macf1* in neuronal activity and plasticity by regulating cell signalling, cell migration, tissue integrity, maintenance and axonal extension. Unsurprisingly, the role of the cytoskeleton in neuronal function and plasticity has been studied and shown to be important in LTP and LTD (Gordon-Weeks & Fournier, 2014).

Several studies have looked into the roles of *Macf1* and found that it is crucial for normal neuronal functioning. For example, it was reported that knocking out of MACF1 in mice is lethal, which results in death at E10 stage. However, conditional knockout of MACF1 can produce viable animals, but they die within 24-36 hours post-partum (Goryunov et al., 2010). Similarly, the knockdown of MACF1 using Cre-Loxp system results in severe developmental defects in the mouse brain (Goryunov et al., 2010) and primary cultures (Sanchez-Soriano et al., 2009). Furthermore, siRNA knockdown of *Macf1* in Neuro2A cells produced a higher percentage of axons with unbundled and looped microtubules (Sanchez-Soriano et al., 2009). Additionally, corroborating our finding, mutagenesis studies have found that a reduction in *Macf1* is correlated with reduced filopodia numbers in Drosophila neurons and N2A cells (Sanchez-Soriano et al., 2009). In our experiment, we also found that the proportion of filopodia is reduced after the overexpression of mir-483-5p in primary neuronal cultures. These studies show that *Macf1* is important for normal neuronal development and function. This is also reflected in the identification of *Macf1* in several neurological conditions. For example, it is implicated in decreased sensitivity to PTSD in maternally separated female mice (Sun, Tu, Shi, Xue, &

Zhao, 2014) and schizophrenia interactome (Camargo et al., 2007). Additionally, misregulation of *Macf1* is also implicated in Parkinson's disease and schizophrenia (Hu et al., 2017; Moffat, Ka, Jung, Smith, & Kim, 2017). These phenotypes through MACF1 regulation might be due to its involvement in WNT singling. WNT signalling is a well-investigated pathway that has important roles in gene transcription, cytoskeletal manipulation and intracellular calcium dynamics. MACF1 has been shown to be important for the WNT signalling induced translocation of AXIN, APC, ß-catenin and GSK3B (Goryunov et al., 2010). Additionally, studies have shown that shRNA silencing of *Macf1* resulted in WNT signalling inhibition through down-regulation of *ß-catenin* (Chen et al., 2006). Furthermore, MACF1 is shown to be regulating the several aspects of pyramidal neurons by controlling the microtubule dynamics and GSK3 signalling (Ka, Jung, Mueller, & Kim, 2014). Interestingly, another putative function of MACF1 is suggested to be involved in the transportation of GPI-linked proteins from the trans-Golgi network (TGN) to the cell periphery (Kakinuma, Ichikawa, Tsukada, Nakamura, & Toh, 2004). TGN is a collection of several protein complexes involved in the transportation of gene products to the appropriate cellular locations (Guo, Sirkis, & Schekman, 2014). Our data show that stress increases the expression of mir-483-5p, which downregulates *Macf1* in the amygdala. Based on our data and the literature, *Macf1* may have a regulatory role in stress-induced dendritic spine formation, which results in potential synaptogenesis. Whether this mechanism is through WNT signalling, GPI-linked protein transportation or both is yet to be determined.

Box 4: *Pgap2*

Our study revealed *Pgap2* (Post GPI attachment protein 2) as one of the three genes that is targeted by mir-483-5p. Unlike *Gpx3* and *Macf1*, *Pgap2* demonstrated reduced expression in both total and synaptosomal fractions (Figure 4.8, p. 185). PGAP2 is an important protein in the cascade of processes leading to glypiation, one of the post-translational modifications that come under the category of glycosylation. Over 100 rare genetic disorders of glycosylation are known so far, several of them being neurological in nature (Freeze, Eklund, Ng, & Patterson, 2012, 2015). In mammals, nine monosaccharides are distributed among ten different pathways involved in glypiation (Ikezawa, 2002). Interestingly most of the pathways are associated with one or other form of genetic defects. Glypiation has been shown to be important in axon guidance, synaptic adhesion, cytoskeletal remodelling and localised signalised transduction. These processes are important for the *de novo* synaptogenesis and synapse maintenance. This is unsurprising given the importance of glypiation, which is an essential step required for the proper transport of proteins to the lipid raft (Um & Ko, 2017). Studies have found that depletion of the lipid raft at synapse destabilises GluRs and results in loss of synapses. Several other proteins like EphrinA, Sema7A, Netrin-Gs (NGRs), Glypicans (GPCs) and Contacticans undergo glypiation and are implicated in axonal growth and navigation. NGRs and GPCs are shown to be important in neuronal regeneration and repair. Some glypiated proteins are shown to be necessary for synapse modification. For example, NGRs and 7-cadherin have been shown to negatively regulate synaptic plasticity while Sema7a, Nectrin-Gs and GPCs have been shown to regulate synaptic physiology positively. Research is slowly unveiling glypiation as a necessary factor for efficient synaptic/neuronal plasticity. The next paragraph describes the process of glypiation and the role of PGAP2 in this process.

Glypiation, addition of GPI anchors, is a reversible process done *en bloc* by an enzyme transaminidase in the endoplasmic reticulum (ER). It is essential for the sorting of the proteins for transportation to membrane/lipid raft (Hansen et al., 2013). Lipid rafts are specialised areas of the plasma membrane where several signalling molecules and ligands are covalently bound to the membrane into specialised microdomains (Um & Ko, 2017). These include several trans-

membrane proteins that extend through the membrane multiple times and other proteins that are then exposed to the ECM. GPI anchors are added to the C-terminus of the proteins with a specific signal. These proteins then undergo further remodelling which involves the removal of acyl phosphate and ethanolamine phosphate chains (Hansen et al., 2013). Following this process, GPI anchored proteins (GPI-APs) are transferred to the Golgi apparatus where further modification is taken place prior to signalling the transport to and incorporation into the plasma membrane. This modification and transportation are performed firstly by transferring GPI-APs to the Golgi apparatus. This transfer is facilitated by de-acylation of the anchor by PGAP1 (Tanaka, Maeda, Tashima, & Kinoshita, 2004). PGAP1 removes acyl group from the inositol before reaching the Golgi apparatus. While in the Golgi, fatty-acid remodelling of the GPI-APs occurs – removal of unsaturated fatty acid by PGAP3 and addition of saturated fatty acid by PGAP2 (Hansen et al., 2013; Tashima, 2005). This addition of the saturated fatty acid is essential for further processing to GPI-APs. Studies have found that in PGAP2 mutant cells lines, there is a significant reduction in the expression of GPI-APs on the plasma membrane raft. Two mutually inclusive mechanisms have been proposed to explain these observations. Firstly, the lack of PGAP2 results in the incorrect processing of GPI-anchors in Golgi and results in the incorporation of GPI-Aps in a non-specific manner on the plasma membrane. As a result of this 'incorrectly' placed GPI-Aps are exposed to the phospholipase D (PLD), an enzyme expressed abundantly in serum, that cleaves the anchor and releases proteins to the extracellular environment. Secondly, the GPI-Aps that did not undergo processing by *Pgap2* are more sensitive to this cleavage by PLD, even after the correct incorporation into membrane rafts. Some evidence supporting this hypothesis comes from human subjects that have a mutation in *Pgap2* that results in Mabry syndrome, also known as hyperphosphatasia with mental retardation syndrome (HPMRS) (Fujiwara et al., 2015; Hansen et al., 2013; Howard et al., 2014; Krawitz et al., 2013). This syndrome is characterised by intellectual disability, distinctive facial features and elevated levels of alkaline phosphatase (ALP) in the blood. In normal subjects, alkaline phosphatase is attached to the plasma membrane, but in HPMRS patient these ALPs are released into the plasma. This is because of incorrect processing

of ALP anchors by PGAP2, which results in the release of ALP to the serum. Further evidence of this comes from comparing the patients that have a heterozygous or homozygous mutation in *Pgap2*. Patients with a heterozygous mutation in *Pgap2* have exhibited mental retardation with more severity and relatively increased ALP levels when compared to those with a homozygous mutation. This shows that the level of PGAP2 is essential and the level of glypiated proteins in the lipid rafts could be mediated through the regulated expression of PGAP2. However, more evidence is required to understand if this mechanism might have any role to play in the stress-mediated modification of synapse. Interestingly, studies have reported slightly elevated ALP levels after stress, and this process is essential for axonal guidance. However, there is insufficient evidence to suggest whether this elevation is partly or wholly through the down-regulation of PGAP2.

4.8.3 *Pgap2*, *Gpx3* and *Macf1*: Three diverse proteins working in synchrony?

Our data suggest that mir-483-5p targets *Pgap2, Gpx3* and *Macf1* in different cellular compartments to buffer for the stress-induced plasticity changes. Whether this effect is cumulative, through mechanisms involving three genes separately, or synergic, through a combination of two more of genes, is unclear. The above section speculates on the possible mechanisms that make the cumulative effect possible. However, there is also a possibility of a synergic effect where all three targets or the combination of any two targets contribute to a particular effect. This contribution subsequently brings about the stress-buffering effect. One possible mechanism through which this can be achieved is through the combination of *Gpx3*-downregulation induced relative increase of H_2O_2 at juxta-synaptic regions when compared to other cellular regions, and the reduced expression of GPI-linked protein on the plasma membrane. The reduced expression of GPI-Aps can be accomplished in multiple ways. One mechanism could involve a reduction in the transportation of these membranes to the cell periphery through the down-regulation of *Macf1* and *Pgap2*. Another mechanism might include reducing the number of GPI links to the proteins and sensitising these links to the PLD by further down-regulating *Pgap2* in synaptosomal areas. In agreement with this possibility, previous studies have suggested that down-regulation of *Pgap2* increases the ALP levels and other independent studies that have found elevated levels of ALP in the serum after stress. Furthermore, studies have found that in hippocampal neurons, elevated levels of ALP is shown to regulate axonal growth (Diez-Zaera et al., 2011). Also, the dynamic role of H_2O_2 as a neuroprotective chemical makes this possibility closer to the ‘likely' end of the probability spectrum. Further studies are required to validate the cumulative

or synergic effect of the down-regulation of these target genes by mir-483-5p. Currently, the hypothesis suggested in this discussion is closer to a speculation based on current literature rather than empirical evidence.

4.9 Conclusion

In this chapter, we reported the identification and verification of three genes *Pgap2, Gpx3* and *Macf1* as the targets of mir-483-5p in amygdalar neurons. We did this using bioinformatics and experimental approach. Firstly, we determined 12 putative targets of mir-483-5p by exploiting the *in silico* prediction algorithm offered by TargetScan, Eimmo and AmiGo. Secondly, we demonstrated the down-regulation of three of these genes in N2a cells when mir-483-5p was overexpressed in the presence of Dex. Thirdly, using reporter assay, we showed a direct interaction of mir-483-5p at the predicted 3'UTRs of these three genes. Finally, we showed that these genes are indeed down-regulated in the amygdala following 6hRS in total cellular fractions. Moreover, we report that *Pgap2*, a gene involved in the GPI anchoring, is down-regulated in total fraction and synaptosomal fractions. The down-regulation of these genes is correlated with the up-regulation of mir-483-5p. Taken together, mir-483-5p targets these three genes in the amygdala following 6hRS. The action of mir-483-5p extends to synaptosomes where it targets *Pgap2*.

Further research is necessary to decipher the biochemical consequences of the down-regulation of these three genes. Nonetheless, our research using primary neuronal cultures indicates that the neuronal plasticity could be affected by regulating dendritic arborisation and spine dynamics. While understanding the limitation of extrapolating from primary cultures to *in vivo* action, it is likely that the overexpression of mir-483-5p will have a similar effect *in vivo.* The exact

biochemical pathways responsible for this potential effect require further investigation. However, current literature about these three targets shows these genes could work individually or together. In either case, the resulting change will involve modulation of ROs, cytoskeletal transport and GPI anchoring. Previous research indicates that it is indeed possible that these genes could regulate neuronal plasticity. However, further experimental evidence is necessary to obtain a definite conclusion. Irrespective of the mechanisms involved in the changes we observed *in vitro* experiments (Chapter 3), it is imperative to check if these biochemical changes are correlated with meaningful changes in animal behaviour. The next chapter will focus on the behaviour of rodents after the overexpression or mir-483-5p or knockdown of *Pgap2* in the amygdala region.

Figure 4.1 Different types of canonical seed sites

Canonical seed sites are classified into four main types; 8mer, 7mer-M8, 7mer-A1 and 6mer. 8mer has an Adenosine present at the position one of the mRNA seed site pairings and forms complete Watson-Crick pairing from position two to eight. 7mer-M8 has no Adenosine at position one but has a perfect pairing like an 8mer. 7mer-A1 has adenosine at position one, but only six perfect seed pairings. 6mer has six perfect seed pairing without an Adenosine present at position one. The figure above shows mir-483-5p forming hypothetical seed pairings with mRNA seed sites. The seed sequences are capitalised. See section 4.4 for more details.

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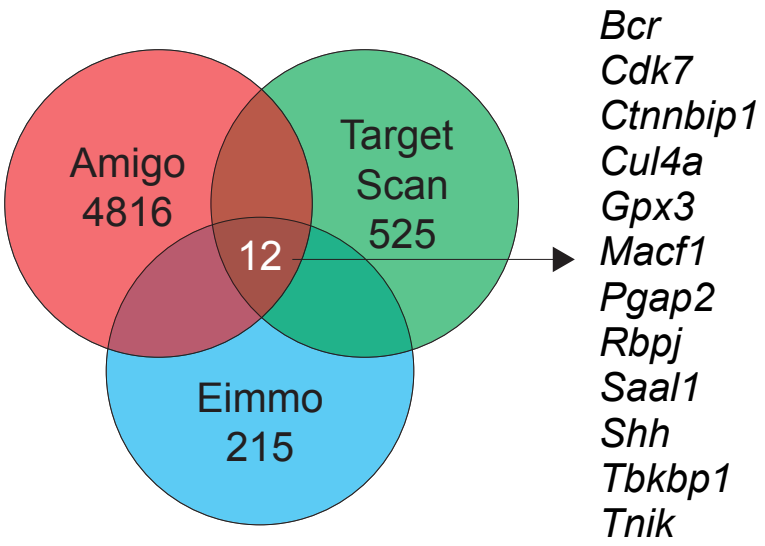

Figure 4.2 Identification of putative targets of mmu-mir-483-5p
We have used a combination of databases to cross-compare the list of genes to identify the targets of mir-483-5p that are more likely to be the genuine targets, expressed in the amygdala and change in response to stress. TargetScan was used to get the list of mir-483-5p targets which produced 525 hits. Eimmo database was used to get the mir-483-5p targets that are expressed in the amygdala (215 hits) while Amigo database was used get the list of genes that 'respond to stress' (4,816 hits). Twelve genes were common in all three lists. Only these genes (listed on the right side) were considered for further analysis.

