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Mutation Screen and Association Analysis of the Glucocorticoid Receptor Gene (*NR3C1*) in Childhood-Onset Mood Disorders (COMD)

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Depressive disorders are highly heterogeneous psychiatric disorders involving deficits to cognitive, psychomotor, and emotional processing. Considerable evidence links disruption to the hypothalamic–pituitary–adrenal (HPA) axis to the etiology of depression, with specific deficits reported in glucocorticoid receptor (GR)-mediated negative feedback. Given the role of GR-mediated negative feedback in mediating response to stress, and the clear link between stress and depression, it is plausible that polymorphisms in the GR gene (*NR3C1*) act to increase susceptibility. Maternal behavior in rats epigenetically alters a NGF1-A transcription factor binding-site in the promoter region of the GR gene, providing a mechanism by which environmental cues can regulate GR expression and thus response to stress. The analogous region of the human GR gene (*NR3C1*) has not been studied, but it is possible that polymorphisms in this region may alter the binding of transcription factors known to regulate GR expression. In this study, we have performed bioinformatic analyses on the promoter region of *NR3C1* to identify conserved promoter sequences and predicted transcription factor binding sites. These regions were screened with denaturing high-performance liquid chromatography (DHPLC) and direct re-sequencing, and several novel polymorphic variants were identified. We genotyped nine polymorphisms across *NR3C1* in a large sample of Hungarian nuclear families ascertained through affected probands with a diagnosis of childhood-onset mood disorders (COMD). Single-marker analysis provided little evidence for an association of this gene with COMD, but multi-marker analysis across a region of high linkage disequilibrium revealed modest evidence for the biased transmission of several haplotypes.

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INTRODUCTION

Depressive disorders are highly heterogeneous psychiatric disorders involving deficits to cognitive, psychomotor, and emotional processing. They are extremely common, ranking second in the global burden of disease in developed countries [Murray and Lopez, 1997]. Etiological studies have focused on the interplay between genetic and environmental factors, with increasing evidence also suggesting an epigenetic contribution to susceptibility [Mill and Petronis, 2007]. Depressive disorders strongly aggregate in families [Sullivan et al., 2000], and twin studies demonstrate that this familial clustering contains a significant genetic component, with severe, recurrent, and early onset forms of the disorder demonstrating elevated heritability [Thapar and McGuffin, 1994; Rice et al., 2002; Thapar and Rice, 2006].

There is substantial evidence that disruption to the brains' hypothalamic–pituitary–adrenal (HPA) axis is a common etiological feature in depression [Neigh and Nemeroff, 2006]. The HPA axis is a complex set of neuroendocrine interactions between the hypothalamus, the pituitary gland, and the adrenal gland. The HPA axis is integral to an individual's response to stress, and given the link between both chronic and acute forms of life-stress and the onset of depression [Kessler, 1997], dysfunction to this system is an obvious etiological candidate for depressive disorders. This notion is supported by the action of many antidepressants, which restore normal HPA axis activity in depressed individuals [Barden, 2004]. Of particular importance to the etiology of mood disorders is evidence for specific impairment to glucocorticoid receptor (GR)-mediated negative feedback on the HPA axis in patients with major depression [Parker et al., 2003]. While not universally observed in studies of early onset depression [Dahl et al., 1992], recent investigations detect significant HPA axis disruption following exposure to psychosocial stress in children "at risk" for [Ashman et al., 2002] or with [Luby et al., 2003] depressive symptoms. Recent genetic evidence linking the HPA axis disruption to early onset mood disorders comes from a study from our group reporting the vasopressin V1b receptor gene (*AVPR1B*), encoding a G protein-coupled receptor that binds arginine vasopressin (AVP), a protein with a crucial role in modulating the HPA axis under stress, is strongly associated with childhood-onset mood disorders (COMD) in the Hungarian sample studied here [Dempster et al., 2007].

The GR is a ligand-activated intracytoplasmatic transcription factor with high affinity to the glucocorticoids that play an integral role in HPA axis signaling and the stress-response pathway. The GR gene, also known as *nuclear receptor subfamily 3, group C, member 1* (*NR3C1*) is located at 5q31–q32 and comprises nine exons, with at least three alternative first exons [Breslin et al., 2001]. A number of polymorphisms have been characterized in *NR3C1*, including a few single nucleotide polymorphisms (SNPs) putatively linked to glucocorticoid function: a *Tth*IIII restriction-site polymorphism (rs10052957) located 5′ to the coding sequence; a *Bcl*I restriction-site polymorphism located in intron 2; and two relatively rare non-synonymous SNPs located in exon 2, ER22/23EK (rs6190) and N363S (rs6195). An intensive screen of the gene for additional variants has yet to be reported; while a screen of exon 1a was unable to detect any variation across this region [Stevens et al., 2004], the remaining promoter regions have yet to be assessed.

