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Converging Evidence Does Not Support *GIT1* As an ADHD Risk Gene

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Attention-Deficit/Hyperactivity Disorder (ADHD) is a common neuropsychiatric disorder with a complex genetic background. The G protein-coupled receptor kinase interacting ArfGAP 1 (*GIT1*) gene was previously associated with ADHD. We aimed at replicating the association of *GIT1* with ADHD and investigated its role in cognitive and brain phenotypes. Gene-wide and single variant association analyses for *GIT1* were performed for three cohorts: (1) the ADHD meta-analysis data set of the Psychiatric Genomics Consortium (PGC, N = 19,210), (2) the Dutch cohort of the International Multicentre persistent ADHD Collabora-Tion (IMpACT-NL, N = 225), and (3) the Brain Imaging Genetics cohort (BIG, N = 1,300). Furthermore, functionality of the rs550818 variant as an expression quantitative trait locus (eQTL) for *GIT1* was assessed in human blood samples. By using *Drosophila melanogaster* as a biological model system, we manipulated Git expression according to the outcome of the expression result and studied the effect of *Git* knockdown on neuronal morphology and locomotor activity. Association of rs550818 with ADHD was not confirmed, nor did a combination of variants in *GIT1* show association with ADHD or any related measures in either of the investigated cohorts. However, the rs550818 risk-genotype did reduce *GIT1* expression level. *Git* knockdown in *Drosophila* caused abnormal synapse and dendrite morphology, but did not affect locomotor activity. In summary, we could not confirm *GIT1* as an ADHD candidate gene, while rs550818 was found to be an eQTL for *GIT1*. Despite GIT1's regulation of neuronal morphology, alterations in gene

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expression do not appear to have ADHD-related behavioral consequences. © 2015 Wiley Periodicals, Inc.

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INTRODUCTION

Attention Deficit/Hyperactivity Disorder (ADHD) is a common and highly heritable neuropsychiatric disorder (heritability 70–80% [Faraone et al., 2005; Burt 2009]), with prevalence rates of 5–6% in childhood [Polanczyk et al., 2007; American Psychiatric Association 2013]. Clinically, ADHD is characterized by two core symptom domains: inattention and hyperactivity/impulsivity [American Psychiatric Association, 2013]. At least 15% and up to 60% of all patients diagnosed in childhood still meet full ADHD criteria when they reach adulthood; prevalence rates of persistent ADHD in adults range between 2.5 and 4.9% [Simon et al., 2009]. The clinical manifestation of adult ADHD may differ from that of childhood ADHD, i.e., by less obvious symptoms of hyperactivity and impulsivity [Haavik et al., 2010; Buitelaar 2011]. However, adult individuals with ADHD might be the most severe cases, given the lifelong impairment [Franke et al., 2012 ;Dalsgaard et al., 2015].

Despite its high heritability, identifying ADHD risk genes has been difficult [Franke et al., 2009a; Gizer et al., 2009], Due to the disorder's complex genetic background [Franke et al., 2012]. Because of the high prevalence of ADHD in the population, the search for genetic factors has mainly focused on common genetic variants (mainly single nucleotide polymorphisms (SNPs)) that occur quite frequently and have generally small effect sizes [Neale et al., 2010b; Li et al., 2014]. Hypothesis-free genome-wide association studies (GWAS) have thus been the main approach to study the genetics of ADHD during the last ten years. However, with eleven GWAS published to date [Lasky-Su et al., 2008a,b; Lesch et al., 2008; Sonuga-Barke et al., 2008; Mick et al., 2010; Neale et al., 2008, 2010a; Hinney et al., 2011; Stergiakouli et al., 2012; Yang et al., 2013; Sanchez-Mora et al., 2014], no genome-wide significant hit has yet been identified for ADHD [Neale et al., 2010b; Li et al., 2014]. A review of the first five hypothesis-free GWAS approaches for ADHD reported only limited overlap between the different studies, except for an association with cadherin 13 (*CDH13*) [Franke et al., 2009b]. So far, only a handful of susceptibility genes have been identified through meta-analysis, all of which confer only small increases in disease risk [Gizer et al., 2009]. Recently, the G protein-coupled receptor kinase interacting ArfGAP 1 gene (*GIT1*; Gene ID 28964), was suggested as a novel candidate gene for ADHD [Won et al., 2011]. The *GIT1* gene comprises 21 exons and spans 16,123 base pairs. It is located on chromosome 17p11.2 and plays an important role in the regulation of cell migration [Penela et al., 2014], neurite outgrowth [Albertinazzi et al., 2003; Za et al., 2006] and synapse formation [Kim et al., 2003; Zhang et al., 2003, 2005; Segura et al., 2007; Saneyoshi et al., 2008; Menon et al., 2010]. In this, the finding of association of *GIT1* with ADHD fits well with earlier work of our group, showing convergence of top-findings from five genome-wide association studies in ADHD on the biological process of neurite outgrowth [Poelmans et al., 2011]. Out of 27-tested single nucleotide polymorphisms (SNPs), the intronic SNP rs550818 in the *GIT1* gene was associated with ADHD in a Korean childhood sample (N = 388; adjusted odds ratio = 2.66) [Won et al., 2011]. The authors reported that the minor allele of this SNP caused a reduction of *GIT1* transcription in a luciferase reporter assay in HEK293 cells, indicating that it is a functional variant [Won et al., 2011]. In the same report, *Git1*-deficient mice displayed ADHD-like symptoms, such as hyperactivity, but also enhanced theta rhythms, and impaired memory. All of these symptoms were reversed by amphetamine, a stimulant medication used for ADHD treatment [Won et al., 2011]. However, the association between the SNP rs550818 and ADHD risk was not replicated in a Brazilian childhood and adolescent ADHD sample (N = 646) [Salatino-Oliveira et al., 2012]. To our knowledge, no other replications of the finding has been published yet, although a recent review listed the *GIT1* association as a reproducible genetic association for ADHD [Hawi et al., 2015].

In the current study we investigated the role of *GIT1* (including SNP rs550818) in ADHD risk and related traits. First, we attempted to replicate the association between *GIT1* and ADHD in the largest data set available, the Psychiatric Genomics Consortium's (PGC; http://www.med.unc.edu/pgc/) ADHD data (N = 19,210). We then assessed the effect of *GIT1* variation on ADHD-related neurocognition, brain volume measures and white matter integrity in adult ADHD patients and controls. We further examined whether SNP rs550818 alters *GIT1* mRNA expression in blood cells from patients with ADHD and controls. Lastly, we characterized the effects of downregulating expression of *Git* in *Drosophila melanogaster*, using synaptic and dendritic morphology and locomotor activity as read-outs.

