Infant attachment and stress regulation : a neurobiological study

Luijk, P.C.M.

5 Attachment genes? Associations of dopaminergic, serotonergic, oxytonergic and neuroplasticity candidate genes with attachment security and disorganization

Maartje P. C. M. Luijk, Glenn I. Roisman, Henning Tiemeier, Cathryn Booth-LaForce, Marinus H. van IJzendoorn, Jay Belsky, Andre G. Uitterlinden, Vincent W.V. Jaddoe, Albert Hofman, Frank C. Verhulst, Anne Tharner, and Marian J. Bakermans-Kranenburg. Manuscript submitted for publication.

Abstract

In two birth cohort studies with genetic and attachment data of more than 1100 infants in total, we tested main effects of candidate genes involved in the dopamine, serotonin, oxytocin, and neuroplasticity systems on attachment security and disorganization. We found no additive genetic associations for attachment security and attachment disorganization, assessed with the Strange Situation Procedure. However, specific tests for dopamine and serotonin system genes revealed a co-dominant risk model for COMT Val158Met, very consistent across both samples. Carriers of the Val/Met genotype showed higher disorganization scores (combined effect size $d = 0.20$, $CI = 0.09; 0.32$, $p = .001$). This unexpected finding might be explained by a broader range of plasticity in heterozygotes, which may increase susceptibility to environmental influences. The current study provides uniquely robust results in combining the two largest attachment cohorts with molecular genetic data to date. Future directions in research on the genetics of attachment are discussed.

Introduction

Attachment is defined as the child's need to seek proximity to a favorite, protective caregiver in times of stress (e.g., illness, danger) and to derive comfort from the attachment figure in stressful settings (Cassidy, 2008). Insecure and especially disorganized attachments elevate risk for psychopathology in adolescence and adulthood (Sroufe, Egeland, Carlson, & Collins, 2005). Formation of an attachment relationship, considered essential for offspring survival (Bowlby, 1969/1982; Suomi, 2008), is influenced mainly by the interactive history of an infant and its caregiver

and, to a lesser extent, socio-demographic factors and psychosocial characteristics of the parents (Belsky & Fearon, 2008). An emphasis on environmental origins of attachment-related individual differences is consistent with behavior-genetic studies of twins, which estimate the contribution of genetic factors to attachment security and disorganization to be negligible (Bokhorst et al., 2003; O'Connor & Croft, 2001; Roisman & Fraley, 2008).

Nevertheless, much-cited work by Lakatos and colleagues (2000) a decade ago presented evidence of a direct genetic effect on disorganized attachment involving a 48 base pair variable number tandem repeat (VNTR) in the promoter region of the Dopamine D4 receptor gene (DRD4). In a homogeneous sample of 90 low-risk Caucasian children, the 7-repeat allele was associated with higher risk for disorganized attachment. These results stimulated several replication efforts (Bakermans-Kranenburg & Van IJzendoorn, 2004; Spangler, Johann, Ronai, & Zimmermann, 2009), but none reproduced evidence of a direct association between DRD4 and disorganized attachment (see Bakermans-Kranenburg & Van IJzendoorn, 2007 for a review). Later, Spangler and colleagues (2009) reported a direct genetic association between the short allele of the serotonin transporter gene 5-HTT and increased risk for attachment disorganization. Their findings in 96 low-risk Caucasian infants call for replication in larger samples.

In two large cohorts of infants, we assessed polymorphisms in the dopaminergic, serotonergic, oxytonergic, and neuronal plasticity systems, to examine whether these are associated with the quality of infants' attachment behavior. The dopaminergic system is involved in attentional, motivational, and reward mechanisms (Robbins & Everitt, 1999). Common variations in dopaminergic genes DRD4 48 bp VNTR, DRD4 -521C/T, DRD2/ANKK1 and COMT Val158Met are associated with regulation of dopamine levels (D'Souza & Craig, 2006). Behaviorally, carrying the minor allele of these polymorphisms (respectively, DRD4 48 bp 7-repeat; DRD4 -521 C; DRD2/ANKK1 T[A1]) has been related to variations in infant temperament (Ebstein, 2006) and ADHD (Faraone & Khan, 2006). A protective effect has been reported for COMT heterozygotes (Val/Met) showing dopamine levels associated with optimal neurobehavioral outcomes, compared with both homozygous groups (Wahlstrom, White, & Luciana, 2010).

