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Effects of bipolar disorder-associated single nucleotide polymorphism on Adenylyl cyclase II protein function and on mouse behaviour

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Abstract

Bipolar Disorder (BD) is a multifactorial disease with both genetic and environmental factors contributing to it. The single nucleotide polymorphism (SNP): rs13166360 located at 5p15.31 was identified to be one of the polymorphisms that is associated with genome-wide significance with BD. This SNP is a deviation from the major allele G to T (minor allele) that encodes a Valine to Leucine missense codon in adenylate cyclase 2 (ADCY2). This polymorphism is speculated to cause functional variation in the protein that may affect BD susceptibility. However, the precise effect of this polymorphism on protein function and its influence on BD susceptibility is still not known.

In order to study the differences in function of the major and the minor allele of ADCY2, we cloned the two variants of ADCY2 and transfected them into HEK293 cells. We used a time-course and an end-point cAMP assays to compare the differences their activities. In both of these assays, we observed that ADCY2 carrying the SNP of interest (ADCY2-L151), produced less cAMP, suggesting that this variant had a diminished activity.

Since this difference in activity might result from altered localization or trafficking, we assessed if the localization of the two variants of ADCY2 differed in Cos7 cells. We observed an increased localization of ADCY2-L151 in the early, recycling and late endosomes and a decreased localization at the plasma membrane. Taken together, the SNP of interest diminishes ADCY2 activity, which is potentially due to reduced plasma membrane localization.

To address the function of ADCY2 in vivo we generated a V151L mouse line mimicking this desired polymorphism in mice using the CRISPR/Cas9 system ($Adcy2^{L151}$). In order to investigate the influence of this polymorphism on BD susceptibility, we screened for differences in anxiety, locomotion, coping behavior, sociability and cognition under basal housing conditions. We observed that $Adcy2^{L151}$ mice displayed decreased anxiety, an active coping behavior and hyperlocomotion after acute amphetamine administration. They also took longer to habituate, were more sociable but lacked preference for social novelty. Furthermore, these mice also displayed cognitive deficits and had a decreased long-term potentiation (LTP). Upon long-term treatment of these mice with lithium chloride, these mice had normalized levels of anxiety and a normalized coping strategy Taken together, the $Adcy2^{L151}$ mice displayed mania-like characteristic under basal housing conditions; some of these characteristics were normalized after long-term mood stabilizer administration.

Since an interplay of genetic and environmental factors lead to the development of BD, we tested the effect of chronic social stress on the $Adcy2^{L151}$ mice. We observed that $Adcy2^{L151}$ mice had increased susceptibility to chronic stress and switched from an active to a passive coping strategy after exposure to chronic social stress. Overall, the behavioral tests validate the functional significance of the disease-associated SNP in ADCY2 and its interaction with stress, in the development of BD

Zusammenfassung

Bipolare Störung (BD) ist eine multifaktorielle Erkrankung, zu der sowohl genetische als auch Umweltfaktoren beitragen. Der Einzelnukleotidpolymorphismus (SNP): rs13166360 bei 5p15.31 wurde als einer der Polymorphismen identifiziert, der mit einer genomweiten Bedeutung für BD assoziiert ist. Dieses SNP ist eine Abweichung vom Hauptallel G zu T (Nebenallel), das ein Valin-Leucin-Missense-Codon in Adenylatcyclase 2 (ADCY2) codiert. Es wird vermutet, dass dieser Polymorphismus funktionelle Veränderungen im Protein verursacht, die die BD-Suszeptibilität beeinflussen können. Die genaue Auswirkung dieses Polymorphismus auf die Proteinfunktionen und seinen Einfluss auf die BD-Suszeptibilität ist jedoch noch nicht bekannt.

Um die Funktionsunterschiede des Haupt- und des Nebenallels von ADCY2 zu untersuchen, klonten wir die beiden Varianten von ADCY2 und transfizierten sie in HEK293-Zellen. Dabei verwendeten wir ein Zeitverlaufs- und ein Endpunkt-cAMP-Assay, um die Unterschiede ihrer Aktivitäten bei der cAMP Produktion zu vergleichen. In beiden Tests beobachteten wir, dass ADCY2, welches SNP (ADCY2-L151) trägt, weniger cAMP produzierte, was auf eine verminderte Aktivität hindeutet.

Da dieser Unterschied in der Aktivität auf eine veränderte Lokalisierung oder einen veränderten Transportweg zurückzuführen sein könnte, haben wir anschließend untersucht, ob sich die Lokalisierung der beiden Varianten von ADCY2 in Cos7-Zellen unterscheidet. Dabei fanden wir eine erhöhte Lokalisation von ADCY2-L151 in den frühen, Recycling- und späten Endosomen und eine verringerte Lokalisation an der Plasmamembran. Dies bedeutet, dass das SNP ADCY2-Aktivität reduziert, was möglicherweise auf eine verringerte Lokalisierung der Plasmamembran zurückzuführen ist.

Um die Funktion von ADCY2 in-vivo zu untersuchen, haben wir Mäusen unter Verwendung des CRISPR / Cas9-Systems ($Adcy2^{L151}$) eine V151L-Mauslinie erzeugt, die den gewünschten Polymorphismus nachahmt. Um den Einfluss dieses Polymorphismus auf die BD-Anfälligkeit zu untersuchen, überprüften wir Unterschiede bei Angstzuständen, Fortbewegung, Bewältigungsstrategien, Geselligkeit und Kognition unter basalen Wohnbedingungen. Dabei beobachteten wir, dass $Adcy2^{L151}$ Mäuse nach akuter Amphetaminverabreichung verminderte Angstzustände, aktive Bewältigungsstrategien und eine Hyperlokomotion zeigten. Des Weiteren brauchten sie auch länger, um sich and neue Situationen zu gewöhnen, waren geselliger, bevorzugten aber keine sozialen Neuheiten. Darüber hinaus zeigten diese Mäuse kognitive Defizite und hatten eine verminderte Langzeitpotenzierung (LTP). Bei Langzeitbehandlung dieser Mäuse mit Lithiumchlorid hatten zeigte sich ein normalisiertes Angstniveau und eine normalisierte Bewältigungsstrategie. Zusammengenommen zeigten die $Adcy2^{L151}$ Mäuse unter basalen Unterbringungsbedingungen Manie ähnliche Eigenschaften. Einige dieser Eigenschaften wurden nach langfristiger Verabreichung eines Stimmungsstabilisators normalisiert.

Da eine Kombination von genetischen und Umweltfaktoren zur Entwicklung von BD führt, haben wir die Wirkung von chronischem sozialem Stress auf die $Adcy2^{L151}$ Mäuse getestet. Wir fanden, dass $Adcy2^{L151}$ Mäuse eine erhöhte Anfälligkeit für chronischen Stress aufwiesen und nach Exposition gegenüber chronischem sozialem Stress von einer aktiven zu einer passiven Bewältigungsstrategie wechselten. Insgesamt bestätigen die Verhaltenstests die funktionelle Bedeutung des krankheitsassoziierten SNP in ADCY2 und seine Wechselwirkung mit Stress bei der Entwicklung von BD.

1. Introduction

1.1. Psychiatric disorders

While records of mental illnesses date back to ancient Mesopotemia (Black & Green, 1992), little is known about underlying disease causes and we largely lack adequate treatments of these disorders until recently. Since the late 20th century, these disorders were identified based on very specific diagnoses as described in various diagnostic classification systems (ICD-11, WHO, 1993; DSM-5, American Psychiatric Association, 1994). This division in diagnosis has led to fragmentation of treatment as well as research for each disorder, impeding its progress. (Assary, Vincent, Keers, & Pluess, 2018; Kader, Ghai, & Maharaj, 2018). Additionally, the social stigma associated with mental disorders has also hampered better diagnosis and understanding of these disorders (Klin & Lemish, 2008). However, with an increase in awareness over the last few decades, mental disorders account for one third of all disabilities (World Health Organization, 2004);(Assary et al., 2018; Wittchen et al., 2011). ICD -11 distinguishes currently 21 different categories of mental disorders (Table 1.).

1.1.1. Identifying candidate genes associated with psychiatric disorders

Unlike a lot of other disorders where the pathophysiology of the disorder is well known (making it easy to recognize the underlying genetic variation), little is known about the pathophysiology of psychiatric disorders. In the past, candidate genes for some psychiatric disorders, like schizophrenia and major depression, were identified. Most of these candidate genes were identified based on the effect of psychiatric medication on monoamine pathways. However, most of the identified functional polymorphisms in candidate genes linked to these pathways were not significantly associated with psychiatric disorders (Allen et al., 2008)

The alternative strategy for identifying candidate genes is based on detection of disease-associated sequences in the whole genome, considering only gene's location within the genome. Some of the earlier methods included genome-wide linkage studies (GWLS) and linkage disequilibrium mapping (LD) (The Psychiatric GWAS Consortium, 2009).

Disorder	Examples
Neurodevelopmental disorders	Rett's syndrome, ADHD
Schizophrenia and other primary psychotic disorders	Schizophrenia, Schizoaffective disorder
Mood disorders	Depressive disorders, Bipolar disorders
Anxiety and fear-related disorders	Generalized anxiety disorder, Panic disorder
Obsessive-compulsive and related disorders	Obsessive-compulsive disorder, Hypochondriasis
Disorders specifically associated with stress	PTSD, Adjustment disorder
Catatonia	Catatonia associated with another mental disorder, Secondary catatonia
Dissociative disorders	Trance disorder, Dissociative identity disorder
Feeding and eating disorders	Anorexia nervosa, Bulimia nervosa
Elimination disorders	Enuresis, Encopresis
Disorders of bodily distress and bodily experience	Bodily distress disorder, Body integrity dysphoria
Disorders due to substance use and addictive behaviours	Alcoholism
Impulse control disorders	Pyromania, Kleptomania
Disruptive behaviour and dissocial disorders	Oppositional defiant disorder, Dissocial disorder
Personality disorders	Histrionic personality disorder
Paraphilic disorders	Exhibitionistic disorder, Pedophilic disorder
Factitious disorders	Factitious disorder imposed on the self, Factitious disorder imposed on another
Neurocognitive disorders	Amnestic disorder, Dementia
Mental and behavioural disorders associated with pregnancy, childbirth and the perperium	
Psychological and behavioural factors affecting disorders or diseases classified elsewhere	
Secondary mental or behavioural syndromes associated with disorders	

Table 1. Categories of mental disorders as classified by ICD-11

iple

members have the disorder. However, this strategy was not able to successfully identify

many candidate genetic variants in complex disorders like psychiatric disorders. (Lewis et al., 2003; McQueen et al., 2005). LD uses population-wide correlation between sequence variants. When two single nucleotide polymorphisms SNPs occur close to each other in the genome, then both variant alleles are usually inherited together by offspring. This non-random association between the two variant alleles is known as linkage disequilibrium. Previously, it was thought that mapping this non-random association could be used to identify candidate genes linked to disorders. These studies, as well as meta-analyses of these studies have identified a few candidate genes for psychiatric disorders (Lewis et al., 2003; Norton, Williams, & Owen, 2006)

The small sample size in the earlier studies affected their statistical significance. Therefore, an unbiased approach was proposed to identify small sequence variations with greater statistical power known as genome-wide association study (GWAS). In GWAS, several sequence variants spread in a large patient population (usually around 10,000 individuals) is genotyped. These are compared to sequences from a similar number of healthy controls. In addition to SNPs, GWAS also allowed the detection of disease-associated copy number variations of particular genes, as well as disease-associated rare SNPs (Altshuler, Daly, & Lander, 2008); (Cross Disorder Phenotype Group of the Psychiatric GWAS Consortium, 2009). However, there is still a lot of debate on the role of genetic variants in the development of psychiatric disorders. It is debated whether a large number of common sequence variants, each with a small effect, act together to manifest psychiatric disorders or if few sequence variants with strong effects are underlying these disorders (Goldstein, 2009; Malhotra & Sebat, 2012). Additionally, the fact that one genetic variant can manifest in different psychiatric disorders (Kida & Kato, 2015) and the interactions of these genetic variants with environmental factors make it more difficult to understand their role in the development of psychiatric disorders.

1.1.2. Interaction of genetic and environmental factors underlying psychiatric disorders

Twin studies also suggest that there is a heritable component to the development of psychiatric disorders (McGuffin et al., 2003). However molecular heritability of polymorphisms that could be associated with psychiatric disorders in the general population is much lower than heritability found in twin studies. This gap in heritability is also best explained by environmental factors (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). This suggests that psychiatric disorders develop due to a complex interplay between genetic and environmental factors (Uher, 2014).

Additionally, as per the theory of evolution, genetics that reduce evolutionary fitness, as in the case of psychiatric disorders, should be pruned out of the genomic pool, and consequently the frequency of these disorders should diminish over time. The reason that this is not the case also suggests that both genetic and environmental factors are needed for the full development of psychiatric disorders, and the genetic factors by themselves only slightly affect reproductive fitness (Uher, 2014).

Recent studies have identified various environmental factors playing of role in the development of psychiatric (Teicher & Samson, 2013). These environmental factors include stressors like early childhood adversity (Bradley et al., 2008; McGowan et al., 2009) and other stressful life events (Taylor & Kim-Cohen, 2007)

1.1.3. Molecular mechanisms underlying gene-environment interaction

Genetic polymorphisms that are associated with psychiatric disorders might alter how certain environmental factors functionally modify the same gene or the functions of other genes or proteins. Epigenetic modifications refer to changes in gene expression without affecting the DNA sequence itself. A polymorphism can result in epigenetic modifications of the same or other genes (Klengel & Binder, 2013). One classical epigenetic modification that is associated with several psychiatric disorders is aberrant DNA methylation (D. Kim et al., 2016; Ota et al., 2014; Puglia, Lillard, Morris, & Connelly, 2015). Previous studies show that experience changes DNA methylation patterns (Kinnally et al., 2011; Weaver et al., 2004). Additionally certain genetic variations near or in CpG sites (where methylation

typically occurs) and SNPs in DNA methyltransferase also alter the methylation status and result in these anomalous methylation patterns. However, the exact mechanism connecting DNA methylation and psychiatric disorders is still unknown

Other well studied epigenetic mechanisms include post translational modifications (PTM) of histones. These include histone methylation, acetylation, phosphorylation, biotinylation, ubiquitination, sumoylation and ADP-ribosylation (Stadler et al., 2005; Tessarz & Kouzarides, 2014). These histone modifications can activate or repress gene expression, depending on the site of modification.

1.2. Bipolar Disorder

Bipolar disorder (BD) is a severe mood disorder characterized by rapid transitions between manic, hypomanic and a depressive phases (Grande, Berk, Birmaher, & Vieta, 2016; Vieta & Phillips, 2007). Manic or hypomanic phases are characterized by elevated mood, increased motor activity that only last for a certain length of time and can vary in severity. In addition, a manic phase can involve social and occupational impairments and sometimes might also be accompanied with psychotic symptoms. Patients may need hospitalization during this phase. Another characteristic symptom is the patient's reduced need for sleep. During hypomanic phases, occupational impairments may be visible to others, but typically the impairments are not as severe as during manic episodes. The depressive phase is marked by symptoms like depressive mood, anhedonia, significant weight loss or gain, insomnia or hypersomnia, slower motor activity (DSM-5, American Psychiatric Association, 2013)

BD spectrum is known to affect 2.4% of the population globally, regardless of their ethnic background or socioeconomic status. Within this spectrum bipolar disorder I (BD I), which is characterized by full manic phases, affect 0.4% of the population and bipolar disorder II (BD II), characterized by more hypomanic phase affects 0.6% of the population(Merikangas et al., 2011). BD I affect men and women equally, however BD II is more prevalent among women (Nivoli et al., 2011). BD is known to have a high heritability of 80% based on twin studies (Maletic & Raison, 2014). However, the heritability of associated SNPs is only 20-30% (Uher, 2014).

BD can lead to severe impairments with 9%-15% of patients committing suicide (Medici, Videbech, Gustafsson, & Munk-Jorgensen, 2015). This disorder is treated by acute stabilization of the different phases, and relapse prevention which aims at minimizing symptoms in the long run and improving general function. Acute treatment is difficult as one mood phase can rapidly switch to another mood phase. Antipsychotic drugs such as Olanzapine, risperidone, and haloperidol are reported to be the most effective short term treatments for manic episodes. Typically anti-depressants are used to stabilize depressive episodes (J. R. Geddes & Miklowitz, 2013). However, the efficacy of antidepressants in treating bipolar depression is often poor. A recent meta-analysis of antidepressants in bipolar depression reported no significant effect compared to placebo (Sidor & MacQueen, 2012).

Relapse treatment is typically done using drugs with minimal long term side effect. At the moment lithium is the best available option for such a treatment. A meta-analysis of five placebo controlled lithium treatments demonstrates that lithium reduces the risk of manic relapse by 38% and depressive relapse by 28% (J. Geddes et al., 2004). However meta-analysis of lithium's toxicity reveals that it has several adverse effects and a low therapeutic index (McKnight et al., 2012). Valproate is another commonly prescribed mood stabilizer for long term maintenance treatment. But the evidence of its efficacy in long-term prevention of mood episodes remains limited. Additionally, the use of valproate is also reported to be associated with various adverse effects like increased sedation and infection (A. Cipriani, Reid, Young, Macritchie, & Geddes, 2013; Hayes et al., 2011; Macritchie, Geddes, Scott, Haslam, & Goodwin, 2001).

Integration of pharmacological intervention and psychotherapy is often reported to have the best treatment outcome (Miklowitz & Otto, 2007; Scott et al., 2009). These psychotherapies include cognitive-behavioural therapy, family-focused therapy, interpersonal and social rhythm therapy and group psychoeducation. However, psychotherapy for long term maintenance often poses a high cost(J. R. Geddes & Miklowitz, 2013). Like for many psychiatric disorders, the lack of understanding of the aberrant molecular mechanisms underlying BD has hampered the identification of biomarkers or development of better pharmacological interventions (Y. Kim, Santos, Gage, & Marchetto, 2017)

1.2.1. BD associated candidate genes identified by GWAS

GWA studies have helped to identify several new susceptible genes and loci. Identifying these genes has helped to generate novel hypotheses with regards to possible aberrant mechanisms underlying BD. Studying these possible aberrant mechanisms could help to identify better potential drug targets for treating BD.

Earlier GWA studies could identify very few BD associated SNPs that were statistically significant (Wellcome Trust Case Control Consortium, 2007). However, a meta-analysis combining datasets from multiple studies proved to be a more useful method for identifying BD associated SNPs (Sklar et al., 2008). This is because most of the earlier studies only had 1500 to 3000 individuals per group and there was a relatively small chance of the positive findings to be statistically significant genome-wide in any one GWAS.

Meta-analysis of GWA studies found strong evidence implicating SNPs in CACANA1 gene, encoding voltage gated L-type calcium channel and in ANK3, a neuronal scaffolding protein, to BD (Ferreira et al., 2008). Since then much larger GWA studies, like the Psychiatric GWAS Consortium study in 2011, with many more of patients and healthy controls and have been carried out and these studies have been able to identify many more risk loci for BD (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011). One of the largest GWAS study on Bipolar Disorder, consisting of more than 24,000 patients and healthy control, identified two novel polymorphisms in 2014 (Muhleisen et al., 2014). This group identified SNPs in five previously identified risk loci including ANK3, ODZ4 and TRANK1. They also identified SNPs in two new risk loci: ADCY2 and the region between MIR2113 and POU3F2 (Fig 1.) Since then another recent GWAS once again identified ADCY2 and POÚ3F2 as risk loci for BD (Stahl et al., 2019). Additionally, this study has identified 20 new loci that are genome-wide significant for BD. Studying the consequences of these candidate genes might help generate novel hypotheses on possible dysfunctions underlying BD.

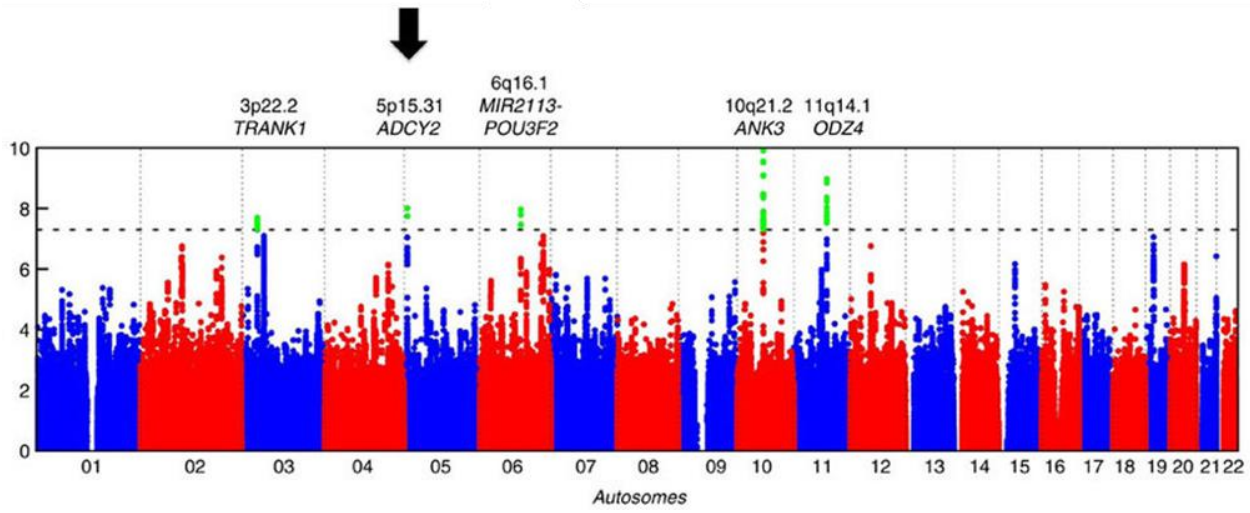


Figure 1. Manhattan plot indicating identified BD associated risk loci. (From Mühleisen T.W. et al, 2014 with permission)

1.2.2. Potential mechanisms causally linked to BD

While the exact mechanisms underlying BD are not fully understood, several hypotheses attempt to explain the underlying dysfunctions. Most of these hypotheses are based on abnormalities identified in patient studies and some of them are based on association studies. The following are several existing hypothesis about aberrant mechanisms underlying BD.

1.2.2.1. Environmental factors underlying BD

Patient studies have implicated several environmental factors to the development of BD (Fig. 2). There are several environmental factors that have been identified repeatedly in several patient studies and meta-analysis. One of these factors is infection in both prenatal and perinatal stages of development.

Maternal influenza has been associated with the development of BD in offspring (Aldinger & Schulze, 2017; Canetta et al., 2014; Parboosing, Bao, Shen, Schaefer, & Brown, 2013). The association of influenza infection in adulthood to BD has not been well studied, and the use of small sample sizes in the few patient studies make it difficult to draw any conclusion about the effect of influenza infection in adulthood to the development of BD (Aldinger & Schulze, 2017).

Pre-natal *Toxoplasma Gondii* (*T. Gondii*) infection has also been repeatedly associated with BD. Some studies have also associated *T. Gondii* infection during adulthood with the development of BD (Rowland & Marwaha, 2018; Sutterland et al., 2015). One confounding factor in these studies in that the exact time of exposure is unclear. Studies in mice and humans have also demonstrated behavioural changes related to exposure to *T. Gondii (Kocazeybek et al., 2009; Webster, 2007)*. For instance a study demonstrates that *T. Gondii* infection alters dopamine metabolism and enhances dopamine release from dopaminergic neurons (Prandovszky et al., 2011). Aberrant dopamine metabolism has been implicated in BD (see below); therefore this mechanism could explain the association of *T. Gondii* infection with the development of BD.

	Exposure	Bipolar disorder
Prenatal	Season of birth	++
	Inadequate nutrition	++
	Infection during pregnancy	++
	Prenatal stress	+
Perinatal	Preterm birth	+++
	Perinatal infection	+
Childhood	Maltreatment	+++
Adolescence	Cannabis	+
Adulthood	Stressful life events	++
	T. Gondii infection	+
	Poor social support	++

Table 2. Environmental factors causally linked to BD. *The number of plus signs indicates the strength of evidence for association: +++ indicates consistent evidence from multiple studies or a meta-analysis;* ++ indicates evidence from *several* studies or a strong association in a high-quality study; + indicates evidence from a single study or multiple low quality studies (Modified from Uher R, 2014; Rowland R.A. and Marwaha S., 2018)

Childhood maltreatment is another environmental factor that is strongly correlated with the development of BD. However, this factor is also strongly correlated to the development of other psychiatric disorders, such as schizophrenia or major depression (Uher, 2014). Among the different types of childhood maltreatment, emotional abuse was most closely associated with the development of BD (Palmier-Claus, Berry, Bucci, Mansell, & Varese, 2016). Childhood maltreatment is also associated earlier onset of BD and unfavourable clinical outcomes in the treatment of BD (Agnew-Blais & Danese, 2016). However it is still not well understood how childhood trauma leads to the development of BD. Childhood trauma is however linked to an increase in brain-derived neurotrophic factor (BDNF) and inflammatory cytokines, and an alteration in the hypothalamic–pituitary–adrenal (HPA) axis function (Aldinger & Schulze, 2017). It is possible that these alterations interact with genetic factors to lead to the development of BD.

Psychological stressors are also strongly associated with the development of BD. However, this factor is also known to influence the development of other psychiatric disorders, and is again not specific for BD (Tsuchiya, Byrne, & Mortensen, 2003; Uher, 2014). Exposure to stressful life events is associated both with the onset and relapse of BD-associated manic or depressive episodes (Kessing, Agerbo, & Mortensen, 2004; Lex, Bazner, & Meyer, 2017). Of the different psychological stressors, some studies have associated the loss of a parent and childbirth to BD (Lex et al., 2017; Tsuchiya et al., 2003). In general, the interaction between genetic factors and environmental stressors are hypothesized to act together in the development of BD.

1.2.2.2. Circadian rhythm disruption

A characteristic symptom in patients with BD is a disruption of sleep as described in DSM-5. This suggests that the molecular mechanisms underlying regulation of circadian rhythm might be disrupted in BD. This is further supported by patient studies that demonstrate patients with BD have abnormal levels of melatonin (Nurnberger et al., 2000; Robillard et al., 2013). Therefore, it is not surprising that several smaller linkage studies have identified genes involved in circadian rhythm to be associated with BD. These studies have associated genes like Per3 (Brasil Rocha, Campos, Neves, & da Silva Filho, 2017), clock genes (Lee et al., 2010; Soria et al., 2010), Arntl I (Mansour et al., 2006; Soria et al., 2010) and CRY2

(Mansour et al., 2006) to name a few. However, circadian rhythm disruptions have been reported in other psychiatric disorders like schizophrenia as well (Ekman et al., 2017).

1.2.2.3. Metabolic disruption

Several patient studies have reported metabolic dysfunctions in patients with BD (Weber et al., 2013). While the mechanisms underlying metabolic dysfunctions and BD remain unclear, there are several hypotheses concerning this link. It is possible that these metabolic dysfunctions are also a result of circadian rhythm disruptions (Boudebesse et al., 2015). Additionally, some studies suggest that the metabolic symptoms might result from administration of mood stabilizers and antipsychotics, low activity and poor diet during depressive phase (McElroy et al., 2002). Other studies suggest that metabolic disruption might be linked to mitochondrial dysfunction (Kato & Kato, 2000).

Patient studies suggest a less efficient metabolism of affected brains. The rate of the inefficient glycolysis in mitochondria is hypothesized to increase, while the rate of the more efficient oxidative phosphorylation decreases (Figure 2) (Y. Kim et al., 2017). This is because the rate of the tricarboxylic acid (TCA) cycle, a step in anaerobic respiration, seems to decrease. This is supported by an increase in pyruvate, the end product of glycolysis in serum of patients with BD (Yoshimi et al., 2016). Additionally, decreased intracellular pH and elevated levels of lactate in BD patient brains and cerebrospinal fluid further support this hypothesis (Chu et al., 2013; Dager et al., 2004; Regenold et al., 2009).Moreover, post-mortem brains of BD patients also show a decreased expression of genes encoding subunits of the electron transport chain complexes (Andreazza, Shao, Wang, & Young, 2010; Konradi et al., 2004; Sun, Wang, Tseng, & Young, 2006).

Mitochondria are also involved in regulating intracellular calcium levels. In some BD patient samples elevated intracellular calcium concentrations were detected (Dubovsky, Daurignac, & Leonard, 2014; Perova, Wasserman, Li, & Warsh, 2008). This would suggest that BD might disrupt mitochondria-related calcium homeostasis. Some mitochondrial DNA polymorphisms, involved in mitochondrial calcium signalling, were associated with BD in smaller association studies (Kato, Kunugi, Nanko, & Kato, 2001). It was hypothesized that these BD associated polymorphisms affected mitochondrial metabolism by disrupting intra-cellular calcium signalling. However, those polymorphisms could not be verified in

larger GWA studies (Hou et al., 2016). It is possible that even larger GWA studies with higher statistical power might be need to identify this association. Other polymorphisms such at that in CACNA1C has been associated with BD in GWA studies (Ferreira et al., 2008). Studies suggest that aberrant calcium signalling might play a role in the development of BD (Berridge, 2014). Dysfunctions in calcium regulation would affect intracellular signalling pathways, as well as neurotransmitter release (Neher & Sakaba, 2008).

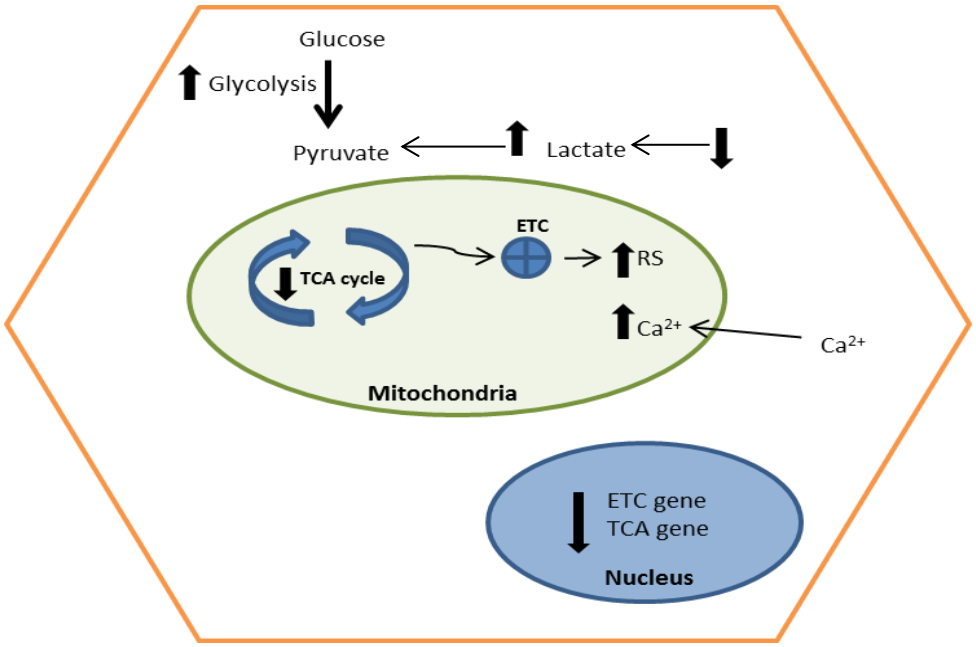


Figure 2. Energy metabolism disruption in mitochondria underlying BD. The hypothesis states that the rate of oxidative phosphorylation decreases and the rates of glycolysis increases. Typically increased glycolysis will be accompanied by increased intracellular pyruvate, increased mitochondrial calcium levels and increased reactive oxygen species (RS). Additionally increased intracellular lactate and a decrease in intracellular pH would be observed. Electron transport chain (ETC) and TCA associated gene transcription should be repressed. (adapted from Kim Y. et al, 2017)

1.2.2.4. Changes in neurotransmission

Some studies implicate disturbances in monoamine transmission in BD. One such hypothesis implicates dysregulation in dopamine. This hypothesis originates from the fact that administration of dopamine precursor, L-DOPA, as well as amphetamine (which increases dopamine at synaptic clefts) produce hypomanic episodes in patients with BD. Additionally, earlier studies have associated polymorphisms in the dopamine transporter to BD (Greenwood, Schork, Eskin, & Kelsoe, 2006; Horschitz, Hummerich, Lau, Rietschel, &

Schloss, 2005). The increased dopaminergic transmission during the manic phase could lead to a decreased dopamine receptor sensitivity over time, which in turn promotes the depressive phase of the disorder (Berk et al., 2007).

Another hypothesis on monoamine disturbances implicates disruptions in serotonin transmission. Some patient studies have associated a decreased serotonin transporter level in the midbrain with the depressive phase of BD (Cannon et al., 2006; Oquendo et al., 2007). However, other patient studies have contradicted this finding (Chou, Hsieh, Chen, Lirng, & Wang, 2016). Moreover, it is not known whether this decrease is the underlying cause of the depressive phase or it is the result of the patients being in the depressive phase.

Additionally, GWA studies have linked polymorphisms in ionotropic glutamate receptors to BD (Le-Niculescu et al., 2009). Studies have also reported NMDA receptor dysfunction in patients. The studies found a decrease in expression of NMDA scaffolding proteins like PSD95 in the thalamus of BD patients (Clinton & Meador-Woodruff, 2004). A reduction in expression of NMDA receptor subunits has also been reported in the hippocampus of BD patients (Beneyto, Kristiansen, Oni-Orisan, McCullumsmith, & Meador-Woodruff, 2007; Law & Deakin, 2001; McCullumsmith et al., 2007). Another study also identified increased glutamate in the frontal cortical regions of BD patients (Hashimoto, Sawa, & Iyo, 2007). However, the mechanism underlying the dysregulation of glutamate neurotransmission remains to be elucidated.

Some studies have implicated dysfunctions in GABAergic neurotransmission to BD. These studies have reported decreased numbers of parvalbumin- and somatostatin-positive GABAergic interneurons with altered maturation (Gandal, Nesbitt, McCurdy, & Alter, 2012; Wang et al., 2011). These analyses also showed significant changes of RNA and protein levels in the GABAergic system in brains of BD patients (Torrey et al., 2005). Additionally, GABA receptor variants have been associated in smaller association studies with BD (Horiuchi et al., 2004; Otani et al., 2005). However, a major confounding factor is that similar changes have also been observed in brains of patients with other psychiatric disorders like schizophrenia. Furthermore, the molecular mechanisms underlying these changes that lead to the aberrant behavioural endophenotypes related to BD in are still

unknown. It is possible that the dysregulation of excitatory and inhibitory circuits lead to an excitation/inhibition imbalance that promotes the development of BD.

Taken together, various changes in neurotransmission are associated with BD. The mechanisms underlying these dysfunctions still remain to be elucidated. Furthermore, it is still unclear if these dysfunctions are the underlying cause of BD or simply an effect of other aberrant mechanisms underlying BD.

1.2.2.5. Disruption of intercellular signalling cascades

The psychotropic drugs used to treat bipolar disorder are thought to affect various intracellular signalling cascades, which eventually lead to changes in gene expression, neural plasticity and neurotransmission. Additionally, recent GWAS have identified SNPs that affect various genes involved in different intracellular cascades (Maletic & Raison, 2014). But in the context of BD, the underlying maladaptive pathways are not well studied.

Some BD studies that combine GWAS data with gene expression data have identified dysfunctions in hormonal regulations, second messenger systems and glutamatergic signalling, histone and immune pathways (Nurnberger et al., 2014). The precise effect of these polymorphisms is still unknown. Understanding the effects on signalling cascade would not only provide a better understanding of the underlying dysfunctions, but also could lead to the development of more effective therapeutic strategies.

1.3. Understanding molecular mechanisms underlying BD in-vitro

The molecular dysfunctions in BD are also studied *in-vitro* in cell culture. These cellular tools have been used to better understand the pathophysiology of BD. One way of studying this mechanism is by using induced pluripotent stem cells (iPSCs) differentiated from patient fibroblasts and comparing them to those from healthy controls (H. M. Chen et al., 2014). These iPSCs were differentiated into forebrain neurons and transcriptionally profiled demonstrating their deviation from healthy controls. Cells derived from patients showed increased expression of various membrane-bound receptors and ion channels. Furthermore, lithium treatment of patient cells significantly changed their calcium transients and wave amplitude. Additionally, transcription factors regulating dorsal/ventral patterning cues were significantly altered in cells from patients (H. M. Chen et al., 2014).

Some studies used a family based approach for studying BD mechanisms *in-vitro*. In this approach iPSCs are generated from family members who are either healthy or have developed BD. One such family based approach was used to generate iPSCs which were developed into neural progenitor cells (Madison J.M. et al, 2015). No difference was observed in the iPSCs generated from BD patients and those generated from healthy family members. However, the neural progenitor cells from BD patients revealed differences in neurogenesis and a different expression profile of genes involved in plasticity. This included genes involved in the Wnt pathway and those encoding for ion channel subunits (Madison et al., 2015).

Another method is to compare cells from patients carrying a disease associated genotype to cells from patients heterozygous for the disease-associated variant and patients carrying the non-disease associated variant. One such study uses neurons induced from fibroblasts derived from patients carrying the rs1006737 polymorphism in the CACNA1C gene, which is associated with BD. This study identified an increase in CACNA1C mRNA and an increase in calcium current density in cells from patients homozygous for the polymorphism compared to those heterozygous for the polymorphism and those that did not carry this polymorphism (Yoshimizu et al., 2015).

Studying BD in in-vivo systems provide some insight into the pathophysiology underlying BD. However, these models are limited their ability to correlate particular molecular dysfunctions with aberrant behaviours observed in BD patients. Furthermore, these in-vitro studies suffer from the lack of genetic heterogeneity, as the genetic background of each patient is different. These differences in genetic background could also influence the observed mechanistic differences between the test samples and controls. This issue can be better controlled for in animal models, where the genetic backgrounds of the test and control animals remain similar, while the SNPs of interest can be introduced, e.g. using Crispr/Cas9 technology. The effect of molecular dysfunctions on particular disease-related endophenotype is better examined in animal models of BD.

Bipolar Mania related endophenotype	Rodent paradigm
Reduced anxiety, increased novel exploration	Elevated plus maze; dark–light box; open field arena; social interaction test, novelty-suppressed feeding
Reduced depression	Forced swim test, tail suspension test, learned helplessness test
Impaired sensory processing	Paired-pulse inhibition (PPI)—sensorimotor gating
Risk-taking behavior	Iowa Gambling Task (IGT)
Psychostimulant-induced hyperactivity	Cocaine or amphetamine injection, repeated injections for locomotor sensitization
Hedonia	Sucrose preference; cocaine or amphetamine self-administration; intracranial self-stimulation (ICSS) of medial forebrain bundle; two-bottle free-choice alcohol drinking; cocaine-conditioned place preference (CPP)
Hyperactivity	Open field arena; homecage monitors; wheel-running; behavioral pattern monitor (BPM)
Reduced or disrupted sleep	EEG; circadian activity; sleep–wake monitoring
Disrupted circadian rhythms	Circadian wheel-running activity; temporal patterns of behavior

Table 3: Modelling human bipolar disorder related phenotypes in rodents. (Modified from Logan R.W. and McClung C.A., 2016)

1.4. Animal Models of BD

Typically animal models of psychiatric disorders have been used to get better insight into the molecular mechanisms underlying the disorder. Most of these models are genetic models, where risk loci detected in GWAS or predicted in patient studies have been altered in mice, in an attempt to capture BD-associated behaviour. Furthermore, some of the mechanistic differences identified in patient studies, such as disruptions in circadian rhythm and disruptions in dopaminergic transmission, are also observed in these mouse models. Usually, certain behavioural tests are used in rodents to test for these BD endophenotypes (Table 3). However, most mouse models have been limited by their inability to mimic both mania-like behaviour and depression- like behaviour (Harrison,

2016). Most rodent models of BD recapitulate mania-like behaviour. Some newer BD models however claim to have captured this switch between the manic and depressive phase (Young & Dulcis, 2015). Furthermore, most of these models fail to demonstrate face validity, construct validity and predictive validity.

1.4.1. Issues with face, construct and predictive validities in models of BD

When constructing animal models of disorders, having good face, construct and predictive validities are important. Face validity is the extent to which an animal model captures the characteristic features of the disorder. Construct validity is the degree to which a test using the animal model measures what it is meant to measure. It measures the relevance of the translational aspect of the model. Predictive validity is the extent to which the animal models respond to treatments that are used in human patients.

Modelling BD in animals poses several issues of face validity. This stems from the fact that the criteria for psychiatric disorders are primarily based on qualitative enquiries and thus not very clearly defined (Chesler & Logan, 2012). Another aspect of face validity is the extent the models recapitulate the pathophysiological and biochemical aspects of the disorders. In the context of BD, this can be better evaluated by comparing data obtained from animal models, with those from in-vitro studies as well as patient studies (Logan & McClung, 2016).

