Adenylyl Cyclase and Anxiety in Bipolar Disorder

In the search for a specific treatment target for bipolar disorder

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Cover image: Part of 'Self portrait, 1889', painted by one of the world's most famous bipolar disorder patients: Vincent van Gogh.

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Abstract

Bipolar disorder (BPD), also known as manic depressive illness, is a complex brain disorder common under adults as well as adolescents. Patients with BPD disorder suffer from dramatic mood swings, which severely hamper a patient's life. Symptoms vary from hyperactivity and psychosis during mania to self-deprecatory and anxious behaviour during depression. These contrasting symptoms hinder the search for a cause and treatment for BPD. Currently used medicines are often anti-depressants with many unwanted side effects, and of which the exact working mechanism are unclear. Therefore, there is a need for a specific treatment for BPD. Recent studies with knockout mice pointed at the enzyme adenylyl cyclase subtype 8 as potential treatment target for BPD, but further research has to confirm this. In this thesis, the potential of different adenylyl cyclase subtypes as treatment targets for BPD is studied. This is approached by studying the BPD symptoms and their underlying molecular mechanisms, and by linking characteristics of adenylyl cyclase subtypes to BPD symptoms. These approaches lead to the finding that adenylyl cyclase subtype 1 and adenylyl cyclase subtype 8 play an important role in BPD pathology. This is shown by their role in neural growth and other neural processes, their expression in the cerebellum and hypothalamus, and their involvement in the hypothalamic-pituitary- adrenal -axis. In addition, AC8 is crucial for the generation of behavioural stress responses, and it induces anti-depressive or manic like behaviour. Furthermore, the findings in this thesis indicate a major amplifying role for anxiety in the development and course of BPD. Also anxiety generation involves AC1 and AC8, which supports the strong relation between anxiety and BPD. Taken together, these results point at anxiety, AC1 and AC8 as high potential treatment targets for BPD.

Introduction

In the last decade, the number of Bipolar disorder (BPD) patients in the US increased drastically. Bipolar disorder, also known as Manic-depressive illness, is a brain disorder common under adults as well as adolescents (Moreno, 2007). Although BPD was first mentioned by Hippocrates (460-377 BC), and its occurrence has been increasing ever since, it still appears to be very difficult to recognize and diagnose this disease.

BPD is a severe brain disorder characterized by dramatic mood swings. These recurrent episodes of depression and mania hamper a patient's life in very many ways, and can even lead to suicide. The most prominent symptoms of a manic episode are increased energy, restlessness, euphoric mood, extreme irritability, bad concentration capacity, increased sexual drive, racing thoughts, aggressive behaviour and impulsive and unrestrained behaviour (Goodwin 2007; Kato 2008). On the contrary, depressive episodes are characterized by decreased energy, fatigue, anxiety, sad mood, feelings of guilt and worthlessness, loss of interest in once enjoyable activities (including sex), sleep disturbances, bad concentration capacities, change in appetite and weight, chronic pain and thoughts of suicide (Goodwin 2007; Kato 2008). The disease knows several courses, which are Bipolar I Disorder, Bipolar II Disorder and rapid cycling. Bipolar I Disorder is known as the 'classic' Manic-Depressive illness with recurrent manic and depressive episodes, whereas Bipolar II Disorder involves depressive episode alternating with episodes of hypomania (mild to moderate mania) instead of severe mania. Rapid cycling Bipolar Disorder is the case when four or more episodes occur within one year. Patients can even experience several episodes within a week or a day (Goodwin, 2007; Kato, 2008).

Like with other subtypes of depression, diagnosis of BPD is based on rather subjective descriptions of behavioural symptoms. To reduce variance in diagnosis between clinicians, the American Psychiatric Association (2000) has developed a Diagnostic and Statistical Manual with several criteria. Although one can debate about the accuracy of these diagnostic criteria (Wong & Licinio, 2001; Bowden, 2008) it is a good start. However, it would be more substantial to have measurable biological criteria which would make it possible to distinguish the different sub types of BPD. Unfortunately, there are no such criteria. The many different ways of manifestation of the disease and the lack of sophisticated knowledge on the pathophysiology of BPD make it very difficult to find such criteria (Wong & Licinio, 2001). It is not just the diagnosis of BPD that is nonspecific; also the treatment lacks specifity. Many of today's medications for BPD are deduced from medications for other disorders. They are aspecific and their exact pharmacodynamica are often unknown. They vary in effect and efficiency, and because of their aspecifity its use results in many side effects (Gould et al, 2004). To improve diagnosis and treatment of BPD, detailed knowledge about the pathophysiology of BPD is necessary.

Current knowledge on the pathophysiology does not show a clear cause for BPD. It seems that a combination of many factors cause this disease. Environmental factors like early life events, stress and nutrition are known to elevate the risk for BPD (Goodwin, 2007). On the molecular level several hypotheses and mechanisms are being studied. Results suggest dysfunctioning of the Hypothalamic-Pituitary-Adrenal (HPA)-axis and monoamine (dopamine, serotonin, and noradrenalin) regulation as an underlying cause for BPD (Wong & Licinio, 2001; Bowden 2008). Although several twin studies have shown a strong heritability of BPD, causative

genes still have to be identified. Evidence indicates there is no single gene responsible for BPD and the influence of these single genes on BPD is marginal (Bowden, 2008). More likely, a complex combination of genetic and environmental factors is the cause of BPD.

In order to identify a cause for BPD, and thus to identify a specific treatment target, several approaches can be used. One of these approaches is studying the pharmacodynamics of currently available anti-depressants including lithium, valproate, diazepam and carbamezepine (Gould et al, 2004). For instance, diazepam works via the receptors of serotonin, a hormone involved in regulating emotion and sleep (Griebel et al, 2000). Another possible approach to obtain more knowledge about BPD pathology is studying the different symptoms and their underlying (molecular) mechanisms. The latter approach will be used in this thesis, in order to identify a potential specific treatment target for BPD. Specific treatment is most likely to succeed when interfering with molecular targets with specific functions. One of these targets can be adenylyl cyclase (AC). Although AC is part of the ubiquitous cAMP pathway (cyclic adenosine monophosphate), the nine AC subtypes might give AC the specificity needed for a specific treatment target. Recent studies point at AC subtype 8 as important factor in BPD pathology (Schaefer et al, 2000; deMooij-Malsen et al, 2009). The aim of this thesis is to study the potential of AC8 and the other AC subtypes as treatment target for BPD.

Part I Behaviour

1. Endophenotypes of Bipolar Disorder

Bipolar Disorder

BPD is a brain disorder that causes unusual changes in a patient's mood. Symptoms vary from disturbed behavioural patterns to decreased cognitive capacities (Table 1). The most prominent symptoms of a manic episode are increased energy, restlessness, euphoric mood, extreme irritability, bad concentration capacity, increased sexual drive, racing thoughts, aggressive behaviour and impulsive and unrestrained behaviour (Goodwin, 2007; Kato, 2008). On the contrary, depressive episodes are characterized by decreased energy, fatigue, anxiety, sad mood, feelings of guilt and worthlessness, loss of interest in once enjoyable activities, decreased sexual drive, sleep disturbances, bad concentration capacities, change in appetite and/or weight, chronic pain and thoughts of suicide (Goodwin, 2007; Kato, 2008). In both states, patients suffer from an increased irritable mood and memory problems (Bowden et al, 2007). BPD manifests itself differently per patient. Furthermore, the prevalence of the different symptoms varies (Table 1). This makes BPD a complex and variable disorder, which hinders the search for a clear cause and treatment.

The complexity of BPD lies in its variable appearance and contrasting symptoms. The appearance of BPD is rarely the same: it varies from person to person and within persons from time to time. Symptoms during mania and depression are in sharp contrast which makes it even more difficult to study this disease and to find a biological cause for it. A way to simplify this complex disorder is to study endophenotypes, which are defined as components of a complex behavioural phenotype and involve a simpler behaviour or an underlying endocrine- or neural mechanism (Bowden, 2008). Characterization of the endophenotypes of BPD at different levels can reveal new, detailed information about the pathology of BPD. Furthermore, treating these endophenotypes or symptoms separately results in specific treatment of BPD for each patient, irrespective the variable appearance among BPD patients. Because it would be beyond the scope of this thesis to discuss all the endophenotypes, the focus will be on one of them: anxiety. This symptom of BPD will turn out to be an interesting actor in the course of BPD.

Anxiety: endophenotype or causal factor?

Of all BPD patients, about forty percent suffers from an anxious mood during depression. Anxiety is described as a psychological and physiological state of long term nervousness and worry (Rosen & Schulkin, 1998; Goodwin, 2007). It is closely related to fear, though they both are two distinct phenomena. While fear is the response to a specific and direct threatening stimulus, anxiety is the response to a situation of relative permanent, uncontrollable, unpredictable and unspecific

	Symptoms during Mania	Occurrence in patients (%)	Symptoms during Depression	Occurrence in patients (%)
Mood	Irritable	71	Irritable	85
	Euphoric	63	Melancholic	100
			Anxious	40
Cognition & Perception	Racing Thoughts	76	Decreased Cognitive ability	91
	Distractibility	75	Self deprecatory & accusatory	95
	Memory confusion	29	Poor memory	50
	Psychosis	53		
Activity & Behaviour	Hyperactivity	90	Fatigue	76
	Hyperverbosity & Speech	89	Lack of activity	80
	Sleep disorder	83	Sleep disorder	100
	Hypersexuality	51	Hyposexuality	75
	Psychomotor activation		Psychomotor retardation	80
	Impulsive behaviour	56	Social withdrawal	100
	Aggressive/Violent behaviour	47		

Table 1. Prevalence of BPD symptoms BPD knows a variety of symptoms, occurring during many or depression (column 2 and 4). Percentages indicate the symptoms' prevalence (column 3 and 5). (*Adapted from Goodwin, 2007*)

threat (Rosen & Schulkin, 1998). Fear and anxiety have in common that they are natural states which prepare an individual to respond to a threatening situation, and consequently lead to adaptations to its environment. In mammals, anxiety is characterized by freezing (phasic immobility), autonomic changes, startle (increased reflect responses to sensory stimuli), hypoalgesia and increased urination and defecation (Rosen & Schulkin, 1998; Goodwin, 2007). Anxiety in humans results in symptoms including agitation, accelerated thought processes, restlessness and irritability (Goodwin, 2007). Under normal circumstances, these symptoms are healthy and even necessary for survival. However, anxiety can reach pathological proportions. Patients with an anxiety disorder, as well as BPD patients with anxiety symptoms, suffer from exaggerated and irrational worry. This pathological mood severely hampers a patient's normal life, with all its consequences.

