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### ► To cite this version:

Paul Gerson Unschuld, Marcus Ising, Darina Roeske, Angelika Erhardt, Michael Specht, et al.. Gender specific association of galanin polymorphisms with HPA-axis dysregulation, symptom severity and antidepressant treatment response.. *Neuropsychopharmacology*, 2010, n/a (n/a), pp.n/a-n/a. 10.1038/npp.2010.30 . hal-00518313

**HAL Id: hal-00518313**

**<https://hal.science/hal-00518313>**

Submitted on 17 Sep 2010

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**Gender specific association of galanin polymorphisms with HPA-axis dysregulation,  
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**Running title:**

Galanin SNP associations in anxiety and depression

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## **Abstract**

*Galanin (GAL)* is an estrogen inducible neuropeptide, highly expressed in brain-regions reported to be involved in regulation of mood and anxiety. *GAL* possibly has a direct modulatory effect on hypothalamic-pituitary-adrenal (HPA) -axis regulation. Recent data from pharmacological and genetic studies indicate a significant role of *GAL* in stress-related disorders.

By using a tag SNP approach covering the locus encoding *GAL* (*PPGAL*), earlier findings of female-specific associations of polymorphisms in this locus with panic-disorder were expanded to a larger sample of 268 outpatients with anxiety-disorders (AD). Within a larger sample of 541 in-patients with major depressive disorder (MDD), we then tested associations of one *PPGAL* tag SNP with specific depression symptom clusters and HPA-axis activity assessed by the combined dexamethasone-suppression / CRH-stimulation test both at in-patient admission and discharge ( $n=298$ ). Gender-specificity as well as dependence of the association on levels of circulating estrogens was analyzed.

Genotyping revealed high LD in the promoter area of the *PPGAL*-gene, which includes several estrogen response elements. Confirming earlier results, rs948854, tagging this promoter region, was associated with more severe anxiety pathology in female AD-patients, but not in males. In premenopausal female MDD- patients, the same allele of rs948854 was associated with more severe vegetative but not cognitive depressive symptoms at discharge and worse treatment response on antidepressant medication. Furthermore this allele was associated with higher HPA-axis activity at admission. No significant case-control associations could be observed. However, due to power limitations of both patient samples, small effects can not be excluded.

The reported associations in independent samples of AD and MDD support an estrogen-dependent role of *GAL* in pathophysiology of anxiety and depression, affecting response to antidepressant treatment.

**Keywords:** Galanin; association; genetics; anxiety; depression; response

## Introduction

Human *galanin* (*GAL*) is a 30 amino-acid neuropeptide, proteolytically processed from *preprogalanin* (*PPGAL*) (Evans and Shine 1991; Schmidt *et al*, 1991). *PPGAL* is a single-copy gene located on chromosome 11q13.3-13.5, spanning over 6kb of genomic DNA and organized into 6 exons (Rokaeus and Brownstein 1986; Vrontakis *et al*, 1987). Estrogen response elements (ERE) in the promoter-region have been proposed to be responsible for estrogen-induced regulation of *GAL* expression (Howard *et al*, 1997; Kaplan *et al*, 1988). For *GAL* so far three G-protein coupled receptors (*GALR1*, *GALR2*, *GALR3*) have been described, and are expressed in both the central and peripheral nervous system (reviewed in Mitsukawa *et al*, 2008). Genetic associations of *PPGAL* and the genes encoding *galanin receptor 3* (*GALR3*) have been reported for psychiatric phenotypes (Belfer *et al*, 2006; Belfer *et al*, 2005; Levrán *et al*, 2008; Unschuld *et al*, 2008). In animal studies *GAL* has been shown to modulate anxious and depressive behavior (reviewed in Kuteeva *et al*, 2008). Intraperitoneal application of a nonpeptide *galanin* receptor agonist showed antidepressant-like effects in rats (Bartfai *et al*, 2004) and the intravenous application of *GAL* has been reported to have an antidepressant effect in patients with depression under standard antidepressant treatment (Murck *et al*, 2004).

In humans, besides being expressed in anxiety-relevant brain regions such as the locus coeruleus (LC) and amygdala, particularly high concentrations for *GAL* have been reported for the hypothalamus and the pituitary (Gentleman *et al*, 1989; Michener *et al*, 1990), with strong evidence for a regulatory role of *GAL* in the control of anterior pituitary function (Lopez *et al*, 1991; Otlecz *et al*, 1988). The regulatory effect of *GAL* on neuroendocrine stress reactions and its involvement in anxious and depressive symptomatology have been thoroughly reviewed (Kuteeva *et al*, 2008; Mechenthaler, 2008; Picciotto, 2008; Wrenn and Holmes, 2006). It has been hypothesized that *GAL* affects stress related behavior by interacting with monoaminergic

