DRD4 VNTRs, observed stranger fear in preschoolers and later ADHD symptoms

Irene Pappa\textsuperscript{a,b}, Viara R. Mileva-Seitza\textsuperscript{a,b}, Eszter Szekely\textsuperscript{b,c}, Frank C. Verhulst\textsuperscript{b,c}, Marian J. Bakermans-Kranenburg\textsuperscript{d}, Vincent W.V. Jaddoe\textsuperscript{e,f}, Albert Hofman\textsuperscript{e}, Henning Tiemeier\textsuperscript{b,c}, Marinus H. van IJzendoorn\textsuperscript{a,b,d,e}

\textsuperscript{a} School of Pedagogical and Educational Sciences, Erasmus University Rotterdam, The Netherlands
\textsuperscript{b} Generation R Study Group, Erasmus Medical Center, Rotterdam, The Netherlands
\textsuperscript{c} Department of Child and Adolescent Psychiatry/Psychology, Erasmus University Medical Center-Sophia Children’s Hospital, The Netherlands
\textsuperscript{d} Centre for Child and Family Studies, Leiden University, PO Box 9555, 2300 RB Leiden, The Netherlands
\textsuperscript{e} Department of Epidemiology, Erasmus University Medical Center-Sophia Children’s Hospital, The Netherlands
\textsuperscript{f} Department of Pediatrics, Erasmus University Medical Center-Sophia Children’s Hospital, The Netherlands

\textbf{A R T I C L E  I N F O}

Article history:
Received 14 July 2014
Received in revised form 29 August 2014
Accepted 6 September 2014
Available online 16 September 2014

Keywords:
Candidate gene 
Mediation 
Dopamine receptor D4 
Temperament 
Attention 
Inhibitory control 
Social anxiety

\textbf{A B S T R A C T}

Fear of strangers is a developmental milestone in childhood that encompasses behavioral inhibition and decreased novelty seeking. Children with attention deficit/hyperactivity disorder (ADHD) often exhibit fearless and impulsive behaviors, similar to those observed in children with atypically low levels of stranger fear. It is currently unknown whether these behaviors share common underlying biological mechanisms. Polymorphisms in the dopamine receptor 4 gene (DRD4) have been implicated in the risk for developing ADHD symptoms in childhood. Here we investigate whether (1) DRD4 variable number tandem repeats (VNTRs) are associated with both stranger fear and ADHD symptoms, and (2) stranger fear in preschoolers mediates the link between DRD4 VNTRs and ADHD in later childhood. Stranger fear was observed in a large sample (N = 589) of 3-year-old Caucasian children and ADHD symptoms were assessed by a validated, mother-rated questionnaire at 6 years. We found evidence that longer DRD4 variants were associated with increased ADHD symptoms at 6 years, and that this relationship was partially mediated by lower levels of observed stranger fear at 3 years. Our results suggest a common underlying neurobiological mechanism in the association between low stranger fear and ADHD symptoms; variation in DRD4 may be an important contributor to this mechanism.

\textcopyright 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Preschoolers show significant individual variation in expressed fear when facing unfamiliar adults (Brooker et al., 2013). These patterns of behavior are often attributed to child temperament, and are suggested to be heritable (Gagne and Goldsmith, 2011; Saudino, 2005) and relatively stable over time (Durbin et al., 2007). The development of stranger fear is an important milestone, emerging at 6 months of age and increasing before 12 months (Sroufe, 1977). Lack of stranger fear has been associated with inattention and low inhibitory control in later childhood (Bakermans-Kranenburg et al., 2011), and extreme stranger fear has been associated with greater social anxiety in adulthood (Degnan and Fox, 2007). Stranger fear is a multidimensional trait that encompasses behavioral inhibition, lack of impulsivity and novelty seeking, and shares conceptual overlap with attention deficit/hyperactivity disorder (ADHD), a common neurodevelopmental disorder characterized by inattention, impaired inhibitory control and hyperactive/impulsive behavior (Schneider et al., 2006). Yet the underlying biological link between stranger fear and ADHD has not been previously established.