NR3C1 polymorphisms have been associated with several glucocorticoid-related phenotypes including metabolic parameters [van Rossum and Lamberts, 2004], body mass index [van Rossum et al., 2003], cortisol responses to stress [Wust et al., 2004], and glucocorticoid sensitivity [Huizenga et al., 1998; Stevens et al., 2004]. Studies of *NR3C1* in depression have, to date, utilized clinically ascertained case–control adult depression samples. van West et al. [2006] genotyped four SNPs in two large samples from Belgium and Sweden. While they identified modest evidence for association, different polymorphisms, alleles, and haplotypes appear to be associated with depression in the two samples. In another large dataset, van Rossum et al. [2006] found that homozygous carriers of the *Bcl*I polymorphism and ER22/23EK-carriers had an increased risk of major depressive disorder (MDD). A recent pharmacogenetic study found that the *Bcl*I polymorphism was related to treatment response in patients with MDD [Brouwer et al., 2006].

Given the likely role of epigenetic factors in the etiology of depression [Mill and Petronis, 2007], an interesting observation is that maternal behavior in rats epigenetically alters a NGF1-A transcription factor binding-site in the promoter region of the rat GR gene [Weaver et al., 2004]. Weaver et al. were able to demonstrate that differences in maternal care correlated with GR expression in the hippocampus [Weaver et al., 2001], resulting from altered DNA methylation in the promoter region of the GR gene [Weaver et al., 2004]. This provides a mechanism by which stress-related environmental cues can regulate GR expression, altering the function of the HPA axis and its reaction to stressful-stimuli. Given the links between stress, the HPA axis, and depression, these findings provide additional support for a role of the GR in mood disorders. The analogous promoter region of the human GR gene has not been widely studied. While a preliminary epigenetic analysis failed to identify between-individual variation in DNA methylation [Mill et al., 2005], it is plausible that sequence differences in this region may disrupt the binding of transcription factors, such as NGF1-A, which are known to regulate GR expression and increase the risk for depressive disorders.

METHODS

Subjects

The sample used in this study is part of a multi-disciplinary program project researching multiple risk factors in COMD [Liu et al., 2006; Kiss et al., 2007]. Our initial genotyping was performed on 466 affected children from 384 nuclear families recruited from 23 mental health facilities across Hungary. Following an initial analysis, two markers (SNP5 and SNP6) were genotyped in an additional set of families, and in total 778 affected individuals from 638 families were included for these polymorphisms. A full description of sample recruitment and assessment can be found in Liu et al. [2006] and Kiss et al. [2007]. Briefly, the probands and affected siblings met DSM-IV criteria for either depressive or bipolar disorders with onset prior to 14.9 years. At the time of ascertainment, less than 1% of the sample had a diagnosis of bipolar disorder, although this proportion is likely to increase through the life-course given the clear genetically mediated non-independence of

depressive and bipolar disorders [McGuffin et al., 2003]. The Interview Schedule for Children and Adolescents Diagnostic Version (ISCA-D), which is an extension and modification of the ISCA [Sherrill and Kovacs, 2000], was the instrument used for diagnosis. The child and the parent informants were interviewed individually on two separate occasions approximately 1 month apart by two different trained clinicians. A best-estimate consensus diagnosis taken from both clinicians was used as the final diagnosis.

