Genetic and environmental risk factors, informative subtypes and putative endophenotypes in major depressive disorder

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Timea Anna Bianchi-Rimay, M.D.

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Timea Anna Bianchi-Rimay, M.D.

Faculty of Medicine

University of Szeged

Supervisors:

Ágnes Vetró, M.D. Ph.D.

Krisztina Kapornai, M.D. Ph.D.

Department of Child- and Adolescent Psychiatry

Department of Pediatrics and Child Health Center

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V. **T. Rimay**, G. Daróczi, E. Kovács, I. Benák, Á. Fehér, A. Juhász, Á. Vetró, M. Kovacs: Brain-derived neurotrophic factor Val66Met polymorphism in depressed children and adolescent population Clinical Neurogenetics from Diagnosis to Therapy, Hungarian Society of Clinical Neurogenetics, VIIIth Conference, Visegrád, Hungary, 4-5. December 2009. p.:38.

VI. A. Vetró, J. Gádoros, I. Baji, K. Kapornai, E. Kiss, **T. Rimay**, L. Mayer, M. Kovacs: What is News in Childhood Onset Depression in Hungary International Conference Sponsored by European Society for Child and Adolescent Psychiatry, Budapest, Hungary, 22-26. August 2009. p.:23.

VII. **T. Rimay**, G. Daróczi, E. Kovács, I. Benák, Á. Fehér, A. Juhász, Á. Vetró, M. Kovacs: Brain-derived neurotrophic factor Val66Met polymorphism in depressed children and their siblings Hungarian Child Neurology, Child Neurosurgery and Child and Adolescent Psychiatry Association 34rd Annual Meeting, Kecskemét, Hungary, 23-25. April 2009. p.:76.

ABSTRACT

Introduction and hypotheses: Gene polymorphisms that are suspected of playing a role in the development of childhood onset depression are examined in our work. Furthermore, we examine symptoms or symptom groups that can be considered as homogeneous types or endophenotypes of depression, and can be associated with the investigated genotypes or alleles. Oxytocin (OXT) and prolactin (PRL) are neuropeptide hormones that interact with the serotonin system and are involved in the stress response and social affiliation. In human studies, serum OXT and PRL levels have been associated with depression and related phenotypes. Both brain-derived neurotrophic factor (*BDNF*) polymorphisms and interferon-gamma +874 T/A polymorphism have been examined for their contribution to depression with equivocal results. More homogeneous phenotypes might be used to improve our understanding of genetic liability to depression.

The aim of our work was to 1) determine if single nucleotide polymorphisms (SNPs) at the loci for *OXT* and *PRL* and their receptors, *OXTR* and *PRLR*, were associated with childhood-onset mood disorders (COMD). 2) to test for association between *BDNF* Val66Met polymorphism and childhood onset melancholic depression and 3) to investigate the interferon-gamma +874 T/A polymorphism and the somatic type of depression.
We also examined the interactive effects of stressful life events (SLE) and the Val66Met polymorphism on the risk of childhood onset melancholic depression.

Materials and Methods: Using 678 families in a family-based association design, we genotyped 16 SNPs at *OXT, PRL, OXTR* and *PRLR* to test for association with COMD. Examining the *BDNF*, in the preliminary studies and the following case-control studies, first subsets, then a sample of 583 depressed probands were involved (55 from the somatic type of depression, 162 of the melancholic subtype). Diagnoses were derived via the Interview Schedule for Children and Adolescents - Diagnostic Version and life event data were collected by means of an Intake General Information Sheet.

Results: No significant associations were found for SNPs in the *OXTR, PRL,* or *PRLR* genes. Two of three SNPs of the *OXT* gene were associated with COMD (p=0.02), significant after spectral decomposition, but were not significant after additionally correcting for the number of genes.

In the „preliminary *BDNF* study", regarding the melancholic subtype, *BDNF* Val66Val genotype had a significantly higher frequency in the melancholic depressed group than in the controls (p=0.024). In the study examining the whole sample of depressed probands, 27.8% of the participants met criteria for melancholy. In the melancholic group the proportion of the females was higher (53.1%), though there were more males in the overall depressed sample. We detected no significant differences in genotype or allele frequency between the melancholic and the non-melancholic depressed group. *BDNF* Val66Met polymorphism and stressful life event interaction was not significantly associated with the melancholy outcome.

Regarding the association of somatic type of depression and the interferon-gamma +874 T/A polymorphism, the frequencies of the A allele containing genotypes were higher in the somatic subtype, than in the comparison group, without statistically significant difference (T/A: SB (sickness behaviour) group: 60.0%, comparison group: 51.1%; A/A: SB group: 21.8%, comparison group: 13.3%; p=0.122). The comparison of allele frequencies also did not reveal statistically significant differences (T: SB group: 48.2%, comparison group: 61.1%; A: SB group: 51.8%, comparison group: 38.9%; p>0.1).

Conclusion: In our study, females were more prone to develop the early-onset melancholic phenotype. The present work is the first genetic analysis to examine the association between variants in the oxytocin and prolactin neuroendocrine genes and depression with onset in childhood. To our knowledge, this is also the first study to investigate the differentiating effect of the genotype and the G×E interaction on melancholic phenotype in a large sample of depressed young patients. We didn't find association between the melancholic subtype of major depression and the *BDNF* genotype and stressful life event interaction and the somatic subtype of childhood-onset major depression and the interferon-gamma +874 T/A polymorphism in this large sample, which is representative to the Hungarian clinic-referred population of depressed youths.

List of abbreviations

AVP	Arginine Vasopressin
AVPR1B	Vasopressin V1b Receptor Gene
BED	Best Estimate Diagnosis
BDNF	Brain- Derived Neurotrophic Factor
CDI	Children Depression Inventory
COD	Childhood-Onset Depression
COMD	Childhood-Onset Major Depression
D	Depression
DNA	Deoxyribose Nucleic Acid
DSC	Depressive Symptom Checklist
DSM-III	Diagnostic and Statistical Manual of Mental Disorders 3rd Edition
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders 4th Edition
FET	Fisher´s Exact Test
G×E	Gene–Environment Interaction
GIS	General Information Sheet
HPA	Hypothalamic–Pituitary–Adrenal
IFN-γ	Interferon-gamma
IGIS	Intake General Information Sheet
IGR	Intergenic Region
ISCA	Interview Schedule for Children and Adolescents
ISCA-D-PL	Interview Schedule for Children and Adolescents Diagnostic Version Present and Lifetime
LD	Linkage Disequilibrium
LRS	Likelihood Ratio Test Statistic

MCN	Magnocellular Neuron
MDD	Major Depressive Disorder
MEL	Melancholic
MFU	Mail-Follow-up Test Package
NIMH	National Institute of Mental Health
NMEL	Nonmelancholic
OXT	Oxytocin
OXTR	Oxytocin Receptor
PCR	Polymerase Chain Reaction
POE	Parent-of-origin Effects
PRL	Prolactin
PRLR	Prolactin Receptor
RR	Relative Risk
SD	Standard Deviation
SLE	Stressful Life Event
SNP	Single Nucleotide Polymorphism
SNPSpD	Single Nucleotide Polymorphism Spectral Decomposition
SR-GIS	General Information Sheet-Short version
STOD	Somatic Type of Depression
Tr	Transmitted
TDT	Transmission Disequilibrium Test
Un	Untransmitted
5-HTTLPR	Serotonin-Transporter-Linked Polymorphic Region

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1. Introduction

1.1. Background

Major depressive disorder (MDD) is a major public health problem representing the leading cause of disability in developed countries and the fourth leading cause of disability worldwide. Lifetime risk for developing MDD is estimated to be approximately 15% (Kessler et al., 2003). Mortality risk of depressed patients is two times greater than that of the general population due to direct (suicide) and indirect causes (Cuijpers and Smit, 2002).

It is well documented that MDD is most likely resulted from complex interactions of genetic, epigenetic, environmental and developmental factors, nevertheless the exact mechanisms underlying the disease are still largely unknown (e.g. Ebmeier et al., 2006). There is fairly consistent evidence that childhood onset depression (COD) has familial determinants (Rice et al., 2002). For example, in a large twin study the hereditability for MDD was 29% in males and 42% in females, respectively (Kendler et al., 2006). Levinson (2006) estimated the genetic contribution to the development of MDD as 40-50%.

To achieve the goal of developing more effective treatment for MDD, a greater understanding of the neurobiology of psychopathology and the identification of the risk and resilience factors are needed.

1.2. Putative genetic liability to depression

1.2.1. Oxytocin and prolactin genes

Molecular genetic studies of depression-related phenotypes in paediatric samples are relatively few in number, with many focused on serotonin system genes. Genetic association studies of variants in the serotonin transporter (SLC6A4) and serotonin receptor (e.g. 5HT1A, 1B, 2A) genes have found weak, inconsistent associations with depression (Lemonde et al., 2003; Parsey et al., 2006; Huang et al., 2004; Caspi et al., 2003; Lotrich and Pollock, 2004; Surtees et al., 2006; McMahon et al., 2006). This suggests that genes outside of the serotonergic system, may also be important in the aetiology of depressive disorders such as childhood-onset mood disorder (COMD). Oxytocin (OXT) and prolactin (PRL) are neuropeptide hormones that have been

shown to be associated with antidepressant activity, to interact with the serotonin system and to be involved in the stress response and the formation of social bonds.

Oxytocin has been proposed to balance the suppression of the serotonergic system by the hypothalamic–pituitary–adrenal axis (HPA) following stressful events (Legros, 2001) and reduce anxiety (Domes et al., 2007). Evidence from animal and human investigation supports the notion of a relationship between oxytocin and serotonin systems (Emiliano et al., 2007; Lee et al., 2003; Amico et al., 2004). Published studies suggest that stress and fear induce compensatory (Windle et al., 2004; Ebner et al., 2005; Kirsch et al., 2005) changes in OXT expression. Human biomarker studies provide another line of evidence implicating oxytocin in depressive symptoms (Zetzsche et al., 1996; Bell et al., 2006; Scantamburlo et al., 2007; Cyranowski et al., 2008; Costa et al., 2009).