The serotonin system is involved in affect and emotion. A 44 bp insertion/deletion segment of the serotonin transporter gene 5-HTT (5-HTTLPR) is associated with less efficient transcription and serotonin uptake in the synapse (Greenberg et al., 1999; Heils et al., 1996), and the short allele is related to psychiatric disorders (Ebstein, 2006; Rutter, 2006). The oxytonergic system is related to social and parenting behaviors, and both oxytocin levels and polymorphisms in the oxytocin receptor gene (OXTR rs53576 and rs2254298; in particular for the minor A-allele) are associated with the formation of social bonds in both human and animal studies (Bakermans-Kranenburg & Van IJzendoorn, 2008; Carter, Boone, Pournajafi-Nazarloo, & Bales, 2009; Feldman, Gordon, Schneiderman, Weisman,

& Zagoory-Sharon, 2010; Insel, 2010). Finally, brain-derived neurotrophic factor (BDNF) is a protein associated with neuronal growth and survival (Gizer, Ficks, & Waldman, 2009). The gene coding for this protein, also called BDNF, contains a polymorphism influencing secretion of BDNF in the brain. This polymorphism (especially the minor Met-allele) is associated with ADHD (Gizer et al., 2009) and responses to stress and adversity; children with the Met-allele exposed to early deprivation manifest increased anxiety (Casey et al., 2009).

Combining the two largest attachment cohorts to date provides a unique opportunity to explore effects of candidate genes involved in the dopamine, serotonin, oxytocin, and neuroplasticity systems on attachment security and disorganization. The use of a standardized assessment in two independent, well-powered cohorts of Caucasian infants may lead to robust findings.

Materials and Methods

Setting

This report is based on two investigations, the Generation R Study, a prospective cohort study investigating development from fetal life into young adulthood in Rotterdam, the Netherlands (see Jaddoe et al., 2007; 2008), and the NICHD Study of Early Child Care and Youth Development (SECCYD), a prospective study carried out in 10 sites in the USA following children from birth to age 15 years (NICHD, 2005).

Detailed studies were performed in an ethnically homogeneous sub-sample of children of Dutch national origin from the Generation R Study. These children, their parents and their grandparents were born in the Netherlands, which was a selection criterion in order to reduce the risk of confounding (population stratification) by ethnicity. Children participating in this cohort were born between February 2003 and August 2005. Children visited the research center regularly for various assessments. Detailed measurements of child development also were obtained in the SECCYD, which followed an ethnically diverse sample, though the focus of the present inquiry was on the sub-set of Caucasian participants. Participating children were born in 1991 and regularly visited the local universities that recruited them. Written informed consent was obtained from parents of all participants in both studies, which were approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam and the Internal Review Boards of the SECCYD participating universities, respectively.

Study population

In the Generation R study, DNA was collected from cord blood samples at birth. To check for contamination with maternal blood, gender was determined in male participants. Contamination occurred in < 1% of cases, which were excluded. SECCYD DNA was obtained from buccal cheek cells when children were 15 years old. In both studies infants and their parent participated in the Strange Situation

Procedure (SSP) at age 15 months. Quality of attachment was available for 829 (Generation R) and 1191 (SECCYD) parent-child dyads; availability of genotype information ranged from n = 640 to n = 690 for specific SNPs in Generation R. In SECCYD, DNA was collected from n = 711 participants, 478 to 522 of whom were Caucasian, provided pertinent genotype information *and* completed the SSP in infancy. Non-response analysis indicated significant differences between the groups with and without genotypic data in Generation R mainly on perinatal variables. Children without genotypic data had lower gestational age, birth weight and Apgar scores (ps < .01) and mothers were more often nulliparous (p < .05). These births may have been more problematic, raising logistical difficulties to sample cord blood for DNA. SECCYD non-response analysis indicated that Caucasians with genotypic and infant attachment data differed from Caucasians lost to follow-up before age 15 years or who did not provide genetic data; those in the current analysis were more likely to be female (p < .05) and have mothers who were somewhat older (p < .01) and more educated (p < .01) at study onset. Table 1 presents characteristics of both samples.