Mutation Screening of the NR3C1 Promoter Region

Bioinformatic analyses were performed on the upstream region of *NR3C1* to identify a region homologous to the NGF1-A transcription-factor binding site (sequence GCGGGGGCG) found to be epigenetically altered in the rat by Weaver et al. [2004]. We identified a region of the human *NR3C1* gene that shows considerable overall similarity with rat exons 1–7, and including a putative NGF1-A binding sequence. In addition, we identified three other conserved putative promoter sequences, corresponding to the three known alternative first exons of *NR3C1*. Primers were designed to span these four regions using Primer Express software (PE Applied Biosystems, Foster City, CA). Oligonucleotide primer sequences used for the mutation screen can be seen in Table I. All PCRs were optimized to work under standard conditions, with an initial 5 min denaturing step at 95°C followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension phase of 72°C for 10 min. We screened an average of 32 individuals for each amplicon giving a probability of >0.95 for the detection of variants with a minor allele frequency of 0.05. Heteroduplex molecules were formed by denaturing the PCR products in a thermal cycler for 3 min at 95°C, and then gradually re-annealing them from 95 to 65°C for 30 min. Heteroduplexed PCR products were analyzed using the WAVE DNA Fragment Analysis System (Transgenomic, Oak Ridge, TN) using column gradient conditions determined by software provided with the WAVE System. The run temperature(s) for each amplicon were determined primarily by using the denaturing high-performance liquid chromatography (DHPLC) Melt program (http://insertion.stanford.edu/melt.html). In a number of cases, alternative melting temperatures were also determined using Transgenomic's WAVEMAKER software. Column temperatures can be seen in Table I. Individual DNA fragment elution profiles were compared by visual inspection, and overlaid to allow for the easy detection of variants. Samples containing probable mutations (see Table II) were amplified by PCR and sequenced in both directions using the same primers as were used for DHPLC. Sequencing traces were analyzed using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), and multiple samples aligned to aid mutation detection.

Genotyping of NR3C1 Polymorphisms

DNA was extracted from whole blood using a high salt extraction method [Miller et al., 1988]. SNP2 (rs10482605) was genotyped by digesting DHPLC amplicon *NR3C1*-NGF1A with the restriction enzyme *Bsl*I (New England Biolabs, Ipswich, MA) overnight at 55°C, and visualizing bands on a 2% agarose gel stained with ethidium bromide under UV light. The remaining eight SNPs were genotyped using the TaqMan 5′ nuclease assay (Applied Biosystems, Foster City, CA). Where available, primers and probes were supplied as "Assay-on-Demand" assays from Applied Biosystems. SNP3 (*Tth*IIII), SNP4 (ER22/23EK), and SNP6 (*Bcl*I) were genotyped using primer and probe sequences designed using Primer Express software (PE Applied Biosystems). Primer and probe sequences for these three SNPs are given in Table III. TaqMan assays were read on the ABI 7900-HT Sequence Detection System

TABLE I. Primer Sequences and DHPLC Column Temperatures for the *NR3C1* Promoter Amplicons Screened With DHPLC

Amplicon	Chr 5 position[a]	Primer sequences	DHPLC temperatures (°C)
NR3C1-1A-A	142795003–142795361	F: CTTCCGTTGGACACATGCG R: TTTTTCTGGCACCTGCTTGAT	59
NR3C1-1A-B	142795273–142795673	F: CCACACAATAGGAGGAAATGAAAA R: GGATGGAATCCGTTCTCGAG	55
NR3C1-1A-C	142795614–142796077	F: GCTTGTATTACATGGCCAAATTGA R: TTTCTACTTCTTAAGTAAAACATCACCACTTAA	52, 57
NR3C1-1B-A	142764100–142764556	F: AGGGACGGGATAGCGGG R: GAAGAAGAGGTCAGGAGTTTCGG	54, 63
NR3C1-1B-B	142764415–142764713	F: CGAGCTGGATTTCTTTGCACT R: TGGCATTTTAAAGCTGCCTGT	56, 62
NR3C1-1B-C	142764638–142764916	F: CGTTGCTCACCCTATCACGTT R: CCGCACAAGGTAGGAGGCT	58, 64
NR3C1-1C	142763045–142763479	F: GGAGCGCGTGTGTGCGAGTG R: CGCAGAAGGAGCAGGAGG	61, 66
NR3C1-NGF1A	142763531–142763997	F: GAACGATGCAACCTGTTGGT R: GACACACTTCACGCAACTCG	66

[a]March 2006 UCSC genome assembly.

TABLE II. Polymorphisms Detected by DHPLC Analysis

Amplicon name	Position in amplicon and chromosome 5[a]	Polymorphism	dbSNP status	>5% MAF
NR3C1-1A-B	243, 142795515	A/G SNP	Not present	N
NR3C1-1B-A	44, 142764143	G I/D	rs5871845	Y
NR3C1-1B-A	221, 142764320	A/G SNP	rs3806854 (unverified)	N
NR3C1-1B-A	223, 142764322	A/C SNP	rs3806855 (unverified)	N
NR3C1-1B-C	169, 142764806	A/G SNP	rs10482603	N
NR3C1-1B-C	270, 142764907	C I/D	Not present	N
NR3C1-1C	272, 142763209	G I/D	rs10482610	N
NR3C1-1C	275, 142763206	C/T SNP	rs10482611	N
NR3C1-NGF1A	184, 142763714	G/A SNP	rs10482605	Y

[a]March 2006 UCSC genome assembly.

using the allelic discrimination end-point analysis software version 2.0 (Applied Biosystems).