Anxiety an sich can reach pathological states, but it can cause other pathological symptoms as well. For instance, anxiety results in social withdrawal. This correlation is shown in rats (Overstreet et al, 2002) and occurs in 100% of BPD patients (Goodwin, 2007). A severe form

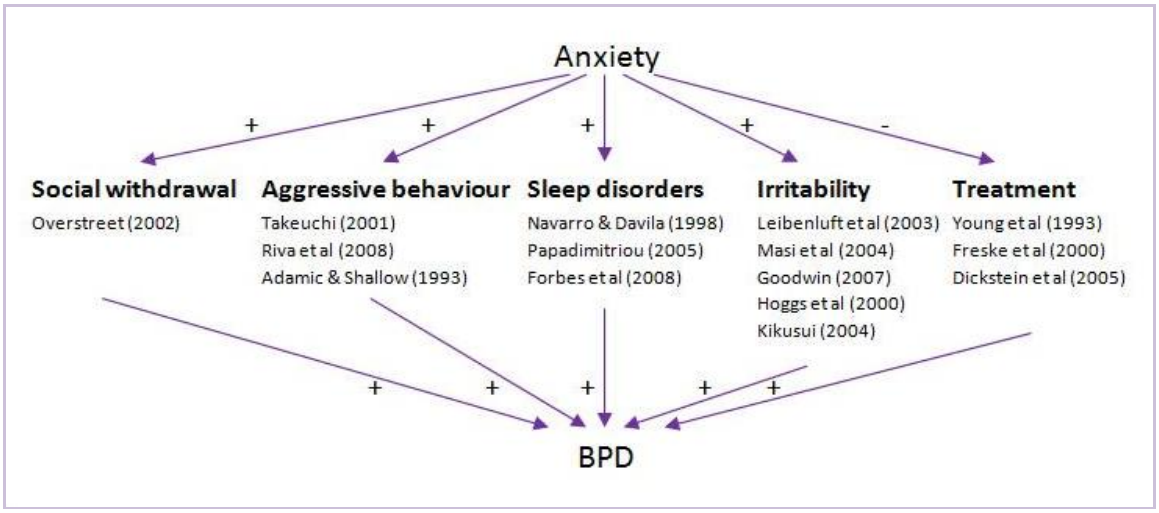

Figure 1. Possible relationship between anxiety and BPD Evidence from the literature suggests an amplifying or causal role for anxiety in BPD

of social withdrawal caused by anxiety is known as the psychiatric illness social phobia, or social anxiety disorder (DSM, American Psychiatric Organization). Furthermore, anxiety can lead to aggressive behaviour. This is extensively shown in studies on dogs, where anxiety aggression is a major behavioural problem (Takeuchi, 2001; Riva et al, 2008). This relation between anxiety and aggression is confirmed in studies under laboratory conditions with rats (Adamec & Shallow, 1993; Hogg et al, 2000; Kikusui, 2004). In addition, anxiety is correlated with sleep disorders (Navarro & Davila, 1998; Papadimitriou, 2005; Forbes et al, 2008). Problems like longer sleep latency, increased time awake and reduced sleep efficiency are significantly higher in anxious patients. Another major symptom of anxiety disorders is irritability: about 75% of anxious patients suffer from irritability (Leibenluft et al, 2003; Masi et al, 2004). These symptoms; social withdrawal, aggression, sleep disorders and irritability, also occur in many BPD patients (Table 1; Goodwin, 2007). Taken together, anxiety results in other symptoms of which some are strongly related to BPD. This points at anxiety as an important component of BPD pathology.

Above this causal effect of anxiety on behaviour, clinical and epidemiological studies have shown a comorbidity of different types of anxiety disorders with BPD (Chen et al, 1995). Anxiety symptoms and disorders occur in 40% of the BPD patients (Table 2; Mc. Elroy et al, 2001). A possible reason for this comorbidity could be a common cause of both disorders. However, it is shown that BPD patients with anxiety symptoms have a more severe course of the disease, a higher risk for suicide an alcohol abuse, and a worse response to medical treatment then BPD patients without anxiety symptoms (Young et al, 1993; Feske et al, 2000). This does not indicate a common cause for both disorders, but more likely a causal relationship between them. Again, anxiety seems a very important link in the chain of BPD.

An early indication for a relation between anxiety and other mental disorders came from studies on the correlation between anxiety and unipolar depression. Already in the early seventies, Paykel et al (1971) showed a severe and long term course of depression in anxious patients compared to other patients. Depressive and anxious patients tend to have a more chronic course of the illness and respond only weakly to antidepressants (Maser & Cloninger,

1990). An early study on anxiety in BPD came from Young et al (1993). Eighty BPD patients were divided in groups of high or low anxiety. Highly anxious patients had more symptoms of cyclothymia, more suicide attempts, were more likely to abuse alcohol and responded less to lithium. Additionally, Feske et al (2000) showed in a more recent study that anxious BPD patients are less tolerant for side effects of medication than non-anxious patients. So anxiety in BPD patients is correlated with a more severe course of the illness, a less effective treatment and more side effects. Although Feske et al (2000) doubt if there is a causal relationship between anxiety and these aspects of BPD, the results above suggest otherwise. This is strongly confirmed by results from Dickstein et al (2005), who showed a high prevalence of anxiety in children with BPD. An even stronger confirmation is their finding that the onset of anxiety predates the onset of BPD. Furthermore, the onset of BPD in anxious patients is significantly earlier in life than in patients without anxiety. Taken together, this indicates a causative or at least an amplifying role for anxiety in BPD (Fig. 1).

Summary

To summarize, BPD is a complex disorder with different and contrasting symptoms, within as well as between patients. These symptoms can be described as multiple endophenotypes. Studying these endophenotypes separately simplifies the search for knowledge of BPD pathology and treatment possibilities. One of these endophenotypes is anxiety. Anxiety results in several different behaviours. BPD patients with anxiety symptoms have a more severe course of the illness then patients without anxiety symptoms. Furthermore, the onset of anxiety predates the onset of BPD. Taken together, these findings indicate a causal or amplifying role for anxiety in BPD (Fig. 1). Since anxiety can be treated well by psychotherapy (Schneider, 2006), insights in the relation between BPD and anxiety can lead to new and efficient treatments of BPD. Future studies have to find out if anxiety is more than just a marker for BPD and have to confirm the hypothesis of a causal relationship between anxiety and BPD.

Part II Molecules

Adenylyl Cyclase

In order to develop an effective and specific treatment for BPD, molecular agents involved in this disease have to be identified. One of these agents can be adenylyl cyclase, a membrane bound enzyme which is part of the G-protein Coupled Receptor (GPCR) machinery (Fig. 2). The GPCR is a common type of receptor in the vertebrate body. A G-protein exists of three subunits: α, β and γ. After ligand binding, the receptor is activated and an αβγ-complex binds to the GPCR, together with Guanosine Diphosphate (GDP). This GDP is replaced by the high energy compound Guanosine Triphosphate (GTP) which forms a complex with the α-subunit. The α-GTP complex dissociates from the receptor and is the effective molecule, which can activate several molecules like effector proteins. One of these effector proteins is adenylyl cyclase.

Adenylyl cyclase is a membrane associated enzyme which converts ATP to the second messenger cyclic Adenosine Monophosphate (cAMP). Binding of α-GTP to adenylyl cyclase activates adenylyl cyclase which produces many molecules of this second messenger cAMP. In turn, cAMP molecules have several effectors, like phospholipases or protein kinases. Each of these effectors has its own function and can regulate for example gene expression, and hence regulate behaviour or emotions. Consequently, adenylyl cyclase can be an important factor in regulating behaviour. There are nine subtypes of adenylyl cyclase in mammals (Sunahara & Taussig, 2002): AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8 and sAC, of which sAC is a soluble, non-membrane bound AC. These nine mammalian subtypes of adenylyl cyclase each have their own specific characteristics and enzyme kinetics, and are distributed specifically through the body. In order to give better insights in to the role of adenylyl cyclase in BPD, all subtypes will be discussed. But first, a general top down approach is used to discuss the role of adenylyl cyclase in BPD symptoms.