The dopamine receptor D4 (DRD4) gene contains a widely studied 48-bp variable number tandem repeat (VNTR), with 2–11 repeats on exon III. The 7-repeat allele is a known risk factor of ADHD (Wu et al., 2012) and children with ADHD are often characterized as being fearless and impulsive. “Long” DRD4 variants have also been associated with increased novelty seeking in healthy 3-years olds (Ebstein et al., 1996). Children with long DRD4 VNTRs who are at risk for ADHD might also exhibit a less fearful response to facing a stranger during a laboratory setting. Only two small studies (Auerbach et al., 2001, Lakatos et al., 2003) have examined this association, showing no main genetic effects.
The aims of the present study are (1) to examine the association between different DRD4 VNTR groups and observed levels of stranger fear during a well-validated laboratory task in a typically developing, non-clinical sample (N = 589) of 3-year-old Caucasian children, and (2) to assess the association of DRD4 VNTR with ADHD symptoms at 6 years and examine whether stranger fear at 3 years mediates the link between DRD4 VNTRs and later ADHD symptoms.

2. Methods

2.1. Setting

This study was conducted in a subsample of children participating in the Generation R Study, referred to as the Generation R Focus Study (Tiemeier et al., 2012). The design and sample characteristics of the Generation R Study have been described in detail elsewhere (Jadot et al., 2012). The current study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from parents of the participating children.

2.2. Study population

All children of the Generation R Focus Study are of Dutch origin. DNA was obtained from cord blood samples at birth. At age 36 months (M = 37.5, S.D. = 1.5), N = 589 children (52.8% boys) visited our lab where stranger fear was assessed using the Stranger Approach episode from the Laboratory Temperament Assessment Battery [Lab-TAB, Goldsmith et al., 1999].

2.3. Genotyping

The DRD4 48 bp VNTR was amplified using primers D4-F-GGCACTACCGGG-TCTACTGC and D4-R-AGGACCCTGATGGGCGTG. Reactions were performed in a 384-well format in a total reaction volume of 10 µl containing 10 ng DNA, 1 pmol/µl of each primer, 0.4 mM dNTPs, 1 µM betaine, 1 × GC buffer I (Takara Bio, Inc., Otsu, Japan) and 0.5 U/µl LA Taq (Takara Bio, Inc.). PCR cycling consisted of initial denaturation of 1 min at 94 °C, and 34 cycles with denaturation of 30 s at 95 °C, annealing of 30 s at 58 °C and extension of 1 min at 72 °C. PCR fragments were size-separated on the Labchip GX (Caliper Life Sciences, Hopkinton, MA) using a HT DNA 5K chip (Caliper Life Sciences). The number of DRD4 repeats was determined using the size of the PCR fragments. Ambiguous genotypes were re-genotyped. The main genotypes (2/4, 4/4 and 4/7-repeats) were in Hardy–Weinberg equilibrium (χ² = 0.01, p = 0.91).

2.4. DRD4 VNTR classifications

Due to ongoing uncertainty about DRD4 VNTR classification (Das et al., 2011), we assessed the most common classifications found in developmental research: (1) 7-repeat carriers vs. noncarriers, (2) (2–5) vs. (6–11)-repeat carriers, (3) (2–4) vs. (5–11)-repeat carriers, and (4) (2–6) vs. (7–11)-repeat carriers. We also used a more conservative classification, based on evidence of biochemical differences only between 2-, 4- and 7-repeat variants (Van Craenenbroeck et al., 2005). For this functional approach, we used the classification (5) (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes.

2.5. Stranger fear

Stranger fear was measured using the Stranger Approach episode of the Lab-TAB Preschool version (Goldsmith et al., 1999). The rationale was to measure social fear at age 3 years old. During the observation the child was left alone in a specially designed room, with toys. After some time, the task began and was divided into nine epochs during which a female stranger (disguised with a hat, sunglasses, and a coat) approached the child and asked standardized questions (i.e. "Are you having a good time here today?", "Are you playing with a lot of toys?"). Intensity of fear expression, distress vocalizations, activity decrease, approach, avoidance, gaze aversion, verbal hesitancy and nervous fidgeting were scored in each epoch from DVD recordings according to the original coding system by trained coders, blind to children's genetic information and ADHD status (mean intra-class correlation coefficients ranging from 0.70 to 0.92, n = 25). Averages were computed for each child response or parameter across epochs. We computed a stranger fear composite score by first reversing the approach scale average, converting all scale averages into z-scores, and taking the mean of all standardized averages.