MATERIALS AND METHODS

Cohorts

PGC ADHD meta-analysis. Data from nine studies including 5,621 cases and 13,589 controls were available for analysis. Samples were of Caucasian or Han Chinese origin and met diagnostic criteria according to the DSM-IV (Supplementary Table SI). The meta-analytic data used in this study were available as summary statistics, including genome-wide SNP data with corresponding *P*-values and odds ratios.

Dutch cohort of the International Multicentre persistent ADHD CollaboraTion (IMpACT-NL). A total of 225 individuals (115 adult ADHD patients, 110 healthy control subjects matched for age, gender, and IQ) from IMpACT-NL [Franke et al., 2010a; Franke and Reif 2013] participated in this study. Participants were recruited from the department of Psychiatry of the Radboud university medical center in Nijmegen or through advertisements. Patients were included if they met DSM-IV-TR criteria for ADHD in childhood as well as in adulthood. Participants had a mean age of 37.42 years (range 18–63), and 43.1% of the sample was male. For genetic data analysis, subjects were not allowed to be genetically related to each other. The study was approved by the regional ethics committee. Written informed

consent was obtained from all participants. A more detailed description of the IMpACT-NL cohort can be found in the supplementary material.

Brain imaging genetics study (BIG). The study sample consisted of healthy adult volunteers taking part in the diverse studies conducted at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen, The Netherlands [Franke et al., 2010b]. Genome-wide genotyping data and structural Magnetic Resonance Imaging (MRI) data was available for 1,300 subjects [Guadalupe et al., 2014; Hoogman et al., 2014]. Participants were highly educated (80% with a bachelor student level or higher), of Caucasian descent, and had no self-reported neurological or psychiatric history. The mean age was 22.9 years (range 18–40 years), and 42.7% of the participants were males. All participants gave written informed consent and the study was approved by the regional ethics committee.

Demographic characteristics of the PGC, IMpACT-NL, and BIG cohorts are presented in Table I.

Neuropsychological Data

Data on cognitive functioning was available for participants of the IMpACT-NL cohort. They were assessed with a neuropsychological test battery composed to cover multiple cognitive domains earlier found affected in ADHD (Mostert et al., submitted). This included executive functioning, timing of motor output, reaction time, delay aversion, impulsivity, inhibition, attention, vigilance, working memory, motor speed, and set shifting. The neuropsychological tests were always administered in the same order across ADHD patients and healthy controls. The following tasks and variables were selected for association analyses with the *GIT1* locus, because related tasks were either studied by Won and colleagues (continuous performance task; [Won et al., 2011]), or were affected in *Git1* knockout mice (working memory [Won et al., 2011]): (1) sustained attention dots (SAD) task ([Huijbregts et al., 2008], variables: mean series completion time, standard deviation (SD) series completion time, SD series errors and the response bias) and (2) Digit span task ([Wechsler 1997], variables: raw scores on forward and backward condition). Additionally, we explored the effect of the *GIT1* locus on the following tasks and variables, because performance on these cognitive domains was shown to be different between ADHD patients and controls in our IMpACT-NL cohort (Mostert et al., submitted): (1) Flanker task ([Huijbregts et al., 2002], variable: total SD of reaction time (RT)), (2) Sustained Attention to Response Task (SART, [Smit et al., 2004] variable: SD of RT for hits), (3) Delay discounting task ([Dom et al., 2006], K100), and (4) Trail-making task ([Kortte et al., 2002], variables: time to complete part A and B). The following variables were log-transformed to achieve a normal distribution: SAD task standard deviation (SD) series completion time, SAD task SD series errors, SART SD of reaction time (RT), delay discounting task K100. For more detailed information on the tasks and variables see Supplementary Table SII.

Neuroimaging, MRI Acquisition, and Data Processing

Because altered brain volumes have been consistently found to be associated with ADHD [Castellanos et al., 2002; Frodl and Skokauskas 2012], and *Git1* was shown to affect neurite outgrowth [Albertinazzi et al., 2003; Za et al., 2006], spine morphogenesis [Zhang et al., 2005; Segura et al., 2007], and synapse formation [Kim et al., 2003; Zhang et al., 2003; Zhang et al., 2005; Segura et al., 2007; Saneyoshi et al., 2008; Menon et al., 2010] in mice, we aimed to investigate the role of genetic variation within the *GIT1* locus on total brain volume, gray and white matter volume and white matter integrity in two different cohorts.

IMpACT-NL. T1-weighted MRI images were acquired previously and details of acquisition and processing are described in the supplementary material and elsewhere [Onnink et al., 2014]. For 203 samples (101 ADHD patients and 102 healthy controls) both MRI and genetic data was available.

BIG. Anatomical T1-weighted whole brain MPRAGE scans were either acquired at a 1.5 Tesla scanner (Sonata and Avanto, Siemens Medical Systems, Erlangen, Germany) or at a 3 Tesla scanner (Trio and TrioTim, Siemens Medical Systems, Erlangen, Germany) at the Donders Centre for Cognitive Neuroimaging (Nijmegen, The Netherlands). The imaging protocols of the T1 scans included slight variations, because images were acquired during several studies. Details of these variations on the protocol used in the IMpACT-NL study and parameters are described in the supplementary material and elsewhere [Hoogman et al., 2014]. For 1,300 subjects both MRI and genetic data were available.

Genetic Data

PGC. We obtained access to genome-wide summary statistics from the most recent PGC ADHD meta-analysis. Detailed procedures of DNA isolation, whole-genome genotyping and imputation were described previously [Neale et al., 2010b]. Shortly,

TABLE I. Demographic Characteristics of the Different Cohorts

	PGC (N = 19,210)	IMpACT-NL (N = 225)	BIG (N = 1,300)
Age[a]	NA	37.42 (10.94), 18–63	22.9 (3.82), 18–40
Gender	NA	43.1% male	42.7% male
Cases/controls	5,621/13,589	115/110	—

[a]Data are shown as mean (standard deviation), minimum–maximum.

genome-wide data was obtained from different genotyping arrays (Supplementary Table SI) and was imputed using 1000 Genomes data as a reference panel (Phase I integrated variant set release (v3) in NCBI build 37 (hg19) coordinates) for autosomal SNPs [Genomes Project et al., 2010]. Meta-analytic data were processed through a stringent quality control pipeline applied at the PGC [Neale et al., 2010b].

IMpACT-NL. From all IMpACT-NL participants, DNA was either isolated from saliva using Oragene containers (DNA Genotek, Ottawa, Ontario, Canada) or from EDTA blood samples according to manufacturer's protocol at the department of Human Genetics of the Radboud university medical center. Genome-wide genotyping of 235 IMpACT subjects (122 cases, 113 controls) was performed using the Human CytoSNP 12 version 2 genotyping BeadChip (Illumina Inc., San Diego, CA). Details on data quality control and imputation procedure can be found in the supplementary material.

