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Research report

Polymorphisms in the galanin gene are associated with symptom–severity in female patients suffering from panic disorder

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Abstract

Background: Galanin (GAL) is a neuropeptide, which is expressed primarily in limbic nuclei in the brain and mediates miscellaneous physiological processes and behaviors. In animal studies, both the application of GAL and antagonism of its receptors have been shown to affect anxiety-like and depression-related behavior. In humans, intravenous administration of the neuropeptide galanin has been reported to have fast antidepressant efficacy. Furthermore, GAL is involved in hypothalamic–hypophysiotropic signalling and cosecreted with luteinizing hormone-releasing hormone (LHRH), possibly acting as a mediator of estrogen action.

Methods: In this study six single nucleotide polymorphisms (SNPs) within the gene coding for GAL were analyzed for possible associations with diagnosis and severity of symptoms in 121 male and female patients suffering from panic disorder (PD).

Results: Our results suggest an association between genetic variations in the GAL-gene and severity of PD-symptoms in female patients. The most pronounced effects could be observed for two haplotypes containing the closely linked, non-protein-coding SNPs rs948854 and rs4432027. Both polymorphisms are located within CpG-dinucleotides in the promoter region of GAL and thus might be involved in epigenetic regulation of the GAL-gene.

Limitations: A relatively small patient sample was analyzed in this study, the herein presented results need to be validated in independent studies.

Conclusions: The results of this study underline the potential of further genetic research concerning GAL and a possible role of this neuropeptide in the pathogenesis of female PD. In this regard, GAL and its receptors appear to be a promising target for pharmacological therapy of anxiety and affective disorders.

Keywords: Panic disorder; Genetics; Galanin; Association study

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1. Introduction

Patients suffering from panic disorder (PD) present themselves with recurrent attacks of intense fear, typically accompanied by somatic symptoms such as accelerated heart rate, sweating, dyspnea, nausea or abdominal distress. PD may be accompanied by agoraphobia, which is defined by a set of phobias mainly concerning fears of having a panic attack in places, where escape may appear to be difficult. As uniform as the cluster of symptoms underlying PD may seem, the extent of fears and the resulting limitations for private and job-related life show significant variations between individual patients.

Epidemiologic studies on PD in different countries show life-time prevalences up to 2.9% (Goodwin et al., 2004; Narrow et al., 2002; Weissman et al., 1997), and family studies suggest a reasonable impact of genetical heredity on predisposition and course of disease (Hettema et al., 2001; Kessler, 2002). Several studies describe genetic associations with PD, so far mainly focussing on the serotonergic system (Erhardt et al., 2007; Freitag et al., 2006; Inada et al., 2003; Maron et al., 2005; Rothe et al., 2004, 2006; Strobel et al., 2003; Thoeringer et al., 2007; Unschuld et al., 2007).

Galanin (GAL) is a 30-amino acid non-C-terminally amidated peptide, expressed by a gene that is located at 11q13.3–q13.5 (Evans et al., 1993; Nicholl et al., 1995). From animal studies an involvement of GAL is known for different physiological and behavioral functions, such as cognition, pain perception, feeding behavior, neuroendocrine control, sleep, depression-like and anxiety-related behavior (Barrera et al., 2006; Holmes et al., 2003; Kuteeva et al., 2005, 2007; Murck et al., 2004; Steiger, 2007; Steiger and Holsboer, 1997; Swanson et al., 2005; Vrontakis, 2002). A recently published study describes antidepressant-like activity of a systemically administered galanin receptor type-3 (GALR3) antagonist in mice and rats (Barr et al., 2006).

GAL is widely expressed both in the peripheral nervous system (PNS), the endocrine system and the central nervous system (CNS). It is closely associated with ascending monoamine pathways and colocalization has been described amongst others with the neurotransmitters noradrenaline (NA) and serotonin (5-HT) (Fuxe et al., 1998). Galaninergic transmission has been shown to modulate activity of NA- and 5-HT responsive neurons in limbic areas that are involved in the regulation of emotionality and anxiety-related behavior, such as the ventral tegmental area, the dorsal raphe nuclei and locus coeruleus (Melander et al., 1986; Skofitsch and Jacobowitz, 1985; Weiss et al., 1998; Wrenn and Crawley, 2001; Xu et al., 1998b).