neurotransmitters such as serotonin (5-HT) and noradrenaline (NA), but also with gamma-aminobutyric acid (GABA) (Fuxe *et al*, 1998; Kehr *et al*, 2002; Khoshbouei *et al*, 2002a; Ma *et al*, 2001; Sharkey *et al*, 2008; Tyszkiewicz *et al*, 2008; Xu *et al*, 1998). *GAL* expression in the LC and limbic nuclei may be stimulated by stress (Holmes *et al*, 1995; O'Neal *et al*, 2001; Sweerts *et al*, 1999) and appears to have a significant impact on HPA-axis signalling (Brogan *et al*, 1999; Khoshbouei *et al*, 2002b; Lopez *et al*, 1991; Malendowicz *et al*, 1994). In preclinical and clinical studies corticotropin-releasing hormone (CRH) and vasopressin (AVP) have been demonstrated to be main effectors of HPA-activity, important for the mediation of anxious and depressive-like behavior (Britton *et al*, 1986; Holsboer, 2000; Landgraf, 2006; Nemeroff *et al*, 1984; Pepin *et al*, 1992; Stenzel-Poore *et al*, 1994; Strohle *et al*, 1998). There is both an anatomical and functional connection between *GAL* and the CRH/AVP system, as some hypothalamic neurons in the paraventricular nucleus co-express these peptides (Arvat *et al*, 1995; Ceccatelli *et al*, 1989; Leibowitz, 1998; Mazzocchi *et al*, 1992) and *GAL* inhibits stress-induced ACTH secretion, presumably by altering CRH and/or AVP release from nerve terminals in the median eminence (Hooi *et al*, 1990).

This concatenation of data thus suggests that *GAL* may be important in anxiety- and depression-related behavior and also HPA-axis regulation. In an earlier study, we could show a gender-specific association of *PPGAL*-promoter polymorphisms with severity of anxious pathology in a small sample of female panic-disorder patients (Unschuld *et al*, 2008). The aim of this study was to expand our initial finding in female panic-disorder patients using an increased patient sample with panic disorder as well as other anxiety disorders and a sample of patients with major depressive disorder (MDD). Since our initial report identified female gender-specific associations and EREs have been identified in the *PPGAL* promoter area, we hypothesized that the female specific effects in our previous report (Unschuld *et al*, 2008) might be related to differences in

circulating estrogen levels and therefore stratified our analyses not only by gender but also menopause-status, which was possible in the larger MDD sample. Because of the observed genetic associations with anxious depression in this analysis and the reported worse antidepressant treatment response in patients with anxious depression (Fava *et al*, 2009), we also tested whether *PPGAL* polymorphisms would associate with treatment response, using the HAMD rating scale. Finally, we examined whether menopause status-specific associations can also be observed on the neuroendocrine level, using the combined dexamethasone-suppression / CRH-stimulation test (Dex-CRH) test. As described above, interactions of the *GAL* system on the HPA-axis have been reported.

## **Materials and Methods**

### **Study population**

The present study protocol was approved by the local ethics committee and is in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Only individuals over 18 years were included, written informed consent was obtained from all participating subjects. Ethnicity was recorded using a self-reported sheet for perceived nationality, language and ethnicity of the subject itself, parents and grandparents. The level of anxiety and depression symptoms is described in table 1.

### **Anxiety disorder (AD)-patients**

The AD-outpatient sample included 268 individuals (62% females, 80% of the females younger than 46 years), recruited at their first visit with the following anxiety disorders at the Max-Planck-Institute of Psychiatry (MPI): panic disorder with agoraphobia (71.6%), panic disorder without agoraphobia (12.3%) and social phobia (8.1%), agoraphobia (2.4%), specific phobia (3.3%) and generalized anxiety disorder (2.4%). These include the 121 panic disorder patients analyzed in Unschuld *et al*, 2008. All patients underwent a clinical examination including EEG and ECG. Exclusion criteria were anxiety disorders in the context of substance abuse, a medical or neurological disorder and a concurrent major depressive, bipolar or axis II disorder. Affective and anxious pathology was assessed at the patient's first presentation using Hamilton depression (HAMD) rating scales (Hamilton, 1960) (table 1). As a measure of severity of anxious pathology, indicating particularly phobic avoidance and impairment of life-quality, the Bandelow-panic and agoraphobia subscale B (PAS-B) was used (Bandelow, 1995). In our previous study a different scale was used, assessing severity of panic attacks (Unschuld *et al*, 2008). 89% of the AD-



patients were German Caucasians, other nationalities were Central European countries and the U.S. (with German Caucasian ancestry).