BIG. DNA isolation, whole-genome genotyping, and imputation were described previously [Guadalupe et al., 2014; Hoogman et al., 2014]. Shortly, saliva was collected using Oragene containers (DNA Genotek, Ottawa, ON, Canada). Whole genome genotyping was done using Affymetrix GeneChip SNP, 6.0 (Affymetrix Inc., Santa Clara, CA). For imputation, the 1000 Genomes data was used as a reference panel (Phase 1.v3 EUR [Genomes Project et al., 2010]) and the imputation of autosomal SNPs was done following the Enhancing Neuro Imaging Genetics Through Meta Analysis (ENIGMA) protocol (according to NCBI build 37 (hg19) coordinates; http://enigma.ini.usc.edu/).

Association of the GIT1 Locus With ADHD and ADHD-Related Quantitative Traits

Association analyses between *GIT1*, ADHD, and related traits were done in two ways. First, we performed a single SNP association between the earlier described ADHD-risk SNP rs550818, ADHD status, and/or ADHD-related quantitative traits. Second, we analyzed the association of the *joint* effect of all common genetic variants in the *GIT1* locus with ADHD status and/or ADHD-related quantitative traits.

Single-SNP analyses. The SNP rs550818 lies within intron 20 of the *GIT1* gene on chromosome 17, at base pair position 27901975 (hg19/build 37). The A-allele has been reported to be the risk allele. The minor allele frequency (MAF) and the R^2 estimates for rs550818 in the different samples are shown in Supplementary Table SIV.

For the PGC data, the association p-value for rs550818 and ADHD status was extracted from the summary statistics. For the IMpACT-NL sample, association analyses for the self-reported symptom counts (hyperactivity/impulsivity, inattentive and combined symptoms) and the *GIT1* locus were performed in cases only ($N = 115$), given the known case-control differences for these phenotypes. We applied a linear regression with an additive genetic model and a missing data likelihood score test in SNPTEST (version 2.4.1) [Marchini et al., 2007]. Age and gender were used as covariates for all analyses. For the neuropsychological data, analyses were performed in the same way, including age, gender, and diagnostic status as covariates in the model ($N \geq 178$). For the analysis of MRI-derived traits, age, gender, and total white matter volume (when analyzing gray matter) or total gray matter volume (when analyzing white matter) were included as covariates for the association analyses ($N = 203$). Diagnostic status was not used as a covariate, because we found no differences in brain volume between ADHD patients and healthy controls (Supplementary Table SV). For the BIG sample ($N = 1{,}300$), association analyses for the *GIT1* locus were performed using linear regression for total brain volume, gray and white matter by using genotypic data and the "linear" command in PLINK (version 1.07) [Purcell et al., 2007]. Age, gender, magnetic field strength, and total white matter volume (when analyzing gray matter) or total gray matter volume (when analyzing white matter) were used as covariates. Association *P*-values for rs550818 were extracted from regression results of the individual analyses.

To test the effect of rs550818 genotype on local gray and white matter volumetric and integrity differences, we performed a voxel-based morphometry (VBM; [Ashburner and Friston 2000]) analysis on the T1 ($N = 1{,}261$) and DTI data ($N = 255$) in the BIG cohort. The genotypes of SNP rs550818 were coded to represent a linear allelic additive effect (0, 1, or 2). Age, gender, and magnetic field strength were used as covariates. Gray and white matter cluster extent was analyzed separately and tested across the entire brain using a $P_{FWE} < 0.05$ and a cluster-forming threshold of $P_{uncorrected} < 0.001$ [Hoogman et al., 2014]. Fractional anisotropy (FA) and mean diffusivity (MD) were tested in the same manner, except that FA comparisons were restricted to voxels having anisotropy > 0.1.

Gene-based analysis. The *GIT1* locus was defined as the *GIT1* gene $\pm$ 25 kb flanking regions in order to capture regulatory elements [Bralten et al., 2011]. The gene range was selected according to the UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/). Gene-based tests of the *GIT1* locus were performed using the offline version of the versatile gene-based test for genome-wide association studies (VEGAS) software [Liu et al., 2010]. This program uses SNP names (rs-numbers) and *P*-values as input to estimate gene-based effects. The approach takes LD between markers in a gene into account by using simulations based on the LD structure of a custom set of reference individuals [Liu et al., 2010]. As a reference panel we used genotypic data from BIG [Guadalupe et al., 2014] imputed with 1000 Genomes Phase 1.v3 EUR reference panel [Genomes Project et al., 2010]. VEGAS assigns SNPs to autosomal genes according to their position in hg19/build 37. A corresponding gene list was downloaded from http://www.biomart.org/biomart/martview Multiple testing was based on the number of simulations per gene and was set to 10,000.

For the PGC ADHD meta-analysis data set, SNPs were included in this analysis if they showed an imputation score (R^2) ≥ 0.6 and MAF ≥ 0.01 in unaffected subjects and Hardy-Weinberg equilibrium (HWE) $P > 10^{-6}$. Out of 126 common genetic variants within the *GIT1* locus, 97 SNPs had valid rs-numbers and were considered in the subsequent analysis (Supplementary Table SIII). In the data from IMpACT-NL, we analyzed the association of the *GIT1* locus (52 SNPs) with self-reported symptoms counts (total number of symptoms, number of inattentive symptoms, number of hyperactive/impulsive symptoms), neuropsychological variables, and MRI derived traits, such as total brain volume and gray and white matter

volume (Supplementary Table SIII). Subsequent gene-based tests used the results from the individual regression analyses as input for VEGAS described above. For the data from the BIG cohort, we analyzed the association of the *GIT1* locus with MRI-derived traits, i.e., total brain volume, gray and white matter volumes. SNP data selected required an imputation score (R^2) $\geq$0.3 and MAF $\geq$0.01. Forty-three SNPs within the *GIT1* locus were considered in subsequent analyses (Supplementary Table SIII). Gene-based tests of the *GIT1* locus were performed with the offline version of VEGAS using the results from the individual regression analyses as described above. The multiple testing-corrected p-value for significance of the analyses described above, derived from 10,000 permutations, was determined as 0.05 divided by the number of tested variables.

Power calculation. The Genetic Power Calculator (GPC) [Purcell et al., 2003] was used to define the power our samples had at either a range of genotype relative risks (GRR, for the PGC ADHD meta-analytic data, testing for case-control discrete trait) or additive QTL variances (for the IMpACT-NL and BIG cohort, testing for quantitative association) at $\alpha = 0.05$. We used a disease prevalence of 5% (as estimated by Polanczyk et al. [Polanczyk et al., 2007], and a multiplicative model (power calculation based on the allelic test). The actual risk allele frequencies of SNP rs550818 for the individual cohorts were included in the power analysis.