Procedures and measures

Strange Situation Procedure. In both studies, parent-infant dyads were observed in the Strange Situation Procedure (SSP, Ainsworth, Blehar, Waters, & Wall, 1978) when the infant was about 15 months old. In the Generation R study, SSPs were conducted with the primary caregiver; 87% mothers (n = 721) and 13% fathers (n = 108). In SECCYD, SSPs were conducted with mothers. The SSP is a well-validated, widely used procedure to measure the attachment quality. It consists of seven 3-minute episodes designed to evoke mild stress to trigger attachment behavior (Ainsworth et al., 1978). To make it fit a tight time schedule in Generation R (only), two (pre-) separation episodes were shortened by one minute, keeping the critical reunion episodes intact (Luijk et al., 2010).

Attachment behaviors may be categorized as secure (B) or insecure (A, C, D; Main & Solomon, 1990). When stressed, secure (B) infants seek comfort from their mothers, which proves effective, enabling the infant to return to play. Avoidant (A) infants show little overt distress, while turning away from or ignoring mother on reunion. Resistant (C) infants are distressed and angry, but ambivalent about contact, which does not effectively comfort and allow the child to return to play. Examples of disorganized/disoriented (D) behaviors are prolonged stilling, rapid approach-avoidance vacillation, sudden unexplained affect changes, severe distress followed by avoidance, and expressions of fear or disorientation upon return of mother.

Attachment behavior was coded from DVD (Generation R) and videotape (SECCYD) recordings according to established coding systems (Ainsworth, et al., 1978) by two or three highly-trained, reliable coders. Inter-coder agreement was calculated on 70 SSPs in Generation R and 1191 double-coded SSPs in the

SECCYD. For ABCD classification, inter-coder agreement was 77% and 83% (κ = .63 and .69); agreement on disorganized versus non-disorganized attachment classification was 87% and 90% (κ = .64 and .64), respectively.

Richters and associates (1988) developed a method to score attachment in a continuous way. Van IJzendoorn and Kroonenberg (1990) adapted their algorithm, producing a valid Attachment Security Scale which has been widely used (e.g. Kochanska, Aksan, Knaack, & Rhines, 2004). Higher security scores indicate a more secure attachment relationship. Continuous scores for disorganization were derived directly from coding, with higher scores indicating more disorganized behavior. Intercoder reliability (intraclass correlation coefficients [ICC]) for the continuous attachment security and disorganization scales were .88 and .88, respectively, in Generation R (n = 70) and were .92 and .84, respectively, in SECCYD (n = 1191).

Genotyping. Genotyping was performed for genes in the dopaminergic system; DRD4 48 bp VNTR, DRD4 -521C/T (rs1800955), DRD2 (rs1800497), COMT Val158Met (rs4680), the serotonergic system; 5-HTTLPR, and the oxytonergic system; OXTR (rs53576 and rs2254298), and a gene involved in neuroplasticity; BDNF (rs6265). Table 2 displays minor allele frequencies (MAF). Frequency distributions conformed to the Hardy-Weinberg equilibrium (HWE), except for OXTR rs53576 (χ^2 = 4.90; p = .03) in Generation R and DRD4 48 bp VNTR (χ^2 = 14.17; $p < .001$) in SECCYD. The appendix provides detailed information about extraction and genotyping procedures.

Statistical analyses. Preliminary ANOVA and correlational analyses evaluated whether demographic variables were related to genotype and attachment security. Associations between the pertinent gene polymorphisms and attachment security and disorganization were tested using regression analyses applying additive genetic models. For DRD4 48 bp VNTR, DRD2, COMT, and 5-HTT VNTR previous studies have suggested increased risk for carriers of the DRD4 48 bp 7-repeat (Ebstein, 2006), the A1 allele of DRD2 (Berman, Ozkaragoz, Young, & Noble, 2002), and the short allele of 5-HTT (Lesch et al., 1996; Philibert et al., 2007), and a beneficial effect for COMT heterozygotes (Wahlstrom et al., 2010). These models were tested in additional ANOVAs. Attachment security and disorganization, as orthogonal constructs (Van IJzendoorn, Schuengel, & Bakermans-Kranenburg, 1999), were analyzed separately. Assuming a power of 0.80 and significance level of .05 (2-sided) (using Quanto 1.2.4 software, http://hydra.usc.edu/GxE), we were able to detect genetic effects of 1% of explained variance in both outcomes in Generation R and approximately 1.5% in SECCYD.

