HUMAN GNAL, C18ORF2, AND MPPE1 GENES

Genomic organization of the human GNAL gene and characterization of two novel genes, C18orf2 and MPPE1, on chromosome 18p11.2, a susceptibility region for schizophrenia and bipolar disorder

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Abstract

The genomic organization and mRNA expression of the human GNAL gene on chromosome 18p11.2, a region that has been associated with bipolar disorder and schizophrenia, was determined. The GNAL gene was shown to span over 80 kb and consist of twelve exons, and its structure was very similar to adenyl cyclase stimulating G protein GSα. The start site of transcription was revealed by 5'-RACE. Two polyadenylation signals were found, and 3'-RACE assay was used to verify the functional site. The GNAL gene was expressed as approximately 6 kb transcripts in various regions of the human brain, and no alternative splicing was detected. One informative CA-dinucleotide repeat of 11 alleles and 74% heterozygosity was found in intron 5, and two single nucleotide polymorphisms in introns 3 and 10 were detected by SSCP.

During characterization of the GNAL gene, two previously unknown genes were found. A novel intronless gene C18orf2 coding for a functionally unknown protein was localized to intron 5 of the GNAL gene. By semiquatitative RT-PCR, C18orf2 mRNA was found to be moderately expressed in all tissues studied here. Another novel gene, metallophosphoesterase MPPE1, was found to reside adjacent to the 3'-end of the GNAL gene in a tail-to-tail orientation. The deduced amino acid sequence revealed a highly conserved metallophosphoesterase motif gDxH..(16-60)..GDxxdr..(13-34)..GNH[DE], which is typical for various phosphate hydrolyzing enzymes, especially serine/threonine protein phosphatases. The MPPE1 gene contained fourteen exons and spanned about 27 kb. MPPE1 was expressed as a single mRNA of 2.2 kb in various regions of the human brain but not in any other tissues. Four different alternatively spliced forms of MPPE1 were detected by RT-PCR, and each transcript was shown to partially overlap with the 3'-untranslated region of the GNAL gene.

Keywords: G-protein, metallophosphoesterase, psychiatric genetics

to the memory of Atte Juhani Vuoristo

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Abbreviations

bp	base pair(s)
cAMP	cyclic adenosine monophosphate
C18orf2	gene of unknown function on chromosome 18
CSGE	conformation sensitive gel electrophoresis
DRD	dopamine receptor
EST	expressed sequence tag
GABA	gamma aminobutyric acid
GDP	guanosine diphosphate
GNAL	G_{olf} gene
GNAS	G_S gene
G_{olf}	G olfactory protein
G protein	guanine nucleotide binding protein
G_S	stimulatory G protein
GTP	guanosine triphosphate
kb	kilobase(s)
MPPE1	metallophosphoesterase gene
mRNA	messenger RNA
nt	nucleotide(s)
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RT	reverse transcription
SSCP	single-stranded conformation polymorphism

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Berrettini WH, Vuoristo J, Ferraro TN, Buono RJ, Wildenauer D, Ala-Kokko L. (1998) Human G(olf) gene polymorphisms and vulnerability to bipolar disorder. Psychiatr Genet 8:235-238.

II Vuoristo JT, Berrettini WH, Overhauser J, Prockop DJ, Ferraro TN, Ala-Kokko L. (2000) Sequence and genomic organization of the human G-protein Golfα gene (*GNAL*) on chromosome 18p11, a suseptibility region for bipolar disorder and schizophrenia. Mol Psychiatry 5:495-501.

III Vuoristo JT, Berrettini WH, Ala-Kokko L. (2001) *C18orf2*, a novel, highly conserved intronless gene within intron 5 of the *GNAL* gene on chromosome 18p11. Cytogenet Cell Genet 93:19-22.

IV Vuoristo JT, Ala-Kokko L. (2001) cDNA cloning, genomic organization and expression of the novel human metallophosphoesterase gene *MPPE1* on chromosome 18p11.2. Cytogenet Cell Genet 95:60-63.

In addition, some unpublished data are presented.

Contents

1 Introduction

Bipolar disorder and schizophrenia are common and often debilitating familial psychiatric disorders with a lifetime prevalence of 1-2% and 1%, respectively. These deseases present a major public health problem throughout the world.

There is firm evidence from family, twin, and adoption studies of the importance of the risk genes involved in the etiology of bipolar disorder and schizophrenia. Although there may be a small number of families where major genes are involved, the mode of inheritance of both disorders is complex and poorly understood. In addition to environmental factors, currently available evidence suggests that a number of genes, each having a minor role, contribute to the vulnerability for bipolar disorder and schizophrenia.

A vast number of linkage and association studies have more or less conclusively defined candidate regions in practically every chromosome. One of the most attractive regions is a locus near the centromere on chromosome 18p11.2, which has been indicated by several linkage and association studies as a susceptibility region for schizophrenia and bipolar disorder. Within the locus resides a gene for adenylyl cyclase stimulating guanine nucleotide binding protein $G_{olf\alpha}$ (GNAL), a putative candidate gene for both illnesses. When the work on this thesis was initiated, there were also implications that other, yet unknown genes may be present in the immediate vicinity of the GNAL gene.

In order to facilitate mutation detection of the GNAL gene and further studies of the 18p11.2 region, it was necessary to define the genomic structure of the GNAL gene and carefully analyze the adjacent regions.

This work describes the genomic organization and mRNA expression of the GNAL gene, the characterization of the complete genomic structure and mRNA expression of two previously uncharacterized genes, functionally unknown C18orf2 and metallophosphoesterase MPPE1, and the identification of several polymorphisms within the gene cluster.

2 Review of the literature

2.1 Schizophrenia

Schizophrenia is a severely debilitating psychiatric disorder that affects about 1% of the world's population with similar prevalence throughout different geographic areas (Thaker & Carpenter 2001). The illness is characterized by a variety of symptoms including hallucinations, delusions, severely inappropriate emotional responses, disordered thinking and concentration, erratic behavior, and social and occupational deterioration. The typical age of onset of schizophrenia is at early adulthood (Andreasen 1995).

Schizophrenia has been an evolving clinical entity since the term "Demence precoce" was first coined by the French psychiatrist Benedict Augustin Morel in 1857. In his approach to classify mental disorders, influential German psychiatrist Emil Kraepelin described two major classes of psychosis: manic-depressive insanity and dementia praecox, and divided the latter one into hebephrenic, catatonic, and paranoid subtypes (Kraepelin 1899). Hebephrenic or disorganized dementia praecox is characterized by disorganized speech, disorganized behavior, and flat or inappropriate emotions. The catatonic type of dementia praecox is dominated by physical symptoms such as immobility, excessive motor activity, or the assumption of bizarre postures. Typical symptoms of the paranoid form of the illness are a preoccupation with delusions or auditory hallucinations, while disorganized speech and inappropriate emotions are less prominent.

In 1911 Swiss psychiatrist Paul Eugen Bleuler emphasized that the illness known by the name dementia praecox was not an actual dementia and did not always begin at early age, and renamed the disorder to schizophrenia (Bleuler 1911). Later on, schizophrenia was further divided into two additional subtypes, residual and undifferentiated. The residual subtype is characterized by a lack of current active phase symptoms, but the definite experience of at least one schizophrenic episode in the past and the continued presence of milder forms of some of the symptoms, such as unusual behavior, odd beliefs, and marked eccentricity. In the undifferentiated subtype, disorganized behavior, hallucinations, or prominent delusions are common, but the diagnostic criteria for

hebephrenic, catatonic, or paranoid schizophrenia are not met. (American Psychiatric Association 1994).

2.1.1 Biological mechanism

The biological events leading to schizophrenia are still largely unknown. Several mechanisms involving acetylcholine, dopamine, glutamate, and their corresponding receptors and factors that are linked to subsequent events have been proposed to explain the biochemical phenomena behind the illness (for review, see Pearlson 2000). Acetylcholine is a neurotransmitter that binds to the same nicotine acetylcholine receptors (nAChRs) as nicotine. Observations of an extraordinarily high prevalence of smoking among schizophrenic patients, along with evidence that nicotine can improve some psychophysiological dysfunction in schizophrenics, have raised an interest towards acetylcholine and its role in the pathogenesis of schizophrenia (Stassen *et al.* 2000). However, probably partially due to negative results from association studies of nicotinic receptors with schizophrenia (Lai *et al.* 2001), the theory of an acetylcholinergic mechanism as a primary cause of schizophrenia has not gained wider acceptance. In the light of currently available evidence, the most appealing theories involve abnormalities in either dopaminergic (Coyle 1996) or glumamatergic pathways (Seeman 1987).

2.1.2 Dopaminergic hypothesis in schizophrenia

Dopamine acts as a neurotransmitter between dopaminergic neurons. In the central nervous system, there are several dopaminergic pathways. About 80% of dopamine is synthesized in pigmented cells in the substantia nigra, and dopamine synthesis from the amino acid tyrosine involves a number of enzymatic reactions. Key elements of the synthesis are tyrosine hydroxylase, which converts tyrosine to a dopamine precursor L-dopa, and aromatic amino acid carboxylase that, in turn, converts L-dopa to dopamine (Figure 1).

Dopaminergic neurons project from the substantia nigra to the striatum and other surrounding areas of the brain. Dopamine is released from synaptic vesicles into the neural junction where it binds to the postsynaptic dopamine receptors. Downstream effects can include the regulation of adenylyl cyclase or other signaling systems such as phospholipase C or the polyphosphoinositide cycle. Of the five known types of dopamine receptors, types 1 (DRD1) and 5 (DRD5) have stimulatory effects on adenylyl cyclase, whereas types 2 (DRD2), 3 (DRD3), and 4 (DRD4) either inhibit or have no effect on adenylyl cyclase activity. Reuptake of dopamine occurs and is achieved by the dopamine transporter. The recycled dopamine is incorporated into synaptic vesicles and either reused as a transmitter or degraded by monoamine oxidases A or B (MAO-A and MAO-B), but preferentially by MAO-B. (Missale *et al.* 1998).

Amphetamine has been shown to act via an interaction with the vesicular monoamine transporter 2 (VMAT2) causing a redistribution of monoamines, especially dopamine, back into the cytoplasm (Sulzer *et al.* 1995). Amphetamine has complex psychotropic

effects, which may be partially explained by its ability to also affect serotonin (5-HT) and noradrenaline transporters (Seiden *et al.* 1993, Kuczenski & Segal 1997). The notion that antipsychotic drugs potently block the psychostimulant actions of amphetamines in animals and humans (Randrup & Munkvad 1965) suggests that dopaminergic signal transduction may play a role in the development of schizophrenia. This hypothesis was supported by biochemical studies that revealed that neuroleptic drugs act as antagonists at DRD2 (Creese *et al.* 1975, Seeman *et al.* 1976). Further evidence was provided by positron emission tomography (PET) studies demonstrating an increased occupancy of DRD2 by endogenous dopamine in patients with schizophrenia (Abi-Dargham *et al.* 2000). DRD1 has a significantly lower affinity for neuroleptic drugs and has not gained much attention as a target for schizophrenia-related research. However, recent experiments demonstrated that DRD1 knockout mice had less ketamine-induced behavioral responses than wild-type mice, and that treatment of wild-type mice with selective DRD1 antagonist, SCH23390, inhibited these ketamine-induced behavioral changes (Miyamoto *et al.* 2001). Therefore, the role of stimulatory dopamine receptors DRD1 and DRD5 in the etiology of schizophrenia cannot be ruled out.