Functional Characterization of GIT1: Effect of rs550818 on GIT1 mRNA Expression

We specifically tested for the effect of rs550818 genotype on mRNA expression of *GIT1* in human blood samples from the IMpACT-NL cohort. From 148 consecutive IMpACT-NL participants blood samples for RNA isolation were collected in PAXgene Blood RNA Tubes (produced by QIAGEN GmbH for PreAnalytiX GmbH, Hombrechtikon, Switzerland) at the Radboud university medical center.

Validation of rs550818 genotype by TaqMan genotyping assay. Rs550818 genotypes from the genome-wide genotyping array were validated for the IMpACT-NL samples prior to this analysis. Allelic discrimination of rs550818 was performed using Taqman® SNP Genotyping assay (Life Technologies, Nieuwerkerk a/d IJssel, The Netherlands; Assay ID: C_2416538_10). For a detailed description of the TaqMan genotyping assay conditions see the supplementary material.

RNA isolation and cDNA synthesis. Total RNA was extracted from PAXgene blood RNA tubes at the department of Human Genetics of the Radboud university medical center using the Qiagen PAXgene Blood RNA Kit (produced by QIAGEN GmbH for PreAnalytiX GmbH) according to manufacturer's protocol. RNA integrity was assessed by gel electrophoresis. The cDNA was synthesized from 500 ng RNA in a reaction volume of 20 μl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories B.V. Veenendaal, The Netherlands) according to manufacturer's protocol. For the expression analysis a 1/3.75 dilution was used.

Gene expression analysis using Taqman assays. *GIT1* mRNA gene expression was assessed using Taqman gene expression analysis (Taqman assay Hs01063104_m1 for *GIT1* [Life Technologies]) according to manufacturer's protocol. Glucuronidase beta (*GUSB*), was taken along as reference gene (Taqman assay Hs00939627_m1 for *GUSB* [Life Technologies]). For a detailed description of the gene expression analysis conditions see the supplementary material. All measurements were performed in triplicate, and blanks were taken along as quality control during mRNA expression assessment. Results were analyzed with the 7500 Software v2.0.6 (Life Technologies) using an automatic threshold. Only samples with standard deviations of the triplicates $\leq$0.25 were considered for subsequent analysis, which resulted in 121 samples. As a calibrator sample the mean ΔCT of all control samples with the major genotype was used. Data was visualized using GraphPad prism (version 5.03), and the mean and a 95% confidence interval are shown.

Statistical analysis. *GIT1* mRNA expression data was normally distributed (Supplementary Figure S1). We determined the effect of rs550818 genotype on *GIT1* mRNA expression based on three genotype groups (independent variable) using linear regression analysis with an additive genetic model. We also assessed whether *GIT1* mRNA expression levels differed between healthy controls and participants with ADHD using a two-tailed Student's t-test. All data analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 20.0 Armonk, NY: IBM Corp.).

Functional Characterization of Git in Drosophila

Genetics and breeding. Conditional knockdown of the *Drosophila GIT1* ortholog *Git* (CG16728) in all or specifically in multidendrite neurons was achieved with the UAS-GAL4 system [Brand and Perrimon 1993] using promoter lines w; *UAS*-Dcr-2; *elav*-GAL4 and *477*-GAL4, *UAS*-mCD8::GFP; *ppk*-GAL4, respectively [Dietzl et al., 2007]. The *Git UAS*-RNAi line (vdrc108123 *UAS-RNAi^Git^/CyO*) and its genetic background control (vdrc60100) were obtained from the Vienna *Drosophila* RNAi Centre (VDRC, [Dietzl et al., 2007]. For synapse and dendrite experiments, stock vdrc108123 was rebalanced with CyO-GFP to allow for selection of knockdown larvae. Crosses were cultured according to standard procedures at 28°C.

Neuronal morphology of synapses at the neuromuscular junction. Synapses at the type 1b neuromuscular junctions (NMJs) of muscle 4 were analyzed as described previously [Schuurs-Hoeijmakers et al., 2012]. Male L3-stage larvae of the genotypes *UAS-RNAi^{Git}/UAS*-Dcr-2; +/*elav*-GAL4 and the respective control +/*UAS*-Dcr-2; +/*elav*-GAL4 were dissected following a dorsal midline incision [Brent et al., 2009]. Dissected larvae were fixed in 3.7% paraformaldehyde for 25 min, washed in PBS containing 0.3% Triton X-100 (PBST), stained with 1:125 anti-brp (nc82), washed in PBST, and stained with 1:500 Goat anti-Mouse Alexa Fluor 488 and 1:25 anti-dlg1 antibody covalently coupled to Goat anti-mouse Alexa Fluor 568 IgG1 (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA; Zenon® Antibody Labeling Kit, Life Technologies). The larvae were mounted in Prolong anti-fade Gold (Life technologies). Images were taken with a Zeiss Axio Imager Z2 microscope (63× magnification), subsequently stacked and synaptic area, branches and active zones were analyzed in Fiji [Schindelin et al., 2012; Schuurs-Hoeijmakers et al., 2012]. . For the *Git* RNAi genotype at least 19 synapses and for the control genotype at least 29 synapses were analyzed. Statistical analysis was per-

formed in Graphpad prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com).

Dendritic morphology of class IV dendritic arborization neurons. Dissection and immunostaining was performed as described above, but for imaging the dorsal dendritic arborization C (ddaC) class IV dendritic arborization neurons larval were opened along the ventral midline [Brent et al., 2009]. Genotypes analyzed were *Git* RNAi: *UAS-RNAi*^{Git}/*477*-GAL4, *UAS*-mCD8::GFP; +/*ppk*-GAL4), and the control: +/*477*-GAL4, *UAS*-mCD8::GFP; +/*ppk*-GAL4. The *477* and *ppk* promoters simultaneously drive RNAi and expression of mCD8::GFP in a tissue-specific manner. Antibodies used were 1:100 Rat anti-mCD8 primary antibody and 1:200 Goat-anti-Rat Alexa Fluor 488. Z-stack images were taken at a Zeiss LSM 510 confocal microscope with a 20x objective. Z-stacks were imported into NeuronStudio (version 0.9.92, http://research.mssm.edu/cnic/tools-ns.html) for generation of neuronal reconstructions and Sholl analysis (10 μm interval) [Wearne et al., 2005]. Tracing files were analyzed with L-Measure (version v5.2, [Scorcioni et al., 2008]) and significance was analyzed using the Student's (equal variance) or Welch's t-test (unequal variance).