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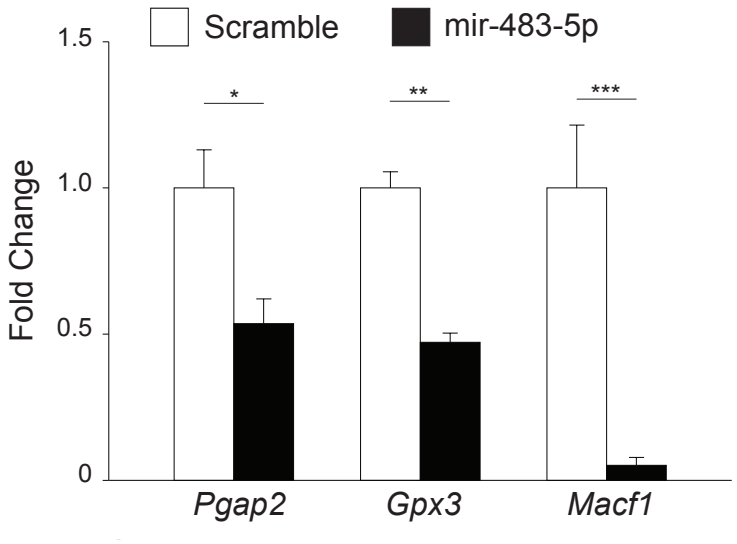

Figure 4.3 mir-483-5p down-regulated three predicted targets in N2a cells.

To identify if mir-483-5p can down-regulate the predicted gene targets, N2a cells were transfected with mir-483-5p or scramble vectors and treated with dexamethasone. Subsequently, mRNA levels of *Pgap2*, *Gpx3* and *Macf1* were quantified using qRT-PCR and normalised the ß-actin mRNA levels. The results show that relative to control samples, *Pgap2*, *Gpx3* and *Macf1* levels were significantly down-regulated N2a cells over-expressed with mir-483-5p, demonstrating that the mir-483-5p over-expression is correlated with down-regulation of these mRNAs in a biological system. Data are shown as fold change relative to respective controls. T.test was performed to compare the means. n=4-6 per group. *p<0.05, **p<0.01, ***p<0.001

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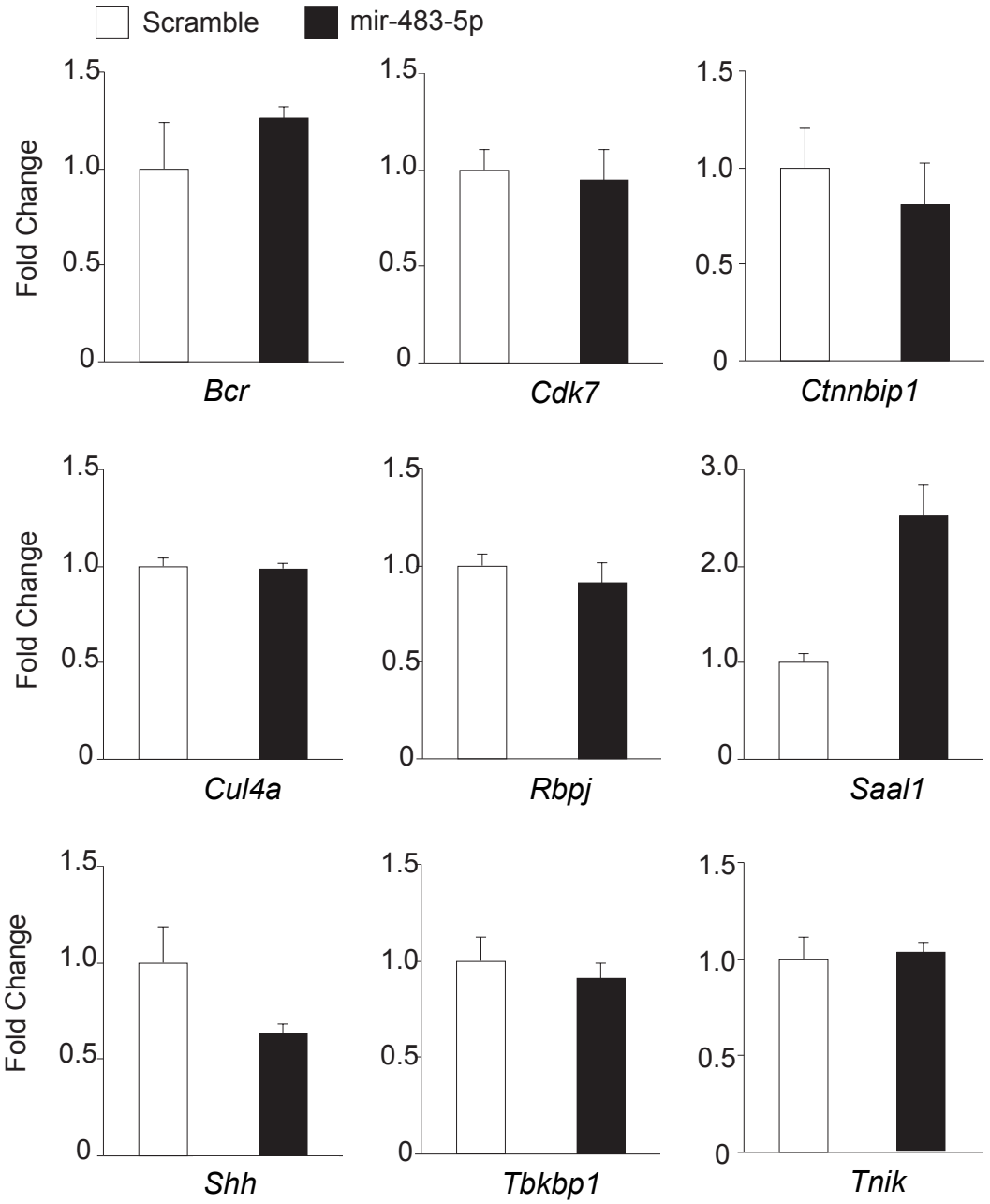

Figure 4.4 mir-483-5p failed to down-regulate nine of the predicted targets in N2a cells.

To check if the targets predicted by the algorithm is down-regulated by mir-483-5p, we over-expressed mir-483-5p (or a control scramble) vector in N2a cells and treated the cells with dexamethasone. Majority of the targets did not show any significant reduction in their mRNA levels when analysed by qRT-PCR. Data are shown as fold change relative to appropriate controls. T.test was used to compare the means. n=4-6 per group. These nine genes were subsequently excluded from further analysis.

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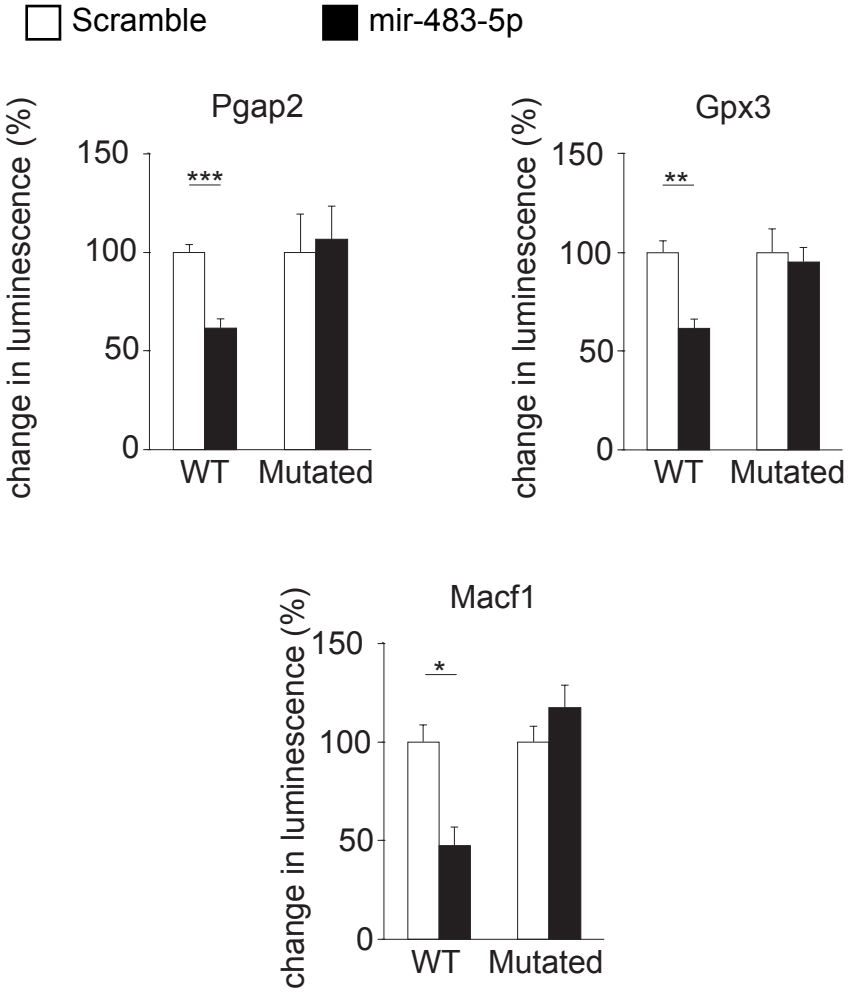

Figure 4.5 Luciferase assay confirms a direct interaction of mir-483-5p at the 3'UTR of *Pgap2*, *Gpx3* and *Macf1*

Dual-Glo® luciferase assay system from Promega was used to investigate if mir-483-5p interacts at the predicted 3'UTR of the *Pgap2*, *Gpx3* and *Macf1*. We double transfected N2a cells with pmiRGLO Dual-Luciferase vector that has the wild-type (WT) or mutated 3'UTRs downstream of the luciferase gene and with mir-483-5p over-expressing (or a scramble sequence) plasmids. Luciferase activity from the reporter *Firefly* luciferase was quantified and normalised to *Renilla* luciferase luminescence which served as a control. We found a significant reduction in luciferase activity in WT sequences, but not in mutated sequences of all three targets. Data are shown as the percentage change in luminescence relative to respective controls that are transfected with scramble sequences. T.test was used to compare the means. *$p<0.05$, **$p<0.01$, ***$p<0.001$

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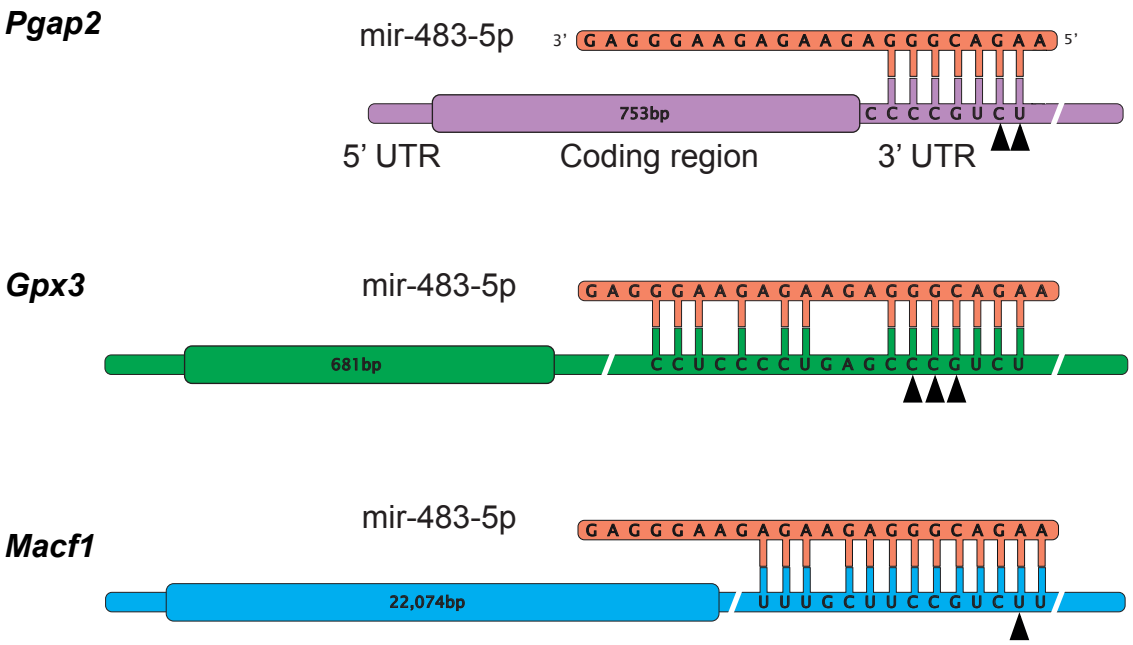

Figure 4.6 Schematic representation of mir-483-5p interacting at the 3'UTR of confirmed targets

Experiments using N2a cells and Dual-Glo® luciferase assay (fig 4.5) system revealed that mir-483-5p (orange) forms a 7mer-M8 type of interaction with the predicted 3'UTR of *Pgap2* (purple), *Gpx3* (green) and *Macf1* (blue). mir-483-5p is shown in 3' to 5' direction while the targets are shown in 5' to 3' direction. The black triangles show the mutations that were made in the 3'UTR seed site regions to establish a direct interaction using luciferase assay.