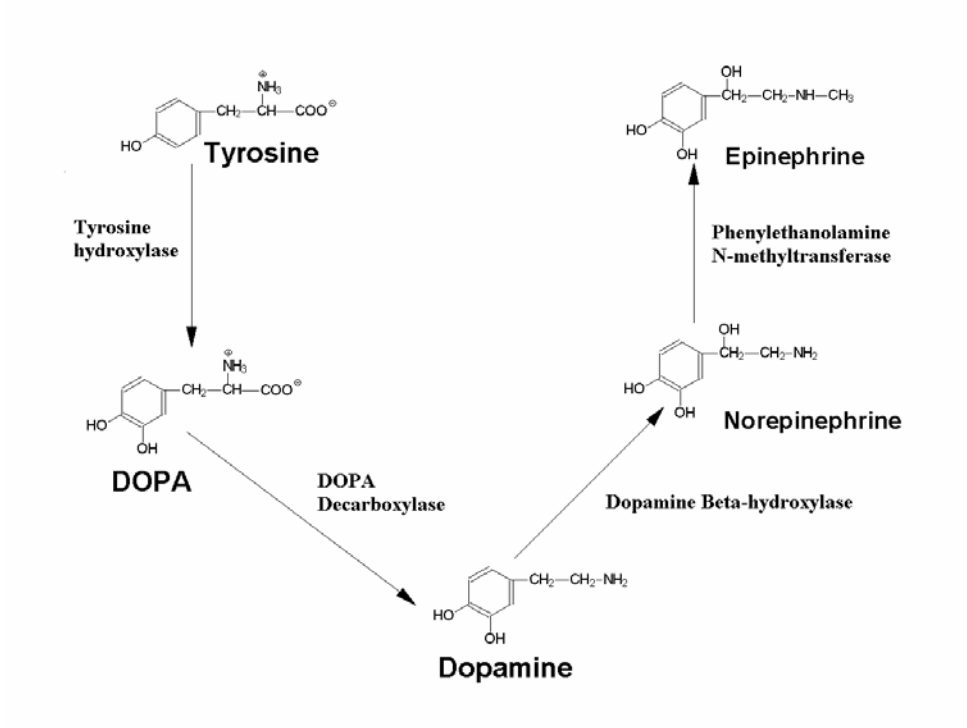

Fig. 1. Schematic presentation of synthesis of catecholamines from tyrosine.

2.1.3 Glutamatergic hypothesis in schizophrenia

By itself, the dopaminergic hypothesis cannot account for the observation that negative symptoms, such as flat emotions and decreased motivation, often do not improve with antipsychotics targeting the dopaminergic pathway. Also, despite a supposedly quickly responding dopaminergic system, several weeks of treatment with typical antipsychotic drugs are required for clinical response. (Meador-Woodruff & Healy 2000). Interestingly, amphetamine has been shown to actually diminish the negative symptoms such as flattening of emotions, alogia, anhedonia and attentional impairment in some patients with schizophrenia (Andreasen & Olsen 1982, Angrist *et al.* 1982, van Kammen & Boronow 1988).

Glutamate is a major excitatory neurotransmitter in the brain with up to 40% of all synapses being glutamatergic. Although glutamatergic neurons are distributed throughout the nervous system, they are prominently represented in the cerebral cortex and limbic regions of the brain (Cotman & Monaghan 1987). It has been proposed that glutamate has a significant role in the control of dopamine transmission in the striatum. Dopamine transmission occurs in two different temporal modes, phasic and tonic. Phasic release of dopamine is transient and rapidly terminated, and it selectively affects only the receptors that are within or near the synapse. Phasic transmission is primarily dependent on the activity of the dopaminergic neurons. Tonic release of dopamine results in a constant level of dopamine in the extracellular, extrasynaptic space and is regulated mainly by glutamate (Grace 1991, 1993).

Glutamate acts on four major classes of glutamate receptors: ionotrophic N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate receptors, which are glutamate-gated ion channels that cause neuronal depolarization; and a class of metabotropic receptors, whose intracellular actions are mediated by G-proteins. Glutamatergic neurotransmission has been implicated in schizophrenia largely as a result of the psychotomimetic effects of NMDA receptor antagonists (Krystal *et al*. 1999). Clinical investigations have shown that phencyclidine (PCP) or “angel dust”, which is an uncompetitive antagonist of the NMDA receptor, and the related drug ketamine can produce a syndrome quite similar to schizophrenia, including positive symptoms such as prominent delusions, hallucinations and persistently bizarre behavior as well as negative symptoms, cognitive impairment, and alterations in body image. In addition to producing these symptoms in normal individuals, PCP and ketamine can stimulate psychosis in schizophrenia (Andreasen & Olsen 1982, Lieberman *et al.* 1987, Krystal *et al.* 1994, Lahti *et al.* 1995).

2.2 Bipolar disorder

Bipolar disorder, or manic-depressive illness, is a common, recurrent, and severe psychiatric illness characterized by episodes of mania or depression with an estimated lifetime prevalence of 0.5 to 1.5%. Average onset of the disease is around the age of 21 years with similar rates in males and females throughout the world (Smith & Weissman 1992). Bipolar disorder is associated with a high level of morbidity, and it has been

estimated that approximately 15% of patients eventually die by suicide (Guze & Robins 1970).

Bipolar disorder was first categorized as an illness by Falret, who in 1851 described the disease “folie circulaire” (circular madness) defined by manic and melancholic episodes separated by symptom free intervals. In 1899 Kraepelin, in his approach to further classify mood disorders, developed the term “manic-depressive insanity”, which included a broad variety of mood disorders from single episodes of mania or depression to recurrent depression. However, Kraepelin himself later came to the conclusion that manic-depressive illness may, in fact, be more heterogeneous than previously thought (Kraepelin 1913). Despite that, and several publications in which the distinction between mania, depression, and bipolar disorder were maintained (Ballet 1903, Pilcz 1901, Ziehen 1902), Kraepelin’s original unification of affective disorders set the basis for classification for several decades, leading to the publication of a number of research reports in which the authors, as late as in 1968, failed to distinguish between depression, mania, and bipolar disorder (Bratfos & Haug 1968). Currently, bipolar disorder can be roughly divided into two main categories: Bipolar disorder type I (BPI) and Bipolar disorder type II (BPII) (American Psychiatric Association 1994). The most characteristic features of BPI are episodes of a severe form of mania interspersed with periods of depression. BPII is generally less debilitating, with hypomania replacing the manic episodes (Goodwin & Jamison 1990).

2.2.1 Biological mechanism

As is the case with schizophrenia, the neurobiology of bipolar disorder remains poorly understood. Neurochemical abnormalities involving serotonin, norepinephrine, second messenger pathways, and to a lesser extent dopamine, acetylcholine, gamma-amino butyric acid (GABA) have been implicated (for reviews, see Whybrow & Parange 1981, Diehl & Gershon 1992, Owens & Nerneroff 1994, Petty 1995, Ghaemi *et al.* 1999).

Lithium (Li^+), a monovalent cation that belongs to a group of alkali metals has been a common treatment of bipolar disorder for over 50 years and it has putative effects on all of the neurotransmitter systems mentioned above, yet the mechanism by which it exerts its therapeutic actions is unknown (for review, see Lenox & Hahn 2000). Administration of the antihypertensive drug reserpine, which depletes aminenergic neurotransmitters such as serotonin, norepinephrine, and dopamine, leads to an increased incidence of the affective disorder. Conversely, tricyclic antidepressants are thought to exert their effects by increasing norepinephrine and serotonin levels through competition with their respective transporters. The increase in neurotransmitters could act via the cyclic adenosine monophosphate (cAMP) and phosphoinositol second messenger signal cascade system (Vawter *et al.* 2000).

2.2.2 Serotonin and norepinephrine in bipolar disorder

Serotonin has an important role in mood and perception of pain, and in many basic activities such as feeding, motor activity, sexual behavior, sleep-wake cycle, and the regulation of temperature.

The synthesis of serotonin in neurons proceeds by biochemical steps that are analogous to those of the dopamine biosynthesis pathway. The starting material is tryptophan, which is hydroxylated by tryphophan hydroxylase to 5-hydroxytryptophan, which is then carboxylated by dopamine decarboxylase to serotonin (Figure 2). Like dopamine, serotonin is degraded by monoamine oxidases, preferentially MAO-A. Of the known 14 types of serotonin receptors, all but 5-HT3, which is a ligand-gated ion channel, are G-protein coupled receptors. (Parks *et al.* 1998).

In neurons that synthesize the neurotransmitter norepinephrine, dopamine is transported into vesicles in where dopamine beta-hydroxylase catalyzes the hydroxylation of the ethylamine side chain of dopamine to form norepinephrine (Figure 1.). The effects of serotonin and norepinephrine are transmitted via G-protein coupled receptors.

It has been postulated that serotonin and norepinephrine deficits occur in parallel (Hsiao *et al.* 1987). This theory was supported by the observation that when depressed patients were treated with a combination of the serotonin and norepineprin uptake blockers fluoxetine and desipramine, respectively, a more rapid antidepressant response was achieved than with desipramine treatment alone (Nelson *et al.* 1991). Furthermore, the administration of the norepinephrine precursor tyrosine potentiated the antidepressant effect of 5-hydroxytryptophan, the precursor of serotonin (van Praag 1983). Mongeau *et al.* (1997) proposed that antidepressant treatment works, at least in the hippocampus, by increasing and decreasing serotonin and norepinephrin neurotransmission, respectively. Lithium has been shown to increase serotonergic transmission, possibly by producing a subsensitivity of presynaptic inhibitory 5-HT1A receptors, and thus increasing the net release of serotonin per impulse (Hotta & Yamawaki 1988). The effect of lithium on signal transmission by norepinephrine is inconclusive. However, there is some evidence that lithium prevents neurotransmitter depletion induced supersensitivity of beta-adrenergic receptors (Hermoni *et al.* 1980).

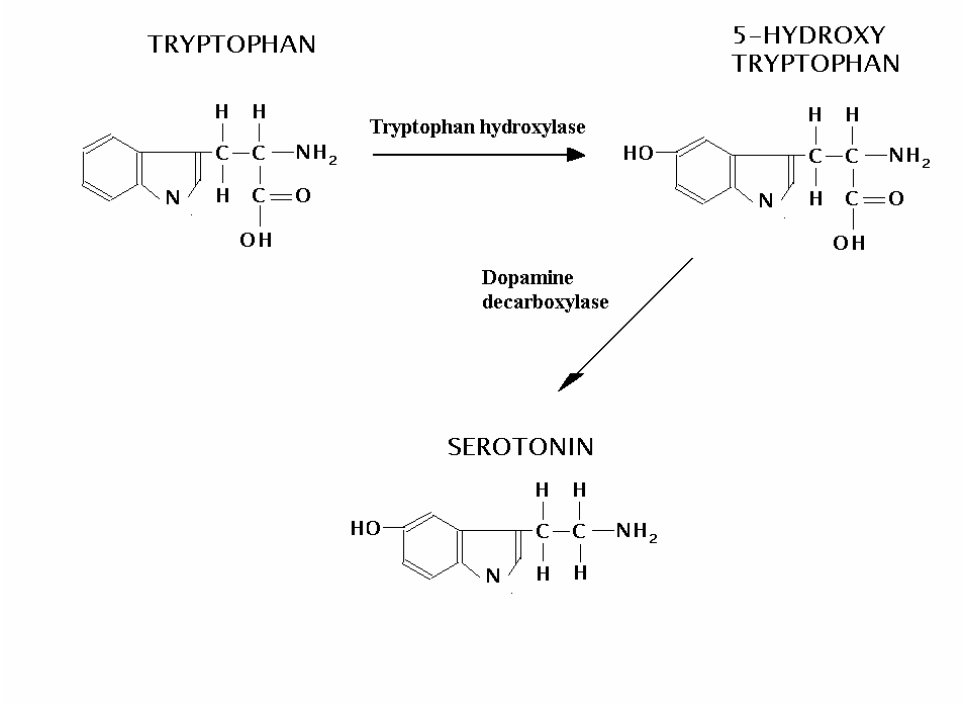

Fig. 2. Schematic presentation of serotonin synthesis.