### **Major depressive disorder (MDD) patients**

The MDD-patient sample consisted of 541 patients (59% females, 44% of the females younger than 46 years), all in-patients at the MPI of Psychiatry with the diagnosis of unipolar depression (25.9% first episode, 74.1% recurrent) and participants of the MARS-study (Hennings *et al*, 2009). All patients were included within the first three days of their stay in this study and underwent clinical examination including EEG and ECG. Exclusion criteria were known manic or hypomanic episodes, mood incongruent psychotic symptoms, substance abuse and a major internal medical or neurological disorder. Affective pathology was assessed using Hamilton depression (HAMD) rating scales, 21 item scale (Hamilton, 1960) and further characterized using the factorial HAMD subscales for cognitive (HAMDCog) and vegetative depression (HAMDVeg) established by Rhoades and Overall, 1983. Psychometric assessments were performed at in-patient admission and discharge, at the time points at which the combined Dex-CRH test was used for analysis (table 1). Time from admission to psychometric assessment was used as a covariate in association calculations. Additionally, for each patient completing the study, the individual difference of overall HAMD at admission and discharge was calculated (HAMDDiff). A HAMD-score at discharge below 10 was used as a threshold for remission as described earlier (Ising *et al*, 2009). During their hospital stay, all MDD in-patients were treated according to the doctor's choice using a combination of psychopharmacological and cognitive-behavioral therapy efforts. Antidepressant treatment at the time-point of the initial Dex-CRH test was mainly monotherapy (56.2%), of which 25.1% were on selective serotonin re-uptake inhibitors (SSRIs), 12.1% on tricyclic antidepressants (TCA) and 14.7% on Mirtazapine as the three largest groups. 8.1% of the patients did initially not receive psychopharmacological treatment. 42.2%

concomitantly received benzodiazepines, 2.1% mood-stabilizers (except Carbamazepine) and 3.7% neuroleptic medication. A previous study had shown that number and length of ineffective antidepressant treatment trials prior to hospitalization and type of medication at the time point of the test other than Carbamazepine did not influence the endocrine response in the Dex—CRH test at admission (Kunzel *et al*, 2003).

### **Healthy controls**

The control sample used in this study consisted of 541 individuals (52% females), who were matched for ethnicity, sex and age to the MDD sample. All controls were screened for the absence of anxiety disorders, depression or substance dependence using the Composite International Diagnostic Screener (Wittchen, 1999) and current depressive symptomatology was recorded using the Beck-Depression-Inventory (BDI) (Beck *et al*, 1961) (table 1). Only individuals without any severe neurological or medical disorder and axis I disorder were included in the study. 91.7% were German Caucasians, other nationalities were Central European countries and the U.S. with German Caucasian ancestry.

### **Dex-CRH test**

The Dex-CRH test was performed as described in detail earlier (Heuser *et al*, 1994b). Patients were administered the test within the first 10 days of admission and the last 10 days of discharge. Briefly, patients were orally pre-treated with 1.5 mg of dexamethasone at 23:00h. The following day a venous catheter was placed at 14:30h and blood was drawn at 15:00h, 15:30h, 15:45h, 16:00h and 16:15h into tubes containing EDTA and Trasylol (Bayer Inc., Germany). At 15:02h 100µg of human CRH (Ferring Inc., Kiel, Germany) was administered IV. For the area under the curve (AUC) of the cortisol (CAUC) and ACTH (AAUC) response, the area under the concentration- time course curve was computed using a trapezoidal integration for the test at

admission as well as at discharge. Time from admission to the first Dex-CRH test was used as a covariate in association calculations, however there was no correlation with CAUC and AAUC at admission ( $r = -0.024$ ;  $p = 0.63$  and  $r = -0.031$ ;  $p = 0.52$ , respectively) and no significant difference between genders.

Dex-CRH testing was performed in 298 MDD-patients (53% females), at a mean of 6.04 (SD = 2.15) days after admission, and 245 of these patients additionally in the last week before discharge, after a mean hospital-stay of 11.7 (SD = 8.6) weeks.

### **Grouping of female MDD patients according to menopausal status.**

Women younger than 46 years at the time-point of the Dex-CRH test were categorized as high-estrogen premenopausal and women older than 50 years as low-estrogen, postmenopausal. This categorization concurs with earlier reports of incidence of menopause in western industrialized countries (Matthews *et al*, 1989). Women aged between 46 and 49 years were excluded from these analyses. In a subgroup of patients, plasma sex-hormone levels were assayed for samples taken at the time of the admission Dex-CRH test and subdivided in premenopausal and postmenopausal women. Estrogen levels were significantly higher, LH and FSH levels lower in premenopausal ( $n=28$ ) than in postmenopausal ( $n=27$ ) MDD-in-patients (*mean* (SEM): estradiol pg/ml = 69.32 (11.81) vs. 27.83 (4.63),  $p = 0.01$ ; FSH  $\mu$ U/ml 20.93 (7.07) vs. 70.03 (10.19),  $p = 0.001$ ; LH  $\mu$ U/ml 10.53 (2.33) vs. 30.82 (4.68),  $p = 0.002$ ).

### **Hormone assays**

Briefly, for the measurement of plasma cortisol concentrations, a radioimmunoassay (RIA) kit (ICN Biomedicals, Carson, CA) was used where the detection limit was 0.3 ng/ml. For plasma ACTH concentrations an immunometric assay without extraction (Nichols Institute, San Juan Capistrano, CA, U.S.) was used with a detection limit of 4.0 pg/ml. In 59 female MDD-patients

additionally to Dex-CRH testing, plasma estradiol, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations were measured by commercially available ELISA. The intra-assay variations were between 5.6 and 8.2%, the interassay variations were in the range of 6.6 - 8.7%.