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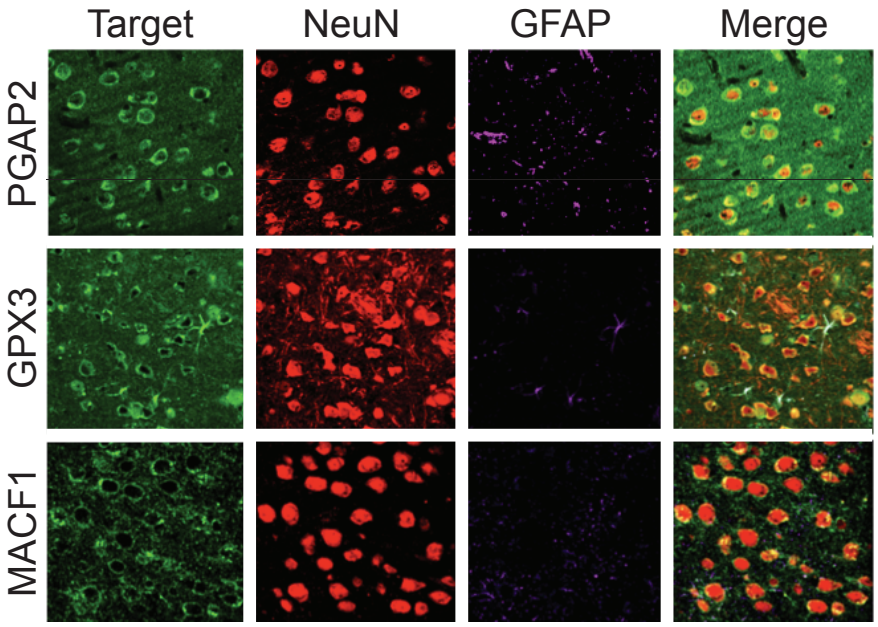

Figure 4.7 mir-483-5p targets are expressed in BLA neurons
To investigate if the *PGAP2*, *GPX3* and *MACF1* were expressed in the basolateral amygdala (BLA) of WT mice, we conducted multi-label immunostaining using antibodies against the target proteins, NeuN (neuronal marker) and GFAP (astrocyte marker). Our data show that all three target proteins can be detected in BLA neurons while GPX3 can be detected in both BLA and astrocytes. Moreover, all of the three targets proteins show relatively high expression in the cell membrane instead of homogeneous diffusion in the cytosol.

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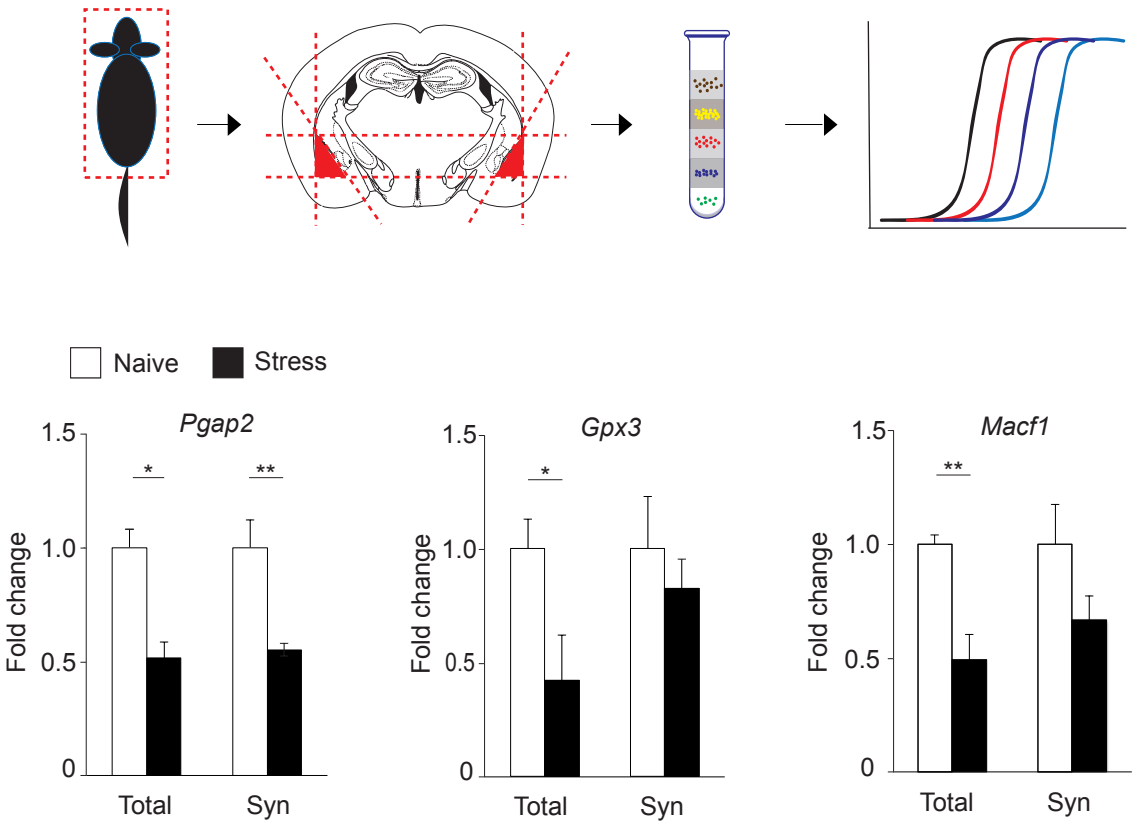

Figure 4.8 mir-483-5p targets are down-regulated in the amygdala after 6h restraint stress.

(Top) To investigate if the gene expression of *Pgap2*, *Gpx3* and *Macf1* were reduced in the amygdala after 6 hours restraint stress (6hRS), we assayed the mRNA levels of these three genes in total cell homogenate and synaptosomes made from the amygdala of mice exposed to 6hRS and compared against stress naive mice. (Bottom panels) Our results show that all of the three genes showed a significant reduction of mRNA levels in total homogenate following 6hRS. However, only *Pgap2* showed a significant reduction in both total and synaptosomal homogenate. Data are shown as fold change relative to stress naive mice. T.test was used to compare the means. *p<0.05, **p<0.01, ***p<0.001

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CHAPTER 5 miR-483-5p induced anxiolytic behaviour is mediated via *Pgap2*

5.1 Introduction to anxiety-like behaviours in animals

Anxiety is defined as “the prolonged state of tension, worry, and apprehension regarding uncertain and potentially negative future events” (Duval et al., 2015, p. 116). Our research uses rodent model of stress-induced anxiety like behaviours to understand the role of mir-483-5p and its targets in the amygdala. Firstly, it is crucial to differentiate anxiety as experienced by humans and ‘anxiety-like behaviours' in animals. Unavailability of methods to study the subjective aspects of anxiety in non-human animals makes the animal models of anxiety prone to errors and potential anthropomorphism. However, there is an overlap of certain characteristics associated with anxiety in all animals. These characteristics can be ethologically assayed to inform us about the anxiety profile of an organism at any given time. These include increased vigilance, freezing or hypo-activity, elevated physiological responses in heart rate & breathing and anhedonia, as seen by suppressed food consumption and reduction in motivation to explore novel objects or arena (Lezak, Missig, & Carlezon, 2017). Behavioural correlates of these characteristics can be observed in animal behaviour that can be considered as anxiety-like behaviours. In this thesis, when ‘anxiety' is used to refer to behaviour in animals, it refers to anxiety-like behaviours measured using one or more ‘anxiety tests' described in the following sections. Anxiety tests in animals exploit several of these ethological characteristics to quantify ‘anxiety'. One such behavioural characteristic is the approach-avoidant behaviours. Approach-avoidant behaviours exploit the animal's tendency to perceive an environmental stimulus as neutral or threatening. It also looks at the animals’ tendency to avoid circumstances that expose it to predation (Lezak et al., 2017). One of the other characteristics of rodents that can be exploited in these tests is

their natural inclination towards the dark and enclosed spaces (Bailey & Crawley, 2009; Crawley, 1981). These behaviours partly depend on baseline anxiety of the subject at the time. Several behavioural assays can be employed to assay the anxiety level of rodents. Next sections briefly describe some of these tests.

5.1.1 Common rodent anxiety tests

One of the most commonly used tests that exploit both of these characteristics is the elevated-plus maze (EPM) (Belzung & Griebel, 2001); see section 2.2 (p. 70) for more details. In this test, the animal is placed in the centre of the maze and number of entries to the open lit arms and time spent exploring the open arms are taken as ethological indicators of anxiety. 'Anxious' animals tend to enter less often or spend less time in the open arms relative to the closed or the dark arms (Lezak et al., 2017; Lister, 1987; Pellow & File, 1986). Novel object recognition test is another paradigm that can be used to measure the anxiety level of rodents. In this test, the subject is presented with a novel object in an arena. The latency to approach the object and the time spend with the object are used as the correlates of anxiety-like behaviour. Animals experiencing anxiety tend to take longer to approach a novel item and spend less time with the item. In another test, Light-Dark box test, the subject is placed inside a dark arena where an exit is available to enter the lit area. The latency of animals to enter the lit area and time spent in that area is measured. Relatively short latency to enter and increased time spent in the lit area is normally taken as an indication of anxiolytic behaviour. Social interaction test utilises the approach-avoidance behaviour in a social context (File & Hyde, 1978; File & Seth, 2003). In this test, a single subject is first placed in the arena, and the baseline activity is measured by timing the duration spent in a 'marked zone'. The subject is removed and another animal, a

‘social partner', is introduced to the marked zone, and then the target subject is reintroduced to the arena. After reintroduction, the latency to enter the marked zone, that now houses the social partner, and the duration spent in the zone is measured. Data are typically presented as relative to baseline measurements. Anxious animals tend to spend less time in the zone that has a social partner and take longer to approach the partner. Researchers have proposed this behaviour is reminiscent of the human tendency to avoid social contact while experiencing anxiety (File, 1985; File & Seth, 2003). However, the social interaction test has invited criticisms due to its inability to differentiate between the anxiety or the reward of the social interaction (Lezak et al., 2017). Nonetheless, experiments after administering anxiolytic drugs have shown that animals indeed spend more time with the social partner compared to the control animals that were not given the drug.

Another test that exploits the approach-avoidance behaviour of animals is the open field test (OFT). In this test, the animal is placed on a large open well-lit arena that has a centre zone. This test exploits the tendency of rodents to avoid well-lit open areas where they are most susceptible to predation. Animals tend to enter relatively less into the centre arena, and they spend the majority of the time close to the walls or away from the centre. More anxious animals tend to exhibit longer latency to enter the centre zone and spend less time in the centre compared to the normal subjects. OFT is also used to measure locomotive behaviour of the animals (Lezak et al., 2017). Assaying locomotion is essential to inform about the anxiety of any animals. If animals experience locomotive impediments due to the experimental intervention, this can potentially confound the data and misrepresent the anxiety profile of the animals.

Another useful test that is used to quantify anxiety is hyponeophagia test or novelty suppressed feeding. This test measures the tendency of rodents to avoid consuming novel food items. In this test, the animal is placed on an open field and presented with a novel and very palatable food item, such as sweetened condensed milk. The latency of the animals to approach and consume the food is measured and used as an indicator of anxiety (Deacon, 2011). This test can be altered slightly to measure a similar tendency. Instead of measuring the apprehension of animals to consume novel food in an open arena, latency of animals to consume familiar food in a novel arena can be measured. In this test, animals are first familiarised with a palatable food item before it is presented to the animal in a novel arena (Dulawa & Hen, 2005). One advantage of using this test is the lower inter-trial variation relative to other tests. Moreover, animals can be repeatedly measured by varying the test arena or food items used. Several important factors need to be considered when using these tests to measure anxiety-like behaviours. These tests can be highly sensitive to the test conditions like light levels, habituation and background noise which could influence the test and function as confounders (Bourin, 2015; Bourin et al., 2007; Lezak et al., 2017).

Another method of quantifying anxiety is by studying defensive behaviours, behaviours that are elicited by specific stimuli reminiscent of predation. These behaviours can be measured when a predator (rat, cat) is present (fear-related behaviours) or when cues (smell, sound) related to predators are present (anxiety-related behaviour) (Blanchard & Blanchard, 1989; Grillon, 2002; Lezak et al., 2017). Responses that are measured in these tests are the startling response (flinch-like behaviour) or freezing (suddenly extended

immobility). Acoustic startle test measures flinch like behaviour and freezing to a sudden burst of white noise. Studies have shown that administering CRF (Corticotropin releasing factor) to rodents elicits measurable differences in freezing and startle response (Y. Lee, Schulkin, & Davis, 1994; Risbrough et al., 2009). This is interesting, since CRH (Corticotropin releasing hormone), the human CRF equivalent, also promotes the stress response in humans. Startle response is considered an isomorphic behaviour because it can also be studied in humans. Measuring these endophenotypes is extremely useful since they allow relatively higher translational ability to humans and allow the preliminary testing of the efficacy and mechanisms behind anxiolytic drugs.

5.2 Methods of eliciting anxiety in rodents

5.2.1 Prenatal stress

In the ethological analysis of anxiety studies, it is essential to elicit anxiety in rodents unless basal anxiety is what is being measured. Several paradigms exist for this purpose, and some of these methods are used because of the similarities experienced by humans while some methods are species-specific environmental manipulations that result in severe stress response in animals. The nature of the stressor used could be developmental, chronic or acute. Developmental stressors could be further classified into prenatal or postnatal. In prenatal models of anxiety, stress is typically applied to the mother during gestation. This can have long-term enduring anxiety-like behaviours in the offspring. Prenatal stress has been shown to induce long-term devastating effects in human subjects as well as in rodents (De Weerth, Van Hees, & Buitelaar, 2003; Huizink, Robles De Medina, Mulder, Visser, & Buitelaar, 2002; Weinstock, 2008). In rodent models of prenatal

stress, the stressor is typically applied during the last week of the pregnancy (Lezak et al., 2017; Weinstock, 2008). Despite individual differences, consistent activation of the HPA axis, increased anxiety-like behaviours and reduced ability to experience pleasure are observed among the offspring (Weinstock, 2008). Human infants, where the mother has suffered from severe stress, display similar issues like increased irritability, hypersomnia and a higher incidence of affective disorders during childhood and adolescence (De Weerth et al., 2003; Huizink et al., 2002).