2.2.3 Gamma-amino butyric acid

Gamma-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous system. It modulates the activity of several neurotransmitters including dopamine, serotonin, and norepinephrine. GABA is synthesized in a single step from its precursor glutamate by glutamic acid decarboxylase. GABA is metabolized by successive transamination and oxidation to yield succinic semialdehyde and succinic acid, respectively. As a part of the transamination reaction, a recycling system is formed in which α-ketoglutaric acid is converted to the GABA precursor glutamate by GABA-glutamic acid transaminase. (Figure 3).

GABA acts via binding to its receptors, which can be divided into two families, $GABA_A$ and $GABA_B$. The more abundant and extensively studied $GABA_A$ is a ligand-gated chloride channel receptor. Opening of the $GABA_A$ receptor leads to hyperpolarization by an increased chloride conductance, which then inhibits the firing of neurons (Macdonald & Olsen 1994). The less abundant and not very well known $GABA_B$ is a G-protein coupled receptor that causes inhibitory effects via increased K^+ conductance or decreased voltage dependent Ca^{2+} currents (Ong & Kerr 2000).

The decrease in glutamic acid decarboxylase activity and the reduction of GABA release and uptake has been reported in a number of studies of post mortem brain samples

from schizophrenic patients (Simpson *et al.* 1989, Reynolds *et al.* 1990, Sherman *et al.* 1991). Decreased levels of GABA have also been found in the brain, cerebrospinal fluid, and plasma of patients with bipolar disorder (Berrettini *et al.* 1983, Petty *et al.* 1993). Additionally, many antidepressants and antipsychotic drugs, including clozapine, as well as some of their metabolites, reverse the inhibitory effect of GABA (Squires & Saederup 1991).

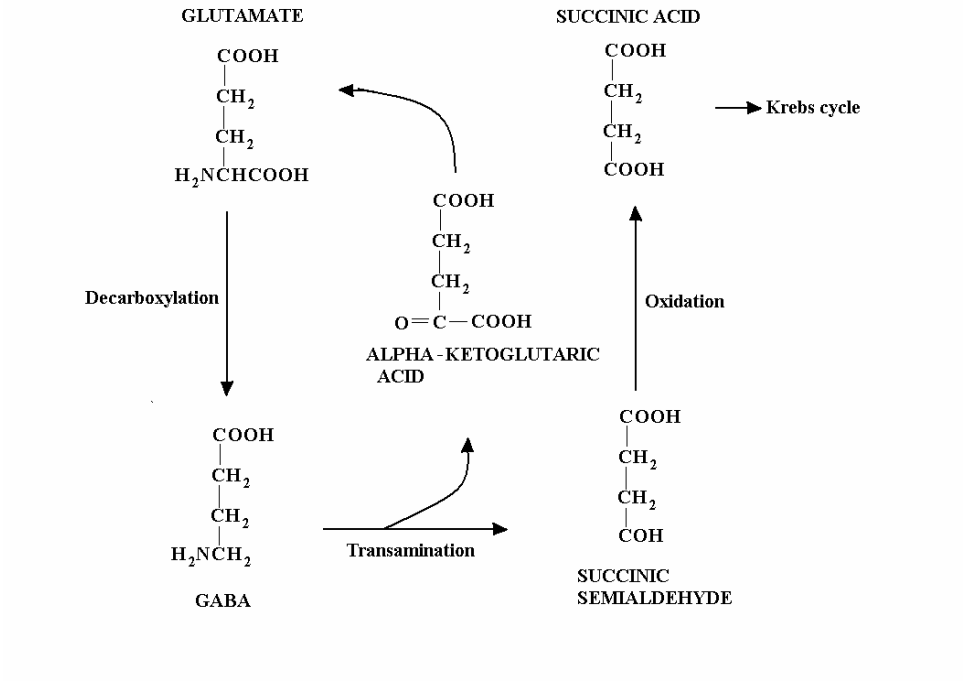

Fig. 3. Schematic presentation of GABA synthesis.

2.3 Other relevant biological mechanisms

2.3.1 G-proteins

G proteins are a large family of cytosolic proteins that bind guanine nucleotides and are coupled to receptors that have seven membrane-spanning domains, including receptors for dopamine, glutamate, norepinephrine, gamma-aminobutyric acid, and serotonin. They play a crucial role in the transduction of many extracellular signals (Gilman 1987,

Simon *et al.* 1991, Spiegel 1992, Neer 1995, Sprang 1997, Hamm 1998). The proteins are heterotrimeric and comprised of α-subunits of 39 to 52 kDa, β-subunits of 35 to 36 kDa, and γ-subunits of 8 to 10 kDa. Each of the G-proteins shows considerable amino acid sequence similarity. Signal transduction by the G-protein coupled receptors is initiated by ligand binding, which stabilizes an alternate conformational form of the receptor and thus transmits information across the cell membrane by activation of the heterotrimeric G-protein. Interaction of the G protein with the activated receptor promotes the exchange of GDP for GTP on the α-subunit. The GDP/GTP exchange dissociates the α-GTP complex from the βγ heterodimer. The free α- and βγ-subunits each activate target effectors such as ion channels and enzymes that generate regulatory molecules or second messengers. Second messengers such as adenosine 3',5'-cyclic monophosphate (cAMP), in turn, generate intracellular changes including protein phosphorylation, gene transcription, cytoskeleton reorganization, secretion, and membrane depolarization. Termination of the signal occurs when the intrinsic GTPase activity of the α-subunit hydrolyzes the bound GTP to GDP. The α-subunit then re-associates with the βγ-complex.

The involvement of G-proteins in many of the major signal transduction systems makes them very attractive candidates in search of mechanisms behind bipolar disorder and schizophrenia. Alterations in the levels of various G-proteins have been detected in patients with bipolar disorder (Schreiber *et al.* 1991, Young *et al.* 1991, Mitchell *et al.* 1997, Spleiss *et al.* 1998). In patients with schizophrenia, recent studies have found increased levels of stimulatory G-protein Gs (GNAS) and regulator of G-protein signaling 4 (RGS4) (Avissar *et al.* 2001, Mirnics *et al.* 2001). In addition, there are several reports of lithium induced alteration of G protein function (Avissar *et al.* 1988, Watanabe *et al.* 1990, Manji *et al.* 1995).

2.3.2 cAMP

cAMP is widely distributed throughout the nervous system and plays an important role in various signaling pathways. The cytosolic concentration of cAMP is determined by the balance of its synthetic enzymes, adenylyl cyclase, which converts ATP into cAMP and pyrophosphate, and cyclic nucleotide phosphodiesterase, which is responsible for the rapid hydrolysis of cAMP to 5'-AMP. Due to this rapid hydrolysis, the increase in cAMP concentration produced by adenylyl cyclase is usually very brief, lasting only seconds to minutes. The activities of adenylyl cyclase and cyclic nucleotide phosphodiesterase are regulated by G proteins and calcium concentration, respectively.

Lithium has been shown to inhibit adenylyl cyclase activity. This inhibitory effect was antagonized by Mg^{++}, suggesting that a possible mechanism behind this phenomenon is direct competition with Mg^{++} (Mork & Geisler 1989). However, in other experiments Mg^{++} did not antagonize the effect of lithium. Instead, the inhibition of adenylyl cyclase activity was reversed by increasing the concentration of GTP, implying that lithium may not target directly adenylyl cyclase but related G proteins (Newman & Belmaker 1987). The principal mediator of cAMP action in the central nervous system is cAMP-dependent protein kinase A (PKA). Some post-mortem studies have shown reduced cAMP binding

and increased PKA activity in the temporal cortex of patients with bipolar disorder (Rahman *et al.* 1997, Fields *et al.* 1999).

2.3.3 Phosphatidylinositol signaling

In the phosphatidylinositol signaling pathway, agonist stimulated activation of phospholipase C (PLC) leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Hydrophilic IP3 diffuses in the cytosol, where it releases calcium from internal stores. If there is a sufficient Ca^{2+} concentration, plasma membrane-bound hydrophobic DAG activates protein kinase C (PKC). PKC, in turn, has a variety of functions including the gating of ion channels and desensitization of receptors. DAG and IP3 are recycled to PIP2 via a complex pathway. First, DAG is phosphorylated to phosphatidic acid (PA), which then combines with cytidine triphosphate (CTP) to produce CTP-PA. Simultaneously, IP3 is sequentially dephosphorylated to inositol bisphosphate (IP2), inositol monophosphate (IP1), and inositol. Combined inositol and CTP-PA are then sequentially phosphorylated to phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI2), and PIP2.

Dephosphorylation of IP1 to inositol is catalyzed by inositol monophosphatase (IMPA), which is a putative target of lithium. Generally, lithium has little effect on healthy people, but it has been demonstrated to inhibit the activity of IMPA in conditions where neurotransmitter systems are hyperactive. Therefore, the components of the phosphatidylinositol signaling pathway should be considered as candidates for bipolar disorder. (For review, see Atack 1996).

2.3.4 Protein phosphorylation

Protein phosphorylation-dephosphorylation represents one of the preeminent molecular mechanisms for modulating the functional properties of proteins. It takes place in all living cells and has been employed for the regulation of practically every aspect of cellular existence, including a vast number of cellular signal transduction pathways. The protein phosphorylation-dephosphorylation process includes two thermodynamically favorable reactions; the water-driven hydrolysis of phosphoester, phosphoramide, or acylphosphate bonds by protein phosphatases, and a high-energy compound-driven phosphorylation by protein kinases (Figure 4). In contrast to more or less permanent covalent modifications of the proteins, this reversible phosphorylation-dephosphorylation reaction is a fast and economical way to change the properties of proteins in a desired manner in the cellular environment. (Westheimer 1987). In order to sustain reasonable target specificity among thousands of phosphoproteins, a network of hundreds, if not thousands, of protein kinases (Hanks & Hunter 1995) and protein phosphatases (Charbonneau & Tonks 1992) is required. (For review, see Kennelly 1999).

There are several reports of altered phosphorous metabolism such as changes in phosphomonoester and phosphodiester levels in the brains of patients with schizophrenia (for review, see Keshavan *et al.* 2000) and to a lesser extent, bipolar disorder (Deicken *et*

al. 1995). Due to these observations and the universal role of reversible protein phosphorylation in cellular signaling, protein phosphatases and kinases cannot be ruled out in search of candidate genes for schizophrenia and bipolar disorder.

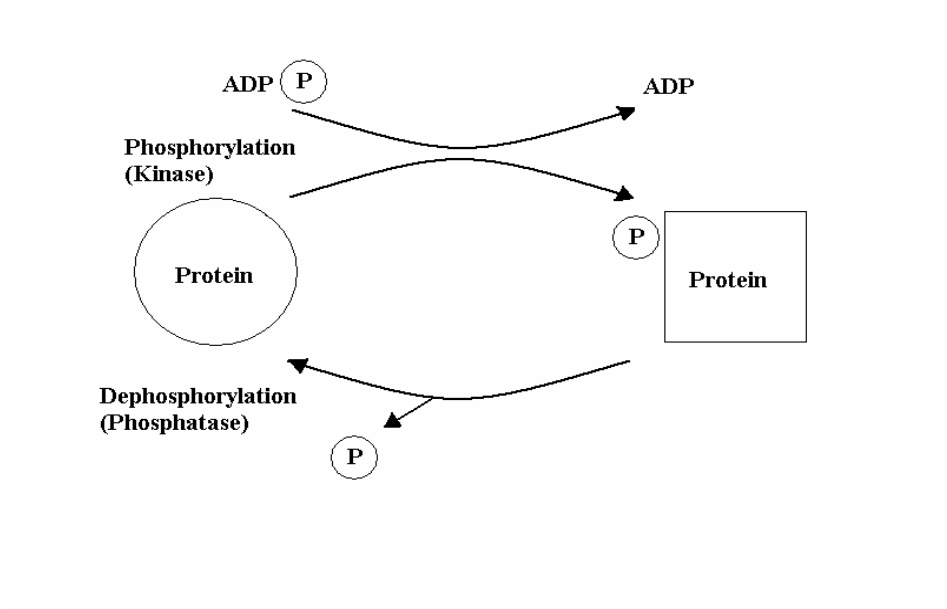

Fig. 4. Schematic presentation of reversible protein phosphorylation.