### **DNA preparation, SNP selection and genotyping**

On enrollment in the study 40ml of EDTA blood was drawn from each subject and DNA was instantly extracted using a standardized procedure (Puregene whole blood DNA-extraction kit; Gentra Systems Inc., MN, U.S.). 6 SNPs (rs2187331, rs1893679, rs3136540, rs3136541, rs1042577, rs6591350) genotyped using the Illumina Sentrix Human-1 Genotyping 100K and 300K BeadChip systems (Illumina Inc., San Diego, CA, U.S.) are located within the *PPGAL* locus and were included in the analysis. The SNP rs948854, which is not included in the HapMap CEU dataset and thus the Illumina arrays, was assessed using the MALDI-TOF Sequenom (Sequenom Inc., San Diego, CA, US) genotyping platform as described earlier (Binder *et al*, 2004). rs948854 was included as it had shown associations with the tested phenotypes in an earlier report of our group (Unschuld *et al*, 2008). These seven SNPs located within the *PPGAL* locus and 5.6 kb 5' flanking region were genotyped in 541 healthy controls. The call rate for each SNP was >99.6% (*mean* 99.9%), and minor allele frequencies all exceeded 15%. No SNP showed a significant deviation ( $p < 0.05$ ) from Hardy-Weinberg equilibrium. SNP genotyping was performed at the Center for Applied Genotyping (CAGT), Munich, Germany. The program HaploView 4.0 (Barrett *et al*, 2005) was applied to determine linkage disequilibrium (LD). By using a cut-off for  $r^2 > 0.8$ , three tag SNPs (rs2187331, rs948854 and rs1042577) covering *PPGAL* were identified (figure 1) and selected for the genetic analyses to be performed in 268 AD patients. These three selected SNPs cover 100% of the genetic variation described in HapMap version II in the region chr11:68202000-68216000 at  $r^2$  greater than 0.8 (cut-off for

minor allele frequencies greater than 0.05, <http://www.hapmap.org>) in the Caucasian CEU population. As rs948854 is not included in the HapMap dataset, the adjacent polymorphism rs1893679, which is predicted in our control sample by rs948854 at  $r^2$  0.95, was used together with the tag-SNPs rs2187331 and rs1042577 calculate to the genetic coverage of the 3 tag SNPs for the *PPGAL* region in the CEU population.

### **Statistical data analysis**

Statistical analyses were performed using the WG-Permer software, a tool for rapid analysis of large scale genome association studies (Ripke, 2007, [www.wg-permer.org](http://www.wg-permer.org)) and the commercial SPSS-statistics package, version 16.0 (©2007 SPSS inc.). As the AD-patient sample was – in contrast to the MD-sample - not matched for age and sex with the control sample, age and sex were considered as covariates in the case-control analyses by using logistic regression. In the MDD samples, genetic association with psychopathology and the cortisol and ACTH response in the Dex-CRH test were also analyzed stratified by gender and presumed menopausal status. This resulted in three subgroups of MDD-patients (n-counts: psychopathology at admission; at discharge; Dex-CRH test performed): premenopausal women (18-45 years;  $n=102$ ; 91; 61), postmenopausal women (50-80 years,  $n=150$ ; 134; 81) and male MDD-patients ( $n=221$ ; 200; 140). The three groups did not significantly differ in basal cortisol levels or CAUC and AAUC, indicating no effect of gender as described earlier (Hennings *et al*, 2009). There were also no significant differences in any of the psychometric measures.

For the analysis of linkage disequilibrium (LD) and selection of tagging-SNPs, the program HaploView 4.0 (<http://www.broad.mit.edu/mpg/haploview>, Barrett *et al*, 2005) was used. Blocks were defined using the confidence interval method described by Gabriel *et al*, 2002. The association analysis for SNPs and phenotypes was conducted by applying allelic and carrier-

frequency-models. The phenotypes describing severity of anxious- and depressive pathology (PAS-B, HAMD) showed normal distribution in the analyzed samples. However, to increase robustness of the analysis, permutation-based-estimates of p-values were used in patients and controls. The minimum P method of Westfall and Young (WY) with 100,000 permutations was used to correct for multiple testing of tag SNP- and phenotype-associations (Westfall and Young, 1993) both in case-control and dimensional analyses, taking into account possible correlation between predictors. P-value adjustment was performed as follows in the particular samples:

1.) Tests for case-control associations in the AD- and MDD-sample were adjusted for testing of three tag-SNPs in three genetic models (allelic, carriers of the frequent and rare allele respectively) according to Westfall and Young (Westfall and Young, 1993).

2.) Tests for PAS-B associations in the AD sample were adjusted for SNPs and three genetic models, according to Westfall and Young (Westfall and Young, 1993).

3.) For analyses in the MDD sample only rs948854 was analyzed by applying solely an allelic model of genetic testing. Adjustment of p-values for multiple testing was performed using Bonferroni correction (Bland and Altman, 1995) for the number of tests:

a) Interaction terms on HAMD were corrected for testing pre- and post- treatment values.

Interaction terms on Dex/CRH response were corrected for testing pre- and post treatment and additionally ACTH and cortisol.

b) Consecutive exploratory tests for significant HAMD-interactions were corrected for testing the subcategories of assumed estrogen status (premenopausal versus postmenopausal and males) and additionally testing both overall HAMD and HAMDdiff, its subcategories vegetative HAMD and cognitive HAMD. In the consecutive exploratory tests for significant Dex-CRH test interactions, nominal p-values were adjusted for testing the subcategories of assumed

estrogen status and the laboratory values cortisol and ACTH.