Prolactin secretion is also dependent on the serotonergic system. Human serum PRL levels have been shown to reflect serotonergic function via D-fenfluramine challenge (Lee et al., 2003; Mann et al., 1995). Studies show that the prolactin response to D-fenfluramine and serotonergic agents is blunted in depressed individuals compared to controls (Mann et al., 1995; Heninger et al., 1984; Golden et al., 1992; Anderson et al., 1992; Papakostas et al., 2006). Several papers show stress-induced increases in PRL (Drago et al., 1990; Bodnár et al., 2004; Torner et al., 2004). The postpartum antidepressant-like effects of lactation may be mediated by the parity of the mother (Sibolboro Mezzacappa and Endicott, 2007). Plasma PRL levels in response to tryptophan challenge are associated with neuroticism, a correlate of depressive disorders (Brummett et al., 2008). Still other results support an OXT-PRL feedback loop in the hypothalamus (Liu and Ben-Jonathan, 1994; Kokay et al., 2006).

1.2.2. *BDNF* gene

Numerous other candidate genes have been studied as potential risk factors for COD (Rice, 2010), including the *BDNF* locus. *BDNF* Val66Met is a SNP of nucleotide 196 in exon 5, resulting in a Val/Met amino acid change in codon 66, affecting the pro-BDNF sequence, with no functional effect on the mature BDNF, but causing changes in its cellular transport and secretion.
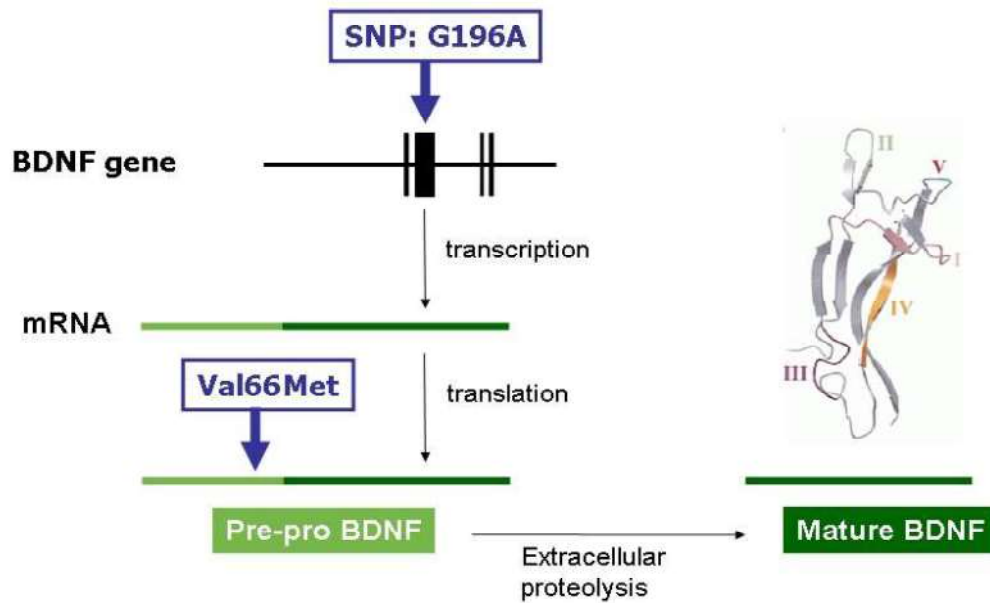
Figure 1. *BDNF* **polymorphism**

The encoded protein is a neurotrophin, playing a role in hippocampal dendritic morphology and synaptic function. It has been implicated in depression, evidenced by decreased hippocampal volume and hippocampal and serum BDNF levels associated with the disease. (Savitz and Drevets, 2009; Sen et al., 2008, Cardoner et al., 2013). The presence of the Val66Met Met allele has been associated with inefficient secretion of BDNF regardless of gender (Egan et al., 2003; Ozan et al., 2010) and decreased serum BDNF levels were found in patients with depression (Ozan et al., 2010).
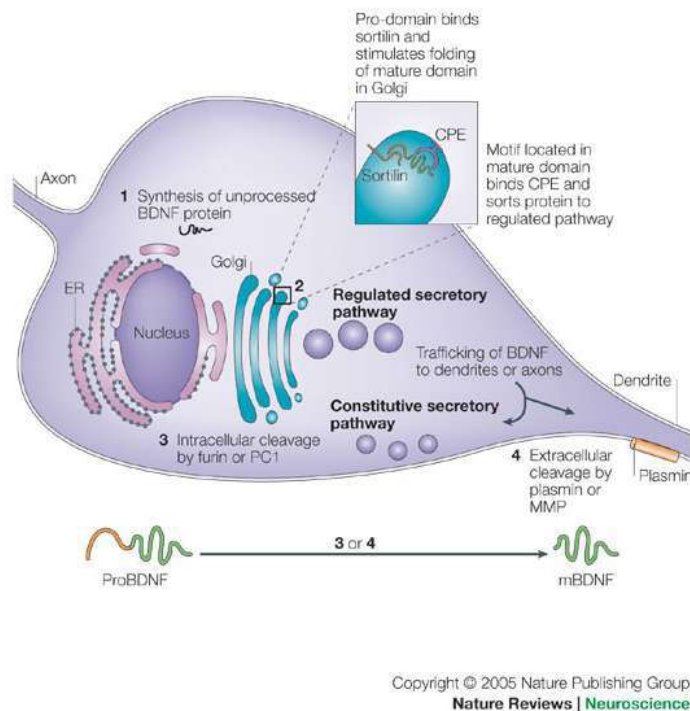
Figure 2. Cellular transport and secretion of BDNF

In a sample of adults with a history of childhood onset mood disorder (COMD) (Strauss et al., 2004) the *BDNF* Val66Met-$(GT)_n$ two marker Val/short (<170 bp) haplotype was associated with COMD, but no significant association was found between the examined *BDNF* polymorphisms and childhood-onset mood disorder by allelic or genotypic analysis. In another study, the same authors examined a sample of 258 Hungarian trios including juvenile probands with COMD and found the *BDNF* Val66Met Val allele to be associated with the disorder (Strauss et al., 2005). Importantly, the present study is performed on a subset of the same Hungarian sample. Noteworthy, a meta-analysis of Chen et al. (2008) did not confirm the association between Val66Met polymorphism and depression. Also, in contrast to the often reported association between the *BDNF* polymorphism and depression, the meta-analysis of Verhagen et al. (2010) suggests only a gender-dependent role of the polymorphism in the development of depression, as significant association was found only in males.

1.2.3 Inflammatory cytokine gene variant

It is known that the genetic variants of inflammatory cytokines can play a role in the appearance of depression (Misener et al., 2008). In a previous study, increased interferon-gamma (IFNγ) level was found in a sample of adolescents with major depression (Gabbay et al., 2009). Another study showed that the increase in the level of interferon-gamma correlates with the interferon-gamma +874 T/A single nucleotide polymorphism (Pravica et al., 2000).

1.3. Factors which may influence the link between genetic liability and depression

1.3.1. Melancholic phenotype of depression

In this work a number of factors are considered that could contribute to and/or moderate the liability to depression. The limited success of genetic studies might arise from current classification schemas including DSM-IV, since they are based on clusters of syndromes in a heterogeneous patient group (Hasler et al., 2004). MDD as defined in DSM-IV is a heterogeneous disorder with regard to etiology, symptoms and level of functioning and response to treatment (Domschke et al., 2010).

One of the clinical subtypes of MDD that showed distinct clinical (Kessler et al., 1997b) and biochemical (Maes et al., 1990) features was melancholic depression (Gold et al. 2002). Schotte et al. (1997) provided the validation through cluster analysis of melancholic and non-melancholic subtypes of unipolar depression.

A promising attempt has been made to determine the nosological status and diagnostic strategies to melancholia (Parker and Paterson, 2014). Furthermore, several biological markers have been suggested to identify this putative endophenotype. The preliminary results of Bracht et al., (2013) suggest that the melancholic subtype of MDD is characterized by white matter microstructure alterations of the medial forebrain bundle compared to healthy controls, while it did not differ between the healthy controls and the overall MDD sample. Though some studies claim that psychomotor retardation is a feature of depression (Widlöcher et al., 1983, Dantchev, 1998), some authors suggested that psychomotor retardation allows determining putative endophenotypes such as melancholy (Bennabi et al., 2013, Parker et al., 1996). Preliminary evidence suggests that genetic factors may discriminate melancholic and non-melancholic depression in a putatively interesting preliminary study of Quinn et al. (2012) with low sample

size. The authors (Quinn et al., 2012) found that the *BDNF* rs6265 Met allele predisposes non-melancholic depression and the interaction of the *BDNF* rs6265 Met allele and stressful life event (SLE) predicts non-melancholia over main effects. Furthermore, in a study of Patas et al. (2014) investigating the association between serum brain-derived neurotrophic factor and plasma interleukin-6 in major depressive disorder, plasma interleukin-6 was found to be a positive predictor of BDNF only in the melancholic sample. According to Harkness et al. (2006) subjects with severe melancholic depression are more sensitive to stress, with episodes being influenced by more minor stressors than those of non-melancholic patients.

1.3.2. Somatic type of depression

Another putatively interesting phenotype is the somatic type of depression (STOD). Inflammatory cytokines induce behavioural symptoms, known as sickness behaviour (SB), that are similar to symptoms seen in depression (fatigue, altered sleep patterns, psychomotor retardation, social withdrawal, appetite loss, anhedonia and impaired cognitive function). INF-γ is primarily secreted by T-lymphocytes, it usually provokes fatigue, malaise, headache, lack of appetite, weight loss, weakness, lethargy and decreased concentration, which can all be symptoms of depression (Fent et al., 1987, Triozzi et. al., 1990). This similarity led to the theory that the imbalance of inflammatory cytokines can contribute to the development of depression.