Most models of psychiatric disorders like BD also have several issues with construct validity. This stems from the lack of definitive knowledge on which genetic factors, molecular mechanism, environmental factors or circuits underlie BD. As GWAS studies associate certain genetic variants with BD, modelling these genetic variants in transgenic animal models does have a certain level of construct validity. However, how these genetic variants might interact with other genes or environmental factors, to underlie the endophenotypes of BD is still not fully understood (Logan & McClung, 2016). Furthermore, naturally occurring polymorphisms are often just SNPs, or variants in non-coding regions of the genome rather than a complete knockout of a certain gene, as is done in various mouse models. Growing understanding of the role of these coding and non-coding SNPs would be mandatory to better understand their significance. Technologies like Crispr/Cas9, which

can be used to introduce SNPs to mice or other model organisms, would improve construct validity of modelling BD.

Predictive validity in most animal models, as well as some cellular models is tested using the response to drugs like lithium. Most animal models are able to show a reduction in mania-like phenotypes after administration of mood stabilizers. However, to what extent the altered behavioural phenotypes reflect a reduction in mania-like behaviour is unclear as it does not take into account side effects of the treatment. The predictive validity could be improved by taking more cross species approaches, that have been done in some BD models (Logan & McClung, 2016; van Enkhuizen, Minassian, & Young, 2013; Young & Light, 2018).

1.4.2. Genetic Models of Bipolar Disorder

Genetic animal models include introduction of SNPs, as well as single gene mutation, deletion or overexpression. These genetic models give some insight into the roles of these genes in the development of BD. However, complex disorders like BD involve multiple genetic and environmental factors (Pena, Bagot, Labonte, & Nestler, 2014). Therefore, single gene variants in these models often do not paint a complete picture of the underlying cause of BD. In the following some well-established genetic mouse models of BD will be discussed.

1.4.2.1. The Clock Δ19 mouse

The Clock Δ19 mouse carries a mutation in the Clock gene. This gene in involved in the regulation of circadian rhythm. Previous patient studies have associated polymorphisms in this gene with BD (K. Y. Lee et al., 2010; Soria et al., 2010). These mice have a single base pair mutation in exon 19 of the Clock gene, which in turn results in a splice variant that lacks the activation domain required for Clock-mediated transcription (King et al., 1997). The Clock Δ19 mouse model was reported to display mania-like behaviour during the day. Additionally, these mice also display somatosensory deficits in the PPI test. However, at night these mice demonstrate normal behaviour (McClung, 2007). Some studies have tested the effect of this gene using a variety of different model organisms (cross-species

testing). These studies have also validated some of these BD-related phenotypes (van Enkhuizen et al., 2013).

Increase activity of dopaminergic neurons in the ventral tegmental area of these mice is associated with the demonstrated mania-like behaviour during the day. This is because the Clock gene represses the transcription of tyrosine hydroxylase. Tyrosine hydroxylase is involved in dopamine synthesis (Sidor et al., 2015). With a lower expression of the Clock gene in the Clock Δ19 mouse, an increased tyrosine hydroxylase expression leads to an upregulation of dopamine levels. The increase in dopamine, observed during the manic phase in BD patients (Berk et al., 2007), further supports this interplay between circadian rhythm dysfunction and dopamine levels in BD patients. Other studies have identified ClockΔ19 mutant mice to have other aberrant molecular mechanisms like increased propionic acid mediated excitatory response at the nucleus accumbens (Parekh et al., 2018).

1.4.2.2. The Shank3 mouse

Shank3 is a member of the Shank protein family. This is a family of scaffolding protein that forms a macromolecular complex at the post-synaptic density of excitatory synapses (Sheng & Kim, 2000). Polymorphisms at the SHANK protein have been associated with autism and schizophrenia (Gauthier et al., 2010; Moessner et al., 2007). Duplication of the 22q13 region of SHANK3 has been associated with Asperger's syndrome and schizophrenia (Durand et al., 2007; Failla et al., 2007). A few patient studies have eventually linked SHANK3 dysfunction to BD (Vucurovic et al., 2012).

However, the Shank3 mouse model, overexpressing this protein, demonstrates mania-like behaviour, aberrant circadian rhythm and seizures indicative of an excitation/inhibition imbalance (Han et al., 2013). The mania-like behaviour is reduced by the mood stabilizer: valoproate. The behavioural phenotype closely resembles the manic phase of BD. The excitation/inhibition imbalance is a result of a disruption in SHANK3's interacting partner the Arp2/3 complex, which in turn aberrantly increases the F-actin level (Han et al., 2013).

1.4.2.3. The Ank3 mouse

Genes identified through GWA studies have also been used to model BD in animals. ANK3 was associated with BD in GWAS with an odds ratio of 1.35 (Sklar et al., 2011). This gene encodes various isoforms of the scaffold protein: Ankyrin G. It is involved in various different cellular mechanisms including the formation and maintenance of the axon initial segments and the localization of GABA synapses at axon initial segments of excitatory neurons (Ango et al., 2004; Bennett & Lambert, 1999).

Mice heterozygous for Ank3 demonstrated mania-like behaviour and increased reward seeking behaviour. Knockdown of Ank3 by RNA-interference at the dentate gyrus demonstrated similar mania-like behaviour. Chronic administration of lithium chloride reduced this mania-like endophenotype (Leussis et al., 2013). This behavioural phenotype closely the mania like behaviour observed in BD patients. Furthermore the mice heterozygous for Ank3 demonstrate depression-like behaviour post exposure to chronic stress by chronic isolation. Additionally an increased cortisol level was observed after exposure to chronic stress. This suggests an increased vulnerability to stress in this mouse model (Leussis et al., 2013).

1.4.2.4. DAT knock down

Dysfunctions in dopamine neurotransmission have been previously associated with BD. This includes association studies that link polymorphisms in the dopamine transporter (DAT) to BD (Greenwood et al., 2006; Horschitz et al., 2005). DAT knockdown mice, with higher extracellular levels of dopamine exhibit mania-like behaviour (Young et al., 2010b; Young, van Enkhuizen, Winstanley, & Geyer, 2011; Zhuang et al., 2001). These mice however lack somatosensory deficits in the PPI test (Ralph-Williams, Paulus, Zhuang, Hen, & Geyer, 2003). Nevertheless, complete DAT knockout mice (DAT-KO) do demonstrate these somatosensory deficits that are characteristic of bipolar mania (Powell, Young, Ong, Caron, & Geyer, 2008). The somatosensory deficits in DAT-KO mice are alleviated by antipsychotic drugs like Clozapine.

1.4.2.5. Ncan knockout mouse

Another candidate gene identified through GWAS studies is the NCAN gene (Cichon et al., 2011). NCAN encodes the protein neurocan that is involved in cell adhesion and migration. It has been shown to have a specific expression pattern in the cortex and hippocampus. Since these areas both play a role in cognition, this particular candidate gene was modelled (Miro et al., 2012). The Ncan knockout mice demonstrated mania-like behaviour, hyperactivity and amphetamine hypersensitivity. The mania-like behaviour normalized after lithium administration. These behavioural endophenotypes closely resembles the manic phase of BD. Patient studies further support this model as patients carrying the NCAN risk allele demonstrate mania-like behaviours.

1.5. Future directions for understanding BD

Since the mechanisms underlying BD are still poorly understood, models based on clinical observations provide the most insight into these mechanisms. However, integration of different approaches like studying mechanisms in *in-vitro* cell cultures and in animal models as well as patient studies, are needed to better understand a complex disorder like BD. Furthermore, large genome-wide association studies, with higher statistical powers have hugely benefited the field of BD research by identifying various different BD associated polymorphism (Harrison, 2016; Muhleisen et al., 2014). While these studies have associated polymorphisms in common proteins like ADCY2 to BD, the exact molecular dysfunctions underlying these polymorphisms are not understood. Investigating how these identified polymorphisms interact with other polymorphisms as well as environmental factors to affect molecular mechanisms as well as behavioural endophenotypes would help understand BD better.

The SNP identified in the Mühleisen et al, 2014 study: rs13166360T lies in the coding region of Adcy2 gene. Since its identification, the Adcy2 locus has been identified multiple times as a risk locus for BD (Douglas, McGuire, Manzardo, & Butler, 2016; Stahl et al., 2019). Moreover dysfunctions in other ADCYs (ADCY1 and ADCY5) have been previously identified to result in aberrant behavioural phenotypes; this makes this SNP particularly interesting (Bosse et al., 2015; H. Kim et al., 2017; Sethna et al., 2017). Additionally, since ADCY2 is the second most abundant ADCY in the brain, it might play a significant role in various regulatory mechanisms in the brain. This is also suggested by the fact that human and

mouse ADCY2 share 95% homology, i.e. the SNP is conserved between species. Mühleisen and colleagues (2014) hypothesized that the SNP of interest might result in a functional variance in ADCY2, however the exact effects of this SNP on protein function and its implication in BD phenotypes have not been investigated in details.

1.6. The adenylyl cyclase family

Adenylyl cyclases are enzymes involved in the conversion of ATP into cyclic AMP (cAMP). cAMP acts as a second messenger molecule that targets various different downstream signalling pathways in cells. At a cellular level, cAMP is known to regulate growth and differention (Borland, Smith, & Yarwood, 2009). cAMP signalling is involved in many different mechanisms including altering gene expression patterns, regulating hormone levels and learning and memory (Szaszak, Christian, Rosenthal, & Klussmann, 2008).

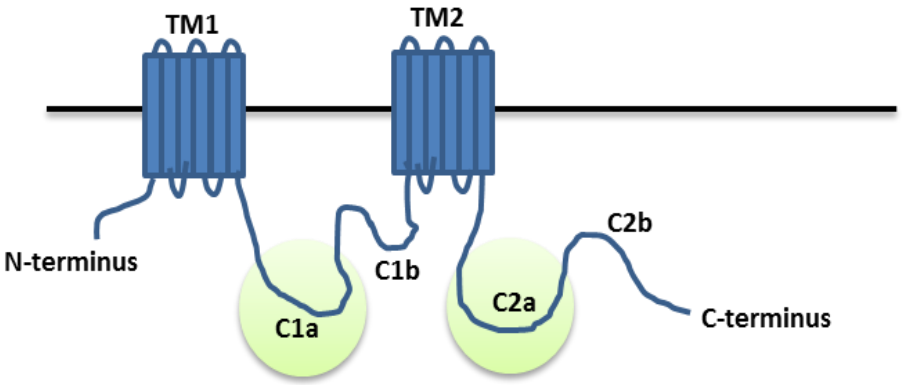


Figure 3. Structure of membrane bound adenylyl cyclases (ADCYs). ADCYs consist of two hydrophobic transmembrane domains (M1 and M2). Each of these domains consists of 6 transmembrane spans linked by an intracellular domain containing the C1a and C1b regions. The C-terminus contains the second cytosolic domain consisting of the C2a and C2b regions. The highly conserved C1a and C2a regions (green) associate to form the catalytic cleft. The sequences of the N-terminus, C1b and C2b can vary. ATP and agonists like forskolin act on the formed synaptic cleft. (Modified from Halls M.L. and Cooper D.M., 2017)

There are 10 mammalian isoforms of adenylyl cyclase and 9 of these isoforms (ADCY1 to ADCY9) are membrane bound. Membrane bound adenylyl cyclases consist of two transmembrane domains which compromises of six transmembrane spans each, and two cytosolic domains. This results in a pseudosymmetrical protein. The two cytoplasmic domains come together to form the catalytic cleft, which is subject to different mechanisms of intracellular regulation, depending on the isoform. Both the cytoplasmic domains are therefore necessary for catalytic activity, as well as for interaction with the agonist forskolin

(Fig 3.) (Halls & Cooper, 2011; Hanoune & Defer, 2001). While the nine isoforms are relatively conserved at the catalytic domain (around 60%), they diverge more in other regions (Halls & Cooper, 2011).

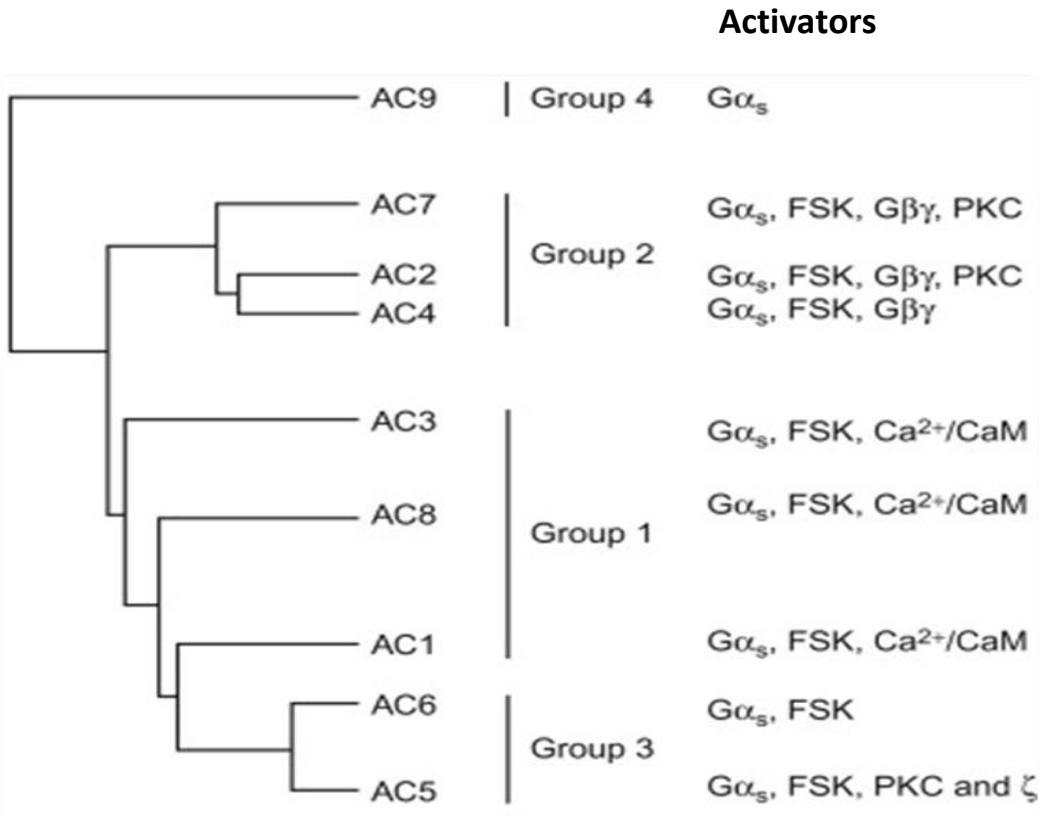


Figure 4. Phylogenetic tree of mammalian adenylyl cyclase. Isoforms closer to each other have similar functions and thus are place in the same functional group (1-4). The activators of each of these ADCYs are listed on the right. (Modified from Patel. et al., 2001)

Even though there are 9 different isoforms of ADCYs, each of them has a unique expression pattern (Table 4.). (Defer, Best-Belpomme, & Hanoune, 2000). The regulatory properties of these ADCYs also vary substantially as each of these isoforms can be activated in various different ways, including different G-proteins, calmodulin, calcium, protein kinases etc. ADCYs that are phylogenetically more closely related have similar activators and mechanisms of actions (Fig. 4) (Hanoune & Defer, 2001; Patel, Du, Pierre, Cartin, & Scholich, 2001).

	Brain	Heart	Kidney	Lung	Liver	Adrenal	Uterus	Skeletal Muscles	Testis
Adcy1	XX	-	-	-	-	X	-	-	-
Adcy2	XX	X	-	X	-	-	X	XX	-
Adcy3	X	X	-	X	-	-	X	-	X
Adcy4	X	X	X	X	X	-	X	-	-
Adcy5	X	XX	X	X	X	X	X	-	X
Adcy6	XX	XX	XX	XX	XX	X	X	X	X
Adcy7	X	X	X	X	X	-	X	XX	X
Adcy8	XX	X	-	XX	-	X	X	-	X
Adcy9	XX	X	X	XX	X	X	X	XX	-

Table 4. mRNA expression pattern of mammalian adenylyl cyclases in general. The x represents the organs that express each Adcy, xx represents organs with relatively higher expression of each Adcy and – represents organs with no expression of each Adcy. (modified from Defer N.. et al., 2000)

The effect of differences between the different isoforms at the transmembrane has not been studied so far(Halls & Cooper, 2011). For ADCY8 it has been shown that the proper interaction between the two transmembrane domains is required for proper trafficking and the correct functional assembly of the catalytic cleft (Gu, Cali, & Cooper, 2002). While the classical models of ADCY action assumes that ADCYs do not dimerize, it has been previously demonstrated for ADCY5, ADCY6 and ADCY8 that transmembrane regions can also dimerize to enhance its assembly, activity and trafficking (Ding, Gros, Chorazyczewski, Ferguson, & Feldman, 2005; Gu et al., 2002).

Plasma membranes are heterogeneous due to differences in their lipid components. Parts of the membrane often form lipid rafts, which are sections of the membrane having a higher concentration of cholesterols, phospholipids with unsaturated side-chains and gangliosides and proteins like caveolin (Pike, Han, Chung, & Gross, 2002). Lipid raft separation and immunoblot analysis shows that ADCY3, ADCY5, ADCY6 and ADCY8 are present in lipid rafts, while ADCY2, ADCY4 and ADCY7 are not present (Ostrom & Insel,

2004). It has been shown that ADCY5 can bind to caveolin and this inhibits ADCY5 activity. Additionally, a binding peptide from the caveolin-1 domain inhibits the localization and activity of ADCY5 and ADCY3, but not ADCY2. However, peptides from caveolin-2 domains did not elicit a similar effect. This selective partitioning and activity regulation of ADCYs in lipid rafts might play a role in the compartmentalization of cAMP signalling (Ostrom & Insel, 2004). Furthermore, ADCY 5, ADCY 6 and ADCY8 have also been shown to be associated with actin cytoskeleton (Ayling et al., 2012; Head et al., 2006). While the mechanism for ADCY trafficking is still not fully understood, it has been suggested that these interactions of ADCYs with lipid rafts and cytoskeleton might play a role in trafficking.

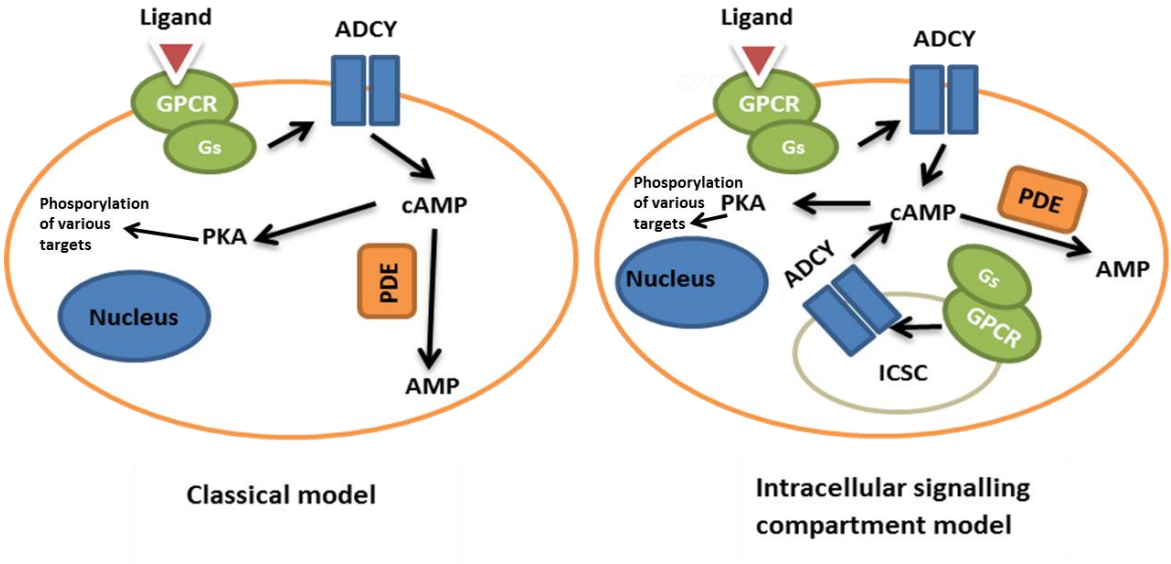


Figure 5. Intracellular signalling compartment model. The classical model assumes that the ADCY remains at the plasma membrane and ATP, cAMP and PDE freely diffuse in the cytosol. The intracellular signalling compartment model assumes that the GPCR, the bound ligand, as well as the ADCY are internalized where they continue to signal. (Modified rom Calebiro et al, 2009)

The localization of ADCYs only in the plasma membrane has brought up the question of how cAMP can reach different parts of the cell. The presence of phosphodiesterase (PDE) in the cytoplasm, which breaks down cAMP makes the fact that cAMP can still reach various parts of the cell illusive (Houslay & Milligan, 1997). One hypothesis is that ADCYs are internalized with the upstream GPCR and continue to produce cAMP inside the cytoplasm of cells. Another study provide some evidence of the internalization of thyroid stimulating hormone receptor post stimulation, along with the Gαs and ADCY (Calebiro et al., 2009). They proposed an intracellular signalling compartment model (Fig. 5). Using mathematical

modelling, they also demonstrated that such a model of intracellular signalling compartments will more closely mimic the cAMP dispersion within the cell than the classical model of membrane bound ADCYs. Additionally, immunohistochemical studies detect copious amounts of ADCY in the cytosolic region supporting such a model (Bogard, Adris, & Ostrom, 2012). However, another theory of cAMP diffusion claims that GPCR activation leads to the coupling of β-arrestin with GPCR. This is in turn leads to the internalization of β-arrestin, which activates the ERK pathway. The ERK pathway in turn leads to the phosphorylation and inhibition on PDE. This would stop cAMP from breaking down, and would help it diffuse to different parts of the cell (Baillie & Houslay, 2005). A third hypothesis attributes soluble ADCYs to be the internal source of cAMP (Zippin et al., 2013). Further work is needed to better understand how the diffusion of cAMP occurs throughout cells.

1.7. Adenylyl cyclase type II

Adenylyl cyclase type II (ADCY2), is calcium insensitive, membrane-bound mammalian ADCY isoform. It is encoded by the ADCY2 gene in chromosome 5 in humans. It is usually activated by $G_{\beta\gamma}$ (βγ subunit of G protein coupled receptors) as well as $G_{\alpha s}$. When GPCRs are activated, the $G_{\beta\gamma}$ activates ADCY2. Once activated, it is involved in the conversion of ATP into cAMP and pyrophosphate (Stengel et al., 1992). The cAMP acts as a second messenger and activates a large number of downstream targets, including the activation of protein kinase A and the transcription factor cAMP response element-binding protein (CREB). CREB in turn alters the expressions of various target genes (Fig. 6). ADCY2 activation is also known to increase interleukin 6 productions (Ding et al., 2004).

Like other group 2 ADCYs, ADCY2 is stimulated by the Gβγ subunits of the G proteins; as long as the active α subunit (Gsα) of the stimulatory GTP binding protein Gs is also present (Feinstein et al., 1991). Adcy2 is also activated by phosphorylation via PKC. Therefore, in addition to forskolin, the PKC activator: Phorbol 12-myristate 13-acetate (PMA), can also be used to pharmacologically activate ADCY2. Similarly, PKC inhibitors like staurosporine, can reduce PKC mediated ADCY2 activation (Jacobowitz & Iyengar, 1994). Like all other membrane bound ADCYs, p-site inhibitors, which are adenine nucleoside 3’

polyphosphates, inhibit ADCY2 activity in a non-competitive manner (Dessauer & Gilman, 1996).

Adcy2 is the second most abundant ADCY isoform in the brain. It is highly expressed in the cortex, the amygdala complex, the hippocampus, the thalamic reticular nucleus and the striatum (Sanabra & Mengod, 2011). This pattern of expression is different from the expression patterns of other ADCYs in the brain, suggesting that ADCY2 might have a unique function (Furuyama, Inagaki, & Takagi, 1993).

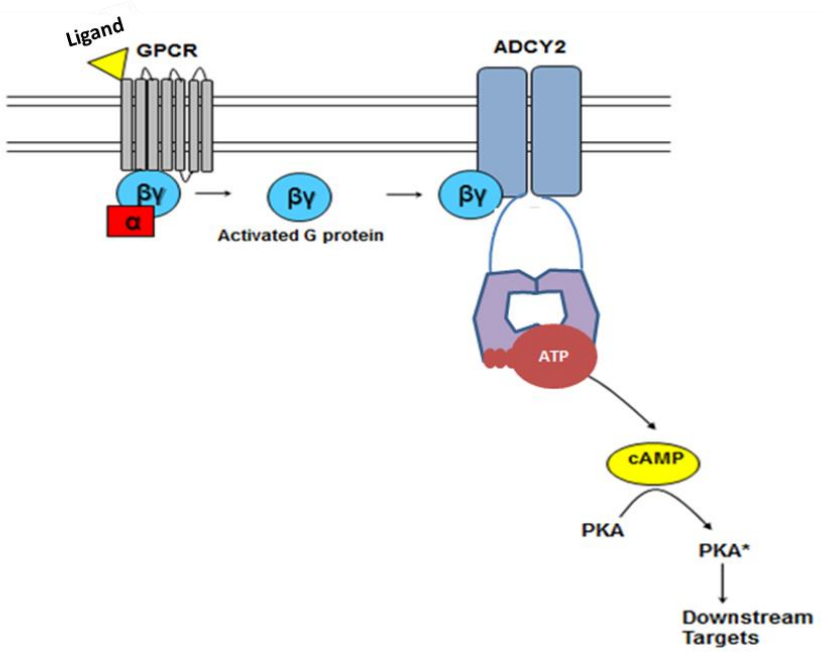


Figure 6. Signalling mechanism of adenylyl cyclase type II. When a G protein coupled receptor (GPCR) is activated by ligand, the $G_{\beta\gamma}$ subunit of the GPCR activates ADCY2, which in turn converts ATP into cAMP and pyruvate. cAMP has various downstream targets including protein kinase A (PKA) and cAMP response element-binding protein (not shown)

Adcy2 is also expressed in relatively lower amounts in the lung and skeletal muscle. In addition to its association with BD, polymorphism in the ADCY2 gene has been associated with chronic obstructive pulmonary disease in one association study (Hardin et al., 2012). However, the functional consequences of this SNP are unknown.

The identified BD associated SNP: rs13166360, in ADCY2 is located in the first transmembrane domain at the fourth transmembrane span (Muhleisen et al., 2014). The ADCY2 locus also carries several other BD-associated non-coding SNPs, which are in linkage

disequilibrium (Stahl et al., 2019; van Hulzen et al., 2017). Rs13166360 lies in exon 3 coding for amino acid number 147 in the human adenylyl cyclase 2 (ADCY2) proteins and causes a missense mutation. It results in an amino acid substitution at this position from a Valine to a Leucine. This results in a slightly less hydrophobic Leucine in the minor T allele instead of a slightly more hydrophobic Valine as in the major G allele (Biswas, DeVido, & Dorsey, 2003). Several other non-coding Adcy2 SNPs

While the effects of SNPs in the transmembrane domains of ADCYs are not well studied, it has been previously shown that improper interaction of these transmembrane domains could affect ADCY trafficking as well as the catalytic cleft formation (Gu, Sorkin, & Cooper, 2001). These deficits might in turn alter the protein activity. (Ding et al., 2005; Gu et al., 2002).

Understanding the impact of this SNP on protein function and any aberrant mechanisms underlying potential changes in protein activity *in-vitro* would help elucidating the effect of this SNP at a molecular level. Furthermore, since this SNP is associated with BD, studying how this SNP impacts behaviour in an animal model would help better understand its role in the development of BD. Unlike other BD associated SNPs, which are also associated with other psychiatric disorders like schizophrenia, this SNP has only been associated with BD so far. This makes the Adcy2 SNP particularly interesting, as understanding its effect would help us elucidate aberrant mechanisms specific to BD.

Since mice and humans share a 95% homology in the ADCY2 protein, a mouse model would help to better understand the effect of this SNP in the context of BD. One issue of using a mouse model is the fact that they typically only study a complete loss-of-function, reduced function and gain-of-function of a certain locus of interest (Ellegood et al., 2014; Horev et al., 2011; Niwa et al., 2010; Sigurdsson, Stark, Karayiorgou, Gogos, & Gordon, 2010) However, the new Crispr/Cas9 technology allows the rapid introduction of a conserved SNP into the mouse genome (Doetschman & Georgieva, 2017; Mianne et al., 2017). Using such technology to insert disease-associated point mutations, like rs13166360, would help scientists and clinicians better understand the role of such minor allele in the development of the disease.

2. Aims

Previous GWA studies have demonstrated a genome-wide significant association of the SNP rs13166360 in the *ADCY2* locus with BD. However, it is currently unclear how this SNP might be causally involved in the development of BD. Therefore, the aim of this thesis was to address the impact of the SNP on protein function and its potential role in the development of BD-related phenotypes in mice. In particular, the following questions had to be addressed:

1. Is there a direct effect of rs13166360 on ADCY2 protein function?
2. If yes, what is the mechanism underlying any difference in protein function?
3. What is the effect of this SNP on mouse behaviour under basal housing conditions?
4. What is the effect of this SNP in conjunction with exposure to chronic stress?

As there is no good antibody against Adcy2 available, we constructed vectors carrying the wild-type HA-tagged Adcy2 and HA-tagged Adcy2 carrying rs13166360. We then explored the effect of this SNP on cAMP production *in-vitro* using an end-point measurement assay as well as a time course assay. Moreover, we explored any differences in localization or trafficking of Adcy2 using immunohistochemistry.

Furthermore, using the Crispr/Cas9 technology we created a mouse model carrying the disease-associated SNP rs13166360. We tested these mice for various endophenotypes of BD under basal housing conditions. These included tests for anxiety-related behaviour, stress coping behaviour, social behaviour and cognition. Finally to test for the gene x environment interaction we investigated these with regards to behavioural consequences following exposure to chronic stress.

3. Materials and Methods

3.1. Materials

3.1.1. Buffers for agarose gel electrophoresis

1 x Tris acetate EDTA (TAE) buffer
4.84 g Tris(hydroxylmethyl)-aminomethane (TRIS)
1.142 ml Acetic acid
20 ml 0.05 M Ethylendiaminetetraacetate (EDTA), pH 8.0
800 ml H_2Obidest
adjust pH to 8.3 with acetic acid
ad 1 l H_2O bidest

6 x DNA loading buffer (orange)
1 g Orange G
10 ml 2 M TRIS/HCL, pH 7.5
150 ml Glycerol
ad 1 l H_2O_{bidest}

3.1.2. Solutions for InSitu Hybridization:

H_2O-DEPC
1 ml Diethylpyrocarbonate (DEPC) (Sigma-Aldrich)
add 1 l H_2O_{bidest}
2 x autoclave
10 x Phosphate buffered saline (PBS)
1.37 M NaCl
27 mM KCl
200 mM Na_2HPO_4 x 12 H2O (Merck, Darmstadt, Germany)
20 mM KH_2PO_4 (Merck)
pH 7.4
add 1 ml DEPC/l, ad H_2O_{bidest}
incubate overnight (o.n.), 2 x autoclave

20% Paraformaldehyde (PFA)
20% w/v Paraformaldehyde
in 1 x PBS-DEPC
pH 7.4

10 x Triethanolamine (TEA)
1.0 M TEA

pH 8.0
add 1 ml DEPC/l, ad H_2O bidest
incubate o.n., 2 x autoclave

20 x Standard saline citrate (SSC)
3 M NaCl
300 mM Sodium citrate
pH 7.4
add 1 ml DEPC/l, ad H_2O bidest
incubate o.n., 2 x autoclave

Hybridization mix (hybmix)
50 ml Formamide
1 ml 2 M TRIS/HCl, pH 8.0
1.775 g NaCl
1 ml 0.5 M EDTA, pH 8.0
10 g Dextran sulphate
0.02 g Ficoll 400
0.02 g Polyvinylpyrrolidone 40 (PVP40)
0.02 g Bovine serum albumin (BSA)
5 ml tRNA (10 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany)
1 ml carrier DNA (salmon sperm, 10 mg/ml, Sigma-Aldrich)
4 ml 5 M dithiothreitol (DTT, Roche)
store as 1 to 5 ml aliquots at -80°C

Hybridization chamber fluid
250 ml Formamide
50 ml 20 x SSC
200 ml H_2O bidest
5 M DTT/DEPC
7.715 g DTT
4 ml H_2O-DEPC
shake falcon tube until the powder is nearly solved
ad 10 l H_2O-DEPC

5 x NTE
146.1 g NaCl
50 ml 1 M TRIS/HCl, pH 8.0
50 ml 0.5 M EDTA, pH 8.0
add 1 ml DEPC/l, ad 1 l H_2O bidest

3 M NH_4OAc
3.0 M Ammonium acetate (NH4OAc)

ad H_2O $_{bidest}$

Alcohol dehydration solutions

Alcohol (dehydration)-solutions alcohol conc.	vol. of EtOH 100 % (ml)	vol. of 3 M NH4OAc (ml)	$H2O_{bidest}$ (ml)
30% EtOH/ NH_4OAc	150	50	300
50% EtOH/ NH_4OAc	250	50	200
70% EtOH/ NH_4OAc	350	50	100
96% EtOH	480	0	20
100% EtOH	500	0	0

Oligonucleotides for generation of Riboprobes

Name	**Sequence**
Adcy2ISH F	5'-gcgtcataggggctcaga-3'
Adcy2ISH R	5'-aatggatggcaacgtgct-3'
SP6	5'-atttaggtgacactatag-3'
T7	5´-taatacgactcactataggg- 3´

3.1.3. Immunohistochemistry solutions:

Blocking solution
5 % BSA
0.1 % Triton

Solution for antibodies
5 % BSA
0.01% Triton

3.1.4. Reagents for Bacteria Culture:

Lysogeny broth (LB) medium
1% (w/v) bacto-tryptone (BD, Heidelberg, Germany)
0.5% (w/v) bacto-yeast-extract (BD)
1.5% (w/v) NaCl
pH 7.4 with NaOH (Karl Roth)
autoclave

LB agar plates

1% (w/v) bacto-tryptone
0.5% (w/v) bacto-yeast-extract
1.5% (w/v) NaCl
1.5% (w/v) bacto-agars (BD)
pH 7.4 with NaOH
autoclave

3.1.5. Solutions for Western Blotting:

RIPA Buffer
50 mM Tris HCl, pH 8.0
150 mM NaCl
1% NP-40
0.5% sodium deoxycholate
0.1% SDS

10 x TBS
200 mM TRIS/HCl
1.35 M NaCl
ad H_2O bidest
pH 7.6

TBS/T
100 ml 10 x TBS
1 ml Tween20 (BioRad)
ad 1 l H_2O bidest

Blocking solution and solution for antibodies
5% Milk Powder (Applied Biosystems)
0.1 % Tween-20
ad 1 x PBS
pH 7.4

Medium stripping buffer
15 g glycine
1 g SDS
10 mL Tween 20

Lämmli 4x
40 % Glycerol (v/v)
0.25 M TRIS/HCl
8 % SDS

0.008 % Bromphenoleblue
ad H2Obidest
add 7.5 % ß-mercapto-EtOH prior to use.

Running buffer
25 mM TRIS/HCl
190 mM Glycine
0.1 % Sodium dodecyl sulfate (SDS)
ad H_2O bidest

3.1.6. Reagents for genotyping:

Oligonucleotides for genotyping

Name	Sequence	Amplicon
Adcy2 Fw	5’-ccaccgccaatgcttcctgc-3’	323 bp
Adcy2 Rw	5’-aacaggtgctccttggcccc-3’	

3.1.7. Reagents for cloning:

Oligonucleotides for cloning

Name	Sequence	Amplicon
ADCY2 cloning F1	5’-aggcggtaggtgaccaca-3’	4037 bp
ADCY2 cloning R1	5’-gggcactagacaccaaagtacg-3’	
ADCY2NestedCloning F	5’-gcggtaggtgaccacagag-3’	3570 bp
ADCY2NestedCloning R	5’-actactctgcaagggctcca-3’	

Oligonucleotides for mutagenesis

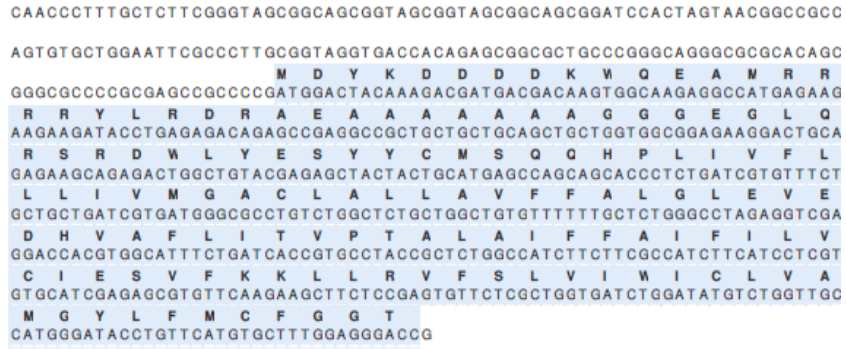
Name	Sequence
g210c1	5'-gggaagcatggtgtacagcacgaagatgatgaaga-3'
g210c	5'-tcttcatcatcttcgtgctgtacaccatgcttccc-3'
MoreActiveAC2 Fw	5'-cgtcataggggctcagaacccacaatatgacatct-3'
MoreActiveAC2 Rv	5'-agatgtcatattgtgggttctgagcccctatgacg-3'

Oligonucleotides for qRT-PCR:

Name	Sequence
AC1 HSA-F	5’-tgaacatgcgtgtgggtctg-3’
AC1 HSA-R	5’-ggccaaggtcacatcattgg-3’

AC2 HSA-F	5’-atgtggcaggaggcgatg-3’
AC2 HSA-R	5’-ggctcatgcagtagtaggactcg-3’
AC3.1 HSA-F	5’-gcttcatgcggctgactttc-3’
AC3.1 HSA-R	5’-aagaccaccagcaccagcag-3’
AC3.2 HSA-F	5’-tcctctacctgtgcgccatc-3’
AC3.2 HSA-R	5’-ttcatcttcacctccagcgact-3’
AC4 HSA-F	5’-gatgcgtccatcactgctga-3’
AC4 HSA-R	5’-aggggtggaggtggacacag-3’
AC5 HSA-F	5’-ctgtctgtccttccccgtca-3’
AC5 HSA-R	5’-tcacgttgtcatgtttctggatg-3’
AC6 HSA-F	5’-gctgctgttcctctgcacca-3’
AC6 HSA-R	5’-ctggatgtaaccgcgggtct-3’
AC7 HSA-R	5’-tgaccaagatgcgctctacga-3’
AC7 HSA-F	5’-ggctgaaggcaatgatgatga-3’
AC8 HSA-F	5’-agtctgctgtccttgcctga-3’
AC8 HSA-R	5’-aagggcagttcagggctcca-3’
AC9 HSA-F	5’-aactcagaacgggctcctca-3’
AC9 HSA-R	5’-ccctgcccaccttcccttct-3’
AC10 HSA-F	5’-tgatcaagaccctggcaaccc-3’
AC10 HSA-R	5’-ggcttcagagacaaggaacga-3’
AC1-RT F	5’-ccttttggtcaccttcgtgt-3’
AC1-RT R	5’-acgccatacatgttcacacc-3’
AC2-RT F	5’-cgagtgttctcgctggtgat-3’
AC2-RT R	5’-tccctcatgttgaagggaag-3’
AC3-RT F	5’-aaacaagatggaggctggtg-3’
AC3-RT R	5’-ttggaggcaatgatgaggt-3’
AC4-RT F	5’-gagccaacagtacccactgc-3’
AC4-RT R	5’-cagagagaagccacccaaag-3’
AC5-RT F	5’-tctccacttggccatctctc-3’
AC5-RT R	5’-gatacactcccgggtctcct-3’
AC6-RT F	5’-cccgtgttcttcgtctaca-3’
AC6-RT R	5’-ttcagcagggtagtgtgtgc-3’
AC7-RT F	5’-acactacatgcccgacaaca-3’
AC7-RT R	5’-ggtcaaacttcccaaacagc-3’
AC8-RT F	5’-ccaggccttcctggagac-3’
AC8-RT R	5’-aggtgctcatcctccacatt-3’
AC9-RT F	5’-catatctgaggccactgcaa-3’
AC9-RT R	5’-acctcaaagccagaaagcag-3’
AC10-RT F	5’-agttcagcacggccatgta-3’
AC10-RT R	5’-ttgctttcgttccactttcc-3’

3.1.8. Synthesized DNA fragment:

Name	Sequence	Company
HA-Adcy2 Fragment		Integrated Device Technology (Munich, Germany)
FLAG-Adcy2 Fragment		Integrated Device Technology (Munich, Germany)

3.1.9. Kits used for study

Item	Company
RNeasy Mini Kit	Qiagen (Hilden, Germany)
QIAquick Spin Miniprep Kit	Qiagen (Hilden, Germany)
QIAGEN Plasmid Midi Kit	Qiagen (Hilden, Germany)
Nanobit Protein Protein Interaction System	Promega (Mannheim, Germany)
cAMP-glo Assay	Promega (Mannheim, Germany)
DIG Nucleic Acid Detection Kit	Roche (Mannheim, Germany)
DIG RNA Labeling Kit (SP6/T7)	Roche (Mannheim, Germany)
TOPO TA Cloning Kit pCRII TOPO	Invitrogen (Darmstadt, Germany)
Quick Change II Mutagenesis Kit	Agilent(Waldbronn, Germany)
TOPO XL PCR Cloning Kit	Thermo Fischer Scientific (Planegg, Germany)
Expand Long Template PCR System	Sigma Aldrich (Taufkirchen, Germany)
SYBR Green I Master	Roche (Mannheim, Germany)
QIAGEN PCR Purification Kit	Qiagen (Hilden, Germany)
QIAGEN Gel Extraction Kit	Qiagen (Hilden, Germany)

Lipofectamine 3000 Kit	Thermo Fischer Scientific (Planegg, Germany)

3.1.10. Enzymes, nucleotides, compounds and nucleic acid

Item	**Company**
T4 DNA Ligase	Roche (Mannheim, Germany)
Taq DNA Polymerase	ABgene (Hamburg, Germany
Proteinase K	Sigma-Aldrich (Taufkirchen, Germany)
T7-, SP6-plymerase (20U/µl)	Roche (Mannheim, Germany)
Oligo$(dT)_{12}$ Primer	Roche (Mannheim, Germany)
DNase I, RNAse free	Roche (Mannheim, Germany)
Restriction enzymes with 10x buffer	New England Biolabs (Frankfurt am Main, Germany)
DNAse I, RNAse	Roche (Mannheim, Germany)
RNAse H	Sigma-Aldrich (Taufkirchen, Germany)
Desoxynucleotides	Roche (Mannheim, Germany)
[α-thio-^{35}S]-UTP	PerkinElmer (Groningen, Netherlands)
DIG RNA labeling mix	Roche (Mannheim, Germany)
XcmI	New England Biolabs (Frankfurt am Main, Germany)
BamHI	New England Biolabs (Frankfurt am Main, Germany)
HindIII	New England Biolabs (Frankfurt am Main, Germany)
XhoI	New England Biolabs (Frankfurt am Main, Germany)
XbaI	New England Biolabs (Frankfurt am Main, Germany)
BbsI	New England Biolabs (Frankfurt am Main, Germany)
NruI	New England Biolabs (Frankfurt am Main, Germany)
NdeI	New England Biolabs (Frankfurt am Main, Germany)
AluI	New England Biolabs (Frankfurt am Main, Germany)
Poly-D-Lysine	Sigma-Aldrich (Taufkirchen, Germany)
Forskolin	Sigma-Aldrich (Taufkirchen, Germany)

3.1.11. Antibodies

Name	**Company**
HA polyclonal Rabbit	Abcam (Cambridge, UK)
FLAG polyclonal Mouse	Sigma-Aldrich (Taufkirchen, Germany)
Alexa 488 α-Mouse	Life Technologies (Darmstadt, Germany)
Alexa 594 α-Rabbit	Life Technologies(Darmstadt, Germany)
HPR α-Rabbit	Cell Signalling (Frankfurt am Main)
Actin polyclonal Rabbit	Cell Signalling (Frankfurt am Main)

3.1.12. Cell culture medium

Name	Company
Dulbecco's Modified Eagle Medium	Thermo Fischer Scientific (Planegg, Germany)
Opti-MEM I Reduced Serum Media	Thermo Fischer Scientific (Planegg, Germany)
Dulbecco's Phosphate-Buffered Saline	Thermo Fischer Scientific (Planegg, Germany)

3.2. Methods

3.2.1. Molecular methods

3.2.1.1. Polymerase Chain Reaction (PCR)

Desired DNA sequences were amplified using PCR using genomic DNA, cDNA or plasmid DNA as templates. For a regular 25 µl PCR reaction 0.5-5 µg genomic DNA, 0.1-1 µg cDNA or 20-200 ng plasmid DNA were used. The reaction was prepared as follows:

x µl template (as stated above)

2.5 µl 10 x reaction buffer IV (ABgene)

1.5 µl 25 mM $MgCl_2$ (ABgene)

0.5 µl 10 mM dNTPs (Roche)

0.5 µl 10 µM primer forward

0.5 µl 10 µM primer reverse

0.25 µl Thermoprime Plus DNA polymerase (5 U/µl, ABgene)

adjust to 25 µl with H_2O_{bidest}

In general, the following standard PCR reaction was carried out: 95°C 5 min, 35 cycles of 95°C for 30 sec, 57-60°C for 30 sec, 72°C for 1 min per 1 kb of DNA, followed by 72°C for 10min and subsequent hold at 4°C. If required, the annealing and elongation temperatures were adjusted for a specific DNA product. PCR products were analysed via gel electrophoresis in a 1-2% agarose gel (1 x TAE), containing ethidium bromide. For this, a small aliquot of the PCR (1-5µl) was mixed with 6x Orange loading dye and loaded onto the gel, which was subsequently analysed with a UV-transilluminator and a BioDoc II gel documentation system from Biometra or the Quantum gel documentation system 1100 from Vilber Lourmat.

