Title: Monoamine and neuroendocrine gene-sets associate with frustration-based aggression in a gender-specific manner

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Short title: Genetic mechanisms of aggression subtypes

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Abstract

Investigating the molecular basis of aggression can provide insights into aggression biology and may lead to new prevention and treatment options. In the current study, we evaluated the taxonomy of aggression and examined genetic mechanisms underlying aggression subtypes in healthy males and females. Confirmatory Factor Analysis (CFA) was used to replicate a recently reported three-factor model of the Reactive Proactive Questionnaire (RPQ) in healthy adults (n=661; median age 24.0 years; 41% male). Gene-set association analysis of a combination of three molecular pathways previously implicated in aggression, i.e. serotonergic, dopaminergic, and neuroendocrine signaling was conducted with MAGMA software in males and females separately (total n=395) for aggression subtypes. The three-factor CFA model of the RPQ provided a good fit for the data. Using competitive tests, we found a significant female-specific association of reactive aggression due to internal frustration with genetic variation in the combined gene-set. Both the neuroendocrine and serotonergic gene-sets contributed to this association. We confirm the existence of three RPQ-based aggression subtypes in healthy adults. We identify variation in genes involved in neuroendocrine and serotonergic signaling as biological risk factors for frustration-based reactive aggression in females. By aggregating the effect of multiple genetic variants in implicated biological systems, we boosted statistical power for finding reliable genetic association. Our genetic findings are subtype- and sex-specific, stressing the value of efforts to reduce heterogeneity in research of aggression etiology. Furthermore our work highlights opportunities for sample size maximization offered by population-based studies of aggression.

Keywords: Aggression; Genetics; Factor Analysis; Serotonin; Dopamine; Neuroendocrine System

1. Introduction

Aggression has been defined as any behavior directed toward the goal of causing harm or injury to others (Baron and Richardson, 1994). From an evolutionary perspective, aggressive behaviors can be adaptive and have an important role in survival and competition for resources (Georgiev et al., 2013). In modern societies, aggression often is maladaptive and associated with negative consequences, causing psychological and somatic burden to victims as well as to aggressive individuals themselves (Fergusson et al., 2005; Reef et al., 2010). Aggression poses a substantial financial burden on society, for example caused by increased legal costs and work absence (WHO, 2007). A better understanding of the taxonomy and etiology of aggression is needed to facilitate prevention and to improve treatment options (Fergusson et al., 2005). Given that about half of the variance in aggressive behaviors may be explained by genetic influences (Tuvblad and Baker, 2011; Veroude et al., 2016), studying the molecular genetics underlying these behaviors can provide important mechanistic insights. Research into aggression etiology is, however, complicated by several factors, including considerable phenotypic heterogeneity and the existence of sex differences in aggressive behaviors (Baker et al., 2008; Georgiev et al., 2013). In addition, genetic heterogeneity is of importance.

Subtypes of aggression

Heterogeneity in the etiology of aggression may be parsed by considering subtypes. Different classification systems have been proposed; one based on biological hypotheses is the distinction of proactive and reactive aggression (Dodge and Coie, 1987). Proactive aggression, also referred to as instrumental aggression, is goal-oriented, organized behavior often associated with low autonomic arousal and affect. Reactive aggression on the other hand, is also known as impulsive or affective aggression, and occurs in response to provocation or a negative emotional state (Raine et al., 2006; Stanford et al., 2003). Importantly, the subtypes have been associated with distinct behavioral, neurocognitive, and neural characteristics. For example, proactive aggression has been related to psychopathic traits and delinquent