Statistical Analysis

All data were screened for Mendelian errors using PEDSTATS, and MERLIN to detect for any crossovers between markers [Abecasis et al., 2002]. Thus, our data set was free of any detectable Mendelian errors and none of the markers genotyped deviated from the Hardy–Weinberg equilibrium. Single-marker and multi-marker haplotype association analyses were performed using UNPHASED [Dudbridge, 2003]. UNPHASED comprises of a suite of programs for association analysis of multi-locus haplotypes from UNPHASED genotype data, and is freely available for download from (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/). For haplotype analysis, only haplotypes with a frequency greater than 5% were analyzed. Linkage disequilibrium (LD) between the markers was calculated using Haploview v 3.2 [Barrett et al., 2005].

RESULTS

Mutation Screen

In total, eight amplicons were screened (Table I), and nine polymorphisms (six SNPs and three single-base insertion/deletions) were identified following DHPLC and the direct sequencing of selected amplicons (Table II). Of these polymorphisms, two were not present in the dbSNP database, and two were unverified. Most of the variants were present at a frequency <5% (ascertained from either subsequent screening or information contained in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), and were thus not carried forward for individual genotyping in the COMD sample. Our results for exon 1a were in agreement with those of Stevens et al. [2004], who also did not detect any variants in this region with a MAF >5%. Two of the detected polymorphisms did have a MAF >5%, and were selected for further investigation. Of particular interest is SNP rs10482605 (G/A), located less than 20 bp downstream of the predicted NGF1-A transcription factor binding site.

Single-Marker Association Analysis With COMD

One of the polymorphisms identified in the mutation screen with a MAF >5% (rs5871845) could not be genotyped successfully and was thus dropped from the study. In total, nine SNPs across *NR3C1* were successfully genotyped in this study. SNPs were selected on the basis of putative functional effects, previous association studies, and their position in the gene. All genotypes were found to be in Hardy-Weinberg equilibrium, and a block of fairly strong linkage disequilibrium covered the whole region. The location of the nine SNPs, and the LD relationships between them, can be seen in Figure 1. Single-marker TDT analysis for each SNP failed to identify any significant evidence of biased transmission of alleles to offspring

TABLE III. Primer and Probe Sequences for the Three SNPs Not Genotyped Using ABI Preoptimized Assays

Polymorphism	Primer sequences	Probe sequences
3-A/G (*Tth*III)	F: GCAGAGGTGGAAATGAAGGTGAT	Vic-TATTCAGACTCAGTCAAGG
	R: GGAGTGGGACATAAAGCTATGACAA	Fam-TTCAGACTCAATCAAGG
4-G/A (ER22/23EK)	F: TCCAAAGAATCATTAACTCCTGGTAGA	Vic-ACATCTCCCTTTTCCTGAGCAAGC
	R: GCTCCTCCTCTTAGGGTTTTATAGAAG	Fam-CATCTCCCCTCTCCTGAGCAAGC
6-C/G (*Bcl*I)	F: GCACCATGTTGACACCAATTCC	Vic-CTCTTAAAGAGATTGATCAGC
	R: CAGGGTTCTTGCCATAAAGTAGACA	Fam-CTCTTAAAGAGATTCATCAGC

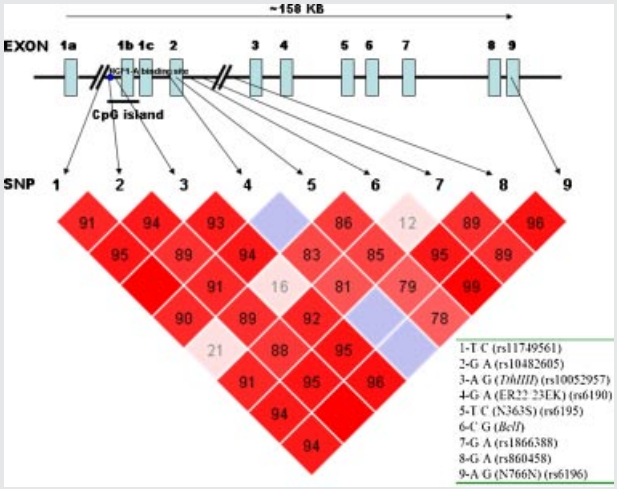

FIG. 1. Structure of the *NR3C1* gene showing the location of the nine genotyped SNPs and LD relationships (measured by D′) between them. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

affected with COMD (Table IV), although the N363S (rs6195) non-synonymous SNP showed a strong trend toward significance after being genotyped in the extended sample set (62T vs. 41NT; $\chi^2 = 3.51$; $P = 0.06$) with the T allele being over-transmitted.