Drosophila locomotor activity. Locomotor activity of individual male flies was recorded with the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Waltham, MA) [Suh and Jackson 2007; Catterson et al., 2010] to assess whether *Git* pan-neuronal knockdown flies displayed hyperactive behavior or sleep regulation defects. Activity of 3–5 days old male flies was recorded over 4 days on a 12-h light:dark cycle and the average daily activity of at least 25 flies for each genotype was calculated. Locomotor activity data were analyzed in pySolo [Gilestro and Cirelli 2009], modified to analyze activity and sleep (the latter defined as 5-min of inactivity [Rosato and Kyriacou 2006]) between 120–540 min relative day and 840–1260 min relative night to reflect the stable locomotor activity in those intervals. Statistical analysis was performed in Graphpad prism (version 5.00 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com). T-tests were performed on summarized statistics.

RESULTS

Association Between the GIT1 Locus and ADHD

Demographic characteristics of the different cohorts are presented in Table I. Testing whether variation in the *GIT1* locus (including SNP rs550818) altered ADHD risk we found that neither the SNP rs550818 ($P = 0.49$; odds ratio 1.022; 95% confidence interval (CI) 0.959–1.088) nor the *GIT1* locus showed association with ADHD in the PGC ADHD meta-analysis data (N = 19,210, $P = 0.465$, Fig. 1). Power analysis showed that the test was highly powered to detect an association with a genotype relative risk (GRR) ≥1.1 (for range of GRRs see Supplementary Table SVI).

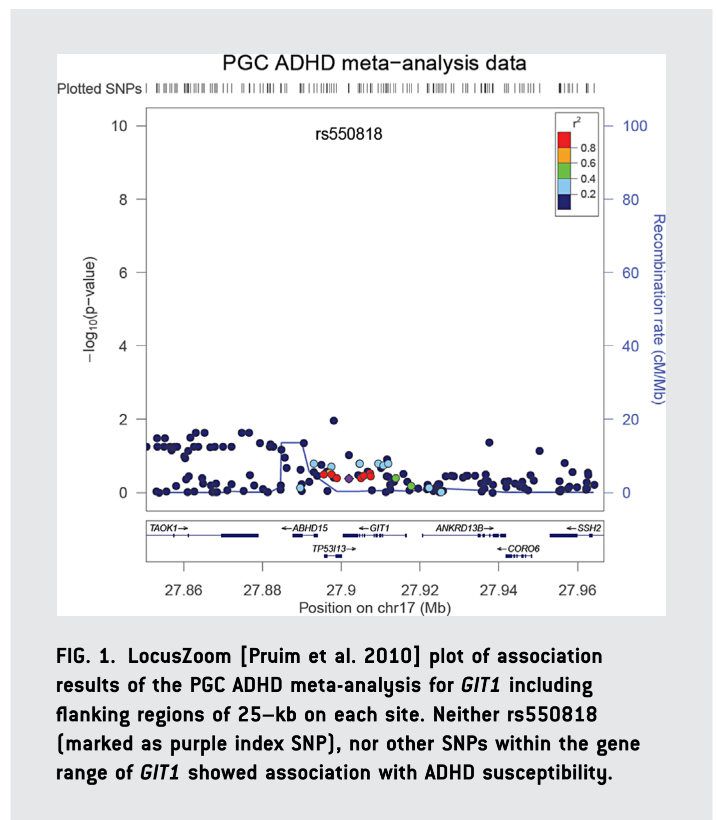

FIG. 1. LocusZoom [Pruim et al. 2010] plot of association results of the PGC ADHD meta-analysis for *GIT1* including flanking regions of 25–kb on each site. Neither rs550818 (marked as purple index SNP), nor other SNPs within the gene range of *GIT1* showed association with ADHD susceptibility.

Association Between the GIT1 Locus and Quantitative Measures Related to ADHD in the IMpACT-NL Cohort

We did not find an association of SNP rs550818 or the *GIT1* locus with self-reported hyperactivity/impulsivity or inattentive symptom counts (N = 115, $P_{corrected} > 0.05$, and Table II) in the IMpACT-NL cohort (for details on demographics see Supplementary Table SVII). Previously, Won and others investigated the effect of rs550818 on sustained attention in a continuous performance task and they studied the effect of *Git1* deficiency in mice on working memory. Therefore, we tested the effect of rs550818 genotype and the *GIT1* locus on neuropsychological performance in the same cognitive domains. The association results between our genetic variables and outcomes of the SAD task (mean series completion time, SD series completion time, SD series errors, and the response bias) or the Digit Span task (forward and backward condition) were not significant (all *P*-values >0.05, Table II). Testing neuropsychological measurements in additional domains of cognitive functioning (reaction time, sustained attention, inhibition, impulsivity, delay aversion, motor control, and set shifting; for description of variables see Supplementary Table SII) did not provide evidence for association with the rs550818 genotype or the *GIT1* locus ($P_{corrected\ for\ all\ tests} > 0.05$, Supplementary Table SVIII). However, power of these analyses was limited; the IMpACT-NL sample provided 32% power to detect an association explaining 1% of the variance (see more elaborate power analysis in Supplementary Table SIX).

Association Between the GIT1 Locus, Brain Volume and White Matter Integrity of Microstructure

We investigated the effect of the *GIT1* locus on brain volume measurements in the case-control sample IMpACT-NL (N = 203) and the population-based cohort BIG (N = 1,300). Given the

TABLE II. Results of Single-SNP and Gene-Based (rs550818 and *GIT1*) Association Analyses for Self-Reported ADHD Symptom Counts, Sustained Attention Dots (SAD) Task and Digit Span Task in the IMpACT-NL Cohort

Trait	Variable	N (HC/ADHD)	P rs550818	β	95% CI	P GIT1[c]
Self report symptom score[a]	Hyperactivity/impulsivity	−/115	0.413	0.134	−0.191–0.458	0.477
	Inattention	−/115	0.593	0.088	−0.237–0.413	0.944
	Total	−/115	0.395	0.140	−0.186–0.466	0.614
Sustained attention dots task[b]	Mean series completion time	99/95	0.445	−0.091	−0.324–0.143	0.696
	Standard deviation series completion time*	99/95	0.142	−0.173	−0.405–0.059	0.563
	Standard deviation series errors*	99/95	0.439	−0.089	−0.316–0.138	0.808
	Response bias*	99/95	0.150	−0.163	−0.387–0.061	0.424
Digit span task[b]	Forward score raw*	100/98	0.511	−0.076	−0.304–0.152	0.433
	Backward score raw	100/98	0.941	0.008	−0.217–0.233	0.831

[a]Age and gender were used as covariates and 52 SNPs were considered for the gene-based analysis.
[b]Age, gender and diagnostic status were used as covariates. 52 SNPs were considered in the gene-based analysis.
[c]Effect sizes and 95% confidence intervals could not be estimated for the gene-based association tests.
*Variables that are significantly different between adult ADHD patients and healthy controls after correction for multiple testing (Mostert et al., submitted).

known involvement of *Git1* in neuronal development [Zhang et al., 2005; Za et al., 2006; Segura et al., 2007], we tested associations of genetic variation in *GIT1* with global brain measures for gray matter, white matter, and total brain volumes. None of these analyses yielded significant associations ($P_{\text{corrected for all tests}}$ >0.05, and Table III). Additionally, we performed exploratory voxel-wise brain-wide analyses of gray and white matter volume, and of microstructural integrity in the BIG cohort for rs550818 to identify potential local effects of *GIT1* variation. Neither the VBM analyses for gray or white matter volume, nor the voxel-wise analyses for mean diffusivity and fractional anisotropy showed significant associations with rs550818 genotype (data not shown). While the IMpACT-NL sample again only provided limited power for this analysis (Supplementary Table SIX), the analyses in the BIG cohort were highly powered to detect associations explaining between 1% (>95%) and 0.5% (>72%) of variance (Supplementary Table SIX).