Another prenatal stress paradigm uses a maternal immune activation model. In this rodent model, an immune reaction is triggered in the gestating female by administering bacterial or viral mimetic. Studies have shown that offspring from these mothers experience anxiety-like behaviours (Babri, Doosti, & Salari, 2014; Estes & McAllister, 2016). This type of stress also has high translatability value since studies have recently shown that infection in human mothers makes infants more likely to experience stress and anxiety disorders (Murphy et al., 2017). A link between immune system response and stress-related disorders has been noted in animal studies and humans. Similarly, a stressful experience around the time of puberty has been shown to increase the likelihood of stress and anxiety disorders in mice (Giovanoli et al., 2013) and humans (Morrison, Narasimhan, Fein, & Bale, 2016). Genetic changes in the amygdala are partly responsible for the peri-puberty stress induced affective disorders (Tzanoulinou, Riccio, de Boer, & Sandi, 2014). Moreover, administering SSRIs prenatally can increase the incidence of anxiety in adulthood in humans and rodents (Glover & Clinton, 2016). Interestingly, like humans, rodents also show postpartum anxiety (Miller, Piasecki, & Lonstein, 2011). These observations

show that there are mechanistic overlaps in the neurology of anxiety-like behaviours in humans and other animals.

5.2.2 Postnatal stress

Another stress model that is commonly used is the postnatal stress paradigms. Exposure to severe postnatal trauma results in long-term neuropsychiatric conditions in both rodents and humans (Aas et al., 2016; Heim & Nemeroff, 2001; Lupien, McEwen, Gunnar, & Heim, 2009; Nemeroff, 2004; Teicher et al., 2003). One of the models used is the maternal separation model (Nishi, Horii-Hayashi, & Sasagawa, 2014). In this model, pubs are removed from the mother after birth for a predetermined duration (hours to weeks). These pubs typically show increased anxiety-like behaviour when they reach adulthood. Another feature of these animals is the hyper-excitability of the HPA axis (Nishi et al., 2014; Pryce, Rüedi-Bettschen, Dettling, & Feldon, 2002). Interestingly, the HPA responsiveness, measured by ACTH response to mild stress, depends on the time of removal of the pubs (Van Oers, De Kloet, & Levine, 1998). If pubs were removed at post-natal days (PND) 3-4, animals showed a marked increase in ACTH response at day 20. Interestingly, if pubs were removed at PND 11-12, they showed a significant reduction in ACTH response at day 20. In another model to study early life stress, pubs are left with the mother, but only impoverished caging conditions were provided. Suboptimal levels of resources are provided for nesting materials from PND 2-9 (Molet, Maras, Avishai-Eliner, & Baram, 2014). As a result, the maternal care received by the pubs is inadequate and results in anxiety-like behaviours in adulthood (Dalle Molle et al., 2012; Ivy, Brunson, Sandman, & Baram, 2008).

5.2.3 Stressors used on adult mice

Manipulation of the environment is one of the easiest ways to induce anxiety in rodents. The crux of the manipulations is to expose the animals to the stress of varying intensity and assay anxiety using one or more of the tests described earlier (section 5.1.1, p. 189). The exact nature of stress used is not necessarily translatable to stress experienced by humans. Nonetheless, by using species relevant stressors, manipulating the stressors, duration and intensity it is possible to model some aspects of the stress and concomitant neurobiological changes in the rodent brain. The type of stressors used in rodent models of anxiety research includes social defeat, foot-shock, forced swim, restraint, elevated pedestal, sleep deprivation, home cage disturbances, irregular cage disruption, predator odour etc. Combinations of the above stressors with varying intensity have been used to elicit anxiety.

When the stressors applied are relatively mild and administered over a period of weeks, it is considered chronic mild stress. This could be the same stressor or a series of different stressors over time (chronic unpredictable stress). Chronic unpredictable stress is performed over 10-21 days, but the stressor used is randomised so that the animals do not habituate to the stress process (Pêgo et al., 2008; Ajai Vyas et al., 2002; Wilson, Grillo, Fadel, & Reagan, 2015). One encouraging feature of this paradigm is the similarity with daily stressors experienced by human subjects. While earlier studies have doubted the inter-lab reproducibility of chronic stress paradigms (Willner, 2005, 2017), recent meta-analysis has shown that chronic stress is a consistent and robust way to elicit depressive states in rodents, and the heterogeneity observed in the literature is due to the variations, such as diurnal and gender differences, within the protocol

applied (Antoniuk, Bijata, Ponimaskin, & Wlodarczyk, 2019). Furthermore, some level of heterogeneity in effects is expected among individual animals since humans also show significant personal differences in stress resiliency and predisposition (Matar, Zohar, & Cohen, 2013).

In another chronic stress model, chronic immobilisation stress, animals are immobilised using straps for 2hrs over ten consecutive days (Ajai Vyas et al., 2002). Other Common stressors used in this paradigm are cage tilt, changing cage mates, overnight illumination and restraint stress. Administering stressors over the weeks are sufficient to trigger enduring anxiety and depression. One classical characteristic of animals treated this way is anhedonia, and these effects seem to be alleviated by the administration of antidepressants (Krishnan et al., 2007; Lezak et al., 2017; Willner, 2017). A variant of chronic stress is social instability stress. In this test, subjects' cages mates are changed every day for a pre-determined number of days (Tsai et al., 2014). Regularly changing cage mates induce social instability potentially due to the lack of consistent hierarchy and from experiencing physical threat from the new cage mates that already have an established hierarchy.

Another variant of the chronic stress model is the chronic social defeat test. In this test, the subject is exposed to an aggressive intruder for 10 minutes per day for a few days/weeks. As a result, the subject will start to show anxiety-like behaviours and depression-like symptoms. The duration of the psychological effects after the removal of mice can vary from individuals. Similar to humans, some subjects recover quicker than others (Donahue, Muschamp, Russo, Nestler, & Carlezon, 2014). One critique of the neuroscientific study of behaviours using animals is inter-subject variability and gender variability. For example,

aggressive behaviours by the intruder have less effect on females (see the limitations section in Chapter 6, p. 237).

5.2.4 Single stress paradigms

In the above examples of chronic stress, stressors are administered over several days. However, other paradigms exist where a stressor is applied from minutes to a few days. These stresses could be classified under acute stress models; however, scientists have used the exact name of the stress to describe these models instead generally calling it acute stressors. One of the models that involve single stress is the single prolonged stress paradigm (SPS). This was first developed to study PTSD (Yamamoto et al., 2009). In this model, animals are exposed to sequences of severe stressors that include restraint stress, forced swim and electric shock. Behaviours are measured days after the exposure to a stressor. These animals after the exposure to stress display signs of several neuropsychiatric disorders and impaired learning (Lezak et al., 2017).

In another model, single unpredictable prolonged stress is performed for a day, but the animals are exposed to a variety of stressors one after the other followed by a recovery period before behavioural assay (Wilson et al., 2015). This is considered to be closer to the heterogeneity of stressors experienced by humans in their daily life. Another model that is commonly used is the restraint stress or immobilisation stress model (Campos, Fogaça, Aguiar, & Guimarães, 2013). It can be applied for five minutes to six hours for one day or up to 21 days (Bennur et al., 2007; Mucha et al., 2011; Skrzypiec et al., 2013; Wilson et al., 2015). Studies have shown that these models reproducibly produce heightened anxiety-like behaviours in rodents (Padovan & Guimarães, 2000). Unfortunately, there is a lack of concordance within the neuroscientific community about the

duration of restraint session. Researchers have used various time points that are not necessarily comparable. In our experiments, we have used 6hRS since this method was sufficient to observe changes in anxiety-related behaviours and morphological changes in the amygdala (Attwood et al., 2011; Skrzypiec et al., 2013).

Chapters three and four reports the identification of mir-483-5p as one of the highly up-regulated miR in response to 6hRS. mir-483-5p is also up-regulated in a compartmentalised manner, relatively more expressed in synaptosomes vs cytosolic fractions. Moreover, mir-483-5p is demonstrated to be correlated with altered spine dynamics in primary neuronal cultures. Using a bioinformatics and data mining approach, we identified few targets of mir-483-5p, three of which, *Pgap2*, *Gpx3* and *Macf1*, are directly targeted by mir-483-5p. These targets are also down-regulated in the amygdala following 6hRS where *Pgap2* shows further down-regulation in synaptosomal fractions. The purpose of this chapter is to present evidence from experiments that were designed to study the phenotypic effects of mir-483-5p overexpression in rodent anxiety-like behaviours. Moreover, this chapter will consider *Pgap2* as a likely candidate for the regulation of behaviour through mir-483-5p. This is mainly because *Pgap2* is the only confirmed target of mir-483-5p that showed a reduction in its mRNA levels in synaptosomes and total fractions. Therefore, the effect of *Pgap2* is more likely to be involved in mechanisms that are global and also in synaptosomes. Moreover, the role of *Pgap2* or GPI anchoring was not explored in a behavioural context before. We hypothesise that given the significant up-regulation of mir-483-5p in the amygdala, it is likely that changes in anxiety-like behaviours will be observed in the animals after the lentiviral induced up-regulation of mir-483-5p. Moreover,

this effect can be recapitulated by down-regulating one of its targets, *Pgap2* using shRNA knockdown. Testing these hypotheses is the primary aim of the experiments described in this chapter.

5.3 Results

5.3.1 Lentiviral particles; synthesis and validation

In order to test if anxiolytic behaviours can be mediated via the interaction with mir-483-5p or *Pgap2*, it is essential to manipulate the levels of mir-483-5p or *Pgap2* in BLA and assay the anxiety-like behaviours in mice. To test the role of mir-483-5p, we used lentiviral particles to overexpress mir-483-5p or a control sequence in the amygdala. Similarly, we used shRNA-mediated knockdown of *Pgap2* on a separate cohort of mice to explore if the effect from mir-483-5p could be recapitulated. A mir-483-5p overexpressing virus is synthesised in the lab (section 2.13.1, p. 82) while *Pgap2* shRNA virus was commercially obtained. The titre of mir-483-5p lentiviral particles are estimated to be $9.9x10^7$ particles/ml. The company reported the concentration of shRNA particle to be $1.07x10^7$ IFU/ml. Cell cultures were transduced either with mir-483-5p lentiviral particles or *Pgap2* shRNA lentiviral particles, and the RNA levels were quantified and compared against relevant controls. Our results showed that transduction using mir-483-5p overexpressing lentiviral particles resulted in close to 3 times (277% ±88) increase in mir-483-5p levels ($t(4) = 2.95$, $p<0.05$, Figure 5.1, p. 209) compared to the controls. *In vitro* transduction with *Pgap2* shRNA lentiviral particles produced ~60% reduction (38% ±7) in *Pgap2* levels ($t(3) = -5.8$, $p<0.01$, Figure 5.2, p. 211). Our experiments showed that the lentiviral particles are able to overexpress mir-483-5p or down-regulate *Pgap2 in vitro*. Next, we bilaterally injected these particles into the BLA of mice and then studied the resulting behavioural changes.

5.3.2 Anxiolytic effect of bilateral stereotaxic injection

Our experiments hitherto showed that mir-483-5p is up-regulated in the BLA upon stress. We have also identified *Pgap2* as its target molecule down-regulated in the BLA. Additionally, luciferase experiments have shown a direct interaction of mir-483-5p at the predicted seed regions in the 3'UTR of *Pgap2* mRNA. Moreover, overexpression of mir-483-5p in primary neuronal cultures resulted in structural changes in dendritic arbour and spines morphology that is reminiscent of anxiolysis (Chapter 3). These data lead us to suspect if the up-regulation of mir-483-5p could produce any measurable changes in anxiety like behaviour in the animals. Moreover, because *Pgap2* is a target of mir-483-5p and *Pgap2* is down-regulated in cellular compartments where mir-483-5p is up-regulated, shRNA induced down-regulation of *Pgap2* in BLA could also have a similar behavioural effect on animals. In order to test this, we made lentiviral particles to overexpress mir-483-5p or down-regulate *Pgap2* using shRNA. These particles (total volume of 1.5 µl per injection) were injected bilaterally into the basolateral amygdala of mice, Figure 5.3, p. 213 (see the section 2.13, p. 81, for more details). Following the injection animals were left to recover for three weeks before starting the behavioural experiments.

After the behavioural experiments, animals were sacrificed, and brains were extracted and fixed in PFA. Using GFP antibody bound to a fluorophore, we examined the injection sites of the animals. Animals that showed GFP signal in at least one BLA were included in the data analysis (Figure 5.4, p. 215 & Figure 5.7, p. 221). We found that when mice were injected with mir-483-5p overexpressing lentiviral particles, they entered more often to the open arms of the EPM ($t(12.66) = -4.07$, $p < 0.01$, Figure 5.5, p. 217), suggesting an anxiolytic

effect when compared against the animals injected with scramble expressing lentiviral particles. Total number of entries (Figure 5.5, p. 217), total distance travelled, and the speed of the animals (Figure 5.6, p. 219) were not different between the groups. Corroborating with our hypothesis, when animals were injected with *Pgap2* shRNA lentiviral particles, we observed that animals entered the open arms of the maze more frequently ($t(10.2) = -3.23$, $p<0.01$, Figure 5.8, p. 223). In scramble conditions and *Pgap2* shRNA conditions, the total number of entries, total distance and the speed was unaffected (Figure 5.9, p. 225). These results show that when animals were injected with mir-483-5p or *Pgap2* shRNA lentiviral particles, they exhibited anxiolytic effect. Our experiments show that mir-483-5p overexpression or down-regulation of *Pgap2* shRNA has an anxiolytic effect on animals when assayed using EPM. Based on the experiments mentioned in the previous chapter and this data, we hypothesise that the anxiolytic effect of mir-483-5p could be mediated, at least partially, by down-regulating *Pgap2*.