2.3.4.1 Protein kinases

Protein kinases are phosphoryltransferases that catalyze the transfer of a phosphoryl group from a high-energy compound, such as ATP, to a nucleophilic acceptor group located on the amino acid side-chain(s) of a protein. Possible acceptor sites are the hydroxyl groups of serine, threonine, and tyrosine, the carboxylate groups of aspartic and glutamic acids, and the nitrogen atoms on the side-chains of histidine, lysine, and arginine. Protein kinases are loosely classified according to the regulatory agent that activates the enzyme. For example, protein kinases that are activated by cAMP, by cGMP, or by calcium, are referred to as cAMP-dependent, cGMP-dependent, and calcium-dependent kinases, respectively.

Structurally, protein kinases can be divided into three superfamilies: histidine kinases, cAMP-dependent protein kinase like kinases (cAPK), and other protein kinases. (For review, see Kennelly 1999). The direct role of several protein kinases has been implicated in bipolar disorder or schizophrenia including the reduced activity of protein kinase C (PKC) and the increased activity of serine/threonine kinase Akt-1 by lithium, elevated levels of mitogen-activated protein kinases (MAPK) in the brains of schizophrenic

subjects, and the role of glycogen synthase kinase-3beta (GSK-3) in brain development (Chalecka-Franaszek & Chuang 1999, Hahn & Friedman 1999, Kyosseva *et al.* 1999, Kozlovsky *et al.* 2002).

2.3.4.2 Protein phosphatases

Protein phosphatases are phosphoesterases that catalyze the hydrolytic removal of a phosphate group from the hydroxylated amino acid residue of a protein. Protein phosphatases can be divided into four superfamilies: PP1/PP2A/PP2B, PP2C, His-Arg-Thiolate (HAT), and other protein phosphatases (Kennelly 1999). The PP1/PP2A/PP2B superfamily consists of heterodimeric serine/threonine phosphatases. Members of this superfamily differ in their metal ion dependence, sensitivity to inhibitors, and substrate specificity, but they share one of the highest degrees of amino acid homology among conserved enzyme families (Cohen & Cohen 1989). PP2C is another metal ion (Mg^{2+}) dependent protein phosphatase. Mammalian PP2C is a monomer and does not share any amino acid homology with other protein phosphatases. Therefore, it is considered to be in a separate superfamily (Bork *et al.* 1996). HAT protein phosphatases are a large heterogeneous group of tyrosine or dual specific phosphatases that dephosphorylate both seryl/threonyl and tyrosyl residues. HAT protein phosphatases do not share significant sequence homology other than their active site signature motif His-Cys-X_5-Arg. The fourth superfamily, other protein phosphatases, consists of low molecular weight acidic phosphotyrosine phosphatases that contain an active-site motif similar but not identical to that of HAT phosphatases. Thus far, there is no clear evidence for a role of any specific protein phosphatase in the etiology of bipolar disorder or schizophrenia.

2.4 Heritability

2.4.1 Schizophrenia

In 1899 Kraepelin described over 70% of dementia praecox cases as familial. On average, modern twin and adoption studies have revealed concordance rates of approximately 50% in monozygotic and 14% in dizygotic twins (Tsuang 2000). Even though biological and nonbiological components, such as intrauterine fetal hypoxia or malnutrition, viral infections, and psychosocial factors, such as dysfunctional family communication, may increase vulnerability to schizophrenia, it has been well established that a hereditary component has a significant role in schizophrenia (Tienari *et al.* 1994, Wahlberg *et al.* 1997, Jones & Cannon 1998, for review, see Tsuang 2000).

The mode of inheritance of schizophrenia is far from clear. Early studies concentrated on the idea of monogenic inheritance. Both recessive and more or less irregular dominant models were suggested (Book 1953, Kidd & Cavalli-Sforza 1973). However, typical Mendelian inheritance patterns are generally not observed in schizophrenia. The genetic

complexity of schizophrenia, including incomplete penetrance and low phenocopy rate, may be at least partially explained by the effects of a combination of multiple genes and nongenetic components (multifactorial inheritance model), or by the epistasis model in which a few genes acting jointly cause the illness (Risch 1990). Further complexity is added to the inheritance models of schizophrenia by observations of anticipation, a phenomenon in which successive generations within schizophrenic families seem to have an earlier age of onset of the illness (Bassett & Honer 1994).

Dynamic mutations involving the expansion of trinucleotide repeats have been shown to have deteriorating effects on DNA stability (Richards & Sutherland 1992). The expanded repeats are unstable between generations and somatically, giving rise to unusual patterns of inheritance, particularly anticipation. Trinucleotide expansions are associated with several diseases including fragile X syndrome, myotonic dystrophy, and a number of neurodegenerative disorders (Sutherland & Richards 1995). The role of unstable DNA in the etiology of schizophrenia has been suggested, and studies of unrelated patients with schizophrenia have shown a modest increase in the number of trinucleotide repeats relative to controls (O'Donovan *et al.* 1996). However, in a very extensive review, Vincent *et al.* (2000) conclude that unstable trinucleotides are unlikely candidates to have a major role in psychosis.

2.4.2 Bipolar disorder

Bipolar disorder is a highly heritable illness with concordance rates of 65% to 70 % and approximately 14% in mono- and dizygotic twins, respectively (Gurling 1995). The mode of inheritance of bipolar disorder is not clear. McMahon *et al.* (1995) found evidence of maternal transmission of the illness suggesting the effect of imprinted genes or mutations on mitochondrial DNA. Attempts to find mutations in the mitochondrial DNA of bipolar disorder patients have failed. However, by comparing polymorphisms in the mitochondrial DNA of bipolar patients and a control group, Kirk *et al.* (1999) were able to find suggestive evidence of selection against maternal lineages in bipolar disorder. Evidence of genomic imprinting on the transmission of bipolar disorder has been observed by several research groups (Stine *et al.* 1995, Collins & Go 1997, McMahon *et al.* 1997). However, other studies have yielded negative results regarding the parent-of-origin effect on the transmission of bipolar disorder (Kato *et al.* 1996, Murphy *et al.* 2001).

2.4.3 Overlapping diseases?

Even though the current diagnostic criteria clearly separates bipolar disorder from schizophrenia, Kraepelin already came to the conclusion that his original distinction of these disorders may have been too strict: "It is becoming increasingly clear that we cannot distinguish satisfactorily between these two illnesses, and this brings home the suspicion that our formulation of the problem may be incorrect" (Kraepelin 1920). Some epidemiologic features that seem to be generally shared are the rate of occurrence, age of

onset, degree of heritability, often episodic course of illness, and increased risk for suicide. There are neither reports of an increased risk for bipolar disorder among the first degree relatives of patients with schizophrenia nor of an increased risk for schizophrenia among first degree relatives of bipolar probands. However, there are several reports of an increased risk for recurrent unipolar disorder and schizoaffective disorder among the first degree relatives of probands with either schizophrenia or bipolar disorder (Gershon *et al.* 1988, Maier *et al.* 1993, Winokur *et al.* 1982, 1995). Recurrent unipolar disorder is characterized by recurring depression without intervening episodes of mania, and schizoaffective disorder typically shares features from both bipolar disorder and schizophrenia. Thus, functional psychoses may constitute a continuum of disorders ranging from schizophrenia to affective psychoses with an overlap of symptoms and genetic factors (Crow 1990, Squires & Soederup 1991, Cardno *et al.* 2002, for review, see Berrettini 2000a, 2000b). At least three confirmed susceptibility loci, shared by bipolar disorder and schizophrenia, have been reported on chromosomes 13q32 (Lin *et al.* 1997, Ginns *et al.* 1996), 18p11 (Berrettini *et al.* 1994, Schwab *et al.* 1998a), and 22q11-13 (Lachman *et al.* 1997, Kelsoe *et al.* 2001).

2.4.4 Chromosomal rearrangements

Several chromosomal rearrangements have been implicated in both bipolar disorder and schizophrenia. Velo-cardio-facial syndrome is associated with small deletions of chromosome 22q11. Interestingly, a recent study demonstrated that of 48 patients with velo-cardio-facial syndrome, 12 fulfilled criteria for schizophrenia and 6 were diagnosed with major depression (Murphy *et al.* 1999). Examples of other chromosomal rearrangements of interest are a balanced translocation t(1:11)(q43,q21) and inversion inv(18)(p11.3,q21.1). The translocation between chromosomes 1 and 11 has been associated with schizophrenia, schizoaffective disorder and recurrent major depression (St Clair *et al.* 1990). The inversion on chromosome 18 has been associated with bipolar disorder in one Danish family and schizophrenia in a Scottish family (Mors *et al.* 1997). These reports suggest that corresponding chromosomal locations may harbor genes that are involved in the etiology of psychiatric disorders.

The role of chromosomal rearrangements in the etiology of disorders with unusual patterns of inheritance may be more complex and more common than previously thought. It has been estimated that approximately 5% of human genome consists of interspersed duplications. Many of these duplications exhibit a nucleotide sequence identity of over 95% and are the substrate for the formation of chromosomal rearrangements such as duplications, inversions and translocations (Eichler 2001). Recently, Giglio *et al.* (2001) described unequal crossovers between two olfactory receptor gene clusters on chromosome 8p that were responsible for the formation of four different chromosome rearrangements. Their observations indicated that at least some of the recurrent rearrangements are due to specific low-copy repeats in the genome. A potential assosiation of this inversion polymorphism to neuropsychiatric diseases has lately become a topic of intensive research (Ophoff et al 2001).

If a significant portion of the human genome is involved in relatively common rearrangement processes, it would be tempting to assume that such processes may have a role in unusual patterns of inheritance and partially explain difficulties in linkage and association studies in complex diseases such as schizophrenia and bipolar disorder.

2.4.5 Mapping

It seems likely that a genetic factor behind bipolar disorder and schizophrenia is a combination of a number of genes, each having a small contribution to relative risk (Risch 1990, Chakravarti 1999, Gershon 2000). Together with environmental or random variance, this oligogenic model of inheritance makes attempts to map susceptibility genes very difficult. To detect such genes, an extremely large number of small families are, if not required, at least preferred (Badner *et al.* 1988).

All but three autosomes (14, 17, 19) and the Y chromosome have been more or less suggestively connected either to schizophrenia or bipolar disorder by linkage and association studies (Table 1). Other research groups have been able to confirm these results in several cases. An association or linkage to bipolar disorder has been confirmed for chromosomes 1, 4, 5, 6, 12, 13, 15, 18, 21, and X (Stancer *et al.* 1988, Pekkarinen *et al.* 1995, Gershon *et al.* 1996, Kelsoe *et al.* 1996, Ewald *et al.* 1998a, 1998b, Papadimitriou *et al.* 1998, Detera-Wadleigh *et al.* 1999, Morissette *et al.* 1999, Blackwood *et al.* 2001, Liu *et al.* 2001a, 2001b). An association or linkage to schizophrenia has been confirmed for chromosomes 1, 5, 6, 8, 10, 13, 18, and 22 (Schwab *et al.* 1998a, 1998b, Brzustowicz *et al.* 1999, 2000, Li *et al.* 2000, Blackwood *et al.* 2001, Ekelund *et al.* 2001, Gurling *et al.* 2001, Lindholm *et al.* 2001, Yoshikawa *et al.* 2001). However, in most cases, the number of negative or exclusive association or linkage results far exceeds the number of these positive findings (for examples, see Goldin *et al.* 1982, De bruyn *et al.* 1994, Adams *et al.* 1997, Vallada *et al.* 1998, Curtis *et al.* 1999, McQuillin *et al.* 1999, Schwab *et al.* 1999, DeLisi *et al.* 2000a, 2000b, Nancarrow *et al.* 2000, Muir *et al.* 2001, Maziade *et al.* 2002).