The level of significance for all statistical analyses was set to 5% after correction for multiple testing.

Calculations of power to detect case-control associations in the AD-patient and MDD sample respectively were performed using the program Quanto, Version 1.2.4 (<http://hydra.usc.edu/gxe/>) with a level of significance 0.006 (correcting for SNPs and three models of genetic testing), disease allele frequency of  $>0.15$ , an additive genetic model and disease prevalences as reported in the US National Comorbidity Survey Replication (Kessler et al, 2005). Power to detect a genotype relative risk of 1.7 (additive model) in the AD-sample was 88% and in the MDD-sample 98%. Power to detect a female specific genotype relative risk of 1.7 in the AD-sample was 63% and 82% in the MDD sample.

Information about predicted promoter positions, gene-exon boundaries and LD in other populations was retrieved from publicly accessible online-databases ([www.genecards.org](http://www.genecards.org), [www.genome.cse.ucsc.edu](http://www.genome.cse.ucsc.edu), [www.hapmap.org](http://www.hapmap.org), [www.snpper.chip.org](http://www.snpper.chip.org)).

## Results

### **Associations with severity of female AD – expansion of results presented in Unschuld *et al*, 2008.**

In this expanded AD sample, we could support the previously reported association of severity of anxiety symptoms with *PPGAL* SNPs in female patients only. rs948854, but not the two other tag-SNPs showed gender-specific association with anxiety severity as measured using the PAS-B subscale. Female carriers of the rare G-allele of rs948854 showed significantly higher PAS-B scores, when compared with carriers of the more frequent A-allele (PAS-B rs948854 G-allele: 7.6, SE 0.28 and A-allele: 6.86, SE 0.20, allelic model: *nominal*  $p = 0.008$ ,  $p < 0.05$  after correction for multiple testing; carrier-A and carrier-G models not significant after correction for multiple testing). When analyzed separately, carriers of the G-allele of rs948854 show higher severity measures in both the female panic-patients reported in our earlier publication (cohort 1) (PAS-B *means* (SEM): A:6.75(0.42); G:7.54(0.42)) and the newly recruited female patients with different anxiety disorders (cohort 2) ((PAS-B *means* (SEM) A:6.12(0.62); G:7.81(0.41)). No association of severity could be observed for male patients, when an allelic and carrier-model was applied. Stratification by menopausal status was not performed, due to the low N in the postmenopausal group and thus power limitations.

### **Dependence of association with anxiety severity in female patients on menopausal status – evidence from an MDD sample.**

We then tested whether the G-allele of rs948854 was also associated with depression or anxiety symptom severity at admission or discharge in the MDD sample stratified by gender and menopausal status, as this was possible in the larger MDD sample. Using an ANCOVA with HAMD as outcome and rs948854 allele, group (pre-, vs. postmenopausal women or male) and



interaction of SNP by group as predictors and time from admission to discharge as covariate, we observed a significant interaction between SNP and group for HAMD at discharge, but not at admission (HAMD at discharge, allelic model: *nominal*  $p = 0.001$ ,  $F_{1,849} = 10.26$ ,  $p < 0.01$  after correction for multiple testing). There were no significant main effects of the rs948854 allele-status or patient group.

Secondary exploratory testing revealed, that at discharge premenopausal female MDD-in-patients showed a significantly higher HAMD score in carriers of the G-allele of rs948854 compared to carriers of the A-allele (11.55, SE 1.28 vs. 7.34, SE 0.56, allelic model: *nominal*  $p = 0.002$ ;  $p < 0.05$  after correction for multiple testing) and worse response (HAMDDiff, *means*(SEM) G: 16.16(1.41); A: 19.31(74), *nominal*  $p = 0.040$ ). The percentage of patients with HAMD-score at discharge below 10 as a threshold for remission differed in the subgroup of premenopausal patients depending on rs948854 allele status (95% CI): A:61% (48.25-72.44); G:40.25% (32.94-48.02). OR for premenopausal carriers of the rs948854 G-allele for non-remission at discharge was 2.3 (95% CI: 1.26-4.29; *nominal*  $p = 0.006$ ). This may be a reflection of higher vegetative anxious-, but not cognitive depressive symptoms, as analyzed using the HAMD-subcales described above. Premenopausal female carriers of the G-allele of rs948854 showed nominally significant higher values for the vegetative depression subscale, than carriers of the frequent allele (HAMDVeg: 0.94 SE 0.15 and 0.61 SE 0.06, allelic-model: *nominal*  $p = 0.039$ ) but no significant difference in the subscale cognitive depression. These differences were neither observed in postmenopausal women nor in male patients (figure 2).