5.4 **Discussion**

Several methods are available to study the role of specific genes or microRNAs in specific tissue types. Previous studies have used lentiviral injections to specific areas of the brain and quantified the resulting complex behaviours (Barbash, Hanin, & Soreq, 2013; Haramati et al., 2011; Issler et al., 2014; Scott et al., 2015). These studies have already provided the proof of concept for using lentiviral technology to understand ethological effects of specific gene products or lack thereof. In our experiment, we used lentiviral particles to either overexpress mir-483-5p or knock down one of its targets, *Pgap2,* in mice BLA. Before the *in vivo* injections, the lentiviral particles were used to transduce cell lines and the resulting overexpression of mir-483-5p or reduction in *Pgap2* mRNA was confirmed. Our results, Figure 5.1 (p. 209), showed that these lentiviral particles were sufficient to produce an increased expression of mir-483-5p or a reduction of *Pgap2* mRNA (Figure 5.2, p. 211). Our previous experiments have shown that exposing mice to 6hRS can induce measurable anxiety-like behaviours. Additionally, our data also shows that mir-483-5p is significantly up-regulated in the amygdala. Moreover, when mir-483-5p is overexpressed in the primary neuronal cultures, the resulting changes in the spine dynamics were reminiscent of anxiolytic features.

When animals were bilaterally injected with mir-483-5p overexpressing lentiviral particles, an increase in open arm entries and duration spent in open arms were observed. The total number of entries was unaffected showing that both groups of animals showed similar exploratory behaviour. Similarly, the total distance travelled, and speed was also similar showing the locomotion is unaffected. Increased open arm preference in rodents treated with anxiolytics is

taken as an indicator of anxiolytic properties. Similarly, animals overexpressed with mir-483-5p in the amygdala showed anxiolytic behaviour. However, the exact mechanisms through which this effect is mediated require further studies. The pathway responsible for this effect is likely to involve the regulation of *Pgap2*, see sections below. Previous studies have reported the stress-buffering effects of miRs, and their role in adaptive measures against stress has been proposed (Mannironi et al., 2017, 2013; O'Connor et al., 2012).

Our previous experiments have shown that mice exposed to 6hRS exhibit increased anxiety-like behaviours. This poses another interesting question. If mir-483-5p overexpression induces anxiolytic behaviour and if mir-483-5p is significantly up-regulated in the amygdala following 6hRS, then why do the animals exposed to 6hRS show anxiety-like behaviours? One reason for this could be due to the time point when these measurements were taken. mir-483-5p levels were extracted soon after the termination of 6hRS while the behaviour of animals was measured the day following 6hRS. Moreover, mir-483-5p is only one of the miR that is up-regulated among 100s of other miRs, mRNA and proteins (O'Connor et al., 2012; Scott et al., 2015; Skrzypiec et al., 2013). Therefore, mir-483-5p alone might not be sufficient to compensate the entire adverse effects of 6hRS or the expression of mir-483-5p might be temporally restricted and the development of anxiety-like behaviours are triggered overnight or 24hrs after stress. This is in line with our behavioural data that shows mir-483-5p overexpression induced anxiolytic behaviours in mice, in the absence of severe stress. One useful analogy to explain the role of mir-483-5p is the production of endogenous opioids in response to pain. Opioids are produced in response to pain; however, their function is to relieve pain. During severe injuries,

the endogenous opioids production become insufficient to deal with the pain from the injury. Similarly, mir-483-5p could be transcribed in response to severe stress as a way to deal with the negative consequences of the stress, but the expression of mir-483-5p in response to stress at the biological level might not be sufficient to overcome all of the consequences of stress. Further experiments to measure anxiety-like behaviour after the administration of 6hRS post overexpression of mir-483-5p will shed more light into the efficacy of mir-483-5p even in the presence of stress.

Another question that warrants discussion is about the mechanisms responsible for the observed behavioural effects. From our data, it is difficult to unequivocally state if mir-483-5p is involved in the maintenance of homeostasis of amygdala architecture after 6hRS or in the reversal of stress-induced changes. Altering the anxiety and changing the spine dynamics might seem to suggest the latter role. However, it is also possible that in the absence of aversive stimuli the mir-483-5p overexpression is uncompensated for and as a result, the anxiolytic behaviours and neuronal morphology was surfaced. Further experiments are required to analyse whether mir-483-5p is important for homeostasis maintenance or has an active role in the reversal of anxiety-like behaviours. Interestingly, in another study when DICER is knocked down in the amygdala, an increase in anxiety-like behaviours was observed in mice (Haramati et al., 2011). Lack of DICER results in a global reduction of miR induced translational silencing and this resulted in anxiety-like behaviour. This shows the overall function of miRs could be in the maintenance of neuronal functions. Contrastingly, some studies have shown that certain miRs are significantly down-regulated following 2hRS (Mannironi et al., 2017, 2013). How then does global knockdown of DICER

leading to anxiety-like behaviours require further research? Interestingly other studies have also shown that overexpressing certain miRs in the CeA can be anxiolytic (Haramati et al., 2011).

5.4.1 Potential role of *Pgap2* in anxiolysis

Previous chapters report that *Pgap2* is one of the targets of mir-483-5p and the level of *Pgap2* is down-regulated in the total and synaptosomal amygdala homogenate following 6hRS. Moreover, when *Pgap2* is knocked down in the amygdala, mice exhibited anxiolytic behaviour. This behaviour was recapitulated by the overexpression of mir-483-5p in the amygdala. Taken together, our data suggest that the behavioural effects induced by mir-483-5p could be mediated through *Pgap2*. Exactly, how this is done is not clear and required further study. Some possible pathways are suggested in chapters 4 and 6.

Pgap2 is involved in the addition of GPI anchoring to the proteins, a crucial step required for the successful translocation of protein to membrane lipids rafts (Tashima, 2005). Several proteins that are involved in the neuronal maintenance and synaptic plasticity are GPI anchored. These proteins include proteases, adhesion molecules, membrane receptors and protein involved in signal transduction (Ikezawa, 2002; Tsui-Pierchala, Encinas, Milbrandt, & Johnson, 2002). By temporally controlling the down-regulation of *Pgap2* by mir-483-5p, it is possible to transiently attenuate the additions of GPI anchored proteins to the membrane rafts. This, in theory, could result in the significant changes in the lipid rafts dynamics by disproportionally affecting specific receptors. Moreover, the selective down-regulation of *Pgap2* in synaptosomes suggests that this mechanism could undergo targeted spatial regulation at extrasynaptic regions.

5.5 Conclusion

Previous studies have shown some miRs involved in anxiety-like behaviours (Haramati et al., 2011; Mannironi et al., 2017). However, these miRs were affecting proteins and pathways that were already implicated in stress response. In this chapter we have seen, for the first time, that overexpression of a miR, miR-483-5p, in BLA is sufficient to influence anxiety-like behaviour in mice. Moreover, knockdown of one of its targets, *Pgap2*, recapitulates the behavioural pattern suggesting that the behavioural changes could be via the regulation of *Pgap2*. This study adds mir-483-5p to the repertoire of miRs that can regulate anxiety-like behaviours and a novel gene, *Pgap2*, which can function on its own to produce a measurable effect in anxiety-like behaviours. Moreover, this thesis reports the plausible identification of a novel pathway, mir-483-5p induced regulation of GPI anchoring by spatially biased down-regulation of *Pgap2*, that could be important in the effective adaptive measures against stress-induced anxiety-like behaviours.

Our research warrants further investigation into GPI anchoring mechanisms in order to understand anxiety disorders. Moreover, our study also shows that this approach to identifying genes and miRs in anxiety will be beneficial for the identification of several previously unidentified genes that form a functional network to orchestrate these changes. Furthermore, the importance of incorporating the functions of miRs to understand the complex phenomenon like anxiety behaviours is emphasised. Such an approach is likely to positively drive our discoveries and open the scientific community to novel therapeutic interventions in response to stress and anxiety disorders.

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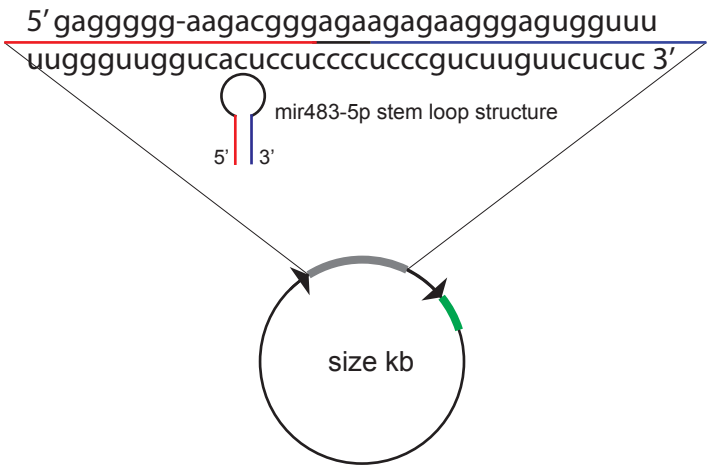

pLenti 5'-EF1ap-mmu-mir-483

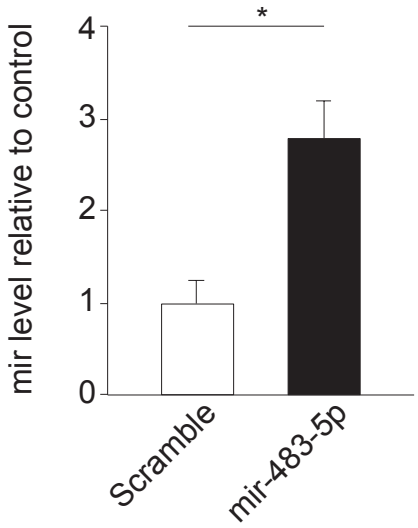

Figure 5.1 Validation of lentiviral particles to over-express mir-483-5p
mir-483-5p sequence or a scramble sequence was used to produce lentiviral particles, pLenti-UbC-eGFP-mmu-mir-483 where the mir-483-5p insert was placed upstream of an enhanced GFP sequence and UbC promoter (top). Lentiviral particles were used to transduce N2a cells. Total RNA was isolated and qRT-PCR was performed. Transduction resulted in 3-fold increase when compared against the control ($p<0.05$, $n=5$) (right). Data are shown as fold change relative to control. *$p<0.05$

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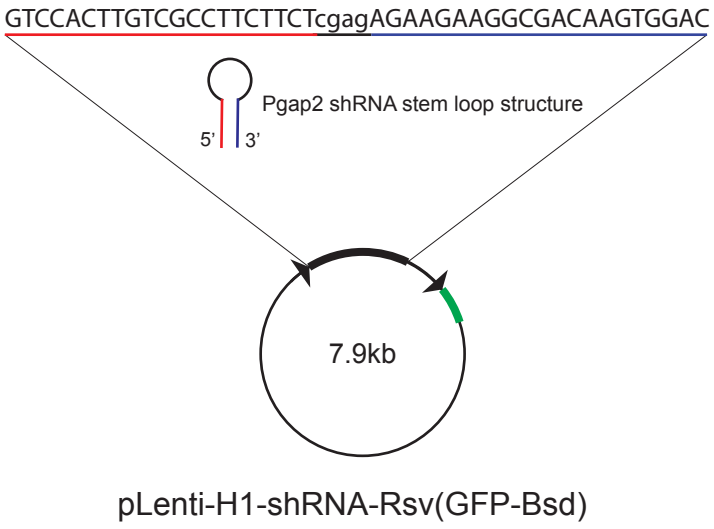

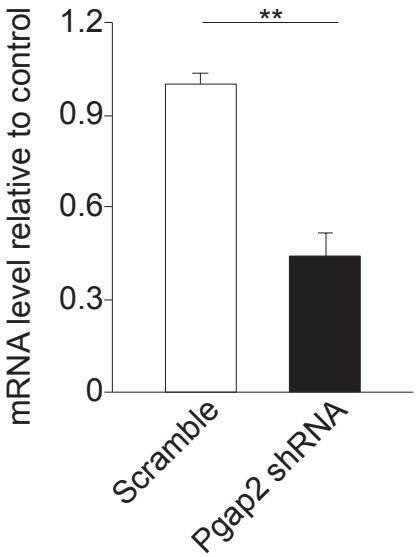

Figure 5.2 Validation of lentiviral particles to knockdown *Pgap2*
(Top) Lentiviral particle, pLenti-H1-shRNA-Rsv(GFP-Bsd), was used to knockdown *Pgap2* were purchased from AMSBIO. Expression of shRNA sequences was driven using H1 promoter upstream of the sequence. GFP-Bsd were used as markers. (Bottom) Transduction in HEK cells with the shRNA lenti particle resulted in ~60% reduction on *Pgap2* mRNA cells, quantified using qRT-PCR ($p<0.01$, $n=5$ per group). Data are shown as fold change relative to the control.