Results

Distribution of attachment

Distribution of attachment classifications was as follows in Generation R and SECCYD: 58.6% and 69.8% secure (n = 486 and n = 370), 18.2% and 15.7% insecure-avoidant (n = 151 and n = 83), 22.4% and 14.5% insecure-resistant (n = 186 and n = 77). In Generation R, no classification could be assigned for n = 6 (0.7%) children (All SECCYD participants were assigned to their best fitting category). Of all children, 21.0% and 13.4% were classified as disorganized (n = 174 and n = 71), 79.0% and 83.2% were non-disorganized (n = 655 and n = 441). SECCYD excluded 18 (3.4%) difficult to classify cases from the ABCD groupings. Mean Attachment Security Scale scores in Generation R and SECCYD were 0.24 (SD = 2.58) and 1.21 (SD = 3.17); mean disorganization scores were 3.37 (SD = 1.91) and 2.39 (SD = 2.01). Of all background characteristics (see Table 1), only breastfeeding at six months was associated both with attachment quality (security: $p < .05$ and disorganization: $p < .05$) and genotype ($p < .01$) in the Generation R sample. Children breastfed at six months were more secure and less disorganized, and less often carried the minor Val allele of COMT. Taking breastfeeding into account as a covariate did not change the Generation R results. None of the demographic variables in Table 1 was associated with both attachment quality and genotype in SECCYD.

Table 1. *Sample characteristics for Generation R and NICHD SECCYD*

Child characteristics	Generation R	NICHD SECCYD
Child gender, % female	49.3	51.5
Parity, % nulliparous	63.5	47.7
Birth weight in grams	3514 (540)	3537 (496)
Gestational age in weeks	40.0 (1.8)	39.3 (1.4)
Apgar score, % < 7	4.8	--
Parental characteristics		
Age at intake mother	31.9 (3.8)	29.4 (5.3)
Maternal educational level, % low/medium	33.7	22.6
Hours working, mother	28.8 (12.4)	22.5 (19.6)
Marital status, % single	4.3	6.8
Smoking during pregnancy, %	12.3	--
Alcohol during pregnancy, %	58.1	--
Breastfeeding at 6 months, %	30.4	51.8

Note. Unless indicated otherwise, values are Mean (SD). -- = Not measured.

Attachment genes

Using an additive genetic model, in both samples none of the genetic associations for attachment security and attachment disorganization reached significance (Table 2). Table 3 presents results of additional ANOVAs testing a recessive or co-dominant effect for DRD4 48 bp VNTR, DRD2, COMT, and 5-HTT VNTR. DRD4 associations were non-significant. For 5-HTT, short-allele carriers were more often securely attached and DRD2 A1 carriers showed higher disorganization scores, but only in Generation R. For COMT, no associations with attachment security emerged. However, COMT heterozygotes were more disorganized in both samples, see Table 3 (combined effect size $d = 0.20$, *95% CI* = 0.09; 0.32, $p = .001$).

Discussion

In both studies, no evidence emerged for additive effects of candidate genes putatively involved in attachment security and disorganization. Thus, the 'usual suspects' (Ebstein, Israel, Chew, Zhong, & Knafo, 2010) in the dopamine, serotonin, oxytocin and neuroplasticity systems were not related to attachment quality. Furthermore, proposed risk models for DRD4, DRD2, and 5-HTT failed to provide unequivocal results. No effects were found in either study for insecure or disorganized attachment in carriers of the DRD4 48 bp 7-repeat. And although DRD2 minor-T(A1)-allele carriers showed increased disorganization and 5-HTT short-allele carriers proved more securely attached in Generation R, neither finding was replicated in SECCYD.

However, a co-dominant effect of the COMT Val/Met proved replicable across studies (a small effect of $d = 0.20$). In carriers of the Val/Met genotype, disorganization scores were higher compared to both Val/Val and Met/Met carriers, a disadvantage also referred to as negative heterosis (Comings & MacMurray, 2000). Co-dominant effects for COMT Val/Met have been reported for neurobehavioral functioning (Gosso et al., 2008; Wahlstrom et al., 2010) and schizophrenia (for a meta-analysis, see Costas et al., 2010). However, these studies showed evidence of *positive* heterosis. Molecular heterosis is thought to be biologically plausible. Several studies (e.g. Tunbridge, Harrison, & Weinberger, 2006) suggest that there is an inverted U-shape with opposing gene expression occurring in heterozygotes compared to the homozygotes. Alternatively, a greater range of gene expression in heterozygotes compared to homozygotes could play a role. The range of expression of gene products could be greater in heterozygotes, providing a broader window for plasticity or response to stress (Comings & MacMurray, 2000).