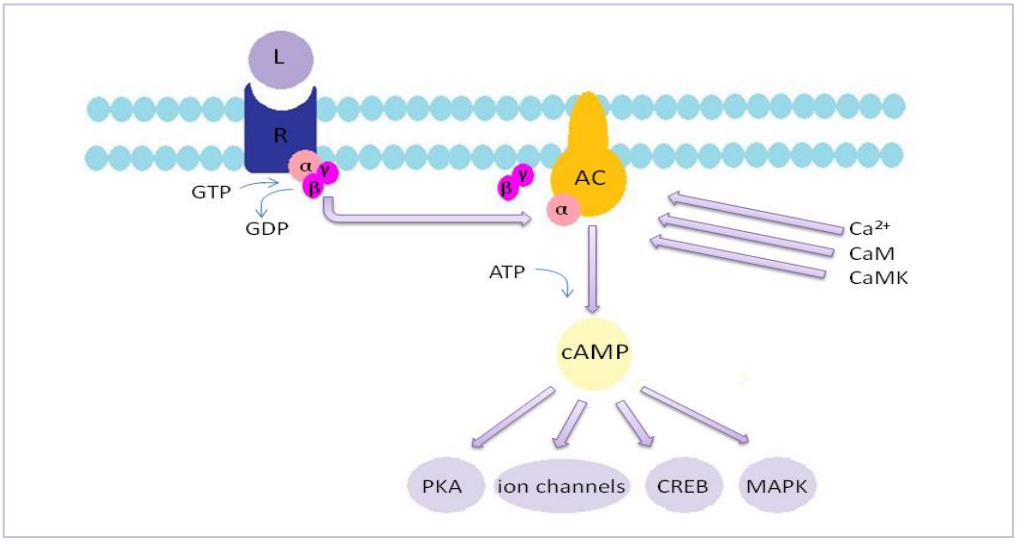

Figure 2. Molecular pathway of AC Schematic overview of the molecular pathways around AC.
Abbreviations: L: ligand; R: receptor; GDP: guanosine diphosphate; GTP: guanosine triphosphate; AC: adenylyl cyclase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PKA: proteinkinase A; CREB: cAMP response element binding; MAPK: mitogen activated protein kinase; Ca^{2+}: calcium; CaM: calmodulin; CaMK: calmodulin kinase

Modulation. Either inhibiting or inducing, depending on AC- and GPCR-subtype.

Transformation

2. Top down: from symptoms to molecules

Behaviour & Adenylyl Cyclase: a link towards BPD?

Adenylyl cyclase (AC) plays an important role in regulation of behaviour, including behavioural symptoms of BPD, as shown by several animal studies. Early results came from studies with invertebrates, like fruit flies, sea slugs and crustacaea, but are confirmed by more recent studies in vertebrates. The literature discussed here illustrates early indications of a relation between AC and behavioural symptoms of BPD and is summarized in Table 2.

Defensive behaviour is equivalent or a possible indicator of aggression, and thus of a BPD symptom. It increases due to AC activity in lobsters and sea slugs (Doernberg, 2001; Glanzman, 1989). Furthermore, Glanzman et al (1989) used the withdrawal reflex of the siphon of the sea slug *Aplysia californica* as a parameter for defensive behaviour. He and his colleagues showed that serotonin plays a major role in dishabituation and sensitization of the withdrawal reflex. Interestingly, the receptor of this neurotransmitter is coupled with AC. The effect of serotonin on AC is also shown in the for humans more representative rat: serotonin antagonists increase defensive behaviour in rats by interfering with AC in the hippocampus (Griebel et al, 1996). More evidence for linkage between AC, hippocampus and behaviour came from Guillou et al (1998): in a lesion study they showed that hippocampal regulatory systems include AC and that these systems are involved in learning and memory processes. In BPD patients, these learning and memory processes are often disordered.

Also disordered in BPD patients, is sexual behaviour. Arching of the spine in rats, known as lordosis behaviour, is a common used parameter for sexual behaviour. It is inhibited by agonist activation of the 5HT1A serotonin receptors in the ventromedial nucleus (VMN) of the hypothalamus, which inhibits AC activity. This inhibition of lordosis behaviour by serotonin agonists can be enabled by increasing AC and cAMP concentrations (Uphouse, 2000). So an inhibition of AC activity results in inhibition of sexual behaviour. Hence, AC activity in the VMN stimulates sexual behaviour. Already in 1994, Kow showed that complex alterations of AC pathways and IP pathways (inositol phospholipid pathway: a cellular signaling pathway including generation of inositol triphosphate (IP_3) and diaglycerol (DAG) by phospholipase C in the plasma membrane) by hormones like estrogen affect sexual behaviour. Frye et al (2006) did the same for progesterone, and showed increased levels of AC and cAMP in the midbrain of sexual receptive rats. Also in fiddler crabs and sea slugs sexual behaviour is shown to be modulated by serotonin and AC activity (Weissburg 2001; Glanzman, 1989). Other behaviours related to BPD that are modulated by AC include locomotion, feeding and drinking (Glanzman 1989; Weissburg 2001; Castro, 2000). In fruit flies (*Drosophila sp.*) and fiddler crabs (*Uca sp.*) also chemo sensitivity and olfactory responses are inhibited by AC activity (Martin, 2001; Weissburg, 2001).

As discussed above, complicated serotonin mechanisms play a role in regulating behaviour, hence could play a role in behavioural disorders. However, the complexity and dualism of the serotonin system, with different types of receptors and dualistic effects, make it difficult to establish this. The same applies for the dopamine system, another mechanism in regulating behaviour. This neurotransmitter regulates a variety in behaviours. In *Aplysia* it mediates motor behaviour (Flinn, 2001), and in many higher organisms it is known to be

Animal behaviour	Symptom of BPD*		Animal model
	During mania	During depression	
Defensive	Violent/ Assaultive/ Aggressive behaviour	Anxiety	Lobster (Doernberg, 2001) Aplysia (Glanzman, 1989) Rat (Griebel, 1996)
Sexual	Hyper sexuality	Decrease of sexual interest	Aplysia (Glanzman, 1989) Fiddlercrab (Weissburg, 2001) Rat (Kow, 1994; Uphouse, 2001; Frye & Petralia, 2003,2006)
Locomotion	Hyperactivity & Increased psychomotor activity	Fatigue & Psychomotor retardation	Aplysia (Glanzman, 1989; Flinn, 2001)
Feeding		Change of appetite	Aplysia (Glanzman, 1989)
Drinking		Change of appetite	Rat (Castro et al, 2000)
Chemo- & Olfactory sensitivity	Olfactory psychosis		Drosophila (Martin, 2001) Fiddler crab (Weissburg, 2001)
Learning & Memory	Distractibility	Distractibility	Rat (Guillou, 1989)
Stereotypic			Rat (Yeghiayan, 1997)

Table 2. Animal behaviour and BPD symptoms The animal behaviours in this table are modulated by AC. Comparison with behavioural symptoms of BPD indicates a role for AC in BPD.
* Symptoms from Goodwin, 2007

important in the reward system (Wise & Rompre, 1989). By modulating this system it regulates emotions and behaviour. Dopamine works via different GPCR's which can activate or inactivate AC, but there is also evidence for an AC-independent pathway of dopamine (Deveny & Waddington, 1995). Furthermore, dopamine and serotonin interfere with each other (Yeghiayan, 1997; Frye et al, 2006). Taken together, the dopamine and serotonin systems play an important but complicated role in generating emotions and behaviour, and therefore are potential targets for treatment of BPD.

Summary

In summary, comparison of BPD symptoms with animal models suggests a role of AC in BPD pathology: defensive behaviour, sexual behaviour, locomotion, and learning and memory processes are related to BPD symptoms and all modulated by AC (Table 2). Also the dopamine and serotonin system are indicated as actors in BPD pathology.

3. Bottom up: from molecules to symptoms

G-protein machinery

Adenylyl Cyclase (AC) is part of the cAMP-pathway, which starts with a G-protein coupled receptor (GPCR). The G-protein machinery is a crucial part of the molecular processes around AC. Activation of the receptor by binding of an extracellular messenger molecule results in activation of the heterotrimeric G-protein. The of an α, β, and γ subunit existing protein changes conformation, exchanges a GDP for a GTP molecule and dissociates in a βγ- and α-GTP complex (Fig. 2, page 16). In general, the latter is the active component which activates several effectors including AC. In turn, activated AC transforms ATP in to the second messenger cAMP which can bring about a variety of cellular processes, like activation of CREB (cAMP response element binding protein). After phosphorylation by cAMP, CREB binds to the cAMP response element in DNA and activates gene transcription, establishing the effect of cAMP on gene expression.

G-proteins are signal transducers: they connect receptors to effectors and like this they modulate intracellular pathways. GPCR's are receptors for, among others, hormones, neurotransmitters, and para- and autocrine factors. Different G-protein pathways can influence each other indirectly. For example, all G-proteins influence MAPK-pathways (mitogen associated protein kinase, part of intercellular pathway affecting gene expression), either inhibitory or stimulatory (Neves, 2002). Furthermore, duration and rates of responses of several pathways are modulated by the GPCR-cAMP pathway. Short term effects are established via protein kinase A (PKA,) and ion channels, while long term effects are established via MAPK and gene expression (Neves, 2002). Taken together, there is indirect connectivity between the several G-protein pathways, which makes it a complex and diverse machinery that integrates different GPCR signals. One should take this into account when considering a part of the GPCR/cAMP machinery as target for medication, like when testing AC as a target for BPD treatment.

The variance of GPCR-effects does not come only from diversity in ligands, but also from diversity in G-proteins. Although the literature is somewhat contradictory, there are generally four G-protein families: G_s, G_q, $G_{i/o}$, and $G_{12/13}$ (Hamm, 1998; Neves, 2002; Sunahara & Taussig, 2002). They are coupled to distinct receptor subtypes and all have their particular characteristics. All G-protein subtypes influence Rap and Rho, two GTP-ases. By dephosphorylation of GTP to GDP they inactivate the α-GTP subunit and consequently inactivate AC or other effectors activated by the α-GTP complex. With this function they are the time mechanism of AC activation. The G-protein subtypes differ in other functions, for instance, G_s activates AC, while $G_{i/o}$ inhibits AC and G_q activates PLC-β (phospholipase, an enzyme part of the IP pathway). The function of $G_{12/13}$ is unknown. However, the effects of G subunits are not completely separated. This is shown by Hadcock and colleagues (1990), who showed cross linking between G_s and G_i pathways. Persistent stimulation of the G_s pathway resulted in increased G_i and moderated G_s concentrations. This is important to keep in mind when considering AC as possible treatment target, because modulating AC activity can influence other G-protein pathways as well.