2.6. ADHD symptoms

ADHD symptoms begin in childhood but establishing a precise diagnosis at an early age is questionable (Applegate et al., 1997). Thus, we used mother-reported data on children's ADHD symptoms at 6 years (mean age, M = 5.9, S.D. = 0.20) measured by the Attention Deficit/Hyperactivity Problem subscale (six items, alpha = 0.70) of the Child Behavior Checklist [CBCL 1½–5, Achenbach and Rescorla, 2000]. The six items of the Attention Deficit/Hyperactivity Problem subscale are (a) cannot concentrate, cannot pay attention for long, (b) cannot sit still, restless, or hyperactive, (c) cannot stand waiting, wants everything now, (d) demands must be met immediately, (e) gets into everything, and (f) quickly shifts from one activity into another. The reliability and validity of the Dutch version of CBCL 1½–5 have been previously demonstrated (Tick et al., 2007). Although the CBCL Attention Deficit/Hyperactivity Problem subscale is not a diagnostic tool, it shows high diagnostic and discriminating power for ADHD in population-based, non-clinical samples (Chen et al., 1994).

For N = 589 children both stranger fear and ADHD symptoms scores were available. Loss to follow-up children did not significantly differ in the levels of stranger fear [t(8) = 0.66, p = 0.51].

2.7. Statistical analysis

We ran one-way ANOVAs in SPSS Version 20.0 for Windows (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) to compare the mean levels of observed stranger fear across the different DRD4 VNTR genotype groups. We used a four-step mediation analysis approach (Preacher and Hayes, 2004), based on several regression analyses to evaluate the role of observed stranger fear as a potential mediator of the association between DRD4 VNTR genotypes and ADHD symptoms.

3. Results

3.1. Genotypic and phenotypic sample characteristics

In the Generation R sample, allele frequencies were similar to those reported for Caucasian populations (Chang et al., 1996). The most common genotypes were 4/4 (41.1%), 4/7 (25.5%) and 2/4 (13.4%). Total stranger fear scores ranged from −1.11 to 1.76 (M = 0.00, S.D. = 0.40), with higher scores indicating greater fear. The scores on the ADHD symptoms scale ranged from 0.00 to 8.00 (M = 1.35, S.D. = 1.55), with higher scores indicating more mother-reported ADHD symptoms.

In our sample, levels of stranger fear were not associated with child's sex [F(1, 588) = 3.11, p = 0.08)]. However, there was a significant association of child's sex with ADHD symptoms [F(2, 579) = 18.72, p = 0.00] after adjusting for child's age, with girls showing lower ratings.

3.2. DRD4 VNTRs and stranger fear

We used five common genotypic classifications found in the literature and tested for associations with levels of stranger fear in our sample of 3-year-old children. For two genotypic classifications (1) (2–5) vs. (6–11)-repeat carriers and (2) (2–4) vs. (5–11)-repeat carriers, the "long" DRD4 alleles were associated with lower levels of stranger fear (see Table 1). In all other classifications, there was a consistent trend for an association between longer DRD4 VNTRs and lower stranger fear.

3.3. DRD4 VNTRs, ADHD, and stranger fear

Formal mediation analysis procedures were used to determine whether the association of "long" DRD4 VNTRs [(2–5) vs. (6–11)-repeat variants, the contrast that showed the strongest relation to stranger fear] with mother-rated ADHD symptoms at age 6 years was mediated by levels of stranger fear at age 3 years.

First, the direct effect (path a) from DRD4 VNTRs to stranger fear at age 3 years was significant (β = −0.09, p = 0.03); "long" alleles were associated with lower levels of stranger fear. Second,
that multiple genes with relatively small effects will be associated
manifest the ADHD symptomatology are also responsible for the
DRD4 indicator of later ADHD symptoms, and that
suggest that less fearful behavior towards strangers may be an early
to fear recognition in social contexts (Maier et al., 2014). Our results
duals with ADHD have altered neuronal activation patterns related
developmental de"
"fear explained only a modest proportion of the variance in ADHD
indicated partial mediation of the association between
explains part of this association. Controlling for stranger fear
between DRD4 VNTRs and ADHD symptoms at 6 years, although modest,
levels of stranger fear (r = .08, p = .053, R² = .021). The
proportion of explained variance of the association of “long”
DRD4 VNTRs and ADHD symptoms at 6 years, although modest,
was significantly increased when stranger fear at 3 years was also
included in the model (R²-change = .009, p = .03). All analyses
were adjusted for age and sex (Fig. 1).