behavior (Cima and Raine, 2009; Cima et al., 2013), while the reactive subtype of aggression has been associated with impulsivity, anxiety, and hostile interpretation bias (Brugman et al., 2015; Bubier and Drabick, 2009). Twin studies showed slightly higher heritability estimates for proactive than reactive aggression (Baker et al., 2008; Brendgen et al., 2005; Tuvblad et al., 2009). The two aggression subtypes may have partially distinct genetic contributions. Serotonergic and dopaminergic neurotransmission may regulate both reactive and proactive aggression, whereas endocrine signaling seems to be more involved in the regulation of reactive aggression, e.g. through modulation of impulsivity and the stress response (Waltes et al., 2015). Recently, a further subdivision of reactive aggression has been proposed based on an exploratory factor analysis of the Reactive Proactive Questionnaire (RPQ) in an adolescent sample (Smeets et al., 2016). Besides a proactive factor, reactive aggression was further subdivided into a subtype associated with external provocation or threat and another one associated with internal frustration. These reactive subtypes differed in their associated behavioral correlates, which suggests that the three-factor model may further reduce phenotypic heterogeneity and facilitate the search for genes involved in the etiology of aggression.

Sex differences in aggression

The most convincing observation supporting the existence of sex differences in aggression is the difference in crime rate statistics between males and females. Females are vastly less likely to commit serious offenses than males, and males are more likely to display antisocial behavior than females (Stephenson et al., 2014). Males are also overrepresented in aggression-related disorders such as conduct disorder (CD), where the gender ratio is approximately 2.5 (Hill, 2002). Importantly, sex differences are also found in the type of aggressive behavior displayed (Collett et al., 2003). The clear gender-specificity of aggression is thought to have evolved by sexual selection, and to reflect differences in optimal strategies in the competition for resources for males and females (Georgiev et al., 2013). Sex differences in heritability estimates have been observed in some but not all of the aggression twin studies conducted to date, with higher heritability estimates for boys than girls, when self-report measures were assessed

(Baker et al., 2008; Wang et al., 2013). Incorporation of sex in aggression studies may be essential to identify the underlying biological mechanisms of aggressive behaviors.

Biological systems

The biological systems most investigated in the context of aggression phenotypes (as well as related traits such as mood disturbances and impulsivity) are the monoaminergic neurotransmitter systems related to serotonin and dopamine and the neuroendocrine system. Multiple reviews to date discuss these systems in the context of aggression and list the candidate genes that have been investigated for association with aggressive behaviors (Pavlov et al., 2012; Veroude et al., 2016; Waltes et al., 2015).

The serotonergic system is hypothesized to play a key role in aggression due to its influence on functions including social cognition, emotional regulation, and cognitive control (Lesch et al., 2012). While genetic association studies have often produced equivocal results, investigations measuring levels of the serotonin metabolite 5-HIAA in cerebrospinal fluid, e.g. (Brown et al., 1979; Coccaro and Lee, 2010), or manipulating central serotonin function through tryptophan depletion/loading, e.g. (Bjork, 2000), have revealed a highly significant relationship between serotonin availability and aggression (Rosell and Siever, 2015). Dopamine is relevant for understanding aggression because of its effects on reward, motivated behavior, and decision making (Costa et al., 2012). While studies of dopamine manipulation have mostly been conducted in animals, the involvement of dopamine in aggression is also evidenced by the fact that in humans, D2-receptor antagonists have been used effectively to treat aggressive behavior (Nelson and Trainor, 2007). The third system implicated in aggression is the neuroendocrine system, including both stress-related hypothalamic-pituitary-adrenal (HPA) axis signaling and sex-hormone-related hypothalamo-pituitary-gonadal (HPG) axis signaling. As early life stress is known to increase risk for the development of mood and aggression-related disorders (Agid et al., 1999; Éthier et al., 2004; Fonagy, 2006; Heim et al., 2001), the neuroendocrine stress response with its genetic components is a major candidate system for the development of aggressive behaviors. The relation of the HPA axis to aggression has been well established, especially through animal studies (Veenema, 2009). Also in

humans, cortisol levels have been related to aggression repeatedly (Alink et al., 2012; Loney et al., 2006; Popma et al., 2007; Shirtcliff et al., 2005; van Bokhoven et al., 2004). The HPG axis involves signaling between hypothalamus, pituitary, and the gonadal glands, which produce estrogen and testosterone. Testosterone levels have been related to human aggression (Book et al., 2001; Brown et al., 2008; Chichinadze et al., 2010; Yu and Shi, 2009) and it has been hypothesized that especially the interplay between cortisol and sex steroids is important in determining aggression liability (Pavlov et al., 2012; Terburg et al., 2009).