### **Associations of rs948854 with HPA-axis activity in premenopausal MDD**

Subsequently we tested whether the G-allele of rs948854 was associated with the endocrine response in the Dex-CRH test in any of the three MDD-patient groups. An ANCOVA was used

with either CAUC or AAUC as outcome and rs948854 allele, group (pre-, vs. postmenopausal women or male) and interaction of SNP by group as predictors and time from admission to test as covariate. We observed a significant interaction between SNP and group for AAUC and CAUC at admission, but not at discharge (CAUC, allelic model: *nominal p* = 0.014,  $F_{1,559} = 6.14$ ,  $p < 0.05$  after correction for multiple testing; AAUC, allelic model: *nominal p* = 0.020,  $F_{1,559} = 5.47$ , not significant after correction for multiple testing). There were no significant main effects of the rs948854 allele-status or patient group.

Secondary exploratory tests revealed that carriers of the G-allele of rs948854 in the premenopausal women subgroup had significantly higher cortisol responses in the Dex-CRH test (CAUC rs948854: 4825.35, SE 876.15 and 3223.74, SE 320.67, *nominal p* = 0.005, allelic model;  $p < 0.05$  after correction for multiple testing). ACTH response in the premenopausal women subgroup was significantly higher for carriers of the rare allele of the SNP rs948854 (AAUC rs948854: 1568.70, SE 190.68 and 1139.93, SE 71.79, *nominal p* = 0.001, allelic model;  $p < 0.01$  after correction for multiple testing) (figure 3).

### **Case-control associations**

Using allelic and carrier-models, no significant case-control associations could be observed for the AD-sample and the MDD-sample as a whole or stratified by gender after correction for multiple testing for any of the three tag-SNPs.

## Discussion

Expanding earlier findings of our research group on single-locus and haplotype effects including in particular associations of the rare allele of the *PPGAL* SNP rs948854 with more severe pathology in female panic-disorder patients (Unschuld *et al*, 2008), now a more than doubled sample of patients with anxiety-disorders was analyzed using a tag-SNP approach covering the genetic locus encoding *GAL* and 5.6 kb of its 5' promoter region (figure 1). Consistent with the earlier findings, the rare G-allele of rs948854 was associated with more severe anxious pathology in female but not male patients. Severity of anxiety symptoms in general was measured using the PAS-B scale, which reflects phobic avoidance and resulting every-day limitations of the patients (Bandelow, 1995), while in our earlier study a panic-specific version of this scale was used (Unschuld *et al*, 2008). No case-control association could be observed, however our AD-sample lacked power to detect smaller risks, especially if only tested in women.

As *GAL* has been implicated in the pathophysiology of anxiety as well as depression (reviewed in Kuteeva *et al*, 2008; Mechenthaler, 2008; Picciotto, 2008; Wrenn and Holmes, 2006) and because anxiety symptoms are common in patients with MDD (Fava *et al*, 2009), the present study was extended to an MDD-in-patient sample. The use of this larger sample also allowed to stratify the analyses by menopausal status, as we hypothesized that the gender-specific genetic associations observed in the AD sample were driven by circulating estrogen levels. Functional EREs have been described in the promoter region of *PPGAL* (Howard *et al*, 1997; Kaplan *et al*, 1988). In these analyses we could show that association of symptom severity were specific to the group of premenopausal women. At in-patient discharge, premenopausal MDD-patients, carriers of the rare G-allele of rs948854 showed a significantly higher symptom severity as measured by the HAMD and less remission following antidepressant therapy (OR 2.3, 95% CI: 1.26-4.29).

Analysis of HAMD subscales specific for vegetative vs. cognitive symptoms revealed that this difference was presumably carried by an enhanced vegetative but not cognitive depressive pathology with the rs948854 G-allele. The direction of the association was thus similar to the one observed for the AD sample, with more anxious pathology in female G-allele carriers (figure 2). Vegetative depressive symptoms correlate more closely with anxiety symptoms in depression than cognitive symptoms (Rhoades and Overall, 1983). In a study analyzing symptom stability in recurrent major depression, anxious pathology measured according to the subscales defined by Overall and Rhoades appeared as a particular robust category in terms of symptom-stability across episodes and might indicate an anxious subtype of depressive disorder (Oquendo *et al*, 2004). It has been speculated earlier, that anxious depression might depict a subcategory of MDD, which shows particularly poor response to antidepressive therapy (Fava *et al*, 2009; Ising *et al*, 2009; Nemeroff and Owens, 2002) and may be consistent with our findings of high vegetative symptoms and worse overall treatment response at discharge in premenopausal MDD-patients, carrying the G-allele of rs948854.

Case-control associations were negative for MDD as well as AD. However, it has to be taken into account, that group sizes of both samples are small for case-control studies, especially when stratified by gender, thus lacking power to detect small effects. In addition, a number of phenotypes have been tested in the AD and MDD sample. When correcting for multiple testing as described in the methods section, the associations in female AD patients with severity and in MDD premenopausal women with HAMD at discharge, cortisol and ACTH-response at admission remain significant. The associations of the G-allele of rs948854 with the vegetative HAMD subscale at discharge and HAMD improvement after therapy in premenopausal women only show nominal significance, not withstanding correction for multiple testing, so that these associations need to be interpreted with caution. Misclassification of menopausal status based on

age may have reduced power of this study, as individual conditions of altered estrogen-levels or irregular incidence of menopause are possible unnoticed confounders. However, to minimize effects of misclassification, patients who reported hormone replacement therapy or known medical disorders influencing hormone status were excluded from analyses.