Evidence from this inquiry might suggest the latter, with COMT Val/Met carriers possibly being more susceptible to environmental influences, which in turn may increase risk for attachment disorganization. Moreover, COMT Val158Met has been shown to be involved in regulation of emotional arousal (Drabant et

Table 2. *Minor allele frequencies and main effects of candidate genes on attachment security and attachment disorganization for both samples*

Gene	Marker	Minor allele	Generation R						NICHD SECCYD					
			MAF	N	*B*	95% *CI*	*r*	*p*	MAF	N	*B*	95% *CI*	*r*	*p*
Security														
Dopaminergic system														
DRD4	48bp VNTR	7+	19	647	0.11	-0.26; 0.47	.02	.57	12	478	0.19	-0.38; 0.76	.03	.52
DRD4	rs1800955	C	44	682	-0.06	-0.34; 0.22	-.02	.70	-	-	-	-	-	-
DRD2	rs1800497	T	18	641	-0.13	-0.50; 0.26	-.03	.48	19	512	-0.02	-0.54; 0.50	.00	.95
COMT	rs4680	G (val)	48	640	<0.01	-0.28; 0.28	.00	>.99	50	522	0.31	-0.06; 0.68	.07	.10
Serotonergic system														
5-HTT	44bp VNTR	short	44	677	0.21	-0.06; 0.49	.06	.13	59	512	0.05	-0.29; 0.38	.01	.77
Oxytonergic system														
OXTR	rs53576	A	34	687	-0.01	-0.31; 0.29	.00	.93	35	512	0.06	-0.35; 0.47	.01	.77
OXTR	rs2254298	A	12	690	0.16	-0.27; 0.59	.03	.47	11	503	0.13	-0.52; 0.78	.02	.70
Neuroplasticity														
BDNF	rs6265	A (met)	19	688	0.28	-0.08; 0.63	.06	.12	-	-	-	-	-	-
Disorganization														
Dopaminergic system														
DRD4	48bp VNTR	7+	19	647	-0.17	-0.44; 0.10	-.05	.22	12	478	0.28	-0.09; 0.64	.07	.14
DRD4	rs1800955	C	44	682	-0.02	-0.23; 0.18	-.01	.83	-	-	-	-	-	-
DRD2	rs1800497	T	18	641	0.25	-0.02; 0.52	.07	.07	19	512	0.19	-0.14; 0.52	.05	.26
COMT	rs4680	G (val)	48	640	0.13	-0.07; 0.34	.05	.20	50	522	0.10	-0.14; 0.34	.04	.42
Serotonergic system														
5-HTT	44bp VNTR	short	44	677	-0.05	-0.25; 0.15	-.02	.63	59	512	-0.05	-0.26; 0.17	-.02	.67
Oxytonergic system														
OXTR	rs53576	A	34	687	-0.20	-0.42; 0.26	-.07	.08	35	512	-0.11	-0.37; 0.15	-.04	.42
OXTR	rs2254298	A	12	690	0.15	-0.17; 0.47	.04	.35	11	503	0.10	-0.31; 0.52	.02	.63
Neuroplasticity														
BDNF	rs6265	A (met)	19	688	-0.03	-0.29; 0.22	-.01	.80	-	-	-	-	-	-

Note. Additive models are presented. *B* denotes change in security and disorganization scores per unit change in the predictor.

Table 3. *ANOVAs for specific models in DRD4 VNTR, DRD2, COMT and 5-HTT VNTR for both samples*