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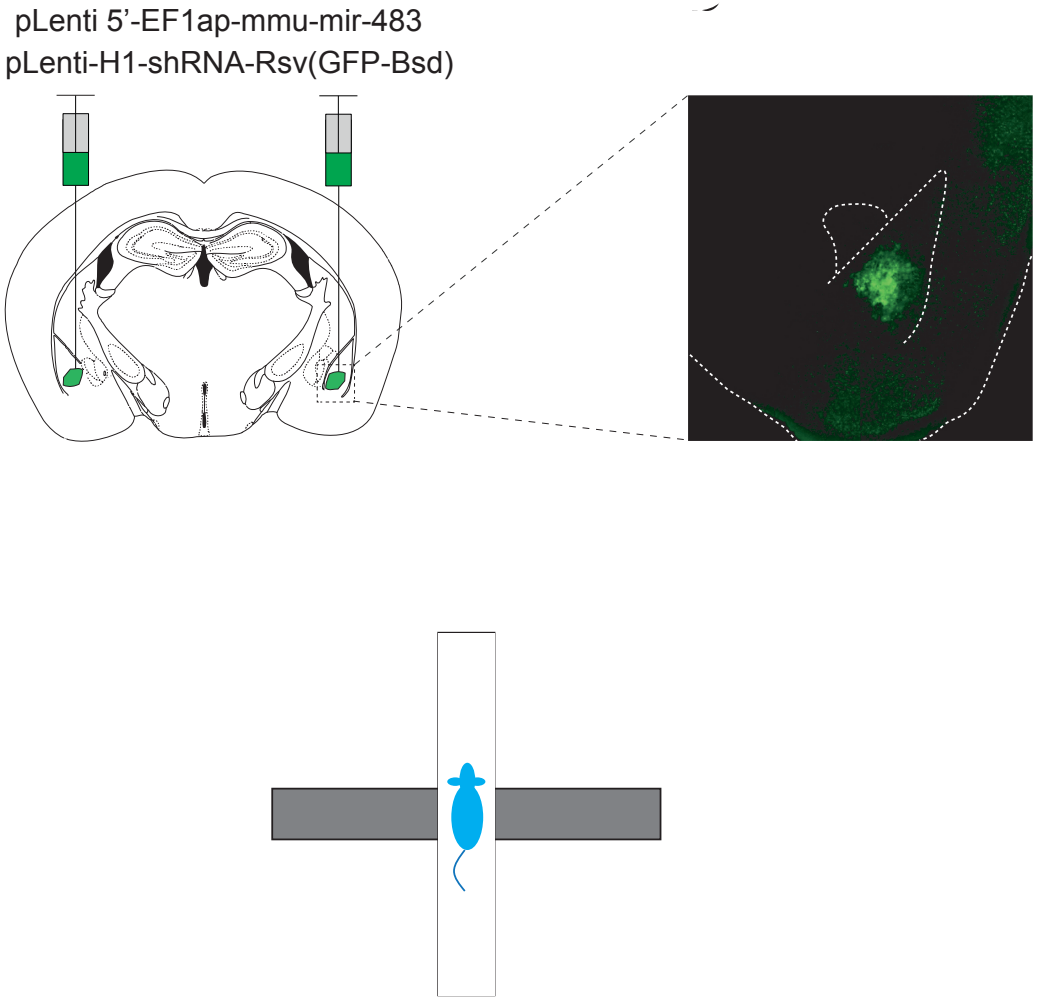

Figure 5.3 Bilateral injection of lentiviral particles to the amygdala
(Top left) After the validation (Figures 5.1 & 5.2) lentiviral particles were bilaterally injected to the mice amygdala. At the end of the experiments, animals were sacrificed and brain extracted. The anti-GFP-conjugated antibody was used to verify the injection sites for all injections (top right). See figures 5.4 & 5.7 to see the injection sites for all the animals used in the data analysis. Animals were given three weeks after the surgery to recover. Following the recovery, anxiety-like behaviours were quantified using the Elevated Plus-maze test (EPM) (bottom).

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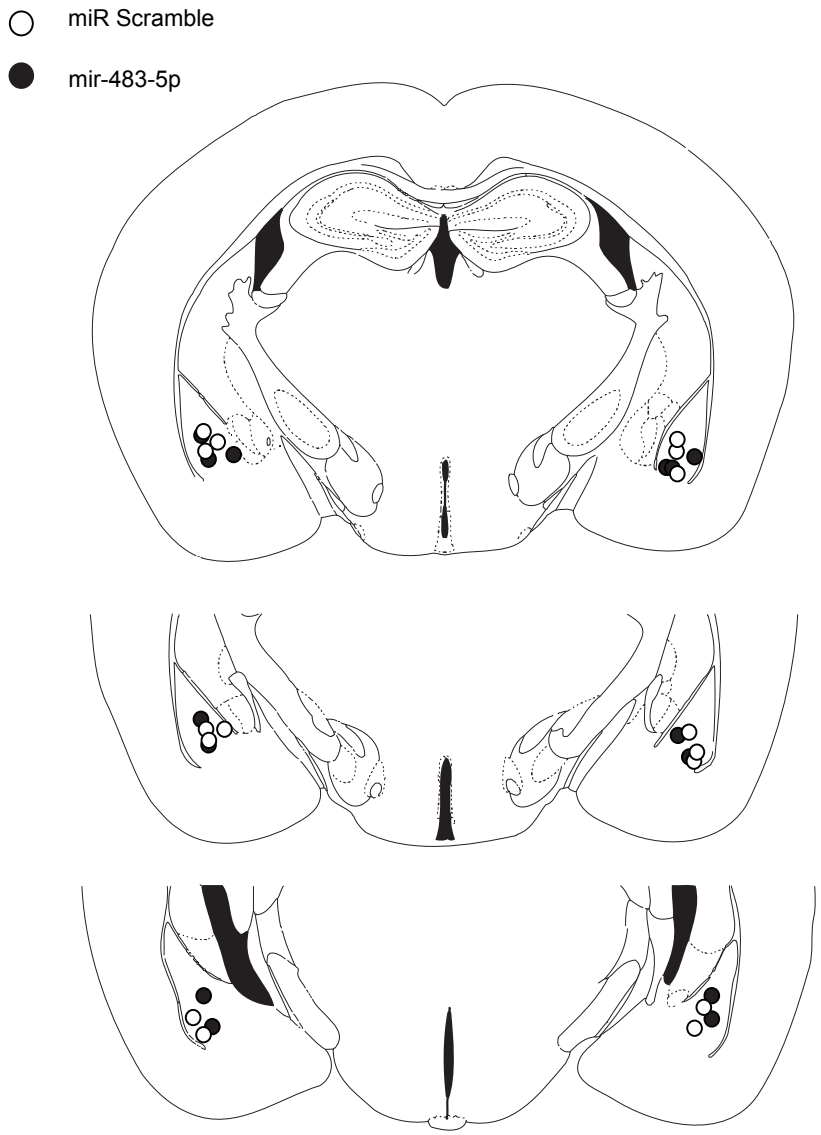

Figure 5.4 validation of the injection sites of mir-483-5p over-expressing lentivirus particles
After the injection of lentiviral particles to over-express mir-483-5p animals were given three weeks to recover. After ensuring complete recovery, subjects were undergone behavioural analysis in EPM. Following behavioural experiments, animals were sacrificed, and the whole brain was extracted after perfusion with PFA to fix the tissue. After an additional overnight fixing in PFA brain sections were cut using a vibratome. Sections then underwent immunohistochemistry with fluorescently tagged anti-eGFP. The sections were visualised using a fluorescent microscope and markings were made on the brain outline. The white circles show the areas of control injections and the dark circles shows the mir-483-5p injection sites identified by GFP signal.

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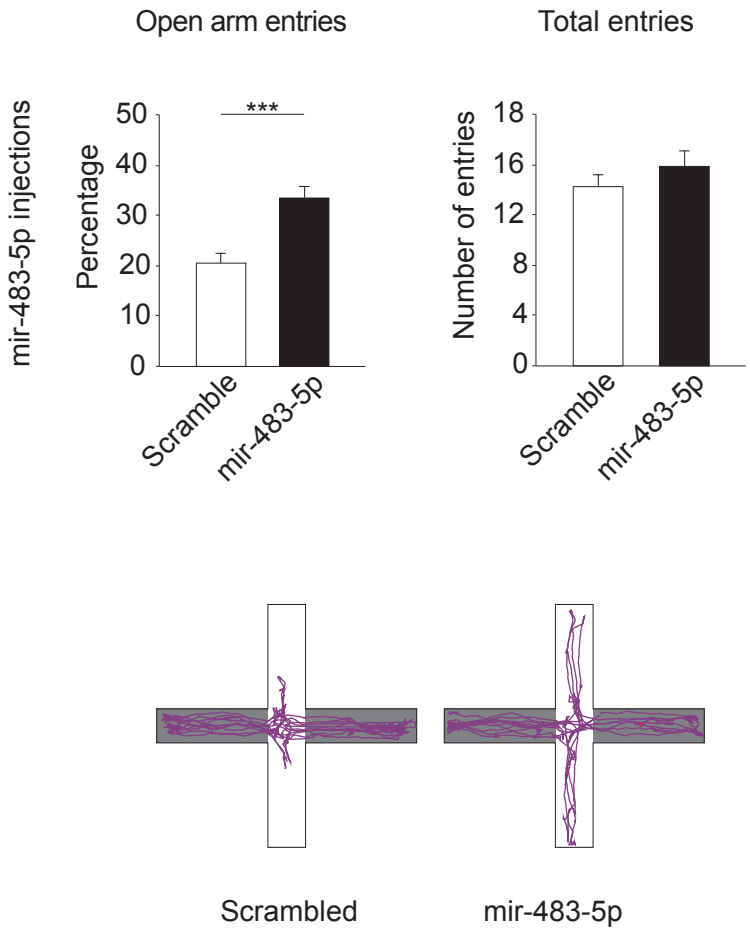

Figure 5.5 mir-483-5p over expression in the amygdala has anxiolytic effect on mice

mir-483-5p is over expressed bilaterally in the amygdala using lentivirus over-expression system. After the stereotaxic injection of lentiviral particles into the amygdala, subjects were left to recover for three weeks. Following recovery, Elevated Plus-maze test was performed to profile the anxiety-like behaviours. Animals injected with mir-483-5p over-expressing lentiviral particles demonstrated anxiolytic behaviour by entering more often to the open arms (top left) ($p<0.001$, n=7-8 per group). Data are shown as the percentage of open arm entries. (top right) The total number of entries were unaffected showing that both groups of mice showed similar exploratory activity. Data are shown as the total number of entries. (bottom) Shows representative track plots of the path of the subjects during the 5 minutes of EPM activity. Data presented as mean±SEM. *$p<0.05$, **$p<0.01$

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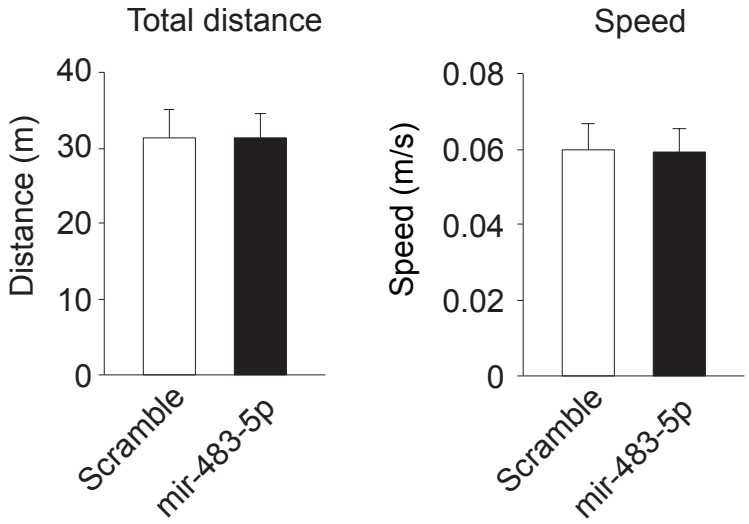

Figure 5.6 Lentiviral over-expression of mir-483-5p in the mice amygdala did not affect locomotor behaviour.
Three weeks after the surgery the behaviours of animals were assayed. In order to check if the locomotor behaviour is affected, animals were placed on an open arena and their activity was assayed semiautomatically using AnyMaze. Total distance travelled in meters (left) and the speed in meters per second (right) were analysed. The result shows that both groups of animals did not show any observable difference in these two parameters showing that their locomotion was not affected. T. Test was used to compare the means of both groups. $p>0.05$, n=7-8 per group. Data presented as mean±SEM.

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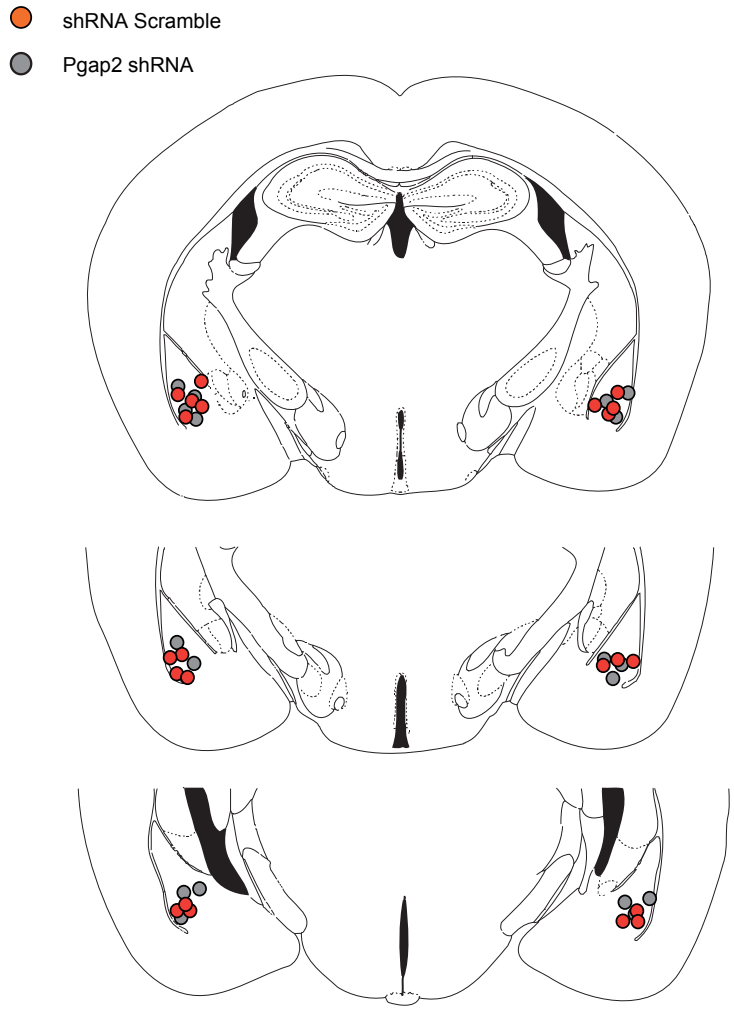

Figure 5.7 Validation of injection sites of *Pgap2* shRNA lentivirus particles
Following the injection of lentiviral particles to knock-down *Pgap2* mRNA, animals were given three weeks to recover. Subsequently, subjects were undergone behavioural analysis in EPM and in an open field to analyse locomotive behaviour. After behavioural experiments, animals were sacrificed, and the brains were extracted. Brain tissues were fixed in PFA overnight and cut using a vibratome. Immunohistochemistry with fluorescently tagged anti-eGFP was used to analyse the injection sites. The sections were visualised using a fluorescent microscope and markings were made on the brain outline. The orange circles show the areas of control injections and the grey circles shows the mir-483-5p injection sites identified by GFP signal.