1.3.3. Role of stressful life events

Indeed, the role of stressful life events has been proven in depression (Kessler et al., 1997a) and several studies showed the effect of gene by environment (G×E) interaction on mood disorders. Many environmental factors have been identified, including early and recent stressful life events (Caspi et al., 2003) and parental depression (Bouma et al., 2008), which may interact with the genetic background (Ebmeier et al., 2006). For example, the short allele of the serotonin transporter 5-HTTLPR polymorphism compared to the long allele exhibited more depression and suicidality in relation to stressful life events in a number of studies (Caspi et al., 2003, Kaufman et al., 2004, Eley et al., 2004). Although this association was not confirmed in the meta-analysis of Risch et al. (2009), it thus seems that MDD is caused by numerous interacting genetic and non-genetic factors.

In a study of Bukh et al. (2009) the Met allele of the *BDNF* Val66Met polymorphism was independently associated with the presence of SLEs prior to the onset of depression and in a paper of Elzinga et al. (2011) *BDNF* Met allele carriers were found to be sensitive to early SLEs. Kim et al. (2007) found significant interaction of stressful life events and *BDNF* Met/Met and Val/Met genotypes on the risk of depression. In a study of Chen et al. (2012) on a sample of 780 pairs of ethnic Han Chinese adolescent twins, authors found that the *BDNF* Val allele modulates the influence of environmental stress on depression. Interaction between *BDNF* Val66Met polymorphism and environmental stress on depression was found even when separating pure environmental factors from the environmental factors under partial genetic control and adopting a prospective longitudinal design (Chen et al., 2013). In a study of Hosang et al. (2014) the Met allele of *BDNF* significantly moderates the relationship between life stress and depression, the association being stronger for an interaction with stressful life events and weaker for interaction of *BDNF* Val66Met with childhood adversity. In the study of Brown et al. (2014) the Met alleles of *BDNF* Val66Met significantly moderated the relationship between recent life events and adult onsets of depression, though *BDNF* did not significantly influence the effect of childhood maltreatment on chronic depression.

An illustrative example of how difficult it is to find replicable gene-MDD associations is provided by a study by Bosker and colleagues (2011). In their meta-analysis of 92 single nucleotide polymorphisms from 57 candidate genes previously reported to be significantly associated with MDD only four SNPs from four genes were found to replicate. Even these associations may turn out to be false positives due to multiple testing.

Genome wide association studies, up to date best suited for detecting variants with minor effect (Shyn et al., 2010) haven't shown genome-wide significant findings, though their non-significant top signals may be promising. Top signals may point to the possible role of synaptic processes in the genetic background of depression (Sullivan et al., 2009, Shyn et al., 2011). A number of classical pathways were associated with depression, firstly those of monoamines, from which the most commonly examined pathways are serotonergic, dopaminergic and noradrenergic pathways and the nitric oxide pathway (Smoller, 2016).

1.4. Aims and hypotheses

1) We tested whether single nucleotide polymorphisms (SNPs) at the loci for *OXT, PRL* and their receptors are associated with childhood-onset mood disorders (COMD).

   Based on our preliminary studies regarding the *BDNF* Val66Met polymorphism in depressed children and their siblings, to improve our knowledge about the potential genetic contributors to the melancholic type of depression, we examined whether an effect of the *BDNF* Val66Met polymorphism, and interaction of the *BDNF* Val66Met polymorphism with stressful life events could differentiate juvenile melancholic and non-melancholic patients.

2) Specifically, we hypothesized that:

   a. Melancholic and non-melancholic depressed probands are characterized by different *BDNF* Val66Met genotype and allele distributions
   b. Past exposure to stressful life events interacts with the *BDNF* Val66Met polymorphism to predict a melancholic depression phenotype

   Furthermore, we examined the possible association between *interferon-gamma* +874 T/A single nucleotide polymorphism and the development of a symptomatically homogenous type of childhood onset depression.

3) We hypothesized that:

   Depressed probands showing and those not showing the investigated group of somatic symptoms are characterized by different *IFN*-γ +874 T/A gene and allele distributions

These studies are innovatively integrating recent work on the molecular genetics of childhood-onset MDD.

2. Methods

2.1. Participants, enrolment and assessment procedures in the Childhood Onset Major Depression study

In our present work we report on three different but connected studies using depressed probands and their unaffected siblings on a large sample, which is representative to the Hungarian outpatient and clinic-referred population of depressed youths as the recruitment sites supported more than approximately 85% of the newly referred cases during the study period.
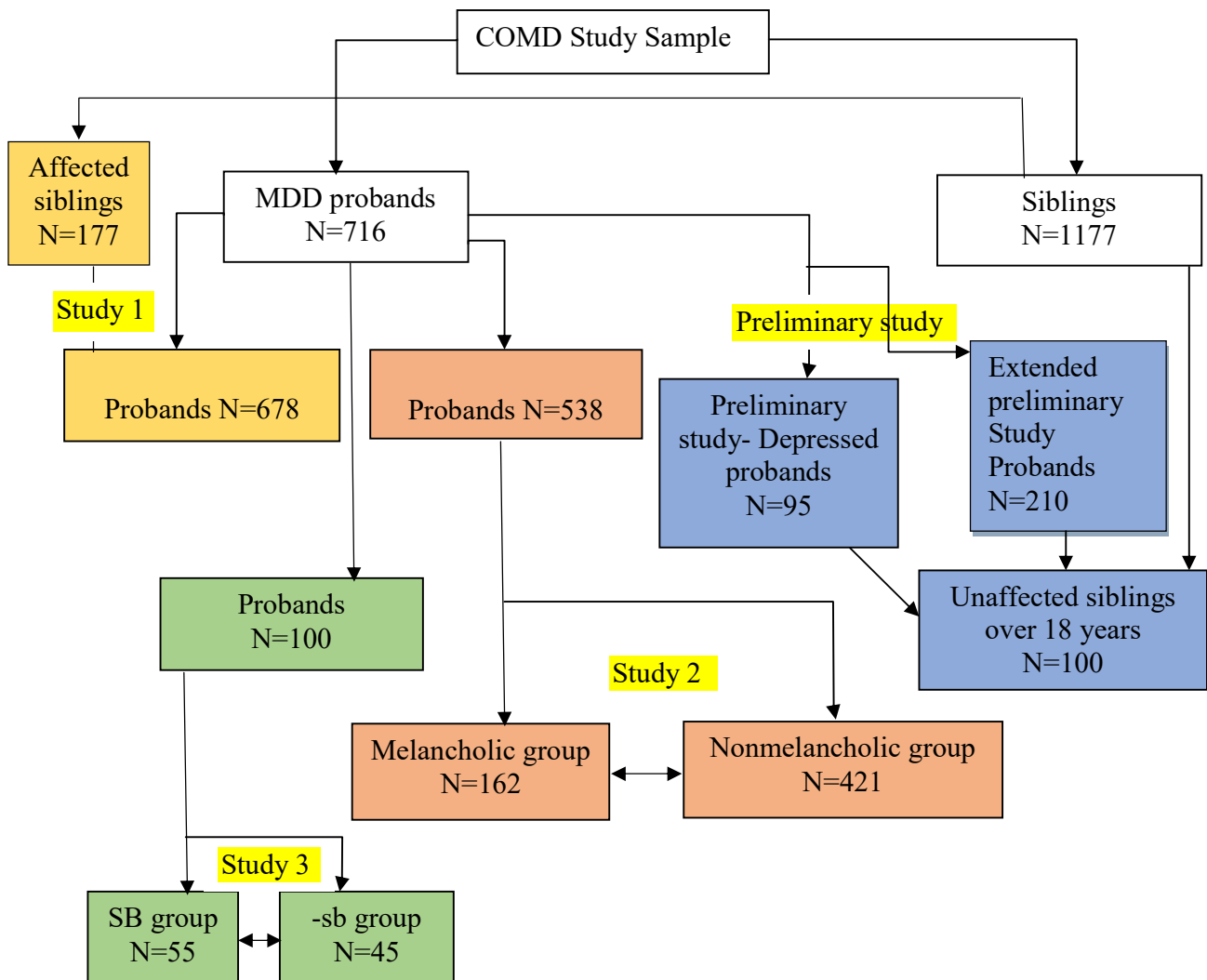

Figure 3. Description of the entire sample and the identified subgroups

Table 1. Descriptions of the samples and hypotheses

	Subsample		Hypotheses
	Group	Comparator group	
Study 1 (oxytocin and prolactin)	678 probands and 177 affected siblings	-	Association of OXT and PRL and their receptors with COMD
Preliminary (BDNF)	95 /210 probands	100 unaffected siblings	Association of BDNF Val66Met with depression and melancholy
Study 2 (BDNF X SLE)	162 melancholic probands	421 non-melancholic probands	Association of BDNF Val66Met with melancholy and role of interaction with SLE
Study 3 (IFNγ +874)	55 probands showing somatic symptoms	45 probands without somatic symptoms	Association of IFNγ +874 with somatic symptoms

The data derive from a multidisciplinary Program Project studying genetic and psychosocial risk factors for COMD. The project was conducted in Hungary, in collaboration with Pittsburgh University, NIMH grant #MH056193. The sample includes in total 716 depressed probands, recruited from 23 mental health facilities across the country, including 7 child and adolescent psychiatry inpatient units. Screening of probands was performed from 01.11.1999 to 01.07.2005. Signed consent from the parents and assent from the children were obtained from all subjects in accordance with the Hungarian National Health and the Regional Human Investigation Review Board guidelines, in accordance with the legal requirements in Hungary and the University of Pittsburgh, Pittsburgh, USA. Table 1 and figure 3 describes the entire sample and shows the identified subgroups. Inclusion criteria were the following: DSM-IV diagnosis of MDD with the onset of the first episode from 7.0 years by age 14.9 and being free of mental retardation and major systemic medical disorders, with at least one biologic parent and a 7 –17.9-year-old sibling available. At first, children were selected on the basis of a depressive symptom screen, the short version of the Children's Depression Inventory (Kovacs et al., 2003), having to reach the cut-off score for inclusion. The instrument was selected during the course of a pilot study to maximize sensitivity and specificity. Screen cut-off and also screening measure used were adjusted during the recruitment phase in order to minimize false positives. The diagnostic procedure included an

audiotaped semi-structured Interview Schedule for Children and Adolescents - Diagnostic Version (ISCA-D), an extension of the Interview Schedule for Children and Adolescents (ISCA) (Sherrill et al., 2000). The interviewers were child- and adolescent psychiatrists and psychologists, who completed 3 months of didactic and practical training in the interview technique (an average of 85% symptom-agreement reached on 5 consecutive videotaped interviews against "gold standard" interview ratings provided by the trainers). Interrater reliability on ISCA-D symptoms was satisfactory.