2.4.6 Candidate genes

Despite the difficulties in collecting optimal samples and the uncertainties in linkage or association analysis, a Herculean effort has been put into the search for susceptibility genes for psychiatric disorders. Some of the putative candidate genes are listed in Table 1 and discussed below.

“Disrupted in schizophrenia” DISC1 and DISC2 genes are located on chromosome 1 in an area that has been shown to contain a translocation breakpoint more often in patients with schizophrenia than in the control population (St.Clair *et al.* 1990). The function of the genes is unknown, but the predicted protein structure of DISC1 shares some similarities with known neuronal proteins such as myosins, which are involved in axon guidance, synaptogenesis, functioning of the synapse, and intracellular transport along axons and dendrites. DISC2 is apparently a noncoding transcript that is antisense to

DISC1. Therefore, DISC2 may have a controlling role in DISC1 expression (Millar *et al.* 2000).

The ASCT1 gene, or solute carrier family 1, member 4 (SLC1A4), is located on chromosome 2 in a region associated with schizophrenia in Micronesian families (Coon *et al.* 1998), and is a neutral amino acid transporter that structurally resembles the sodium-coupled glutamate transporter (GLAST1) (Arriza *et al.* 1993). ASCT1 is functionally associated with chloride channel activity (Zerangue & Kavanaugh 1996). Bennett *et al.* (2000) were not able to find any mutations in the ASCT1 gene of the schizophrenic patients studied by Coon *et al.* (1998).

Table 1. Linkage or association findings in bipolar disorder and schizophrenia and putative candidate genes in corresponding chromosomal regions.

Chromosome	Bipolar disorder	Schizophrenia	Candidate gene(s)
1	Turecki et al. 1995	Hovatta et al. 1999	DISC1, DISC2
2	-----	Coon et al. 1998	ASCT1
3	-----	Pulver et al. 1995	DRD3
4	Blackwood et al. 1996	Muir et al. 2001	DRD5
5	Coon et al. 1993	Sherrington et al. 1988	DRD1, HTR1a
6	Smeraldi et al. 1978	Wang et al. 1995	HLA
7	-----	Ekelund et al. 2000	-----
8	-----	Pulver et al. 1995	PENK
9	Sherrington et al. 1994	-----	DBH
10	Foroud et al. 2000 Cichon et al. 2001	Straub et al. 1998	-----
11	Egeland et al. 1987	Gurling et al. 2001	TH
12	Ewald et al. 1998a	-----	PLA2A, DUSP6
13	Ginns et al. 1996	Lin et al. 1997	5-HT2A
14	-----	-----	-----
15	Ginns et al. 1996	Freedman et al. 1997	GABRA5
16	Eiberg et al. 1993	-----	PGP
17	-----	-----	-----
18	Berrettini et al. 1994	Schwab et al. 1998a	ACTH, GNAL, IMPA2, IMPACT
19	-----	-----	-----
20	Radhakrishna et al. 2001	Gurling et al. 2001	GS
21	Straub et al. 1994	-----	ADARB1
22	Kelsoe et al. 2001	Lachman et al. 1997	GNAZ
X	Baron et al. 1987	Milunsky et al. 1999	G6PD
Y	-----	-----	-----

Proenkephalin A (PENK) (Legon *et al.* 1982) on chromosome 8 has opioid like functions and thus is likely to have an effect on signal transduction in the brain. Konig *et al.* (1996) noticed severe behavioral changes in Penk knockout mice including hiding under the bedding, frantic running or jumping, and prolonged freezing in response to moderate noise. They were more anxious, and males displayed increased offensive aggressiveness. By studying the same mice, Ragnauth *et al.* (2001) concluded that the Penk gene product is a natural inhibitor of fear and anxiety. Several polymorphisms were found in the PENK gene of patients with schizophrenia (Mikesell *et al.* 1996).

The regions that were linked to schizophrenia and/or bipolar disorder have been shown to contain multiple signal transduction associated genes such as dopamine receptors 1 (DRD1) (Dearry *et al.* 1990), 3 (DRD3) (Sokoloff *et al.* 1990), and 5 (DRD5) (Beischlag *et al.* 1995), and other dopaminergic pathway related genes such as tyrosine hydroxylase (TH) (Grima *et al.* 1987) and dopamine beta hydroxylase (DBH) (Kobayashi *et al.* 1989), serotonin receptor genes HTR1A (Fargin *et al.* 1988) and HTR2A (Chen *et al.* 1992), G-proteins Gs (GNAS) (Kozasa *et al.* 1988), GNAZ (Matsuoka *et al.* 1988), and GNAL (Jones & Reed 1989), Gamma-aminobutyric acid receptor, alpha-5 (GABRA5) (Glatt *et al.* 1997), dual-specificity MAP kinase phosphatase 6 (DUSP6) (Furukawa *et al.* 1998), phospholipase A2 (PLA2A) (Seilhamer *et al.* 1986), and myoinositol monophosphatase 2 (IMPA2) (Yoshikawa *et al.* 2000).

Other possible genes of interest include melanocortin 2 receptor (ACTH) (Mountjoy *et al.* 1992), RNA-editing deaminase (ADARB1) (Mittaz *et al.* 1997), and glucose-6-phosphate dehydrogenase (G6PD) (Martini *et al*. 1986). Previous studies have suggested that bipolar patients are supersensitive to light suppression of melatonin, and this justifies the consideration of the ACTH gene as a candidate gene for bipolar disorder (Nurnberger *et al.* 2000). ADARB1 has been shown to be able to edit the glutamate receptor B pre-mRNA by deaminating adenosine residues to inosine. This alters a codon and changes an amino acid that has an effect on the calcium permeability of the AMPA glutamate receptors (Lai *et al.* 1997). Glucose-6-phosphate is a precursor of inositol, and therefore mutations affecting G6PD could have downstream effects on phosphoinositol signaling pathways.

Another gene to consider is a functionally unknown imprinted gene (Impact) (Kosaki *et al.* 2001) on chromosome 18p. Additionally, the major histocompatibility complex (HLA) region (Bach & Amos, 1967) on chromosome 6 has been suggested as a vulnerability locus in both schizophrenia and bipolar disorder. Unfortunately, from the genehunter's point of view, the major histocompatibility complex on chromosome 6p is not an ideal region because it contains approximately 100 genes or expressed sequence tags (EST) (Gruen *et al.* 1996).

Despite all the effort, decisive mutations in candidate genes for schizophrenia and bipolar disorder are yet to be found. So far, the available reports have been either negative or, at best, weakly suggestive (for examples, see Le *et al.* 1994, Cichon *et al.* 1996, Jacobsen *et al.* 1999, Meszaros *et al.* 2000).

2.4.6.1 GNAL

The cDNA for the α-subunit of the G-protein G_{olf} was originally isolated during a screen of a cDNA library from rat olfactory tissue (Jones & Reed 1989). The GNAL gene was localized on chromosome 18p11 (Overhauser *et al.* 1993), on a susceptibility region for both bipolar disorder and schizophrenia (Berrettini *et al.* 1994, Schwab *et al.* 1998a). mRNA from the gene was found in olfactory neuroepithelium but not in brain from which the olfactory bulb had been dissected, kidney, liver, lung, heart, and intestine (Jones & Reed 1989). Therefore, its expression was thought to be specific for olfaction related structures. In further work, $G_{olf\alpha}$ was shown to be expressed in insulinomas as well as in testis, retina, liver, lung, and spleen (Zigman *et al.* 1993, Herve *et al.* 1995, Frayon *et al.* 1999, Ferrand *et al.* 1999). The results, therefore, suggested that the expression was not specific for olfactory neuroepithelium. Instead, it suggested a much wider role for $G_{olf\alpha}$ in cellular signal transduction.

Antisera to the $G_{olf\alpha}$ protein was bound primarily to the olfactory sensory apparatus and neurons. The protein was found to share extensive amino acid identity (88%) with the α-subunit of stimulating G-protein $G_{S\alpha}$ (Jones & Reed 1989) and was shown to stimulate adenyl cyclase in a heterologous system.

Subsequently, $G_{olf\alpha}$ was found to be expressed in the basal ganglia, where it apparently couples dopamine D_1 receptors to adenyl cyclase (Herve *et al*. 1993). Interestingly, contrary to the common belief that adenosine A_{2A} receptors, which are coexpressed with dopamine D2 receptors, are specifically coupled to the stimulatory Gs protein, $G_{olf\alpha}$ was also shown to be colocalized and functionally coupled with adenosine A_{2A} receptors (Kull *et al.* 2000). Further evidence of the diverse role of $G_{olf\alpha}$'s in signal transduction was provided by an experiment with Gnal knockout mice. In addition to being anosmic, mice homozygous for a null mutation in Gnal exhibit hyperactivity and inadequate maternal behaviors (Belluscio *et al.* 1998). Subsequent experiments demonstrated that the Gnal knockout mice are deficient in striatal type 1 dopamine receptor mediated behavioral and biochemical effects. The knockout mice did not show any hyperlocomotor response to D1 agonist SKF-81297 or cocaine, and their striatal type 1 dopamine receptors had a decreased affinity for dopamine (Zhuang *et al.* 2001). These results suggest that G protein Golfα may, in addition to olfactory functions, play some of the roles otherwise attributed to Gs, and have more duties than previously thought in a diverse population of cells in a variety of tissues. Both functional data and chromosomal localization of the GNAL gene makes it an excellent candidate gene for bipolar disorder and schizophrenia.

3 Outlines of the present research

This research project was initiated at Thomas Jefferson University in 1994 when professor Wade H. Berrettini found a positive linkage between bipolar disorder and markers on chromosome 18p11. Of the possible candidate genes, the human GNAL gene was previously localized to that particular chromosomal region. Several other still uncharacterized genes were suggested to reside in close physical proximity to the GNAL gene.

To facilitate confirmation of the linkage findings and mutation analysis of the GNAL gene, the following aims were set:

1. To isolate and characterize the genomic clones for the human GNAL gene.
2. To set up a rapid and sensitive screening method for analyzing sequence variations in the GNAL gene.
3. To search for other putative candidate genes for bipolar disorder and schizophrenia on chromosome 18p11.

4 Materials and methods

4.1 Isolation and characterization of genomic clones for the human GNAL gene (I, II)

To define the genomic structure of the human GNAL gene, a chromosome 18-specific cosmid library (Lawrence Livermore National Laboratory; Livermore, CA) was screened with two cDNA probes covering the entire coding sequence of the GNAL gene according to standard protocols (Sambrook *et al.* 1989). The first probe corresponded to the 3'-coding sequence and the second probe to the 5'-coding sequence of the cDNA. Positive clones were picked out, and DNAs were isolated by the alkaline lysis method or DNeasy DNA isolation kit (Qiagen).

Nucleotide sequencing of the cosmid clones was performed with the dideoxynucleotide method (Sanger *et al.* 1977) and modified T7 DNA polymerase (Sequenase 2.0; U. S. Biochemical Corp.). Additional nucleotide sequencing was carried out by cycle sequencing (dsDNA Cycle Sequencing System; BRL). To accelerate the sequencing process, inserts of some of the cosmid clones were released from the vector by SfiI digestion. SfiI fragments were then purified from agarose gel by electroelution or by commercial kit (QIAEX II Gel Extraction Kit, Qiagen). Eluted inserts were partially digested with EcoRI and/or BamHI, and subcloned into a plasmid vector (pT7 Blue, Novagen Inc). Where applicable, the GNAL introns were amplified by PCR. The exon boundaries in the GNAL cDNA were predicted to be similarly located as in the previously characterized human GNAS gene, and the PCR primers were designed to fit into predicted neighboring exons. Nucleotide sequencing was carried out directly from PCR products (Sequenase PCR Product Sequencing Kit; U.S. Biochemical Corp.)