Functional Characterization: Effect of rs550818 on GIT1 mRNA Expression

Previously, it was reported that the minor allele (A) of the SNP rs550818 caused a reduction in luciferase transcription in HEK293 cells [Won et al., 2011]. We therefore investigated this effect in blood samples of adult ADHD patients and healthy controls from the IMpACT-NL cohort. High quality RNA samples were available for 121 individuals (55 healthy controls and 66 individuals with ADHD); the G allele was the major allele in our European Caucasian sample. Indeed, SNP rs550818 genotype significantly affected *GIT1* mRNA expression in the total sample independent of diagnostic status (N = 121, $b_{\text{standardized}} = -0.220$, $P = 0.015$); carriers of the common allele (GG; N = 63) had highest expression, while heterozygotes (GA; N = 53) had intermediate expression and the carriers of the risk-associated genotype (AA; N = 5) showed lowest expression (Fig. 2A). *GIT1* mRNA expression levels did not differ significantly between healthy controls and participants with ADHD (t = 1,559 df = 119, $P = 0.1217$) (Fig. 2B).

Functional Characterization: Effect of Git RNAi on Neuronal Morphology and Locomotor Activity in Drosophila

The fruit fly *Drosophila melanogaster* is a suitable model to study the behavioral and cellular consequences of genes associated to genetic disorders [van der Voet et al., 2014]. To model the ADHD risk allele and validate the function of *GIT1* in neuronal morphology, we targeted the *Drosophila GIT1* ortholog, *Git*, using conditional RNA interference. The effect of the neuronal *Git* knockdown on synaptic organization was studied at the neuromuscular junction (NMJ). The *Drosophila* larval NMJ is a well-established synaptic model system that shares major features with central excitatory synapses in the mammalian brain [Koh et al., 2000] and has successfully been used for characterizing a number of *Drosophila* models of neurological diseases, including schizophrenia [Dickman and Davis,

TABLE III. Results of Single-SNP and Gene-Based (rs550818 and *GIT1*) Association Analyses for Brain Volumes in the IMpACT-NL and BIG Cohort

	IMpACT-NL cohort[a]				BIG cohort[c]			
	P rs550818	β	95% CI	*P* GIT1[b,e]	*P* rs550818	β	95% CI	*P* GIT1[d,e]
Total brain volume	0.658	0.039	−0.134–0.211	0.563	0.897	0.511	−2.269–3.292	0.415
Total gray matter volume	0.622	−0.035	−0.175–0.105	0.497	0.970	0.069	−0.624–0.761	0.791
Total white matter volume	0.361	0.079	−0.084–0.229	0.154	0.934	0.150	−0.874–1.174	0.453

[a]N = 203 (101 ADHD patients). Adult ADHD patients do not differ in brain volume from healthy controls (Supplementary Table SV).
[b]52 SNPs were considered for the gene-based analysis.
[c]N = 1,300.
[d]Fourty three SNPs were considered for the gene-based analysis. Total brain volume is the sum of total gray and white volume. Age, gender, magnetic field strength, and gray matter when testing for white matter and vice versa were used as covariates.
[e]Effect sizes and 95% confidence intervals could not be estimated for the gene-based association tests.

2009] and intellectual disability disorders [Schenck et al., 2003; Zweier et al., 2009; Bayat et al., 2011; Liu et al., 2011]. Pan-neuronal knockdown of *Git* resulted in a significant decrease in the number of neurotransmitter release sites, so-called active zones, per synaptic terminal compared to controls (0.87 fold, $P = 0.027$), whereas the total area of the neuromuscular junction (NMJ) was not changed ($P = 0.96$) (Fig. 3A + B). Quantitative evaluation of synaptic terminal morphology revealed abnormal branching of synaptic terminals in the *Git* RNAi knockdown condition (Fig. 3B). Both the number of branches and branching points were significantly increased at NMJs of the *Git* RNAi line when compared to control flies (1.49 and 1.86 fold, $P = 0.0002$ and 0.0032, respectively).

Drosophila class IV dendritic arborization (da) neurons are complex and provide a good model for studying dendritic morphology [Jan and Jan 2010]. Knockdown of *Git* in these neurons induced abnormal dendritic complexity (Fig. 3C). Quantification of the traced, reconstructed neurons revealed a reduced number of branches, bifurcations, and terminal tips in the knockdown condition compared to control (0.63 fold, $P = 0.0003$ for all three parameters) (Fig. 3D, Supplementary Figure S2). The average branch length did not differ significantly ($P = 0.061$), but the total branch path length was decreased in the mutant neurons (0.74 fold, $P = 0.0002$) (Supplementary Figure S2, Fig. 3D). These data suggest that *Git* knockdown results in a branching defect. Consistently, the maximum branch order was reduced (0.84 fold, $P = 0.017$) and a Sholl analysis that plots the branch order as a function of soma distance, reveals a reduction in branch order throughout the neuron (Fig. 3D). Other dendritic parameters, namely branch contraction and partition asymmetry, were not significantly different (Supplementary Figure S2).

We have recently demonstrated increased locomoter activity and decreased sleep in ADHD *Drosophila* models [van der Voet et al., 2015]. We therefore assessed whether *Git* pan-neuronal knockdown flies also affect locomotor behavior. No defects in activity levels were found (day: $P = 0.4$ and night: $P = 0.1$, respectively; Fig. 3E). Sleep of *Git* knockdown flies did also not differ from their genetic background controls ($P = 0.4$ and $P = 0.2$, respectively; Fig. 3E). These data suggest that despite a role in regulating synapse and dendrite morphogenesis, *Git* knockdown does not cause increased locomotion.