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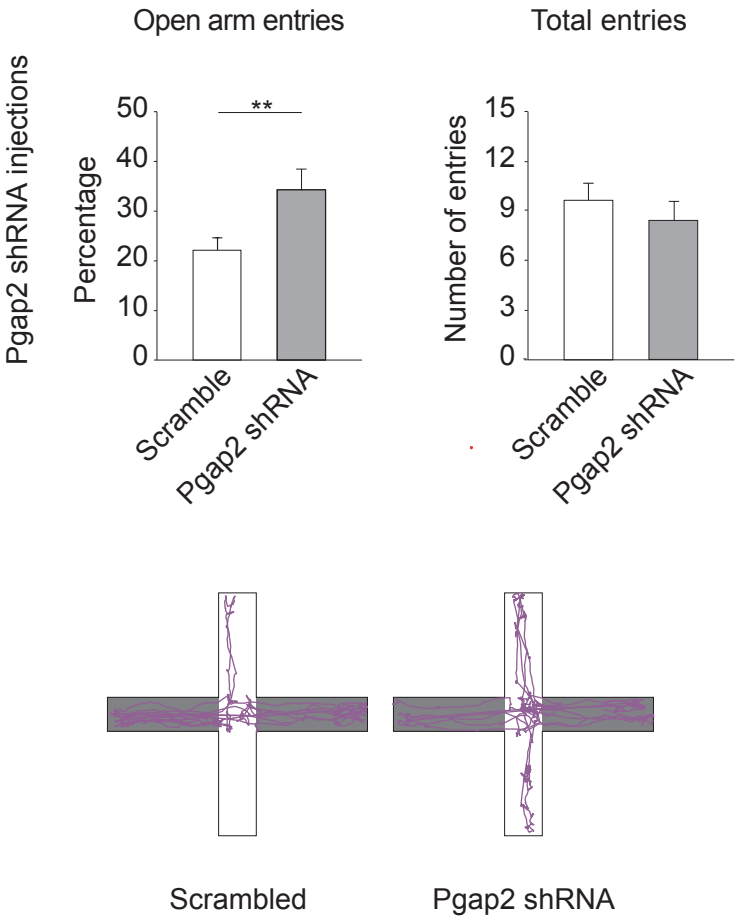

Figure 5.8 shRNA induced knock down of *Pgap2* in the amygdala has an anxiolytic effect on mice

Lentiviral particles purchased from AMSBIO were used to express lentiviral particles to knock down *Pgap2* shRNA. After three weeks of post-surgery recovery time, the anxiety-like behaviours of animals were measured using EPM. Percentage of entries to open arms are shown in top left panel. When shRNA was knocked down in the amygdala, animals enter more often to the open arms demonstrating anxiolytic behaviour ($p<0.001$, n=10-12 per group). The total number of entries for both groups were unaffected showing similar explorative behaviour in both conditions (top right). (Bottom panels) Shows representative track plots of animals activity during a 5 minutes test session. Data are shown as mean±SEM. *$p<0.05$, **$p<0.01$

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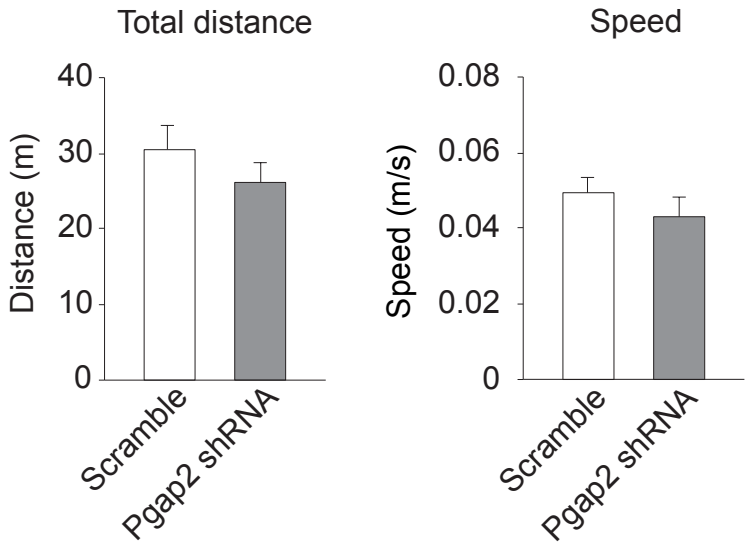

Figure 5.9 shRNA knockdown of *Pgap2* in the mice amygdala does not affect locomotor behaviour.
Lentiviral particles to knock-down *Pgap2* shRNA or a scramble control sequence were bilaterally injected to the amygdala of adult mice. Three weeks after the surgery the anxiety profile of animals was measured using EPM (Fig 5.7). Following the behavioural assay, animals were placed in an open arena to measure their locomotive activity. Distance in meters (left) and speed in meters per second (right) were analysed. None of these parameters showed any difference in both groups. T.test was used to compare the means of both groups. $p>0.05$, n-10-12 per group. Data are shown as mean±SEM.

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CHAPTER 6 Conclusion, future directions and limitations

6.1 What do our experiments show?

miRs have been termed as 'micro-controllers' and 'drivers' of evolutionary adaptations and complex physiological, biochemical and behavioural manifestations. Since their discovery two decades ago, thousands of studies have reported the importance of miRs in diverse processes including complex animal and human behaviours. Several studies and the experiments described in this thesis show that even one single miR can significantly affect complex behaviours like sleep-wake regulation, substance abuse and addiction, alcohol tolerance, anxiety and many more (O'Connor et al., 2012). Knockdown of two miRs, miR-132 and miR-219, in the SCN of mice significantly affected the circadian rhythm of mice (Cheng et al., 2007). Alcohol is shown to induce miR-9 expression which targets the BK channel (Ca^{2+} activated K^{+} channel) (Pietrzykowski et al., 2008). It is hypothesised that alcohol tolerance is partly developed through miR-9 regulated mechanisms. Interestingly, mir-9 was also shown to be involved in protection against synaptic toxicity (Chang et al., 2014) and control of dendritic development by targeting REST (Restrictive element-1 silencing transcription factor), a transcriptional repressor involved in neurogenesis, neuronal differentiation and synaptic plasticity (Giusti et al., 2014; Hwang & Zukin, 2018). Furthermore, studies have unveiled various roles of miRs in several psychiatric conditions, section 1.9. Table 1.1 (p. 51) shows selected examples of hitherto identified miRs in some psychiatric conditions and animal models. One such condition that contributes significantly to the world diseases burden is stress and anxiety disorders (Bandelow & Michaelis, 2015). Animal models are commonly used to study several aspects of these disorders. In our research, we have used mice model of anxiety to understand more about the

changes in miRs that take place in the amygdala following a stress paradigm. The following section lists the conclusions from each chapter and suggests novel pathways that are involved in anxiolytic behaviour in mice.

Firstly, miR microarray from amygdalar homogenate from mice that were exposed to 6hRS was performed (Dr Satyam Pate's doctoral thesis). We identified five miRs that were significantly up-regulated. One of these up-regulated miR that showed the highest increase is mir-483-5p (Figure 3.1, p. 123). In order to understand if mir-483-5p undergoes spatial bias in its localisation, we isolated total RNA from cytosolic fractions and synaptosomal fractions. We found that mir-483-5p is enriched in synaptosomal fractions and the levels of mir-483-5p are significantly up-regulated selectively in synaptosomes following 6hRS (Figure 3.2, p. 125).

Based on the above we reasoned, that mir-483-5p might be involved in the functions within, but not limited to, juxta-synaptic compartments. Previous research has reported changes in dendritic arbour and spine dynamics following 6hRS. In order to address this, we investigated the role of mir-483-5p in dendritic plasticity by overexpressing mir-483-5p in primary neuronal cultures made from P1 mice amygdala. Sholl analysis revealed that distal regions of the neurons overexpressed with mir-483-5p showed reduced complexity of distal dendritic arbour (Figure 3.3, p. 127).

When the proportions and types of dendritic spines were analysed, we saw that mir-483-5p-overexpressing neurons did not have significantly different density of spines (Figure 3.5, p. 131). However, these neurons showed a significant difference in the type of spines (Figure 3.4, p. 129). In neurons where mir-483-5p was over-expressed, an increase in mushroom-like spines and a

decrease in filopodia-like protrusions was observed. Mushroom spines are considered to be mature and more likely to form synapses, while filopodia-like protrusions are considered to be immature and less likely to form synapses. This pattern of morphological change in spine proportions is reminiscent of anxiolysis. This indicated that mir-483-5p could have an anxiolytic effect. We next sought to understand the targets of mir-483-5p. Using bioinformatics and data mining approach, we identified targets of mir-483-5p that are expressed in amygdala and respond to stress (Figure 4.2, p. 173). We identified 12 putative targets for mir-483-5p.

In order to understand if these targets are indeed down-regulated by mir-483-5p in a biological system, we overexpressed mir-483-5p in N2a cells and quantified the mRNA levels of each target in the presence of stress-mimicking synthetic hormone, dexamethasone (Figure 4.3, p. 175 & Figure 4.4, p. 177). We saw that three out of the 12 targets, *Pgap2*, *Gpx3* and *Macf1* showed significant down-regulation following overexpression of mir-483-5p (Figure 4.3, p. 175). These three targets (*Gpx3, Macf1* and *Pgap2*) were subsequently studied. DualGlo luciferase assay was performed with and without mutation in the predicted 3'UTR seed sites. This assay showed that the above targets were indeed down-regulated by mir-483-5p through its interaction at the predicted seed sites (Figure 4.5, p. 179 & Figure 4.6, p. 181). Furthermore, using multi-label immunostaining of brain section of WT mice, we have shown that the three targets of mir-483-5p is expressed in the BLA neurons, Figure 4.7 (p. 183). The pattern of expression showed that the proteins are expressed in cell membrane rather than diffused global expression.

Following the confirmation of the *Pgap2*, *Gpx3* and *Macf1* being the targets of mir-483-5p and demonstrating their expression in BLA neurons, we assayed the mRNA levels of these genes in total and synaptosomal homogenate of amygdala following 6hRS. If mir-483-5p is up-regulated in the amygdala and it targets these three genes, then the mRNA levels of these genes should be significantly down-regulated in these regions. We found that the mRNA level of all three targets was significantly lower in the total amygdala homogenate following 6hRS (Figure 4.8, p. 185). Additionally, only *Pgap2* showed down-regulation in both total homogenate and synaptosomal compartments (Figure 4.8, p. 185). From these observations, we inferred that the function of mir-483-5p in synaptosomal compartments could be mediated via *Pgap2*. Therefore, we focused on *Pgap2* for further investigation into the role of the mir-483-5p pathway in regulating behaviour.

In order to investigate the role of mir-483-5p in anxiety-like behaviour, we overexpressed mir-483-5p in the amygdala using lentiviral technology (Figure 5.3, p. 213 & Figure 5.4, p. 215). We found that the overexpression of mir-483-5p is sufficient to induce anxiolytic behaviour in the subjects when quantified using EPM (Figure 5.5, p. 217). We reasoned that, if the behavioural regulation by mir-483-5p were mediated through *Pgap2,* then a similar anxiolytic behaviour would be unveiled if *Pgap2* was knocked down in the amygdala. Using lentiviral technology, we knocked down *Pgap2* using shRNA (Figure 5.2, p. 211). In agreement with our prediction, these animals also showed reduced anxiety-like behaviours (Figure 5.7, p. 221).

Taken together (Figure 6.1, p. 241), our data suggest that mir-483-5p is up-regulated in the amygdala in response to 6hRS, and it could be functioning by

acting as a molecular buffer to alleviate some of the stress-induced changes in the neuronal architecture by changing the dendritic tree remodelling and shifting the dendritic spine more towards mushroom spines. Overexpression of mir-483-5p is sufficient to induce anxiolytic behaviour in animals, an effect that is recapitulated by the knockdown of *Pgap2*, one of the targets of mir-483-5p. Our study has identified a new miR and its target gene in potential regulation of anxiety-like behaviours in rodents. Further investigation into mir-483-5p or *Pgap2* regulated pathways might help with designing new strategies for therapeutic interventions in order to better control some of the symptoms of pathological anxiety. The next sections propose possible mechanisms and pathways by which mir-483-5p or *Pgap2* interaction can induce anxiolytic behaviours.

6.1.1 Plausible mechanisms and pathways involving mir-483-5p

Research has demonstrated that mir-483-5p expression is induced following status epilepticus (Risbud & Porter, 2013), nicotine admission (Gomez et al., 2015), admission of glucocorticoids (Veronese et al., 2011) and stress (our research). This shows that mir-483-5p is induced during the activation of neurons by both physiological and psychological stress. Furthermore, the host gene of mir-483-5p is *Igf2* (Insulin-like growth factor), an important gene required for memory and synaptic plasticity in the hippocampus (Pascual-Lucas et al., 2014). Recently, *Igf2* has been implicated in trans-generational anxiety in mice (Short et al., 2016). Moreover, mir-483-5p is co-transcribed with *Igf2* suggesting a potential functional partnership. Interestingly, the ‘transfer’ of anxiety trait is thought to be mediated via glucocorticoids (Short et al., 2016).