4.2 Isolation and characterization of the MPPE1 and C18orf2 genes (III, IV)

To isolate the human MPPE1 cDNA, a human brain cDNA library (Clontech) was used as the template in PCR amplifications with EST sequence derived primers and a vector-specific primer from either side of the polycloning site. The conditions for PCR were 94º C initially for 90 s and then 35 cycles of 94º C for 45 s, 56º C for 15 s, and 72º C for 90 s. The PCR products were analyzed by agarose gel electrophoresis, and PCR reactions with 3 or less distinct products were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (EXO)(USB) and sequenced with an ABI377 automatic sequencer (Perkin Elmer) with nested MPPE1-primers, thus eliminating the need for purification and sequencing of the individual PCR products. In view of the sequencing results, further nested primers were designed and PCR, SAP and EXO treatment, and sequencing were repeated to the point at which the 5' and 3'-ends of the MPPE1 cDNA were obtained.

The genomic structure of the MPPE1 gene was defined by sequencing the exon boundaries and most of the intronic sequences from a cosmid clone isolated from a human chromosome 18 specific library (Lawrence Livermore National Laboratory) using primers derived from the MPPE1 cDNA sequence. The cosmid clone was originally obtained in the course of characterizing the human GNAL gene, and covered the entire MPPE1 gene and the 3'-end of the GNAL gene.

The structure of the C18orf2 gene was defined by PCR amplification with EST derived primers and direct sequencing of the PCR products. Cosmid clones covering the GNAL intron 5 and human genomic DNA that was isolated from EDTA anti-coagulated blood (Bell *et al.* 1981) were used as templates. PCR conditions were essentially the same as for the amplification of the MPPE1 cDNA.

4.3 Isolation of RNA and RT-PCR (II, III, IV)

Total RNA was isolated from human brain tissue (Brain Bank, St Elizabeth Hospital, NIMH). About 250 mg of frozen brain tissue wrapped in aluminum foil was pulverized on a -80° C metal block with a liquid nitrogen-cooled carpenter's hammer (Stanley Tools). Total RNA was then isolated from the pulverized tissue according to the manufacturer's protocol (RNeasy RNA Isolation Kit, Qiagen).

To verify the cDNA sequences and to detect possible alternatively spliced mRNAs of the GNAL, C18orf2, and MPPE1 genes, RT-PCR reactions were performed (GeneAmp RNA PCR kit, Perkin Elmer Corp). In brief, about 1 µg of total RNA was used as starting material. The first strand synthesis was carried out with random hexamers or oligo(dT) primers. Subsequent PCR reactions were performed with cDNA specific primer pairs (GeneAmp RNA PCR kit, Perkin Elmer Corp or Thermoscript RT-PCR system, GibcoBRL). The RT-PCR products were analyzed by agarose gel electrophoresis and direct sequencing.

4.4 3´-RACE (II, IV)

3'-RACE analysis was used to define the functional polyadenylation signals in the GNAL and MPPE1 genes. About 1 μg of total human brain RNA was reverse transcribed (GeneAmp RNA PCR kit, Perkin Elmer Corp or Thermoscript RT-PCR system, GibcoBRL) using random hexamers. In the first PCR reactions, the single-stranded cDNA was amplified with an oligo(dT) primer linked to a random sequence, 5'-TAC TGA GCA GCG AAT TCT ACG TCG $C(T)_{20}$, and forward primers designed to hybridize to the 3'-end of the genes. The second round of amplifications were performed with a reverse primer that was identical to the random sequence at the 5'-end of the oligo(dT) primer and with gene specific nested forward primers. The first PCR was in a volume of 50 μl with 10 pmol of reverse primer and 10 pmol of forward primer at 94.5°C for 60 s, 53°C for 60 s, and 72°C for 90 s for 40 cycles. About 1 μl of 50 μl was taken for a second amplification with 10 pmol of reverse primer and 10 pmol of nested forward primer in a final volume of 50 μl. The conditions were 94.5°C for 60 s, 53°C for 60 s, and 72°C for 60 s for 35 cycles. The products of the second PCR were analyzed on agarose gels and by direct sequencing (Sequenase PCR Product Sequencing Kit; U.S. Biochemical Corp.) or with an ABI377 automatic sequencer (Perkin Elmer).

4.5 5´-RACE (II)

To define the transcription initiation site of the GNAL gene, 5'-RACE analysis was utilized. Pooled adapter-ligated cDNAs from human brain were used as templates (Marathon-Ready cDNA Kit, Clontech). The first PCR was performed with a forward primer AP-1 (Marathon-Ready cDNA Kit, Clontech) designed to hybridize to the adapter sequence and a reverse primer complementary to part of the GNAL exon 4. For the second round of amplification, another nested forward primer AP-2 (Marathon-Ready cDNA Kit, Clontech) and reverse primer corresponding to exon 1 were used. The conditions for the PCRs were essentially the same as in the 3'-RACE analysis. The products of the second PCR were analyzed on agarose gels and by direct sequencing (Sequenase PCR Product Sequencing Kit; U.S. Biochemical Corp.) or with an ABI377 automatic sequencer (Perkin Elmer).

4.6 Northern analysis (II, IV)

To assay the mRNA expression of the GNAL and MPPE1 genes, commercially supplied filters containing mRNA from several human tissues (Human Multiple Tissue Northern Blot; Clontech) and various regions of the human brain (Human Brain Multiple Tissue Northern Blot; Clontech) were employed. Each lane on the filters contained about 2 μg of poly(A)-enriched RNA. PCR products from the MPPE1 cDNA corresponding to exons 5 to 10 and PCR products from the 3'-untranslated region of the GNAL gene were labeled with α^{32}P-dCTP by nick translation (Nick Translation System, Gibco BRL) according to

the manufacturer's protocol. The filters were also hybridized and washed according to instructions from the manufacturer (Clontech).

4.7 Expression analysis by semiquantitative RT-PCR (III)

To analyze the mRNA expression of the C18orf2 gene in different tissues, Human Multiple Tissue cDNA Panel I (Clontech) and semiquantitative RT-PCR were employed. In brief, cDNAs from seven different tissues: pancreas, kidney, skeletal muscle, liver, lung, placenta, and brain were amplified with C18orf2 specific primers for forty cycles. To define the optimal number of PCR cycles for linear amplification, an aliquot from each of the samples was removed at 22 cycles, and thereafter at every fourth additional cycle. The human G3PDH specific primers, provided by the manufacturer (Clontech), were used in control reactions. The amplification products were analyzed by agarose gel electrophoresis.

4.8 Detection of polymorphisms (I, II, III, IV)

To identify polymorphisms within the GNAL-C18orf2-MPPE1 gene cluster, random samples of human genomic DNA were used as the templates for PCR amplification. Subsequent analysis was performed by the direct sequencing of the PCR products, or with the single-stranded conformation polymorphism (SSCP) or conformation sensitive gel electrophoresis (CSGE) methods.

4.8.1 SSCP (I, II)

To search for polymorphisms within the GNAL gene, single-stranded conformation polymorphism (Orita *et al.* 1989) analysis was utilized. Each exon of the GNAL gene was amplified using 45 ng of genomic DNA as template. One oligonucleotide from each primer pair was end-labeled with γ^{32}P-dATP using T4 polynucleotide kinase (Gibco BRL). PCR was performed in a total volume of 13 µl with 2 pmol of unlabeled primer and 1 pmol of labeled primer for 35 cycles using an annealing temperature of 55°C. In the presence of loading buffer, PCR products were first denatured at 95°C for 5 minutes and then loaded onto mutation detection enhancement (MDE) gels (FMC Bioproducts). Gels were electrophoresed at 25 W for 15 hours at room temperature, then dried and exposed to radiographic film.

4.8.2 CSGE (II, IV)

As an alternative method to search for polymorphisms within the GNAL and MPPE1 genes, conformation sensitive gel electrophoresis (CSGE) was employed (Ganguly *et al.*

1993, Körkkö *et al.* 1998). PCR primers flanking each exon of the GNAL and MPPE1 genes were designed. The products varied between 150 and 450 bp and contained at least 40 bp of exon-flanking sequences at either side of the exon. From 10 to 50 ng of genomic DNA was amplified in a 30 µl volume at 94°C for 45 s, 53 to 62°C for 25 s, and 72°C for 1 min for 35 cycles followed by an extension at 72°C for 10 min. Heteroduplexing was performed at 95°C for 5 min, followed by 30 min at 68°C. PCR products were loaded onto a CSGE gel, which consisted of 15% polyacrylamide, a 99:1 ratio of acrylamide to 1,4-bis-acryloylpiperazine (Fluka), 10% ethylene glycol (Sigma), 15% formamide (Gibco), 0.1% APS, and 0.07% TEMED in 0.5xTTE buffer (44.4 mM Tris, 14.25 mM Taurine, 0.1 mM EDTA). The gel was electrophoresed on a standard sequencing apparatus at 45 W for 5 hours at room temperature using 0.5xTTE as the running buffer. After electrophoresis, the gel was stained with ethidium bromide and photographed.

4.9 Nucleotide and amino acid sequence analysis (II, III, IV)

Nucleotide sequence data were analyzed using either MacDNASIS (Hitachi) or the Wisconsin Sequence Analysis Package (GCG) version 8.0-UNIX (Genetics Computer Group) (Devereux *et al.* 1984). Promoter analysis was performed with TSSG / Recognition of human PolII promoter region and start of transcription (Soloyev & Salamov 1997). Further analysis of the nucleotide and amino acid sequences was done with the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.ch/) (Wilkins *et al.* 1998) using Translate for translation of the DNA sequences, ScanProsite and Motifscan for analysis of the amino acid sequences, Tmpred for prediction of transmembrane domains, and CLUSTALW for sequence alignment. Additional nucleotide and amino acid sequence comparisons were performed at http://www.ncbi.nih.gov/BLAST/.

5 Results

5.1 Characterization of the human GNAL, C18orf2, and MPPE1 genes (II, III, IV)

5.1.1 GNAL (II)

To characterize the GNAL gene, a chromosome 18 specific cosmid library was screened with two cDNA probes. A total of fourteen cosmids that gave a strong hybridization signal with either one of the cDNA probes were isolated and sequenced with the GNAL cDNA derived primers. The initial sequencing defined the intron boundaries of the first four exons in the 5'-end of the gene and the last seven exons in the 3'-end of the gene. The missing cDNA fragment in the middle region of the cDNA was later characterized from an additional cosmid clone that gave weak hybridization signals with the two cDNA probes. Over 32 kb of the gene that spanned about 80 kb were sequenced. The sequences revealed that the GNAL gene consists of twelve exons. The relatively large size of the gene was explained mostly by the unusually large sizes of introns 4 and 5. Because of their large sizes, these two introns were not completely sequenced here. The exon sizes of the gene were very similar to the related gene G_S, with the exception that exon 1 was 6 bp longer. Also, GNAL did not contain an exon corresponding to the alternatively spliced exon 3 of the G_S gene (Bray *et al.* 1986, Kozasa *et al*. 1988). The intron sequences analyzed included 22 *Alu* repeat sequences that were widely distributed throughout the gene. Two of the *Alu* repeats were in the 3'-nontranslated region of the gene.

About 6 kb upstream of the GNAL, a processed pseudogene was found. It had a high degree of identity with a cDNA for the asparagine synthetase gene (Andrulis *et al.* 1987, Greco *et al.* 1987). The sequence consisted of about 600 bp that extended from nt 167 to nt 740 of the asparagine synthetase cDNA, followed by an *Alu* repeat sequence of about 300 bp and then an additional 250 bp extending to nt 989 of the cDNA. If the *Alu*

sequence was excluded, there was 83% identity with the reported sequences of the cDNA for asparagine synthetase. Several stop codons were found in all three reading frames.