A complicated puzzle: AC subtypes in the G-protein machinery

The different G-protein subtypes differ in the way they effect each AC subtype (Table 3). First, $G\alpha_q$ only inhibits the AC1 subtypes. Second, $G\alpha_s$ stimulates all AC subtypes. It works synergistic with the Calcium(Ca^{2+})-Calmodulin complex for AC1, AC3 and AC8. For AC2, AC4, AC5 and AC6, $G\alpha_s$ works synergistic with forskolin (Sunahara et al, 1996; Sunahara & Taussig, 2002). The latter is a vegetable chemical that directly stimulates AC and is broadly used to increase intracellular cAMP levels in studies on the cAMP pathway (Seamon & Daly, 1981). Forskolin's stimulating effect is additive for AC1, AC3 and AC8 and cooperative with Ca^{2+}-Calmodulin complex activations. Back to the G-subunits, Sunahara and colleagues showed that AC8 is activated by $G\alpha_s$. In contrast, Xia & Storm (1997) showed that AC8 is not activated by $G\alpha_s$, but only by Ca^{2+}. The role of Ca^{2+} will be discussed in more detail in the next section. Third, $G\alpha_i$, the α-subunit of the inhibitory G-protein G_i, $_{\text{inhibits}}$ AC1, AC5 and AC6 (Sunahara, 1996). In case of chronic activation of $G\alpha_i$ coupled receptors (like DA1 and DA4 receptors), $G\alpha_i$ can bring about sensitization of AC1, AC5, AC6 and AC8 (Sunahara & Taussig, 2002). Possible explanations for this finding are increased expression of AC, CREB or PKA, because phosphorylation of some ACs by PKA increases AC activity. This sensitization process can be interesting when looking for an agent in BPD, because disturbed sensitization processes can result in exaggerated or, on the other hand, apathic response. In turn, this can lead to symptoms of BPD. Taken together, AC subtypes differ in their function, what makes them interesting candidates for specific treatment of separate BPD symptoms. However, the different types and effects of G-proteins make this specifity debatable.

Besides the α-subunit, also the βγ-subunit complex influences the cAMP pathway. In co-activation with $G\alpha_s$, Gβγ stimulates AC2, AC4 and AC7 (Hamm, 1998; Sunahara & Taussig, 2002), while it inhibits AC1 and AC8. This inhibiting effect of Gβγ on AC1 and AC8 is the result of inhibition by Gβγ of $G\alpha_s$, which consequently inhibits $G\alpha_s$-activation of AC1 and AC8 (Xia & Storm, 1997). Interestingly, Steiner et al (2006) show several β isoforms families with different influences on AC in combination with γ-subunits. For example, β5 is not able to stimulate MAPK1, and their results suggest that Gβ1γ5 cannot inhibit AC8. It would be beyond the scope of this thesis to discuss all the β isoforms families, but again, it shows the complexity of the G protein machinery and its effect on AC's. Furthermore, Gβγ indirectly affects AC because it inhibits forskolin, Ca^{2+}-Calmodulin complex and $G\alpha_s$. Gβγ also inhibits Na^{+}- and Ca^{2+}-channels , but stimulates K-channels, GPCR kinase, PLCβ and MAPK1 (Hamm, 1998; Sunahara & Taussig, 2002). Taken together, the G-protein machinery is very complex in its function and the effects it has on AC. Further research has to clarify the exact effects of G proteins on the functioning of the different AC isoforms.

The inhibitory $G\alpha_i$ coupled receptors mentioned above include the dopamine receptors DA1 and DA4. Dopamine is a hormone and neurotransmitter with a crucial role in emotions and mood (Wise & Rompre, 1989). Disfunctioning of the dopamine mechanism could result in disturbed emotions, which clearly links to BPD. This idea is supported by a study of Hains & Arnsten (2008). They showed that excessive stimulation of the DA1 receptor leads via the cAMP pathway to disconnection of prefrontal networks. This results in impaired functioning of the prefrontal cortex, which results in weakened regulation of emotion, behaviour and thought; factors standing at the origin of symptoms of BPD. These findings suggest a role in BPD pathology for $G\alpha_i$ and the ACs it sensitizes, which are AC1, AC5, AC6 and AC8 (Sunahara & Taussig, 2002). The inhibitory effect of $G\alpha_i$ on AC are supported by Izquierdo-Claros and

AC	Stimulated by	Inhibited by	Effects	Distribution
AC1	$G\alpha_s$ (synergistic with Ca^{2+}CaM) Forskolin Ca^{2+} CaM	$G\alpha_q$ $G\alpha_i$ $G\beta\gamma$ CaMK	Melatonin, Neural growth, Dopamine	Brain, adrenal medulla
AC2	$G\alpha_s$ (synergistic with forskolin) $G\beta\gamma$			Brain, lung, heart, skeletal muscle
AC3	$G\alpha_s$ (synergistic with Ca^{2+}CaM) Forskolin $(Ca^{2+}CaM)^{?}$ $(Ca^{2+})^{?}$	$(Ca^{2+}CaM)^{?}$ $(Ca^{2+})^{?}$		Brain, olfactory epithelium
AC4	$G\alpha_s$ (synergistic with forskolin) $G\beta\gamma$			Brain, heart, kidney, liver, lung, uterus
AC5	$G\alpha_s$ (synergistic with forskolin)	$G\alpha_i$ Ca^{2+}	Neural growth, Dopamine	Brain, heart, kidney, liver, lung, uterus
AC6	$G\alpha_s$ (synergistic with forskolin)	$G\alpha_i$	Dopamine	Ubiquitous
AC7	$G\alpha_s$ $G\beta\gamma$			Ubiquitous
AC8	$G\alpha_s$ (synergistic with Ca^{2+}CaM) Forskolin Ca^{2+} CaM	$G\beta\gamma$ CaMK	Neural growth, Dopamine	Brain
AC9	$G\alpha_s$			Brain, skeletal muscle

Table 3. AC subtypes and their regulators All AC subtypes have their characteristic stimulators, inhibitors, effects and distribution. Abbreviations: Ca^{2+}: calcium; CaM: calmodulin; CaMK: calmodulin kinase; Ca^{2+}CaM: calcium-calmodulin kinase.
(From Conti et al, 2007; Hains & Arnsten, 2008; Hamm, 1990; Izquierdo-Claros et al, 2002; Kato, 2008; Krishnan et al, 2008b; Matsuoka et al, 1997; Sunahara et al, 1996; Sunahara & Taussig, 2002; Xia & Storm, 1997)

colleagues (2002), who showed decreased G_i and AC activity after four days of melatonin administration. Melatonin synthesis in the pineal gland is thought to be regulated by AC1 (Xia & Storm, 1997), so this indicates inhibition of AC1 by $G\alpha_i$. There are other indications for a role for melatonin in BPD, which will be discussed in detail in chapter 4 of this thesis. Interestingly, Steiner et al (2006) show no independent inhibition of AC8 by $G\alpha_i$. This makes the role of AC8 in the sensitization process unclear. Taken together, these results point at AC1 as an actor in BPD pathology more likely than AC8. Further research has to show the exact mechanism of sensitization of AC subtypes by $G\alpha_i$, and its possible role in BPD.

Another interesting aspect of the AC isoforms is their distribution. Each isoform has its own characteristic distribution through the body, which might say something about their

function. A nice overview of this is given by Sunahara et al (1996). Body tissues where several AC isoforms can be found are the heart, kidneys, lungs and skeletal muscles, described in detail in Table 3. According to Sunahara et al (1996), all AC isoforms can be found in the brain, but it is a prominent side of expression for AC 2, AC 3 and AC 8. However, in a more recent study (Sunahara & Taussig, 2002) they are less explicit about this, and instead they propose AC7 to be excessively expressed in the brain. On the contrary, Matsuoka and colleagues (1997) speak of AC1, AC2 and AC5 as 'the major three brain adenylyl cyclases'. The literature is not consistent about which AC isoform is or isoforms are the most important in the brain. Nonetheless, there are interesting data about AC localization in the brain (Matsuoka et al, 1997). Each AC isoform has its own specific distribution in specific brain areas, some even age dependent. For example, AC 5 expression increases during development and is found in the striatum and nucleus accumbens (Matsuoka et al, 1997). AC1 expression in the cerebral cortex, striatum, thalamus and brainstem decreases drastically after the postnatal stage, while expression in cerebellum and hippocampus clearly increases (Matsuoka et al, 1997). These findings suggest a role in synaptic transmission, cell differentiation and maturation. Further research has to show the role of AC1 and synaptic transmission, cell differentiation and maturation in BPD pathology. Interestingly, AC3 is the only AC isoform involved in the olfactory system (Sunahara & Taussig, 2002). As mentioned in chapter 1, a symptom of BPD during mania is olfactory psychosis (Goodwin, 2007). The fact that AC3 is the only AC isoform in the olfactory system could indicate a role for AC3 in BPD. Further research has to confirm this.