3.2.1.2. Generation of Adcy2-V151L mice

Adcy2-V151L mice were generated using CRISPR/Cas9 system as previously described (Brandl C, *et al*., 2014). The CRISPR/Cas9 technology was used to create a mouse line in which Valine at position 151 was substituted by a Leucine resembling the SNP found to be associated with bipolar disorder. The targeting vector contained 2 silent mutations, one that added a restriction enzyme site (AluI) for genotyping purposes and another that obliterated the PAM sequence (NGG) so that the donor DNA is not cleaved by Cas9 (Fig. 2a). Founders derived from zygote injection were screened by PCR and restriction digests. Subsequently the presence of the V151L substitution was confirmed by sequencing. Founders bred to C57BL/6J mice transmitted the mutation to the next generation. Breeding of heterozyous mice resulted in wild-type, heterozygous and homozygous offspring at Mendelian rates

3.2.1.3. Genotyping

For genotyping PCRs of transgenic mice, tail lysates were prepared by digesting tail cuts in 100 µl 50 mM NaOH for 30 min at 95°C. This was followed by a neutralization step using 30 µl 1 M Tris-HCl (pH 7.0) and stored at 4°C. 2 µl of the tail lysates were used as template for PCRs. For genotyping, a PCR reaction was set up as stated above. The PCR reaction was then digested with 1 µl of AluI enzyme and 3 µl of CutSmart Buffer, by incubating this reaction for 1hr at 37°C. After the digest, 15 µl of the reaction was analysed via gel electrophoresis as stated below:

Enzyme Name	**Variant**	**Size**
AluI	Wild Type (WT)	323bp
	Heterozygous (Het)	172bp+152bp+323bp
	Homozygous (Hom)	172bp+152bp

Alternatively for sequencing, the PCR reaction was purified using Qigen PCR Purification Kit according to manufacturer's instructions. The purified PCR fragment was diluted to 1 ng/µl

and 15 µl of this reaction was sent to Eurofins Genomics for sequencing using Adcy2Fw Primer.

3.2.1.4. Preparation of plasmid DNA

Plasmid DNA was prepared using DNA isolation Kits from Qiagen (Mini-, Midi- and Plasmid Maxi-Kit) according to the manufacturer's instructions. For MiniPreps, a single colony was inoculated in 3-5 ml LB medium with a selective antibiotic overnight (o.n.) on a shaker at 37°C. For MidiPreps, a single colony was inoculated in 100 ml LB medium with a selective antibiotic o.n. on a shaker at 37°C. For MaxiPreps, 500 µl of culture from a single colony that was incubated in 200ml of LB medium with appropriate antibiotics o.n at 37°C. MiniPrep, or the appropriate glycerol-stock of bacteria was added to 250 ml LB medium with the appropriate antibiotic and incubated o.n. on a shaker at 37°C.

3.2.1.5. Transformation

Chemically competent DH5α or Stbl3 E. coli bacteria were shortly thawed on ice. 1-5 µl DNA was added to the bacteria and mixed by gently tapping the tube. Bacteria were incubated for 20-30 min on ice. Competent cells were then heat-shocked at 42°C for 45 secs and subsequently put on ice for 2 mins. This enabled the uptake of the plasmid DNA. 1 ml LB medium was added, and the cells were incubated on a shaker at 37°C for 1-2 h. Subsequently, cells were plated on LB plates containing the appropriate antibiotic for selection (100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated overnight at 37°C. Single colonies were picked and inoculated in 5 ml LB medium containing the appropriate selection marker (100 µg/ml ampicillin or 50 µg/ml kanamycin) and grown overnight at 37°C on a shaker at 250 rpm for subsequent DNA preparation.

3.2.1.6. Glycerol stocks

For long-term storage, 750 µl of an overnight bacteria culture was mixed with 250 µl 80% glycerol and frozen at -80°C.

3.2.1.7. Nucleic acid concentration measurement

DNA or RNA concentrations were measured in a UV photometer (Gene Quant II, Pharmacia Biotech). An optical density (OD) of 1 at a wavelength of 260 nm and a cuvette thickness of 1

cm corresponding to the following concentrations: 50 µg/ml double stranded DNA and 40 µg/ml RNA. Consequently, concentrations of nucleic acid is determined by the following equation: X µg/ml = 260 OD x n x f, with f being the dilution factor and n the default 50 µg/ml double stranded DNA and 40 µg/ml RNA.

3.2.1.8. Restriction digest analysis

DNA fragments were digested using New England Biolabs restriction enzymes and buffers by incubating 10 units/µg restriction enzyme, 1X corresponding buffer dissolved in H_2O_{bidest}. The fragments were analysed using a 0.5%-2% Agarose gel in gel electrophoresis. The

3.2.1.9. DNA gel extraction

The QIAquick Gel Extraction Kit was used according to manufacturer´s instructions to purify DNA fragments out of an agarose gel. DNA was eluted in 30-50 µl of $H2O_{bidest}$. The DNA quality was assessed by gel electrophoresis.

3.2.1.10. PCR purification

Amplified PCR products were purified using the Qiagen PCR purification kit according to the manufacturer's protocol. The DNA quality and concentration was measured using the UV photometer as described above.

3.2.1.11. Ligation

50 ng of linearized plasmid backbone was ligated to 3x to 5x molar excess of insert. The appropriate amount of insert was calculated as following: m insert (ng) = (molar ratio) x 50 ng x (bp of insert / bp of backbone vector). T4 DNA ligase buffer and 5 U of T4 DNA ligase were added in a total volume of 10-15 µl, and incubated aver night at 4°C. On the next day, a heat shock transformation using 2-5 µl of the ligation product was performed in chemocompetent bacteria.

3.2.1.12. TOPO TA cloning

For ligation of products obtained from PCR reactions, inserts were cloned into the pCRII-TOPO vector (TOPO TA cloning Dual promoter Kit, Invitrogen), containing Sp6 and T7 promoters for efficient in vitro transcription. PCR products with 3'-A-overhangs were inserted into a linearized pCRII-TOPO vector with single 3'-T-overhangs. The reaction was set up as per

manufacturer's instruction and was then transformed into chemically competent bacteria as described above and plated on LB-agar plates. The plasmid was extracted using MiniPrep kit as described above and verified by restriction digest analysis and sequencing by Eurofin.

3.2.1.13. TOPO-XL PCR cloning

Fragments longer than 1kb were amplified using the Thermo Fischer Scientific Expand Long Template PCR system according to manufacturer's instructions. The PCR product was purified and the concentration was quantified as described above. A poly-A tail was added to the purified DNA fragment using the following reaction:

X mg template
5 µl 10 x reaction buffer IV (ABgene)
3 µl 25 mM $MgCl_2$ (ABgene)
1mM ATP
0.2 ul Thermoprime Plus DNA polymerase (5 U/µl, ABgene)
adjust to 25 µl with H_2O_{bidest}

The reaction was incubated for 20 mins at 72°C. The DNA fragment was then cloned into pCR-XL-TOPO. 2-5 µl of the reaction product used for this reaction and the reaction was set up according to the manufacturer's instructions of Thermo Fischer Scientific TOPO XL PCR Cloning Kit. The reaction was transformed into chemically competent bacteria as described above and plated on LB-agar plates. The plasmid was extracted using MiniPrep kit as described above and verified by restriction digest analysis and sequencing by Eurofins Genomics, Ebersberg, Germany.

3.2.1.14. Site directed mutagenesis

Two complementary oligonucleotides, that contained the desired mutation, and were flanked by unmodified nucleotide sequence, were synthesized using Metabion's primer order service in Planegg, Germany. Using the template plasmid where the mutation should be introduced, and the synthesized oligonucleotides, the reaction was setup according to the manufacturer's

protocol (Quick Change II Mutagenesis Kit, Aigent). The reaction was incubated for 1 min, 95°C, then 18 cycles of 50 sec at 95°C, 50 sec at 60°C, 8 mins at 68°C, then 7 mins at 68°C. Subsequently the reaction was digested with the restriction enzyme DpnI as per the manufacturer's instruction. The completed reaction was transformed into chemically competent bacteria as described above and plated on LB-agar plates. The plasmid was extracted using MiniPrep kit as described above and verified by sequencing by Eurofins Genomics in Ebersberg, Germany.

3.2.1.15. Sequencing

DNA fragments and plasmids were diluted in H_2O_{bidest} to the concentrations specified by Eurofins Genomics (https://www.eurofinsgenomics.eu/). Samples were then sent to Eurofins Genomics for sequencing where the sequencing reactions were carried out

3.2.1.16. Cell culture

Immortalized cells (e.g. HEK293, Cos7 etc.) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 units/mL penicillin G and 100 µg/mL streptomycin, They were incubated at 37°C with 5% CO_2

3.2.1.17. Cellular transfection

Sterilized coverslips were incubated with 300 µl 0.5mg/ml Poly-D-Lysine solution for 7 hrs at 37°C. The cover slips were washed 2X with $H_2O_{bidest.}$ Immortalized cells were then plated on the coated cover slips and incubated at 37°C with 5% CO_2 overnight or till the cover slips reach 80% confluency. The following amount of Optimem, Lipofectamine 3000, P3000 Reagent and plasmid DNA was added to the growth medium according to manufacturer's instructions:

In 500 µl growth medium:

25 µl Optimem

0.5 µg DNA

1 µl P3000

1.5 µl Lipofectamine 3000

The transfected cell was incubated overnight at 37°C with 5% $CO_{2.}$ After 12 hrs the growth medium was changed. Cells were used for further assays or immunohistochemistry after 1 to 2 days of incubation.

3.2.1.18. RNA Isolation and quantitative real time PCR

RNA was isolated either from HEK293 cells transfected with plasmid of interest, from mammalian cells or from murine tissue using the TRIzol protocol (Invitrogen). cDNA was generated from total RNA using Reverse Trancriptase Superscript II (Invitrogen) and oligo-dT primers according to the manufacture's protocol. The synthesized cDNA was utilized as template for quantitative real time PCR, which was carried out in the Lightcycler 2.0 System (Roche) using the QuantiFast SYBR Kit (Quiagen). The mastermix was prepared as follows (volume/sample):

5 µl 5x PCR mix (QuantiFast SYBR Green PCR Mix)

1 µl 10 µM forward primer

1 µl 10 µM reverse primer

1 µl H_2O (Quiagen, Hilden)

8 µl of the mastermix

Subsequently, 2 µl of diluted cDNA (1/10) were added and the capillaries were closed. Samples were centrifuged for 30 seconds before they were placed into the LightCycler. The same PCR settings were chosen for all runs. At the end of every run a melting curve was measured to ensure the quality of the PCR product. The calculations were conducted by the LightCycler®Software 4.05 (Roche). Threshold and noise band values were set to the same level in all compared runs. Relative gene expression was determined with the 2-ΔΔCT method (Livak and Schmittgen, 2001), using the real PCR efficiency calculated from an external standard curve, normalized to the housekeeping genes hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Tata box protein (TBP) related to the data of control experiments.

3.2.1.19. NanoBiT® Assay

Hek293 cells were grown on clear 24 welled plates and transfected as described above with the plasmid CAG-GFP and either pcDNA3.1-V151, pcDNA3.1-L151 or pcDNA3.1-AC2-KO plasmids as well as the LgBiT plasmid and SmBit plasmid that was provided by the manufacturer. The assay was carried out as per the manufacturer's instructions. The

fluorescence and luminescence signals were recorded using TriStar² LB 942 microplate reader. The ratio of luminescence to fluorescence was calculated by dividing each luminescence value with the fluorescence value and multiplying it by 10000

3.2.1.20. cAMP-Glo™ assay

HEK293 cells were grown on clear 24 well plates and transfected as described above with either pcDNA3.1-V151, pcDNA3.1-L151 or pcDNA3.1-AC2-KO plasmids. The cells were then stimulated with various concentrations of forskolin and incubated for 30 mins. The assay was then carried out as per the manufacturer's instructions. The luminescence signals were recorded using TriStar² LB 942 microplate reader (Berthold Technologies Bioanalytic, Baden-Württemberg, Germany).

3.2.1.21. Bradford assay

5x Bradford Reagent (BioRad) was diluted 4:1with water. A 96-welled clear bottomed microwell plate was used for the assay. A BSA stock of 1 mg/mL was created. Then the following concentrations of samples for the standard were created:

Sample 1 (0.0 mg/mL): 0 uL BSA + 30 uL buffer.
Sample 2 (0.2 mg/mL): 6 uL BSA + 24 uL buffer.
Sample 3 (0.4 mg/mL): 12 uL BSA + 18 uL buffer.
Sample 4 (0.6 mg/mL): 18 uL BSA + 12 uL buffer.
Sample 5 (0.8 mg/mL): 24 uL BSA + 6 uL buffer.
Sample 6 (1.0 mg/mL): 30 uL BSA + 0 uL buffer.

Standards were plated in triplicate (10 uL per well) in the 96 welled plate. Column 1 blank was left blank; samples were pipetted from column 2. Samples were plated in triplicate (10 uL per well). Since the standard curve was from 0-1 mg/mL (for BSA), it was ensured that the samples should be in this range. An O.D. reading of the sample was taken and it was generalized that 1 O.D. = 1 mg/mL to get a ballpark value. The samples were diluted appropriately and 200 uL of diluted Bradford reagent was added to each well, and it was incubated at RT for 5 mins. The 595 nm filter was inserted into the TriStar² LB 942 microplate reader (Berthold Technologies Bioanalytic, Baden-Württemberg, Germany) and the values of the standard and the diluted

samples were read. The results were used to plot the standard curve (Axes labeled as y=A, 600 nm and x=mg/mL). The standard curve and the data from bradford assay were used to determine unknown protein concentration.

3.2.1.22. Western blot

Cells were grown and transfected as described above. They were then lysed with 1 ml RIPA buffer containing protease inhibitors (Roche) per 1 well in a 24-well plate. Protein samples were separated by 5% SDS-PAGE and transferred to 0.45-µm PVDF membranes (Millipore). The membranes were then incubated with anti-HA Tag primary antibody and a secondary HRP-conjugated antibody. Chemiluminescence signals were visualized in a ChemiDoc station (BioRad) and analysed using Image Lab (Bio-Rad).

3.2.1.23. Immunohistochemistry (IHC)

Immortalized cell lines were grown on sterilized cover slips and transfected with specific plasmids as described above. The cells were then incubated with 4% PFA (w/v) in PBS, pH 7.4, for 20 mins in order to fix the cells. The cells were then washed 3 x with PBS and permeabilized with PBS-TritonX-100 0.1% for 30 mins at RT. Cells were washed 3 x PBS-TritonX-100 0.01% and blocked at RT for 1 h in 5% BSA (w/v) in PBS-TritonX-100 0.01%. The cells were then incubated o.n. at 4°C with the primary antibody, which was diluted in 5% BSA (w/v) in PBS-TritonX-100 0.01%. After a 3 x 10 min washing step with PBS-TritonX-100 0.01%, the selected secondary antibody diluted in PBS-Triton 0.01%, was added and incubated for 2 h at RT. Cells were washed 3 x with PBS, stained with DAPI and mounted with anti-fading fluorescence VectaShield medium (Vector Labs). For Phalloidin staining, Thermo Fischer Scientific's Alexa Fluor™ 488 Phalloidin was used. The Phalloidin was diluted in DMSO to create the stock solution as per manufacturer's instruction. 0.1 µl of stock solution per 100 µl PBS-Triton 0.01%, containing secondary antibody, was diluted. This solution was used for staining for secondary antibody as described above. The steps following secondary antibody staining, as described above, were also carried out for cells stained with Phalloidin.

3.2.1.24. Image Acquisition and Analysis

Images were taken using a Zeiss confocal microscope (LSM800) and digitalized using Zeiss Zen Microscope software and Fiji's Image J. Image analysis were carried out using Fiji's Image J and Image J plugin JACoP.

3.2.1.25. Brain sectioning and *In situ* hybridization (ISH)

ISH was performed as previously described (Refojo et al., 2011). Male mice (age 8-12 weeks) were anaesthetized with isoflurane (Floren®, Abbott) and sacrificed by decapitation. Brains were immediately dissected, and shock frozen on dry ice. Frozen brains were cut on a cryostat in 20-µm thick sections and mounted on SuperFrost Plus slides. Slides were stored at -20°C until further processing. Before pretreatment, slides were thawed approximately for 30 min at RT. Fixation and pretreatment of slides were performed at room temperature as per the following:

Steps	Duration (mins)	Solutions	Comment
1. Fix	10	4% PFA in 1x PBS / DEPC	On ice
2. Wash	2 X 5	1x PBS / DEPC	
3. Acetylate	10	0.1M triethanolamine-HCl in 1 TEA, pH 8.0	Add freshly 600 µl acetic anhydride per 200 ml TEA and stir
4. Wash	2 X 5	2x SSC / DEPC	
5. Dehydrate	1 1 1 1 1 1	60% Ethanol/DEPC 70% Ethanol/DEPC 96% Ethanol/DEPC 100% Ethanol CHCl3 100 % Ethanol	
Air dry slides in a dust free, RNase-free area			

Specific riboprobes were generated by PCR applying T7 and SP6 primers using plasmids containing the required template cDNA (Table 9). Radiolabeled sense and antisense cRNA

probes were generated from the respective PCR products by in vitro transcription with 35S-UTP (PerkinElmer) using T7 and SP6 RNA polymerase. After 20 min of DNase I (Roche) treatment, the probes were purified using the RNeasy-Mini Kit protocol (Qiagen) and measured in a scintillation counter. The sections were hybridized overnight at 57°C with a probe concentration of 7 x 106 counts per minute (cpm)/µl. Subsequently they were washed at 64°C in 0.1 x saline sodium citrate (SSC) and 0.1 mM dithiothreitol. The washing steps were carried out as follows:

Step	Temperature (°C)	Duration (mins)	Solutions	Comment
1.Wash	RT	4 X 5	4 X SSC	
2.RNAse	37	20	1x NTE / 500 µl RNaseA (20 µg / ml)	Add RNase freshly
3.Wash	RT	2 X 5	2x SSC / 1mM DTT	Add DTT freshly
4.Wash	RT	10	1x SSC / 1mM DTT	Add DTT freshly
5.Wash	RT	10	0.5x SSC / 1mM DTT	Add DTT freshly
6.Wash	64	2 X 30	0.1x SSC / 1mM DTT	Add DTT freshly
7.Wash	RT	2 X 10	0.1 X SSC	
8.Dehydrate	RT	1 1 1 1 2 x 1	30% Ethanol / 300mM NH4OAc 50% Ethanol / 300mM NH4OAc 70% Ethanol / 300mM NH4OAc 95% Ethanol 100% Ethanol	
Air dry slides in a dust free area				

In order to visualize hybridization signals, dried slides were exposed to a special high performance X-ray film (Kodak, BioMax) for different time intervals.

3.2.2. Behavioural tests

3.2.2.1. Animals Housing Conditions

All animal experiments were conducted in accordance with the Guide of the Care and Use of Laboratory Animals of the Government of Bavaria, Germany. Mice were group housed in IVC cages under standard laboratory conditions (22 ± 1°C, 55 ± 5% humidity) and were maintained on a 12 h light-dark cycle (lights on between 6:00 a.m. and 6:00 p.m.) with food and water ad libitum.

3.2.2.2. Animal handling prior to and during behavioural tests

At weaning mice were numbered by ear-punching, and a small tail-tip cut was taken for genotyping. Animals were transported at least four days prior to the behavioural testing into racks near the experimental rooms. Mice were single housed two days prior behavioural testing. Animals were monitored for scars/ wounds prior to behavioural testing and cages were not changed during behavioural testing weeks. The old bedding was replaced with fresh bedding when the bedding became dirty during the behavioural testing weeks.

3.2.2.3. Open Field (OF)

This test was used to assess differences in locomotion as well as anxiety-related behaviour. The open field boxes (50 x 50 x 60 cm) were made up of grey polyvinyl chloride (PVC) and evenly illuminated (10-15 Lux). The apparatus was virtually divided into an outer an inner zone (15 x 15 cm). All mice were placed into a corner of the apparatus at the beginning of the trial. The test duration varied between 10-30 min depending on the experimental setup. Parameters assessed included the total distance travelled, immobility time, number of inner zone entries and inner zone time.

3.2.2.4. Dark-light box (DaLi)

The dark-light box test also assesses anxiety-related behavior. The apparatus consisted of a secure black PVC compartment (15 x 28 x 27 cm) and an aversive, illuminated white PVC

compartment (48 x 28 x 27 cm), which are connected by a small tunnel (5 x 7 cm). The illumination in the dark compartment was < 5 lux and 800 lux in the lit compartment. Animals were placed into the tunnel facing the illuminated compartment and allowed to freely explore the test arena for 5 min. Parameters assessed included time spent in the lit compartment, number of lit compartment entries and time spent at the entrance of the lit compartment.

3.2.2.5. Elevated plus maze (EPM)

The elevated plus-maze is used to assess anxiety-related behavior in mice. The EPM setup was made of grey PVC and consisted of a plus-shaped platform, which is elevated 50 cm above the floor, with four intersecting arms. Two opposing open (30 x 5 x 15 cm) and closed arms (30 x 5 x 15 cm) are connected by a central zone (5 x 5 cm). The illumination was 30 lux in the open arms and < 10 lux in the closed arms. Animals were placed in the center of the apparatus facing the open arm and allowed to freely explore the maze for 10 min. Parameters measured included time spent in the open arms, open arm entries and total distance travelled.

3.2.2.6. Novel object exploration

This test investigates exploratory behaviour of a novel object in a familiar environment, which is considered to reflect bipolar disorder associated traits (Perry et., 2009). In an initial open field trial, mice were first allowed to habituate to an OF arena (50 x 50 x 60 cm high, 10 lux) for 10 min. Subsequently a novel object made of Lego pieces object was introduced on one side of the arena and the animals were allowed to explore the object for an additional 15 min (object trial). The distance travelled as well as the time spent in the zone containing the object were measured in both trials. Additionally, the time spent interacting with the object was assessed.

3.2.2.7. Social interaction test

This test was used to assess if the sociability of the animals of the two genotypes differed. The apparatus is evenly illuminated with 10 lux. Each animal was placed into the OF arena (50 x 50 x 60 cm high, 10 lux), which it was previously habituated to, with a novel conspecific of the same genotype for 10 mins. The time the two mice spent interacting with each other was measured manually.

3.2.2.8. 3 Parted chamber test

The apparatus contained a three chamber grey PVC box (50 x 25 cm) with one center and two outer chambers (left and right chamber 19 x 25 x 40 cm; center chamber 12 x 25 x 40 cm). Two small openings with doors served as access points from one chamber to the other. The apparatus was filled with bedding and evenly illuminated with 10 lux. The paradigm consists of three separate stages.

First stage:

In the first stage the animals were habituated to this setup for 15 mins, when they were allowed to freely explore the arena.

Second stage:

In the second stage, a novel adult conspecific was placed in a wire mesh cup in the left chamber and an empty wire mesh cup was placed on the right chamber. This stage also lasted 15 mins and the number of entries into the left and the right chamber was measured, as well as the time spent sniffing the left and the right chamber was measure. The ratio between the time sniffing the cup containing the novel conspecific and the time spent sniffing the empty cup was measured to assess sociability.

Third stage:

In the third stage of the test, the mouse previously placed in the left chamber was now placed in the right chamber. A novel adult conspecific was placed in the left chamber in a wire mesh cup. This stage also lasted 15 mins and the amount of time the test mice spent in the left and the right chamber was measured. Additionally, the amount of time the test mice spent sniffing the known conspecific on the right chamber and the unknown conspecific in the left chamber was measured. Ratio of the amount of time the test mouse spent sniffing the novel conspecific to the amount of time it spent sniffing the old conspecific was calculated to access their preference for social novelty

3.2.2.9. Marble burying test

Fresh home cages of animals were filled with bedding and 10 marbles were placed on the bedding in an equally spaced manner. This test was also carried out with a illumination of 10 lux. The mice were placed on the centre of the cage and a lid partially covered the apparatus to prevent the mice from escaping. The mouse was left in the apparatus for 90 mins and the number of marbles that the mouse buried in the 90 mins was counted.

3.2.2.10. Amphetamine induced hyperlocomotion

The OF arena (50 x 25 cm) was placed under 10 Lux lighting condition. Mice were habituated to the arena for 30mins by letting them freely explore the arena for 30 mins. The next day the mice were introduced to the arena for 10 mins were they were allowed to freely explore the arena once more. They were then injected with Amphetamine (2-4mg/kg of body weight) or saline solution and placed back in the OF arena where they were allowed to freely explore for the next 50 mins.

3.2.2.11. Forced swim test (FST)

The forced swim test was used to assess active versus passive stress-coping as previously described in the literature (Slattery & Cryan, 2012). Each animal was placed into a two liter glass beaker (radius: 11 cm, height; 23.5 cm) filled up to a height of 15 cm with tap water. The water beakers were left o.n. so that the water temperature reached room temperature at 25°C Three behavioural parameters were assessed for 6 min including time struggling, swimming and floating. A mouse was considered to be floating once it stopped any movements expect those that were necessary to keep its head above water. Vigorous swimming movements involving all four limbs of the mouse, with the front paws breaking the surface of the water, usually at the walls of the beaker, were regarded as struggling. Behaviour involving movement of only two limbs in a goal directed manner, was considered swimming.

3.2.2.12. Morris water maze

A gray PVC cylinder with a diameter of 150 cm and a height of 60 cm is filled with water until the water is 30 cm high. The cylinder is divided into east, west north and south quadrants. A transparent PVC platform of 10cm x 28cm is submerged just beneath the water at the southwest quadrant. The four walls of the test room have four different patters hung from the wall to help the mice orient themselves while swimming in the apparatus. The test has

three separate stages and all the stages are carried out in the apparatus, which is evenly illuminated with 10 lux:

Training phase:

In the training phase, each mouse is released at different quadrants of the cylinder in a randomized order and trained 4 times each day over 5 days in 3 mins sessions to find the platform in the southwest quadrant. The inter trial interval each day was 1hr. Once the mouse reaches the platform in the southwest, the mouse is removed from the quadrant, dried and placed back in its home cage. Parameters measured include the latency to reach the platform and the distance travelled.

Probe Trial:

This phase lasts one minute and it is performed the day after the last training day. The platform in the southwest quadrant is removed from the cylinder and the mouse is released from the northeast quadrant of the cylinder. The mouse is allowed to freely swim in the cylinder and the time the mouse spends in each quadrant and the distance it swims is measured

Long term memory test:

This phase lasts one minute and it is performed the seven days after the last training day. The platform in the southwest quadrant is removed from the cylinder and the mouse is released from the northeast quadrant of the cylinder. The mice is allowed to freely swim in the cylinder and parameters measured include the time spent in each quadrant and the total distance travelled.

3.2.2.13. Lithium chloride administration

Lithium chloride was mixed into the drinking water of mice at 600 mg/liter for 10 days according to (Dehpour et al., 2002; Roybal et al., 2007). This dosage was chosen as previous described in the literature. Behavioural testing stated between day 10 and day 12 throughout which LiCl was

continuously administered. Control mice received normal water. The behavioral tests were OF and FST. These tests were performed as described above.

3.2.2.14. Home cage activity

Activity in a novel home cage and subsequently in a familiar environment of the home cage was monitored by an automated infrared tracking system (Mouse-E-Motion 2.3.6, Infra-E-Motion, Hagendeel, Germany). Each mouse was tracked for at least 93h to obtain an accurate measure of activity during the light and dark cycle.

3.2.2.15. Chronic social defeat stress (CSDS)

The chronic social defeat was performed as previously described in the literature (Wagner K.V. et al., 2015). Mice were submitted to chronic social defeat stress for 21 consecutive days. They were introduced into the home cage of a CD1 resident that was trained to be aggressive. Once the test animals showed defeat by raising its two front paws, they spent 24 hours in the same cage as the resident mouse, separated by a wire mesh partition that enabled sensory but not physical contact. Every day experimental mice were exposed to a new unfamiliar resident. Control animals were single housed in their home cages throughout the course of the experiment. All animals were weighed every 3 days and their fur status was evaluated as described in the literature (Mineur, Prasol, Belzung, & Crusio, 2003). Behavioral tests were carried out during the last week of the paradigm and included the social avoidance (SA), OF, DaLi and FST. Animals were weighed and then sacrificed by decapitation 7 days after the chronic social defeat stress. Adrenal glands and thymus of each animal were collected. The additional tissues surrounding the thymus and the adrenal glands were removed and these organs were then weighed.

3.2.2.16. Social avoidance test

This test was carried out as described in the literature (Wagner et al., 2015). This test is typically carried out to assess if the test mice undergoing CSDS paradigm has learned to avoid the aggressive resident CD1 mice. The test is carried out in the OF apparatus which is evenly illuminated with 10 lux. The test mouse is first exposed to the apparatus with an empty wire cup on one side of the apparatus and allowed to freely explore the apparatus and the cup for

15 mins. The amount of time the test mouse spends in the zone containing the wire cup is measured. A CD1 mouse is then placed in the wire cup and the test mouse is again allowed to freely explore the apparatus and the wire cup containing the CD1 mouse. The amount of time the test mouse spends in the zone containing the wire cup containing the CD1 mouse is measured. The ratio of the time the test mouse spends exploring the empty wire cup to the time the test mouse spends exploring the wire cup containing the CD1 mouse is calculated and taken as the social avoidance ratio.

3.2.2.17. Blood collection post-acute stress and under basal levels of stress following CSDS

On the 21st day of CSDS the test mice and the controls underwent FST as described above. 30 mins post FST, blood samples were collected from the tail tips of mice. 7 days after the last day of CSDS mice were sacrificed and trunk blood was collected. Corticosterone plasma concentrations were measured with a radioimmune assay according to the manufacturer's instructions (MP Biomedicals Inc; sensitivity 6.25 ng/ml).

3.2.3. Electrophysiology

Hippocampal long-term potentiation (LTP) was investigated by studying changes on classical LTP measurements. Mice were deeply anesthetized with isoflurane and decapitated shortly after. The brains were removed and quickly transferred into ice-cold carbogenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF). Sagittal hippocampal slices (350 µm) were obtained using a vibratome (HM 650V, Microm International, Walldorf, Germany). The slices were allowed to recover for at least 1h at 34°C before being transferred to the recording chamber where they were continuously superfused with aCSF at a rate of 5 ml/min. The aCSF contained: NaCl, 124 mM; KCl, 3 mM; NaHCO3, 26 mM; CaCl2,2 mM; MgSO4, 1 mM; D-glucose, 25 mM; NaH2PO4, 1.25 mM, and was saturated with a mixture of O_2/ CO_2 (95% O_2/5% CO_2, final pH7.3). Field excitatory postsynaptic potentials (fEPSPs) at synapses between the Schaffer collateral-commissural pathway (SCCP) and CA1 pyramidal cells were recorded extracellularly in the stratum radiatum of CA1. High-frequency stimulation (HFS) of 1 x 100 Hz/100 pulses to the SCCP were delivered to induce LTP. The recordings were amplified, filtered (3 kHz) and digitized (9 kHz) using a laboratory interface board (ITC-16, Instrutech), and stored with the acquisition program Pulse, version 8.5 (Heka Electronik). Data were analyzed offline with the analysis program IgorPro v.6 (WaveMetrics) software.

Measurements of the amplitude of the fEPSP were taken and normalized with respect to the 30 min control period before tetanic stimulation.

3.2.4. Statistical analysis

Data output and statistical analysis were performed using GraphPad Prism 5.0. All results were shown as means ± standard error of the mean (SEM). Differences were considered significant at $p<0.05$. To examine NanoBit and cAMP-Glo assay data two-way ANOVA repeated measures was employed. Analysis of Mander's co-localization coefficient was performed using Student's t-test. For analysis of behavioural results under basal levels of stress either Student's t-test or one-way ANOVA was employed. For analysis of the 4 groups in behavioural and endocrine measurements during CSDS paradigm two-way ANOVA was used.

4. Results

4.1. Expression of Adcy2 in the mouse brain.

From the 10 ADCYs, ADCY2 and ADCY5 are the two most abundant forms expressed in the brain. The phylogenetic tree of ADCYs, however, shows that these two isoforms are not closely related suggesting distinct functional properties. The fact that each ADCY has a unique expression pattern in the brain (compared to Allen Brain Atlas, 2008, http://mouse.brain-map.org/experiment/show?id=68192998), also suggests that each of these enzymes has a unique function. In situ hybridization using a specific riboprobe for Adcy2 was done to unravel its spatial distribution in the brain (Fig. 7). The *In Situ* hybridization shows strong expression throughout the cortex, amygdala complex, hippocampus, thalamic reticular nucleus and striatum.

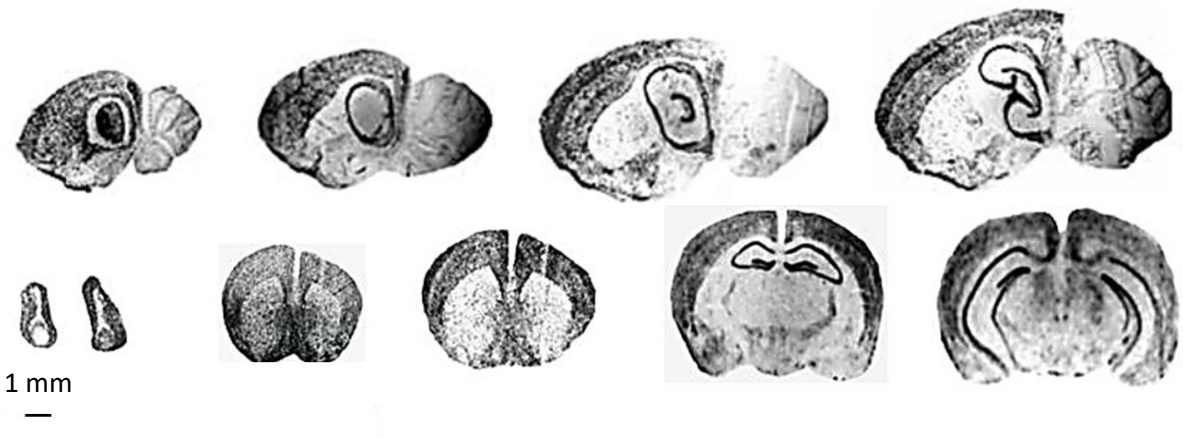


Figure 7. Adcy2 is expressed throughout the murine brain. *In situ* hybridization detecting Adcy2 expression in the mouse brain using sagittal and coronal sections of a wild-type mouse brain. Expression (dark dots) is seen throughout the cortex, amygdala complex, hippocampus, thalamic reticular nucleus and striatum.

4.2. Influence of rs13166360 on ADCY2 protein function

The effect of the missense mutation rs13166360 on protein function is unknown. However, this SNP was speculated to affect protein function (Muhleisen et al., 2014). Thus we aimed to study if there was indeed any impact on protein function.

4.2.1. Construction of Adcy2 expression vectors

The aim was to clone the cDNAs of Adcy2-151V and Adcy2-151L into an expression vector and test if the presence of the V151L substitution in the protein results in a difference in protein function, e.g. on the levels of cAMP production. However, this was problematic so far due to the large size of the cDNA and a GC rich stretch that was difficult to amplify. Amplifying the Adcy2 cDNA from mouse brain RNA always resulted in a truncated PCR product missing 72 base pairs (most of which is GC rich). This truncated product is still in frame and as the full product was never amplified from the brain cDNA it is possible that this is a naturally occurring splice variant just missing 24 amino acids at position 9-32. Thus the GC content of the GC rich region was reduced by codon optimization, and the optimized fragment was fused with a HA tag at the N-terminus. This fragment was synthesized externally by Integrated Device Technology (Munich, Germany). The fragment was inserted via restriction endonuclease digestion at the N-terminus of the, previously amplified truncated cDNA to construct pcDNA3.1-Adcy2-V151-HA (Adcy2-V151). The product was completely sequenced to verify the correct construction (Fig 8a.)

Attempts to introduce the V151L mutation into pcDNA3.1-Adcy2-V151-HA using a mutagenesis kit failed most likely due to the large size of the plasmid. Therefore, the missense mutation was introduced in the vector carrying the HA-tagged codon optimized N-terminus. After verification of the presence of the amino acid substitution by sequencing, the codon optimized fragment carrying the SNP of interest was inserted at the N-terminus of the truncated plasmid. The whole plasmid was sequenced to verify that the construction of pcDNA3.1-Adcy2-L151-HA (Adcy2-L151) was correct (Fig 8b.). The mutagenesis protocol was modified and optimized for introducing point mutations in larger plasmids. Then as a negative control, two previously described missense mutations that were previously described to result in a loss of protein function (Yan S-Z., et al, 1997), were introduced to the vector pcDNA3.1-Adcy2-V151-HA using the mutagenesis kit to generate pcDNA3.1-Adcy2-KO (KO). The presence of the SNPs of interest and the correct construction was verified by sequencing (Fig 8c.)