Extensive reviews of aggression candidate gene studies have recently been published (Fernandez-Castillo and Cormand, 2016; Pavlov et al., 2012; Veroude et al., 2016; Waltes et al., 2015). Although a moderate number of studies has been conducted, a meta-analysis of individual candidate variants did not reveal any significant associations with aggressive behavior (Vassos et al., 2014). One reason for this may be the complex genetic background of aggression in most people. While a few monogenic aggression disorders caused by rare genetic variations with a high effect size exist (Brunner et al., 1993), aggression in the population has a complex and polygenic genetic background, which can be aggravated by environmental factors (Veroude et al., 2016).

In the current study, we assessed the genetic mechanisms underlying aggression subtypes in the general population. Firstly, we aimed to verify the existence of three aggression subtypes in adult males and females from the general population based on the RPQ. Second, we aimed to assess the association of common genetic variants in the three biological systems with most evidence for a role in aggression, i.e. the serotonergic system, the dopaminergic system, and the neuroendocrine system with the different subtypes. We aimed to maximize power for finding genetic associations by (1) parsing phenotypic heterogeneity through differentiating between subtypes, (2) by assessing males and females separately, and (3) by combining genetic variants in a gene-set analysis (Bralten et al., 2011; Bralten et al., 2013; Naaijen et al., 2017).

2. Methods

Sample

The investigated sample consisted of participants of the Brain Imaging Genetics (BIG) study conducted at the Donders Institute for Brain, Cognition and Behaviour (Franke et al., 2010). The BIG study consists of self-reported healthy adults, who participated in smaller-scale imaging studies at the institute and gave consent to be included in the BIG study. Saliva samples for genetic testing were collected, and an internet-based test-battery of questionnaires was applied. The Reactive Proactive Questionnaire (RPQ; Raine et al., 2006) was available for 661 participants (age range 18-45 years). Of those, 395 participants had genome-wide genotyping data available.

All participants were of Caucasian descent and were screened using a self-report questionnaire for the following exclusion criteria before study participation: a history of somatic disease potentially affecting the brain, current or past psychiatric or neurological disorder, medication (except hormonal contraceptives) or illicit drug use during the past 6 months, history of substance abuse, current or past alcohol dependence, pregnancy, lactation, menopause, and magnetic resonance imaging contraindications (Gerritsen et al., 2012). All participants gave written informed consent, and the study was approved by the regional ethics committee.

Aggression Questionnaire

The Reactive Proactive Questionnaire (RPQ) was used to assess subtypes of aggression (Raine et al., 2006). The RPQ is a self-report questionnaire consisting of 23 items. For each item, subjects are asked to indicate, how often they have engaged in a given type of behavior. Items are rated on a three-point Likert scale (‘never’ =0, ‘sometimes’ =1, ‘often’ =2). Responses were summed to yield the three factors that best described the RPQ in an earlier exploratory factor analysis (Smeets et al., 2016): ‘proactive aggression’,

'reactive aggression due to internal frustration', and 'reactive aggression due to external provocation'. Items relating to each subtype can be found in Supplementary Table 1.