Ca^{2+}-CaM sensitive ACs

Besides the G-protein subunits there are other intracellular effectors that influence AC. One of them is calcium (Ca^{2+}), which is crucial in many cellular processes, especially in neural processes. Ca^{2+} inhibits all AC isoforms in very low concentrations, while under normal in vivo conditions only AC5 and AC6 are inhibited (Conti et al, 2007). Furthermore, Ca^{2+} affects AC1, AC3 and AC8: while the effect on AC3 remains rather unclear, it is shown that Ca^{2+} stimulates AC1 and AC8 (Xia & Storm, 1997; Sunahara & Taussig, 2002; Conti et al, 2007). Interestingly, Sunahara & Taussig (2002) show that Ca^{2+} from the IP-pathway does not influence AC activity. Within the intracellular calcium machinery there is another agent affecting AC1, AC3 and AC8, which is calmodulin (CaM). This calcium-binding protein regulates a variety of proteins and cellular processes, like apoptosis, memory and neural growth (Lawrence, 2005). CaM stimulates AC1 and AC8, while the exact relation between AC3 and CaM is still unclear (Sunahara & Taussig, 2002). CaM and the CaM-sensitive ACs form a negative feedback loop controlling Ca^{2+} mediated cell signaling, which is brought about via Ca^{2+}-Calmodulin dependent protein kinases (CaMK): CaMK phosphorilates AC 1 and AC8, thereby inhibiting AC 1 and AC8 (Xia & Storm, 1997). This calcium signaling plays an important role in neural processes. Since the origin of BPD lies in the brain and interfering in neural processes could be a good treatment (Goodwin, 2007), an interesting possibility to find an agent of BPD treatment lies in the Ca^{2+}-sensitive ACs: AC1, AC3, AC5 and AC8.

AC1 and AC8 are the only ACs clearly stimulated by Ca^{2+}, which gives them a unique role in neural processes like learning and memory (Sunahara & Taussig, 2002). This makes them potential actors and treatment targets in BPD. Supporting findings for this hypothesis come from Kato (2008). Although he proposes a causative role of BPD for the IP and GSK-3β pathways

(Glycogen synthase kinase; involved in neuronal development and metabolism), his results indicate a role for Ca^{2+}-CaM sensitive AC's as well. The bottom line of his review is that disturbed Ca^{2+}-signaling results in vulnerable neurons. Loss or dysfunction of neurons with a mood stabilizing function then results in BPD. Since AC1 and AC8 are part of the Ca^{2+} signaling cascade, these findings indicate a role in BPD for them as well. This is supported by Krishnan and Nester (2008a), who showed changed BDNF functioning in AC1, AC5 and AC8 KO mice. Since BDNF (brain derived neurotrophic factor), regulates neural growth and thus vulnerability and loss of neurons, these results indicate a role for AC1, AC5 and AC8 in neural growth. Taken together, AC1, AC5 and AC8 seem to be involved in causing BPD via disturbed neural growth.

More supporting evidence for a role of Ca^{2+}-CaM sensitive ACs in BPD comes from Krishnan et al (2008b). In a behavioural study with AC5 knockout mice and AC1/8 double knockout mice they investigated the influence of these AC isoforms on endophenotypes of BPD. Behaviour of the knockout mice was measured during a forced swim task, at the elevated plus maze, in a social interaction test and sucrose preference test. Also locomotor activity was measured. The AC1/8 dKO mice showed less activity then the wild type controls, increased social interactions and decreased sucrose preference. On the contrary, AC5 KO mice were hyperactive, showed anxiolytic behaviour and decreased social interactions. All these behavioural changes mark symptoms of BPD: hyperactivity, anxious mood and social withdrawal. Therefore, results of Krishnan et al (2008b) indicate a role for AC1 and/or AC8, as well as of AC5 in causing these symptoms. In addition, AC5 KO mice show decreased social interactions, which is characterizing for a depressive state, and AC1/8 DKO mice show increased social interactions, which is characterizing for a manic state. Furthermore, changes in sucrose preference fit the observation of changed appetite in depressive BPD patients and therefore suggest a role for AC1 and/or AC8. Taken together, these results show a complicated effect of AC1, AC5 and AC8 on emotional behaviour and indicate that AC1 and AC8 induce anti-depressive or manic behaviour while AC5 induces depressive behaviour.

Summary

As discussed in this chapter, the G-protein machinery is a complex cellular messenger system with a variety of functions and effects. AC is part of this machinery. The combination of four G-protein subtypes and nine AC subtypes results in a complex puzzle of inhibiting and stimulating pathways (Table 3). Following these pathways leads to strong indications for Ca^{2+} sensitive AC's as actors in BPD, since these AC subtypes have unique roles in neural processes. Studies with knockout mice point at AC1 and AC8 as the most potential AC's for BPD, because of their role in neural growth and anti-depressant behaviour, and their distribution in the brain.

4. High potentials: AC1 and AC8

As discussed in the former chapter, the Ca^{2+}-depended AC's AC1 and AC8 are the AC's with the best characteristics for a potential treatment target for BPD. Fundamental studies on the localization of AC subtypes give other interesting information about AC1 and AC8 as actors in brain function and BPD. In a study with knockout mice combined with immunohistochemistry, Conti and colleagues (2007) gave insight in the functional roles of AC1 and AC8. A distinct regional and subcellular, age dependent distribution in the brain was shown, which confirmed the same earlier findings of Matsuoka et al (1997). AC1 is strongly expressed during embryonal stages, while AC8 is expressed mainly during adulthood (Nicol, 2005). Onset of BPD occurs mostly later in life, during the adolescent period, when AC1 expression is relatively low. Based on this finding, it is more likely that AC8 is involved in the onset of BPD then AC1. However, if AC1 expression is deregulated during adulthood, also AC1 could play a role in BPD onset. Empirical research has to study the possible relation between temporal expression of AC1 and AC8 in the brain and onset of BPD, but there are already other indications for both AC1 and AC8 as actors in BPD.

AC1 and BPD

The first of the two major brain AC's, AC1, is expressed in the striatum, dorsal thalamus, cerebellum, hippocampus, basolateral amygdala and cortex of the neonatal brain (Nicol et al, 2005). In the adult brain, expression is restricted to the cerebellum, cortex, dentate gyrus and CA1 region of hippocampus, olfactory bulb and pineal gland (Conti, 2007; Nicol, 2005). Specifically, AC1 expression in the hippocampus decreases during development, as well as AC1 expression in the cortex. In the hippocampus, AC1 expression is concentrated in the CA3 region. AC1 expression in the cerebellum remains unchanged during development. In the adult brain, AC1 is abundantly expressed in the molecular layer of the cerebellum. Generalized expression is found in the thalamus (Conti et al, 2007). Taken together, AC1 is abundantly present in as well the neonatal brain as the adult brain.

The expression of AC1 in the pineal gland suggests another link with BPD and AC1. This small endocrine gland in the mammalian brain produces the hormone melatonin. In most mammals, melatonin is released from the pineal gland at night only, and in humans it regulates circadian rhythms and sleep (Lawrence, 2005). Furthermore, since melatonin reduces arousal and facilitates sleep (Zisapel, 2001), disturbance of melatonin levels results in disturbed circadian processes and sleep disorders. Exogenous melatonin is already used as therapeutic against sleep disorders (Pandi-Perumal et al, 2008). Disturbed sleep patterns are a common symptom of BPD (Goodwin, 2007), so there might be a role for melatonin in BPD as well. Melatonin synthesis is regulated by nor-epinephrine (NE) and cAMP, and AC1 plays a crucial role in this process. AC1 shows a rhythmic expression with high concentrations during midday and low concentrations during the night, regulated by NE and environmental light conditions. A rhythmic regulation of AC activity can be found as well: at midday, AC1 is inhibited by Ca^{2+}, and at midnight it is markedly activated by the Ca^{2+}-CaM complex. These findings indicate a crucial role for AC1 in regulating the circadian rhythm, by being the pivot between Ca^{2+} and cAMP

signals (Tzavara et al, 1996; Xia & Storm, 1997). Interfering in the melatonin secretion and regulation pathway could be a good treatment for sleep disorders, and thus for a symptom of BPD. AC1 seems a perfect candidate for this kind of treatment, because it has a crucial and integrating role in regulating circadian rhythms. Taken together, AC1 could be a good target for partly treating BPD.

AC8 and BPD

The expression of the second major brain AC, AC8, shows other localization and development patterns then AC1. Neonatal expression of AC8 is shown in the cortex, CA1 region of the hippocampus, cerebellum, olfactory bulbs, hypothalamus, amygdala and basal ganglia (Nicol et al, 2005). The expression remains during adulthood in the CA1 region of the hippocampus, cerebellum, olfactory bulb, hypothalamus, habenula, piriform and cerebral cortices (Cali et al, 1994). More specifically, AC8 expression in the cerebellum is moderate and constant during lifetime. It slightly decreases in the cortex during development. In adulthood, an intense AC8 expression is found in the CA1/CA2 region of the hippocampus and in the thalamus (Conti et al, 2007). So, also AC8 is abundantly present in both the neonatal and adult brain but in different areas then AC1.

An important function of AC8 lies in long term potentiation (LTP), which in turn is crucial for learning and memory processes. Since BPD patients suffer from decreased learning and memory capacities (Goodwin, 2007), AC8 and LTP might be useful in treating BPD. LTP can be defined as activity depending strengthening of synaptic activity (Xia & Storm, 1997). Stimulation of the neuron with bursts of electrical stimuli results in long term changes in the synapse, with as a consequence an increased response on normal stimuli. LTP occurs after activation of glutamate receptors and/or voltage mediated Ca^{2+}-channels, which result in increased postsynaptic Ca^{2+}-concentrations. This Ca^{2+} signal is coupled to cAMP and the gene transcription machinery by calcium sensitive AC's. The increased postsynaptic Ca^{2+} levels activate AC 1 and/or AC8, which results in increased cAMP levels. cAMP activates CREB, which in turn alters gene expression. This establishes long term effects in the cell and, in this case, results in LTP. Again, Ca^{2+} sensitive AC's are a crucial connection between Ca^{2+} signals and other pathways with large effects in physiology. According to Conti et al (2007), AC8 is expressed presynaptic while AC1 is expressed postsynaptic. However, Xia & Storm (1997) show that AC8 is essential for postsynaptic LTP, in contrast with AC1. Despite this contradiction in the literature (Lisman et al, 2003), the major importance of AC8 in establishing transcription dependent LTP has been shown repeatedly (Xia & Storm, 1997; Wong et al, 1999; Wang et al, 2003; Conti et al, 2007). LTP is the cellular underlying of synaptic plasticity needed for learning and memory. This brings us back to BPD: learning and memory problems are one of the symptoms of BPD (Goodwin, 2007). The crucial role for AC8 in LTP and thus in learning and memory makes it a possible modulator of these processes, and thus of one of the symptoms of BPD.