Moreover, galaninergic-messaging has been described to be interlinked with the hypothalamic–pituitary–adrenal (HPA)-axis in different ways. In the paraventricular nucleus (PVN) of the hypothalamus GAL is coexpressed with corticotropin releasing hormone (CRH) and Vasopressin (Mazzocchi et al., 1992) and has been suggested to affect plasma ACTH- and cortisol-concentrations in humans (Arvat et al., 1995). Depending on the site of its administration in the CNS, GAL has been described to exert influence on HPA-axis responses to stress (Hooi et al., 1990; Khoshbouei et al., 2002; Malendowicz et al., 1994). Changes in HPA-axis response to stress seem to be a central finding also in PD (Erhardt et al., 2006; Heuser et al., 1994; Schreiber et al., 1996). GAL has been shown to be colocalized with luteinizing hormone-releasing hormone (LHRH) in the hypophysis. It has been suggested to influence HPA-mediated reproductive functions and possibly acts as a mediator of estrogen action, regulating activity of LHRH neurons in the context of ovulation (Lopez et al., 1991; Merchenthaler, 2005).

Although results from animal studies imply a substantial influence of GAL on anxiety-like and depression-related behavior (Barr et al., 2006; Holmes et al., 2003; Kuteeva et al., 2005, 2007), there is little information about the effect of genetic variations within GAL on the extent of psychiatric diseases in humans. Polymorphisms in the GALR3-gene and GAL haplotypes, respectively, have been described to be associated with alcoholism, possibly by increasing vulnerability in the context of anxiety-related personality traits (Belfer et al., 2006, 2007). To our knowledge, no further genetic associations of GAL-polymorphisms with psychiatric diseases have been described so far.

Therefore, we investigated genetic associations between 6 SNPs, equispacedly located in the GAL-gene and the clinical diagnosis of PD as well as severity of symptoms in 121 German patients suffering from PD. Because of the reported involvement of GAL in female reproduction via effects on LHRH signalling (Lopez et al., 1991; Merchenthaler, 2005), a secondary analysis for gender-specific effects was also performed.

2. Methods

2.1. Patients

121 patients from our Anxiety Disorders Outpatient Clinic with the primary psychiatric diagnoses PD with agoraphobia (87.4%) or PD without agoraphobia (12.6%) (Table 1) were included. Exclusion criteria were anxiety disorders in the context of a medical or neurological

Table 1
Composition of the examined patient and control sample, matched for age and gender

Sample		N	Mean age	SD	Mean PAS-score	SD	p-value case–control (FPM)	p-value PAS (FPM)
Females	Controls	161	39.28	12.46	–	–	n.s.	0.019
	PD patients	81	41.46	13.23	31.52	9.63		
Males	Controls	62	36.81	10.77	–	–	n.s.	n.s.
	PD patients	40	41.78	9.80	28.85	10.45		

Displayed are resulting *p*-values for case–control and symptom–severity (PAS) associations (FPM, 1000 permutations). Non-significant *p*-values are abbreviated "n.s.".

condition. Furthermore, comorbid generalized anxiety disorder, depression or axis II disorders at the time the panic attacks started led to exclusion. Clinical diagnosis was ascertained with the German version of the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1997; Wittchen, 1999) by trained senior psychiatrists. All patients underwent a clinical examination including EEG, ECG, detailed hormone laboratory assessment and most received cranial nuclear magnetic resonance imaging. The "Panic and Agoraphobia scale" (PAS) total score, reflecting the symptoms during the worst period (Bandelow, 1995), was used for the assessment of severity of PD. Self-reports about nationality, first language and ethnicity of the subject and all four grandparents were obtained from every patient. All patients were Caucasian, and 82% of German origin.

2.2. Controls

The control sample used in this study consisted of 223 individuals, who were matched for ethnicity, sex and age according to the PD-patient sample (Table 1). All controls were selected randomly from a Munich-based community sample and screened for the absence of anxiety and affective disorders using the Composite International Diagnostic Screener (Wittchen, 1999). Only individuals negative in the screening questions for the above-named disorders were included in the sample. The study protocol was approved by the ethics committee of the Ludwig-Maximilians-University in Munich and written informed consent was obtained from all subjects.