4. Discussion

In the current study we firstly investigated the association
between DRD4 VNTR genotypes and stranger fear in preschoolers
by systematically exploring previously used genotype classifications.
More specifically, we found a significant association between
“long” DRD4 genotypes (> 5-repeat variants) and lower levels of
stranger fear. Secondly, we replicated prior reports of an association
between DRD4 genotype and ADHD symptoms in our sample of
6-years old children (N = 589). Recent research shows that indi-
viduals with ADHD have altered neuronal activation patterns related
to fear recognition in social contexts (Maier et al., 2014). Our results
suggest that less fearful behavior towards strangers may be an early
indicator of later ADHD symptoms, and that DRD4 variation explains
part of this association. Controlling for stranger fear indicated partial mediation of the association between DRD4 and
ADHD symptoms. Although DRD4 genotypes and levels of stranger fear explained only a modest proportion of the variance in ADHD
symptoms, our results suggest a significant biological link between “temperamental” behaviors and a neurodevelopmental disorder, in
a non-clinical, population-based sample of children. It is possible that
developmental deficits beginning in early childhood and manifest the ADHD symptomatology are also responsible for the
lower levels of stranger fear, an important developmental milestone
(Sroufe, 1977)

ADHD is a common but complex disorder, and as such, it is likely
that multiple genes with relatively small effects will be associated
with ADHD symptoms (Gratten et al., 2014). Indeed, previous
molecular genetic studies have explored the association of common
genetic variation with increased ADHD symptoms (Li et al., 2014).
The dopaminergic pathway has been involved and several candidate
genes have been identified, including DRD2, DRD5, and DAT1 (Kirley
et al., 2002). In this study, we were specifically interested in the association of DRD4 VNTRs with ADHD symptoms. It is possible
however that more variation within this gene or other genes of the
same pathway would explain a larger proportion of the individual
differences in ADHD symptoms among children.

We investigated the hypothesis that individual variation in
stranger fear partially mediates the association between “long”
DRD4 VNTRs and ADHD symptoms in children. It is possible that
other temperamental [i.e. high neuroticism and surgency, Martel
et al., 2014], parental [i.e. parenting behavior and parent depression,
Tung et al., 2014] and environmental [i.e. media use, Nikkelen et al.,
2014 and family income, Larsson et al., 2014] factors mediate the link
between genetic variation and ADHD symptoms. The integration of
all these factors in one viable model of ADHD is the ultimate goal, but
lies beyond the scope of this study, addressing the various factors
previously associated with ADHD symptoms.

The two previous studies on the genetics of stranger fear in
small samples of children have failed to show a main effect of
DRD4 on stranger fear (Lakatos et al., 2003; Auerbach et al., 2001).
It is possible that lack of power and/or the use of different
genotypic classifications of DRD4 VNTRs may have influenced
these findings. Our study illustrates that although effect sizes
can be very consistent across different classifications of the same
underlying group of DRD4 variants, the interpretation of the
significance tests can depend on the choice of the classification.
The use of the functional approach [i.e. comparing only the DRD4
VNTRs for which evidence of biological differences exist] was also
in line with the classical “short” vs. “long” classifications, although
it yielded no statistical significant results. One explanation could
be the smaller sample size of this group (N = 508), comparing to
the total group (N = 589). Another possibility could be that there is
true biological difference of the rare DRD4 VNTRs and that this can
be better captured by the “short” vs. “long” classifications. More
studies are required before we can draw definitive conclusions about the functionality of rare DRD4 VNTRs.

In our sample, girls show lower levels of mother-rated ADHD
symptoms at 6 years. Previous research supports this finding, in
both clinical (Gershon, 2002) and population-based samples
(Holbrook et al., 2014). Furthermore, we found no evidence of
gender differences in levels of stranger fear among 3-year-olds,
which is consistent with a recent meta-analysis in children (Else-
Quest et al., 2006). Given that our study indicates stranger fear as a
partial mediator of the link between DRD4 VNTRs and ADHD
symptoms, it is possible that other dimensions of temperament
[i.e. low inhibitory control] may be responsible for the gender
differences in ADHD symptoms. Indeed, previous research has
associated lower levels of effortful control with increased attention
problems in boys (Nigg et al., 2004).