AC1 and AC8: a joint venture?

A closer look of AC1 and AC8 functioning in the brain leads to strong indications for a role of AC1 and AC8 in BPD. Both are expressed in relative constant concentrations during life time in the

cerebellum (Cali et al, 1994; Nicol et al, 2005; Conti et al, 2007). The cerebellum is thought to play a role in affect, emotion, thought and psychosis (Schmahmann, 2000; Bailleux et al, 2008). It is connected with several brain structures that are known to regulate aspects of affect, for example the hypothalamus, limbic system and some cortical areas, which makes the cerebellum an essential node in the neural circuitry of affect and emotion. Recent studies on cats showed that the cerebellum influences arousal, autonomic behaviour and emotional responsiveness (Schmamann, 2000). Furthermore, the cerebellum is shown to modulate aggression and mood in adults as well as in children. In addition, patients with cerebellar atrophy or degeneration show disordered emotions and behaviour (Schmamann, 1991). Arousal, emotional responsiveness, aggression and mood are all aspects of BPD and are all modulated in the cerebellum. Taken together, this indicates a role for the cerebellum in BPD. This is confirmed by Soares & Mann (1997), who showed a decreased cerebellum size in BPD patients. Another symptom of BPD is motor retardation, and the cerebellum plays a major role in motor regulation as well (Bailleux et al, 2008). This confirms the role of the cerebellum in BPD. Since AC1 and AC8 are expressed in the cerebellum and could regulate effects of the cerebellum on emotion and mood, interfering with AC1 and AC8 could modulate symptoms of BPD. More research to the exact distribution and function of AC1 and AC8 in the cerebellum and its effect on BPD has to establish the value for BPD treatment of AC1 and AC8 in this specific brain area.

Another brain structure where AC1 and AC8 both are expressed is the hypothalamus (Cali et al, 1994). This structure might be of greater importance than the other corresponding structures of AC1 and AC8 because AC1 and AC8 are the only ACs expressed in the hypothalamus, which gives them an absolute unique role in neuroendocrine activities (Conti et al, 2007). The hypothalamus is a coordinating center that integrates autonome and endocrine processes, in order to regulate a cascade of processes, from vital functions to emotional states. It is part of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the stress response and thus fight, flight and fright behaviour. These behaviours are disturbed in BPD, which results in symptoms like aggression and anxiety (Goodwin, 2007). In other disorders, like major depression and anorexia nervosa, a deregulated HPA-axis activity is implicated as a cause for these disorders (Gold & Chrousos, 2002). A role for the hypothalamus in BPD is confirmed by several clinical and fMRI studies (Hendrick et al, 1998; Fagiolini et al, 2008; Malhi et al, 2004), which show increased activation of the hypothalamus in depressed BPD patients during negative affect generation, compared to healthy controls (Malhi et al, 2004). The fact that the Ca^{2+}-CaM modulated AC's are the only AC's expressed in the hypothalamus makes them unique in processes regulating certain aspects of BPD and therefore possible specific treatment targets with less side effects. However, the hypothalamus has such a diverse function that it is hard to determine the exact processes involved, which probably reduces the specificity of AC1 and AC8 in the hypothalamus as treatment targets. Still, it's worth it to do further research in order to reveal exact AC1 and AC8 functions in this interesting brain structure.

A first step in studying the exact function of AC8 in the HPA axis is made by Brewer et al (2003). With recombination techniques in mice, the role of AC8 in modulation of the HPA-axis and in the behavioural stress response is studied. Corticotropin releasing hormone (CRH), released by the hypothalamus and inducing glucocorticoid synthesis via the pituitary gland and adrenal gland, is of major importance in realizing the adrenocortical response on physiological and psychological stressors. Interestingly, CRH deficient mice show impaired adrenocortical stress responses, but normal behavioural responses (Brewer et al, 2003). Since these behavioural stress responses are CRH-receptor dependent, these results suggest there is a CRH-

like peptide or other molecular pathway linked to CRH-receptors that can bring about a behavioural stress response. In contrast to CRH, AC8 deficient mice have a normal adrenocortical stress response but impaired behavioural responses (Brewer et al, 2003). This indicates a crucial role for AC8 in establishing behavioural stress responses. This crucial role for AC8 in the HPA axis (Brewer et al, 2003), and the fact that the HPA axis is thought to play a central role in BPD (Goodwin, 2007) points at AC8 as a crucial factor in BPD pathology. This is another indication for AC8 as a potential target for therapeutic treatment of BPD. Finally, AC8 and not AC1 is expressed during the onset of BPD (Nicol, 2005), further

Summary

In summary, there are strong indications for a role for AC1 as well as for AC8 in the pathology of BPD. Both are important in the cerebellum, a brain structure involved in affect and emotion and degenerated in BPD patients. Another shared role for AC1 and AC8 lies in the HPA-axis, pointing at a role in generating stress responses. This is already proven for AC8, which is shown to be crucial for generation of behavioural responses on stress. Furthermore, AC8 is highly expressed during onset of BPD, and it is crucial for learning and memory processes, which are often disturbed in BPD patients. On the other hand, AC1 is shown to be crucial in the melatonin system and thereby in generating sleep disorders, also a symptom of BPD. Taken together, both AC1 and AC8 seem to be involved in BPD and both are pointed at as potential treatment targets.

5. AC and BPD's leading actor: Anxiety

AC and Anxiety

The important and maybe causal relationship between BPD and anxiety (as discussed in Chapter 1, this thesis), makes it worth looking for a role for AC1 and AC8 in anxiety disorders. Patients with an anxiety disorder, as well as BPD patients with anxiety symptoms, suffer from exaggerated and irrational worry in a way that severely diminishes normal life (Goodwin, 2007). Rosen and Schulkin (1998) postulate that this pathological anxiety is caused by hyperexcitability and sensitization of neural circuitries related to fear and anxiety. Normal stimuli would bring about an increased neuronal response and result in pathological anxiety. In their comprehensive review, they suggest a postsynaptic and intracellular signaling cascade which may underlie sensitization, and thus pathological anxiety. Via transcription factors like CREB, the activity of Ca^{2+}, DAG and cAMP would increase expression of several neuropeptides, growth factors and other peptides that modulate cell-excitability. According to Abel et al (1998) sensitization involves increased levels of second messengers and activation and suppression of gene transcription. Although not very exhaustive yet, it is a first indication for the cAMP pathway and AC to be involved in sensitization and thus pathological anxiety.

More sound evidence for AC as actor in anxiety and anxiety disorders comes from studies on the serotonin receptor subtype 5HT1a. This receptor is shown to be involved in pathology of anxiety and depression: anxiolytic and anti-depressant drugs work via this receptor (Griebel et al, 2000; Lesch et al, 2003); in patients with panic disorder or depression the responsivity of 5HT1a receptors is reduced (Lesch et al, 2003) and there is a decrease in 5HT1a ligand binding in the brain of depressed patients (Cheetham et al, 1999; Lesch et al, 2003). Furthermore, when 5HT1a receptors are suppressed or absent, for instance in knockout mice, anxiety related behaviour increases (Lesch et al, 2003). Interestingly, AC is an important effector of 5HT1a receptors: via G-proteins, the 5HT1a receptor inhibits AC. Taken together, these findings support a role for AC in generation of anxiety disorders. However, which AC subtype still has to be identified.

Studies on the working mechanisms of anti-depressant and anxiolytic drugs revealed regional specific differences in 5HT1a receptor sensitivity (Hensler, 2003). While the hippocampus and cortex are very sensitive to 5HT1a agonists, the amygdala and hypothalamus are less sensitive to these anxiolytic drugs (Hensler, 2003). It is thought that this region specific sensitivity is partly caused by changes in the level of AC, but exact mechanisms are still unclear (Hensler, 2003). Further research has to establish if AC is the determining factor for this region dependent sensitivity, and thus if it is a possible target for treatment of anxiety. This is also supported by Savitz et al (2009), who suggest that the expression of AC and its effector cAMP-dependent PKA are indirect markers for 5HT1a receptor functioning. Reduced levels of AC in the temporal lobe and occipital cortices in the brain of depressed suicidal patients support this hypothesis (Reiach et al, 1999; Cowburn et al, 1994; Savitz et al, 2009), as well as reduced levels of AC in the prefrontal cortex and nucleus accumbens (Dwivedi et al, 2004; Savitz et al, 2009). Taken together, the 5HT1a receptor marking function of AC, the AC-level dependent region specific sensitivity of 5HT1a agonists and the effect of 5HT1a receptors on anxious behaviour, point at AC as an interesting target for treatment of pathological anxiety.