Gene	Risk model	Genotype (*M*, *SD*)			Generation R			Genotype (*M*, *SD*)			NICHD SECCYD		
		aa *N*	Aa *N*	AA *N*	*F*	*r*	*p*	aa *N*	Aa *N*	AA *N*	*F*	*r*	*p*
Security													
DRD4 VNTR	7+	0.19 (2.59) 420	0.42 (2.40) 208	-0.14 (3.29) 18	0.75	.04	.39	1.11 (3.22) 377	1.66 (2.88) 85	0.67 (3.74) 16	1.19	.06	.28
DRD2 rs1800497	T (A1)	0.35 (2.60) 433	0.04 (2.54) 185	0.68 (2.53) 22	1.24	-.05	.27	1.19 (3.05) 333	1.29 (3.38) 167	0.58 (3.83) 12	0.02	.01	.88
COMT rs4680	homozygote	0.21 (2.53) 175	0.34 (2.63) 315	0.19 (2.54) 149	0.49	-.03	.49	0.77 (3.32) 140	1.31 (3.00) 245	1.39 (3.29) 137	0.74	-.04	.39
5-HTT VNTR	short	-0.10 (2.46) 215	0.52 (2.58) 323	0.23 (2.73) 139	6.32	.11	.01	1.03 (3.31) 135	1.34 (3.09) 147	1.17 (3.15) 230	0.40	.03	.53
Disorganization													
DRD4 VNTR	7+	3.45 (1.91) 421	3.34 (1.88) 208	2.81 (1.80) 18	0.88	-.04	.35	2.33 (1.97) 377	2.75 (2.27) 85	2.56 (2.45) 16	2.89	.09	.09
DRD2 rs1800497	T (A1)	3.29 (1.87) 434	3.62 (1.91) 185	3.50 (2.17) 22	3.99	.08	.05	2.31 (1.96) 333	2.56 (2.11) 167	2.33 (2.02) 12	1.68	.06	.20
COMT rs4680	homozygote	3.08 (1.97) 176	3.60 (1.83) 315	3.32 (1.92) 149	7.57	-.11	.01	2.12 (1.77) 140	2.58 (2.13) 245	2.31 (1.97) 137	4.20	-.09	.04
5-HTT VNTR	short	3.42 (1.90) 215	3.35 (1.86) 323	3.33 (2.00) 139	0.25	-.02	.62	2.47 (2.10) 135	2.27 (2.00) 147	2.35 (1.95) 230	0.53	-.04	.47

Note. df = 1. aa: homozygous for wildtype, Aa: heterozygous, AA: homozygous for minor allele. Models tested are all dominant (aa compared to Aa combined with AA), except for COMT, which is codominant (AA combined with aa compared to Aa). F, r, and p-values all refer to this two-group comparison.

al., 2006), which is considered central to disorganized attachment. Disorganized infants inability to regulate stress and emotions in arousing situations is striking, and their dysregulation is an early predictor of later psychopathology (Fearon, Bakermans-Kranenburg, Van IJzendoorn, Lapsley, & Roisman, 2010; Sroufe et al., 2005).

Genetic pathways are frequently indirect and subject to numerous biological and environmental influences (Ebstein et al., 2010; Kendler, 2005). Including environmental factors was beyond the scope of the current study, but gene-environment interactions may prove important. Several attachment GxE studies suggest that genetic effects may be contingent upon gene-environment co-action (Gervai et al., 2007; Spangler et al., 2009; Van IJzendoorn & Bakermans-Kranenburg, 2006; see also Rutter, 2006). Moreover, studies on GxE interaction in attachment could benefit from a shift from a conventional model of vulnerability genes, or 'risk alleles', to a focus on plasticity or susceptibility genes (Belsky et al., 2009). From this perspective, certain genes are thought to render individuals more responsive than others to both positive *and* negative environmental experiences (Belsky, Bakermans-Kranenburg, & Van IJzendoorn, 2007).

Previously reported associations for genes involved in attachment (DRD4 48 bp VNTR, 5-HTT) could not be replicated in the two cohorts. Current results thus confirm Burmeister and colleagues' (2008) conclusion that "testing plausible candidate genes for genetic association (…) has led to many false positives and irreproducible reports", something probably caused by a variety of factors (e.g., small samples, publication bias). Also population stratification, sufficient power and accurate assessment of the phenotype are crucial methodological aspects (Ebstein, 2006; Ioannidis, 2007; Little et al., 2009). Here the study populations were selected for Caucasian ethnicity only, securing an ethnically homogenous sample. Although only small single-gene effects were anticipated (i.e.,~1%; Plomin & Davis, 2009), power was sufficient to detect such small effects. Furthermore, the phenotype was assessed carefully, as the SSP is the gold standard for assessing attachment quality. Finally, direct replications were made possible by using the two largest attachment cohorts with molecular genetic data to date.