To identify sequences in the GNAL promoter region that might be involved in the regulation of transcription, about 3 kb of DNA upstream from the ATG translation start codon was sequenced and analyzed for definition of conserved sites for the binding of transcription factors (MacDNASIS program, Hitachi) at 100% homology between the human sequences and previously published rat sequences (Wang *et al.* 1993). One or more conserved sites were found for nine different transcription factors. As was previously reported for the promoter of the rat *Gnal* gene (Wang *et al.* 1993), the promoter for the human gene did not contain a consensus CCAAT box or a consensus TATA box. The 800 bp of sequences from the human promoter region had about a 75% degree of identity with the rat promoter. Further analyses suggested that the gene had multiple start sites for transcription. A 5'-RACE assay indicated that some transcripts extended 100 nt upstream of the start of translation. Assays by RT-PCR suggested that some transcripts extended even further upstream, since a product was obtained with a 5'-primer that was 216 nt upstream of the start of translation. A cDNA previously isolated from a human insulinoma extended 255 nt upstream of the start of translation (Zigman *et al.* 1993). Additional analysis of the GNAL promoter sequence was performed by Recognition of Human PolII Promoter Region and Start of Transcription (Soloyev and Salamov, 1997) through the WWW. The sequence analysis predicted an additional transcription initiation site 318 nt upstream of the start of translation.

Over 5 kb of the 3'-nontranslated region of the mRNAs were sequenced from the genomic clones. The region included two *Alu* sequences and two polyadenylation sequences of -AATAAA- at about 4.2 and 4.5 kb beyond the translation termination codon for translation were defined. Also, a third polyadenylation sequence of -ATTAAA- was found at about 2.4 kb beyond the translation termination codon and located between the two *Alu* repeats. In addition, over 10 variants of the consensus polyadenylation signal (Sheets *et al.* 1990) were present. To confirm that the polyadenylation signals were used, 3'-RACE assay was performed. The total human RNA isolated from caudate nucleus was reverse transcribed using random hexamers. The cDNA was then amplified with a series of forward primers specific for different regions of the 3'-nontranslated region and an oligo(dT) primer that contained a predefined random sequence at its 5'-end. The cDNA amplified in the first step was then re-amplified with a nested forward primer and a reverse primer with the random sequence found in the oligo(dT) primer. Apparently because of the poly-(A) sequences within the *Alu* repeats of the 3'-nontranslated regions, most of the PCR products were anomalous. However, a product of the expected size was obtained with the two primers that were closest to the most distal polyadenylation signal. Sequencing of the products demonstrated that transcription of GNAL is terminated at the most 3' polyadenylation signal in human brain. Thus, the predicted total length of the message is about 5.9 kb.

5.1.2 C18orf2 (III)

During the sequencing of intron 5 of the GNAL gene, a BLAST search revealed a DNA fragment of about 1.6 kb in length that was highly homologous to a number of human and mouse ESTs. The novel gene was localized to about 10 kb upstream of GNAL exon 6 (Figure 5). Additional homologous sequences were found on chromosomes 1 and 17 by BLAST searches through a high throughput genome sequence database (HTGS). In further analysis, the translated C18orf2 sequence of 199 amino acids in length was found to be similar to the *Drosophila melanogaster* gene product CG4108 (GenBank accession no. AAF49241), a hypothetical *C. elegans* protein, which bears some similarity to the putative human breast adenocarcinoma marker protein *BC-2* (GenBank accession no. AF106583), and a number of developmental and hypothetical proteins from *Dictyostelium discoideum*, *Schizosaccharomyces pombe,* and *Saccharomyces cerevisiae* (GenBank accession nos. AF081802, AL022600, and 6322887, respectively). The C18orf2 amino acid sequence also shared a very high degree of homology with a previously published putative metallopeptidase sequence (PRSM1) on chromosome 16q24.3(Scott *et al.*1996). This similarity was observed only after translating the PRSM1 sequence in a +1 reading frame in relation to the one originally suggested.

The C18orf2 promoter sequence was analyzed by the Recognition of Human PolII Promoter Region and Start of Transcription program (Soloyev and Salamov 1997), which predicted a transcription initiation site 88 bp upstream of the translation initiation codon. Further comparisons with the ESTs supported this prediction. The promoter area lacks the TATA-box consensus sequence, which is typical of many housekeeping genes. Two AATAAA polyadenylation signal sequences were found, at +1166-+1171 and +1590-+1595. The functional role of the poly-(A) signals was supported by comparisons with the ESTs. Of 29 highly homologous EST sequences, 13 ended in the immediate vicinity of either one of the polyadenylation signals.

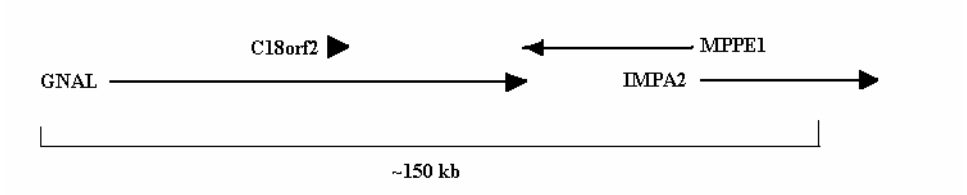

Fig. 5. Schematic representation of the organization of the GNAL-C18orf2-MPPE1-IMPA2 gene cluster. The 5'-3' orientation of the genes is indicated with an arrow.

5.1.3 MPPE1 (IV)

In the process of characterizing a cosmid clone that contained the 3'-UTR of the human GNAL gene, a previously unknown transcript was discovered. A database search revealed several brain-derived ESTs corresponding to this, and it was characterized by amplification of the entire coding sequence with lambda lysate from a human brain cDNA library and EST-derived primers.

The amino acid sequence deduced from the cDNA sequence was 396 amino acids in length, the first 45 amino acids at the N-terminus being recognized by the Genefinder program as forming a signal sequence. According to the ProfileScan analysis, the signal sequence was followed by a 240-amino acid metallophosphoesterase domain containing a gDxH..(16-60)..GDxxdr..(13-34)..GNH[DE] motif, which is highly conserved in a variety of protein phosphatases across several species (Koonin 1994; Zhuo *et al.* 1994), and a putative 24-amino acid transmembrane domain at the C-terminus. An extensive BLAST search revealed that the coding region of the cDNA and amino acid sequence of the human MPPE1 were 96 and 94% identical to Macaca fascicularis brain cDNA library-derived cDNA and the amino acid sequence of a hypothetical protein, respectively (GenBank accession No. AB047842). Human sequences coding for hypothetical protein FLJ11585 (GenBank accession No. XP018376, NP075563) and a protein similar to FLJ11585 (GenBank accession No. BC002877) partially corresponded to the sequences of the MPPE1, suggesting that these sequences were incomplete forms of the MPPE1.

To define the genomic organization of the MPPE1 gene, cDNA-derived primers were used to sequence it from the cosmid clone containing the entire MPPE1 gene and a partial sequence of the GNAL gene. The initial sequence analysis revealed that the gene consisted of eleven exons and spanned about 27 kb of genomic DNA. All the exon boundaries followed the GT-AG rule. The first two exons appeared to be non-coding since several stop codons were found in all three reading frames. The predicted translation initiation codon was located 93 bp downstream from the 5'-end of the first coding exon.

Further analysis revealed three additional exons, bringing the total number of MPPE1 exons to fourteen. The new apparently non-coding exons 3 and 4 were found only by sequence comparisons and not detected with RT-PCR. The third additional exon 13 was found by RT-PCR. When present, the alternatively spliced exon 13 introduced a novel translation termination codon AUG into the sequence. This transcript coded for a truncated 340 amino acid MPPE1 polypeptide lacking the C-terminal transmembrane domain.

Further sequence analysis revealed that the alternatively spliced exon 13, exon 14, and the 3'-nontranslated region of the MPPE1 gene overlapped with the 3'-nontranslated region of the GNAL gene (Fig. 2.).

Sequence analysis revealed two AATAAA-polyadenylation signals 148 and 358 bp downstream of the translation termination codon in exon 14. 3'-RACE assay demonstrated that in the human brain transcription termination occurs at the extreme 3' polyadenylation signal.

5.2 Expression analysis (II, III, IV)

5.2.1 GNAL (II)

Northern blot analysis revealed that the GNAL gene is expressed as a single mRNA of about 6 kb in size in human brain. The highest levels of expression were detected in the caudate nucleus and amygdala.

No alternatively spliced forms of the GNAL gene were detected by RT-PCR experiments with several primer pairs that produced overlapping cDNA fragments.

5.2.2 C18orf2 (III)

RT-PCR analysis revealed that *C18orf2* was moderately expressed in all the tissues studied: heart, brain, placenta, lung, liver, skeletal muscle, kidney, and spleen. Two control primer pairs were used to test the presence of possible genomic DNA contamination. No amplification products were detected.

5.2.3 MPPE1 (IV)

Northern blot analysis of the MPPE1 gene demonstrated that it is expressed as a single mRNA of about 2.2 kb in size in the human brain, but not in any other tissues studied: heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas. RT-PCR assay revealed that the MPPE1 gene is expressed in the human brain as four species of mRNA. The four species of mRNA that were seen as RT-PCR products proved to have resulted from alternative splicing. The most abundant transcript consisted of exons 1,2,5-12, and 14, and another splice variant contained exons 1, 2, and 5-14. The inclusion of exon 13 introduced a premature translation termination codon, UAG, at amino acid 340. The two rare splice variants corresponded to the first two splice forms, but lacked the sequences for exon 10.

5.3 Analysis of the gene sequences for polymorphisms (I, II, III, IV)

To facilitate the use of the GNAL-C18orf2-MPPE1 gene cluster in future association and linkage analyses, possibly informative sequence repeats and single nucleotide polymorphisms were searched by direct sequencing, SSCP, and CSGE.

Direct sequencing and analysis of the genomic sequences revealed a dinucleotide repeat located in GNAL intron 5. This CA-repeat (401 nt from the 3'-end of the intron) was found to be useful for linkage analysis because at least 11 alleles were present. The

heterozygosity was 74%, and the allele frequencies were 106 nt, 3%, 108 nt, 17%, 110 nt, 1%, 112 nt, 5%, 114 nt, 17%, 116 nt, 40%, 118 nt, 5%, 120 nt, 2%, 122 nt, 1%, 124 nt, 17%, and 126 nt, 3% in 212 chromosomes.

Another polymorphism identified by direct sequencing of PCR products from random human genomic DNA samples was a single C to T polymorphism at 72 bp downstream of the translation termination codon TGA of the C18orf2 gene.

Two single nucleotide polymorphisms in GNAL introns 3 and 10 were found by SSCP analysis. Minor allele frequencies of intron 3 A/G (35 bp downstream from exon 3), and intron 10 T/G (7 bp upstream from exon 11) polymorphisms were 31% and 16%, respectively.

Partially to search for additional polymorphisms, but mainly to facilitate future mutation analysis within the GNAL and MPPE1 genes, CSGE analysis was set up. Primers were designed and PCR conditions were optimized for amplification of each GNAL and MPPE1 exon. Initial analysis did not reveal any additional polymorphisms from the GNAL gene. Two polymorphisms causing amino acid substitutions were found within the MPPE1 coding sequences, including a G/C substitution in exon 11 (57 bp downstream from the 5'-end of the exon), changing alanine to proline, and a G/A substitution in exon 7 (23 bp downstream from the 5'-end of the exon), changing valine to isoleucine. In addition, several apparently common intronic polymorphisms were found within the MPPE1 gene.

6 Discussion

The pericentromeric region of chromosome 18p11.2 has been a target of interest since the observation of positive linkage with bipolar disorder (Berrettini *et al.* 1994). Even though reports with opposing results exist (Mynett-Johnson *et al.* 1997, Knowles *et al.* 1998), Berrettini's linkage findings were confirmed in several studies (Haghighi *et al.* 1997, Nothen *et al.* 1999), which is rather unusual with association or linkage studies of psychiatric illnesses.