Ratings were obtained both for current and past symptoms of DSM-IV Axis I diagnoses and major DSM-III disorders (e.g. depression with melancholic features). The evaluation consisted of 2 different parts, conducted by different clinicians, approximately 6 weeks apart. The diagnostic assessment for the participants enrolled in this study has been described further in detail previously (Kapornai et al., 2007; Kiss et al., 2007; Liu et al., 2006). Importantly, to establish depressive episodes and symptoms, we performed two independent semi-structured diagnostic interviews during the intake procedure rated by trained clinicians, and subsequently evaluated by Best-Estimate Diagnostic Procedure (Maziade et al., 1992). The "Mood Disorder Module" of the semi-structured diagnostic interview was administered first, together with the Intake General Information Sheet (IGIS). A time-line with named anchors was created during the interview, in order to identify present and past depressive episodes (with onset and offset dates as punctual as possible) and to document the occurrence of any of a range of significant life events. The anchors consisted of both general and personal events.

If the child had met DSM criteria for mood disorder at the first evaluation, the full semi-structured diagnostic interview and a completion with additional self-rated scales were administered. Not only the child was interviewed about him/herself, but also the parent about the child. Pairs of senior child psychiatrists, trained as Best Estimate Diagnosticians (BEDs), separately reviewed all material, and then together derived consensus diagnoses and decided whether the child entered the "Proband" status.

2.1.1. Study 1. Investigating the oxytocin or prolactin gene variants

In the investigation of oxytocin or prolactin gene variants, we included 678 families (Figure 3) with at least one biological parent and a proband in the statistical analyses. In Study 1, there were 855 affected participants - probands (n = 678) and affected siblings (n = 177) met DSM-IV criteria for either unipolar depression (n = 838) prior to age 14 years or bipolar disorder (n = 17) prior to age 17 years. The mean proband age at study entry was 12.6 years [S.D. 2.32 years]. Probands were 55% male. Bipolar cases were included in the analyses since a significant proportion of unipolar children will switch as they mature (Kovacs et al., 1997; Geller et al., 2001) - less than 1% of the entire sample had a bipolar spectrum diagnosis at intake (Dempster et al., 2007) -, and evidence that unipolar and bipolar illnesses share inherited risk (McGuffin et al., 2003).

2.1.2. Preliminary studies testing the *BDNF* Val66Met polymorphism in depressed children and their unaffected siblings

The aim of our preliminary study on the *BDNF* Val66Met polymorphism was to examine the relationship between the polymorphism and childhood-onset depression, additionally, melancholy as an alternative phenotype, the somato-vegetative symptoms as phenotypic characteristics and stress related life events as environmental factors. Ninety-five depressive patients and their 100 healthy siblings without psychiatric history above 18 years of age were involved in this study. Probands were 50,5% and siblings were 48% male. The average age of the probands from this first sample was 11,35±1,8 years and that of the siblings was 18,4±1,3 years. Depressive episodes and symptoms were examined with semi-structured interview (ISCA-D). Melancholic phenotype was separated on the basis of the additional question of ISCA-D referring to the different quality of depressed mood.

Extending our analysis, we compared 210 depressed (Figure 3) probands and their 100 unaffected siblings. Therefore, our largest case number regarding the preliminary studies is 210. Screening of probands was performed from 01.11.1999. to 01.07.2005, and siblings from 01.11.1999 to 31.05.2008. Distribution of probands by gender and age was 116 males (55.2%), mean age: 13.08 ±2.3 years.

In this study we defined subgroups within depression according to the presence of melancholic symptom on the basis of an item of the diagnostic interview referring to the distinct quality of depressed mood. (This feeling of sadness since … is/was this different than the feeling you get when a friend moves away? Is/was this like a 'missing feeling' or a 'lonely feeling? How is it different? Is it like being in a dark cave? Has anyone close to you died? A pet? Can you remember how you felt then? How is/was your feeling now? Did you feel any different? In what way…?). Ratings were obtained for both current and past symptoms. The distribution of melancholic and non-melancholic phenotypes between the depressed group was the following: 24 (11.4%) participants currently melancholic, 16 (7.7%) with a past melancholic depressive episode and 4 (1.9%) both current and past melancholy.

Siblings of child probands were subjected to the same research screening procedure and in case of positive screening, to the same comprehensive diagnostic assessment procedure, detailed above. Both probands and siblings were followed up by a yearly mail-follow-up test package (MFU), including: a) parental report of interim medical and psychosocial events (short version of our IGIS = SR-GIS) and a 26-item DSM depressive symptom checklist (DSC) and b) each child's self-rated depression scale (short CDI). In case of positive screening, a diagnostic assessment procedure followed. All the siblings who haven't screened positive on the follow-up package before 18 years of age, were invited for assessment. 100 siblings over 18 years of age were involved in the study as controls (48 males (48%), mean age: 18,04±1,3 years), whose assessment procedure excluded the existence of current as well as lifetime episode of major depression or any other major psychiatric morbidity.

2.1.3. Study 2. Investigating the melancholic and the nonmelancholic depressed groups

Studying the *BDNF* Val66MET polymorphism on an even larger sample, the depressed group was divided into two groups on the basis of an improved classification method investigating the presence of the melancholic subtype. From the COMD study sample 583 depressed probands were included in Study 2 (Figure 3). Melancholic subtype of depression was estimated by ISCA-D, on the basis of the criteria for melancholic features of the DSM-IV. The differentiating symptoms were pervasive, nonreactive anhedonia, distinct quality of depressed mood, depression worse in the morning, early morning awakening, marked psychomotor retardation or agitation,

anorexia, weight loss and excessive and inappropriate guilt. 135 subjects met criteria for melancholic depression in the current time-frame, and 44 subjects had such features during a past episode. There were overlaps between the two groups given that some subjects had melancholy in both time frames. Thus, 162 subjects met criteria for melancholic depression in at least one time frame. The mean age of depressed subjects at first interview was 11.72 (SD=2.01) years. The melancholic group (N=162) had a mean age of 11.71 (SD=2.01) and the non-melancholic group (N=421) had a mean age of 11.76 (SD=2.01). The distribution by gender was 55.6% male (n=324) in the total depressed group, 46.9% male (n=76) in the melancholic group and 58.9% male (n=248) in the non-melancholic group (see Table 2 for further details).

Table 2. Characteristics of the total depressed group and the melancholic and nonmelancholic patients

	Depressed group 2 (n=583)	MEL (n=162)	NMEL (n=421)
Age of onset (years)	11.7±2.0	11.7±2.0	11.7±2.0
Male/female	324/259	76/86	248/173
SLE (mean±SD)	6.07±2.86	5.84±3.03	6.15±2.78

MEL, melancholic; NMEL, nonmelancholic; SLE, stressful life event.

Life events were abstracted from a pre-coded demographic data sheet, the Intake General Information Sheet (IGIS), completed by clinicians as part of the intake psychiatric interview, using parents as informants. On the basis of the study of Mayer et al. (2009), 22 of the 26 stressful life events were separated into five clinically meaningful groups: "Parental health", "Death of close relatives", "Sociodemographic" and "Intrafamilial". Miscellaneous events formed the fifth group containing items reflecting extrafamilial life events and abuse. A continuous weighted total score reflecting the number of stressful life events was also computed from the 26 items. Both the total and the grouped scores of stressful life event items were weighted by age (age in years at the intake ISCA-D interview).

2.1.4. Study 3. Investigating the interferon-gamma +874 T/A polymorphism

100 Hungarian patients with childhood onset depression were involved in this study (55 probands in the sickness behaviour (SB) group and 45 probands in the comparison group) (Figure 3). Diagnostic procedure included the above detailed Interview Schedule for Children and Adolescents - Diagnostic Version, a semi- structured interview, based on DSM-IV. Covering

the subject's current episode and lifespan, we examined six somatic symptoms that characterize sickness behaviour (SB) and depression, too (fatigue, altered sleep patterns, psychomotor retardation, appetite loss, anhedonia and impaired cognitive function).

We did not examine social withdrawal, as it is not listed in the diagnostic symptoms of major depressive disorder (according to DSM-IV).

In the presence of at least five of the somatic symptoms, that were frequent, considerable, and noticeable and interfered with functioning, we categorized the subject into the SB group. We compared this SB group with those depressed subjects who did not fulfil these criteria (-sb: comparison group) (Table 3).

Table 3. Characteristics of the subgroups

Depressed group 3	Male (%)	Female (%)	Total	Average age (year)
SB group	30 (54%)	25 (46%)	55	13.25±4.09
-sb group	25 (55%)	20 (45%)	45	13.68±5.17

2.2. Diagnostic tools and questionnaires

2.2.1. Intake General Information Sheet for Children and Adolescents (IGIS)

The IGIS is a fully structured interview containing pre-coded item response choices. In the IGIS the parent is interviewed about the child's socio-demographic/family background, developmental, educational, and health history, and major life events at the intake in the investigation.