One other plausible pathway involving mir-483-5p is Wnt/ß-catenin pathway. Wnt/ß-catenin signalling is essential in synaptic and neuronal plasticity in the

amygdala (Maguschak & Ressler, 2011; Maguschak & Ressler, 2008; Teo et al., 2018) and the highest level of ß-catenin is detected in the amygdala (Maguschak and Ressler, 2008). Moreover, excessive *Wnt* signalling leads to brain defects (Zoltewicz et al., 2009). Studies have shown that ß-catenin is transcribed in response to stress, which also targets the transcription of mir-483 (Veronese et al., 2011). Moreover, ß-catenin is triggered by corticoids and results in up-regulation of mir-483 (Veronese et al., 2011). mir-483 is also postulated to interact with ß-catenin where a negative feedback loop regulates the transcription of mir-483 (Veronese et al., 2011). Furthermore, ß-catenin can translocate to the spines from dendritic shafts after depolarisation of neurons to promote synaptic plasticity (Murase, Mosser, & Schuman, 2002). Translocation of mir-483-5p to synaptosomal compartments was observed in our experiments. ß-catenin targets *Dicer* and increases the level of miRs (Teo et al., 2018). Additionally, studies have shown that shRNA silencing of *Macf1* (a target of mir-483-5p) resulted in *Wnt* signalling inhibition through down-regulation of *ß-catenin* (Chen et al., 2006). Altogether, these observations suggest that mir-483-5p could interact with ß-catenin to regulate its activity to affect synaptic plasticity in response to stress. However, it is not clear if this is a *Pgap2* dependent or independent pathway. Moreover, it could also be the case that the stress-induced overexpression of mir-483-5p in our experiments was mediated through ß-catenin and the effect we observed is independent of the pathways suggested here.

Another plausible pathway could be mediated via the phosphorylation of ERK1, which is proposed to be a direct target of mir-483-5p in glioma (Wang et al., 2012). Phosphorylation of ERK1 is shown to be essential for the development of depressive symptoms, and the suppression of ERK1 is shown to reverse

depression-like behaviours in rodents (Todorovic et al., 2009). However, further research is required to establish a direct interaction of mir-483-5p and ERK1. Furthermore, the evidence is lacking to link ERK1 with anxiety via mir-483-5p.

Finally, several studies have now demonstrated the utility of mir-483 as a potential biomarker in a variety of conditions. This suggests a possible common mechanism by which mir-483 is transported outside the cell. Moreover, miRs have been identified in exosomes. These studies and others have shown that miRs could function as an intercellular signalling molecule (Yoshioka, Katsuda, & Ochiya, 2015). Studies have identified some evidence of miRs having different signalling effects pre-synaptically and post-synaptically. These observations show that mir-483-5p could function as an intercellular signalling molecule in response to stress. However, it is important to note that further experiments are required to investigate whether this is the case and to investigate another possibility where mir-483 is expressed under several promoters. Furthermore, being one of the highly expressed miRs in response to stress, it could be released out of the cell which then diffuses into the circulation.

6.2 Limitations and future directions

6.2.1 Experimental limitations and future directions

One of the uncertainties that shadow the current investigation is the efficacy of mir-483-5p to function as a stress buffer in reducing anxiety-like behaviours. We have shown that the overexpression of mir-483-5p or the down-regulation of its target *Pgap2* is sufficient to reduce anxiety-like behaviours in mice. However, innate up-regulation of mir-483-5p in the amygdala is not sufficient to eliminate anxiety-like behaviour completely. This is evident from the observation that 6hRS induced the up-regulation of mir-483-5p in the amygdala and also promoted anxiety-like behaviours. Performing behavioural analysis after the ectopic overexpression of mir-483-5p and then exposing the animals to restraint stress will inform us if mir-483-5p alone can buffer the effect of stress. Additionally, repressing the innate expression of mir-483-5p in the amygdala and then exposing these animals to stress should show increased anxiety in mir-483-5p-repressed animals. By using miR protectors, it is also possible to prevent *Pgap2* from being repressed by mir-483-5p. Assaying the behaviour of animals after 'protecting' *Pgap2* from repression will further enhance the strength of evidence supporting the mir-483-5p – *Pgap2* interaction being important for anxiety-like behaviours.

In the current experiment, levels of mir-483-5p were only assayed at one time point, soon after the 6hRS. This does not inform us about the temporal dynamics of mir-483-5p in the spatial context studied. By performing another series of experiments where the mir-483-5p level in the amygdala is quantified at various time-points during/after stress, it would be possible to establish if a dose response relationship exists between the severity of stress and mir-483-5p up-regulation.

A similar experiment with *Pgap2* can also be performed to see if *Pgap2* levels are correlated with mir-483-5p levels. Furthermore, this would also inform us about the dynamics of mir-483-5p translocation to synaptosomes.

Translating the changes in spine dynamics from studies in primary cultures to *in vivo* neurons is another potential limitation of the current study. The changes in spine dynamics and dendritic arbour were studied in primary cultures, which was then extrapolated to *in vivo* response. Performing a similar analysis *in vivo* will allow us to compare the direction of effect and the effect size in primary cultures and *in vivo*. This will help us to understand if the mechanism involved in the anxiolytic behaviour is mediated through the morphological changes, biochemical changes or a combination of both. Moreover, by assaying the levels of *Pgap2*, it is possible to infer if the morphological changes in dendritic arbour and spines are also dependent on *Pgap2* repression.

Another important limitation of this particular study and animal studies in general is associated with the use of single strain to understand the role of factors involved in the regulation of anxiety like behaviour. To understand the versatility of mir-483-5p in its roles as a buffer of stress-induced changes, it is important to consider these experiments in different strains of mice and also by using different types of stress. This will tell us if the role of mir-483-5p is dependent on a stress type or restricted to particular strains.

Furthermore, the “permissive” role of Dex in the effect of mir-483-5p is unclear. When mir-483-5p levels were overexpressed, and the target mRNAs were assayed, there were no changes in the *Pgap2*, *Gpx3* and *Macf1* levels. The effect was only apparent after the Dex treatment of N2a cells. It is possible that Dex, a synthetic, stress-mimicking hormone, is necessary for the action of mir-

483-5p. Contrastingly, when mir-483-5p was overexpressed in control animals, anxiolysis was observed. Maybe the presence of corticosteroids even in the small amount is sufficient for the function of mir-483-5p. Performing a similar experiment in animals where corticosteroids are knocked out or knocked down will equip us to clarify this question better.

6.2.2 Inherent limitations

One of the inherent limitations is associated with the use of animal models, especially rodent models, to study complex behaviours like anxiety (Belzung & Philippot, 2007). While it is true that several parallels exist between human and mice anxiety (see Chapter 1), it is possible that some of the aspects of anxiety-like behaviours in animals cannot be translated to human anxiety (Joseph LeDoux, 2012). Literature present some conceptual discussions regarding the exact nature of anxiety being measured (See Box 5 ‘State’ anxiety vs ‘trait’ anxiety, p. 239). Some could argue that, when anxiety level of control mice vs mice where a gene is overexpressed/knocked-down is measured, we are comparing state anxiety of the control animals and trait anxiety of experimental animals. Nonetheless, it is important to note that this distinction could be restricted to semantic issues with insignificant biological relevance.

Different tests of anxiety are thought to represent different facets of anxiety. Therefore an integrated approach where several tests are used should be employed to best study anxiety-like behaviours (Ramos, 2008; Ramos, Pereira, Martins, Wehrmeister, & Izídio, 2008). On top of the test-specific aspects of anxiety, it is important to consider the importance of gender specificity. When WT C57 mice were tested in EPM and compared against Swiss-Webster mice and BALB/c mice, the males showed a significant increase in the open arm

preferences. However, this difference was absent in females. This calls the validity of behavioural methods to study different strain- and gender-independent regulators of anxiety-like behaviours into question. Recommendations were made to give more attention to inter-gender, inter-strain and inter-individual differences (Holmes, Parmigiani, Ferrari, Palanza, & Rodgers, 2000).

Additionally, it is important to consider the limitations of the test used to assay the anxiety like behaviour in mice. Even though EPM is the most widely used anxiety test for rodents, some limitations with EPM are discussed in the literature. The test result using EPM is sensitive to even small modifications of the apparatus (Albrechet-Souza et al., 2005). Moreover, Tucker & McCabe, 2017, reported that compared to EPM, WT mice spent more time in the open arms of Elevated Zero Maze (EZM), a test that closely resembles EPM (Tucker & McCabe, 2017). Furthermore, a phenomenon of one trial tolerance, where result of the subsequent tests are affected by the first test, has been described in rodents while testing on EPM (Albrechet-Souza, Borelli, & Brandão, 2008), which is absent in EZM (Tucker & McCabe, 2017). These suggest that the subject could have behaved somewhat differently depending on the test performed. It is unclear what the results would have been if EZM were used in our experiments. It could potentially reveal a more accurate, dampened or amplified difference between the two groups.

Box 5 'State' anxiety vs 'trait' anxiety

State anxiety-like behaviours are elicited by environmental stimuli, such as exposure to a traumatic or painful event (e.g. foot shock or restraint stress). Trait anxiety is a result of the genetic make-up that results in permanent changes in neurological architecture or molecular machinery (Sylvers, Lilienfeld, & LaPrairie, 2011). Examples of trait anxiety models could be strain differences in mice where BALB/c mice demonstrate anxiogenic behaviour compared to C57 strains (Belzung & Griebel, 2001; Griebel, Belzung, Misslin, & Vogel, 1993). Another example of trait anxiety could be mouse transgenic of knock out models of certain receptor or genes, such as 5-HT1A receptors (Ramboz et al., 1998), CRF receptor (Bale & Vale, 2004) and NPY (Guy Griebel, 1999).

Due to these suggested differences, some researchers have questioned and demanded more clarification with using the term anxiety. One reason is the lack of consensus about the definition of 'normal anxiety' and 'pathological anxiety'. It is unclear if the differences between these anxiety states are quantitative or qualitative (Belzung & Griebel, 2001). One interpretation suggests that pathological anxiety is simply an excessive increase in normal anxiety (quantitative difference). However, it is difficult to establish a threshold when the anxiety shifts from being normal to pathological (Belzung & Griebel, 2001; Sartori et al., 2011). Moreover, even if such a threshold is established, it is likely to exhibit high variability among individuals. Others have claimed that the difference between normal and pathological anxiety is of a qualitative nature, *i.e*. there are differences in mechanisms underlying "normal" and "pathological" anxiety (Belzung & Griebel, 2001). Furthermore, it has been argued that the anxiety disorders as experienced by humans is closer to pathological anxiety with a qualitative difference (Belzung & Griebel, 2001). Therefore, advancements in understanding human anxiety disorders can be made by trying to understand the molecular mechanisms that contribute to these qualitative differences. Additionally, it is proposed that mouse models of induced anxiety through exposure to stressors is an example of excessive normal anxiety, and the different strains of mice that express elevated levels of basal anxiety is exhibiting pathological anxiety (Belzung & Griebel, 2001; Belzung & Philippot, 2007).

6.3 **Concluding remarks**

This thesis has reported experimental evidence to show that mir-483-5p is an important player in neuronal adaptation to stress. This effect of mir-483-5p is likely mediated through one of its confirmed targets, *Pgap2*. Firstly, our research corroborates previous findings to show that miRs can have significant effects on complex phenomena, such as anxiety-like behaviours. Therefore, research into complex phenomena at a molecular level should consider the intracellular dynamics introduced by changes in miR repertoire. miRs offer great flexibility regarding temporal expression, spatial bias and targeting multiple gene products. Secondly, our research introduced mir-483-5p to the collection of miRs that are shown to be important for anxiety-like behaviours. To our knowledge, it is one of the first studies demonstrating that the manipulation of single miR can induce anxiolysis in mice. Finally, our research introduces *Pgap2* and GPI anchoring, as a new candidate and mechanism to be investigated in the regulation of neuronal plasticity, stress and anxiety. Finally, we show that by studying miRs it is possible to uncover important roles of previously understudied genes, even in complex phenotypes. We hope that this research will constitute one among the series of studies that slowly but surely unveil the importance of incorporating miRs into our understanding of biological and psychological phenomena.

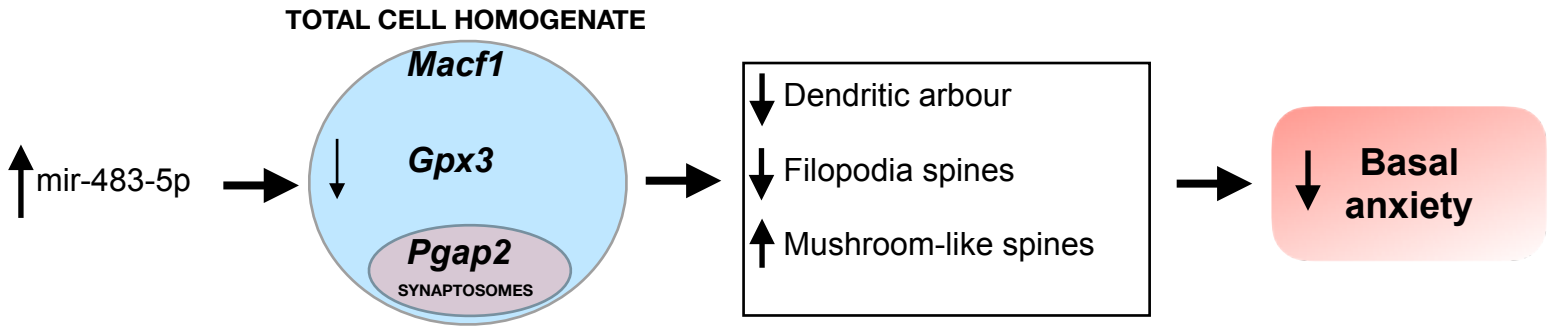

Figure 6.1 Overview of experimental results

Ectopic increase (↑) in mir-385-5p levels leads (⟶) to the reduction (↓) of *Macf1* and *Gpx3* in total homogenate (Fig 4.8) while reduction of *Pgap2* was observed in both total and synaptosomal homogenate (Fig 4.8). Selective increase in mir-483-5p levels in synaptosomal fractions (Fig 3.2) and therefore a reduction of *Pgap2* in synaptosomes led to a reduction of the distal dendritic arbour (Fig 3.3). Furthermore, *Pgap2* reduction in synaptosomes resulted in a decrease of filopodia spines while increasing the proportion of mushroom-like spines (Fig 3.4) without affecting the overall density of spines (Fig 3.5). These changes collectively led to anxiolytic behaviour in mice (Fig 5.5). Anxiolytic effect of mir-483-5p could be mediated via *Pgap2,* one of the target of mir-483-5p (Fig 5.8).. However, the biological elevation of mir-483-5p alone in response to stress (Fig 3.1) is insufficient to reduce the anxiety-like behaviours.

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End of thesis