The gene encoding the alpha subunit of guanine nucleotide binding protein G_{olf} (GNAL) was localized within the susceptibility region for bipolar disorder (Overhauser *et al.* 1993). Due to observations that lithium, a commonly used treatment for bipolar disorder, decreases the affinity with which the G-protein alpha subunit binds GTP (Avissar & Schreiber 1992) and antidepressants modulate the expression of G-proteins in brain (Lesch & Manji 1992), a G-protein expressed in brain would be an interesting candidate gene for bipolar disorder.

The expression of the GNAL gene was originally thought to be specific for olfaction-related structures (Jones & Reed 1989). However, in a subsequent report GNAL gene expression was demonstrated in various regions of the brain, including the nucleus accumbens, striatum, and substantia nigra (Zigman *et al.* 1993). GNAL was also found to be expressed in the basal ganglia, where it apparently couples dopamine D_1 receptors to adenyl cyclase (Herve *et al*. 1993). In addition, the GNAL gene was shown to be expressed in various tissues outside of the brain including testis, retina, liver, lung, and spleen (Zigman *et al.* 1993, Herve *et al.* 1995, Frayon *et al.* 1999, Ferrand *et al.* 1999). These results suggested that the expression was not specific for olfactory neuroepithelium. Together with the expression data, a high degree of amino acid identity with the most abundant, and probably most extensively studied G-protein, adenyl cyclase-stimulating $G_{S\alpha}$, indicates that the $G_{olf\alpha}$ protein may indeed have a more diverse role in cellular signal transduction than previously thought. Further support for similarities with the roles of $G_{olf\alpha}$ and $G_{S\alpha}$was provided by an observation that $G_{olf\alpha}$ was also colocalized and functionally coupled with adenosine A_{2A} receptors (Kull *et al.* 2000). Adenosine A_{2A} receptors, which are coexpressed with dopamine D2 receptors, were previously thought to be specifically coupled to the stimulatory Gs protein. An additional reason for speculation about the true nature of the function of $G_{olf\alpha}$ comes

from a report in which mice homozygous for a null mutation of the Gnal gene were shown to exhibit hyperactivity and inadequate maternal behaviors (Belluscio *et al.* 1998). The Gnal knockout mice were also deficient in striatal type 1 dopamine receptor-mediated behavioral and biochemical effects. The knockout mice did not show any hyperlocomotor response to D1 agonist SKF-81297 or cocaine, and their striatal type 1 dopamine receptors had a decreased affinity for dopamine (Zhuang *et al.* 2001). Both the available functional data and the location of the GNAL gene on chromosome 18p11.2 provide enough evidence to consider GNAL as a reasonable candidate gene for bipolar disorder.

The cDNA sequence for the human GNAL gene has been characterized (Zigman *et al.* 1993), but the genomic structure of the human GNAL gene has not been determined. Therefore, it has been very difficult to detect polymorphisms suitable for verification of the positive linkage and association findings and to develop a mutation screening method for the GNAL gene.

The present work was initiated by the need to define the genomic structure of the human GNAL gene. During characterization of the GNAL gene, we found an informative CA-microsatellite region in intron five. It consisted of eleven alleles with a heterozygosity of 74% in 212 chromosomes. By using this microsatellite, Schwab *et al.* (1998a) showed evidence for its association with schizophrenia in German and Israeli patients. Since deficient olfactory identification performance has been frequently reported in patients with schizophrenia (Moberg *et al.* 1999), this observation by Schwab *et al.* expanded the role of the GNAL gene from a candidate gene for bipolar disorder to a putative candidate gene for schizophrenia as well, and raised again the old question of the possible overlap between these disorders in both inheritance and biological mechanisms.

Nucleotide sequencing of the human GNAL gene revealed that the gene is over 80 kb in length and consists of twelve exons. The exon structure is very similar to the gene for the analogous G protein $G_{S\alpha}$ (GNAS) (Bray *et al.* 1986, Kozasa *et al.* 1988), except that exon 1 is 6 bp longer. Also, the alternatively spliced exon 3 of the GNAS gene was not found in the GNAL gene by complete sequencing of the corresponding intron. In addition, no alternatively spliced exons were found by RT-PCR. The GNAL gene contained a relatively large number of *Alu* repeat sequences in the introns. The frequency of *Alu* repeats was 1 per 1.45 kb, which may increase chances for genomic rearrangements within the GNAL locus. The average frequency of *Alu* repeats in the human genome is approximately 1 per 4 kb (Moyzis *et al.* 1989). Two *Alu* sequences were also found in the 3'-nontranslated region. Our Northern analysis and 3'-RACE results of mRNA from human caudate nucleus indicated that these two *Alu* sequences are part of the full length transcription product of the GNAL gene. *Alu* sequences are found in about 5% of the expressed sequences (Yulug *et al.* 1995).

Together with previously published transcription initiation start sites of human (Zigman *et al.* 1993) and rat *Gnal* (Wang *et al.* 1993), our 5'-RACE analysis suggested that GNAL has multiple transcription initiation start sites located between 318 to 100 nt upstream of the translation initiation codon. To identify putative *cis*-acting regulatory sequences that direct GNAL transcription, we compared human and rat promoter sequences. The comparison revealed several highly conserved transcription factor binding sites, such as SP1 and AP2.

Polymorphisms within the GNAL gene were searched by radioactive single stranded conformation polymorphism (SSCP). Two single nucleotide substitutions were found in introns 3 and 10. Theoretically, both substitutions may be a source of aberrant splicing of the GNAL gene. However, before any conclusions can be made, comparison of allele frequencies between patients with bipolar disorder and healthy controls is required. Since the mutation detection rate of SSCP analysis is relatively low, from 60 to 95% (Cotton 1993, Eng &Vijg 1997), CSGE analysis was set up. CSGE has several advantages over SSCP in that it is simple, does not use radioactivity, requires no special preparation of PCR products, has relatively large capacity, and in optimized conditions, mutation detection sensitivity exceeds 95% (Körkkö et al. 1998). However, no additional polymorphisms were found within the GNAL gene.

During the sequencing of the GNAL intron 5, we found a novel intronless gene C18orf2. Semiquatitative RT-PCR analysis revealed that the mRNA expression of C18orf2 was relatively abundant in all tissues studied here. Database searches indicated that the C18orf2 amino acid sequence is highly conserved across divergent species. Significant homology was found between C18orf2 and a number of developmental and functionally yet unknown proteins. Interestingly, a striking homology was found to a previously published gene encoding a putative metallopeptidase (PRSM1) on chromosome 16q24.3 (Scott *et al.* 1996). PRSM1 does not share significant amino acid sequence homology with any of the GenBank sequences, but when PRSM1 was translated in a +1 reading frame in relation to the originally suggested one, it codes for a 196 aa long polypeptide highly homologous to the predicted C18orf2 protein (55% identity, 76% similarity).

Our results thus suggested that instead of encoding the putative metallopeptidase, the PRSM1 sequence encodes a novel gene closely related to C18orf2. In addition, further HTGS database searches revealed regions homologous to PRSM1 in BAC clones from chromosomes 1 and 17 (GenBank accession nos. AC069482 and AC012300, respectively), suggesting that PRSM1 may be a multicopy gene or a member of a larger gene family. Due to an apparently universal expression and a high degree of conservation throughout the species, C18orf2 is not the most likely candidate gene for bipolar disorder or schizophrenia. However, because of its location within the GNAL gene, possible interactions between the genes during situations when the expression of one or another is abnormally altered cannot be ruled out.

During the analysis of the GNAL 3'-nontranslated sequences, we found homologies with a number of ESTs and RT-PCR products that could not have been explained by possible alternative splicing of the GNAL gene. Extensive sequence analysis revealed a novel gene, MPPE1, in a tail-to-tail orientation with the GNAL gene. We determined its cDNA sequence, genomic structure, expression as mRNA, functional polyadenylation site, and alternative splicing. The MPPE1 gene has 14 exons and spans about 27 kb. All donor and acceptor splice sites have the canonical GT and AG dinucleotides, respectively. CSGE analysis revealed several polymorphisms within the MPPE1 gene. However, before any conclusions of their significance can be made, further experiments with patient and control samples are required.

The results of our Northern analysis showed that the MPPE1 gene is expressed as a single 2.2 kb message in the human brain, but not in any other tissues studied. Three alternatively spliced forms of the MPPE1 were detected only by RT-PCR, suggesting

their low abundance in the brain. The observation that the non-coding exons 3 and 4 can be found in ESTs but not by RT-PCR in brain-derived mRNAs suggests that rare forms of the MPPE1 mRNA containing sequences for exons 3 and 4 may exist in some tissues but in low quantities, because they were not detected here by Northern blotting. This is supported by the finding that only two ESTs (Genbank accession Nos. BE973769, BE465922) containing exons 3 and 4 were found, these being derived from kidney and prostate.

The deduced amino acid sequence for the most abundant form of the MPPE1 is 396 amino acids in length. The first 45-amino acids at the N-terminus form a predicted signal sequence. The C-terminus of the MPPE1 polypeptide consists of a 24-amino acid putative transmembrane domain, which is lacking in the second most abundant splice form. In between the signal sequence and putative transmembrane domain is a 240-amino acid metallophosphoesterase domain that contain a gDxH..(16-60)..GDxxdr..(13-34)..GNH[DE] motif, which is highly conserved in a variety of protein phosphatases across several species (Koonin, 1994, Zhuo *et al.* 1994). The motif contains metal binding and active-sites similar to human serine/threonine phosphoprotein phosphatase catalytic subunits such as protein phosphatase-1 (PP1) (Barker *et al.* 1990), protein phosphatase-2 (PP2A) (Stone *et al.* 1988), and calcineurin (Guerini & Klee 1989). Serine/threonine protein phosphatases are involved in crucial cellular processes, including gene expression, cell growth, and cell differentiation (Cohen 1997). Even though the phosphatase activity or specificity of the MPPE1 protein was not determined, amino acid sequence analysis suggested that the MPPE1 protein is a metal-dependent phosphoesterase and a member of the calcineurin-like phosphoesterase superfamily.

MPPE1 transcription partially overlaps with the GNAL 3'-nontranslated region, and the GNAL polyadenylation signal that is functional in the human brain is located at the 5'-end of MPPE1 intron 12. Thus, all the splice variants of the MPPE1 gene that were detected in the human brain indeed overlap with full-length transcripts of the GNAL gene. There are only a few examples of overlapping genes in humans (Shintani *et al.* 1999). A recent study demonstrated that only a 55 bp tail-to-tail overlap of two genes coding for transcription factor CHOP and methionyl tRNA synthetase (MetRS) has significant control over mRNA stability via antisense-type interactions (Ubeda *et al.* 1999). This overlapping transcription suggests that if GNAL and MPPE1 are expressed in the same brain cells, abnormal expression of one of them may have an effect on the transcription or translation of the other by antisense intervention in the normal processing of the opposing transcript.

Together with the myo-inositol monophosphatase gene (IMPA2), which resides only a few kilobases from the 5'-end of the MPPE1 gene, GNAL, C18orf2, and MPPE1 form a very interesting gene cluster. Both GNAL and IMPA2 have been implicated as candidate genes for bipolar disorder as well as schizophrenia (Berrettini *et al.* 1994, Schwab *et al.* 1998a, Yoshikawa *et al.* 2000, 2001). For future experiments, to either rule out or confirm previous linkage or association findings and determine the role of the GNAL and MPPE1 genes in the etiology of bipolar disorder and/or schizophrenia, we set up a conformation sensitive gel electrophoresis (CSGE) mutation detection system for both genes. Since both disorders apparently result from a complex combination of environmental and genetic factors (Wildenauer *et al.* 1999, Freedman *et al.* 2001), the sample collecting requirements such as diagnostic criteria, the size of the patient groups and variation in

patients' response to medication must be carefully considered for successful mutation screening. However, characterization of the GNAL, C18orf2, and MPPE1 genes here provides a fundamental basis for the mutation screening, analysis of possible interactions between the GNAL and MPPE1 transcripts, and functional and structural analysis of C18orf2 and MPPE1.

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