2.2.2. Interview Schedule for Children and Adolescents – Diagnostic Version Present and Lifetime (ISCA-D-PL)

Major depression was diagnosed using the ISCA-D-PL semi-structured psychiatric interview, assessing both lifetime and current disorders in children and youths (Sherrill & Kovacs, 2000). Furthermore, it includes most DSM-IV Axis-I diagnoses and even some DSM-III symptoms. The clinicians rating is based on both the parent informants and the child's/youth's answer, by deriving a final rating for each symptom. Although the ISCA-D is designed to

provide information even on depression severity, this feature has not been used in the present studies.

Melancholic subtype of depression was estimated by ISCA-D-PL, in case of having the DSM-IV diagnosis of major depression, on the basis of the additional criteria for melancholic features of the DSM-IV: Either of the following, occurring during the most severe period of the current episode:

(1) loss of pleasure in all, or almost all activities

(2) lack of reactivity to usually pleasurable stimuli

Three (or more) of the following:

(1) distinct quality of depressed mood

(2) depression regularly worse in the morning

(3) early morning awakening (at least two hours before usual time before the awakening)

(4) marked psychomotor retardation or agitation

(5) significant anorexia or weight loss

(6) excessive or inappropriate guilt.

2.2.3. Children Depression Inventory-Short Version (CDI-Short Form)

As a part of the initial assessment, the 10-item CDI Short Form was developed. The CDI in its original form is a 27-item self-rated questionnaire for children and adolescents. The Short Form is a multi-rater assessment of depressive symptoms in youth aged 7 to 17 years correlating with the full inventory (r=0,89), administered and scored using paper-and-pencil format in the present study, providing streamline evaluation of depressive symptoms. Each item consists of three choices, keyed 0 (absence of a symptom), 1 (mild symptom), 2 (definite symptom), with higher scores indicating increasing severity. The total score can range from 0 to 20. The cut-off value for clinical depression is 7 points (Mayer et al., 2006).

2.3. Statistical analyses and laboratory methods

2.3.1. Statistical analyses

To study oxytocin and prolactin gene variants, several analyses were performed using Haploview v 3.32 (Barrett et al., 2005), including Hardy-Weinberg equilibrium and Mendelian inheritance, and the transmission disequilibrium test (TDT). A corrected significance threshold was calculated for each gene using Nyholt's (2004) spectral decomposition (SNPSpD) method. Correction by SNPSpD was followed by correction for the number of genes tested, to account for multiple comparisons. Haplotype blocks were defined using the Gabriel et al. (2002) criteria. All haplotypes with a frequency greater than 0.05 were also tested for association with COMD.

To compare melancholic depressed participants to the non-melancholic depressed ones, to compare melancholic depressed participants to their unaffected siblings (with no detectable Axis I DSM-IV disorder) and to compare probands with and without the somatic type of depression, we applied the following statistical methods:

Data analysis was carried out using the software package SPSS Statistics, version 17 (SPSS Inc., Chicago, Illinois, USA). Group characteristics were investigated using an independent-samples t-test and the $\chi 2$ -test. Fisher's exact test was used to compare allele frequencies and the $\chi 2$ - test of statistical significance set at p less than 0.05 was used to compare genotype frequencies among the non-melancholic and melancholic subgroups, furthermore among melancholic probands and their unaffected siblings. Logistic regression models were performed to examine the total weighted and grouped weighted life event scores and *BDNF* Val66Met genotypes. We used these models in the total melancholic group. Main effects and possible interactions were tested using the likelihood ratio test (stepwise regression). In the first model (Model 1), the main effects of the total weighted life event score and Val66Met genotypes were tested. Second, we included the interaction term of total weighted life event scores and Val66Met genotypes. In the second model (Model 2), the genotype and the main effects of grouped life events scores were tested, later adding the interaction terms between grouped life event scores and Val66Met genotypes. The main effects of SLEs were analysed continuously as continuous score yields more information than dichotomous variables, yielding more sensitive results. Effect size was counted for the genotype-by-melancholia interaction and power analysis was carried out using GPower

(Faul et al., 2007). For the power calculation of the logistic regression analysis, the POWER procedure in SAS 9.4 was used.

2.3.2. Laboratory methods

To genotype single nucleotide polymorphisms across the *OXT* and *PRL* genes and their receptors, genomic DNA was isolated from blood for each participant using a high-salt method (Lahiri and Nurnberger, 1991). Four buccal swabs were also collected per individual for quality control purposes including resolution or confirmation of Mendelian inconsistencies. SNPs were selected for each of the four genes based on Caucasian linkage disequilibrium (LD) plots from the International HapMap project database (www.hapmap.org) with a minimum minor allele frequency (MAF) of 20% (Table 4). SNPs were genotyped independently using 50 ng DNA in a standard Applied Biosystems (Foster City, CA) TaqMan® assay-on-demand reaction modified for a 10 µl final volume. Amplified products were analysed on an AB 7500 Sequence Detection System.

Table 4. Oxytocin and prolactin system genetic marker information

Gene	SNP	Chromosome	Position	Observed heterozygosity	HWE p-value	% Genotyped	Minor allele	Minor allele frequency
OXT	rs2740210	20p13	3001255	0.43	0.31	98	A	0.34
	rs2770378	20p13	3001514	0.48	0.50	97	A	0.42
	rs4813627	20p13	3003513	0.49	0.55	98	G	0.47
OXTR	rs237885	3p25	8770543	0.49	0.11	100	T	0.44
	rs2268493	3p25	8775840	0.43	0.32	96	C	0.32
	rs237898	3p25	8783495	0.49	0.37	100	T	0.39
PRL	rs1205961	6p22.3	22393991	0.45	1.00	100	A	0.35
	rs1205960	6p22.3	22396139	0.37	0.46	95	T	0.25
	rs849886	6p22.3	22399346	0.48	0.62	100	T	0.47
	rs1341239	6p22.3	22412183	0.47	0.78	100	A	0.39
PRLR	rs187490	5p13.2	35080884	0.41	0.21	100	C	0.31
	rs249522	5p13.2	35096076	0.22	0.87	98	T	0.13
	rs35614689	5p13.2	35134877	0.51	0.71	95	G	0.48
	rs4703505	5p13.2	35207778	0.40	0.74	92	A	0.27
	rs2047740	5p13.2	35239149	0.43	0.30	100	C	0.33
	rs7727306	5p13.2	35273617	0.48	0.45	100	A	0.40

In our preliminary studies on the *BDNF* rs6265 single nucleotide polymorphism, DNA was isolated from peripheral blood leukocytes and/or buccal swab. Genetic analysis: PCR, PmaCI restriction enzyme digestion, followed by polyacrylamide gel electrophoresis with ethidium bromide staining (Tsai et al., 2003). Statistical analysis was performed using SPSS software.

On the sample of depressed probands in Study 2, the *BDNF* rs6265 single nucleotide polymorphism, also known as Val66Met, was genotyped using the Applied Biosystems TaqMan pre-designed assay C_11592758_10 (Applied Biosystems, Foster City, California, USA). For

each reaction, 20 ng genomic DNA was amplified as per the manufacturer's directions in a total volume of 10 µl in an MJ Research thermocycler (Bio-Rad Laboratories, Hercules, California, USA). Each genotyping plate was analysed after amplification on the ABI Prism 7000 AQ5 Sequence Detection System (software v1.2.3) using the allelic discrimination option. Allele calls were made manually.

Genotyping of the *IFNγ* +874 T/A single nucleotide polymorphism has been carried out by allele specific PCR amplifications, followed by polyacrilamide gel electrophoresis and visualized by ethidium bromide (DNA was isolated from blood sample or buccal swab). After migration of the samples in the electrophoresis gel, a photo of the resulting pattern was taken (Figure 4).
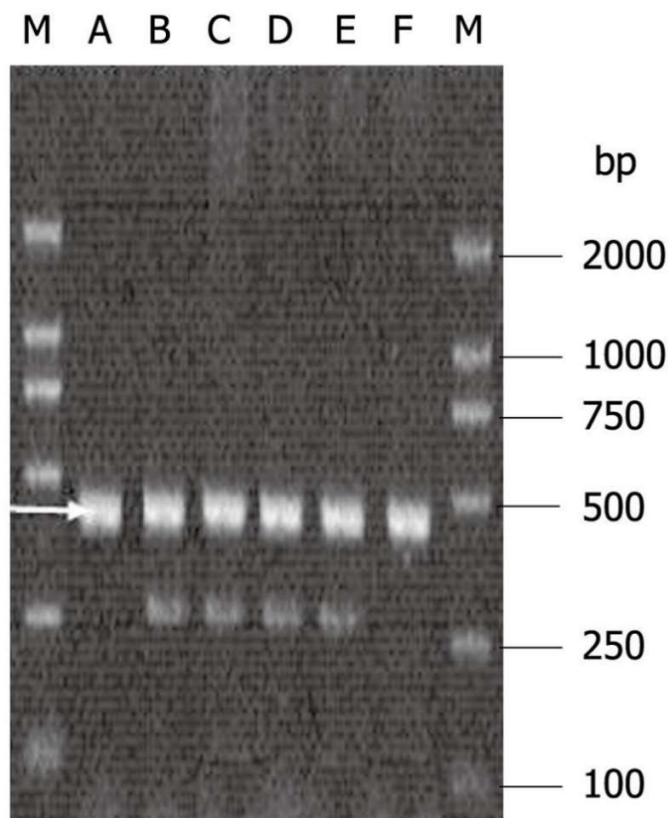

Figure 4. Representative gel picture of IFN-ɤ +874 T/A polymorphism. M, Marker; Lane A & B: AA (low producer) genotype; Lane C & D: AT (medium producer) genotype; Lane E & F: TT (high producer)

3. Results

3.1. Investigating the *OXT, OXTR, PRL*, and *PRLR* genes in child and youth depression

A total of 678 families were genotyped for 16 SNPs across the *OXT, OXTR, PRL*, and *PRLR* genes. All SNPs were in Hardy-Weinberg equilibrium ($p \geq 0.11$). Seventeen families showed Mendelian errors. In 11 of these, inconsistencies were not due to sample handling errors, according to evaluation of buccal swabs taken from the same individual as the blood sample; these families were suspected of nongenetic familial relationships and removed from the analysis. The remaining six families with Mendelian errors showed a discrepancy between the blood and buccal swab samples for one member of the family. In the latter cases, the swab sample was used to genotype the discrepant individual. DNA from blood was used to regenotype 5% of the samples at each marker: error rates were < 1% and were explained by labelling/transcriptional errors.