DISCUSSION

In the original publication of *GIT1* as a risk gene for ADHD, 27 SNPs in a 19 kb region encompassing the *GIT1* gene had been analyzed. Of those, eight SNPs had been shown to be polymorphic in a Korean childhood sample (N = 388), and rs550818 was found associated with ADHD [Won et al., 2011]. In addition, homozygous deficiency of *Git1* in mice resulted in increased locomotor activity [Won et al., 2011]. In this study we performed a multilevel investigation of the role of the *GIT1* locus in ADHD risk and related traits (behavioral and MRI-derived) as well as functional characterization of the *GIT1* gene in humans and in *Drosophila*. Our results clearly show that the *GIT1* locus is not associated with ADHD risk, ADHD symptom counts, neuropsychological performance, or brain volume and white matter integrity variation in large human data sets. However, we demonstrated that rs550818 is indeed functional, as it lowered *GIT1* mRNA expression in human blood samples independently of ADHD diagnostic status. Using *Drosophila* as a model system, we showed that neuron-specific *Git* knockdown altered synaptic and dendritic morphology, whereas locomotor activity parameters remained unchanged.

Using the largest currently available ADHD sample, the PGC ADHD meta-analysis sample ($N_{cases} = 5,621$, $N_{controls} = 13,589$) we analyzed SNP rs550818 as well as the *combined* effects of all SNPs within the *GIT1* locus. Although our study had sufficient statistical power to detect an association, we were unable to replicate the initial finding by Won and coworkers. This is consistent with the results of an earlier replication attempt in a Brazilian childhood ADHD sample [Salatino-Oliveira et al., 2012] Despite the non-significant association, we showed that the effect is in the same direction as previously reported [Won et al., 2011], whereas the Brazilian study reported an odds ratio of 0.749, indicating an opposite directionality [Salatino-Oliveira et al., 2012]. Importantly though, samples used in our and in the Brazilian study consisted (mainly) of participants of Caucasian ethnic origin, while all participants in the first study had an Asian ethnic background [Won et al., 2011; Salatino-Oliveira et al., 2012]. Whereas allele frequencies of the present study and the Brazilian study [Salatino-Oliveira et al., 2012] are consistent with frequencies found in the European population (MAF = 0.27 for allele A), frequencies in Asian populations—including the Korean one

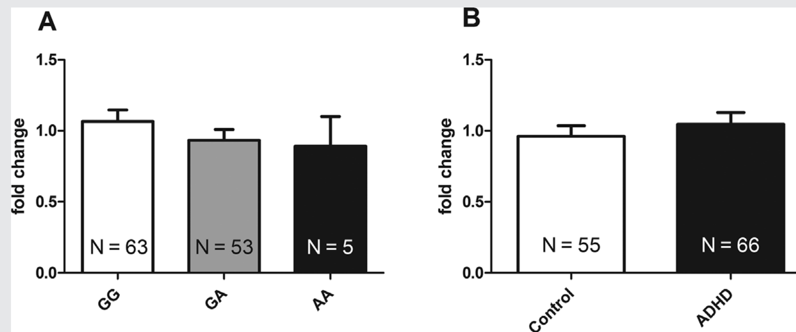

FIG. 2. ***GIT1*** **mRNA expression in human blood samples was dependent on rs550818 genotype but notdiagnostic status in participants from the IMpACT-NL cohort. A: The minor A allele reduced *GIT1* mRNA expression in human blood samples ($N = 121$, $b = -0.116$, $t(119) = -2.462$, $P = 0.015$, $R2 = 0.048$). Bar charts represent mean and 95% confidence interval. B: *GIT1* mRNA expression fold change did not differ in healthy controls compared to individuals with ADHD ($P = 0.1217$; two-tailed Student's t-test). Individuals with ADHD were distributed across the different genotype groups as following: $NG_G = 26$, $N_{GA} = 26$ and $N_{AA} = 3$.**

[Won et al., 2011]—strongly differ from this (MAF between 0.06 and 0.09). Therefore, the lack of replication can be difficult to interpret, as diverse genetic backgrounds and variable environmental exposures may lead to distinct causal genetic variants in different populations [Campbell and Rudan, 2002].

Individuals with ADHD frequently display cognitive deficits, including impairments in inhibition, attentional processing, and increased reaction time variability [Castellanos et al., 2006; Sonuga-Barke et al., 2010; Kofler et al., 2013]. A number of such cognitive domains has also been found impaired in *Git1*-deficient mice. For example, *Git1* knockout mice showed impaired spatial learning and memory in the Morris water maze task and impaired recognition memory during a novel-object recognition task [Won et al., 2011]. Therefore, we tested the *GIT1* locus for association with cognitive performance in relevant domains. However, in concordance with the findings of Won and coworkers, who had applied a continuous performance test in the Korean childhood sample [Won et al., 2011], we did not find an effect of rs550818 or the entire *GIT1* locus on sustained attention in our adult ADHD sample, nor did neuropsychological performance in any of the other tested domains show association with *GIT1*. Additional cognitive deficits observed in *Git1* knockout mice, which were not tested in the current study, include impaired fear response and reduced adaptation to novel and changing environments [Schmalzigaug et al., 2009; Menon et al., 2010]. Tasks quantifying fear response, like the eye blink component of the startle response [Davis 2006; Hajcak et al., 2009], and those measuring reversal learning and tapping into adaptability might therefore be interesting phenotypes for future studies in humans in relation to genetic variation in the *GIT1* locus.

Git1 knockout mice exhibit alterations in dendritic length and spine density [Zhang et al., 2005; Menon et al., 2010; Fiuza et al., 2013]. ADHD has been associated with volume differences in the brain [van Ewijk et al., 2012; Onnink et al., 2014], and we have shown that ADHD symptoms are associated with total brain volume in the general population [Hoogman et al., 2012]. Thus, we investigated the role of *GIT1* in global and voxel-wise brain volume measures and microstructural integrity. However, we could not find an effect of *GIT1* on any brain measurements. In a way, this is consistent with the findings in mice, where changes of neuronal morphology did not translate into structural abnormalities observable at the macroscopic level in 3-month-old mouse brains in *Git1* knockout mice [Menon et al., 2010].

Won and colleagues had shown that the minor allele of rs550818 (A) reduced luciferase signal in an in vitro transcription assay [Won et al., 2011]. In vivo, in human blood samples, we were able to confirm this effect of the A allele of rs550818, showing that *GIT1* mRNA expression was reduced in carriers of the minor allele. Generally, eQTLs can be specific to certain tissues, cells, anatomical regions and diseases (GTex Consortium 2013; [Emilsson et al., 2008]. Therefore, our findings cannot necessarily be translated to other tissue types, e.g., the brain [McKenzie et al., 2014] However, a recent large study shows that there is also overlap between eQTLs from peripheral blood and eQTLs in brain [Wright et al., 2014], which implies that some local regulatory variants might show ubiquitous effects [Kim et al., 2014]. In the case of the *GIT1* eQTL, the fact that consistent effects have been found in vitro and in vivo might indeed indicate that effects are ubiquitous. However, this effect does not seem to be strong enough to modify brain structure, cognitive performance, or ADHD-related behavior.