Anxiety and high potentials: AC1 and AC8

More detailed information about anxiety ad AC subtypes comes from Schaefer et al (2000), who confirmed a role for AC in generating pathological anxiety. They showed a modulating function in anxiety of AC8. AC8 knockout mice lacked stress dependent learning, consequently resulting in decreased chronic stress responses. Their results suggest that AC8 plays a crucial role in translating increased intracellular calcium levels in to PKA- or CREB-mediated changes in gene expression, in turn affecting LTD (Long Term Depression, results in a decreased response on normal stimuli) in the hippocampus. By modulating LTD, stress dependent learning is affected and consequently, also chronic stress responses are affected. More recent studies confirm the role of AC8 in learning and memory formation in the hippocampus (Zhang et al, 2008). Modulating AC8 in the hippocampus could thus indirectly affect stress responses and thereby result in pathological anxiety. Furthermore, BPD patients suffer from impaired memory (Goodwin, 2007) which points at a role for AC8 as well. Sticking with memory formation, a study in the anterior cingulated cortex of the brain showed an unclear role of AC8, but a crucial role for AC1 in LTP (Long Term Potentiation, results in an increased response to normal stimuli) (Liauw et al, 2005). AC1 is crucial for enhancement of synaptic responses in the anterior cingulated cortex, and thus for LTP and long term memory formation. Taken together, recent studies implicate a distinct role for AC1 and AC8 in memory formation and generating anxiety. While AC8 is concerned with episodic memory and stress depended learning (Schaefer et al, 2000; Zhang et al, 2008), AC1 is important for long term memory (Liauw et al, 2005). This distinction points at AC1 and AC8 as possible specific treatment targets for pathological anxiety and memory problems, which both are endophenotypes of BPD.

Another brain area involved in anxiety is the amygdala. It plays a central role in generating fear and anxiety by processing inputs from other brain areas, like the thalamus, cortex and hippocampus, and elicits outputs resulting in hormonal, autonomic and behavioural anxious responses (Rosen & Schulkin, 1998; Phelps & leDoux, 2005; Wu et al, 2008). Furthermore, the amygdala is connected to, among others, the striatum, hypothalamus and brain stem (Wu et al, 2008). There are complex interactions between these brain areas covering other aspects of anxiety, like memory formation, and it is even suggested that the amygdala has a function in emotional influences on attention, perception and social behaviour (Phelps & leDoux, 2005; Wu et al, 2008); all aspects that are deregulated in BPD. Still, the central role of the amygdala in anxiety is clear (Wu et al, 2008). Like discussed for the hippocampus, stress related memory is important for a correct stress response. So is the case for fear responses. Fear memory consolidation in the amygdala requires BDNF signaling (Ou et al, 2007). The expression of BDNF is regulated by calcium stimulated PKA and CaMK resulting in CREB phosphorylation, suggesting a role for calcium depended AC's. Furthermore, increased AC concentrations in the amygdala reduce BDNF concentrations (Ou et al, 2007). Taken together, these results suggest a role for AC1 and/or AC8 in fear memory consolidation. Deregulation of this process can disturb fear memory and consequently result in pathological fear and anxiety. Therefore, AC1 and/or AC8 and the amygdala are good targets for anxiety and BPD treatment.

More supporting evidence for the amygdala and the calcium depended AC's as important actors in pathological anxiety comes from Tzavara et al (2002). Their study on nicotine withdrawal showed that region-selective upregulation of calcium depended AC in the amygdala induces anxious behaviour, showed by rats in an open field test. This indicates a role for AC1 and/or AC8 in generating anxious disorders, of both disorders related to drug abuse as

well as to 'normal' disorders. This relation of calcium depended AC's with drug dependence was already shown for AC8 in 1994 (Matsuoka et al): AC8 increased selectively in the amygdala after morphine treatment. BPD and drug abuse often go together (Goodwin, 2007), so these results point at the amygdala as an important location for specific interference with AC8 and perhaps AC1 in order to treat anxious and depressive symptoms of BPD.

More recent studies support an important role for AC8 in pathological anxiety. According to Wu et al (2008), a variety of molecular targets, including membrane receptors, intracellular signaling proteins and transcription factors, form a balance to generate normal anxiety. Disturbance of this balance would result in pathological anxiety. As discussed earlier (Chapter 4, page 25-26), AC8 is expressed in brain areas involved in generating stress responses. This suggests a role for AC8 in generating these responses and consequently, AC8 can be a good actor for generating or treating abnormal stress responses. This hypothesis is supported by Wu et al (2008), who suggest that this role of AC8 in stress-induced anxiety is due to its role in CREB activation, gene expression and LTD. This is consistent with findings in this thesis (this Chapter, page 29). Empirical evidence for this hypothesis comes from Schaefer et al (2000), already discussed above, and de Mooij-Malsen et al (2009). The latter gave very strong evidence for a crucial role of AC8 in anxiety related behaviour. Using behavioural data of different mouse strains, they identified a chromosome involved in avoidance behaviour: a behavioural marker for anxiety and an endophenotype of BPD. Comparing this genetic information with information from human BPD patients revealed the gene ADCY8, coding for AC8, as involved in avoidance behaviour. Furthermore, mouse strains differing in amount of expression of avoidance behaviour showed region specific differences in expression of AC8 in the brain: a positive correlation of amount of avoidance behaviour and AC8-expression in the ventromedial hypothalamus and piriform cortex. In addition, avoidance behaviour decreased after administration of carbamezepine: a human mood stabilizer working via adenylyl cyclase. Taken together, these results strongly point at AC8 as a crucial actor in generation of anxious behaviour. Identification of the ADCY8-gene might give a good target for specific treatment of anxiety, and thus of an endophenotype of BPD.

Summary

Anxiety and BPD are closely linked to each other. Looking at the molecular pathways involved in pathological anxiety leads to the same actors as in BPD: AC1 and AC8. Both are involved in stress depended learning, fear-memory consolidation, and anxious behaviour related to drug abuse. Stronger evidence is available for AC8, which is shown to be crucial in generating avoidance behaviour. Taken together, AC8 seems to be the most potential AC as target for treatment of pathological anxiety and, consequently, for treatment of BPD.

Conclusions

AC's and BPD

The aim of this thesis was to reveal if AC is a potential treatment target for BPD. Making a study of the behavioural characteristics of BPD and the involvement of AC in these behaviours supported this hypothesis. AC is an important enzyme in the generation of defensive behaviour (Glanzman, 1989; Griebel, 1996; Doernberg, 2001), sexual behaviour (Glanzman, 1989; Kow, 1994; Weissburg, 2001; Uphouse, 2001; Frye & Petralia 2003, 2006), and locomotion (Glanzman, 1989; Flinn, 2001). Also in learning and memory processes (Guillou, 1989), feeding and drinking behaviour (Glanzman, 1989; Castro et al, 2000) and olfactory sensitivity (Martin, 2001; Weissburg, 2001), AC plays an important role. These behaviours and processes all are linked to or representative for symptoms of BPD: violent and assaultive behaviour, change in sexuality, change in activity, disturbed memory capacities, change in appetite and olfactory psychosis. Taken together, these results give a first indication for a role for AC in BPD pathology.

A more detailed look at the molecular processes around AC revealed different mechanisms and functions for each AC subtype. Linking these functions to BPD indicates the potential of the concerning AC subtype as treatment target for BPD (Table 4). This approach revealed that there are no indications that AC4 and AC7 are linked to BPD, and also AC2 is not likely to play an important role in this disorder. In contrast, some results point at AC6 as involved in BPD. AC6 is involved in neural sensitization and the dopamine system. Inhibition of AC6 by $G\alpha_i$ can lead to sensitization and consequently result in disturbed neural responses (Sunahara & Taussig, 2002). In addition, this sensitization process occurs also for the dopamine receptors DA1 and DA4 (Hains & Arnsten, 2008). Since the function of dopamine is concerned with emotion, AC6 might play a role in BPD via the dopamine receptors. However, AC1, AC3, AC5 and AC8 also have this function and above that, other characteristics related to BPD.

AC1, AC3, AC5 and AC8 have in common that they are sensitive for Ca^{2+} (Sunahara et al, 1996; Sunahara & Taussig, 2002; Xia & Sturm, 1997; Conti et al, 2007). AC1 and AC8 are also sensitive for CaM and CaMK (Sunahara et al, 1996; Sunahara & Taussig, 2002; Xia & Sturm, 1997; Conti et al, 2007). The Ca^{2+} signaling machinery plays an important role in neural processes, standing at the origin of generating behaviour and emotion. Therefore, Ca^{2+} and the Ca^{2+}-sensitive AC's are an interesting factor in BPD pathology. In addition, AC3 is the only AC expressed in the olfactory system. A symptom of BPD is olfactory psychosis, which suggests a role for AC3 in BPD. However, more evidence is found for the other AC's. AC1, AC5 and AC8 play a crucial role in neural growth via Ca^{2+} and BDNF (Kato, 2008; Krishnan, 2008a). Degeneration of mood stabilizing neurons is likely to cause BPD (Kato, 2008; Krishnan, 2008a), suggesting AC1, AC5 and AC8 as important factors in BPD pathology and potential targets for BPD treatment. A role for AC1, AC5 and AC8 in BPD is confirmed with behavioural studies in knockout mice, showing that AC5 induces depressive-like behaviour and that AC1 and AC8 induce anti-depressive- or manic-like behaviour (Krishnan, 2008b). Taken together, AC1, AC5 and AC8 are indicated a potential treatment targets for BPD.