Haplotype Analysis

As shown in Figure 1, LD across this region is high, with D′ between most marker combinations being >0.8. A previous investigation by van West et al. [2006] found only limited LD between *NR3C1* markers, but our data are in agreement with a previous investigation of SNPs across *NR3C1* that also reports strong LD [Stevens et al., 2004]. The most consistent previous association findings for polymorphisms in *NR3C1* have been with SNPs surrounding exon 2 of the gene, particularly the N363S (rs6195) non-synonymous SNP (SNP5 in our study) and the *Bcl*I restriction-fragment length polymorphism (SNP6 in our study), but also the low frequency ER22/23EK (rs6190) non-synonymous SNP (SNP4 in our study). Haplotypes containing alleles of SNPs around exon 2 have been associated with a several glucocorticoid-mediated phenotypes including glucocorticoid sensitivity to dexamethasone suppression [Stevens et al., 2004] and adult cortisol concentration in low birthweight individuals [Rautanen et al., 2006]. Recently, van West et al. [2006] have reported an association of *NR3C1* haplotypes, comprising of exon 2 SNPs and SNP rs10482605 (SNP2 in our study), with major depression. We thus had *a priori* evidence to investigate haplotypes of these markers for an association with COMD. Given the low MAF (~2%), the number of informative ER22/23EK (SNP 4) transmissions was too low to be reliably included in haplotype analyses, but analysis of SNPs 2, 5, and 6 uncovered significant over-transmission of the A-T-G haplotype to affected probands (94T vs. 124NT; $\chi^2 = 4.13$; $P = 0.04$) and under-transmission of the A-T-C haplotype to affected probands (157T vs. 123NT; $\chi^2 = 4.13$; $P = 0.04$) (see Table V). To try and pinpoint the specific polymorphisms mediating this effect, a two-marker haplotype analysis was performed sequentially removing one of the three SNPS. We found that dropping SNP2 from the analysis made little difference to the observed results with a similar pattern of biased transmissions as seen with the three-marker haplotype. In fact, extended haplotype analysis using all nine *NR3C1* markers demonstrated that haplotypes containing SNP5 allele T and SNP 6 allele G are consistently over-transmitted to affected offspring. We thus genotyped these two markers in a larger extended sample, and again found evidence that the T-G haplotype is over-transmitted to affected probands (204T vs. 165NT; $\chi^2 = 4.12$; $P = 0.04$) (see Table V). It should be noted, however, that the level of significance is low and none of these results have been corrected for multiple testing.

DISCUSSION

In this study, we performed a mutation screen across four putative promoter regions of the human GR gene and identified nine sequence variants. Subsequent association analysis of nine SNPs across the gene in a large family based COMD sample provided little evidence for the direct involvement of any of the markers

TABLE IV. SNPs Genotyped in This Study and Single-Marker TDT Association Analysis

SNP	rs#	MAF	T/NT[a]	OR	χ^2	*P*
1-T/C	rs11749561	0.47 (T)	198:193	1.02	0.06	0.80
2-A/G	rs10482605	0.17 (G)	97:94	1.03	0.05	0.83
3-A/G (*Tth*IIII)	rs10052957	0.29 (A)	154:140	1.10	0.67	0.41
4-G/A (ER22/23EK)	rs6190	0.02 (A)	14:13	1.08	0.04	0.85
5-T/C (N363S)[b]	rs6195	0.06 (C)	61:42	1.45	3.51	0.06
6-G/C (*Bcl*I)[b]	N/A	0.34 (G)	232:206	1.13	1.54	0.21
7-A/G	rs1866388	0.29 (G)	157:156	1.01	0.00	0.95
8-G/A	rs860458	0.12 (A)	84:72	1.17	0.92	0.34
9-A/G (N766N)	rs6196	0.12 (G)	80:77	1.04	0.06	0.81

[a]Transmitted allele is first allele listed column 1.
[b]Markers genotyped in a larger extended sample.