---

### Table 1

Summary of the one-way ANOVA for stranger fear and alternative groupings of DRD4 VNTRs.

<table>
<thead>
<tr>
<th>Empirical approach</th>
<th>N (589)</th>
<th>Mean</th>
<th>S.D.</th>
<th>R²</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 7-repeat carriers vs. non-carriers</td>
<td>393</td>
<td>0.02</td>
<td>0.48</td>
<td>0.005</td>
<td>0.09</td>
</tr>
<tr>
<td>7-repeat non-carriers</td>
<td>196</td>
<td></td>
<td>0.04</td>
<td>0.73</td>
<td>0.005</td>
</tr>
<tr>
<td>2. (2–5) vs. (6–11)-repeat carriers</td>
<td>382</td>
<td>0.02</td>
<td>0.48</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>(2–5) carriers</td>
<td>207</td>
<td>0.05</td>
<td>0.73</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>(6–11) carriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>3. (2–4) vs. (5–11)-repeat carriers</td>
<td>367</td>
<td>0.02</td>
<td>0.48</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>(2–4) carriers</td>
<td>222</td>
<td>0.04</td>
<td>0.73</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>(5–11) carriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>4. (2–6) vs. (7–11)-repeat carriers</td>
<td>385</td>
<td>0.02</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(2–6) carriers</td>
<td>204</td>
<td>0.04</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7–11) carriers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Functional approach**

| 5. (2/2, 2/4, 4/4) vs. (2/7, 4/7, 7/7) genotypes | 323 | 0.01 | 0.48 | 0.005 |
| (2/2, 2/4, 4/4) genotypes | 185 |      | 0.04 | 0.73 | 0.13 |

---

*In this group, total N = 508 children.*
Strength of the study is that it was conducted with a large and ethnically homogenous sample of children. Second, we replicated previous findings on DRD4 VNTRs and ADHD symptoms and expanded this association, based on the conceptual overlap between stranger fear and ADHD symptoms. Third, by using observational measures of stranger fear at 3 years and mother-rated ADHD symptoms at 6 years, we reduced the risk of potential biases that may arise due to shared method variance.

However, the present study is not without limitations. First, we assessed stranger fear with a laboratory observation task, which, although well-validated, was brief. Second, we were specifically interested in main genetic effects of DRD4 on stranger fear, yet it is possible that environmental factors moderate the genetic influences on fearful behavior (Ellis et al., 2011). Environmental factors were not addressed in the present study. Third, our findings of mediation of DRD4 and ADHD symptoms via levels of stranger fear have to be replicated in independent samples, before generalizing them to larger populations. However, the current study is the largest of its kind to date and provides the first evidence for a common biological underpinning of stranger fear in preschoolers and ADHD symptoms in later childhood. Common genetic variation explaining the association of ADHD symptoms via individual variation in levels of stranger fear during early life may help us better understand the neuropsychology of ADHD etiology.

Declaration of conflict interests

Dr. Verhulst publishes the Dutch translations of ASEBA, from which he receives remuneration.

Acknowledgment

The Generation R Study is conducted by the Erasmus Medical Center in close collaboration with the Erasmus University Rotterdam, School of Law and Faculty of Social Sciences, the Municipal Health Service Rotterdam area, Rotterdam, the Rotterdam Home-care Foundation, Rotterdam, and the Stichting Trombosediënt & Artenlaboratorium Rijnmijd (STAR), Rotterdam. We gratefully acknowledge the contribution of general practitioners, hospitals, midwives and pharmacists in Rotterdam. The first phase of the Generation R Study is made possible by financial support from Erasmus Medical Centre, Rotterdam, Erasmus University Rotterdam and the Netherlands Organization for Health Research and Development (ZonMw, Grant no. 2100.0073 and GeestKracht OOG 100.002.005).

The present study was supported by an additional grant from the Netherlands Organization for Health Research and Development (Grant no. 2100.0074).

References


I. Pappa et al. / Psychiatry Research 220 (2014) 982–986