2.3. DNA preparation

On enrolment in the study, 40 ml of EDTA blood was drawn from each participant and DNA was extracted from fresh blood using the Puregene whole blood DNA-extraction kit (Gentra Systems Inc; MN).

2.4. SNP selection and genotyping

A total of 6 SNPs located as evenly spread as possible within the GAL-gene were selected from public databases (e.g. dbSNP http://www.ncbi.nlm.nih/) (Table 2). A SNP search tool developed at the Institute for Human Genetics, Technical University Munich and GSF-National Research Centre for Environment and Health, was used to download SNP sequences (http://ihg.gsf.de/ihg/snps.html). For all SNPs the July 2005 Human Reference Sequence (hg17, University of Santa Cruz, http://genome.ucsc.edu/) was used. Genotyping was performed on a MALDI-TOF mass-spectrometer (MassArray® system) with the Spectrodesigner software package (Sequenom™; CA) for primer selection and the homogeneous mass-extension process for producing primer extension products (Binder et al.,

Table 2
Analyzed SNPs within the GAL-gene

SNP-ID	DNA-position	Position within GAL	Alleles	HWE-p-value		Minor allele — frequency	
				Controls	Cases	Controls	Cases
rs948854	68206779	Promoter	A/G	0.225	0.677	0.263	0.281
rs4432027	68207823	Promoter	C/T	0.155	0.588	0.26	0.285
rs3136537	68210005	Intron	A/C	1	1	0.034	0.013
rs2513279	68210742	Intron	C/T	0.267	0.641	0.21	0.217
rs3136540	68212986	Intron	C/T	0.806	0.109	0.242	0.111
rs1042577	68215046	mRNA-UTR	A/G	0.472	0.103	0.313	0.335

Positions according to the July 2005 Human Reference Sequence (UCSC Version hg17; http://genome.ucsc.edu/). All SNP-Information was retrieved from dbSNP (http://www.ncbi.nlm.nih.gov/) and GeneCards (http://www.genecards.org).

2004). Genotyping was performed at the Genetic Research Center GmbH, Munich, Germany. All primer sequences are available upon request.

2.5. Statistical analysis

All genotypes were tested for deviations from the Hardy–Weinberg equilibrium (HWE) by applying chi-square tests. HAPLOVIEW (Barrett et al., 2005) was used for pairwise calculations of linkage disequilibrium (LD) and generation of 95% confidence bounds on D-prime (D′) (Gabriel et al., 2002). Fisher's product method (FPM) was used as a multivariate test of associations within GAL-SNPs (Fisher, 1932), haplotype analyses were performed using the COCAPHASE and QTPHASE modules of UNPHASED (Dudbridge, 2003). All reported *p*-values including haplotypes are corrected for multiple testing by permutation tests (1000 permutations for SNPs, 2000 for haplotypes) according to Westfall and Young (1993).

3. Results

We analyzed six polymorphic SNPs located in the GAL-gene with heterozygosity consistent with HWE (Table 2).

Calculation of pairwise LD of the analyzed SNPs provided by the program HAPLOVIEW resulted in two LD blocks within GAL, consisting of the SNPs rs948854–rs4432027 (*r*-square = 0.99, block 1) and rs3136540–rs1042577 (*r*-square = 0.65, block 2), respectively (Fig. 1). As indicated in Fig. 1, pairwise measures between block 1 and 2 result in *r*-square values > 0.5, suggesting a high degree of LD.

None of the analyzed SNPs within GAL turned out to be significantly associated with the diagnosis PD, neither for single SNPs nor in a combined multivariate analysis (FPM). A secondary analysis for gender differences did not show significant case/control associations either (Table 1).

Severity of PD was assessed with the PAS (Bandelow, 1995) and evaluated for associations to the genotyped GAL-SNPs in the PD-patient sample. After performing separate analyses for both genders, a significant combined effect of the GAL-SNPs (FPM) on symptom–severity could be found for female patients ($p = 0.019$ after correction for multiple testing); no association was observed for the male patient sample; male and female patients did not differ in demographic (age) or clinical variables (PAS) (Table 1).