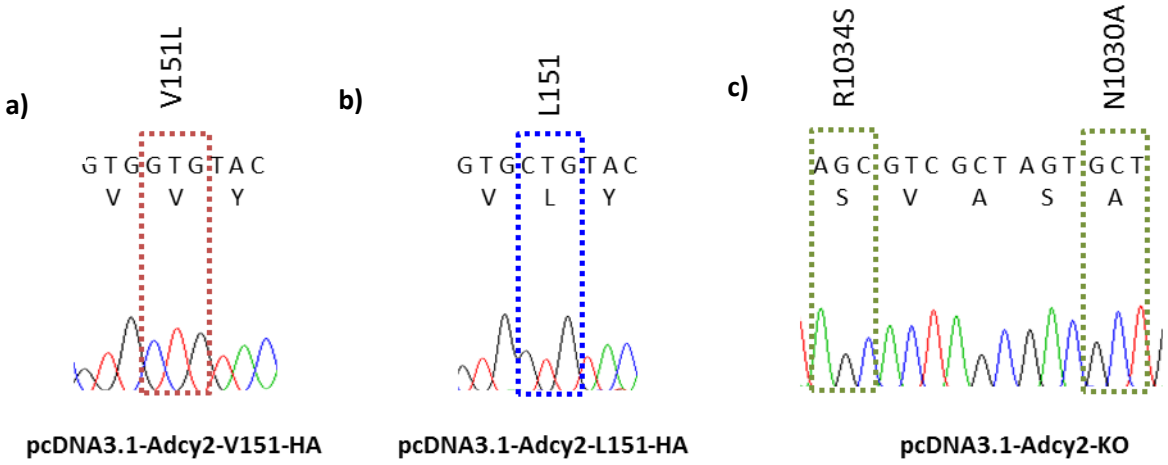


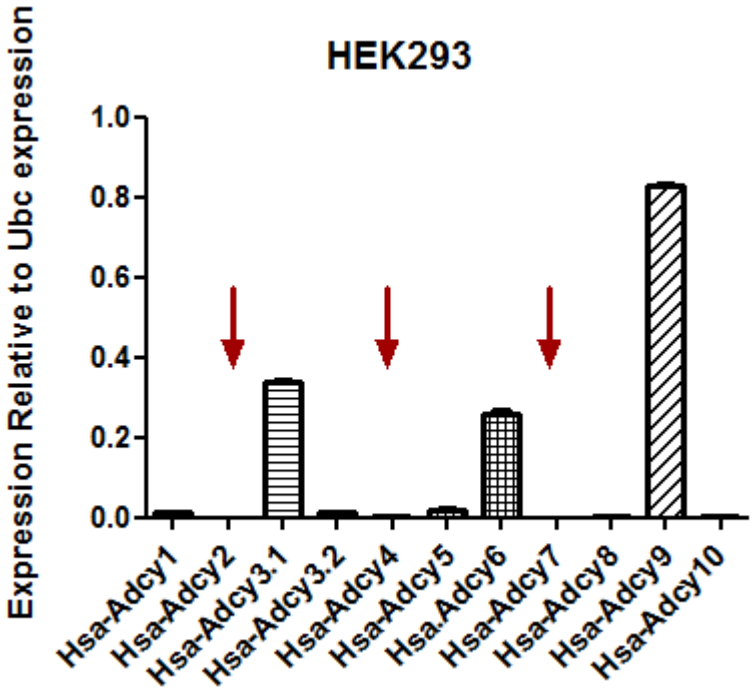
Figure 8. **Validation of Adcy2 expression vectors.** Sequencing results of **a)** pcDNA3.1-Adcy2-V151-HA **b)** pcDNA3.1-Adcy2-L151-HA and. **c)** pcDNA3.1-Adcy2-KO indicate introduced changes to wild-type Adcy2 cDNA sequence

4.2.2. Selecting a cell culture system for *in vitro* analysis of Adcy2

In order to select an immortalized cell line to study differences in protein activity, we had to choose a cell line with low levels of endogenous ADCY expression. This was done by assessing the mRNA expression levels of the 10 mammalian Adcy isoforms in HEK293, a human embryonic kidney cell line and HT22, a mouse hippocampal neuronal cell line (Fig. 9).

Specifically, the aim was to ensure that the cell line had the lowest levels of endogenous ADCY2 activity as well as low levels of activity of the closely related isoforms: ADCY4 and ADCY7. This would minimize background during Adcy2 functional assays. The expression analysis suggested that HEK293 has a relatively low level of endogenous Adcy2, Adcy4 and Adcy7 mRNA expressions. Therefore, HEK293 was the cell line of choice for further *in vitro* studies of activity of the two ADCY2 protein variants.

a)



b)

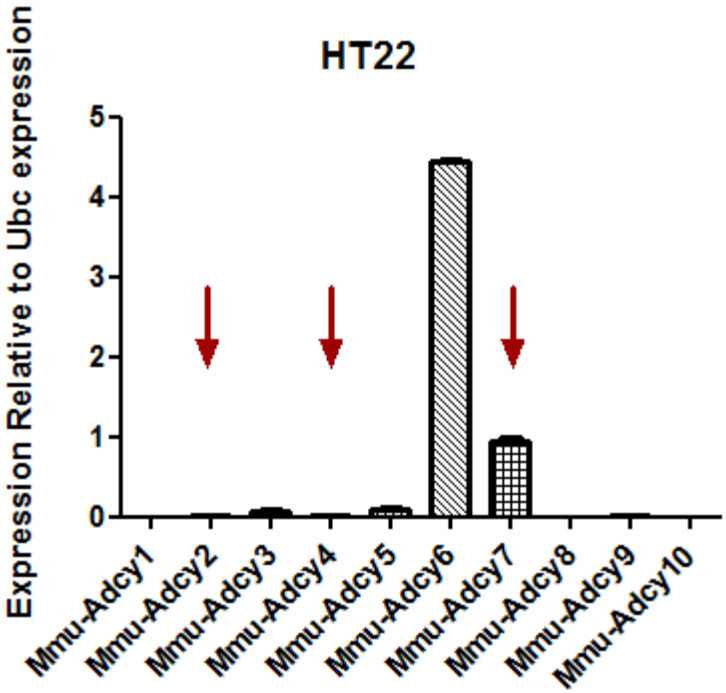


Figure 9. HEK293 cells have the lowest endogenous Adcy expression. The expression levels of endogenous Adcys were assessed in **a)** HEK293 and **b)** HT22 cells by qRT-PCR. The expression was quantified relative to the expression of the housekeeping gene: Ubiquitin C (Ubc). Mmu-Adcys: murine adenylyl cyclases; hsa-Adcys: human adenylyl cyclases. Expression of Adcy2 and its closest relatives, Adcy4 and Adcy7, are marked by red arrows.

4.3. Analysis of Adcy2 overexpression

As a first step, the expression level of exogenously over-expressed Adcy2 was quantified in comparison to endogenous Adcy levels in HEK293 cells. This would allow us to estimate the contribution of exogenous Adcy2 compare to endogenous Adcys with regards to cAMP production.

4.3.1. Expression level of endogenous Adcy2

The levels of endogenous ADCY mRNAs, as well as the level of transfected Adcy2-V151 mRNA were measured using qRT-PCR in expression vector transfected HEK293 cells (Fig.10). The expression analysis demonstrated that the Adcy2-V151-HA is expressed 20 folds higher than any endogenous ADCYs in HEK293 cells. This suggests that the transfected Adcy2 variants will indeed be the dominant contributors to subsequently conducted cAMP production assays

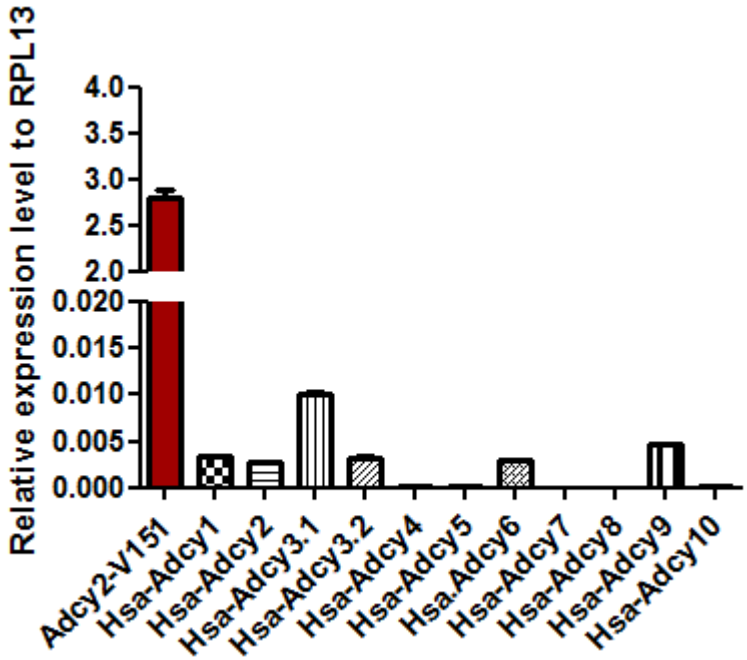


Figure 10. Comparison of transiently transfected Adcy2 expression with endogenous Adcys. qRT-PCR – based quantification of mRNA expression levels of endogenous ADCYs in comparison to the transfected Adcy2-V151. HEK293 cells were transfected with 100 ng of Adcy2-V151. The expression was quantified relative to the housekeeping gene ribosomal protein L13 (RPL13).

4.3.2. Expression of endogenous ADCYs post forskolin stimulation

Since forskolin has been shown to alter the expression levels of endogenous Adcys, the endogenous Adcy levels 30 mins and 3 hrs post forskolin stimulation were also measured using qRT-PCR. The data shows that the levels of endogenous ADCY2, as well as ADCY3, ADCY6 and ADCY9 are significantly altered following forskolin stimulation (Fig. 11). An increased expression of the endogenous ADCYs could significantly contribute to the level of cAMP production following forskolin stimulation in the cAMP assays, unless the level of cAMP produced by the transfected expression vectors still remained significantly higher. Therefore we tested whether the transfected Adcy2 variant expression was still higher than the expression of endogenous ADCYs post forskolin stimulation.

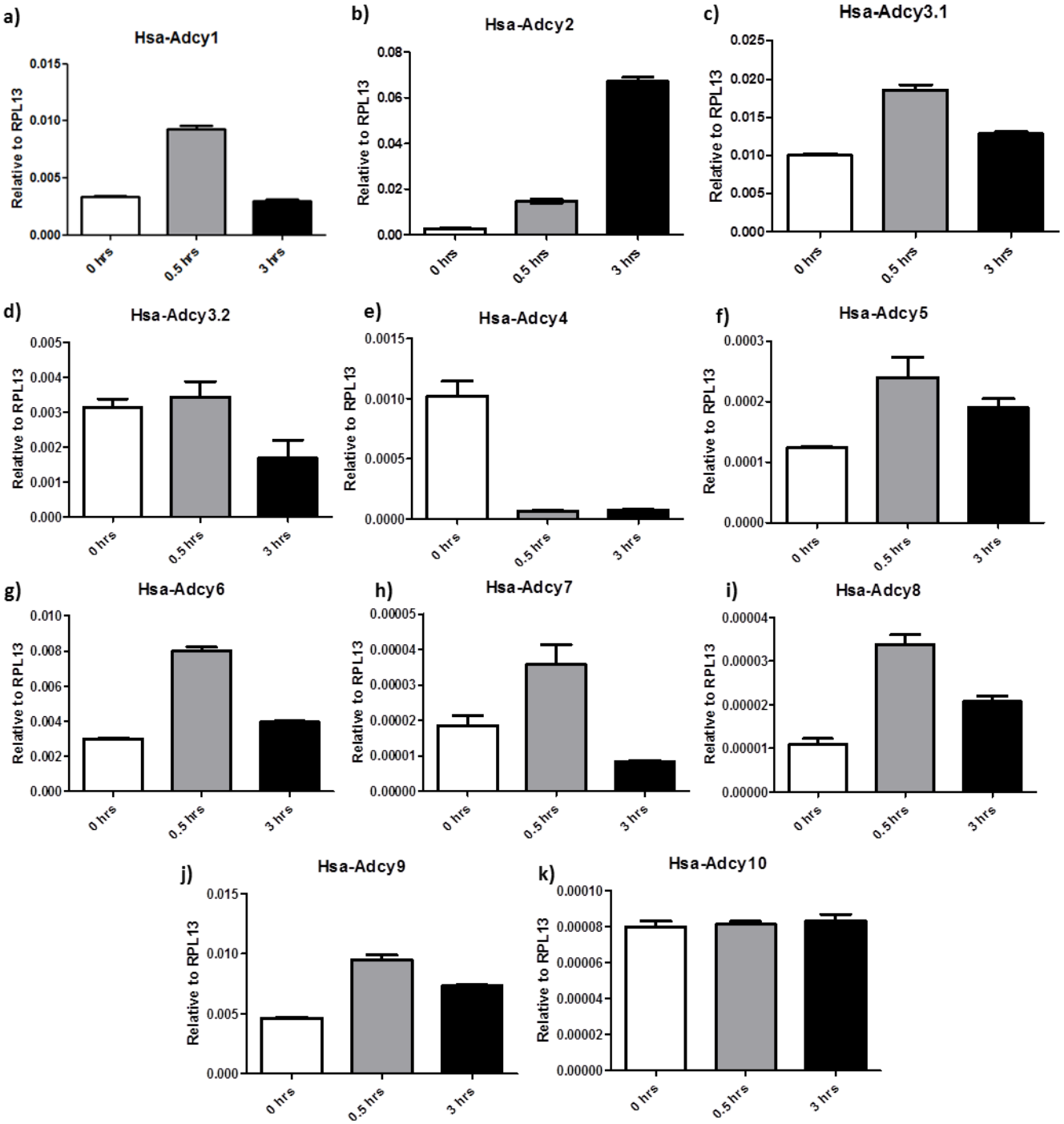


Figure 11. Effect of forskolin stimulation on Adcy expression. qRT-PCR quantification of the mRNA expression level of the endogenous **a)** Hsa-Adcy1 **b)** Hsa-Adcy2 **c) + d)** Hsa-Adcy3 **e)** Hsa-Adcy4 **f)** Hsa-Adcy5 **g)** Hsa-Adcy6 **h)** Hsa-Adcy7 **i)** Hsa-Adcy8 **j)** Hsa-Adcy9 and **k)** Hsa-Adcy10 with no forskolin stimulation, 30 mins post and 3 hrs post 10 µM forskolin stimulation. The expression is quantified relative to the housekeeping gene ribosomal protein L13 (RPL13). The scale of the y-axis was adapted to each Adcy for better visualization of expression changes.

4.3.2.1. Forskolin-dependent regulation of exogenous Adcy2-V151 expression compared to endogenous Adcys

The levels of the endogenous Adcy mRNAs expression, as well as of the transfected Adcy2-V151 were determined by qRT-PCR30 mins and 3 hrs post forskolin stimulation. The data suggests that the expression level of the transfected Adcy2-V151 30 mins post forskolin stimulation was at least 300 fold higher than the expression levels of endogenous Adcys, (Fig 12a). Furthermore, the level of the Adcy2-V151 was at least 600 fold higher than the levels of endogenous Adcy expression 3 hrs after forskolin stimulation (Fig 12b). This verifies that the transfected Adcy2 variants will indeed be the major contributors to cAMP production in any subsequent assays. In general, expression of transfected Adcy2-V151 increases from 30 mins to 3 hrs after forskolin treatment by 7 folds. This increase can be attributed to the fact that the expression vector is driven by a CMV promoter, which harbours cAMP response elements (CREs), resulting in a induction of Adcy2 expression (Wilkinson & Akrigg, 1992).

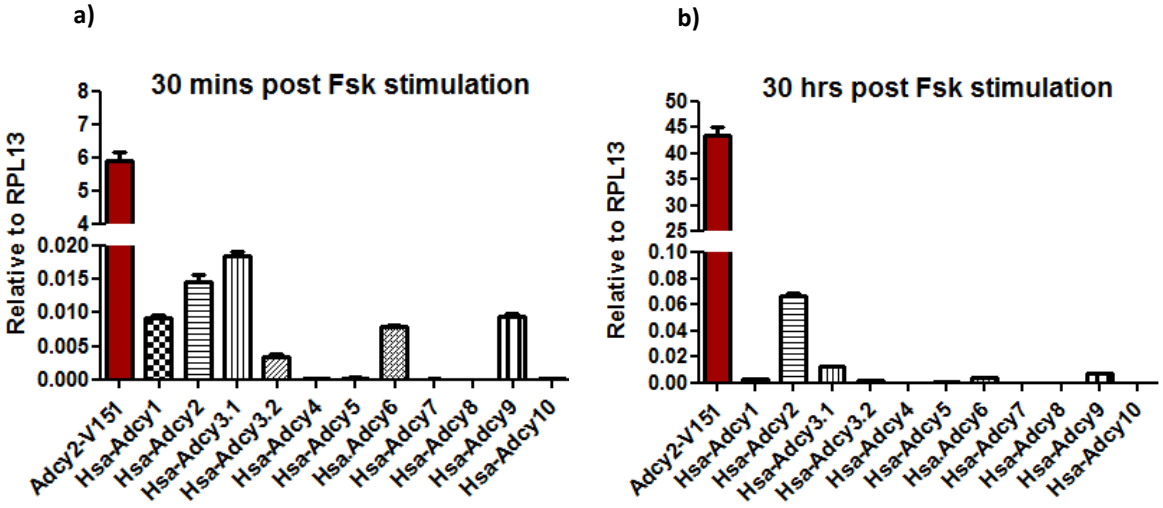


Figure 12. Comparison of transfected Adcy2-V151 expression with endogenous Adcys following forskolin stimulation. qRT-PCR measuring the mRNA expression of the endogenous Adcys and 100 ng of Adcy2-V151 transfected HEK293 cells **a)** 30 mins and **b)** 3 hr post 10 µM forskolin (Fsk) stimulation. The expression is quantified relative to the housekeeping gene Ribosomal Protein L13 (RPL13).

4.3.3. Verifying equal levels of expression of Adcy2 variants

First, we had to ensure potential differences in protein activity are indeed a result of differences in protein function. Therefore, we verified that equal amounts of the transfected expression vectors resulted in comparable expression levels of Adcy2 variants in HEK293 cells.

Levels of mRNA expression by the two expression vectors: pcDNA3.1-Adcy2-V151-HA and pcDNA3.1-Adcy2-L151-HA, were first tested using qRT-PCR. The data suggests that indeed equal levels of the two of the two Adcy2 variants were expressed in transfected HEK293 cells (Fig. 13).

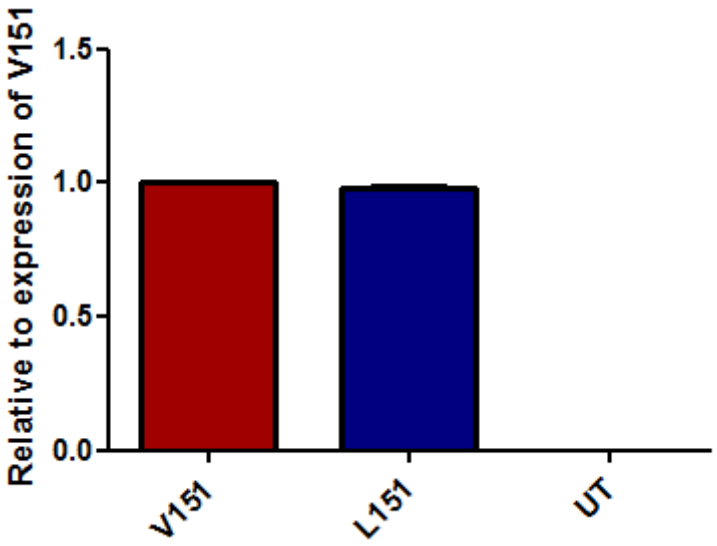


Figure 13. Assessment of Adcy2-V151 and Adcy2-L151 mRNA expression in HEK293 cells. qRT-PCR measuring the Adcy2 mRNA expression in HEK293 cells transfected with 100 ng of pcDNA3.1-Adcy2-V151-HA (red) or pcDNA3.1-Adcy2-L151-HA (blue) or un-transfected HEK293 cells (UT).

While equivalent levels of mRNA expression of Adcy2-V151 and Adcy2-L151 were identified using qRT-PCR (Fig 14), it was still unknown whether this result in equivalent levels of ADCY2 protein variants. In order to test protein levels in HEK293 cells transfected with the 2 transfection vectors, and protein expression was quantified by the Western blot. The Western blot data suggested that indeed equivalent protein levels of ADCY2-151V, ADCY2-151L and ADCY2-KO were expressed in the transfected HEK293 cells (Fig 14), indicating that neither rs13166360 nor the loss-of-function mutation (R1034S, N1030A) have an impact on ADCY2 protein stability or turnover.

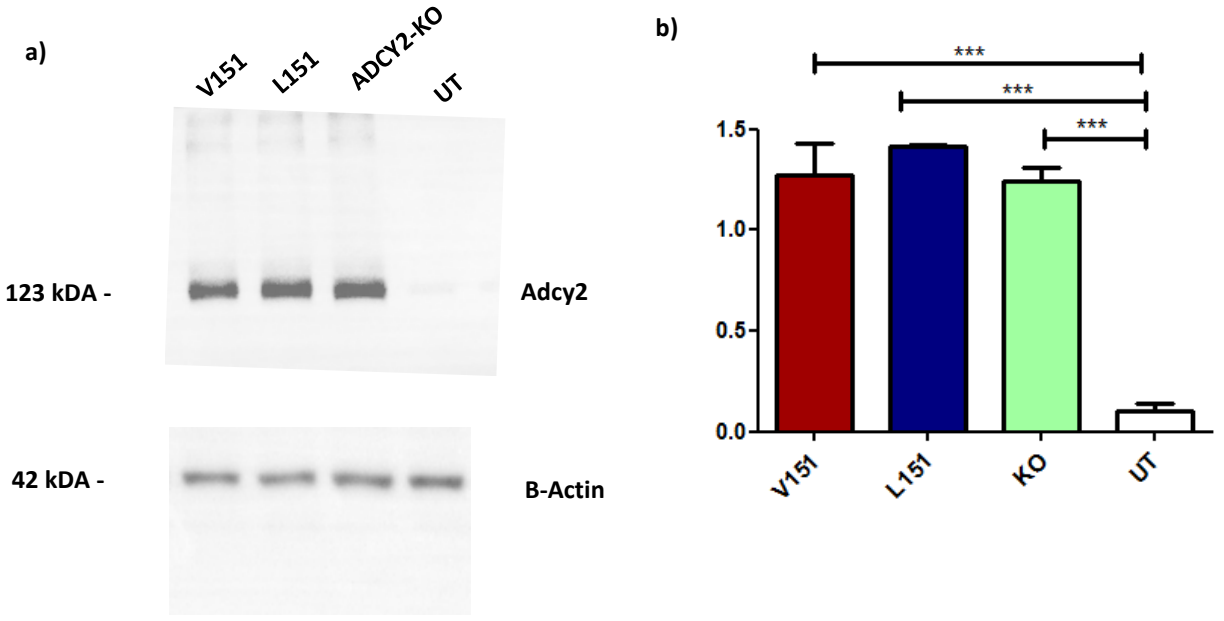


Figure 14. There is no difference in the levels of ADCY2-V151 and ADCY2-L151 protein expression. Western blot analysis verifying that equal levels of ADCY2-V151, ADCY2-L151 and ADCY2-KO are expressed in HEK293 cells transfected with 100 ng of either pcDNA3.1-Adcy2-V151-HA, pcDNA3.1-Adcy2-L151-HA or pcDNA3.1-Adcy2-KO. **a)** ADCY2 protein was detected using an anti-HA tag antibody at 123kDA. β-Actin was used as a loading control. **b)** The levels of ADCY2 protein expression were quantified by normalization to β-Actin using image J. Each protein was isolated, and run on 10% SDS-Page gel and quantified in triplicates. One-Way ANOVA. *** means $p<0.0001$

4.4. cAMP assays to measure difference in protein activity

HEK293 cells transiently transfected with each ADCY2 variant, were used to explore if there is any difference in ADCY2 activity due to the disease-associated SNP of interest. In the process we optimized two assays: the NanoBit assay, which explores the kinetics of cAMP production, and the cAMP Glo assay, which assess any difference in cAMP production after stimulation with different concentrations of the agonist forskolin.

4.4.1. The NanoBit assay

The NanoBit technology is based on a luciferase-like luminescent protein is split into a larger fragment (LgBit) and a smaller fragment (SmBit). The LgBit and the SmBit are fused to two proteins of interest. When these two proteins interact the LgBit and SmBit are brought in close proximity so they can restore the luciferase activity and produce a luminescent signal. In this assay, the LgBit and the SmBit were fused to the regulatory subunit of PKA and the active subunit of PKA, respectively. Thus the inactive PKA produces a luminescence signal, which drops with increasing levels of cAMP and the consequent activation of PKA. The advantage of this assay is that it measures the dynamics of the cAMP production over time. However, it does not quantify the absolute amount of cAMP produced at a particular time point as the signal is not normalized for the transfection levels of the two plasmids.

4.4.1.1. Nanobit assay optimization

Transfection of 200 ng of the Adcy2-expression vector produces low amounts of luminescent signal, even though the amount of signal decreases with increasing forskolin concentration as expected (expected range is 10.000-1.000.000 RLU). This is possibly because the expression vector contains a strong CMV promoter resulting in a high level of Adcy2 expression, thereby potentially limiting the cell's capacity of transcription and translation machinery which is available for expressing the PKA subunits fused to SmBit and LgBit. To overcome this problem, different concentrations of the Adcy2-V151 expression vector, were titrated to identify which concentration is suitable for this assay to use its full dynamic range. 100 ng/well of the expression vector and 50 ng/well of each of the LgBit and SmBit vectors generated a signal within the desired range (Fig. 15). Therefore, 100 ng of expression vector and 50 ng each of LgBit and SmBit per well were used for further experiments.

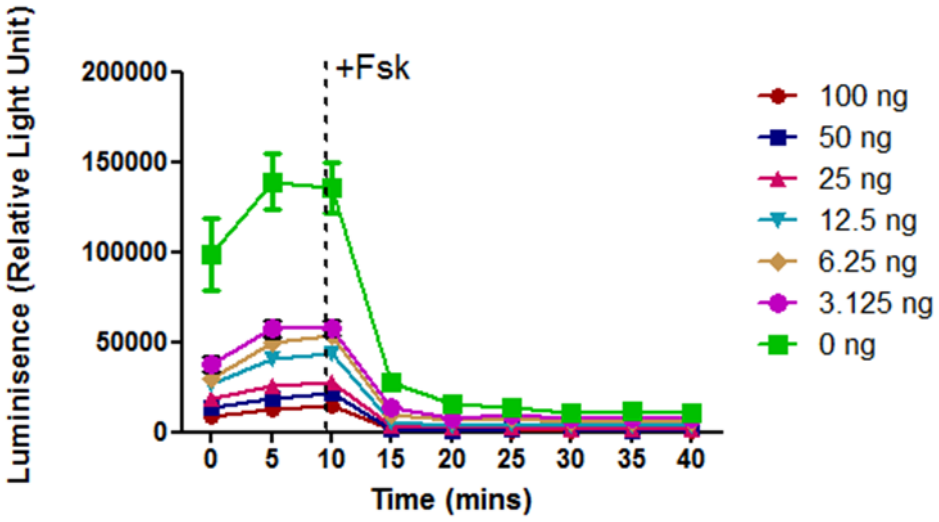


Figure 15. Establishment of Nanobit assay for continuous measurement of cAMP production. Titration of different amounts of the pcDNA3.1-Adcy2-V151-HA expression vector. Each well of HEK293 cells was transfected with the stated amount of the expression vector per well, as well as 50 ng/well of LgBit and 50 ng/well SmBit in a 96-welled plate. After stimulation with 10 µM forskolin (+Fsk), the luminescence signal falls as the amount of cAMP in the cells increase. Each measure was taken in duplicate.

Transfection of HEK293 cells demonstrated that Adcy2-L151 expressing cells generate a higher luminescence signal compared to cells expressing Adcy2-V151. After administration of 10 µM forskolin, the luminescence signals, produced by cells transfected with either of the vectors, dropped to the same level (Two way ANOVA repeated measures, $p < 0.05$) (Fig 16). This observation suggests a lower basal activity of Adcy2-L151. The parallel shift of the luminescence curves resulting from the two Adcy2 variants suggests a similar sensitivity of both variants to stimulation by forskolin. Cells transfected with Adcy2-KO, the loss of function variant of ADCY2, has no activity and does not react to forskolin. However; the Nanobit assay does not quantify the absolute amount of cAMP produced at a particular time point as the signal is not normalized for the transfection levels of the two plasmids. Repeating this assay with varying concentrations of V151 and L151 gave similar results with respect to the kinetics of the cAMP production.

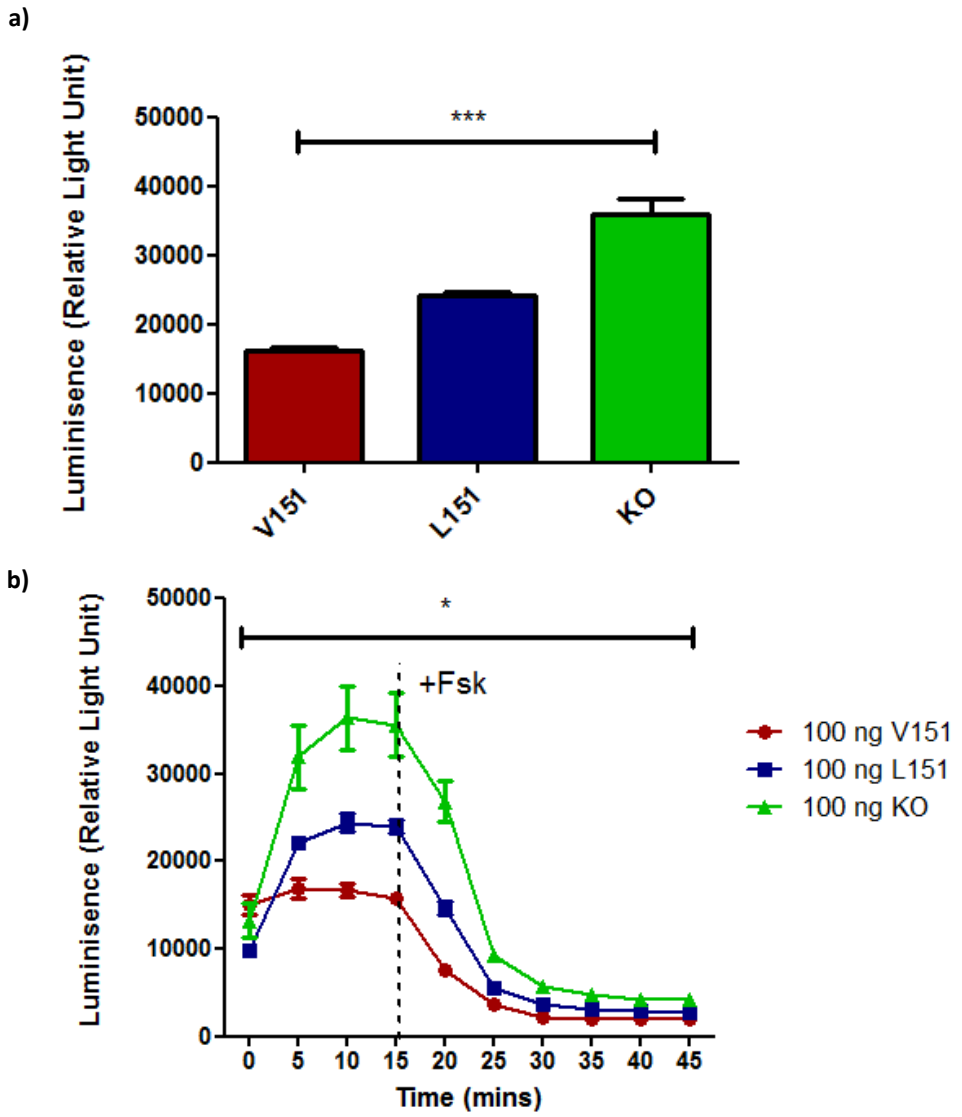


Figure 16. Comparison of cAMP production capacity of ADCY2-V151 and ADCY2-L151. This assay demonstrates that **a)** cells transfected with Adcy2-L151 have a significantly lower cAMP production and therefore higher luminescence signal at basal level.(One way ANOVA, $p<0.01$). **b)** After stimulation with 10 µM forskolin the luminescence signals generated by cells transfected with either of the two plasmids equalize. Two way ANOVA repeated measures, $p<0.05$

Since the NanoBit assay does not quantify the absolute amount of cAMP produced at a particular time point, we aimed normalized the NanoBit assay against a GFP vector controlling for transfection efficiency. The ratio of the luminescence signal to the GFP fluorescence signal was calculated and plotted. The normalized NanoBit assay demonstrates that the normalized luminescence signal of HEK293 cells transfected with Adcy2-KO is much higher than the normalized luminescence signal form cells transfected with either one of the two Adcy2 variants (Two way ANOVA repeated measures, $p <0.05$) (Fig 17a). This reflects the high basal

ADCY2 activity compared to endogenous ADCY activity present in HEK293 cells. Furthermore, the normalized luminescence signal of Adcy2-L151 was significantly higher than the normalized luminescence signal of Adcy2-V151. This suggests that the amount of cAMP produced by Adcy2-L151 was significantly lower compared to Adcy2-V151 (Two way ANOVA repeated measures, $p < 0.05$) (Fig. 17b), confirming that Adcy2-L151 has a lower basal activity than Adcy2-V151. Additionally, stimulation of cells transfected by either one of the two Adcy2 variants by a lower concentration of forskolin (100 nM) causes a similar increase in cAMP production. Only after stimulation of these cells by a much higher concentration of forskolin (10 µM) a ceiling effect is reached, resulting in similar levels of cAMP production.

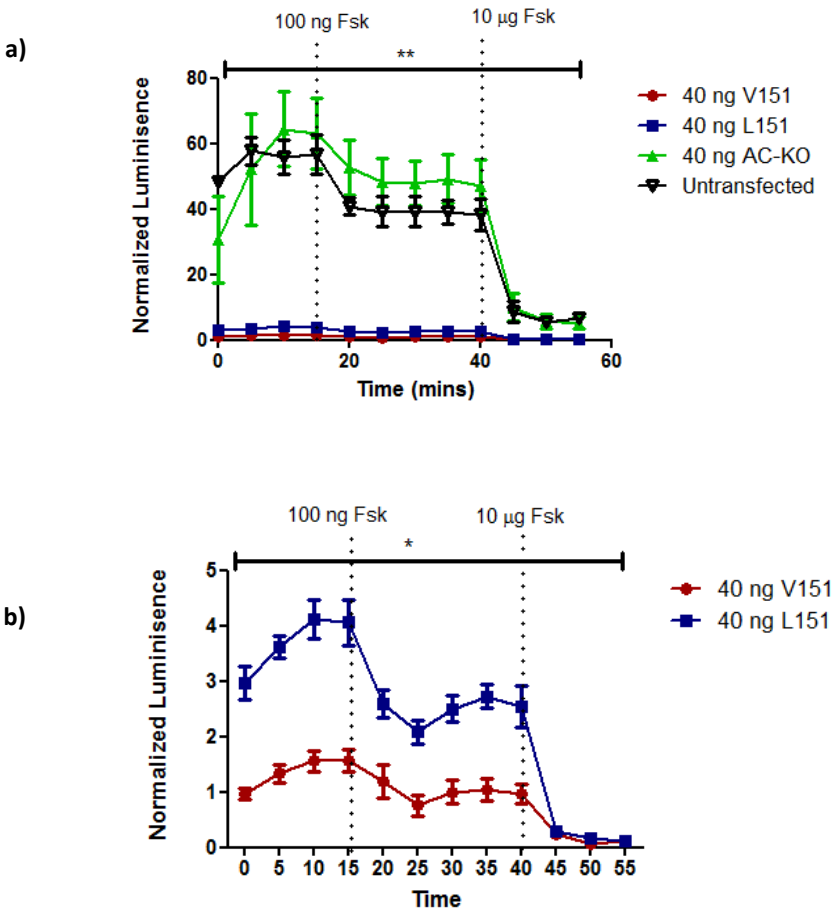


Figure 17. Adcy2-L151 is impaired in its cAMP production capacity. HEK293 cells were transfected with 50 ng of LgBit, 50 ng of SmBit, 10 ng of pCAG-GFP and with 40 ng of V151, L151 or Adcy2-KO. **a)** Normalized luminescence generated by cells transfected with V151, L151 or Adcy2-KO. **b)** Normalized luminescence generated by cells transfected with either Adcy2-V151 or Adcy2-L151. Each data point was measured in duplicates. Two way ANOVA repeated measures, $p < 0.05$

4.4.2. cAMP Glo assay

This assay measures cAMP production at a particular time point after stimulation based on a luciferase that uses ATP in the cell to generate a luminescence signal. As the levels of cAMP increases within the cell, the cAMP activates PKA, and the regulatory subunits undergo a conformational change to release the catalytic subunits. Then the free catalytic subunits catalyse the transfer of the terminal phosphate of ATP to PKA substrates, consuming ATP. Thus as the amount of cAMP increases, the available ATP remaining in the cell for the luciferase to use in the generation of the luminescence signal decreases. This makes the luminescence signal inversely proportional to the amount of cAMP produced in the cell.

Cells were transfected with 200 ng of either pcDNA3.1-Adcy2-V151-HA, 200 ng of pcDNA3.1-Adcy2-L151-HA or 200 ng of pcDNA3.1-Adcy2-KO plasmid. The cells were then stimulated with varying concentrations of the agonist forskolin and the luminescence signal for each concentration was measured. These measurements showed that the cells transfected with ADCY2-L151 produce a higher luminescence signal, indicating lower cAMP production than the cells transfected with ADCY2-V151 (Fig 18) (Two way ANOVA Repeated Measures, $p < 0.05$). This suggests that the Val151Leu substitution decreases ADCY2 function. Cells transfected with pcDNA3.1-Adcy2-KO had a higher signal, and thus a lower level of cAMP production than the cells transfected with ADCY2-L151, suggesting that the disease-associated SNP does not result in a complete loss of function, but rather lowers the amount of cAMP produced.

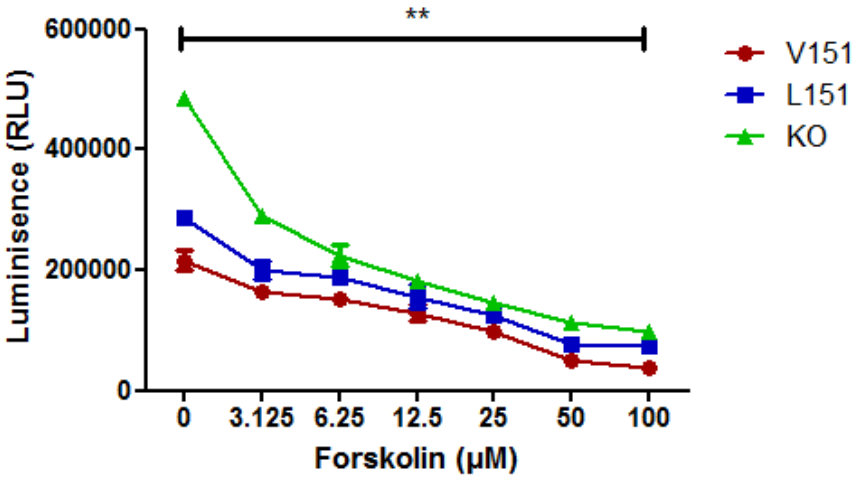


Figure 18. Cells transfected with pcDNA3.1-Adcy2-L151-HA (L151) produce less cAMP than cells transfected with pcDNA3.1-Adcy2-V151-HA (V151). of HEK293 cells transfected with ADCY2-V151, ADCY2-L151 or Adcy2-KO was measured. Each data point was measured in duplicates. (Two way ANOVA, repeated measures, ** indicates p <0.01)

4.5. Investigation of potential mechanisms underlying reduced ADCY2-L151 activity

There could be various different reasons underlying the differences in protein activity observed in the two variants of ADCY2. These differences include alterations in protein structure, stability, turnover as well as protein trafficking or localization. Due to the fact that the disease-associated SNP is located in the transmembrane spanning region of ADCY2, we hypothesized that the amino acid substitution would possibly affect the membrane localization or trafficking of ADCY2.

4.5.1. Subcellular localization of ADCY2 in transfected cells

Cos7 cells were used to study the localization pattern because Cos7 cells possess the advantage of having large cell bodies with an extended cytosolic region so that cytosolic localization and membrane localization could be clearly differentiated.