TABLE V. Haplotype Analysis of SNP2 (rs10482605), SNP5 (rs6195/N363S), and SNP6 (*Bcl*I)

Markers	Haplotype	Freq	T/NT	OR	χ^2	*P*
SNP2–SNP5–SNP6	A-T-C	0.44	94:124	0.76	4.13	**0.04**
	A-T-G	0.33	157:123	1.28	4.13	**0.04**
	A-C-G	0.05	32:36	0.89	0.24	0.63
	G-T-G	0.16	63:64	0.98	0.01	0.93
SNP5–SNP6[a]	T-C	0.34	139:165	0.84	2.22	0.14
	T-G	0.61	204:165	1.24	4.12	**0.04**
	C-G	0.05	36:47	0.85	1.46	0.23

[a]Markers genotyped in a larger extended sample.
Bold values represent $P < 0.05$.

genotyped in the pathogenesis of COMD. Multi-marker haplotype analysis of SNPs previously associated with depression and other glucocorticoid-related phenotypes, however, provided some modest evidence of an association, suggesting that additional variants elsewhere in the gene may play a role in mediating susceptibility to COMD.

Our mutation screen primarily focused on conserved regions of the *NR3C1* gene promoter, including a region with high similarity to rat exons 1–7 containing a predicted NGF1-A transcription factor binding-site analogous to that shown to be epigenetically hypomethylated in the rat following stress-related environmental factors [Weaver et al., 2004]. The high similarity of this region with rat exons 1–7 is interesting given that, to date, only three human *NR3C1* promoter regions have been identified [Nunez and Vedeckis, 2002]. This observation suggests that additional promoter regions may be involved in regulating the complex developmental and tissue-specific expression patterns observed for *NR3C1*, as is the case in other organisms such as the rat [McCormick et al., 2000; Breslin et al., 2001]. Our mutation screen detected nine polymorphisms, including two novel and two previously unverified variants. Seven of these polymorphisms were present at a very low frequency (MAF <5%), and thus not included in our subsequent genotyping study. One of the major factors in determining the success of association studies in common complex phenotypes, such as depression, is the frequency of disease-associated alleles in the population, with rare alleles unlikely to add any additional information in the hunt for susceptibility loci [Risch and Merikangas, 1996]. This is not to say, however, that these rare promoter-region variants are functionally unimportant, and given our incomplete knowledge about the regulation of *NR3C1* expression, future studies should focus on fully characterizing this region of the gene, and investigating transcriptionally important domains for additional sequence variants that could be functionally relevant. In addition, given the findings of Weaver et al. [2004] regarding acquired changes to the DNA methylation status of the NGF1-A binding site and their effect on gene transcription, another obvious focus of future work should be on epigenetic studies of the *NR3C1* promoter, especially given the mounting evidence for an epigenetic component to the etiology of depression [Mill and Petronis, 2007]. Preliminary analysis by Mill et al. [2005] found little evidence for between individual variation in methylation across this region in DNA obtained from lymphocytes and the cerebellum, although given the observation from rats that different promoters are highly tissue-specific [McCormick et al., 2000], this finding is perhaps not surprising.

Our genotyping study of *NR3C1* polymorphisms is, to our knowledge, the most thorough such study yet performed on this gene for a depression-related phenotype, and the first to be performed on a family based sample that avoids the population stratification problems that can affect case–control samples. While no significant evidence was found to implicate any of the genotyped polymorphisms directly in the etiology of COMD, we observed that haplotypes of SNP5 (N636S) and SNP6 (*Bcl*I), were significantly over- and under-transmitted to affected COMD probands. It should be noted, however, that the strength of the association is not large and would not survive correction for multiple testing, and these findings should thus be considered preliminary until replicated in an independent sample. This is, however, an interesting observation given that these are the two most widely investigated *NR3C1* polymorphisms, and have been consistently associated with a range of glucocorticoid-related functions. The N363S polymorphism (rs6195) results in an asparagine to serine amino acid change and has been widely associated with a higher sensitivity to glucocorticoids in vivo [Huizenga et al., 1998], along with a range of metabolic parameters (reviewed in [van Rossum and Lamberts, 2004]). Interestingly, this marker showed a strong trend toward significance when analyzed individually, suggesting that it may explain the association observed for the two-marker haplotype. The *Bcl*I polymorphism has also been linked with glucocorticoid sensitivity [van Rossum et al., 2003] and numerous metabolic phenotypes (reviewed in [van Rossum and Lamberts, 2004]), and has recently also been shown to be associated with major depression in adults [van Rossum et al., 2006] along with medication response in depressed individuals [Brouwer et al., 2006]. In addition, we also observed that three-marker haplotypes containing alleles of the N363S and the *Bcl*I polymorphisms, along with SNP2 (rs10482605) are also marginally significantly associated with COMD in this sample. This is interesting given that rs10482605 has been previously associated with depression by van West et al. [2006], both individually and in conjunction with polymorphisms surrounding exon 2. It should be noted that we found no evidence to implicate a role for another non-synonymous SNP (ER22/23EK) in our COMD sample, but this is not surprising given the very low minor allele frequency (~2%) and the small number of informative

transmissions observed for this marker. Taken together, our data suggest that while none of the SNPs genotyped in the current study are likely to be directly involved in the etiology of COMD, haplotypes across *NR3C1* may incur susceptibility. Future investigations should thus focus on screening the associated haplotype alleles for additional potentially functional sequence variants elsewhere in the gene.