Genotyping and imputation

Genetic analyses were carried out at the Department of Human Genetics of the Radboud University Medical Center. Saliva samples were collected using Oragene kits (DNA Genotek, Kanata, Canada), and genomic DNA was extracted as specified by the manufacturer. Genome-wide genotyping was performed on two different platforms, Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix Inc., Santa Clara, CA, USA) (n=243) and the Infinium PsychArray-24 v1.1 BeadChip (http://www.illumina.com/products/psycharray.html) (n=152). Genotype calling and quality control steps are described in the Supplementary Information. MACH software was used for haplotype phasing and minimac for the final imputation (Howie et al., 2012; Li et al., 2010), with 1000 Genomes Phase 1.v3 reference data (Abecasis et al., 2012).

Data analysis

Factor analysis: Confirmatory factor analysis (CFA) was conducted using Mplus (version 6.11; https://www.statmodel.com/). Results were considered acceptable, when both the Comparative Fit Index (CFI) and the Tucker-Lewis Index (TLI) exceeded .90 (with values closer to 1 indicating better fit), and the Root Mean Squared Error of Approximation (RMSEA) was below .06 (with values closer to 0 indicating better fit) (Hu and Bentler, 1999; Smeets et al., 2016).

Gene-set selection and construction: Gene selection for aggression candidate gene-sets involved in neuroendocrine signaling, dopamine neurotransmission, and serotonin neurotransmission was performed using the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com). The neuroendocrine gene-set contained genes involved in corticotropin-releasing hormone, glucocorticoid, androgen, and estrogen signaling. An overview of selected genes can be found in Supplementary Table 2. All single

nucleotide polymorphisms (SNPs) in or within 100kb flanking regions of the genes (also capturing regulatory sequences; Veyrieras et al., 2008) were selected for analysis.

Gene-set analyses: Genome-wide association analyses for the three subtypes of aggression were performed using Mach2qtl/Mach2dat (Li et al., 2010), adjusting for age, age^2, and four population components derived from multidimensional scaling analysis. For RPQ proactive aggression scores only, scores were dichotomized into high- and low-scoring (score ≥ 2 and score ≤ 1, respectively), because of a highly positively skewed distribution. Separate analyses were run for males and females, and for subjects genotyped on the two different genotyping arrays. SNPs with low imputation quality ($R^2 < 0.6$) and minor allele frequency of less than 1% were filtered out. Resulting SNP p-values for each of the traits were used to run gene-set analysis using MAGMA v1.04 (de Leeuw et al., 2015). SNPs were mapped onto genes using 1000 Genomes Phase 1.v3 reference data followed by computation of gene p-values. Fixed-effects meta-analysis of the output of the two genotyping arrays was run using the weighted Stouffer's Z method as implemented in MAGMA. We first assessed association of all three gene-sets combined on the three aggression subtypes. The MAGMA competitive gene-set analysis was used to assess association, which will correct for confounding due to gene-size, gene density, differential sample size and the log of those values. Results of the self-contained test option in MAGMA, which tests whether a signal is present in the aggregated set of SNPs compared with a signal being present by random chance, are also reported for comparability with previously used methods in literature. This association method does not take into account gene-size and gene density, or whether the association of the gene-set is greater than that of other genes. Results were considered significant if they reached the Bonferroni-corrected *P*-value-threshold for testing of three aggression subtypes and two sexes (*P*-value threshold $=0.05/6 =0.0088$). For significant associations observed in the competitive test, we performed post-hoc tests to localize effects amongst the three separate gene-sets and individual genes within the sets.

3. Results

The general characteristics of our sample of 661 participants and the genotyped sample of n=395 are shown in Table 1. The tree factor model of the RPQ, consisting of a proactive factor, a reactive factor due to internal frustration, and a reactive factor due to external provocation or threat, showed a good model fit in the healthy adults (RMSEA 90% CI: .041-.051, RMSEA: .046, CFI: .915, TLI: .905), Cronbach's alpha = 0.687 (proactive), 0.663 (reactive internal frustration), 0.684 (reactive external provocation). An overview of fit-measures for one-, two-, and three-factor models are provided in Supplementary Table 3. In line with earlier studies, inter-correlations between the three investigated aggression subtypes were moderate and significant in our investigated sample ($.436 \geq r \leq .574$), marking them as distinguishing but correlated dimensions of aggression.