The transfection of Cos7 cells with 250 ng of pcDNA3.1-Adcy2-V151-HA and staining for the HA tag demonstrated that Adcy2 was localized in vesicular structures within the cytosol, as well as at the cell membrane (Fig. 19). This appearance was different from the expected

exclusive membrane localization based on what has been previously described (Kamenetsky et al., 2006; Steegborn, 2014). Due to the strong overexpression of Adcy2-V151-HA). it is possible that this localization pattern is a consequence of over-loading the cellular sorting machinery.

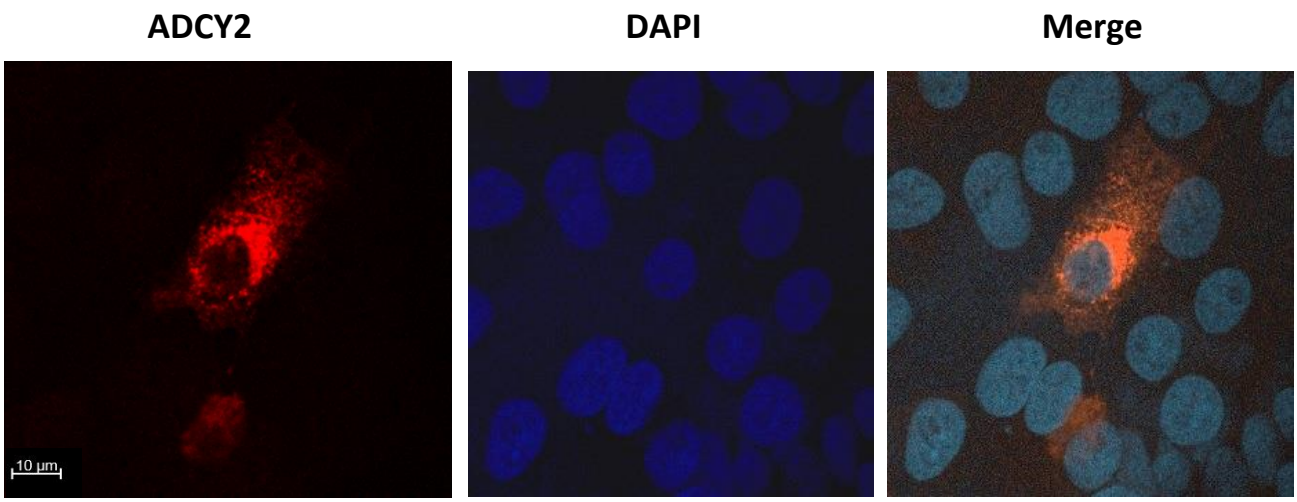


Figure 19. ADCY2 is localized in vesicular structures of the cytosol in addition to membrane localization. Cos7 cells were grown on PDL coated cover slips and transfected with 250ng of pcDNA3.1-Adcy2-V151-HA. ADCY2 was detected with an anti-HA tag antibody and Alexa 594 secondary antibody, cells were counter-stained for DAPI. ADCY2-HA (Red) and DAPI stained Nucleus (Blue). Image was taken in confocal microscope at a 63X zoom

To test this, we transfected Cos7 cells with varying amount of the expression plasmid: pcDNA3.1-Adcy2-V151-HA (Fig. 20). Again we observed a similar vesicular localization of the HA tagged ADCY2 in the cytosol, which was independent of the amount of transfected plasmid. This suggested that indeed some of the ADCY2 protein localizes to intracellular compartments. While such a localization pattern has not been previously described, previous immuno fluorescence staining for ADCY2 also shows similar cytosolic localization, instead of exclusive membrane localization (Bogard et al., 2012)

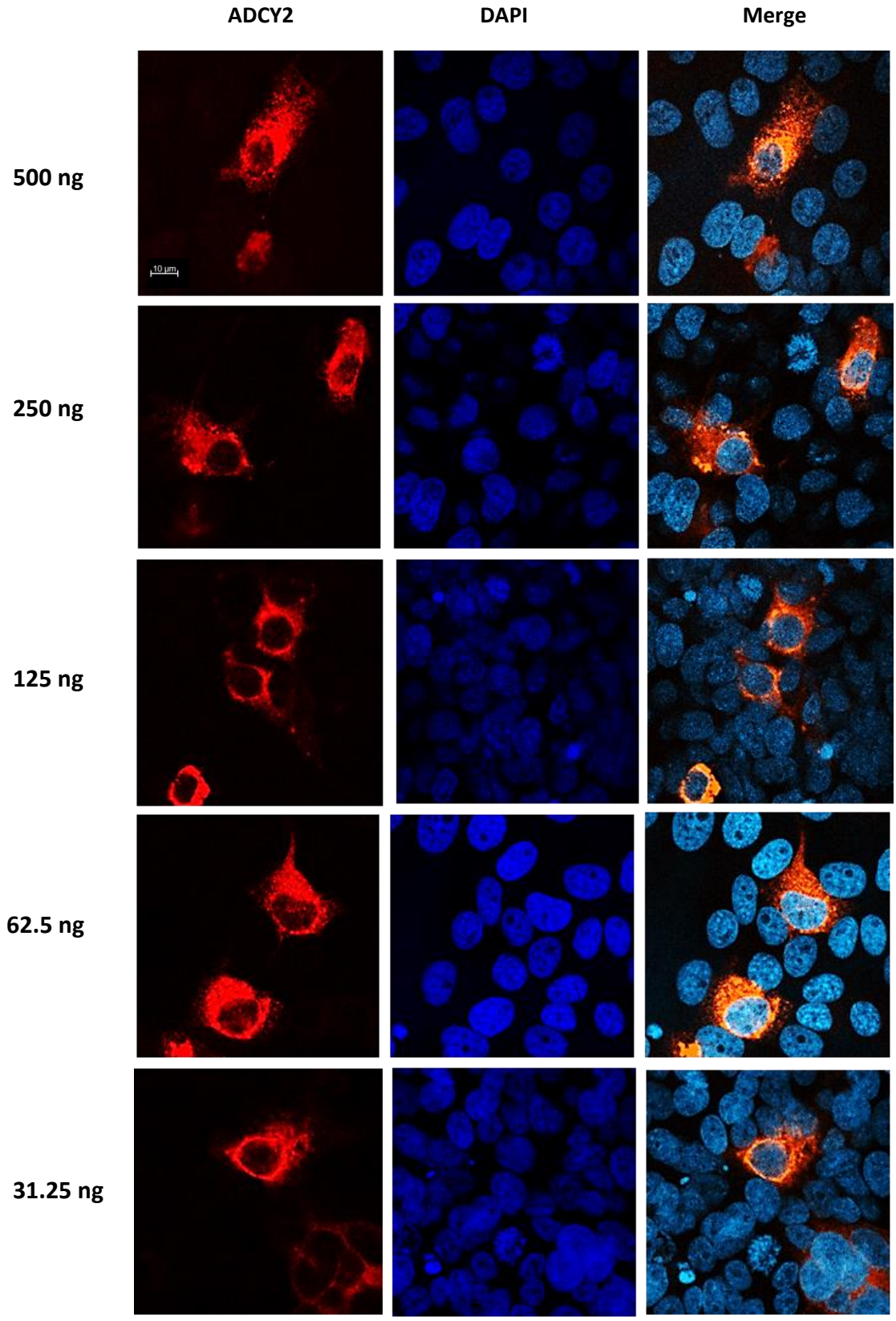


Figure 20. Continued in the next page

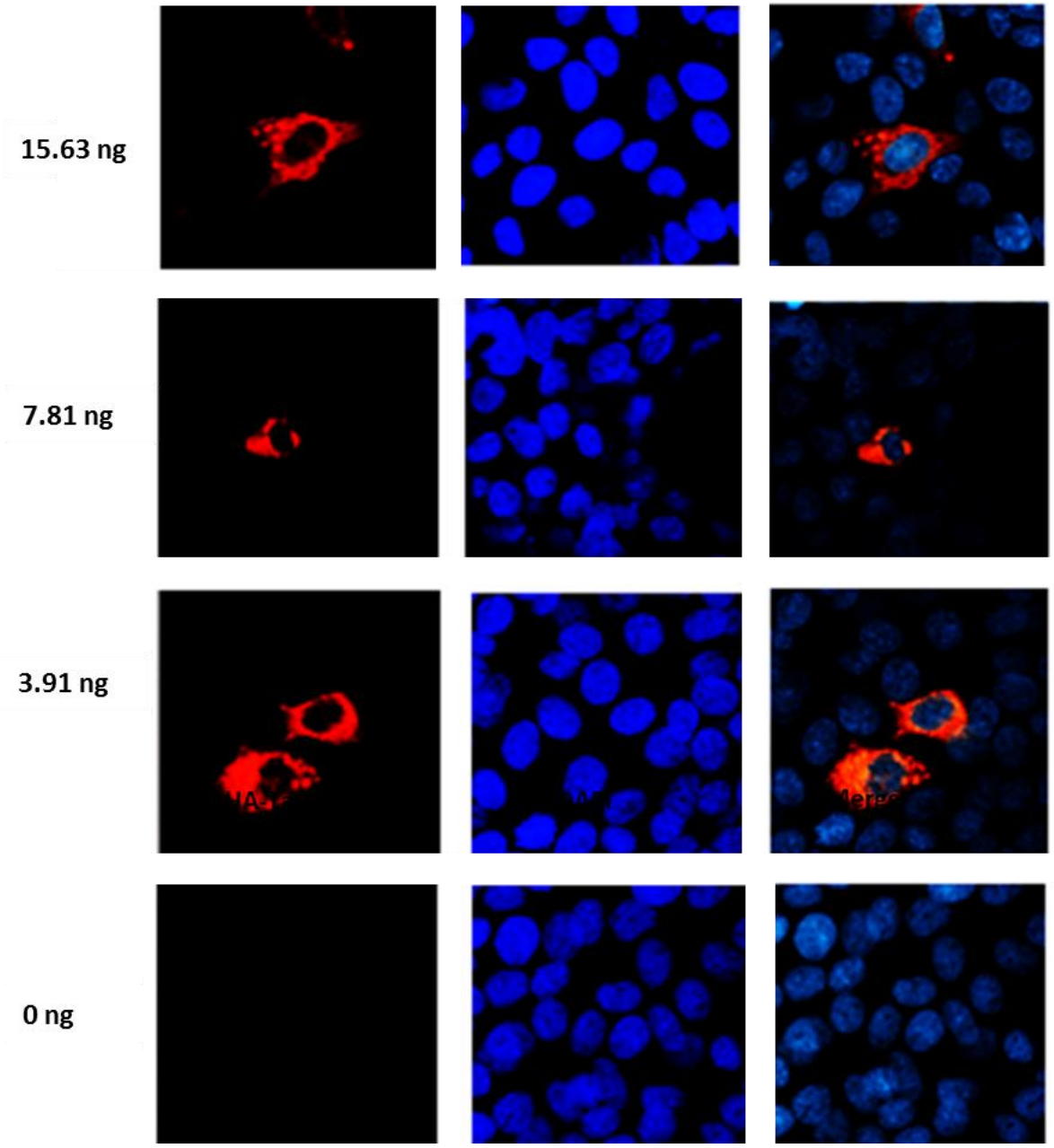


Figure 20. **The localization pattern is independent of the amount of expressed Adcy2.** Cos7 cells were grown on PDL coated cover slips and transfected with varying amounts of pcDNA3.1-Adcy2-V151-HA. ADCY2 was detected with an anti-HA tag antibody and Alexa 594 secondary antibody, cells were counter-stained for DAPI. ADCY2-HA (Red) and DAPI stained Nucleus (Blue). Image was taken in confocal microscope at a 63X zoom

4.5.2. Assessment of differences in localization or trafficking of ADCY2-V151 versus ADCY2-L151

Given that ADCY2 is localized throughout the transfected cells, the next aim was to analyse if there is a difference in localization or trafficking between the wild-type ADCY2-V151 and the mutant ADCY2-L151. Based on confocal microscopy, there were no gross differences in cellular localization observed between the two ADCY2 variants (Fig 20).

Since morphological variance between individual transfected cells were too high, it was difficult to quantify potential differences in localization between cells transfected with pcDNA3.1-Adcy2-V151-HA and cells transfected with pcDNA3.1-Adcy2-L151-HA. Therefore, another expression vector carrying a flag tagged variant of Adcy2-L151 was constructed. The presence of the FLAG tag in this construct (pcDNA3.1-Adcy2-L151-FLAG) was verified by sequencing (Fig. 15). Cos7 cells were transfected with either the combination of pcDNA3.1-Adcy2-V151-HA and pcDNA3.1-Adcy2-L151-FLAG or with the combination of pcDNA3.1-Adcy2-L151-HA and pcDNA3.1-Adcy2-L151-FLAG. These cells were then stained for both HA-tag and FLAG-tag and then the amount of co-localization between these two tags was compared.

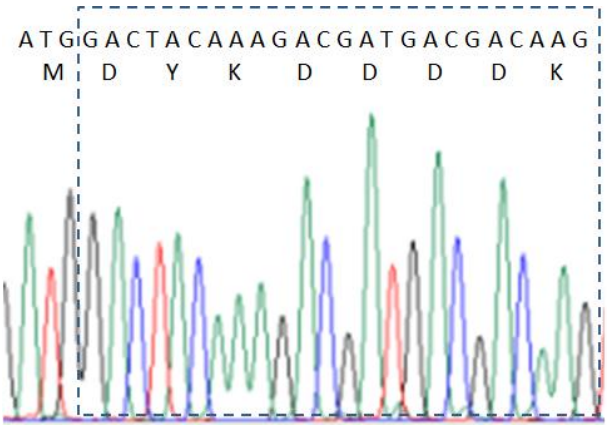


Figure 21. **Verification of pcDNA3.1-Adcy2-L151-FLAG construct by sequencing.** A FLAG-tag containing fragment was used to replace the HA -tag at the N-terminus of Adcy2-L151

Cos7 cells were co-transfected with either Adcy2-L151-FLAG and Adcy2-L151-HA or Adcy2-L151-FLAG and Adcy2-V151-HA. To quantify the co-localization, only cells stained for antibodies against both plasmids, with an intact nucleus were chosen. Furthermore, only cells that had no other cells cluttered around were chosen so that the selected ROI for quantification only contained a single cell (Fig 22a). The Mander's co-efficient correlation test was used to quantify the relative amounts of co-localization. In this test each channel coefficient indicates the proportion of signal in a given channel that co-localizes with the other channel. This test disregards the signal strength, and only focuses on the co-localization in each pixel. This means that the correlation co-efficient is not affected by the exact amount of each plasmid transfected into each cell. The results of this test indicated that the co-localization between for the amount of Adcy2-V151-HA co-localising with Adcy2-L151-FLAG was lower than the extent of co-localization between Adcy2-L151-HA and Adcy2-L151-FLAG (Fig. 22b; Student's T-Test, $p<0.05$). This suggested that the localization of ADCY2-L151 is indeed altered.

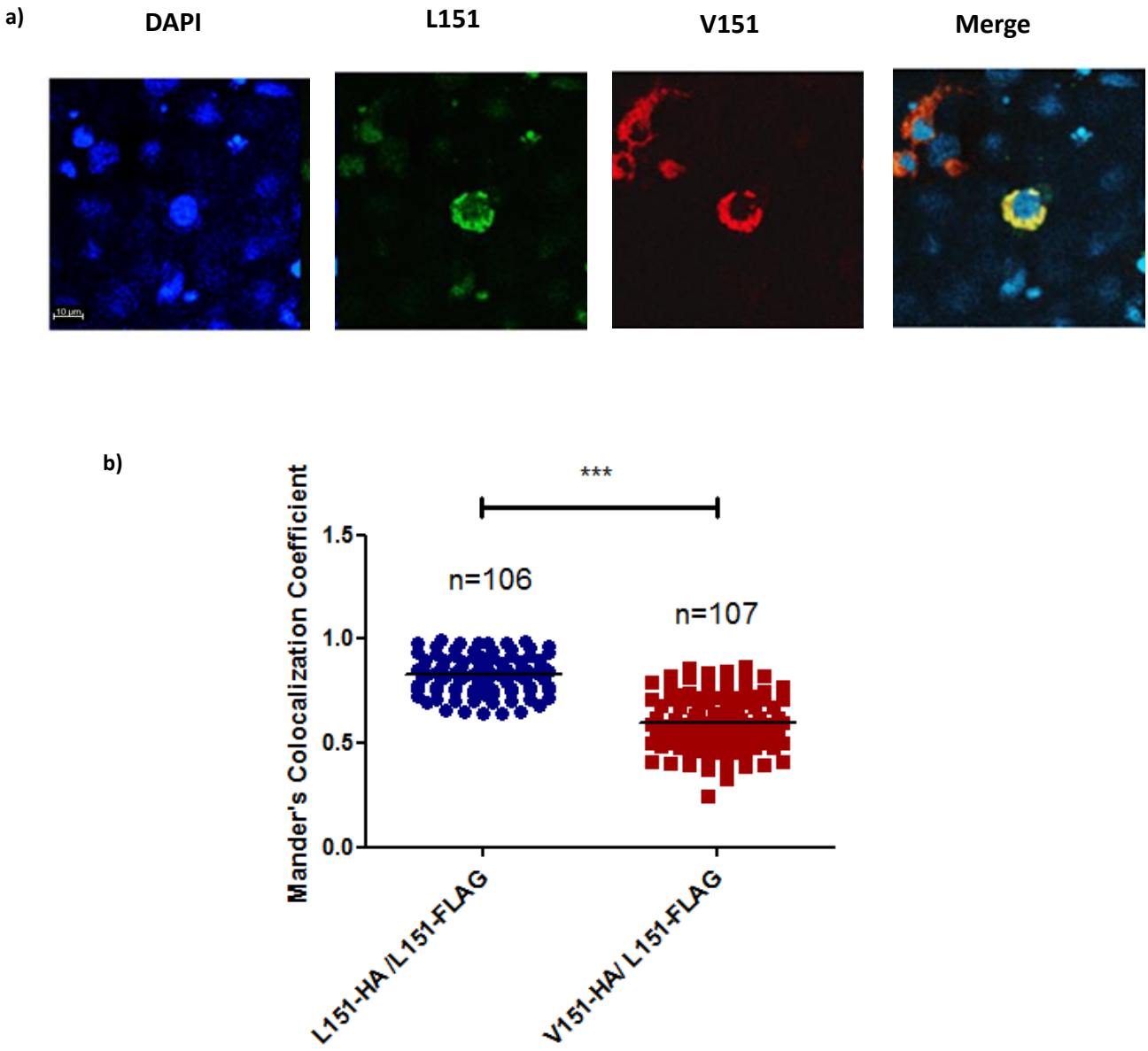


Figure 22. ADCY2-151L have altered localization or trafficking a) Cos7 cells were transfected with either a combination of pcDNA3.1-Adcy2-V151-HA and pcDNA3.1-Adcy2-L151-FLAG or a combination of pcDNA3.1-Adcy2-L151-HA and pcDNA3.1-Adcy2-L151-FLAG. The amount of co-localization between the two tagged variants was quantified**. b)** Mander's Colocalzation coefficient quantifies the amount of co-localization between Adcy2-151L-HA and Adcy2-151L-FLAG (blue) compared to the co-localization between Adcy2-151V-HA and Adcy2-151L-FLAG (red). HA-Tag was stained with Alexa 594 secondary antibody and FLAG-Tag was stained with Alexa 488 secondary antibody. Student's T-Test, $p<0.05$

4.5.3. Localization of ADCY2-V151 and ADCY2-L151

To assess whether the disease-associated SNP has any impact on ADCY2 localization within the cell and thus on ADCY2 function we investigated sub-cellular distribution. To this end the co-localization of ADCY2-151V and ADCY2-151L with markers of intracellular compartments and of the plasma membrane was assessed.

4.5.3.1. Localization of ADCY2-V151 and ADCY2-L151 in intracellular compartments

Fluorescent protein tagged Rab proteins were used as markers of different subcellular compartments. Cos7 cells were transfected with 200 ng of expression vectors for GFP-Rab7, GFP-Rab9, GFP-Rab11 or RFP-Rab5. These cells were also co-transfected with 200 ng of either Adcy2-V151-HA or Adcy2-L151-HA. Furthermore, only cells that expressed both transfected plasmids were selected and which had no other cells cluttered around were chosen for quantification. The Mander's co-efficient correlation test was used to quantify the relative amounts of co-localization.

The Rab7-GFP expression in Cos7 cells co-localized with tagged ADCY2 proteins (Fig. 23a). Since Rab7 is a marker for late endosome, this suggests that Adcy2 is localized in late endosomes. This was further verified by cells transfected with Rab9-GFP and ADCY2-V151-HA as Rab9 is another marker of late endosome (Fig 23b) (Boothe et al., 2016). Additionally, cells transfected with ADCY2-L151-HA and GFP-Rab7 had a higher co-localization than those transfected with ADCY2-V151-HA and GFP-Rab7 (Fig 23c). This suggests that more ADCY2-L151 is localized in late endosomes. This was additionally confirmed by comparing co-localization of ADCY2-L151-HA and GFP-Rab9 and the co-localization of ADCY2-V151-HA and GFP- Rab9 (Fig 23d).

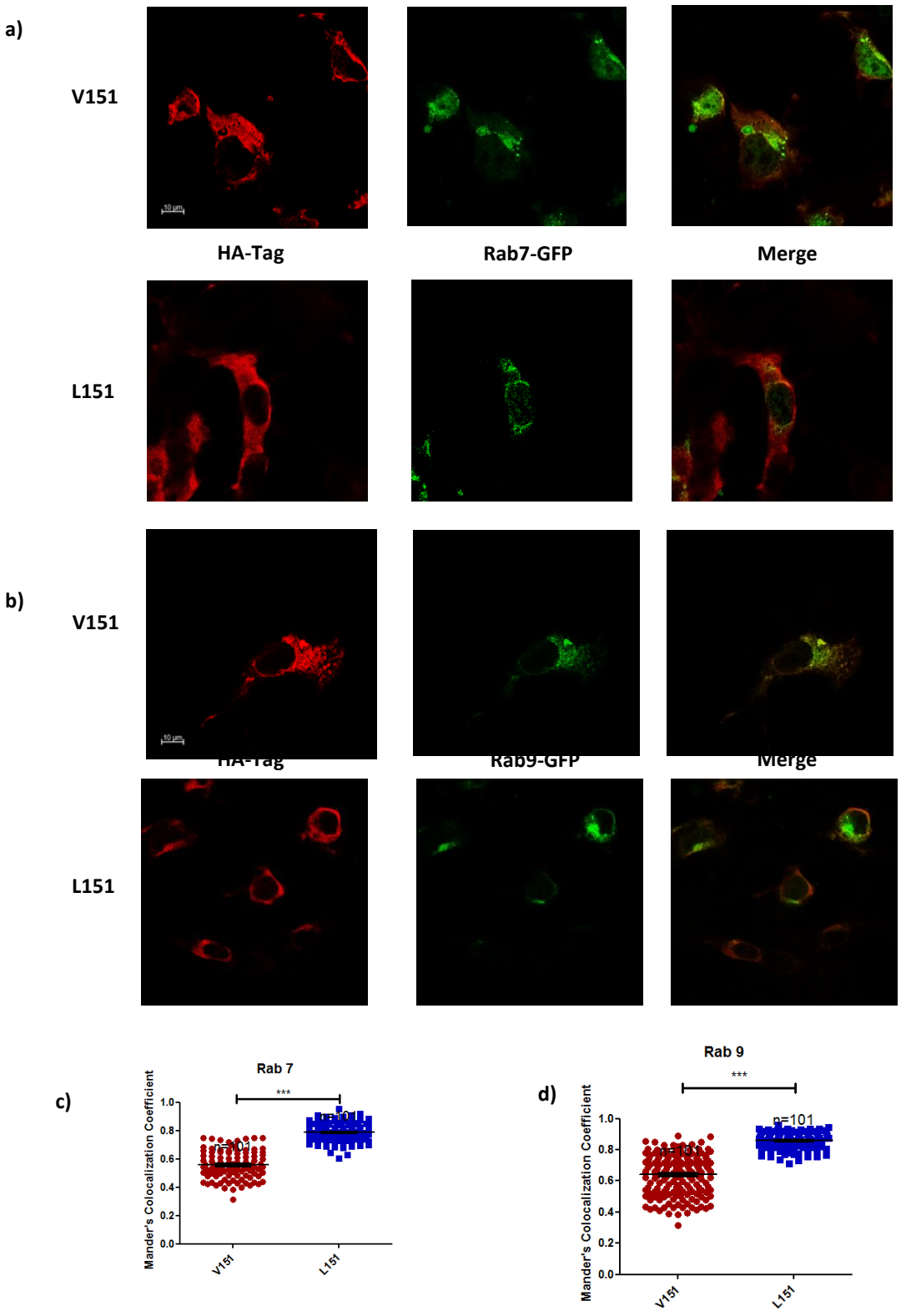


Figure 23. ADCY2-L151 is predominantly localized in late endosomes compared to ADCY2-V151 Cos7 cells were transfected with pcDNA3.1-Adcy2-V151-HA or pcDNA3.1-Adcy2-L151-HA in combination with **a)** Rab7-GFP or **b)** Rab9-GFP**. c)** Mander's Colocalization coefficient quantifies the amount of co-localization between Adcy2-151V-HA and Rab7-GFP (red) compared to the co-localization between Adcy2-151L-HA and Rab7-GFP (blue). Student's T-Test, $p<0.05$ **d)** Mander's Colocalzation coefficient quantifies the amount of co-localization between Adcy2-

151V-HA and Rab9-GFP (red) compared to the co-localization between Adcy2-151L-HA and Rab9-GFP (blue). Student's T-Test, $p<0.05$.

The Rab11-GFP expression in Cos7 cells co-localized with ADCY2-151V-HA protein (Fig. 24a). Since Rab 11 is a marker for recycling endosome, this demonstrates that Adcy2 is localized in the recycling endosomes (Boothe et al., 2016). Additionally, co-transfected cells showed a higher co-localization of ADCY2-L151-HA with GFP-Rab11 than ADCY2-V151-HA with GFP-Rab11. This suggests that more ADCY2-L151 is localized in the recycling endosomes (Fig 24b).

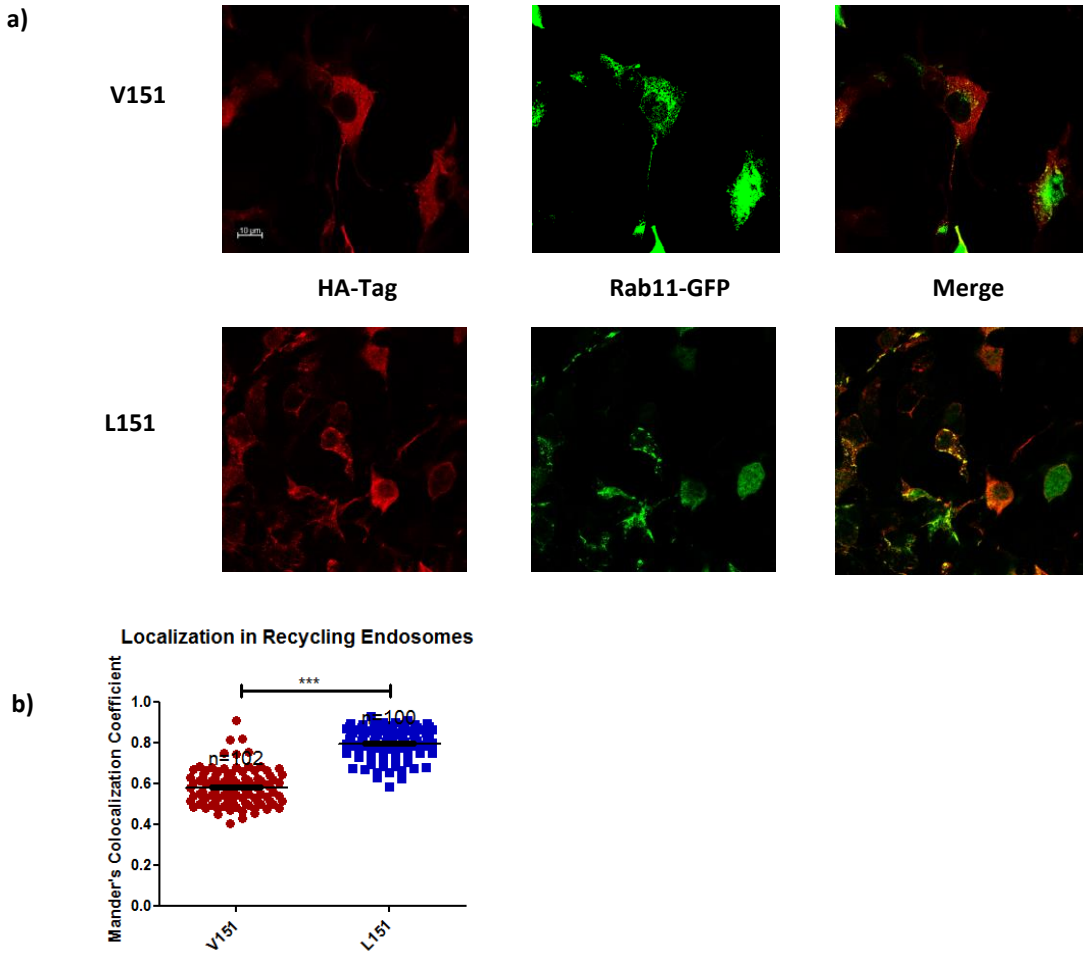


Figure 24. ADCY2-L151 is predominantly localized into recycling endosomes compared to ADCY2-V151. Cos7 cells transfected with pcDNA3.1-Adcy2-V151-HA or pcDNA3.1-Adcy2-L151-HA **a)** in combination with Rab11-GFP. **b)** Mander's Colocalization coefficient quantifies the amount of co-localization between Adcy2-151V-HA and Rab11-GFP (red) compared to the co-localization between Adcy2-151L-HA and Rab11-GFP (blue). (Student's T-Test, $p<0.01$)

The Rab5-RFP expression in Cos7 cells co-localized with ADCY2-151V-HA (Fig. 25a). Since Rab5 is a marker for early endosome, this suggests that Adcy2 is also localized in early endosomes (Boothe et al., 2016). In general, cells transfected with ADCY2-L151-HA and RFP-Rab5 showed a higher degree of co-localization than those transfected with ADCY2-V151-HA and RFP-Rab5. This suggests that more ADCY2-151L is localized in early endosomes compared to ADCY2-V151 (Fig 25c.). Cells transfected with only ADCY2-151V-HA or ADCY2-151L-HA, which were co-stained for endogenous Rab5 using an anti-Rab5 antibody, also demonstrated that more ADCY2-151L-HA co-localized with Rab5, than ADCY2-151V-HA (Fig. 25b and Fig. 25d). This again verifies the fact that more ADCY2-151L is localized in early endosomes compared to ADCY2-V151.

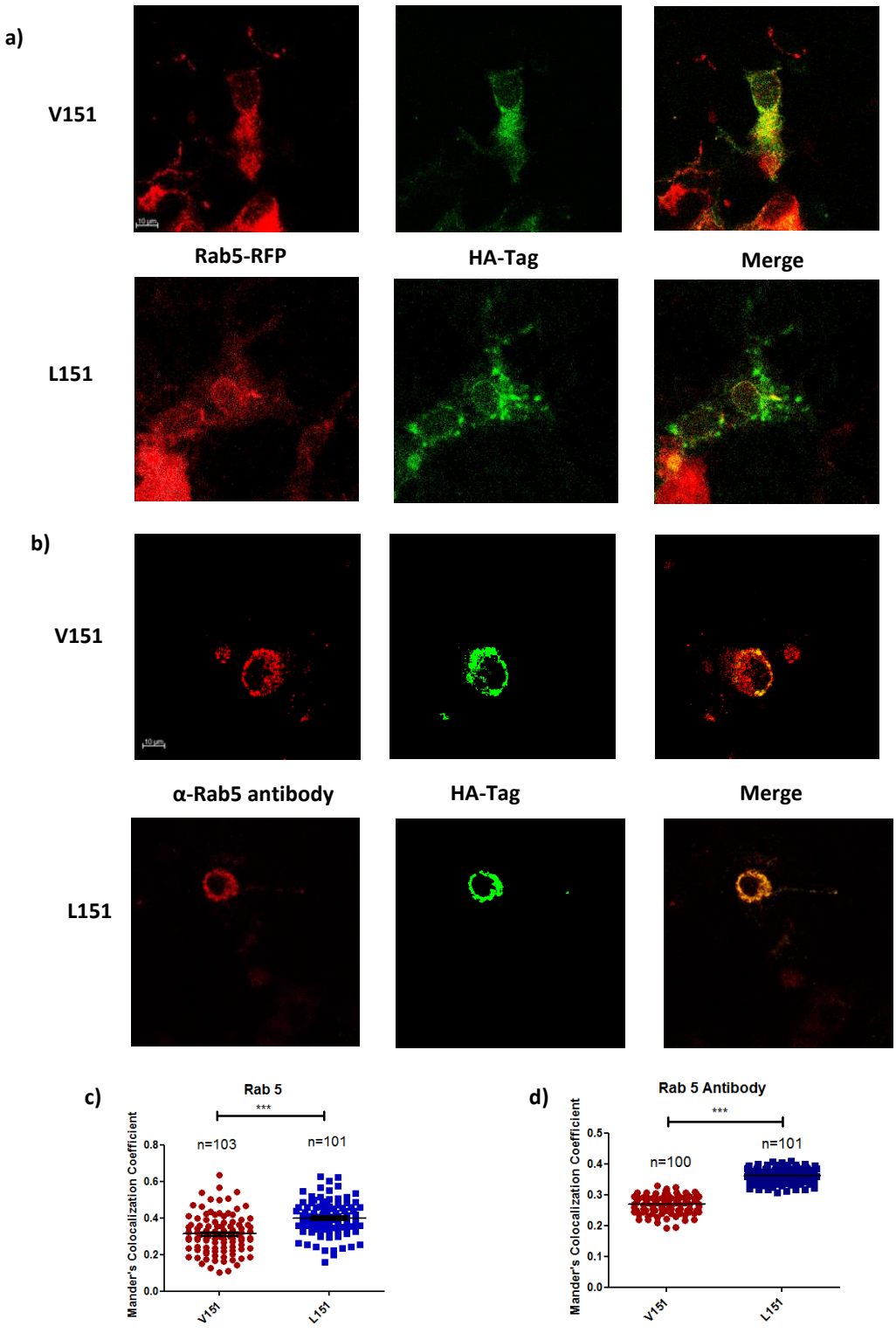


Figure 25. ADCY2-L151 is predominantly localized in early endosomes compared to ADCY2-V151 Cos7 cells transfected with pcDNA3.1-Adcy2-V151-HA or pcDNA3.1-Adcy2-L151-HA **a)** in combination with Rab5-RFP or **b)** stained for endogenous Rab5 using α-Rab5 antibody**. c)** Mander's Colocalization coefficient quantifies the amount of co-localization between Adcy2-151V-HA and Rab5-RFP (red) compared to the co-localization between

Adcy2-151L-HA and Rab5-RFP (blue). Student's T-Test, $p<0.05$ **d)** Mander's Colocalzation coefficient quantifies the amount of co-localization between Adcy2-151V-HA and endogenous Rab5 (red) compared to the co-localization between Adcy2-151L-HA and endogenous Rab5 (blue). (Student's T-Test, $p<0.01$).

The Phalloidin staining of transfected Cos7 cells co-localized with ADCY2-151V-HA (Fig. 26a). Since Phalloidin labels the plasma membrane, this demonstrates that Adcy2 is also localized at the plasma membrane, as previously described (Hanoune & Defer, 2001). Additionally, transfected cells showed a higher co-localization of ADCY2-V151-HA with Phalloidin than of ADCY2-L151-HA with Phalloidin. This suggests that more ADCY2-151V is localized at the plasma membrane compared to ADCY2-L151 (Fig 26b).

Taken together, this suggests that more ADCY2-L151 is retained in different intracellular compartments and less of this variant is present at the cell membrane compared to the wild-type ADCY2-V151, which might explain the observed reduction of cAMP production in cells transfected with the disease- associated variant ADCY2-L151.

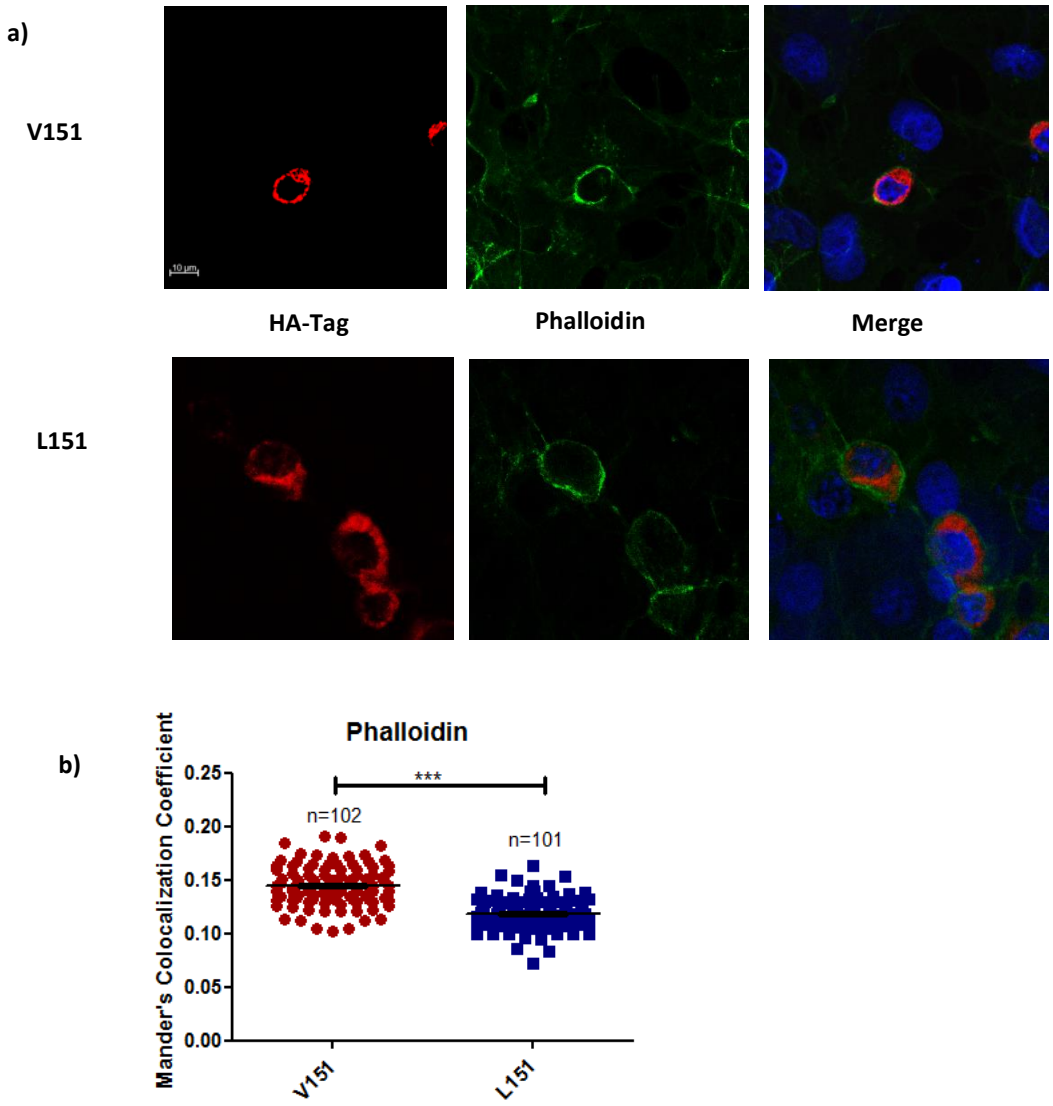


Figure 26. ADCY2-V151 is predominantly localized at the plasma membrane compared to ADCY2-L151 a) Cos7 cells transfected with pcDNA3.1-Adcy2-V151-HA or pcDNA3.1-Adcy2-L151-HA and the plasma membrane was stained with phalloidin. **b)** Mander's Colocalization coefficient quantifies the amount of co-localization between Adcy2-151V-HA and phalloidin (red) compared to the co-localization between Adcy2-151L-HA and phalloidin (blue). (Student's T-Test, $p<0.01$)

4.6. Establishment of Adcy2-L151 mice

In the *in-vitro* assay we could demonstrate that the V151L substitution results in lower ADCY2 activity, which is accompanied by a reduced ADCY2 presence at the plasma membrane. Next we wanted to assess the *in-vivo* relevance of the disease-associated Adcy2 SNP.

The CRISPR/Cas9 technology was used to create a mouse line in which Valine at position 151 was substituted by a Leucine resembling the SNP found to be associated with bipolar disorder. The targeting vector contained 2 silent mutations, one that added a restriction enzyme site (AluI) for genotyping purposes and another that obliterated the PAM sequence (NGG) so that the donor DNA was not cleaved by Cas9. Founders derived from zygote injection were initially screened by PCR and restriction digests. The presence of the V151L substitution was confirmed by PCR and subsequent restriction digest and sequencing (Fig 27).