Two of the three SNPs of the *OXT* gene, showed significant results (rs2740210: allele C overtransmitted, $\chi^2 = 5.32$, $p = 0.02$; rs4813627: allele G overtransmitted, $\chi^2 = 6.01$, $p = 0.01$; see Table 4). After using SNPSpD, both were nominally significant ($M_{eff} = 2.35$, adjusted $\alpha = 0.021$), but they were not significant after further Bonferroni correction by the number of genes tested (adjusted $\alpha = 0.005$). No significant associations were found for any SNPs in the *OXTR, PRL*, or *PRLR* genes ($p > 0.15$ (Table 5). Only one haplotype block, spanning rs1205960 and rs849886 in the *PRL* gene, was identified. None of the haplotypes were significant.

Table 5. Transmission disequilibrium test results for *OXT, OXTR, PRL* and *PRLR* markers

Gene	SNP	Overtransmitted allele	Tr:Un	$x2$	p
OXT	rs2740210	C	260:210	5.32	0.02[1]
	rs2770378	G	271:238	2.14	0.14
	rs4813627	G	299:242	6.01	0.01[1]
OXTR	rs237898	A	278:269	0.15	0.70
	rs2268493	A	229:220	0.18	0.67
	rs237885	A	270:263	0.09	0.76
PRL	rs1341239	T	277:246	1.84	0.18
	rs849886	G	297:268	1.49	0.22
	rs1205960	G	198:193	0.06	0.80
	rs1205961	T	268:229	3.06	0.08
PRLR	rs7727306	A	282:279	0.02	0.90
	rs2047740	C	259:225	2.39	0.12
	rs4703505	T	184:162	1.40	0.24
	rs35614689	T	272:253	0.69	0.41
	rs249522	G	136:121	0.88	0.35
	rs187490	T	242:230	0.31	0.58

Tr: transmitted; Un: untransmitted.

[1]Not significant at adjusted $\alpha = 0.005$.

We performed two additional exploratory analyses to examine parent-of-origin effects (POEs) and proband sex effects for the three SNPs downstream of the *OXT* gene, using the default TDTPHASE sub-option settings of UNPHASED v2.403 (Dudbridge, 2003), followed by a onetailed Fisher's Exact text, based on the hypothesis that oestrogen influences on OXT expression would be more relevant in transmissions to and from females. Three *OXT* SNPs revealed biased maternal transmissions of rs2740210 (allele A undertransmitted, relative risk (RR) = 0.68, CI = 0.46-1.03, likelihood ratio score (LRS) = 7.14, $p = 0.007$) and rs4813627 (allele A undertransmitted, RR = 0.71, CI = 0.49-1.04, LRS = 6.09, $p = 0.01$), while paternal transmissions were not significant; most of the paternal transmissions were in the same direction

as the maternal transmissions, with no parent-of-origin effect by Fisher's Exact Test (p's $\geq$ 0.12). Similarly, all three *OXT* SNPs showed biased transmission to daughters, but proband sex effects were not significant after correction for multiple tests (adjusted α = 0.017) for rs2740210 (FET, p = 0.04) or rs2770378 (FET, p = 0.02) (Table 6).

Table 6. OXT SNP transmissions according to the sex of the parent and proband

SNP	Paternal transmissions					Maternal transmissions					FET (one-tailed)
	Tr	Un	RR	LRS	*p*	Tr	Un	RR	LRS	*p*	
rs2740210	108	95	0.88	0.83	0.36	115	78	0.68	7.14	0.007	0.12
rs2770378	104	100	0.96	0.08	0.78	115	90	0.78	3.06	0.08	0.17
rs4813627	111	88	0.79	2.66	0.1	125	89	0.71	6.09	0.01	0.32

SNP	Transmissions to male probands					Transmissions to female probands					FET (one-tailed)
	Tr	Un	RR	LRS	*p*	Tr	Un	RR	LRS	*p*	
rs2740210	135	128	0.95	0.19	0.66	133	90	0.68	8.34	0.004	0.04
rs2770378	145	152	1.05	0.16	0.68	132	96	0.73	5.71	0.02	0.02
rs4813627	170	151	0.89	1.12	0.29	138	98	0.71	6.81	0.01	0.11

Tr: transmitted; Un: untransmitted; RR: relative risk; LRS: likelihood ratio test statistic; FET: Fisher's Exact test, one-tailed p-value (adjusted α = 0.017).

3.2. The preliminary studies investigating the role of *BDNF* Val66Met polymorphism in childhood onset depression

We did not find significant difference in the frequency of *BDNF* genotypes (p=0.511) and alleles (Figure 5 and 6) between the depressed and the healthy sibling control groups.
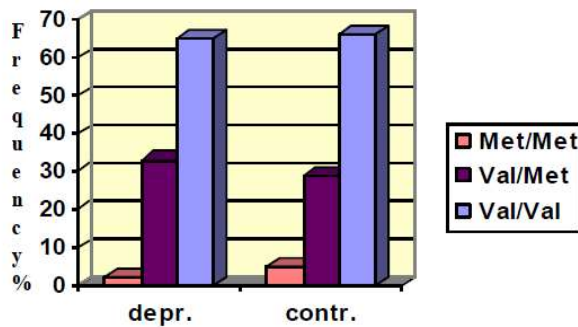

Figure 5. *BDNF* **Val66Met genotype distribution between depressed children and their healthy siblings as controls**
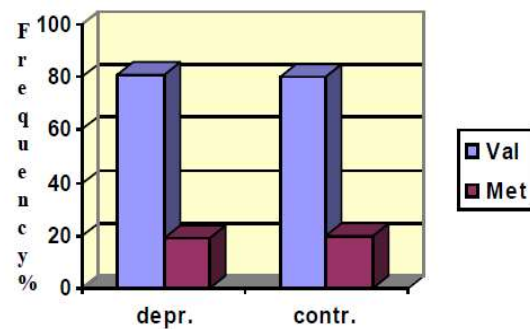

Figure 6. *BDNF* **Val66Met allele distribution between depressed children and their healthy siblings as controls**

Regarding the melancholic phenotype, *BDNF* Val66Val genotype had a significantly higher frequency in the depressed group than in the unaffected siblings (p=0.024).

We did not find significant difference in the frequency of *BDNF* alleles between the melancholic and the non-melancholic group.

From the investigated somato-vegetative symptoms (poor concentration, memory or attention, insomnia, hypersomnia, weight loss or weight gain, psychomotor retardation or agitation) hypersomnia showed association with the frequency of Val66Met genotype ($p < 0.1$).

Regarding the categorized life event groups the occurrence of psychiatric disease of family members showed association with *BDNF* Val allele.

In the extended preliminary study, the melancholic subgroup of depressed probands did not show significantly higher Val/Val frequency than the unaffected control group (p = 0.28 and 0.37, respectively). The Val allele was not associated with the occurrence of melancholic subtype in depressive probands (p = 0.12 and 0.29) (see the Table 7 below).

Table 7. Genotype and allele distributions in melancholic probands and their unaffected siblings

	Unaffected (n=100)	Melancholic subtype (n=24)	Melancholic subtype (n=16)
		Current	Past
Genotype*			***
Met/Met	5 (5%)	1 (4.2%)	-
Val/Met	29 (29%)	11 (45.8%)	7 (43.8%)
Val/Val	66 (66%)	12 (50%)	9 (56.2%)
Allele**			****
Val	95	23	16
Met	34	12	7

* melancholic current vs. unaffected χ^2 = 2.52 p = 0.28

** melancholic current vs.unaffected χ^2=2.47 pVal = 0.12 and χ^2 = 0.32 pMet = 0.57

*** melancholic past vs. unaffected χ^2 = 1.97 p = 0.37

**** melancholic past vs. unaffected χ^2 = 1.1 pVal = 0.29 and χ^2 = 1.7 pMet = 0.28

3.3. *BDNF* Val66Met genotype and allele distributions in melancholic and non-melancholic depressed participants and examining whether the past exposure to SLEs interacts with *BDNF* to predict the melancholic subtype

In this sample of depressed youths, 27.78% fulfilled the criteria for melancholic depression. The mean age at interview did not differ significantly between the melancholic and the nonmelancholic subgroups [t = − 0.28, p (two-tailed) = 0.78], whereas there was a statistically significant difference [t = − 0.262, p (two-tailed) = 0.01] in sex. Further characteristics of the total sample and the subsamples can be found in Table 6 (here we provided an unweighted sum of life events).

Comparison of the total and grouped SLE scores did not show any significant difference between the two patient groups. A nonsignificant difference [t = 1.759, df. = 1, p = 0.079] was found in the intrafamilial SLE scores (M = 17.33 in the nonmelancholic and 15.62 in the melancholic

group, respectively). Table 8 shows the frequency of each life event and the life event grouping, together with the relative *p* - values.