Gene-set association analysis with aggression subtypes was conducted in the 395 subjects with genotyping information available. Males scored significantly higher on proactive aggression than females ($t(393) = 5.97$, $P < 0.001$). A total of 483 unique autosomal genes were selected for the combined dopaminergic, serotonergic, and neuroendocrine gene-set. Twenty additional genes, either located on the X- and Y-chromosome or not captured by the array, could not be included in the analysis (Supplementary Table 2).

Association analysis of all three gene-sets combined with each of the three aggression subtypes was performed for males and females separately (Table 2). In females, the combined gene-set was significantly associated with frustration-based reactive aggression, but not with reactive aggression due to external provocation/threat or with proactive aggression scores. The significant association of the combined set with reactive aggression due to internal frustration as measured by competitive testing was observed for both genotyping arrays ($P_{Affymetrix\_competitive}$ =1.397e-03 and $P_{Infinium\_competitive}$ =2.175e-04, respectively), showing replicability of the finding. In males, the combined gene-set was not associated with any of the aggression subtypes using competitive tests. Self-contained test results were highly significant for proactive aggression scores in both males and females.

For the significant finding for reactive aggression due to internal frustration in females, we subsequently explored contributions of the three separate gene-sets and of individual genes within these sets. As shown in Table 3, these post-hoc analyses showed that the neuroendocrine and the serotonergic gene-set were independently contributing to the association. Separate tests of each of the subsets of the neuroendocrine pathway (corticotropin-releasing hormone, glucocorticoid, estrogen, and androgen signaling cascades) provided evidence for contributions of each of these cascades to the association, with lowest p-values for glucocorticoid and androgen signaling (Table 3). No single genes showed significant associations after Bonferroni correction for 40 (serotonin), 73 (dopamine) and 411 (neuroendocrine) genes tested (Supplementary Table 4). The gene with the strongest association in the serotonergic set was the serotonin transporter (*SLC6A4*, P =0.0098), and the gene with the strongest association in the neuroendocrine set was Cyclin-Dependent Kinase-Activating Kinase Complex Subunit (*CCNH*, P =0.0004).

4. Discussion

In the current study, we investigated genetic mechanisms underlying aggression subtypes in the healthy population. Factor analysis confirmed that three correlated but separate dimensions of aggression can be distinguished in healthy adults, using the self-report scale RPQ. Aggregated analysis of common variants within monoaminergic and neuroendocrine systems confirmed association of these systems with one of two reactive aggression subtypes in females.

Our results confirming the existence of three distinguishable dimensions of aggression in healthy adults are in line with the only previous study investigating alternative factor solutions for the RPQ in adults (Brugman et al., 2016). These authors reported improved fit-indices in exploratory factor analysis for the three-factor model compared to the original two-factor model in a males-only sample recruited partly in forensic psychiatric in- and outpatient clinics and partly from the general population. The current study extends this finding further by showing it to be valid in a highly educated healthy population sample. The scores for both reactive subtypes showed a normal distribution in our general population sample; proactive aggression scores were heavily skewed towards the lower end, reflecting the fact that proactive aggression includes more severe behaviors less prevalent in the general population. The specificity of our finding for one of the subtypes may underscore a biological meaningfulness of the observed three-factor structure.

Our identified association of candidate genetic systems with reactive aggression due to internal frustration in females was driven by variation in serotonergic and neuroendocrine signaling. This finding is in line with literature describing specific effects of serotonin, cortisol, and the sex steroids on aggressive behavior. Indeed, the reported associations of these molecules with aggression often differ as a function of sex and type of aggression studied (reviewed in Rosell and Siever, 2015). For example, higher cortisol reactivity was reported for reactive aggression compared to proactive aggression (Lopez-Duran et al., 2008). One influential theory hypothesizes that a high testosterone/cortisol ratio predisposes to increased aggression, with serotonin modulating the balance between impulsive and instrumental aggression.