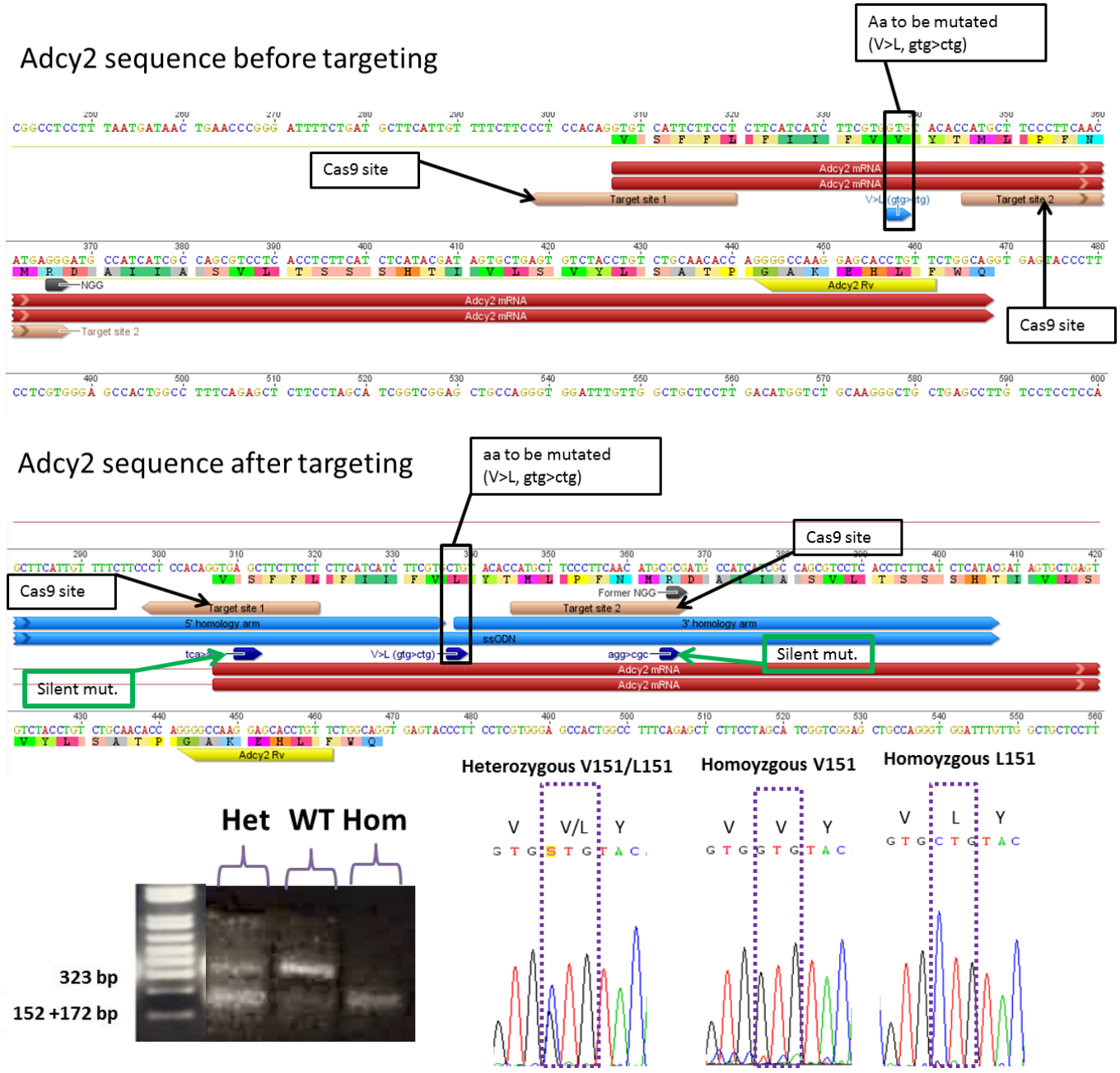


Figure 27. The presence of the SNP of interest in the mouse genome was verified a) Crispr/Cas9 targeting scheme used to introduce the SNP of interest into the mouse genome (Created by Dr. Oskar Ortiz). **b)** Genotyping of mice by amplifying the region of interest. Since a silent mutation also introduces a AluI site in this region, this restriction enzyme digest is used to specify the different genotypes. **c)** The presence of the SNP of interest as well as this genotyping strategy was verified using sequencing.

4.7. Behavioural characterization under basal housing conditions

A battery of behavioural tests was conducted in mice carrying the wild type Adcy2-V151 variant (WT) and mice that were homozygous for Adcy2-L151 (Homozygous). These tests aimed to assess if just the presence of the SNP of interest affected any of the endophenotypes of bipolar disorder. These endophenotypes included changes in anxiety-related behaviour, coping behaviour, novel object exploration, sociability, cognition and locomotion. These particular readouts have been previously described to reflect changes observed in patients with bipolar disorder (Logan & McClung, 2016; Perry et al., 2010)

4.7.1. Adcy2-L151 mice demonstrate increased locomotor activity.

Previous patient studies have demonstrated that hyperactivity is one characteristic of mania-like behaviour (Logan & McClung, 2016). Therefore we decided to measure if this hyper activity phenotype is present in the homozygous mice. Hyper locomotion was taken as a measure of hyperactivity.

To assess acute changes in locomotor activity in a novel environment mice were subjected to an OF test for 30 mins and the distance travelled in this time was measured. The data revealed that there was no significant difference between the total distances travelled by WT and Adcy2-L151 mice in the 30 mins (Student's T-Test, $p > 0.05$; Fig 28).

Next, differences in locomotor activity were screened by observing home cage activity of the wild type (WT) and the Adcy2-L151 (L151) mice (Fig.29). Rodents are nocturnal and therefore the basal levels or activity in these two phases are known to be significantly different. Therefore, two-way ANOVA repeated measures, was performed separately for the activity during the light and the dark phases to ensure that the difference in activity in their active phase (night phase) is captured and is not influenced by their low activity during the inactive phase. Measurement of home cage activity revealed that the Adcy2-L151 mice had a higher locomotion at night than WT mice in the first two nights. However, this difference does not reach significance in the next two nights. This suggests that Adcy2-L151 mice take longer to habituate in a new environment (new home cage).

Amphetamine-induced hyper-locomotion is a typical assay used to address mania-like phenotypes in rodents (Logan & McClung, 2016). To this end, we tested two doses of

Amphetamine to assess whether a similar mania-associated phenotype is observed in Adcy2-L151 mice.

As previously demonstrated, a low dose of amphetamine (2 mg/kg) has only little effect on locomotion (Miro et al., 2012; Shaltiel et al., 2008). However, Adcy2-L151 mice demonstrate a significant increase in locomotion post 2 mg/kg amphetamine compared to the WT mice (Fig 30a). Furthermore, following administration of a higher dose of amphetamine (4 mg/kg), the Adcy2-L151 mice continued to demonstrate a significantly higher increase in locomotion compared to the WT mice. Taken together this data clearly indicates that the Adcy2-L151 mice are more vulnerable to an amphetamine-induced hyper-locomotion phenotype.

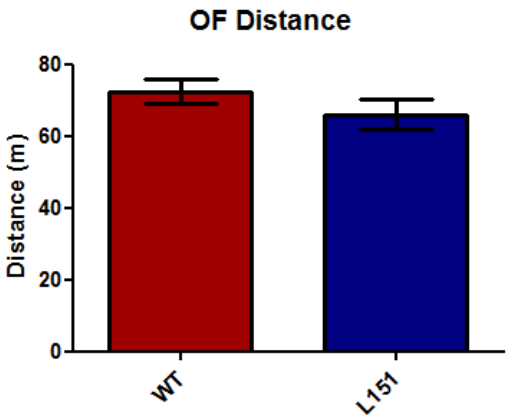


Figure 28. No difference in locomotion observed between Adcy2-L151 and WT animals. ADCY2-V151 (WT, n=12) and Adcy2-L151 mice (L151, n=10) were allowed to freely explore the OF arena under low illumination (10 Lux). The total distance travelled was measured. (Student's t-test, $p>0.05$)

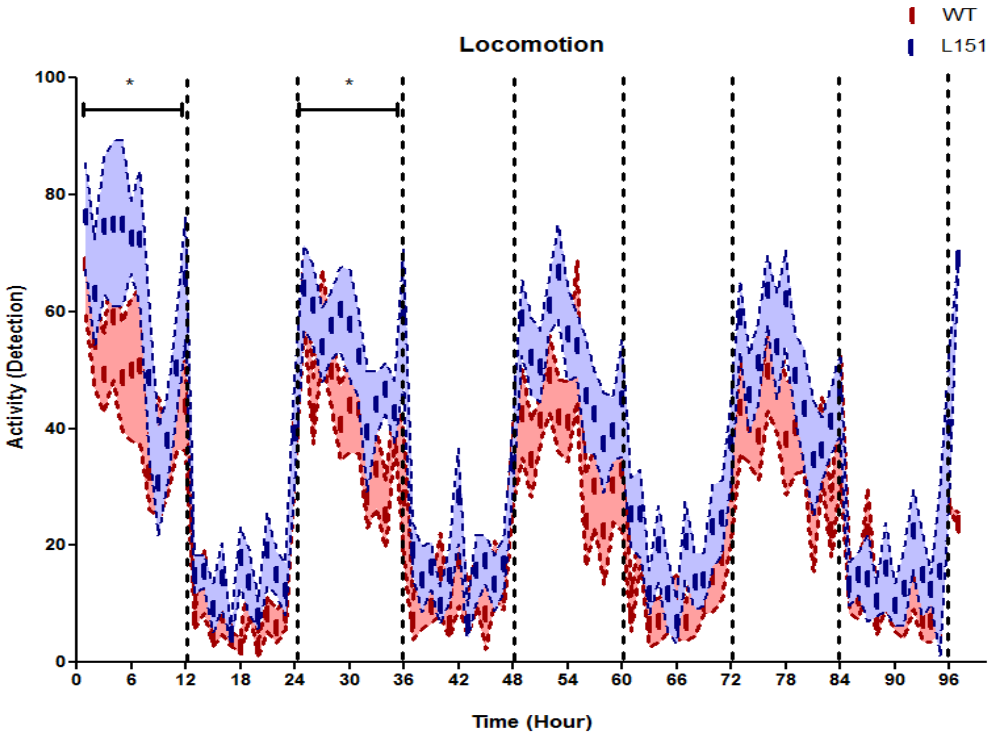
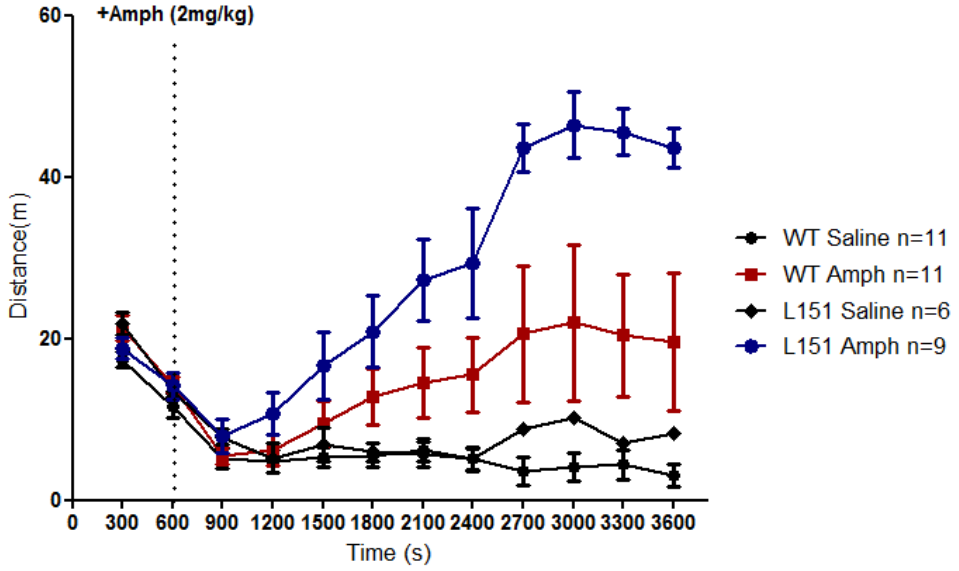


Figure 29. A difference in locomotor activity is observed between the Adcy2-L151 mice and the WT mice in the first two dark phases. Adcy2-V151 (WT, n=9) and Adcy2-L151 (L151, n=9) were placed in a novel home cage at the start of their night phase and tracked by an automated infrared tracking device for 4 consecutive days. (Two-way ANOVA, repeated measures, $p<0.05$).

a)



b)

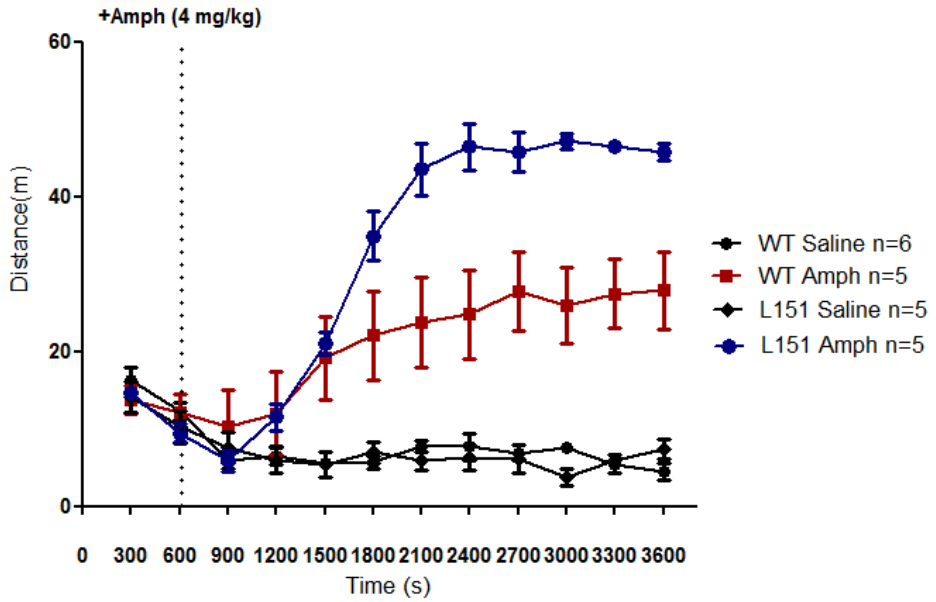


Figure 30, Adcy2-L151 mice show increased amphetamine induced hyper-locomotion. Adcy2-V151 (WT) and Adcy2-L151 mice (L151) were administered **a)** 2 mg/kg amphetamine or 0.9% saline and **b)** 4 mg/kg amphetamine or 0.9% saline and were free to explore the OF. (Two way ANOVA repeated measures, $p<0.01$ (effect of interaction), $p<0.01$ (effect of genotype), $p<0.01$ (effect of amphetamine) for a) and b). a) Interaction $F_{(55,420)}=2.983$, b) Interaction $F_{(33,187)}=19.66$)

4.7.2. Adcy2-151L mice show decreased anxiety-related behaviour

Since mania like behaviour have been previously characterized by lower anxiety behaviour, and depression-like behaviour has been characterized by increased anxiety (Yen et al., 2013), a battery of anxiety tests were performed to test for any difference in anxiety-related behaviour.

We first assessed anxiety-related parameters in the open field (OF) test. The OF test revealed that the Adcy2-L151 mice spent significantly more time in the centre of the OF (Fig 31a; Student's T-Test, $p<0.01$). Furthermore, the number of entries to the centre of the test apparatus was significantly higher in Adcy2-L151 compared to WT mice (Student's T-Test, $p<0.05$; Fig. 31b). Additionally, there was no significant difference between the distanced travelled by mice of each genotype in the OF (Student's T-Test, $p>0.05$) (Fig. 31c). This suggests that anxiety is not confounded by locomotion.

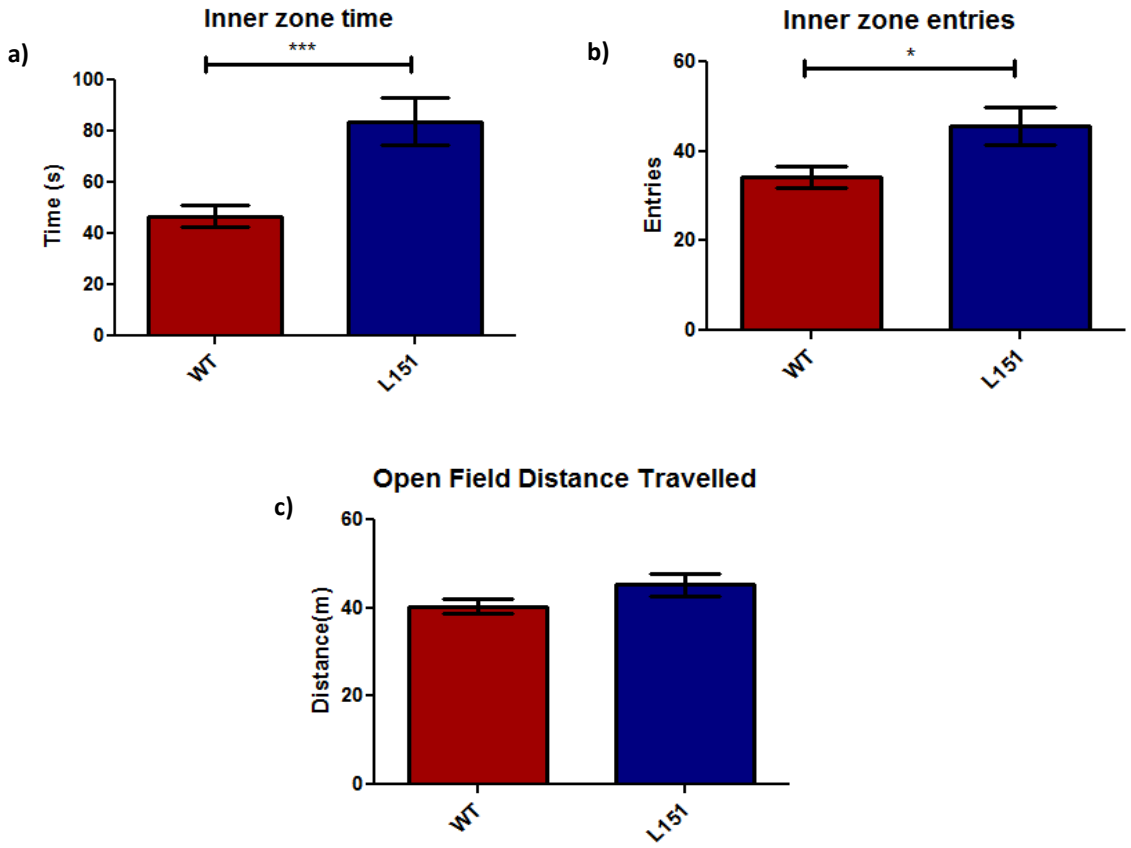


Figure 31. Adcy2-L151 mice enter and spend more time in the centre of the OF arena. ADCY2-V151 (WT, n=10)) and Adcy2-L151 (L151, n=10) were allowed to freely explore the OF arena for 10 mins under low illumination (10 Lux). **a)** Time spent by the animals in the centre of the OF arena (Student's t-test, $p<0.01$). **b)** Number of

entries into the centre of the OF arena (Student's t-test, $p<0.05$) **c)** Distance travelled in the OF. For a)-c) * indicates $p<0.05$ and ** indicates $p<0.01$

Next we carried out the dark light box (DaLi) test which showed that the Adcy2-L151 mice spent significantly more time in the brightly lit chamber than the WT littermates. (Student's T-Test, $p < 0.01$) (Fig 32).

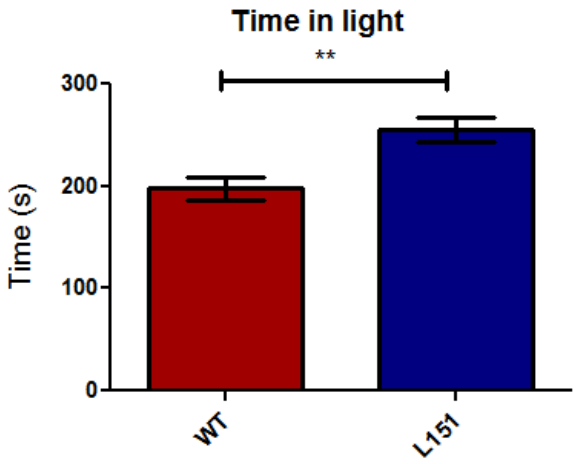


Figure 32. Adcy2-L151 mice spend more time in the brightly lit chamber of the DaLi apparatus than WT littermates. Adcy2-V151 (WT, n=10)) and Adcy2-L151 mice (L151, n=10) were allowed to freely explore the DaLi arena for 10mins.(Student's t-test shows $p<0.01$)

In elevated plus maze (EPM) test no significant difference were observed in any of the assessed parameters, i.e., the time in the open arms or the number of entries to the open arms (Student's t-test, $p>0.05$) (Fig.33).

The marble-burying test is a measure of novelty-induced anxiety. This test also assesses anxiety-like behaviour in a rather locomotion independent manner (Savy et al., 2015). This test revealed that Adcy2-L151 mice buried significantly fewer marbles in the 90 mins than the WT mice (Student's T-Test, $p < 0.05$) (Fig. 34).

Taken together, all anxiety tests, except for the EPM, indicate that the Adcy2-L151 mice have lower levels of anxiety compared to their WT littermates.

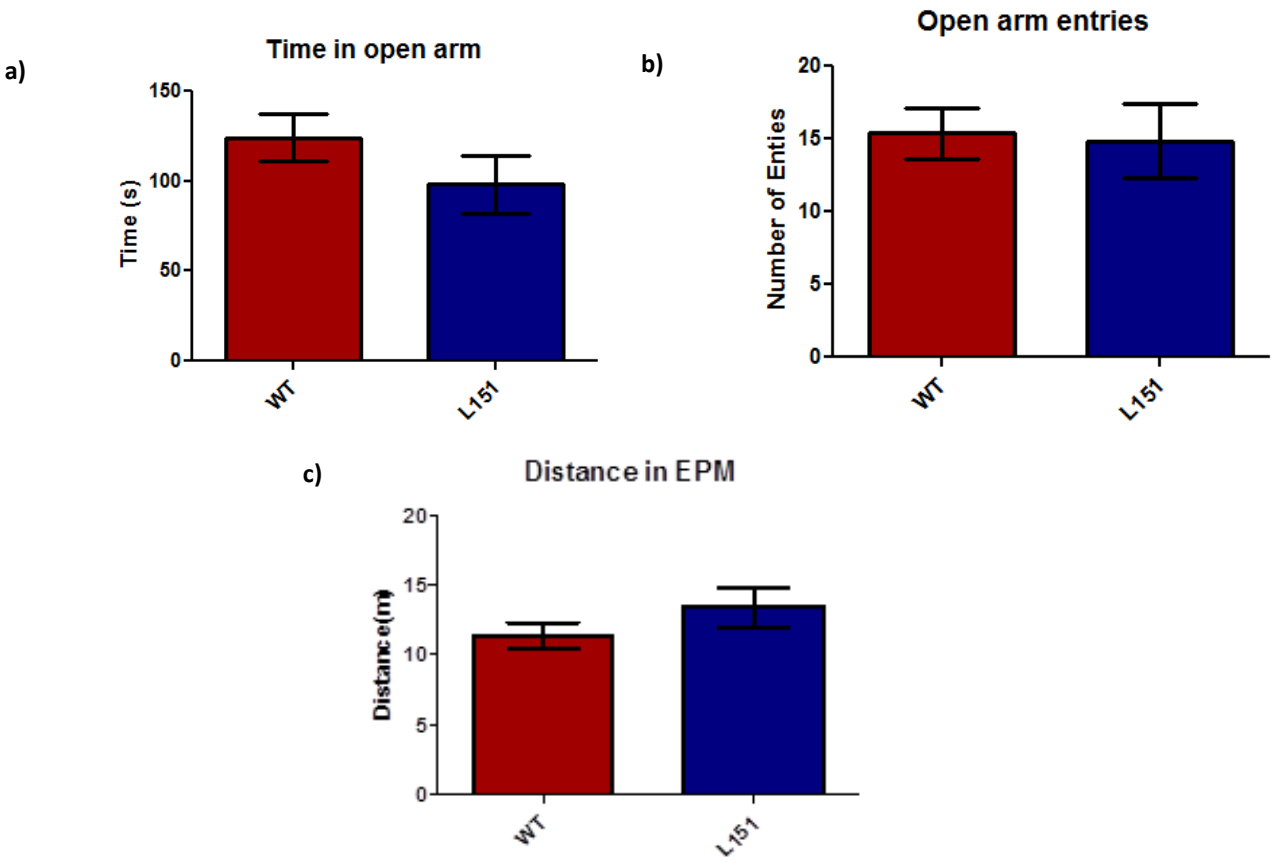


Figure 33 In the EPM Adcy2-L151 mice are indistinguishable from WT littermates Adcy2-V151 (WT, n=10)) and Adcy2-L151mice (L151, n=10) were allowed to freely explore the EPM arena under low illumination (10 Lux) The test measured. **a)** time spent in the open arm **b)** number of entries into the open arm and **c)** distance travelled in the EPM. a)-c) Student's t-test was carried out and revealed $p>0.05$

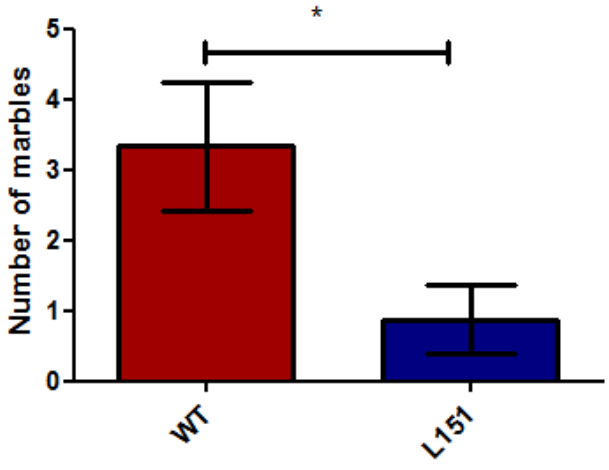


Figure 34. Adcy2-L151 mice burry significantly fewer marbles than WT littermates. Adcy2-V151 (WT, n=11)) and Adcy2-L151 mice (L151, n=11) were placed in a home cage with 10 marbles and were left to freely burry marbles under low illumination (10 Lux). The number of marbles buried after 90 mins was counted. (Student's t-test, $p<0.05$)

4.7.3. Hedonic behaviour

Since mania-like behaviour is often characterized by increased hedonic behaviour (Miro et al., 2012), changes in this phenotype were assessed using the sucrose preference test (SPT). The SPT demonstrated a significant increase in sucrose intake compared to water intake in Adcy2-L151 mice (Two way ANOVA repeated measures, $p<0.05$) (Fig. 35a). However, the maximum preference for sucrose intake was much lower than previously described (Miro et al., 2012; Yen et al., 2013). This difference could be due to switching the side of the bottles during the test. Therefore, the SPT test was repeated without switching the sides of the bottles. The second round of SPT, however, revealed that Adcy2-L151 mice showed a significantly lower hedonic behaviour compared to WT mice (Two way ANOVA repeated measures, $p<0.05$) (Fig. 35b). However, the second round of SPT was confounded by high construction noise. Since Adcy2-L151 mice are more susceptible to stress (see below), it is possible that the stress due to construction noise resulted in the anhedonic behaviour observed in the second round of SPT.

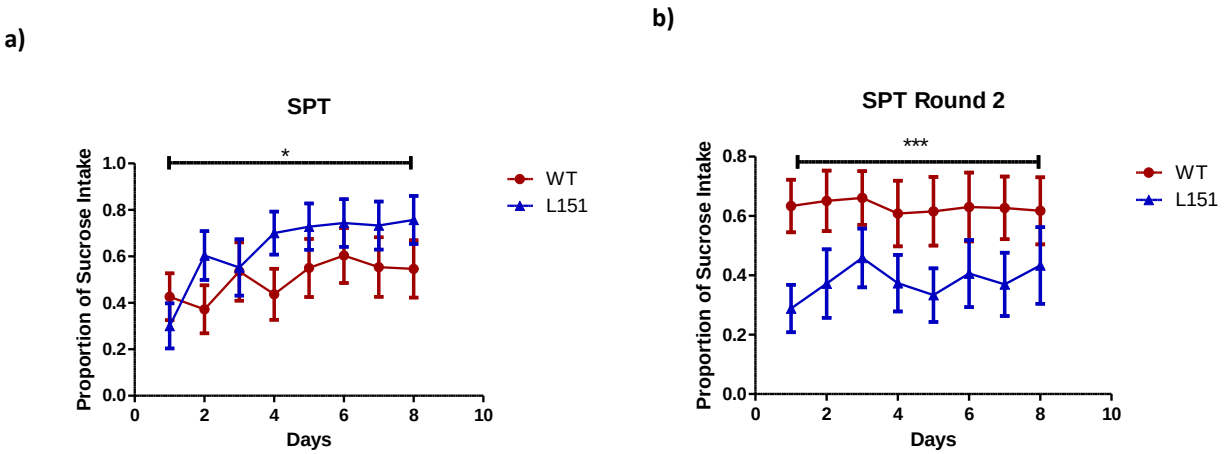


Figure 35. Sucrose preference test. a) The Adcy2-L151 mice (n=10) demonstrate significantly higher sucrose intake compared to WT mice (n=12). Two way ANOVA repeated measures, $p<0.05$. **b)** In the second round of SPT the Adcy2-L151 mice (n=10) demonstrate significantly lower sucrose intake compared to WT mice (n=12). Two way ANOVA repeated measures, $p<0.05$

4.7.4. Adcy2-L151 show increased active coping behaviour

Mania-like behaviour has been previously associated with increased active stress coping behaviour in the forced swim test (FST) (Kromer et al., 2005; Yen et al., 2013). To assess whether stress coping behavior was altered, Adcy2-L151 were subjected to the FST. In this

test, the goal directed search for an escape (swimming behaviour) has been considered as active coping behaviour, whereas the lack of goal directed search while floating was considered as passive coping behaviour. Furthermore, the initial struggling in the water is considered as more panic-like behaviour that is not indicative of a coping style. In the test Adcy2-L151 mice spent more time in a goal directed search for an escape (swimming behaviour) compared to the WT mice (Fig. 36b). Moreover, Adcy2-L151 mice spent significantly less time floating than WT mice (Fig. 36c). Taken together, these measurements in the FST suggest that the Adcy2-L151 mice have an increased active coping behaviour.

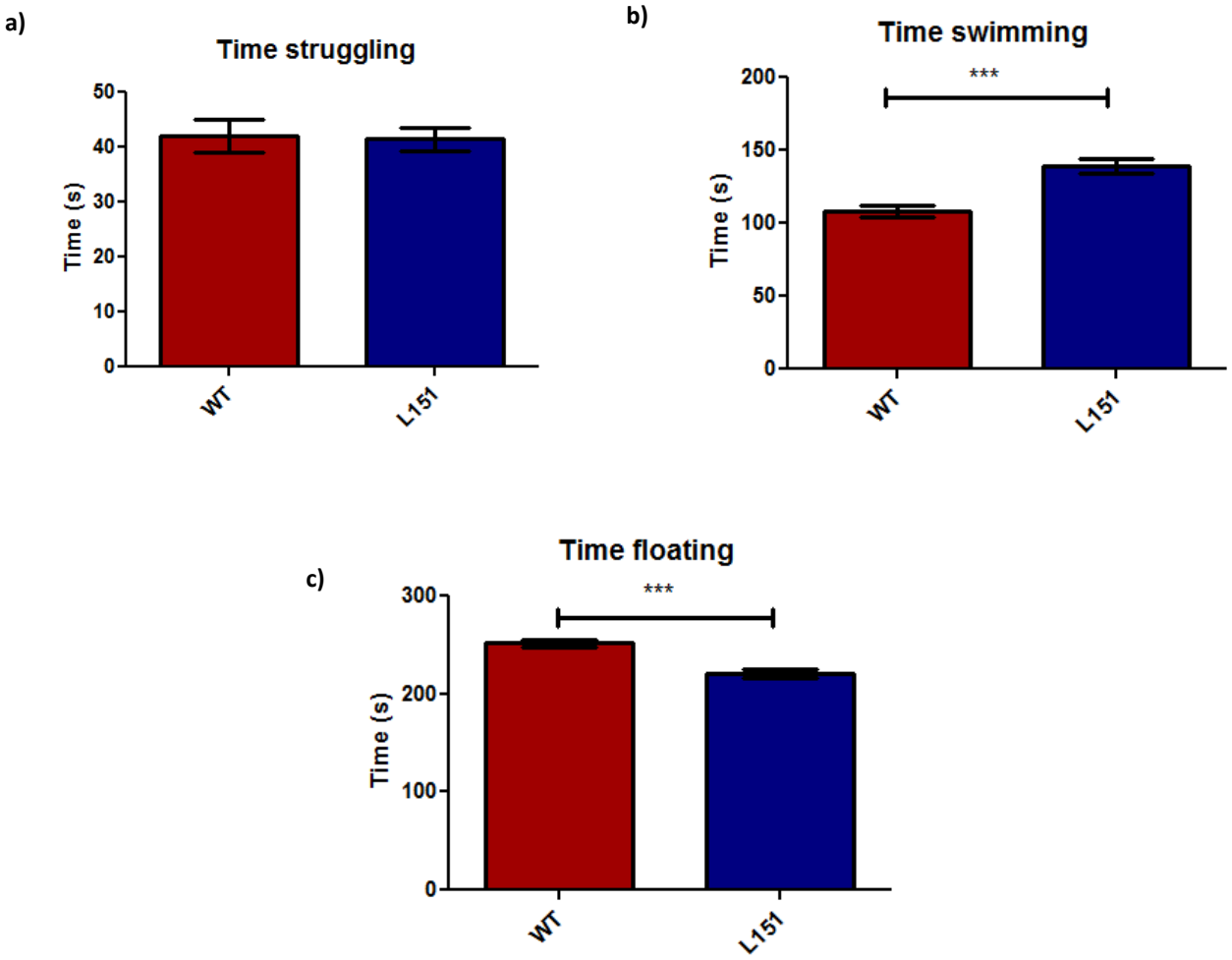


Figure 36 Adcy2-L151 mice show increased active coping behaviour. Adcy2-V151 (WT, n=11) and Adcy2-L151 (-L151, n=10) were placed in the FST arena with water at RT under low illumination (10 Lux). The videos were scored manually. **a)** There was no significant difference between genotypes in the amount of time the mouse spent struggling. **b)** Adcy2-L151 mice spent more time swimming than the WT mice. **c)** Adcy2-L151 mice spent

significantly less time floating compared to the WT mice. Student's t-test was carried out for all tests. For a) $p>0.05$, for b)-c) $p<0.01$.

4.7.5. Adcy2-151L mice show increased object exploration

Previous patient studies have demonstrated that one classical characteristic in patients with bipolar mania is an increased object exploration that is absent in patients with other psychiatric disorders like schizophrenia (Perry et al., 2010). To explore whether this distinct feature of bipolar mania is present in Adcy2-L151 mice, an object exploration test was used. In this test animals that were previously habituated to the OF arena, were place in this arena with a Lego piece. The test was carried out under low illumination (10 Lux). The amount of time mice spent interacting with this object, as well as the number of times the mice entered the zone with the objected was measured. The data shows that Adcy2-L151 mice spent significantly more time interacting with the object (Fig 37a; Student's t-test, $p<0.05$). Furthermore, Adcy2-L151 animals also spent significantly more time in the zone with the object (Fig 37b; Student's t-test, $p<0.05$). This test demonstrates that Adcy2-L151 mice show an object exploration phenotype, which is comparable to patients in a manic phase.

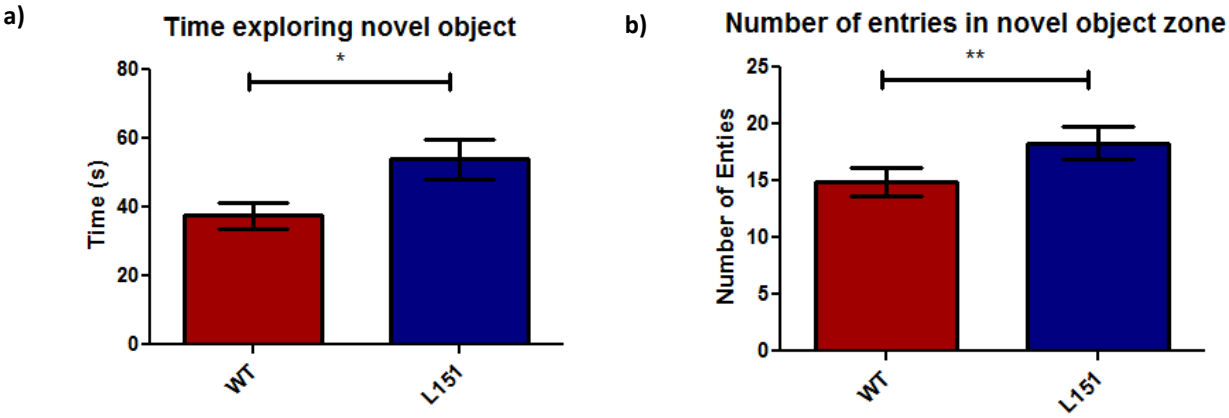


Figure 37. Adcy2-L151 mice show increased object exploration behaviour. **a)** Adcy2-L151 (L151, n=10) mice spent a significantly less time in the zone containing the object compared to Adcy2-V151 (WT, n=11). **b)** The number of times the mouse entered the zone containing the object was also higher in L151 (Adcy2-L151) mice comparted to WT mice. Student's t-test was used in all test. * means $p<0.05$ and ** means $p<0.01$

4.7.6. Adcy2-L151 mice show increased sociability only in a group context and no preference for social novelty

Increased sociability is another mania-like feature that has been previously described (Yen et al., 2013). Therefore, we wanted to test if similar mania-associated increased social behaviour is observed in Adcy2-L151 mice. In a first social interaction test we observed sociability between two freely moving mice of the same genotypes in a group setting. In the second test we used a 3-parted chamber test to test for preferences for social interaction with a novel mouse compared to interaction with a novel object. We also tested the mice's preference for social novelty.

In the sociability test, each mouse was placed in the OF arena to which it was previously habituated to with a novel conspecific of the same genotype. The test was carried out for 5 mins under low illumination. The amount of time the two mice spent in nose to nose touches, following, chasing, body sniffing, anogenital sniffing as previously described (Nadler et al., 2004) were considered as social interaction between the mice in the test. The length of time each pair of mice spent in these social behaviours was manually recorded. The data shows that Adcy2-L151 mice spent significantly more time interacting with the novel conspecific compared to WT mice (Fig. 38) (Student's t-test, $p<0.05$). However, since within each pair, either of the two mice could initiate interaction, the amount of social interaction might be more than that of each individual mouse. Nevertheless, since both mice were of the same genotype, a certain amount of recorded social interaction could be attributed to mice of one of the two genotypes. Thus this test suggests that the SNP of interest results in a phenotype of increased sociability.

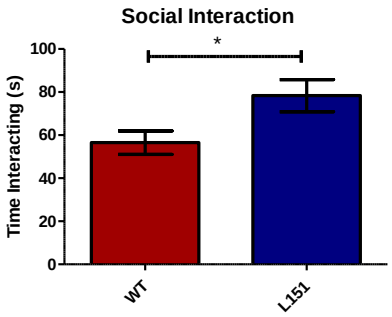


Figure 38. Adcy2-L151 mice show increased sociability. Mice were placed with a novel conspecific of the same gender and genotype in the OF arena and the amount of time spent in social interaction by each mice was measured. The Adcy2-L151 mice (L151, n=10) spent significantly more time interacting with a conspecific compared to Adcy2-V151 mice (WT, n=11). Student's t-test. * implies <0.05

The three-parted chamber test was carried out as previously described (Hartmann et al., 2012; Moy et al., 2004). In this test the results suggest that the Adcy2-L151 mice do not have a significant difference in sociability (Student's t-test, p>0,05)(Fig 39 a). While this seem to contradict our previous finding, the conditions of the social interaction between the two tests are different, which might explain the observed discrepancy (Fig 38). Furthermore, the Adcy2-L151 mice showed no preference for social novelty unlike the WT mice (Fig 33b). This lack of preference for social novelty could be a result of deficits in cognition.