Genetic contributions to attachment may operate in ways not tested in here. For example, epistatic effects could play a role (e.g. Pezawas et al., 2008). Before evaluating these gene-gene interactions, more knowledge is needed about functionality and specific pathways of targeted genes. Also, effects of deletions or multiplications of larger DNA segments—copy number variations (CNVs)—are known to affect protein expression and gene function. These CNVs might act as vulnerability factors for neurodevelopmental phenotypes (Merikangas, Corvin, & Gallagher, 2009). Furthermore, epigenetic processes merit consideration, as these can modify gene expression and neural function without changing nucleotide sequence (Van IJzendoorn, Caspers, Bakermans-Kranenburg, Beach, & Philibert, 2010; Zhang & Meaney, 2010).

Attachment is a developmental milestone and attachment disorganization a major risk factor for later-life psychopathology. Here we found evidence for negative heterosis, with carriers of the COMT Val/Met genotype showing more attachment disorganization than both Val/Val and Met/Met carriers; assuming it is not the result of Type 1 error, this could reflect greater vulnerability to a negative environment. Attachment is a complex behavioral phenotype in which polygenic effects might operate, in combination with environmental factors. The most important genetic effects on attachment might be hidden in interaction with environmental factors. The most promising avenue for future gene-oriented attachment studies is therefore the careful assessment of the interplay between (epi)genetic differences and child-rearing influences.

Appendix

Genotyping information Generation R. Genotyping of polymorphisms DRD4 -521C/T, DRD2, COMT, OXTR, and BDNF was performed using Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA) and Abgene QPCR ROX mix (Abgene, Hamburg Germany). The genotyping reaction was amplified using the GeneAmp® PCR system 9600 (95° C (15 min), then 40 cycles of 94° C (15 s) and 60° C (1 min)). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems) and individual genotypes were determined using SDS software (version 2.3, Applied Biosystems). Genotyping was successful in 97-99% of the samples. To confirm the accuracy of the genotyping results 276 randomly selected samples were genotyped for a second time with the same method. The error rate was less than 1% for all genotypes.

Genotyping of the DRD4 48 bp VNTR was amplified using primers D4-F-GCGACTACGTGGTCTACTCG and D4-R-AGGACCCTCATGGCCTTG. Reactions were performed in a 384-wells format in a total reaction volume of 10 ul containing 10 ng DNA, 1 pmol/ul of each primer, 0,4 mM dNTPs, 1 M betaine, 1x GC buffer I (Takara Bio Inc.) and 0,5 U/ul LA Taq (Takara Bio Inc.). PCR cycling consisted of initial denaturation of 1 min at 94° C, and 34 cycles with denaturation of 30 seconds at 95°C, annealing of 30 seconds at 58°C and extension of 1 minute at 72°C. PCR fragments were size-separated on the Labchip GX (Caliper Life sciences) using a HT DNA 5K chip (Caliper Life sciences). The number of DRD4 repeats was determined using the size of the PCR-fragments. To assure genotyping accuracy 225 random samples were genotyped for a second time. Three samples (1.3%) gave different genotypes. These discrepancies were specific for the repeats longer than 7. The HT DNA 5K chip was unable to accurately distinguish the 7, 8, 9 and 10 repeat. As the frequency of the 8, 9 and 10 repeat is low; all samples with a 7 repeat or longer were analyzed as one group.

Genotyping of the 5-HTTLPR was performed using Taqman allelic discrimination. Primer sequences were taken from Hu et al. (2006). Reactions were performed in a 384-wells format in a total volume of 5 ul containing 2 ng DNA,

120 nM FAM-probe, 80 nM VIC-probe, PCR primers (100 nM each), dimethyl sulfoxide (DMSO) (4% by volume), and 1 x genotyping master mix (Applied Biosystems Inc.). PCR cycling consisted of initial denaturation for 10 minutes at 95° C, and 40 cycles with denaturation of 15 seconds at 96° C and annealing and extension for 90 seconds at 62.5° C. Signals were read with the Taqman 7900HT (Applied Biosystems Inc.) and analyzed using the sequence detection system 2.3 software (Applied Biosystems Inc.). To evaluate genotyping accuracy, 225 random samples were genotyped a second time. No discrepancies were found.