Most pronounced association-effects with female PD could be observed for two haplotypes, containing the

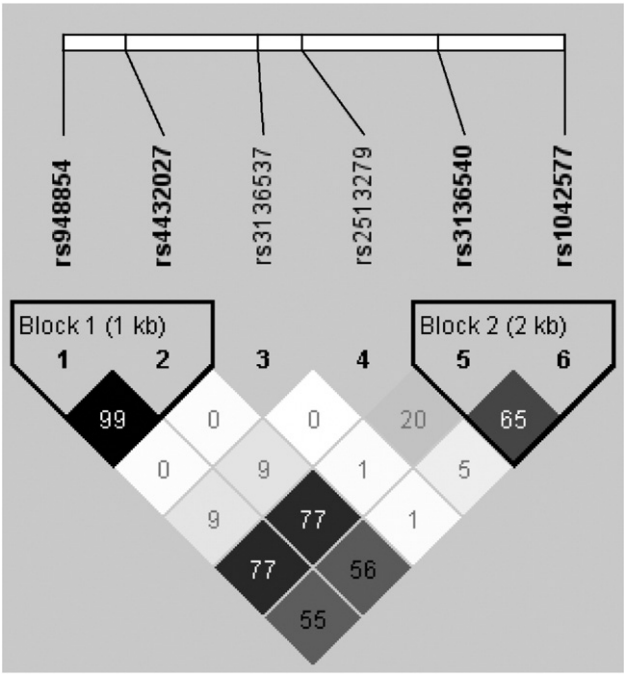

Fig. 1. Linkage disequilibrium (LD) — analysis of six SNPs (Table 2) using HAPLOVIEW. R^2-values are indicated for pairwise measures (Barrett et al., 2005). Pairs of SNPs with evidence for strong LD are colored dark grey, for minor LD light grey and those with evidence for recombination are marked white, numbers indicate R^2-values. LD blocks are displayed representing 95% confidence bounds on D′ (Gabriel et al., 2002).

Table 3
Secondary analysis of female PD patients for single-locus and haplotype effects

SNP	Allele/haplotype	Frequencies	Mean PAS-score	Variance	Likelihood ratio test	Degrees of freedom	Nominal *p*-value
rs948854	A	0.769	29.44	97.8	5.756	1	0.016
	G	0.231	33.62				
rs4432027	C	0.236	33.58	97.78	5.796	1	0.016
	T	0.764	29.42				
rs948854 s4432027	A-T	0.764	29.42	97.77	5.800	1	0.016
	G-C	0.231	33.62				
rs4432027 rs3136537	**C-A**	**0.224**	**34.87**	**92.15**	**9.167**	**1**	**0.0025**
	T-A	**0.764**	**29.53**				
rs948854 rs4432027 rs3136537	**A-T-A**	**0.764**	**29.53**	**92.41**	**9.230**	**1**	**0.0024**
	G-C-A	**0.218**	**34.95**				
rs4432027 rs3136537 rs2513279	C-A-C	0.153	34.18	92.77	9.662	3	0.022
	C-A-T	0.073	36.32				
	T-A-C	0.626	29.77				
	T-A-T	0.136	28.39				

Displayed are only nominally significant associations obtained by application of the program QTPhase. *p*-values in bold are significant ($p<0.05$) after correction for multiple testing by performing 2000 permutations of randomized data-sets (Westfall and Young, 1993).

non-protein-coding SNPs rs948854, rs4432027 and rs3136537 (Table 3). The above-mentioned haplotype associations are significant ($p<0.05$) after correction for multiple testing by performing 2000 permutations of randomized data-sets (Westfall and Young, 1993).

4. Discussion

We observed an association between genetic variations within the gene coding for GAL and severity of PD in German female patients. This association was significant when a multivariate analysis with all of the six analyzed SNPs of the GAL-gene was applied (Fisher's product method, *p*-value = 0.019 (Table 1) or haplotype testing was performed ($p<0.05$). Both results are corrected for multiple testing according to Westfall and Young (1993). As illustrated in Table 3, single-allele and haplotype analysis showed most pronounced effects for haplotypes containing the SNPs rs948854, rs4432027 and rs3136537. The closely linked SNPs rs948854 and rs4432027 (Fig. 1), are both located within CpG-dinucleotides in the GAL-promoter region (http://genome.ucsc.edu/) (Kofler et al., 1995). This finding suggests a possible role in epigenetic regulation of promoter function. None of the associated SNPs is located in a protein-coding region (Table 2). As calculations using the program HAPLOVIEW suggest a high degree of LD within GAL (Fig. 1), LD with a polymorphism in coding regions is imaginable. Absence of significant case/control differences suggests that the GAL-gene polymorphisms are likely to affect disease severity in PD patients rather than the susceptibility to develop PD.