Table 8. Frequency of stressful life events in the melancholic and non-melancholic groups and the p-values of the independent sample t-statistics testing the differences of the SLE scores between those with and without melancholy

Life event groups	Frequency in the melancholic group (N=162)		Frequency in the non-melancholic group (N=421)		
	N	%	N	%	*p*-value
Parental health events					
1. Medical hospitalization of biological mother due to somatic illness	49	30.2	155	36.8	0.59
2. Medical hospitalization of biological father	43	26.9	116	27.6	
3. Medical. hospitalization of stepparent	5	3.1	8	1.9	
4. Physical illness of biological mother	16	9.9	46	10.9	
5. Physical illness of biological father	18	11.2	33	7.8	
6. Physical illness of stepparent	0	0.0	1	0.2	
7. Psychiatric hospitalization of biological mother	31	19.1	63	15	
8. Psychiatric hospitalization of biological father	22	13.7	57	13.5	
9. Psychiatric hospitalization of stepparent	0	0.0	6	1.4	
Death of Close Relatives Events					
10. Death of a parent	10	6.2	17	4.0	0.63
11. Death of a close relative	107	66.0	286	67.9	
Sociodemographic Events					
12. Financial problem	40	24.7	134	31.8	0.40
13. Move	92	56.8	222	52.7	
14. Parent unemployed	73	45.3	203	48.2	
15. Natural disaster	4	2.5	13	3.1	
16. Loss of home	5	3.1	17	4	

Intra-familial Events					
17. Sibling birth	102	63.0	275	65.3	0.079
18. Sibling medical hospitalization	36	22.5	139	33.0	
19. Sibling psychiatric hospitalization	12	7.4	33	7.8	
20. Family arguments	78	48.1	225	53.4	
21. Foster-care of subject	0	0.0	4	1	
22. Divorce of biological parents	61	37.7	160	38.0	
Extrafamilial Events and Abuse					
23. Abuse	44	27.2	114	27.1	0.92
24. Teasing by peers	88	54.3	230	54.6	
25. Police contact	6	3.7	19	4.5	
26. Suspension from school	3	1.9	7	1.7	

Grouping is based on the study of Mayer et al. (2009) Ref. 24

SLE, stressful life event

In both melancholic and nonmelancholic depressed groups, the homozygous Val/Val genotype had the highest occurrence. The homozygous Met/Met genotype was the least common in both groups. Table 9 shows the genotype and allele frequencies in the total depressed group and the subgroups. The genotypes were in Hardy–Weinberg equilibrium, both in the total depressed group and in the subgroups.

Table 9. *BDNF* **genotype distributions and allele frequencies in the melancholic and non-melancholic subgroups**

	Depressed (N=583)	MEL (N=162)	NMEL (N=421)
Genotype [n (%)]			
Met/Met	18 (3.1)	4 (2.5)	14 (3.3)
Val/Met	167 (28.6)	39 (24.1)	128 (30.4)
Val/Val	398 (68.3)	119 (73.5)	279 (66.3)
Allele [n (%)]			
Val	963 (82.6)	277 (85.5)	686 (81.5)
Met	203 (17.4)	47 (14.5)	156 (18.5)

BDNF, brain-derived neurotrophic factor; MEL: Melancholic (either current or past); NMEL: Non-melancholic depressed

Testing our first hypothesis, the allele frequency was not significantly different across the two diagnostic groups (Fisher's exact: $p = 0.121$). The Val/Val genotype frequency did not differ

significantly ($\chi 2$ = 2.80, df. = 2, p = 0.25) between the melancholic and the nonmelancholic group. Because of the low rate of the Met/Met genotype, genotypes were dichotomized on the basis of the presence of the Met allele. There was a trend among the nonmelancholic depressed youth of showing a slightly higher rate of the Met-containing genotype, although not statistically significant (Fisher's exact: p = 0.112). The effect size (W) for our genotype-by-melancholia interaction was 0.12; power on such an effect size with 2 df. was 0.74. Examining our second hypothesis, in the total melancholic group with the Model 1 of the logistic regression, described in the Statistical analysis section, taking Met containing genotypes as the reference category, we did not find either significant main effect or significant interaction effect of the total SLEs and the BDNF polymorphism on the melancholy outcome. When we applied Model 2 (again the Met-containing genotype was considered the reference category) to examine life event groups separately, neither of the events contributed significantly toward the model. Main effects and interactions of BDNF Met-containing genotypes, total SLEs, and SLE groups as predictors in the model of melancholic depression are shown in Table 10. For the logistic regression analysis, power was 0.31 for life events, 0.56 for the presence of a Met allele, and 0.06 for the life events-by-genotype interaction.

Table 10. Main effects and interactions of BDNF Met-containing genotypes, total stressful life events, and stressful life event groups as predictors in the model of melancholic depression in males and females

Dependent variable	Models	Independent variables	OR	*p* value
Melancholy (Females)	Model 1	Met-containing genotypes	-	**.0789**
		Total stressful life events	-	.733
		Met-containing genotypes × Total stressful life events	-	.385
	Model 2	Met-containing genotypes	-	.391
		Parental health	-	.893
		Death of close relatives	-	.187
		Sociodemographic	-	.631
		Intrafamilial	-	.111
		Extrafamilial and Abuse	-	.871
		Met-containing genotypes × Parental health	-	.901
		Met-containing genotypes × Death of close relatives	-	.894
		Met-containing genotypes × Sociodemographic	-	.573
		Met-containing genotypes × Intrafamilial	-	.126
		Met-containing genotypes × Extrafamilial and Abuse	1.05	.021
Melancholy (Males)	Model 1	Met-containing genotypes	-	.073
		Total stressful life events	-	.109
		Met-containing genotypes × Total stressful life events	-	.990
	Model 2	Met-containing genotypes	-	.073
		Parental health	-	.485
		Death of close relatives	-	.074
		Sociodemographic	-	.485
		Intrafamilial	-	.195
		Extrafamilial and Abuse	-	.419
		Met-containing genotypes × Parental health	-	.995
		Met-containing genotypes × Death of close relatives	-	.964
		Met-containing genotypes × Sociodemographic	-	.888
		Met-containing genotypes × Intrafamilial	-	.706
		Met-containing genotypes × Extrafamilial and Abuse	-	.356

BDNF, brain-derived neurotrophic factor; OR, odds ratio

3.4. Investigating the association between interferon gamma +874 T/A polymorphism and the somatic type of depression

In the sickness behaviour (SB) group, the frequencies of the A allele containing genotypes were higher, than in the comparison group, without statistically significant difference (Figure 7) (T/A: SB group: 60.0%, -sb group: 51.1%; A/A: SB group: 21.8%, -sb group: 13.3%; $p = 0.122$).
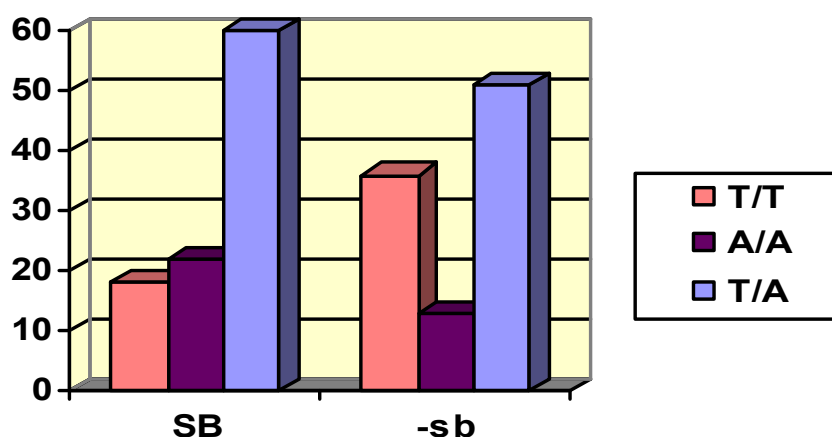

Figure 7. Genotype distribution in probands with and without sickness behaviour
SB, sickness behaviour; -sb, comparison group

The comparison of allele frequencies also did not reveal statistically significant differences (T: SB group: 48.2%, -sb group: 61.1%; A: SB group: 51.8%, -sb group: 38.9%; $p > 0.1$) (Figure 8).
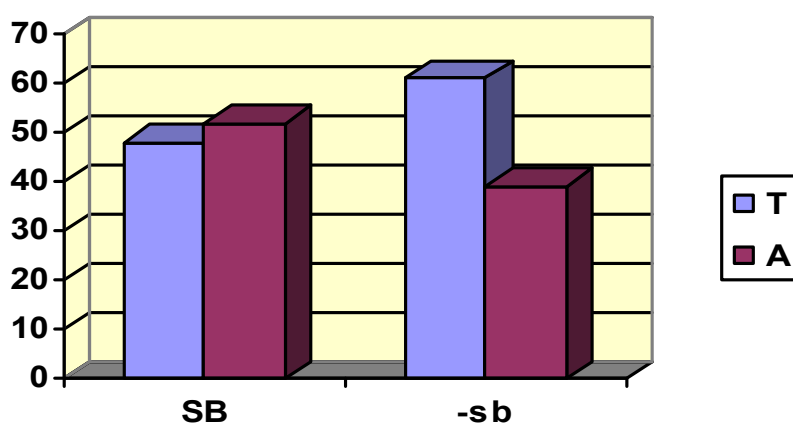

Figure 8. Allele distribution in probands with and without sickness behaviour
SB, sickness behaviour; -sb, comparison group

These results did not reveal association between *IFN-γ* +874 polymorphism and SB. However we observed a tendency, being the genotypes with the A allele more frequent in the SB group.

4. Discussion

Our research is performed in a very large clinical sample of children and adolescents with MDD, even involving their siblings who are affected or not affected by the disorder, as controls. Overall, our results complement a growing body of literature, which suggests that MDD is caused by numerous interacting genetic and non-genetic factors. Even today, gene variants associated with MDD vulnerability have not been clearly identified (Lopizzo et al., 2015).