Genotyping information SECCYD. Extraction for all polymorphisms in the SECCYD was based on adaptations to Freeman et al. (2003). Specifically, buccal mucosa cells were collected with cotton swabs by the subject. The swabs were placed in 15-ml centrifuge tubes containing 2.5 mls of lysis buffer. The tubes were incubated in a water bath at 65°C for 2 hr to activate the proteinase K. After incubation the tubes were centrifuged at 300g for 4 min and the supernatant added to 4ml of isopropanol. Tubes were centrifuged again for 30 min. The supernatant was poured off, the pellet dried and 1 ml of lysis buffer without proteinase K was added. Pellets were resuspended by shaking overnight. The liquid was transferred to a 1,5 ml microfuge tube and 200 µl of an organic deproteinization reagent (ODPR) were added to each tube. The tubes were capped and shaken vigorously by hand. The denatured debris and remaining organic mix were then centrifuged at 5000g for10 min. Supernatant from the tube was transferred to a fresh 1.5-ml tube and 800 ul of isopropanol was added and mixed gently for approximately 1 min. The DNA was collected by centrifugation at 5000g for 10 min. The pellets were dried and washed with 1 ml ethanol 70% (v/v) by centrifugation at 5000g for 10 min. The ethanol wash was discarded, the tubes were inverted, and the pellets were dried for 60 min. The DNA was re-suspended in 250 ul of Tris EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) by rotation in an incubator at 37°C. The DNA was quantified by measuring the absorbance at 260 nm using a Nanodrop spectrophotometer. Samples were aliquoted into storage vials and placed in a -80°C freezer.

The assay for genotyping DRD4 was based on methods developed Sander et al. (1997) and modified by Anchordoquy et al. (2003). The Genomics Core Facility modified it further as the following: 1 x Taq Gold Buffer, 2.25 mM final concentration of MgCl2, 10% DMSO, 0.2 mM dNTPs, 0.1 mM deazo GTP, 0.75 uM primers, 40 ng of DNA and 1 U of Taq Gold (Applied Biosystems, Foster City CA) in a volume of 12 microliters. The primer sequences are: 5'-6-FAM-GCGAC TACGTGGTCTACTCG-3' and reverse, 5'-AGGACCCTCATGGCCTTG-3'. The amplification procedure was as described by Anchordoquy et al. (2003). One microliter was removed and placed in a 96 well plate and 10 microliters of formamide containing LIZ-500 standard (Applied Biosystems, Foster City CA). The plate was run using a Fragment Analysis protocol in the 3730XL DNA Analyzer (Applied Biosystems, Foster City CA). Fragments were analyzed using

Genemapper software (Applied Biosystems, Foster City CA) with PCR products of (in bp): 379, 427, 475 (43), 523, 571, 619 (73), 667, 715, 763, and 811.

In order to genotype DRD2, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

For COMT, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

The assay for 5HTT was a modification of the method of Lesch et al. (1996) and Anchordoquy et al. (2003). The Genomics Core Facility modified it further as the following: 1 x Taq Gold Buffer, 1.8 mM final concentration of MgCl2, 10% DMSO, 0.2 mM dNTPs, 0.1 mM deazo GTP, 0.6 uM primers, 40 ng of DNA and 1 U of Taq Gold (Applied Biosystems, Foster City CA) in a volume of 15 microliters. The primer sequences were: forward, 5'-VIC- GGCGTTGCCGCTCTGAATGC-3' and reverse, 5'-GAGGGACTGAGCTGGACAACCAC-3'. The same amplification protocol as used for DRD4 was used for 5HTLL. One microliter was removed and placed ina 96 well plate and 10 microliters of formamide containing LIZ-500 standard (Applied Biosystems, Foster City CA). The plate was run using a Fragment Analysis protocol in the 3730XL DNA Analyzer (Applied Biosystems, Foster City CA). Fragments were analyzed using Genemapper software (Applied Biosystems, Foster City CA) with PCR products of 484 or 528 bp.

For OXTR rs53576, Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.

Finally, for OXTR rs2254298 Taqman SNP Genotyping Assays were performed using an Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) protocol. Forty nanograms of DNA were combined in a volume of 5 microliters

with 2X Universal PCR Mix (Applied Biosystems) and 1/20 the volume of the Taqman SNP assay in a 384 well plate. A Pre-Read was performed and then PCR as follows: a 10 min hold at 95 C, followed by 40 to 45 cycles of 15 sec at 92 C and then 1 min at 60 C in a 7900HT PCR System. After amplification, a Post-Read was performed to analyze. Automatic and manual calls were made.