Specifically, the high testosterone/cortisol ratio is thought to facilitate the fight-flight response by acting on the amygdala-hypothalamus-periaqueductal gray network, while low serotonin reduces inhibitory control by the prefrontal cortex, together leading to increased impulsive, reactive aggression (Montoya et al., 2012). Our own finding for neuroendocrine and serotonergic signaling was specific to one of the two reactive aggression subtypes, i.e. the frustration-based reactive subtype. One of the characteristics of the frustration-based subtype is thought to be an inflexibility to changes in the environment (Smeets et al., 2016). Our specific finding of strong association of frustration-based reactive aggression with neuroendocrine and serotonergic genes may thus arise (partly) from the function of these genes in stress modulation. However, more research is needed to assess the complex interactions and mechanisms through which the investigated systems lead to aggression-related phenotypes. In this context it will be useful to investigate the effects of early environment on the epigenome and the genetic factors moderating these effects (Provencal et al., 2015). Additionally, imaging genetics studies will be instrumental in investigating the modulation of aggression brain circuitry by aggression risk genes (Bogdan et al., 2017; Thompson et al., 2014).

Our findings were female-specific, a possible explanation for which lies in the idea that the signaling and interaction of the endocrine HPA and HPG axes is different between the sexes. For example, the two axes contribute to androgen production in different proportions in the different sexes (Burger, 2002; Montoya et al., 2012). In general, males and females probably developed different aggression strategies during evolution as a result of sex-specific sex hormone signaling (Georgiev et al., 2013). When using self-contained tests, we found a highly significant association of the gene-set with proactive aggression scores in both sexes. While no biological inferences can be made regarding the tested systems based on self-contained tests, nominally significant competitive association results for proactive aggression in males might nevertheless potentially point towards a role of the investigated systems in proactive aggression risk in males. The sex-specificity of at least some of our findings forms an important starting point into genetic differences in aggressive behavior between males and females. With most studies to date including male

subjects only, the aggression phenotype in females specifically has been understudied and deserves more attention.

Our study should be viewed in the context of specific strengths and limitations. One strength of the current study is the large sample size used to verify the factor structure of the RPQ. Moreover, the study addresses three different types of heterogeneity, tackling issues with phenotypic, sex-related, and allelic heterogeneity. By aggregating the effect of multiple genetic variants relating to the biological processes implicated in aggressive behavior, we were able to boost statistical power for finding genetic association (Naaijen et al., 2017) while remaining close to the assumed underlying polygenic risk model. While our sample size for the genetic study was limited, we find internally consistent associations with aggression. Our study of aggression was performed in healthy individuals. In doing so, we assumed a model in which patients diagnosed with aggression disorders can be seen as the extremes in a distribution of aggressive traits. Several lines of research have already shown that this model is relevant in other psychiatric traits such as attention-deficit/hyperactivity disorder and autism spectrum disorders (Martin et al., 2014; Middeldorp et al., 2016; Riglin et al., 2016; Robinson et al., 2016). We selected genes based on their implication in aggression disorders, and indeed, were able to find association with aggressive traits in the general population. Showing that common genetic variants underlying aggression phenotypes are similar in typical and psychiatric populations, this offers many possibilities for future research. While recruitment of large clinical cohorts often proves challenging, large population-based samples are much easier to investigate, offering important opportunities for sample size maximization.

We provide evidence for the existence of three correlated but separate dimensions of aggression in healthy adults, and identify variation in neuroendocrine and serotonergic signaling as a biological risk factor involved in the etiology of frustration-based reactive aggression in females. To our knowledge, this is the first study investigating the combined effect of common genetic variants related to monoaminergic and neuroendocrine signaling on aggression subtypes. The findings stress the value of reducing phenotypic

and sex-related heterogeneity in research of aggression etiology, and the opportunities offered by population-based studies of aggression.

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