We genotyped single nucleotide polymorphisms across the *OXT* and *PRL* genes and their receptors in a large Hungarian cohort of families with COMD. Our results show a trend towards association of two *OXT* SNPs, rs2740210 and rs4813627, with COMD, not significant after correction for multiple testing. Supplementary analyses initially suggested proband sex effects for *OXT* SNPs rs2740210 and rs2770378, but were not significant after multiple testing corrections. *OXT* lies in a segmental duplication region of human chromosome 20 and shares high sequence homology in the first two exons to the arginine vasopressin (AVP) gene. The *OXT* and *AVP* genes lie tail-to-tail and are separated by a 10 kb intergenic region (IGR); *AVP* and vasopressin V1b receptor gene ((*AVPR1B*) genetic polymorphisms have both been significantly associated with COMD in this sample (Dempster et al., 2007, 2009). The three *OXT* SNPs genotyped in the present study reside within 2 kb of the 3' end of the *OXT* gene. Transgenic rat and mouse studies of the *OXT* and *AVP* genes have shown that the IGR contains regulatory sequences responsible for cell-specific OXT expression in oxytocinergic magnocellular neurons (MCNs) (Murphy and Wells, 2003). The observation that the two *OXT* SNPs showed a nonsignificant trend for affected daughters, is of note in light of OXT sex effects reported in the literature (Richard and Zingg, 1990; Cushing and Carter, 2000). To our knowledge, this is the first genetic association study of *OXT* and *PRL* variants in a pediatric mood disorder sample. Four studies have found associations between *OXTR* gene variations and autism (Wu et al., 2005; Ylisaukko-oja et al., 2005; Jacob et al., 2007; Gregory et al., 2009) and recent evidence has implicated the *OXTR* in maternal sensitive responsiveness with their 2-year-old toddlers

(Bakermans Kranenburg and van Ijzendoorn, 2008). A haplotype of three *OXTR* SNPs that we did not examine has recently been associated with negative affect and loneliness (Lucht et al., 2009). Another investigation noted an association between two *OXTR* SNPs and major depression (Costa et al., 2009); we studied three different loci and provided broader SNP coverage of the *OXTR*, with a childhood-onset sample. There were more missing fathers than missing mothers in our sample. We did not estimate missing parental genotypes. Since a missing parental genotype means the entire family is excluded from the analysis, the relatively larger number of missing fathers will reduce our power, but not influence the relative numbers of parental transmissions tested. We studied a moderately large, well-characterized sample. Statistical methods used were robust to population stratification. Corrections for multiple tests were conservative. We estimate 678 families provide a power of 0.86, given the assumptions of a $\alpha=0.05$, population frequency of 2%, additive model of inheritance, genotype relative risk of 1.4, minor allele frequency of 0.41 (mean minor allele frequency for *OXT* SNPs = 0.41) and complete linkage between risk and disease variants (Purcell et al., 2003). We report evidence that was initially suggestive of possible association between two *OXT* SNPs (rs2740210 and rs4813627) and COMD. The results survived spectral decomposition, but were not significant after correcting for the number of genes tested. *OXTR, PRL* and *PRLR* SNPs were not associated with COMD. In secondary analyses, the relative significances of association for two *OXT* SNPs were increased with parental and proband sex effect analyses. A trend towards proband sex effects to daughters failed to reach statistical significance after correcting for multiple tests. Considering the *OXT* SNPs are located in a putative regulatory region, our exploratory analyses by parent and proband sex are of interest as they may be relevant to epigenetic effects (Kaminsky et al., 2006).

Our second study completes the investigation of the genetic background of depression using alternative phenotypes. We were particularly interested in finding subtypes/alternative phenotypes of depression, as well as finding a trait marker for major depression, because such factors could be helpful in finding susceptibility genes. Given that MDD as defined by the DSM-IV is a complex disorder, finding susceptibility genes may be facilitated using alternative, more homogenous depression phenotypes. However, even factors other than genetic vulnerability and diagnostic procedure must be considered to explain the development of MDD (Hosang et al., 2014). Despite the clinical utility and validity of the melancholic phenotype of depression, a

debate has been ongoing on the relevance of the subtype, suggesting that further research is needed (Hadzi-Pavlovic and Boyce, 2012) to define its biological features. After that the results of our preliminary study showed that the *BDNF* Val66Met polymorphism might play a role in the onset of melancholic type of depression, we decided to perform the analyses on a larger database and with improved criteria for melancholic depression.

Consequently, we studied the contribution of the *BDNF* polymorphism and its interaction with SLE toward early onset melancholic depression. The frequency of melancholia in our sample was in the range of the previously reported prevalences of 20% to almost 50% in juvenile samples (Ryan et al., 1987; Kolvin et al., 1991; Ambrosini et al., 2002). The ratio of females was significantly higher in the melancholic than in the non-melancholic group [t = −0.262, p (two-tailed) = 0.01], suggesting that females are more prone to developing the early-onset melancholic phenotype. The current literature establishes that girls show more depressive symptoms than boys (Wade et al., 2002; Hankin et al., 2007); however, there is some inconsistency in the findings (Chen et al., 2012), which may be because of methodological, age, and ethnic differences in the sample populations. Although in a preliminary study of Quinn et al. (2012) and in the study of Guo et al. (2015), no significant differences were found in the melancholic, non-melancholic, and control groups for sex or age, the difference compared with our findings may be because of their focus on the adult population in addition to a smaller sample size, which decreases the chances of finding a significant difference. Our result may represent an interesting suggestion for future research as the presence of the subtype might influence treatment response (Rush et al., 2008) and complications. According to a study of Gold et al. (2002), common medical complications of different subtypes of depression may occur because of different underlying mechanisms. In the genotypic and allelic association analysis, we found elevated Val/Val genotype frequency in the melancholic group. Our analysis, however, could not be used to discriminate between the melancholic and the non-melancholic groups. The calculated effect size and the power analysis carried out for our genotype-by-melancholia interaction suggests that small to medium effects would have been detected if present. Our results partly correlate with the study of Quinn et al. (2012), showing that no significant relationship was found between the BDNF genotype and groups. However, they found that the gene-by-environment interaction effect is expected to provide a better prediction for melancholic depression. The explanation for the different but not contradictory results is that we examined

biological features discriminating the melancholic versus the non-melancholic phenotype within a depressed sample, whereas Quinn et al. (2012) compared the melancholic and the non-melancholic group of patients with healthy controls. Power analysis for the logistic regression analysis suggests that our sample was adequate to detect the main effects of life events or genotype if they were medium to large, but could only detect interaction effects if they were quite large.

In our third study we investigated the (+874) T/A genotypes given the information that they have previously been studied in connection with the risk of IFN-alpha-induced depression, the presence of T alleles representing a risk for the development of this disease (Oxenkrug et al., 2014). Our case control study investigating directly the role of interferon-γ, performed on a well-defined subgroup of depressed children, however, did not reveal any significant difference in the genotype or allele distribution between subjects with the somatic type of depression and the comparison group. We observed a tendency that failed to attain significance at conventional level, being the genotypes with the A allele more frequent in the SB group. Further investigation may thus be needed with an increased number of subjects.

Since the underlying genetics has proved to be highly complex, identifying the genetic determinants for individual differences in the susceptibility to MDD is amongst the greatest challenges.

An illustrative example on the complexity of the genetic background of depression is given by that interferon treatment may modulate *5-HTT* activity levels (Morikawa et al., 1998). A repeat length polymorphism in the promoter region of this gene has been shown to affect the rate of serotonin uptake and may play a role in depression-susceptibility. In human placental choriocarcinoma cells, a 3-hour treatment with IFN-γ increased levels of 5-HTT mRNA and a treatment with IFN-γ for 3-6 hours also increased 5-HTT uptake activity. These data suggest that IFN-induced psychiatric effects may be modulated by regulation of 5-HTT transcription.

The power and performance of the methods trying to determine the molecular genetic background of depression may be further improved by integrating biological information at the systemic level. To overcome the limitations of the case control approaches, sets of the genes carrying out biochemical processes related to the functioning of neural systems can be investigated in the future.

Strengths and Limitations

Although the sample size would ideally be larger, the above described studies have several methodological strengths. We note that our melancholic group is a well-defined subgroup within the depressed patients thanks to the diagnoses established through a very rigorous procedure, described in the materials and methods section, using clinician administered semi-structured interviews to obtain the optimal collection of biographical and contextual information. Life events were measured by researcher administered questionnaires. The present study on oxytocin and prolactin genes is the first genetic analysis to examine the association between variants in these neuroendocrine genes and depression with onset in childhood. Furthermore, to our knowledge, this is the first study to investigate the differentiating effect of the *BDNF* genotype and the G×E interaction on the melancholic phenotype in a large sample of depressed young patients, which was representative of the Hungarian population.

As the parents were providing information on their children's SLEs, we had limited information on some of them, for example teasing. Though face-to-face interviews by clinically trained assessors, the use of common phrases, and the use of timelines with named "anchors" during the interview may facilitate retrospective recall (Buka et al., 2004).

Given the complexity of Model 2 investigating the interaction of stressful life events with the BDNF Val66Met polymorphism, the particular model would only be able to detect large effects, which represents a limitation of our study.

Conclusions

We found no association between oxytocin and prolactin gene variants and childhood-onset mood disorders. The performed exploratory analyses by parent and proband sex are of interest as they may be relevant to epigenetic effects. We didn't find association between the melancholic subtype of major depression and the *BDNF* genotype and stressful life event interaction and the somatic subtype of childhood-onset major depression and the interferon-gamma +874 T/A polymorphism in this clinic-referred population of depressed youths.

In our study, females are more prone to develop the early-onset melancholic phenotype. This results contributes to the prevention and treatment of the melancholic type of childhood-onset major depression, as consequences can be lessened by focusing on risk groups in early stages. However, additional research and empirical work is needed in this area to further understand the contribution of the early-onset phenotype.

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Figure 1. BDNF polymorphism. The courtesy of Ágnes Fehér.

Figure 2. Cellular transport and secretion of BDNF.From the article titled the Yin and yang of neurotrophin action by Bai Lu, Petti T. Pang & Newton H. Woo (2005). Nature Reviews Neuroscience 6, 603-614.

Appendix

I.

II.

III.