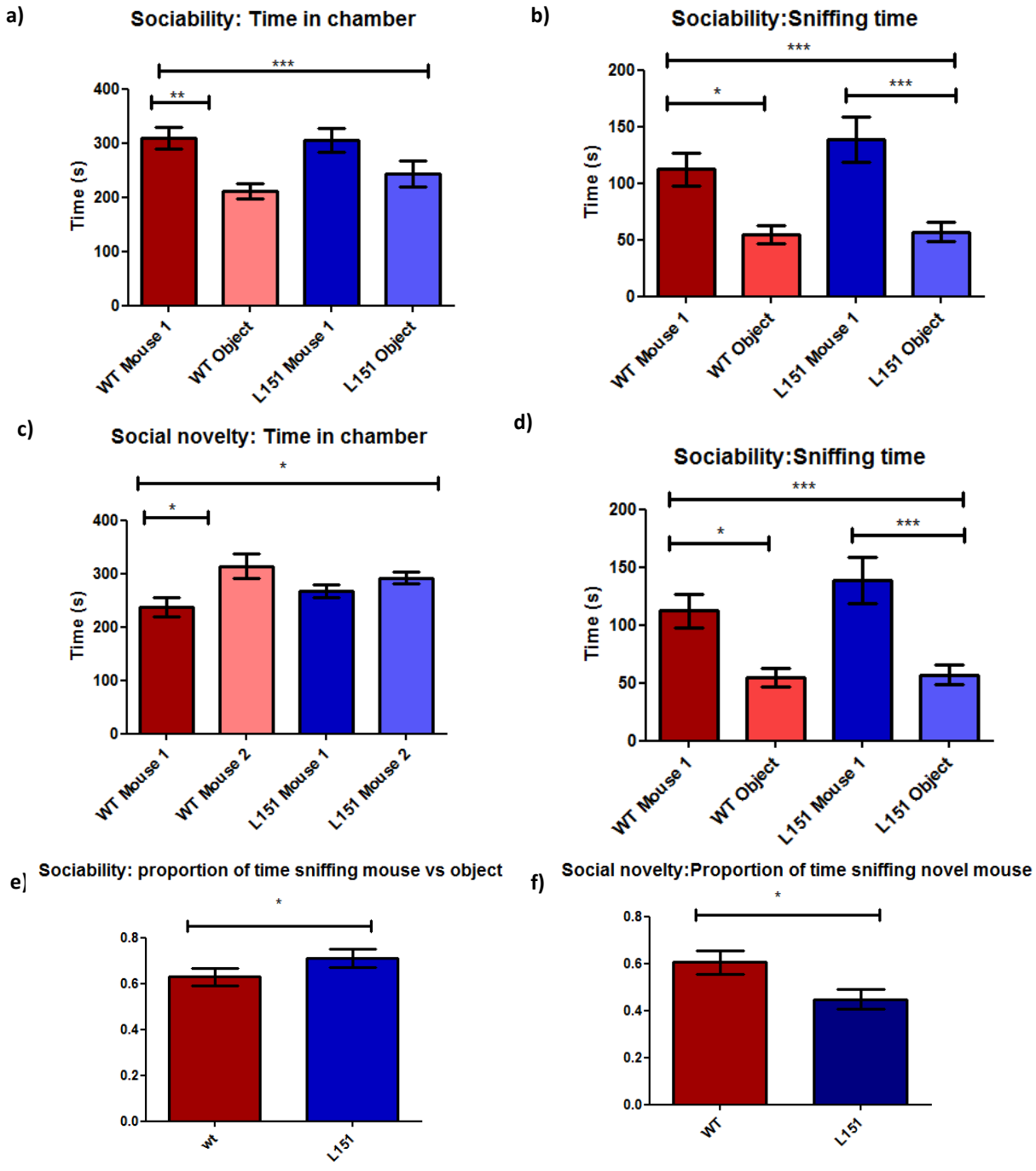


Figure 39. Adcy2-L151 mice show unaltered sociability but lack preference for social novelty a) The amount of time the Adcy2-L151 mice (L151, n=10) and the Adcy2-V151 mice (WT, n=11) spent in the zone with the novel object and novel mouse was measured. **b)** The time the Adcy2-L151 mice (L151, n=10) and the Adcy2-V151 mice (WT, n=11) spent sniffing the familiar conspecific (Mouse 1) and sniffing the novel conspecific (Mouse 2). **c)** Time Adcy2-L151 mice (L151, n=10) and the Adcy2-V151 mice (WT, n=11) spent sniffing the novel object and sniffing the novel conspecific mouse. **d)** Time the Adcy2-L151 mice (L151, n=10) and Adcy2-V151 mice (WT, n=11) spent in the zone with the familiar conspecific (Mouse 1) and the novel conspecific (Mouse 2)). **e)** Adcy2-L151 mice (L151, n=10) showed a trend towards higher interaction with a conspecific than with an object compared to

Adcy2-V151 mice (WT, n=11). **f)** Adcy2-L151 mice (L151, n=10) spent significantly more time interacting with the conspecifics compared to the Adcy2-V151 mice (WT, n=11). One-way ANOVA and Bonferroni post-hoc tests were performed for a)-d). Student's t-test was performed for e)-f) were $p<0.05$

4.7.7. Adcy2-L151 mice show cognitive deficits

Bipolar disorder is often accompanied by cognitive deficits during all phases of the disorder (G. Cipriani, Danti, Carlesi, Cammisuli, & Di Fiorino, 2017; Sole et al., 2017). In order to test for deficits in executive memory, the Morris water maze test was used. Furthermore to test for synaptic correlates of memory deficits, a classical long term potentiation (LTP) measurement was performed using acute hippocampal brain slices.

The classical Morris water maze test was used as previously described (Morris, 1984). This test revealed that the Adcy2-L151 mice were slower learners than WT mice (Two way ANOVA repeated measure, $p<0.05$; Fig 40a). While there was a trend towards a decreased short-term memory as seen from the first test, this did not reach significance (Student's t-test, $p>0.05$; Fig 40c). However, the second test performed 7 days post learning showed a deficit in long-term memory (Student's t-test, $p>0.05$) (Fig 40e). Furthermore, given that there is no significant difference in the distance travelled between the mice of the two genotypes, this difference in time spent in the target quadrant cannot be explained by an increased preference for swimming. Taken together, the Morris water maze demonstrates that Adcy2-L151 mice have cognitive impairments.

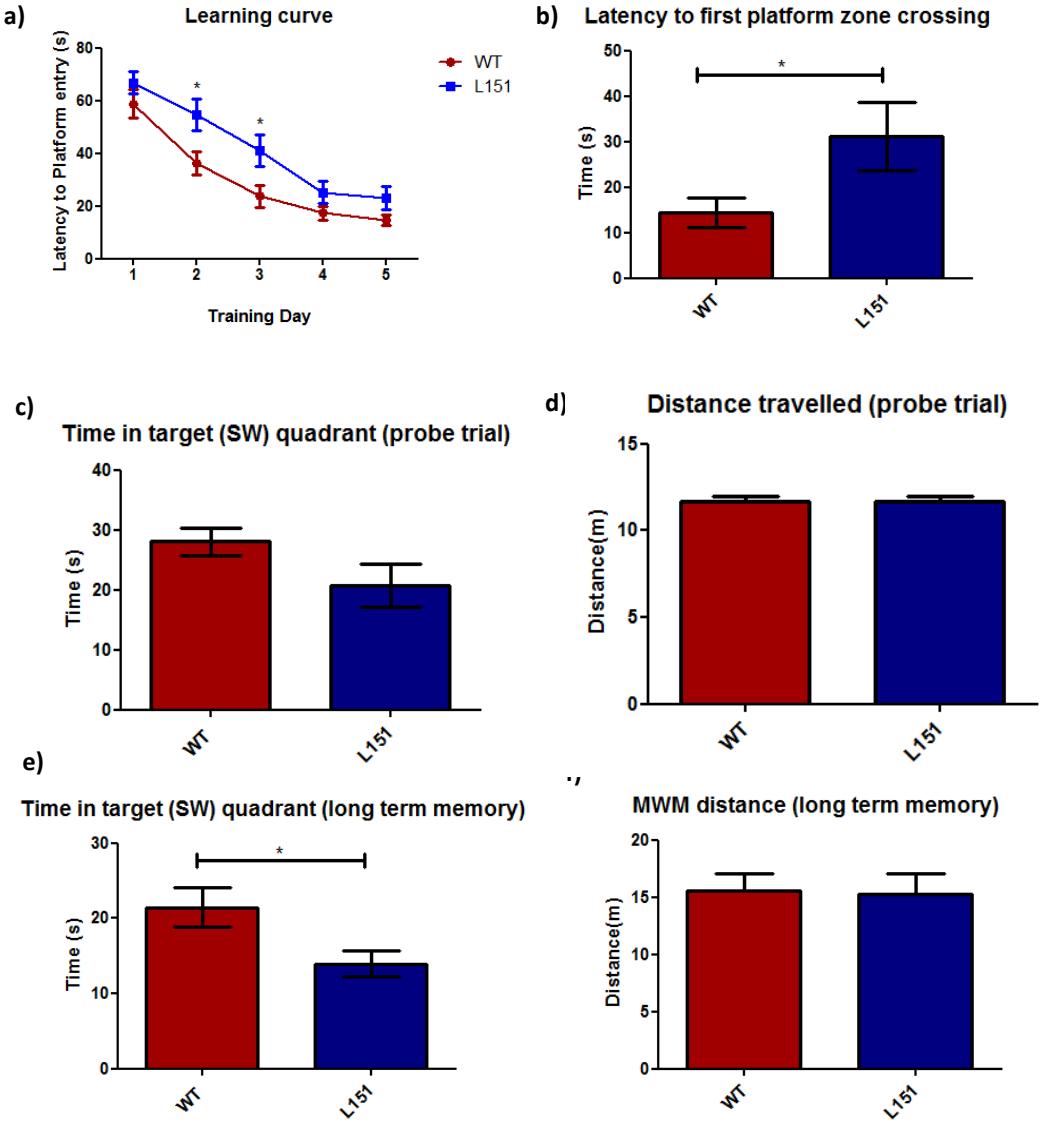


Figure 40. Adcy2-L151 mice show learning and memory deficits. a) Mice were trained to find the platform in the south west quadrant over 5 days, with 4 trials per day. The Adcy2-L151 mice (L151, n=9) were significantly slower learners over the training session compared to Adcy2-V151 mice (WT, n=10). **b)** On the first test it took Adcy2-L151 mice (L151, n=9) significantly longer to cross the zone (SW) where the platform was located during training. **c)** Adcy2-L151 mice showed a trend towards spending significantly less time in the target quadrant (SW) during the probe trial. **d)** The Adcy2-L151 mice show no difference in distance travelled compared to Adcy2-V151 mice during the probe trial. **e)** The Adcy2-L151 mice spent significantly less time in the target quadrant (SW) during the long term memory test. **f)** The Adcy2-L151 mice show no difference in distance travelled compared to Adcy2-V151 mice during the long term memory test. Two-way ANOVA, repeated measure with Bonferroni post-hoc test was used in a) $p<0.05$. Student's t-test was used for b)-f). For b) and e) $p<0.05$

Since the homozygous mice show a deficit in long term memory (Fig. 40e), we evaluated the synaptic correlate for memory, i.e., LTP in Adcy2-L151 mice (Lynch, 2004). Standard LTP measurements were conducted by stimulating the Schaffer collateral pathway while recording from the CA1 region. The LTP measurement demonstrated that the LTP in homozygous mice is significantly lower than that in the WT mice (two way ANOVA, $p<0.05$) (Fig 41). This reaffirms the observation that the Adcy2-L151 mice have memory deficits.

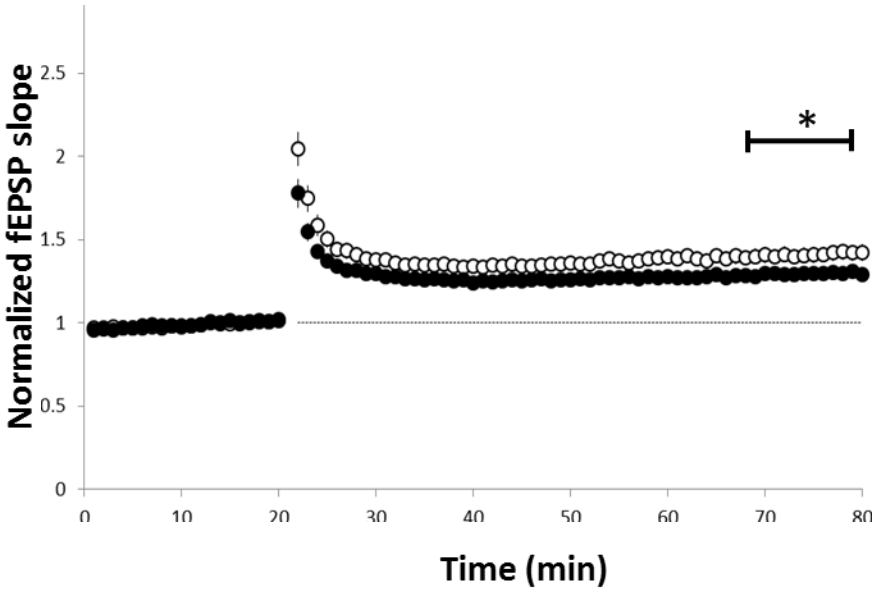


Figure 41. Adcy2-L151 mice show decreased LTP. Standared LTP measurements were conducted using hippocampal slices from Adcy2-V151 (n=5, slices=17) and Adcy2-L151 mice (n=5, slices = 18). The normalized fEPSP was significantly lower in Adcy2-L151 mice compared to WT mice in the final 10 mins of the recording. Two way ANOVA, $p<0.05$. (conducted by scientific core unit electrophysiology (Dr. Matthias Eder)

4.7.8. Lithium treatment reverses mania-like phenotypes in Adcy2-L151 mice

Currently Lithium is the best available long term treatment for BD (J. R. Geddes & Miklowitz, 2013). Previous animal models of BD have been able to treat mania-associated behaviour in mice using Lithium (Roybal et al., 2007). These studies administered 600 mg/L of lithium chloride for 10 days as this complied with drug effectiveness and posed little health concerns as per previous studies (Dehpour et al., 2002). Therefore, in accordance with previous studies, 600 mg/L of Lithium Chloride was administered in the drinking water of Adcy2-V151 and

Adcy2-L151 mice for 10 days. The control mice had access to normal drinking water. These mice were then tested for anxiety and stress coping behaviour.

OF was used to test for anxiety. The results demonstrated that after administration of lithium for 10 days, the homozygous animals spend similar amounts of time in the centre of OF as the untreated wildtype animals (Fig. 42 a). Since there is a significant interaction between lithium administration and genotype, it suggests that lithium administration reduces the reduces the mania-like behaviour or reduced anxiety in Adcy2-L151 mice (Two-way ANOVA repeated measures, interaction $p<0.01$, $F(1,31)=13.29$.)

FST was used to assess coping behaviour. FST shows that after administration of lithium, the homozygous mice spend similar amounts of time floating as the untreated wildtype mice (Fig. 42 b). There was a significant interaction between the use of lithium and the genotype (Two-way ANOVA repeated measures, interaction $p<0.01$, $F(1,32)=324.1$. This once again suggests that long term administration of lithium reduces mania-associated phenotypes like increased active coping in Adcy2-L151 mice.

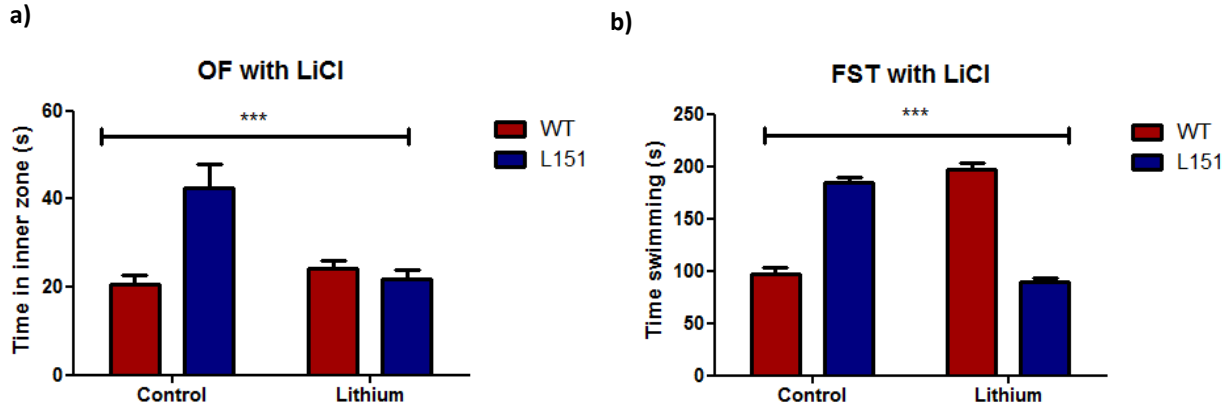


Figure 42. The phenotypes of Adcy2-L151 mice can be recovered by long term administration of lithium chloride a) The amount of time the Adcy2-L151 mice (n=10) and the WT mice (n=9) spent in the centre of open field after administration of lithium for 10 days. WT mice (n=10) and Adcy2-L151 (n=8) that were administered drinking water was taken as controls **b)** The amount of time the Adcy2-L151 mice (n=10) and the WT mice (n=8) spent swimming during FST after administration of lithium for 10 days. WT mice (n=8) and Adcy2-L151 (n=10) that were administered drinking water was taken as controls. Two-way ANOVA with Bonferroni post-hoc test were used for both tests and both show a significant interaction between the effect of lithium and genotype ($p<0.01$). a) has interaction $F(1,31)= 13.29$ and also show a significant difference between genotype ($p<0.01$) and drug administration ($p<0.05$) b) has interaction $F(1,32)=324.1$ and has no significant difference between just the genotype or just the drug administration.

4.8. Effect of environmental factors on behaviour

Typically Bipolar disorder is characterized by the manic and depressive phase. Nevertheless, most mouse models are only able to catch one phase of this disease (Logan & McClung, 2016). Similarly, the homozygous Adcy2-151L mice only demonstrated mania like behaviour under basal housing conditions, and the depressive phase was absent. However both genetic and environmental factors play a role in the development of psychiatric disorders (Uher, 2014). We therefore wanted to test if environmental factors influence the behaviour of Adcy2-151L mice.

4.8.1. Chronic Social Defeat Paradigm

As experiencing stressful events is linked to the development of bipolar disorder (Lex et al., 2017), we decided to assess the impact of chronic stress on Adcy2-151L mice. In order to do so we decided to use the chronic social defeat stress (CSDS) paradigm as has been previously described (Wagner et al., 2015)The first round of carried out in the bigger CSDS cages. However, the physiological parameters and the social parameters revealed that the CSDS did not work. CSDS was repeated using smaller cages increase the sensory queues passed across the barrier overnight. The findings from the second round of CSDS are described below.

4.8.2. Physiological Parameters

Typically following chronic social stress, the adrenal gland weights are described to increase and the thymus weight is described to decrease (Wagner et al., 2015). After this round of CSDS the animals were sacrificed and their thymus and adrenal glands were weighed. The thymus weights of the stressed mice were significantly lower than the unstressed animal (Fig 43a) (One way ANOVA, $p<0.05$). Furthermore the adrenal glands weights of the stressed mice were lower than those from unstressed animals (Fig 43b) (One way ANOVA, post-hoc Bonferroni's Multiple Comparison Test, $p<0.05$). However, there was no difference in the thymus or adrenal gland weights between the two genotypes. Taken together this suggests that the CSDS paradigm worked.

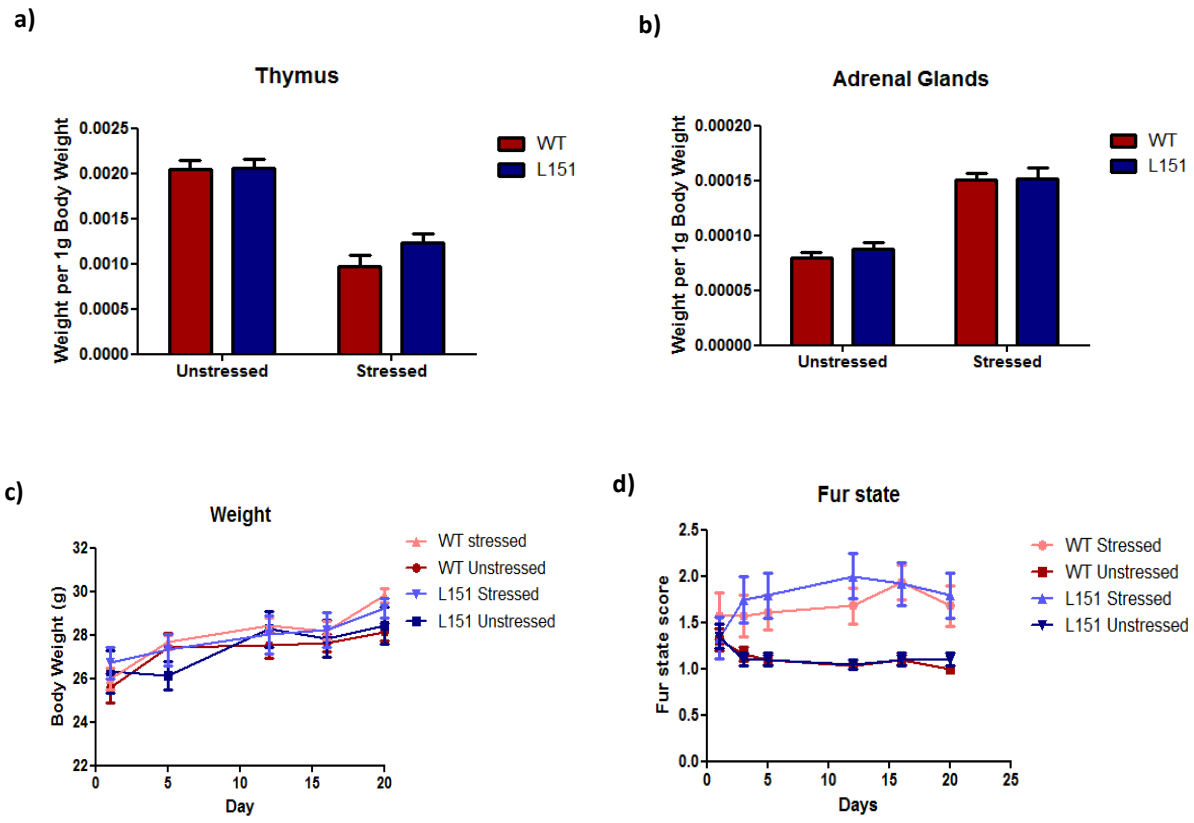


Figure 43 The CSDS paradigm resulted in the predicted physiological changes, however no genotypic differences were observed a) The thymus weight in the stressed animals (WT: n=15, Adcy2-L151: n=11) were significantly decreased compared to unstressed animals **b)** The weights of the adrenal glands were significantly higher in the stressed animals compared to the unstressed controls **c)** There was no significant difference between the body weights of the stressed and the unstressed mice did not have a significant difference. Furthermore there was no difference in the body weights between genotypes. **d)** The fur state changed significantly over time for all animals. While it improved for unstressed animals, it worsened for the stressed animals.. a) - b) Used two-way ANOVA had no interaction and no significant genotype effect ($p>0.05$). There was however a significant stress effect ($p<0.05$). c) Used two-way ANOVA repeated measures and show no interaction, a difference over time ($p<0.05$) however no difference between genotypes and stress conditions ($p>0.05$). d) Used two-way ANOVA repeated measures and show no effect over time, but a significant difference between mice stress conditions ($p<0.01$)

4.8.3. Social Avoidance

The social avoidance test has been previously used as a behavioural verification previously to ensure that the CSDS worked (Wagner et al., 2015). Therefore, this test was also used the WT and Adcy2-L151 mice to obtain a behavioural verification that the CSDS worked. The results demonstrate that the social avoidance ration of the stressed mice was significantly higher than that of the unstressed mice (Two-way ANOVA, for effect of stress: $p<0.05$) (Fig. 44). However, there was no genotype dependent difference in social avoidance observed. Similar to the physiological parameters, the CSDS paradigm affected both WT and Adcy2-L151 in a similar manner.

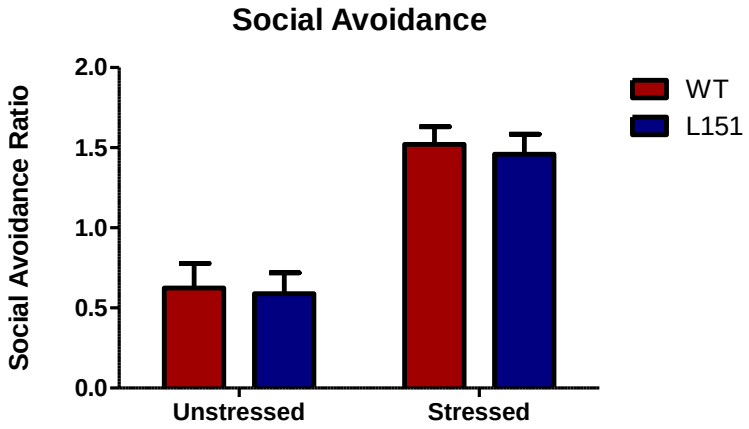


Figure 44. The stressed animals have an increased social avoidance ration compared to the unstressed animals. There is no genotypic difference in social avoidance Social avoidance ratio was calculated as the ratio of time spent in the zone with an empty wire mesh to the time spent in the zone with as wire mesh containing a novel adult CD1 mice (Two-way ANOVA, effect of stress: $p<0.05$, effect of genotype: $p>0.05$, interaction: $p>0.05$).

4.8.4. Anxiety-related behaviour

Under basal levels housing conditions, Adcy2-L151 mice showed reduced anxiety-related behaviour compared to the WT mice. This lower anxiety phenotype can be interpreted as

reflecting mania-like behaviour. After CSDS, the anxiety tests were carried out to assess the anxiety levels in mice post stress.

The OF test was carried out as previously described in the stressed and the unstressed mice. The unstressed Adcy2-L151 mice again spent significantly higher time in the centre of the OF and entered the centre of the OF more often than the WT mice (Fig. 45a).This verifies the previously observed decreased anxiety phenotype of Adcy2-L151 mice (Fig 27).

Interestingly, stressed Adcy2-L151 animals did not differ in the time they spent in the centre of the OF compared to the stressed WT (Two-way ANOVA, Bonferroni's Bonferroni's post-test, interaction $F(1,48)= 19.02$, $p<0.01$, genotype $p<0.01$, stress level $p>0.01$) (Fig 45a). Furthermore, the stressed Adcy2-L151 animals entered the inner zone as often as the stressed WT mice (n=15) (Two-way ANOVA, Bonferroni's post-test, interaction $F (1,50)= 10.25$, $p<0.01$, genotype $p<0.01$, stress level $p<0.01$)

Moreover, the distance travelled by the stressed mice was significantly reduced compared to unstressed mice However, there was no significant difference in locomotion between the genotypes observed and no interaction between the effect of stress and genotype that affected locomotor activity (Two-way ANOVA, interaction $p>0.05$, genotype $p>0.05$, stress $p< 0.05$)(Fig 45c)

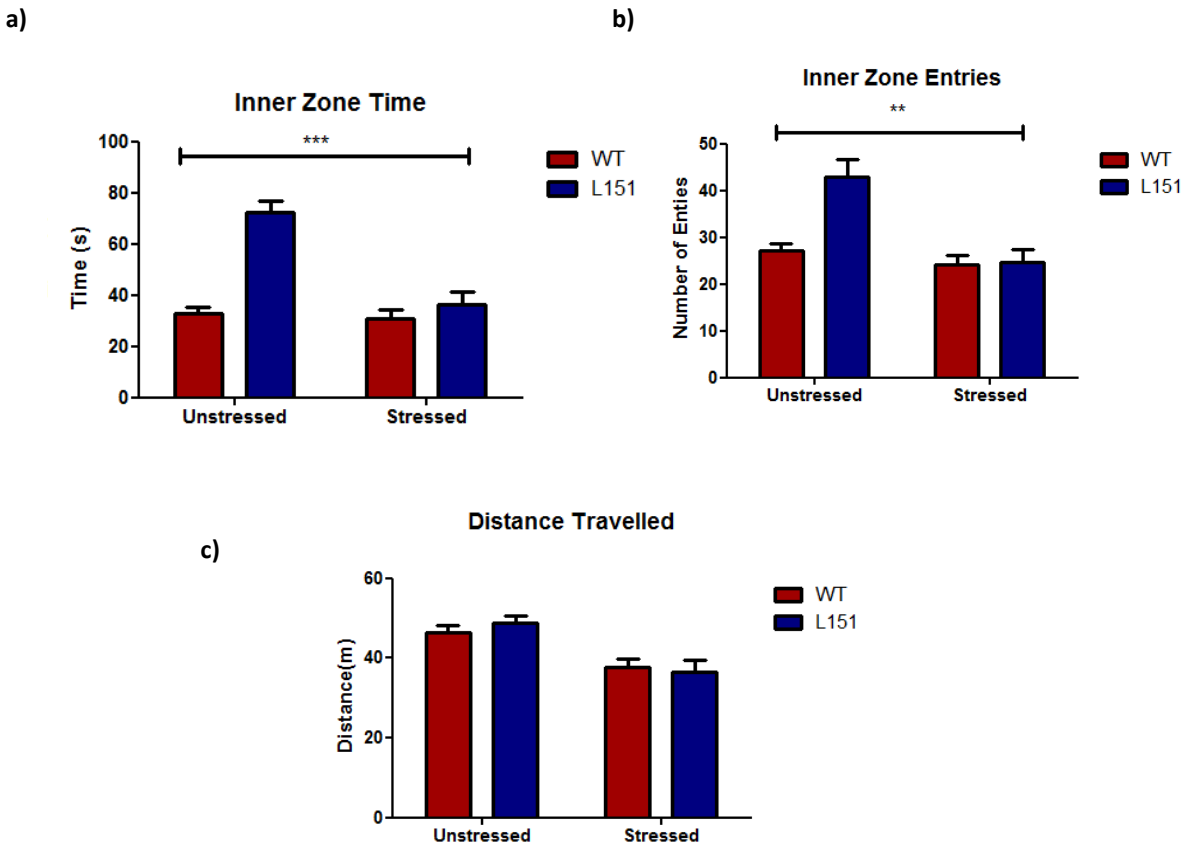


Figure 45. The OF reveals a higher vulnerability of Adcy2-L151 mice to CSDS. 10 mins long OF test was used to assess **a)** The time spent in the Inner Zone **b)** The number of Entries into the Inner Zone and the **c)** Total distance travelled during the test in unstressed WT mice, Adcy2-L151 unstressed mice, WT stressed mice and Adcy2-L151 stressed mice. Two-Way ANOVA, Bonferroni's post-test was applied to a)-c). a) Reveals a significant interaction with $p<0.01$ and $F(1,48)= 19.02$, genotype $p<0.01$, stress level $p<0.01$ b) also reveals a significant interaction with $p<0.01$, interaction $F(1,50)= 10.25$, $p<0.01$, genotype $p<0.01$, stress level $p<0.01$ c) shows no significant interaction between genotype and stress level, $p>0.05$, but a significant difference between stress levels with $p<0.05$

The DaLi test once again demonstrated that the Adcy2-L151 mice under basal housing conditions spend significantly more time in the light compartment, compared to WT animals, verifying the previously observed reduced anxiety of Adcy2-L151 mice. However, after exposure to CSDS, the Adcy2-L151 mice spent similar amounts of time in the light compartment as the stressed WT (Fig.46). Two-way ANOVA reveals that there is a significant interaction between the genotype and the level of stress ($p<0.01$, $F(1,47)=10.73$). However, there is no significant difference between the only the genotypes of or only the level of stress ($p<0.05$) suggesting that these factors alone do not systematically affect the outcome. This

interaction between genotypes and stress levels in both the DaLi and OF test suggests that both genetic and environmental factors interact to influence the anxiety levels in Adcy2-L151 mice.

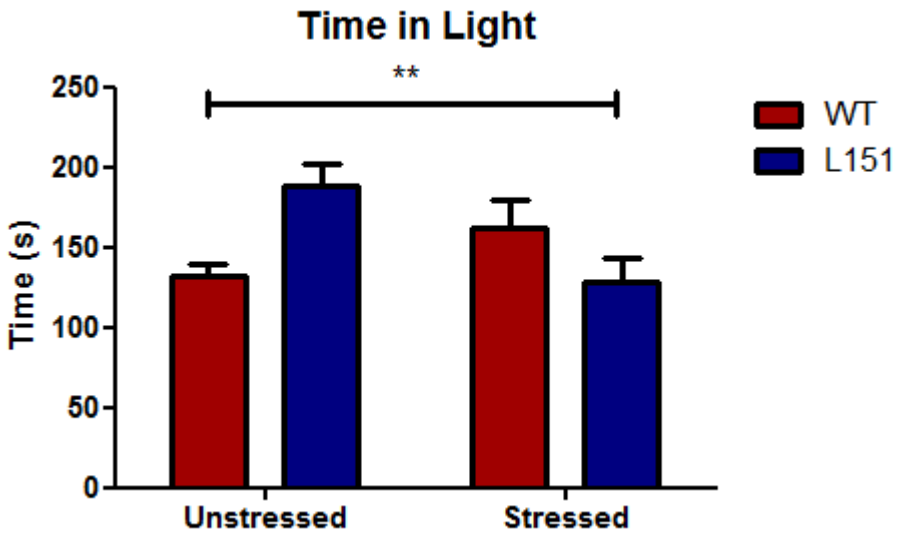


Figure 46. **The DaLi test shows a switch from mania-like low anxiety phenotype to a depressive-like high anxiety phenotype after CSDS**. The 10 mins long DaLi test measure the amount of time the stressed and unstressed WT animals as well as stressed and unstressed Adcy2-L151 animals spend in the light compartment (Two-way ANOVA, repeated measures reveal a significant interaction between stress level and genotype ($p<0.01$). interaction $F(1,47)=10.73$. There was so significant influence of only genotype or stress level ($p<0.05$)

4.8.5. Coping Behaviour

Since increased active coping behaviour is accompanied by mania like behaviour, and a passive coping behaviour is associated with depressive behaviour (Kromer et al., 2005; Yen et al., 2013), we wanted to test if exposure to chronic stress affects coping behaviour. FST demonstrated no significant change in the initial panic behaviour, which was quantified by measuring the initial struggle time, between the stressed and unstressed animals, as well as between genotypes (Two-way ANOVA, repeated measures, interaction, genotype and stress effect all had $p>0.05$) (Fig 47a). The fact that the Adcy2-L151 has a much higher floating time after CSDS, compared to the unstressed Adcy2-L151, suggests that after exposure to stress, these animals adapt a passive coping behaviour. The floating time of unstressed Adcy2-L151 mice and WT mice were significantly different due to the influence of stress levels (Two-way

ANOVA, repeated measures, stress factor p<0.01). Furthermore both the genotype and stress levels significantly interacted to influence the floating time of mice (Two-way ANOVA, repeated measures, interaction p<0.01, F(1,48)=34.55) (Fig 47b). This once again suggests both genetic and environmental factors interact to influence the coping behaviour in mice.. Additionally, there is no significant difference seen in the cortisol level after FST, regardless of the level of stress or genotype (Fig 47c)

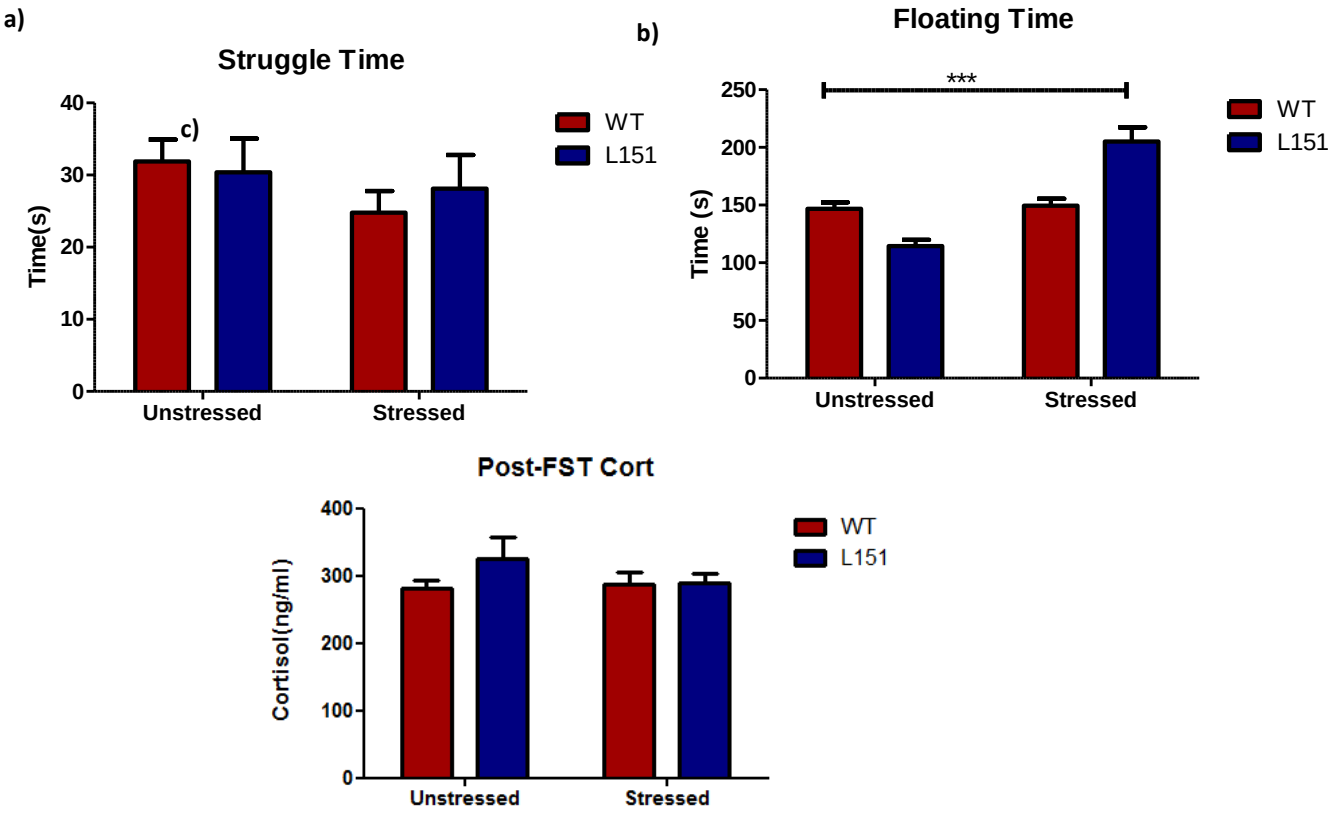


Figure 47 Adcy2-L151 switch from active to passive coping after CSDS exposure a) There is no significant difference in the time spent struggling compared to stressed animals (Two-way ANOVA, interaction, genotype and stress factors have p>0.05). **b)** Stress significantly increases the floating time of Adcy2-L151 animals (Two-way ANOVA, interaction, stress factor p<0.05). The interaction between stress and genotype also significantly affect the floating time of mice (Two-way ANOVA, interaction, interaction p<0.05, F(1,48)034.55) c) There is no significant difference in the cortisol levels between the genotypes and the stress levels of the mice (Two-way ANOVA, interaction, genotype and stress factors have p>0.05)

5. Discussion

Genome-wide association studies have linked the SNP rs13166360 to bipolar disorder (Muhleisen et al., 2014). This SNP lies in exon 3 coding for amino acid number 147 in the human adenylate cyclase 2 (ADCY2) proteins and causes a missense mutation resulting in a Leucine in the minor T allele instead of a Valine as in the major G allele. The associated SNP codes for a missense mutation and the human and mouse ADCY2 share 95% homology, i.e. the SNP is conserved between species. The impact of this SNP on protein function was previously unexplored.

5.1. Understanding the effect of the disease-associated SNP on protein function

While Muhleisen et al., 2014 does not explore the impact of the identified SNP rs13166360 on protein function, they speculate that this SNP might cause a functional variance in the protein. This is a reasonable speculation as the SNP lies in the coding region of Adcy2. Therefore in the first part of this project, we explored the effect of this SNP on protein function, as well as on the localization/trafficking of the protein *in-vitro.*

5.1.1. Construction of expression vectors with HA tagged ADCY2^{V151} and HA tagged ADCY2^{L151}

The lack of reliable antibodies against ADCYs and the comparably low expression levels have always hindered better understanding of ADCY function and localization. Previous studies have overcome this by overexpressing tagged ADCY2 variants to study its function and localization through over-expression studies (Calebiro et al., 2009; Diel, Klass, Wittig, & Kleuss, 2006).

Therefore, for this study the WT Adcy2 mouse cDNA was cloned into the pcDNA3 expression vector in order to overexpress Adcy2 and different variants in a similar manner. Since the aim of the study was to understand the effect of the rs13166360 on protein function, this SNP was introduced. Furthermore, a double mutation previously described to result in a loss of Adcy2 function (N1030A and R1034S) was generated to be used as a negative control (Yan, Huang, Shaw, & Tang, 1997). The constructs were successfully created as verified by sequencing. This type of construct would overcome the hindrance of low expression.

Furthermore, these constructs were HA-tagged at the N-terminus. While the N-terminus has been shown to play a role in the interaction of Adcy2 with AKAP proteins, previous studies have also used an N-terminal tag, which did not interfere with the interaction between Adcy2 and AKAP protein (Piggott, Bauman, Scott, & Dessauer, 2008). This HA-tagging helped stain for Adcy2 using antibodies against the HA-tag and facilitated the visualization of Adcy2 within transfected cells.

5.1.2. Choosing a cell line to study protein activity

While it would have been ideal to measure any difference in Adcy2 activities between the two variants within primary neuronal cultures, the low level of Adcy2 expression typically makes it difficult to detect differences in the protein activity. In particular, there are 10 different mammalian Adcy isoforms and all of them contribute to intracellular cAMP levels (Halls & Cooper, 2011). Since Adcy2 is not the most abundant Adcy isoform, we were not very likely to detect the contribution of Adcy2 to the overall cAMP production on the background of the other Adcys.