To conclude, we have performed a mutation screen across various promoter regions of the human GR gene (*NR3C1*), and identified several novel variants. Association analysis of nine SNPs across the gene failed to find any evidence to implicate this gene with the etiology of COMD, but haplotype analysis of previous implicated variants provided some evidence of an association. Future investigations should focus on replicating these findings in additional samples, further characterizing the promoter region of *NR3C1*, and screening the associated haplotype alleles for additional potentially functional sequence variants elsewhere in the gene.

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REFERENCES

Abecasis GR, Cherny SS, Cookson WO, Cardon LR. 2002. Merlin-rapid analysis of dense genetic maps using sparse gene flow trees. Nat Genet 30(1):97–101.

Ashman SB, Dawson G, Panagiotides H, Yamada E, Wilkinson CW. 2002. Stress hormone levels of children of depressed mothers. Dev Psychopathol 14(2):333–349.

Barden N. 2004. Implication of the hypothalamic-pituitary-adrenal axis in the physiopathology of depression. J Psychiatry Neurosci 29(3):185–193.

Barrett JC, Fry B, Maller J, Daly MJ. 2005. Haploview: Analysis and visualization of LD and haplotype maps. Bioinformatics 21(2):263–265.

Breslin MB, Geng CD, Vedeckis WV. 2001. Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol 15(8):1381–1395.

Brouwer JP, Appelhof BC, van Rossum EF, Koper JW, Fliers E, Huyser J, Schene AH, Tijssen JG, Van Dyck R, Lamberts SW, et al. 2006. Prediction of treatment response by HPA-axis and glucocorticoid receptor polymorphisms in major depression. Psychoneuroendocrinology 31(10): 1154–1163.

Dahl RE, Kaufman J, Ryan ND, Perel J, Al-Shabbout M, Birmaher B, Nelson B, Puig-Antich J. 1992. The dexamethasone suppression test in children and adolescents: A review and a controlled study. Biol Psychiatry 32(2):109–126.

Dempster EL, Burcescu I, Wigg K, Kiss E, Baji I, Gadoros J, Tamas Z, Kennedy JL, Vetro A, Kovacs M, et al. 2007. Evidence of an association between the vasopressin V1b receptor gene (AVPR1B) and childhood-onset mood disorders. Arch Gen Psychiatry 64(10):1189–1195.

Dudbridge F. 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 25(2):115–121.

Huizenga NA, Koper JW, De Lange P, Pols HA, Stolk RP, Burger H, Grobbee DE, Brinkmann AO, De Jong FH, Lamberts SW. 1998. A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 83(1):144–151.

Kessler RC. 1997. The effects of stressful life events on depression. Annu Rev Psychol 48:191–214.

Kiss E, Gentzler AM, George C, Kapornai K, Tamas Z, Kovacs M, Vetro A. 2007. Factors influencing mother-child reports of depressive symptoms and agreement among clinically referred depressed youngsters in Hungary. J Affect Disord 100(1–3):143–151.

Liu X, Gentzler AL, Tepper P, Kiss E, Kothencne VO, Tamas Z, Vetro A, Kovacs M. 2006. Clinical features of depressed children and adolescents with various forms of suicidality. J Clin Psychiatry 67(9):1442–1450.

Luby JL, Heffelfinger A, Mrakotsky C, Brown K, Hessler M, Spitznagel E. 2003. Alterations in stress cortisol reactivity in depressed preschoolers relative to psychiatric and no-disorder comparison groups. Arch Gen Psychiatry 60(12):1248–1255.

McCormick JA, Lyons V, Jacobson MD, Noble J, Diorio J, Nyirenda M, Weaver S, Ester W, Yau JL, Meaney MJ, et al. 2000. 5′-Heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: Differential regulation of variant transcripts by early-life events. Mol Endocrinol 14(4):506–517.