A more detailed look at the role of AC1, AC5 and AC8 in BPD revealed even stronger evidence for AC1 and AC8 as important actors in BPD. First, both AC's are expressed in the cerebellum (Cali et al, 1994; Nicol et al, 2005; Conti et al, 2007). This brain structure is an

AC	Relation with BPD	Reference
AC1	Disturbed neural response via $G\alpha_i$ and sensitization Disturbed emotion via DA1 and DA4 dopamine receptors Expressed in brain Involved in neural processes via Ca^{2+}, CaM and CaMK Involved in neural growth and loss via Ca^{2+} and BDNF Induces anti-depressive or manic-like behaviour Involved in affect and emotion via expression in cerebellum Involved in stress-response via HPA axis in hypothalamus Causing sleep disorders via melatonin secretion	11 3 10, 11 2, 10, 11, 15 4, 5 6, 8 1, 2, 9 1, 7 12
AC2	Expressed in brain	10, 11
AC3	Expressed in brain Involved in neural processes via Ca^{2+} Only AC in olfactory system (possibly involved in olfactory psychosis)	10, 11 2, 10, 11, 15 11
AC4	-	
AC5	Disturbed neural response via $G\alpha_i$ and sensitization Disturbed emotion via DA1 and DA4 dopamine receptors Expressed in brain Involved in neural processes via Ca^{2+} Involved in neural growth and loss via Ca^{2+} and BDNF Induces depressive-like behaviour	11 3 10, 11 2, 10, 11, 15 4, 5 8, 6
AC6	Disturbed neural response via $G\alpha_i$ and sensitization Disturbed emotion via DA1 and DA4 dopamine receptors	11 3
AC7	-	
AC8	Disturbed neural response via $G\alpha_i$ and sensitization Disturbed emotion via DA1 and DA4 dopamine receptors Expressed in brain Involved in neural processes via Ca^{2+}, CaM and CaMK Involved in neural growth and loss via Ca^{2+} and BDNF Induces anti-depressive or manic-like behaviour Involved in affect and emotion via expression in cerebellum Involved in stress-response via HPA axis in hypothalamus Crucial for behavioural stress response Crucial for LTP and learning and memory Expressed during BPD onset	11 3 10, 11 2, 10, 11, 15 4, 5 8, 6 1, 2, 9 1, 7 16 13, 14, 15 9

Table 4. AC subtypes and their relation to BPD Overview of the findings indicating a relation between AC subtypes and BPD.
(References: 1. Cali et al, 1994; 2. Conti et al, 2007; 3. Hains & Arnsten, 2008; 4. Kato, 2008; 5. Krishnan et al, 2008a; 6. Krishnan et al, 2008b; 7. Malhi, 2004; 8. Matsuoka et al, 1997; 9. Nicol et al, 2005; 10. Sunahara et al, 1996; 11. Sunahara & Taussig, 2000; 12. Tzavara et al, 1996; 13. Wang et al, 2003; 14. Wong et al, 1999; 15. Xia & Storm, 1997; 16. Brewer et al, 2003)

essential node in the neural circuitry regulating affect and emotion and shown to play a role in BPD pathology (Schmamann, 2000). The expression of AC1 and AC8 in the cerebellum therefore point at AC1 and AC8 as important factors in BPD. Second, AC1 and AC8 are both expressed in the hypothalamus, the brain structure crucial in the HPA-axis and thus in stress responses (Cali et al, 1994; Malhi et al, 2004). Increased activity in the hypothalamus of BPD patients during negative affect generation also demonstrates the important role of the hypothalamus in BPD (Malhi et al, 2004). Since AC1 and AC8 are the only AC's expressed in the hypothalamus, they are potential treatment targets for BPD. Another plus for AC1 as potential treatment target is found in the pineal gland. This small melatonin secreting gland regulates circadian rhythms and sleep. AC1 expression and AC1 activity in the pineal gland show a rhythmic pattern, suggesting a crucial role for AC1 in generating circadian rhythms and sleep patterns (Tzavara et al, 1996; Xia & Storm, 1997). Disturbed sleep patterns are a symptom of BPD, so the crucial role of AC1 in circadian rhythms make it a specific potential treatment target for disturbed sleep symptoms of BPD. Taken together, AC1 and AC8 are strongly pointed at as actors in BPD pathology and as potential treatment targets.

Although AC1 and AC8 both are potential treatment targets for BPD, there is even more support for AC8. As discussed above, the HPA-axis plays a role in generating stress responses and in BPD. AC8 is shown to be crucial for generation of a behavioural stress response (Brewer et al, 2003), which makes it a very important and specific actor in behavioural responses and thus possibly in behavioural disorders like BPD. Furthermore, AC8 is essential for LTP, a neural state necessary for learning and memory processes. Since BPD patients suffer from disturbed memory formation and decreased cognitive abilities, this is another indication for AC8 as actor in BPD. Finally, AC8 and not AC1 is expressed during the onset of BPD (Nicol, 2005), further underpinning the role of AC8 in BPD pathology. Taken together, AC8 is the most potential AC as target for BPD treatment. Because of the specific and crucial functions of AC8 in several processes related to BPD, it is expected that it is possible to design a symptom-specific treatment. A critical note that has to be made is that the diversity of G-proteins and AC subtypes, and the cross-talk between them, might reduce the apparent specificity of an AC subtype as treatment target. Further research has to establish the interference between the different subtypes of G-proteins and AC's, and what this means for using them as a treatment target for BPD.

Anxiety

BPD is a complex disorder with different and contrasting symptoms. By studying these symptoms, or endophenotypes, separately, more specific knowledge can be earned about BPD pathology and treatment. One of these endophenotypes is anxiety. This thesis showed that anxiety plays a major role in BPD. First of all, anxiety amplifies symptoms of BPD: it results in social withdrawal (Overstreet, 2002), a symptom of BPD occurring in all BPD patients (Goodwin, 2007). Also aggression, sleep disorders and irritability are caused by anxiety (Takeuchi, 2001; Riva et al, 2008; Adamic & Shallow, 1993; Navarro & Davila, 1998; Papadimitriou, 2005; Forbes et al, 2008; Leibenluft et al, 2003; Masi et al, 2004; Goodwin, 2007; Hoggs et al, 2000; Kikusui, 2004). Furthermore, a more severe course of BPD is found in BPD patients with anxiety symptoms (Maser & Cloninger, 1990) and in patients suffering from both BPD and anxiety, the onset of anxiety predates the onset of BPD. In conclusion, anxiety is likely

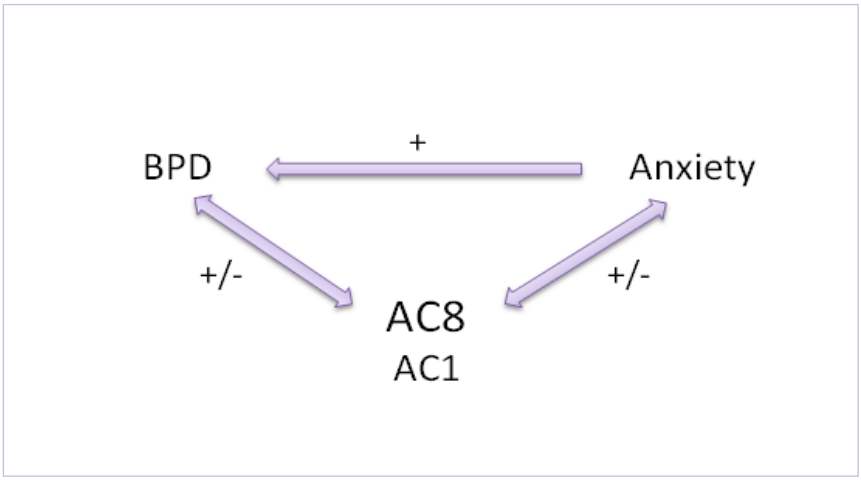

Figure 3. Relationship between BPD, Anxiety and potential treatment targets AC1 and AC8
Treatment of anxiety hypothetically reduces BPD symptoms, while AC1 and AC8 are potential treatment targets for both BPD and anxiety.

to play a causal role in BPD. This shines a new light on treatment of BPD, since pathological anxiety is very treatable with psychotherapy (Schneider, 2006). Based on the findings in this thesis, treating anxiety in BPD patients is now expected to have a positive effect on symptoms of BPD. Future studies have to reveal if this hypothesis is correct.

Taking a look at the molecular background of anxiety and anxiety disorders revealed similar potential treatment targets as for BPD: AC1 and AC8. General support for involvement of the AC- and cAMP pathway is demonstrated by its role in hyperexcitability and sensitization (Rosen & Schulkin, 1998). Via increased neural responses on normal stimuli, hyperexcitability and sensitization in turn are thought to cause pathological anxiety (Rosen & Schulkin, 1998; Abel et al, 1998). Furthermore, AC is inhibited by the 5HT1a serotonin receptor, which is involved in causing pathological anxiety (Cheetham et al, 1999; Griebel et al, 2000; Lesch et al, 2003). Also an AC-dependent, region-specific sensitivity of 5HT1a receptors suggest a role for AC in pathological anxiety (Hensler et al, 2003; Savitz et al, 2009). Which AC subtype is involved in this process still has to be identified, but other studies point at specific subtypes AC1 and AC8 as actors in anxiety.

Also in pathological anxiety, AC1 and especially AC8 are important factors. AC1 and AC8 are involved in learning and memory processes related to anxiety: AC8 is crucial for episodic memory and stress-dependent learning (Schaefer et al, 2000; Zhang et al, 2008), AC1 for long term memory (Liauw et al, 2005). Furthermore, AC1 and AC8 play a role in fear memory consolidation in the amygdala, which is necessary for normal fear responses (Ou et al, 2007). In addition, region-specific upregulation of AC1 and AC8 in the amygdala induces anxious behaviour (Tzavara et al, 2002), supporting the role of AC1 and AC8 in generating pathological anxiety. However, more evidence is found for a role for AC8. A comprehensive study with knockout mice and data of BPD patients showed that AC8 is crucial in generating anxious

behaviour (de Mooij-Malsen et al, 2009). Taken together, both AC1 and AC8 are demonstrated to play an important role in pathological anxiety, but results strongly point at AC8.

Anxiety and BPD are closely related, and studying the molecular underlyings of both disorders revealed the same actors for anxiety and BPD: AC1 and AC8, with AC8 having the most sound evidence. This thesis demonstrates the amplifying relation between anxiety and BPD, and the high potential of AC8 as treatment target for both anxiety and BPD (Fig. 3). Taken together, treating BPD via anxiety and AC8 is a potential new successful specific treatment of BPD, which has to be tested further empirically.

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