Therefore, immortalized cell lines transfected with the different Adcy2 variants were used to explore potential difference in activity between the variants. Since there are 10 different mammalian isoforms of ADCYs, it was important to choose a cellular system with a low endogenous level of Adcy2 expression. Since HEK293 cells had the lowest levels of endogenous Adcy2 expression, as well as of the closely related Adcy4 and Adcy7, this cell line was chosen for studying functional differences between the variants.

Forskolin stimulates most Adcy isoforms. It was not known how exposure to cAMP affects the expression of various endogenous Adcys in the HEK293 cells. In order to study these differences in protein activities between the different variants, it was important that the transfected Adcy2 variants were significantly higher than the endogenous Adcys, as otherwise the assay might reflect the cAMP production by the other endogenous Adcys. The fact that post forskolin stimulation, the transfected Adcy2-HA variant still had a significantly higher expression than the endogenous Adcys solved this problem (Fig. 17). Furthermore, since the expression vector had a CMV promoter, it was also further induced by the increasing cAMP concentration as previously described (Gavin et al., 1992). This resulted in an increased in Adcy2-HA expression just 30 mins post Forskolin stimulation, and a drastic increase in

expression 3hrs post stimulation (Fig. 19). This overexpression of Adcy2-HA post stimulation could confound functional analysis post forskolin stimulation of cells transfected with these plasmids. However, since the plasmids carrying the SNP of interest, as well as the one carrying the loss of function variant also carry this cAMP inducible promoter, a direct comparison of functional difference should not be confounded. Additionally, since most of the assays, used to measure Adcy2 activities, lasts less than 1hr post Forskolin stimulation a drastic increase in Adcy2 expression should not occur.

5.1.3. ADCY2^{L151} has a diminished activity

Some studies associate dysfunctions of other Adcy isoforms to psychiatric disorders. While some of these studies report an aberrant elevation in cAMP levels due to dysfunctions of the Adcy isoforms, other studies report a diminished cAMP level (X. Chen et al., 2016; Hines et al., 2006; H. Kim et al., 2017; Sethna et al., 2017). However, these studies do not associate dysfunctions in the various Adcy isoforms to BD, but rather to autism and depression- like behaviour. Therefore, previously little was known how Adcy activity is altered in the context of BD.

To rule out that the observed difference in cAMP production were not related to differences in transfection, and expression of the different Adcy2 variants, expression levels were assessed on mRNA and protein levels. qRT-PCR and also the Western blot demonstrate that the same amounts of the different Adcy2 were expressed in the transfected cells (Fig 13 and Fig 14.) This suggests that the observed differences in cAMP production are due to functional variance in Adcy2 and independent of factors such as expression level or protein stability.

The continuous measurements and the end point measurements both indicated that the Adcy2 activity is diminished in the presence of the SNP of interest (Fig. 17 and Fig. 18). However, the Adcy2 carrying this SNP of interest does not show a complete loss of function, as the levels of cAMP generated is still higher than in cells transfected with the loss of function variant. However, while there is a basal difference in the cAMP levels between the two Adcy2 variants, the dynamics of these two variants in response to stimulation by forskolin are similar.

The polymorphism of interest is in the transmembrane domain; however the functional consequence was unclear. Previous studies in Adcy8 demonstrate, using various truncated

Adcy8 variants, that the proper interactions between transmembrane domains are essential for correct trafficking and the correct functional assembly of the catalytic cleft (Gu et al., 2001). However, this study only examines truncated variants, and the effect of a SNP could potentially differ.

The SNP of interest does not seem to cause a complete loss of function, but only slightly diminishes the activity of Adcy2 is expected. This is expected as the SNP causes a substitution from a Valine to a Leucine, and Leucine is only slightly less hydrophobic than Valine (Biswas et al., 2003). Furthermore, this is consistent with the fact that psychiatric disorder associated SNPs are expected lead to a small functional variance instead of drastic differences, as polymorphisms leading to drastic changes should be pruned out by evolution (Uher, 2014).

Studying the functional variance of Adcy2 carrying this SNP in an overexpression study is also limiting. Since there are 8 other mammalian isoforms of Adcys, it is not known if this diminished activity in individuals carrying this SNP is compensated for by an increase of expression or activity in other Adcys. However, since Adcys vary in their regulatory properties and have distinct expression patterns this may not necessarily be the case (Dessauer et al., 2017). Adcy4 and Adcy7 are the two isoforms that are closely related to Adcy2 in the phytogenic tree as well as in terms of function (Fig. 5). However, these two Adcy isoforms have very low expression in the brain (Allen Brain Atlas, 2008). This suggests that such a compensatory mechanism is unlikely to occur, and the SNP of interest does decrease the cAMP levels in Adcy2 expressing cells in the brain. However, the cAMP levels in neurons and glial cells carrying this polymorphism of interest needs to be measured to fully understand the physiological effect of the SNP.

When the transfected cells are stimulated with low forskolin (1 ng/µl), this difference in cAMP production is maintained. However, upon stimulation with a higher amount Forskolin (100 ng/µl), this difference in the amount of cAMP between the two variants disappears (Fig. 8). Stimulation with what amount of Forskolin more closely mimics Adcy2 stimulation by GPCR is not clear. Therefore, it is unclear if in the physiological context, a lower absolute amount of cAMP is expected to be produced by cells carrying the SNP of interest, or whether these cells just exhibit a slower rate of cAMP production in response to GPCR stimulation. To explore this

effect, differences in cAMP production in response to GPCR stimulation in primary cells of the two genotypes need to be tested in the future.

Since in the functional study, Adcy2 was directly activated by forskolin, it is still not known if the interaction between GPCR and Adcy2 is also altered due to this SNP. Since various GPCRs usually interact with the same Adcy isoform, this SNP might result in varied effects in different molecular pathways and it is difficult to determine which pathways are affected due to this diminished function that results in the observed behavioural phenotypes.

Just in the cerebral cortex, Adcy2 has the highest expression in astrocytes, nearly equal levels of expressions in neurons and oligodendrocyte precursor cells, and are expressed at a lower level in other glial cells (Zhang et al., 2014). Therefore, this reduction in Adcy2 function due to the SNP of interest might affect the function of any, and possibly all of these cell types. Since the protein function was only studied in transfected HEK 293, it remains unclear which cell types might be affected by this SNP. Understanding which cell types are most affected by this reduction in protein function due to the SNP would give more insight into the underlying aberrant mechanism.

There is a plethora of evidence linking n over activation with BD (Saxena et al., 2017). PKC is also known to inhibit GPCR activity, thus consequently the activation of the downstream Adcy (Rittiner, Brings, & Zylka, 2014). It is possible that the disease-associated polymorphisms in various PKC isoforms (rs1360550, rs12373805 and rs3128396 for instance), that have been identified through GWAS and linkage studies, result in PKC over activation, and affect the same molecular mechanism as the SNP rs13166360T does (Kandaswamy, McQuillin, Curtis, & Gurling, 2012; Perlis et al., 2010). However, further work is needed to understand if this is the case.

5.1.4. ADCY2^{V151} is localized in subcellular compartments

Cos7 cells transfected with HA-tagged Adcy2 shows that Adcy2 is not only present in the plasma membrane but also seems to be present in the cytosolic region (Fig 17). The fact that the cells transfected with different concentrations of the expression plasmid still show Adcy2 expression in the cytosolic region, in addition to the membrane, suggests that this expression is not an artefact due to overexpression. Furthermore the fact that cells transfected with no plasmid show no staining suggests that the antibody against the HA-tag is specific and the

observed staining in the cytosol is not a result of a lack of antibody specificity. Taken together this adds another layer of complexity to the classical model of a plasma membrane bound Adcy2.

The classical model of membrane bound Adcy assumes that they are localized solely in the membrane (Halls & Cooper, 2017). However this type of model does not explain the way cAMP, which is produced at the plasma membrane reaches various different targets in the cell before being degraded by PDE. Previous studies have proposed that membrane bound Adcys might be internalized with the associated signalling GPCR and Adcys continue to signal from the compartments it is internalized in (Calebiro et al., 2009). As per the transfection pattern in our study, Adcy2 does indeed localize in various compartment in the cytosol in addition to the plasma membrane. Furthermore, other similar overexpression studies also detect Adcy2 in the cytosolic region, in addition to the membrane (Bogard et al., 2012). The fact this study did not use antibody against a tag, but rather against the Adcy2 protein after overexpression, suggests that the observed expression in the cytosolic region is not resulting from aberrant localization due to the presence of the HA-tag.

5.1.5. Exploring the localization of Adcy2

Since our data suggested that Adcy2 is also localized in intercellular compartments, we wanted to explore the spatial distribution of Adcy2 in more detail. While previously, there has been evidence of localization of Adcy2 in subcellular compartments, the compartments had not been specified further (Calebiro et al., 2009). Since the endosomal system is one possible compartment these Adcy2 could be localized in, we explored whether this is the case.

Our data suggests that Adcy2 is localized in the endosomal system. This includes early, late as well as recycling endosomes. This suggests that the Adcy2 is internalized, possibly with the activated GPCR, and is trafficked through the endosomal system during signalling. A similar model had been proposed by Calebiro et al, 2009 previously. However, whether the signalling GPCRs are indeed localized in the same subcellular compartments needs to be verified to justify such a model.

5.1.6. Altered trafficking or localization of ADCY2^{L151}

Comparing the localization of Adcy2^{V151} with Adcy2^{L151} suggests that the localization of Adcy2^{L151} is altered. Since the SNP of interest is in the first transmembrane domain, an altered trafficking might be expected as per previous literature (Gu et al., 2001).

We also wanted to investigate how the SNP of interest would alter subcellular localization of Adcy2. Our data suggests that there is an increased Adcy2 expression in the subcellular compartments in cells transfected with Adcy2^{L151} compared to those transfected with Adcy2^{V151}. Given that the expression of Adcy2 was equivalent between cells transfected with the two different Adcy2 variants, a higher intracellular localization would suggest that there is a higher proportion of membrane localized Adcy2^{V151} compared to the amount of membrane bound Adcy2^{L151} (Fig 14). Since a higher amount of Adcy2^{L151} is present in all endosomal compartments, it is possible that more Adcy2^{L151} is degraded in the lysosome. However, since the transfected pcDNA3.1 vector carrying the Adcy2^{L151} cDNA is also constitutively expressed, the degraded Adcy2^{L151} is replenished and no change Adcy^{L151} level was detected by the Western blot analysis compared to Adcy2^{V151}. The amount of Adcy2 of each variant that is localized at the plasma membrane should also needed measured in order to understand whether there is more Adcy2^{V151} retained at the plasma membrane than Adcy2^{L151}.

Staining the plasma membrane with phallloidin and quantifying the co-localization of Adcy2^{V151} and Adcy2^{L151} demonstrated that indeed less Adcy2^{L151} is present on the plasma membrane compared to the wild-type Adcy2^{V151}. The fact that internalized Adcy2 is expected to elevate cAMP levels in the cell contradicts our finding that a diminished amount of cAMP is produced by cells transfected with the Adcy2^{L151} variant compared to those transfected with the Adcy2^{V151}. However, it is possible that the plasma membrane bound Adcy2 has a higher activity compared to the internalized Adcy2. Isolating the plasma membrane bound Adcy2^{L151} and comparing its kinetics with that of Adcy2^{V151} might shed some light on understanding if the plasma membrane bound Adcy2^{L151} does have a lower level of activity, possibly due to altered signalling with GPCR.

However, it is also possible that the Adcy2^{L151} variant has an aberrant trafficking due to the presence of the SNP. This defective trafficking leads to more of the protein accumulating in

the endosomal system instead of being trafficked to the plasma membrane, where it can function efficiently.

Furthermore, it is also possible that the observed difference in cAMP production is primarily due to the aberrant catalytic cleft formation, as might be expected to result from polymorphisms in the transmembrane domain (Gu et al., 2002). The cells might thus attempt to internalize and consequently degrade these Adcy2^{L151} variants that form an aberrant cleft more than the wild type Adcy2^{V151} variant. Since there is some evidence suggesting dimerization of plasma membrane bound Adcys, and the transmembrane domains playing a major role in dimerization, it is also possible that the polymorphism of interest affects dimerization, and the lack of proper dimerization affects its localization and trafficking resulting in a higher level of Adcy2 in the subcellular compartments (Ding et al., 2005; Gu et al., 2002).

5.1.7. Further work to understand the underlying aberrant molecular mechanism

While several aberrant molecular mechanisms are reported in this study, it remains unclear whether these are the cause, a correlated factor or the result of adaptation to other unexplored molecular impairments. Furthermore, the lack of antibodies against Adcy2 and the low Adcy2 expression has prevented the exploration of potential mechanisms in brain tissues.

Since most of the molecular mechanisms were studied in transfected HEK293 cells, producing a cellular model using iPSCs derived from patient samples would help better understand if such aberrant mechanisms are also present in patients. Furthermore, studying primary neuronal as well as glial cultures from mice carrying the SNP of interest would allow disentangling which cell types are the most affected by the SNP of interest. Furthermore, identifying whether excitatory or inhibitory neurons have more Adcy2 expression, as well as studying the differences in CREB expression between mice carrying the WT Adcy2 allele and those carrying the SNP of interest would give a better understand of which circuitries might be affected by this SNP.

5.2. Exploring the consequences of rs13166360 using Adcy2^{L151} mice

While the effect of the disease-associated SNP was understood in-vitro system, its effect of aberrant behaviour still remained to be elucidated. In order to study this impact on behaviour, we introduced this SNP into mice using Crispr/Cas9 technology, and created the ADCY2^{L151} mice. In this mouse model, we tested for various BD-associated endophenotypes under basal conditions, as well as after exposure to chronic stress.

5.2.1. Use Adcy2^{L151} mice to study the effect of SNP of interest

The Crispr/Cas9 system was used to introduce the BD associated SNP rs13166360 into the mouse genome. For the ease of genotyping, two silent mutations were also introduced near the SNP of interest. One of these silent mutations adds an AluI restriction site, which was used to genotype the mice. Since, in the past Crispr/Cas9 system has been known to introduce other off-target mutations, it needs to be ensured that this mouse line does not carry such off target mutation (Zischewski, Fischer, & Bortesi, 2017). One way of ensuring this would be by sequencing the whole ADCY2^{L151} mouse genome. However, since this is an expensive strategy, the mouse genome was searched for other possible recombination sites of the donor DNA. Since no other possible recombination sites were identified, it was assumed that there was no off-target mutation, as the possibility of this occurring was extremely low

One common way of studying mechanisms underlying psychiatric disorders is by using rodent models. This approach uses transgenic mice that either caring a null mutation, a reduction in expression or an over expression of a diseased associated gene. However, with the development of the Crispr/Cas9 system it has become easier to model disease associated SNPs in the mouse. This type of transgenic mouse line generated using Crispr/Cas9 has higher face validity than the traditional gene knock-out, knock down and overexpression models used to study BD, as it introduces the BD associated SNP and more closely mimic the pathophysiology associated with BD patients (Logan & McClung, 2016). This type of animal models also carries several other advantages, such as having a stable expression of the disease-associated SNPs. Additionally, the genetic background of this mice model is unaltered from the wild-type mice, except for the introduced mutation, makes the comparison between wild-type mice, and the mice carrying the minor allele more reliable. Furthermore, the expression of the disease-associated SNP also in the periphery makes this animal model more

similar to patients who also carry this SNP in the periphery. Thus if the influence of the disease-associated SNP in the periphery leads to the manifestation of the disease, this would also be capture in such an animal model.

5.2.2. Adcy2^{L151} mice exhibit mania like behaviour

Typically, mouse models of BD capture one of the two phases of BD. Most well established genetic mouse models of BD have been previously shown to exhibit mania-like behaviour (Han et al., 2013; K. Y. Lee et al., 2010; Leussis et al., 2013; Soria et al., 2010; Young et al., 2010b). Mania is typically characterized by behaviours like reduced anxiety, increased active coping, hyperactivity, impaired sensory processing, psychostimulant induced hyperactivity etc. in patients. These endophenotypes are screened for in rodents using various behavioural tests as described in the results section (Logan & McClung, 2016). The animals carrying the SNP of interest were also tested using several of these described tests and compared to the wild-type littermates, in order to identify BD-associated endophenotypes. Like the previously described genetic mouse models of BD, the mouse homozygous for the SNP of interest also exhibited several mania-like phenotypes but did not show phenotypes emulating the depressive phase of BD under basal conditions.

5.2.2.1. Adcy2^{L151} mice and hyperactivity

Since a characteristic behaviour in patients with mania is hyperactivity, an increased activity in the rodent models of BD is often considered as mania-like behaviour (Logan & McClung, 2016; Miro et al., 2012; Scott, Vaaler, Fasmer, Morken, & Krane-Gartiser, 2017; Sole et al., 2017). However a similar increased in activity level was not observed in the Adcy2^{L151} mice (Fig 29 and Fig. 30). While, an increased locomotion was observed the first two nights of the locomotion detection, this difference was not seen during the subsequent nights. It is possible that this was the case as due to a difference in their pace of habituation to a new home-cage. However, it is also possible that the difference in locomotion was not observed during the subsequent nights due to a high variability in locomotion. Repeating this experiment with a larger sample size would resolve this issue.

It is possible that a difference in locomotion is not observed in Adcy2^{L151} mice as the attention spans vary between the wild-type mice and the Adcy2^{L151} mice. This might be the case as the Adcy2 locus has been associated with early-onset BD that is co-morbid with attention-deficit

hyperactive disorder (van Hulzen et al., 2017). However, since BD is a complex disorder, where often multiple genetic factors as well as environmental factors play a role in its development, it is possible that other genetic or environmental influences are needed to induce any differences in activity (Pena et al., 2014).

The administration of amphetamine led to an increase in locomotion in the Adcy2^{L151} mice that was higher than what was observed in the WT mice (Fig 31). Similar amphetamine hypersensitivity has also been previously reported in other mouse models of BD, as well as in BD patients (Anand et al., 2000; Miro et al., 2012). Therefore this amphetamine hypersensitivity adds to the predictive validity of Adcy2^{L151} mice as a model for BD.

Previously, it has been suggested that an increased level of dopamine at the synaptic cleft might underlie BD (Anand et al., 2000; Horschitz et al., 2005). Mouse models of BD carrying a knockdown or a knockout of the dopamine transporter also exhibited mania like behaviour (Powell et al., 2008; Young et al., 2010a). Amphetamine is known to increase the levels of dopamine at the synaptic clefts. The fact that the Adcy2^{L151} mice exhibit amphetamine hypersensitivity might suggest a similar aberrant increase in dopamine levels at the synaptic cleft. It is possible that the SNP of interest results in an abnormally high dopamine release at the synaptic cleft, or the inability to properly clear the dopamine from the synaptic cleft. Testing the level of dopamine release post amphetamine administration in mice carrying the SNP of interest would give some insight into whether this is the case.

Interestingly, the DAT knockdown mice also showed impaired habituation, which might suggest an impaired adaptability phenotype (Zhuang et al., 2001). The decrease in activity after the first two night phases of Adcy2^{L151} mice in the new home cage could also be explained in terms of decreased adaptability and might be a characteristic feature of patients with BD (Perry et al., 2010).

5.2.2.2. Adcy2^{L151} mice exhibit reduced anxiety

The OF and the DaLi tests reflect that Adcy2^{L151} mice exhibit a lower anxiety phenotype (Fig. 31 and Fig. 32). However, such a difference was not observed in the EPM (Fig. 33). This lower anxiety phenotype might have been absent in the EPM due to a difference in sensitivity between the different test. These test also suggest that the lower anxiety phenotype in Adcy2^{L151} mice is independent of locomotion.

A decrease in the number of marbles buried was observed in the marble burying test (Fig. 34). Conventionally this test is thought to assess novelty induced anxiety levels in rodent models (Savy et al., 2015). The fact that the administration of anxiolytic drugs decreased marble burying further support the fact that this test measures the degree of novelty induced anxiety (Nicolas & Prinssen, 2006).

However, other papers claim marble burying is a measure of repetitive behaviour rather than a test for novelty induced anxiety (Thomas et al., 2009). Previous mouse models of BD have also used this test as a measure for repetitive behaviour instead of anxiety, and have observed an increase in marble burying as opposed to a decrease in marble burying as observed in $Adcy^{L151}$ mice (Miro et al., 2012). However since repetitive behaviour is not a characteristic behaviour of patients with BD, it is unlikely that a mouse model of BD should carry this phenotype. Other well established models of BD like ANK3 mouse also report no increase in repetitive behaviour in their mouse model (van der Werf et al., 2017). Furthermore if marble burying is taken as a test for anxiety, as it is conventionally done, then the decreased marble burying supports the lower anxiety phenotype observed in other tests for anxiety. Therefore we conclude that the $Adcy^{L151}$ mice have a decreased novelty induced anxiety compared to the WT mice.

Since mania like behaviour has been previously associated with lower anxiety levels, this lower anxiety phenotype could indicate mania-like behaviour in $Adcy2^{L151}$ mice (Logan & McClung, 2016). However other patient studies have contradicted this and have observed elevated anxiety levels in the manic, hypomanic as well as the depressive phase (Goes, 2015). Therefore, many studies claim that the relationship between BD and anxiety is still not fully understood. One confounding factor is the co-morbidity of anxiety and BD that is often seen in patients (Mitchell, 2015). This lack of clear understand of the pathophysiology of BD restricts the face validity of animal models of BD.

5.2.2.3. $Adcy2^{L151}$ mice exhibit increased active coping

The Adcy2^{L151} mice spent less time floating in the FST (Fig 36). Similar decrease in floating time has also been observed in other established rodent models of BD (Han et al., 2013; K. Y. Lee et al., 2010; Leussis et al., 2013; Soria et al., 2010; Young et al., 2010b)

There are different interpretations of what this decrease in floating time might mean in the context of complex behaviour. This decreased floating time has previously been considered to reflect a decrease in depression-like behaviour, as the floating behaviour was thought to reflect depression or despair (Logan & McClung, 2016; Yankelevitch-Yahav, Franko, Huly, & Doron, 2015). This would mean that the Adcy2^{L151} mice as well as other mouse models of BD reflect a reduction in depressive behaviour. This reduced depressive behavior would be expected during the manic phase of BD in patients.

However, more recently it has been debated that the floating behaviour in FST reflects passive coping behaviour (Warden et al., 2012; Yen et al., 2013). Therefore a decrease in floating time in the FST as per this interpretation would suggest that the Adcy2^{L151} mice show an increased active coping behaviour or an increased willingness to put effort into finding an escape. However, it has been argued that this interpretation may not be accurate as previously it has been shown that in the long run (2hrs), the animals that float longer tend to conserve more energy, increasing their chances of survival (Nishimura, Tsuda, Oguchi, Ida, & Tanaka, 1988).

A third line of argument suggests that the floating behaviour in the FST is just an adaptive behaviour to the acute stressor (Molendijk & de Kloet, 2015). This interpretation is supported by the fact that animals familiar with the FST setup also display an increased floating time (Borsini, Volterra, & Meli, 1986; Reul, 2014). This would suggest that the Adcy2^{L151} mice as well as other mouse models of BD, show an impaired ability to adapt to stressors. This inability to adapt to stressor could be an indication of cognitive impairment, which is also observed in patients with BD (Sole et al., 2017). Repeating the FST during another consecutive would help elucidate whether the difference in floating time in the FST is due to a cognitive impairment.

The fact that the outcome of the FST is still debated suggests that it is still unclear how the outcome of the FST should be interpreted. Therefore, it is difficult to absolutely determine how this rodent behavioural phenotype might be reflected in patients with BD. More sophisticated methodologies of behavioural testing are needed to determine which aspect of BD is truly reflected by the decrease floating time of Adcy2^{L151} mice.

However, since the conventional interpretation is that FST is a measure of stress coping behaviour, we interpret that this decreased floating time indicates that Adcy2^{L151} mice have an increased active coping.

5.2.2.4. Adcy2^{L151} mice exhibit increased exploratory behaviour

Previous patient studies had identified an increased exploratory behaviour in patients with BD. This increased exploratory behaviour is a unique characteristic of patients with BD that differentiates them from patients with Schizophrenia (Perry et al., 2010). The Adcy2^{L151} mice demonstrated a similar increased exploration of novel objects in a habituated arena (Fig. 37). Similar increased exploratory behaviour was also observed in the DAT knockdown mouse model of BD as well as mice with pharmacologically inhibited DAT function (Perry et al., 2009). This once again suggests that the Adcy2^{L151} mice might have altered dopamine neurotransmission.

5.2.2.5. Adcy2^{L151} mice show increased sociability

Mania is often associated with increased sociability (Yen et al., 2013). Furthermore, twin studies from BD also report an increased sociability in patient with BD (Higier et al., 2014). Therefore, we wanted to see if there is a similar increase in sociability observed in Adcy2^{L151} mice.

The sociability test indicated that there was an increase in sociability in the Adcy2^{L151} mice. (Fig. 38). This difference was not observed in the 3 parted chamber test possibly because this test compares the level of social interaction with another mouse to the amount of interaction with a novel object (Fig 39). However, this difference in the observed sociably can be explained by the fact that the first test measured any difference in sociability in a group setting with two freely moving mice, while the second test only observed sociability of a lone mice which only has limited access to the other novel mouse (along the edge of the wired cup the novel conspecific is placed in).

Some transgenic mouse models that target genes implicated in BD like, such as the GAD67 haplodeficient mice also report impaired sociability (Sandhu et al., 2014). However, these mice report a reduced sociability and an increased aggressive behaviour in these mice. This contradicts the increased sociability phenotype observed in the Adcy2^{L151} mice. Furthermore,

Ank3 mice and glutathione-deficient mice, two well establish mouse model of BD, are reported to have no change in sociability compared to WT animals (Kulak, Cuenod, & Do, 2012; van der Werf et al., 2017). Since multiple genetic and environmental factors contribute to the development of BD, it is possible that decreased expression of either Ank3 or glutathione genes alone does not lead to social impairments observed in patients. Since the sociability of most other well establish BD models are not reported, it is not known if other mouse models of BD also show a similar increase in sociability.

The three parted chamber also suggests that while the WT mice prefer interacting with a novel conspecific, the Adcy2^{L151} mice show no preference for interaction between a familiar and a novel conspecific (Fig 35). The glutathione-deficient mice also show a similar lack of social preference between a novel and a familiar mouse (Kulak et al., 2012). Furthermore, patient studies have reported deficits in facial recognition in patients with BD (Bora & Ozerdem, 2017; Samame, Martino, & Strejilevich, 2015). It is possible that this deficit in social preference is due to impaired social recognition, as identified in patient studies. This would suggest that Adcy2^{L151} mice have cognitive impairments.

These tests of sociability is limited in its assessment of social interaction of these animals as they only reflect the social interaction with a novel conspecific over a limited amount of time, and do not assess the differences in sociability of these mice within their social hierarchy in their home cage. Using more advanced tests that assess rodent behaviour in the home cage in a group setting would provide better insight into any difference in social behaviour (Shemesh et al., 2013).

5.2.2.6. Long term lithium chloride administration attenuates mania-like behaviour in Adcy2^{L151}

Typically, previous rodent models have demonstrated that long-term administrations of mood stabilizers like lithium alleviate this low anxiety-phenotype in mice. Since patients with BD are also often treated with these mood stabilizers, this adds to the predictive validity of these animal models (Han et al., 2013; K. Y. Lee et al., 2010; Leussis et al., 2013; Soria et al., 2010; Young et al., 2010b). Long-term lithium chloride treated Adcy2^{L151} mice displayed similar levels of anxiety and stress-coping behaviour as the untreated Adcy2^{V151} mice (Fig. 42). Since decreased anxiety and active stress-coping behaviour are two classical characteristic of

manic behaviour, this result could be interpreted as an attenuation of mania-like symptoms (Yen et al., 2013). This adds to the predictive validity of this mouse model.

5.2.3. Adcy2^{L151} mice show cognitive impairments

Several patient studies have indicated cognitive impairments in patients with BD (Arts, Jabben, Krabbendam, & van Os, 2009; Balanza-Martinez et al., 2010; Cardenas, Kassem, Brotman, Leibenluft, & McMahon, 2016). Furthermore, the lack of preference for social novelty in the 3 parted chamber test further suggested that the Adcy2^{L151} mice have cognitive impairments. Therefore we decided to test for cognitive deficits using the MWM.

MWM demonstrated that the Adcy2^{L151} mice were slower learners and had deficits in long term memory (Fig. 40). Furthermore, the Adcy2^{L151} mice had a diminished LTP, which is a synaptic correlate of memory compared to wildtype mice (Lynch, 2004). Conditional knockout mutants of CACNA1C demonstrate similar deficits of hippocampus dependent learning (Dedic et al., 2018; Temme, Bell, Fisher, & Murphy, 2016). However, CACNA1C is implicated in the development of both schizophrenia and BD. Similar cognitive deficits have not been reported in most genetic mouse models that are specific to BD. This suggests that the Adcy2^{L151} mice have higher face validity than most well established mouse models of BD.

5.2.4. Adcy2^{L151} mice switch from mania-like behaviour to depression-like behaviour after exposure to chronic stress

Like most psychiatric disorders, BD involves both genetic and environmental factors underlie its development. Previous patient studies have implicated various forms of stressors to the development of BD. These stressors include childhood maltreatment, stressful life events and the lack of social support, to name a few (Bradley et al., 2008; Ellicott, Hammen, Gitlin, Brown, & Jamison, 1990; McGowan et al., 2009; Taylor & Kim-Cohen, 2007; Young & Dulcis, 2015). The underlying cause of the switch between the manic and the depressive phase of BD is still not well understood. However, studies have identified factors like seasonal changes and stressful life events to be correlated to switch between the two phases (Barbini, Di Molfetta, Gasperini, Manfredonia, & Smeraldi, 1995; Ellicott et al., 1990; H. C. Lee, Tsai, & Lin, 2007; Young & Dulcis, 2015)

Since exposure to stressful life event was one factor that were linked to this switch between the two phases of BD, we decided to expose the Adcy2^{L151} mice to chronic social defeat stress using the CSDS paradigm to explore its effect on the BD associated endophenotypes.

The Adcy2^{L151} mice were more vulnerable to chronic stress than the WT mice as was seen in the OF and the DaLi test (Fig. 45 and Fig. 46). A similar vulnerability to chronic stress was also seen in the Ank3 mice, which is a well-established mouse model of BD (Leussis et al., 2013). Furthermore, the mania-like diminished anxiety phenotype switched to higher anxiety levels, which were similar to the stressed wild-type mice. It is possible that there were floor effects in the OF and DaLi tests that failed to capture the difference in anxiety levels between the stressed wild-type and Adcy2^{L151} mice.

5.2.5. ADCY2^{L151} mice exhibit passive coping behaviour post exposure to chronic stress

The exact cause of the switch between the two phases of BD is still unknown. Furthermore, most well established mouse models of BD fail to capture both phases. However, the Adcy2^{L151} mice show a switch from an active coping behaviour to a passive coping behaviour in FST (Fig. 47). This might be a reflection of the mouse switching from the manic phase of BD to the depressive phase and is consistent with previous patient studies that have identified stressful life events to cause a switch between the two phases (Ellicott et al., 1990; Young & Dulcis, 2015). As the molecular mechanism underlying this switch between the two phases is still not completely understood, this mouse model could be used to further investigate this mechanism underlying this switch between the mood states.

Previous patient studies have also indicated that change in photoperiod due to change in seasons could result in a switch between the two phases of BD (Barbini et al., 1995; H. C. Lee et al., 2007). Previous studies have been able to capture only one of the two phases of BD in animal models (van Enkhuizen et al., 2014; Young & Dulcis, 2015). This inability to capture the switching between manic and depressive phases has hampered progress in the field of BD research, and the focus of this field has been aimed at developing an animal model that captures this switching between the two phases (Blumberg, 2012). An Ank3 heterozygous knockout mouse was the first study to develop an *in-vivo* switching model of BD, which captured both the manic and the depressive phase, where a switch between the two phases was observed after exposure to chronic stress (Leussis et al., 2013). Recently, Young et al., has

also been able to switch the mood of DAT hypomorphic mice from mania-like behaviour to depression-like behaviour by switching of photoperiod (Young et al., 2018). This study also identified no change in DAT mRNA levels across photoperiod in the hypomorphic mice, which is typically seen in WT mice. This suggests that aberrant control of dopamine levels might underlie the switch between the two mood phases. Identifying changes in dopamine level between the Adcy2^{L151} mice that are exposed to chronic stress and the unstressed Adcy2^{L151} mice could help understand if an aberrant level of dopamine neurotransmission might underlie this switch between the two mood states.

5.3. Future directions

While the behavioural tests suggest that the SNP of interest results in a wide range of behavioural abnormalities, it is still unclear which brain regions, circuits or molecular mechanisms are affected in-vivo due to this SNP. Whether the SNP of interest leads to a similar decrease in cAMP levels in primary neurons and glial cells of the Adcy2^{L151} mice, as it did in the heterologous *in-vitro* studies, needs to be tested. Furthermore, the behaviour of mice carrying conditional knockout of Adcy2 in specific brain regions and cell types would allow us to identify the circuits and specific cell types that play a role in the development of each of these observed complex behaviours. Furthermore, whether overexpression of the WT Adcy2 allele in these particular brain regions of conditional knockout mice and Adcy2^{L151} mice rescues the WT behavioural phenotype needs to be observed.

6. Conclusion

Genome-wide association studies have linked the SNP rs13166360 to bipolar disorder (Muhleisen et al., 2014). This SNP lies causes a missense mutation resulting in a Leucine in the minor T allele instead of a Valine as in the major G allele. The impact of this SNP on protein function and behaviour was previously unexplored.

This study aimed to understand how the BD associated SNP rs13166360, impacted Adcy2 protein function. Expression of Adcy2^{L151} in a heterologous style in HEK293 cells revealed that this SNP reduces protein function, resulting in a reduction in cAMP production. Since a polymorphism in the transmembrane region of the protein could affect the localization of this protein, we tested whether the localization or trafficking of Adcy2 carrying this SNP of interest is altered. We demonstrated that Adcy2 is localized in subcellular compartments, in addition to being localized in the plasma membrane. Additionally, we showrf that the localization or the trafficking of the Adcy2^{L151} is altered, where fewer Adcy2^{L151} is localized in the plasma membrane and more of the protein is localized in endosomal compartments compared to Adcy2^{V151} (Fig. 48).

This study also explored how the BD associated SNP rs13166360, impacted mouse behaviour under standard housing conditions and after exposure to chronic stress. We demonstrated that at basal levels of stress the Adcy2^{L151} mice display mania-like behaviours. This includes reduced anxiety, increased active stress coping behaviour and hypersensitivity to Amphetamine. While these mice lacked the hyperactivity phenotype in a habituated environment, they demonstrated hyperactivity in a novel environment. These mice also had an increased sociability, no preference for social novelty, increased object exploration phenotype and an impaired cognition (Table 5). Long-term administration of lithium attenuates the displayed mania-like phenotype in Adcy2^{L151} mice (Table 6). After exposure to chronic social stress, these mice also displayed an increased vulnerability to stress, a larger increase in anxiety levels than the wild-type mice and a switch from active coping to passive coping behaviour (Table 7).

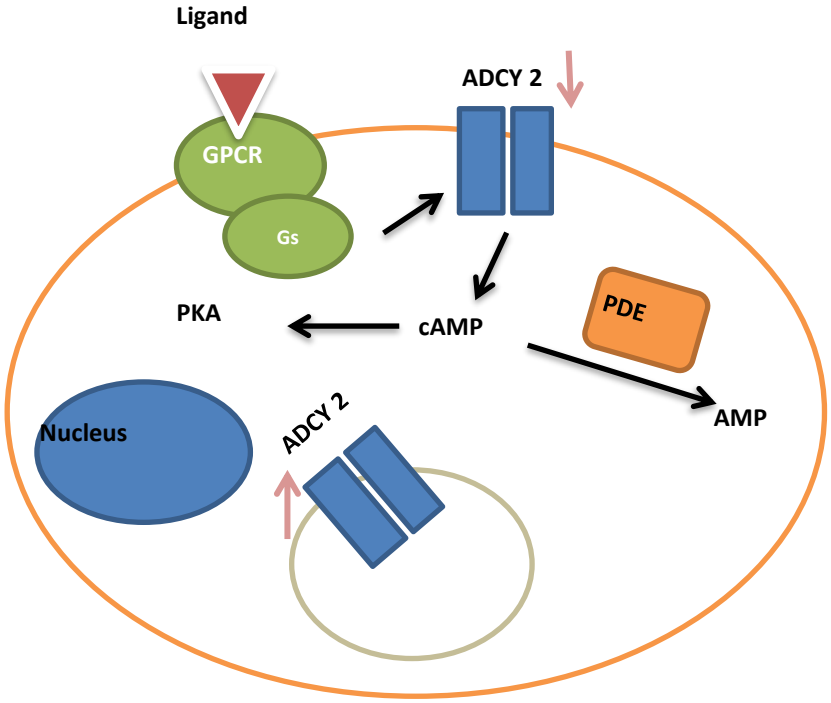


Figure 48. Summary of observed changes in localization/trafficking of ADCY2^{L151}

Phenotype expected based on patient studies during Manic Phase	Tests used on mice	Phenotype of Adcy2^{L151} mice in standard housing conditions	Phenotype of Adcy2^{L151} mice post long term lithium chloride administration	Phenotype of Adcy2^{L151} mice post chronic social stress
Lower Anxiety	Open Field, Dark/Light Box, Elevated Plus Maze,	↓ Anxiety	Normalized	↑ Anxiety
Increased Social Interaction	Social Interaction Test, 3 parted chamber	Unaltered	n/a	n/a
Hyperactivity	Locomotion in Home Cage, Open Field with and without Amphetamine administration	↑ Locomotion in dark phase and post Amphetamine administration	n/a	n/a
Increased Active Coping (Manic phase)	Forced Swim Test	↑ Active Coping	Normalized	↓ Active Coping
Increased Exploratory Behavior	Object Exploration	↑ Exploratory behavior	n/a	n/a
Cognitive Impairments	Morris Water Maze, LTP measurement	Impaired long term memory and slower learning	n/a	n/a

Table 5. Summary of behavioural changes in Adcy2^{L151} mice

7. List of Abbreviation

S35	Isotope 35 of sulfur
5HT	5-hydroxytryptamine, serotonin
ADCY	Adenylyl Cyclase
Adcy2	Adenylyl Cyclase type II
ADP	Adenosine diphosphate
ANK3	Ankyrin -3
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BD	Bipolar Disorder
Ca2+	Calcium
CACNA1C	Gene encoding the Cav1.2 calcium channel
cAMP	Cyclic adenosine monophosphate
Cas9	CRISPR associated protein 9
cDNA	Copy DNA
CLOCK (gene)	Circadian Locomotor Output Cycles Kaput (transcription factor)
CMV	Cytomegalovirus
Cpm	Counts per minute
CRISPR	Clustered regularly interspaced short palindromic repeats
CREB	cAMP response element-binding protein
CSDS	Chronic social defeat stress
DA	Dopamine
DAG	Diacylglycerol
DaLi	Dark-light box
DAPI	4',6-diamidino-2-phenylindole
DAT	Dopamine transporter
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Desoxyribonucleotide triphosphate
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EPM	Elevated plus maze
ER	Endoplasmic reticulum
EtOH	Ethanol
Fsk	Forskolin
GABA	γ-Aminobutyric acid
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
GWAS	Genome-wide association study
GWLS	Genome-wide linkage studies
h	Hour
HA	Human influenza hemagglutinin
Het	Heterozygous
Hom	Homozygous
HPA	Hypothalamic-pituitary-adrenocortical
IEG	Immediate early gene
IHC	Immunohistochemistry

IP_3	Inositol 1,4,5-trisphosphate
ISH	In situ hybridization
Kb	Kilobasepairs
kDa	Kilodalton
KO	Knockout
L151	Adcy2-L151
LB	Lysogeny broth
LD	Linkage disequilibrium
LiCl	Lithium chloride
LTP	Long-term potentiation
L-type	Long-lasting type
M	Molar
MDD	Major depressive disorder
MgCl2	Magnesium chloride
Min	Minute
mRNA	Messenger ribonucleic acid
MWM	Morris water-maze
NMDA	N-Methyl-D-aspartic acid
NMDAR	N-Methyl-D-aspartic acid receptor
NT	Neurotransmitter
o.n.	Over night
OD	Optical density
OF	Open field
ORF	Open reading frame
PAM	Protospacer adjacent motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PKA	Protein kinase A
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
Rpm	Rounds per minute
Rt	Room temperature
S	Second
SA	Social avoidance
SEM	Standard error of mean
SHANK3	SH3 and multiple ankyrin repeat domains 3
SNP	Single nucleotide polymorphism
TAE	Tris acetate EDTA
TEA	Triethanolamine
TM	Transmembrane
Tris	Trisaminomethane
U	Unit(s)
UV	Ultra violet
V151	Adcy2-V151 (wild type Adcy2)
WB	Western blot
Wt	Wild type

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