As described in the introduction, *GAL* is also involved in HPA-axis regulation. To explore whether the observed genetic and psychopathological associations are also reflected by an impaired stress hormone regulation, we analyzed the association of rs948854 with plasma cortisol and ACTH response in the Dex-CRH test. We show that the G-allele of this SNP was associated with enhanced plasma cortisol and ACTH response at admission only in premenopausal women with MDD. This might indicate an enhanced dysfunction of HPA-axis regulation in these G-allele carriers also showing enhanced anxiety and vegetative symptoms which might be a reflection of increased CRH and/or AVP signaling. These two neuropeptides have been linked to anxiety symptoms as well as HPA-axis activation in both humans (Bardeleben and Holsboer, 1989; Heuser *et al*, 1994a; Holsboer, 2000; Nemeroff *et al*, 1984; Strohle *et al*, 1998) and animals (Britton *et al*, 1986; Keck *et al*, 2002; Landgraf, 2006), and are co-expressed in neurons with *GAL* (Arvat *et al*, 1995; Ceccatelli *et al*, 1989; Leibowitz, 1998; Mazzocchi *et al*, 1992).

The tag SNP rs948854 captures the genetic variation within a 6.2kb region starting at -1780bp 5' from the *PPGAL* gene (figure 1). This region includes the so far reported EREs at positions -1162, -527, -365, -361, -122 (Howard *et al*, 1997; Kaplan *et al*, 1988; Kofler *et al*, 1995). rs948854 might thus either directly influence binding and activation of the EREs or be in LD with such a functional variant.

In rodents, estrogen has been demonstrated to significantly induce *GAL* expression in behaviorally relevant loci such as hypothalamus, pituitary and LC (Brann *et al*, 1993; Hemmer

and Hyde, 1992; Kaplan *et al*, 1988; Tseng *et al*, 1997). In humans, significantly higher *GAL* serum levels have been measured in premenopausal than in postmenopausal women (Meczekalski *et al*, 2001). rs948854 might alter the function of EREs in the *PPGAL* promoter and thus the induction of *GAL* by estrogens. As anxiogenic as well as anxiolytic effects of *GAL* have been reported (reviewed in Kuteeva *et al*, 2008), it is difficult to predict whether the G-allele of this SNP is associated with enhanced or reduced *GAL*-induction by estrogen. Possibly estrogens can also moderate the described stress-related induction of *GAL*, providing an additional mechanism for the gender and menopause-status dependent SNP associations.

*GAL* has been suggested earlier to modulate the release of CRH and/or AVP, as it attenuates stress-induced ACTH secretion (Hooi *et al*, 1990). Stress-related increases in CRH and AVP secretion may be counteracted by *GAL* and thereby lead to differences in anxious behavior and HPA-axis activation in the two genetic promoter variants. rs948854 likely influences estrogen-driven *GAL*-induction and by that possibly stifle CRH and AVP activation.

The reported associations of *PPGAL*-polymorphisms in two samples of AD- and MDD-patients with severity of anxiety symptoms and HPA-axis activity thus support a role of *GAL* and its receptors in the pathophysiology of anxiety and depression and demonstrate the importance of gender- and hormone status-specific genetic associations.

## **Disclosures / Conflicts of interest**

All the authors, except FH and EBB, declare that, except for the income received from our primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interests. FH wishes to declare, that he personally received grant support from Bristol Myer Squibb, is founder and share holder of Affectis and share holder of Corcept and Neurocrine. EBB wishes to declare, that she personally received grant support from the National Institute of Mental Health and the Doris Duke Charitable foundation.

## **Acknowledgments**

The authors would like to thank Sabine Damast, Maik Koedel and Susann Sauer for excellent technical assistance. This work has been funded by the Bavarian Ministry of Commerce and by the Federal Ministry of Education and Research (BMBF) in the framework of the National Genome Research Network (NGFN), Förderkennzeichen 01GS0481. The authors are responsible for the contents of this publication.



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## Tables

**Table 1:** Statistics of the analyzed samples, ‘adm.’ stands for test scores at admission, ‘dis.’ for discharge. Hamilton depression rating scale (HAMD, 21 items, Hamilton 1960), Panic and Agoraphobia subscale B (PAS B, Bandelow, 1995), vegetative and cognitive depression subscales (Rhoades and Overall, 1983), Beck-Depression-Inventory (BDI, Beck *et al*, 1961).

## Figures

**Figure 1:** LD chart of the analyzed SNPs within the locus encoding *GAL* (*PPGAL*), displaying linkage of the particular markers ( $r^2$ ) calculated using HaploView 4.0 (Barrett *et al*, 2005), with a MAF cut-off at 5% and an  $r^2$  cut-off greater than 0.8. Tagging-SNPs are marked with ‘\*’.