McGuffin P, Rijsdijk F, Andrew M, Sham P, Katz R, Cardno A. 2003. The heritability of bipolar affective disorder and the genetic relationship to unipolar depression. Arch Gen Psychiatry 60(5):497–502.

Mill J, Caspi A, Dempster E, Williams B, Moffitt T, Craig I. 2005. Methylation analysis of a NGF1-A transcription factor binding-site in the promoter region of the human glucocorticoid receptor gene (NR3C1). Am J Med Genet Part B 138B(1):89.

Mill J, Petronis A. 2007. Molecular studies of major depressive disorder: The epigenetic perspective. Mol Psychiatry 12(9):799–814.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16(3):1215.

Murray CJ, Lopez AD. 1997. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. Lancet 349(9063):1436–1442.

Neigh GN, Nemeroff CB. 2006. Reduced glucocorticoid receptors: Consequence or cause of depression? Trends Endocrinol Metab 17(4):124–125.

Nunez BS, Vedeckis WV. 2002. Characterization of promoter 1B in the human glucocorticoid receptor gene. Mol Cell Endocrinol 189(1–2): 191–199.

Parker KJ, Schatzberg AF, Lyons DM. 2003. Neuroendocrine aspects of hypercortisolism in major depression. Horm Behav 43(1):60–66.

Rautanen A, Eriksson JG, Kere J, Andersson S, Osmond C, Tienari P, Sairanen H, Barker DJ, Phillips DI, Forsen T, et al. 2006. Associations of body size at birth with late-life cortisol concentrations and glucose tolerance are modified by haplotypes of the glucocorticoid receptor gene. J Clin Endocrinol Metab 91(11):4544–4551.

Rice F, Harold GT, Thapar A. 2002. Assessing the effects of age, sex and shared environment on the genetic aetiology of depression in childhood and adolescence. J Child Psychol Psychiatry 43(8):1039–1051.

Risch N, Merikangas K. 1996. The future of genetic studies of complex human diseases. Science 273(5281):1516–1517.

Sherrill JT, Kovacs M. 2000. Interview schedule for children and adolescents (ISCA). J Am Acad Child Adolesc Psychiatry 39(1): 67–75.

Stevens A, Ray DW, Zeggini E, John S, Richards HL, Griffiths CE, Donn R. 2004. Glucocorticoid sensitivity is determined by a specific glucocorticoid receptor haplotype. J Clin Endocrinol Metab 89(2):892–897.

Sullivan PF, Neale MC, Kendler KS. 2000. Genetic epidemiology of major depression: Review and meta-analysis. Am J Psychiatry 157(10): 1552–1562.

Thapar A, McGuffin P. 1994. A twin study of depressive symptoms in childhood. Br J Psychiatry 165(2):259–265.

Thapar A, Rice F. 2006. Twin studies in pediatric depression. Child Adolesc Psychiatr Clin N Am 15(4):869–881, viii.

van Rossum EF, Binder EB, Majer M, Koper JW, Ising M, Modell S, Salyakina D, Lamberts SW, Holsboer F. 2006. Polymorphisms of the glucocorticoid receptor gene and major depression. Biol Psychiatry 59(8):681–688.

van Rossum EF, Koper JW, van den Beld AW, Uitterlinden AG, Arp P, Ester W, Janssen JA, Brinkmann AO, de Jong FH, Grobbee DE, et al. 2003. Identification of the BclI polymorphism in the glucocorticoid receptor gene: Association with sensitivity to glucocorticoids in vivo and body mass index. Clin Endocrinol (Oxf) 59(5):585–592.

van Rossum EF, Lamberts SW. 2004. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res 59:333–357.

van West D, Van Den Eede F, Del-Favero J, Souery D, Norrback KF, Van Duijn C, Sluijs S, Adolfsson R, Mendlewicz J, Deboutte D, et al. 2006. Glucocorticoid receptor gene-based SNP analysis in patients with recurrent major depression. Neuropsychopharmacology 31(3):620–627.

Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ. 2004. Epigenetic programming by maternal behavior. Nat Neurosci 7(8):847–854.

Weaver IC, La Plante P, Weaver S, Parent A, Sharma S, Diorio J, Chapman KE, Seckl JR, Szyf M, Meaney MJ. 2001. Early environmental regulation of hippocampal glucocorticoid receptor gene expression: Characterization of intracellular mediators and potential genomic target sites. Mol Cell Endocrinol 185(1–2):205–218.

Wust S, Van Rossum EF, Federenko IS, Koper JW, Kumsta R, Hellhammer DH. 2004. Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. J Clin Endocrinol Metab 89(2):565–573.