So far particular interest in research on anxiety disorders has been focused on the monoaminergic system and results from genetic association studies suggest, that genes coding for 5-HT and NA may contribute to the susceptibility to PD (Freitag et al., 2006; Inada et al., 2003; Maron et al., 2005; Rothe et al., 2004, 2006; Strobel et al., 2003; Unschuld et al., 2007). Additionally, changes in 5-HT-signalling are connected with specific changes of activity of the HPA-axis (Keck et al., 2005; Van de Kar et al., 2001). Much evidence has been accumulated suggesting that impaired HPA-regulation is involved in causality and course of affective disorders (Binder et al., 2004; Holsboer, 2000).

The neuropeptide GAL has been described to have a hyperpolarizing effect on 5-HT-secreting neurons in the dorsal raphe nuclei- and locus coeruleus-area mediated by the galanin receptor 1 (GALR1) and GALR3 (Branchek et al., 2000; Xu et al., 1998a; Xu et al., 1998b) which results in decreased 5-HT release in the forebrain (Kehr et al., 2002; Yoshitake et al., 2003). Anxiolytic and antidepressant-like effects have been reported in both mice and rats for GALR3 antagonists, which partially reverse the galanin-evoked inhibition of 5-HT secretion (Barr et al., 2006; Swanson et al., 2005). Another study demonstrated an attenuation of depression-like behavior in rats by intracerebrovascular application of a GAL antagonist, which could be reversed by infusion of GAL itself (Kuteeva et al., 2007). In patients, intravenous application of GAL has been reported to exert fast antidepressant efficacy (Murck et al., 2004). Knockout mice for the centrally abundant GALR1 showed an increase of anxiety-like

behavior under different experimental conditions of stress (Holmes et al., 2003). This observation underlines a potential role of GAL in modulating anxiety-like behavior and may be interpreted as an anxiolytic effect mediated by GALR1. Pharmacological agents targeting the GAL-system, have been suggested in this context to hold promise for the treatment of anxiety disorders and depression in humans (Holmes and Picciotto, 2006), future clinical studies may provide further insight towards their potential therapeutic efficacy.

Furthermore, GAL has been described to play an important role in the reproduction process of rats as it is colocalized and coexpressed with luteinizing hormone (LH)-releasing hormone (LHRH) in a subset of LHRH neurons in the hypothalamus (Lopez et al., 1991). Its expression-pattern is sexually dimorphic, involving a higher level of galanin mRNA and peptide expression in females than in males. It has been suggested, therefore, that the role of galanin may be unique to females and may be neonatally determined by an epigenetic mechanism (Merchenthaler, 1998).

Estrogens play the most important role among several other factors controlling the activity of LHRH associated neuronal systems (Herbison and Pape, 2001). A recent study has described a direct effect of estrogens on GAL-expression via estrogen receptor-beta (ERbeta) in LHRH neurons. As GAL is a potent LHRH releasing peptide, it appears to play a central role in mediating pre- and ovulatory estrogen action in the female rat (Merchenthaler, 2005).

The peptide corticotropin releasing hormone (CRH) has been described to suppress gonadal functions in situations of prolonged stress (Almeida et al., 1993; Dudas and Merchenthaler, 2006; Mitchell et al., 2005). The expression of CRH-binding protein however, is positively regulated by LHRH, highlighting the importance of the pituitary gonadotrope as a potential interface between stress and reproductive axes (Westphal and Seasholtz, 2005). We suggest that the neuropeptide GAL may serve as an integrating element, playing a pivotal role in modulating stress-related neural pathways. The fact, that our data indicate an association of polymorphisms within GAL among female PD patients only is in line with a sexually dimorphic function of GAL modulating estrogen and LHRH. However, as a relatively small patient sample was analyzed in this study, the obtained data-set has a preliminary character and the herein presented results need to be validated in independent studies. Particularly in order to identify functional variants, further finemapping of the GAL-gene appears promising. The results of this study underline the potential of further genetic research concerning GAL and its receptors and a possible role of this neuropeptide in the pathogenesis of female PD.

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