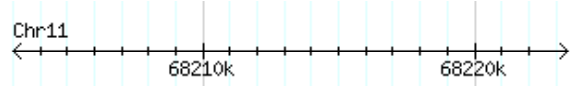
**Figure 2:** rs948854 allele distributions for HAMD (21 items, Hamilton, 1960) and the subscales vegetative and cognitive depression (Rhoades and Overall, 1983) at discharge. Associations significant after correction for multiple testing are labelled with ‘\*\*\*’, nominal significance with ‘\*’.

**Figure 3:** rs948854 dependent levels of Cortisol (ng/ml) and ACTH (pg/ml) measured at admission and discharge of MDD inpatients, displayed separately for the analyzed subsamples: premenopausal women, postmenopausal women and males. At 15:02h IV administration of 100µg CRH was performed, the 15:30h measurement of cortisol and ACTH was the first after stimulation. Solely in the subgroup of premenopausal women at admission significant allele-dependent differences between cortisol and ACTH-levels after stimulation could be observed.

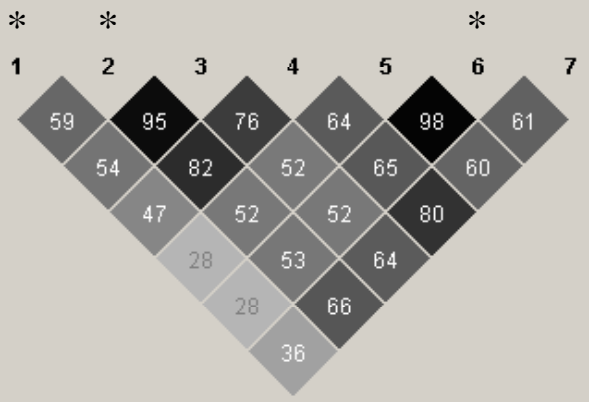
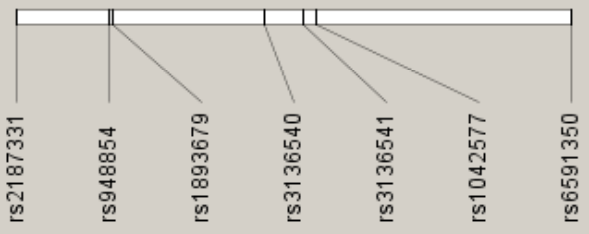


<i>Sample</i>		<i>all patients</i>	<i>females</i>	<i>males</i>
		Mean (SD)	Mean (SD)	Mean (SD)
AD	Age	38.27 (12.09)	39.02 (12.78)	37.05 (10.83)
	Age at onset	27.24 (11.42)	27.65 (11.80)	26.64 (10.82)
	HAMD	13.92 (6.6)	13.64 (6.69)	14.39 (6.46)
	PAS B	6.86 (3.05)	7.06 (2.9)	6.54 (3.27)
MDD	Age	48.38 (13.88)	48.75 (13.96)	47.99 (13.84)
	Age at onset	36.83 (15.62)	35.53 (15.46)	38.20 (15.71)
	HAMD adm.	22.52 (8.89)	23.15 (9.42)	21.9 (8.34)
	HAMD dis.	7.82 (5.88)	8.39 (6.09)	7.27 (5.63)
	Veget. anxiety adm.	2.09 (0.75)	2.18 (0.81)	1.99 (0.67)
	Veget. anxiety dis.	0.68 (0.54)	0.71 (0.57)	0.65 (0.51)
Controls	Age	47.43 (13.46)	47.76 (14.1)	47.04 (12.66)
	BDI	3.74 (3.78)	3.78 (3.70)	3.68 (3.88)

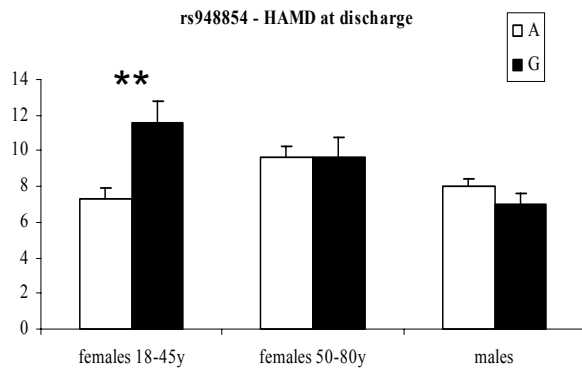
**Table 1.**



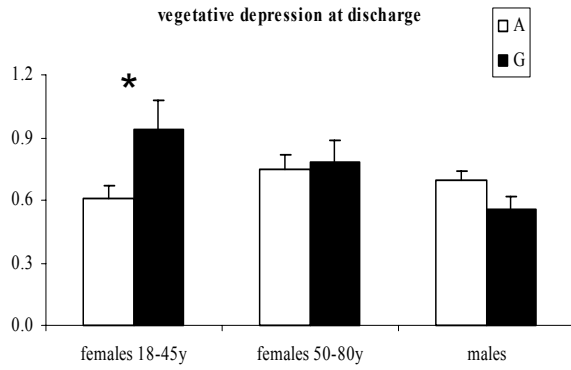
Entrez genes  
 NM\_015973  
 GAL: galanin preproprotein



rs948854 - HAMD at discharge



vegetative depression at discharge



cognitive depression at discharge