Git1 is responsible for recruiting proteins to the synapse, and *Git1* knockout mice displayed decreased dendritic length and spine density [Zhang et al., 2005; Menon et al., 2010]. A recent study identified *Drosophila Git* as a component of the active zone-associated cytomatrix and as a regulator of synaptic vesicle endocytosis and recycling [Podufall et al., 2014], although the actual number of active zones had not been evaluated in this study. Consistent with *Git* being a component of active zones, we did observe a mild but significant reduction in the number of active zones. We further showed that neuronal *Git* RNAi knockdown interferes with synaptic terminal branching and dendrite formation in *Drosophila.* This is consistent with earlier findings showing that various trafficking mutants of genes involved in organelle trafficking processes result in alterations of dendrite

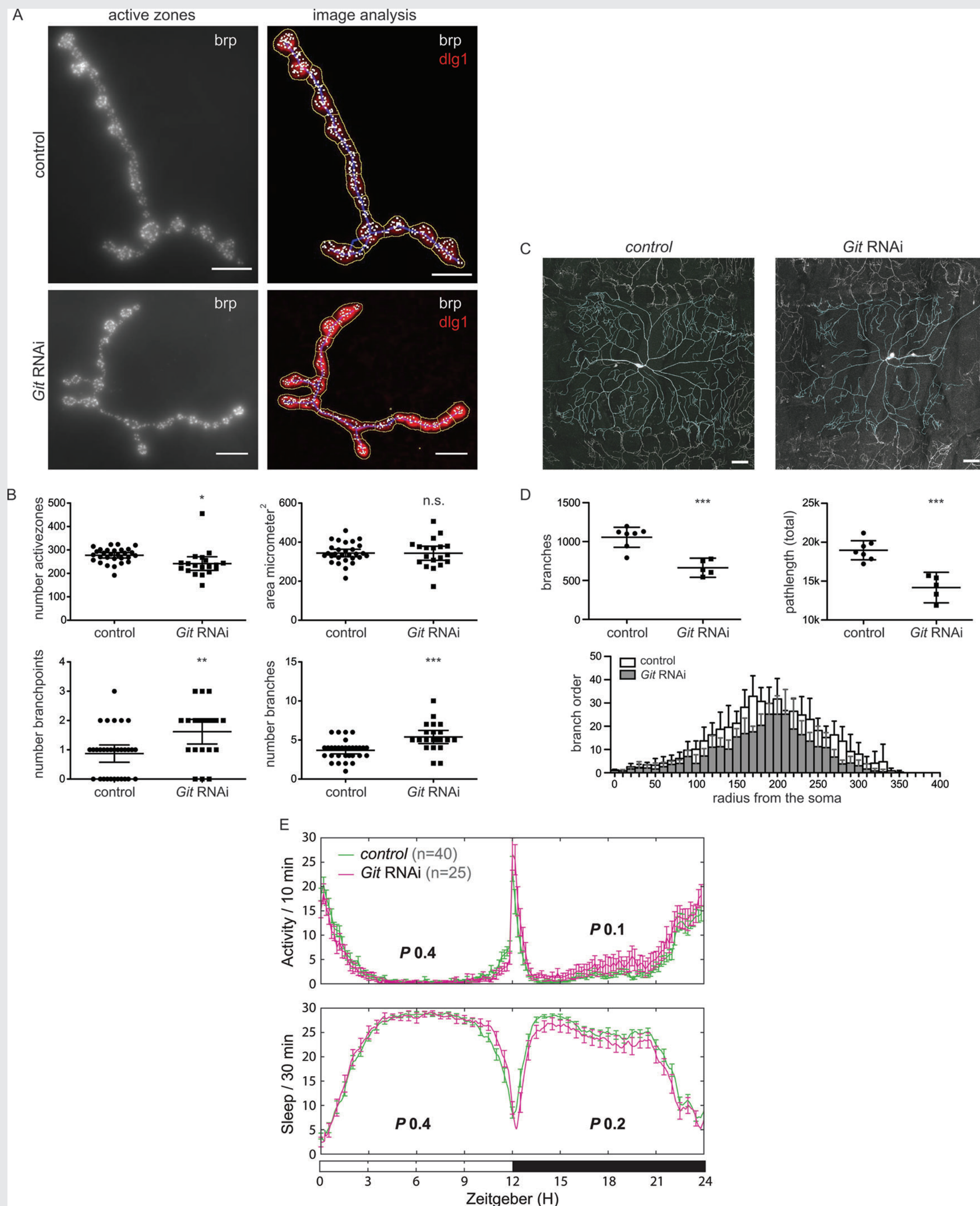

FIG. 3. Git knockdown in Drosophila interfered with synapse and dendrite morphology, but did not alter locomotor activity. A) Representative Drosophila synaptic terminal at the neuromuscular junction (NMJ) for control and *Git* RNAi larvae. Overall morphology of synaptic terminals were visualized with an antibody against the disc large 1 (dlg1) protein, active zones, the presynaptic sites of neurotransmitter release, with an antibody against the active-zone component bruchpilot (brp). Each white foci represents one active zone. Images were quantitatively analyzed using an in house-developed Fiji macro (Schuurs-Hoeijmakers et al. 2012). Scale bar 10 μm. B) Quantitative analysis of NMJs showed a significant decrease in active zone count ($P = 0.027$), increase of branch count ($P = 0.0002$) and branching point count ($P = 0.0032$), while the area was not different ($P = 0.96$). Scatter plots represent individual measurements (*Git* RNAi $N \geq 19$ and control $N \geq 29$), mean and error bars indicate the 95% confidence interval. C) Representative Drosophila class IV da neurons show abnormal dendritic morphology in *Git* RNAi compared to wildtype control animals. Scale bar 50 μm. D) Quantitaive analysis of dendritic trees revealed that *Git* RNAi ($N = 5$) reduces the number of branches ($P = 0.0003$) and total branch path length ($P = 0.0002$), compared to the control ($N = 7$). Sholl analysis reveals that the branch order throughout the neuron is reduced. Scatter plots represent individual measurements. Error bars indicate the 95% confidence interval. E) Locomotor activity profiling of adult *Git* RNAi and control flies revealed normal activity or sleep parameters (values for day (Zeitgeber 0-12h, white bar) and night (Zeitgeber 12-24 h, black bar) periods indicated). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *P*-values were derived from two-sided Student's t-tests, except for not-normally distributed data, then a Wilcoxon-ranked test was performed.

morphogenesis [Corty et al., 2009]. Altogether, these observations support an important role for *Git* in synaptic and dendritic organization. Despite altered neuronal morphology, however, *Git* knockdown did not result in the locomotor hyperactivity that has been observed for other *Drosophila* models of ADHD-associated genes [van der Voet et al., 2015]. Our knockdown is likely not to remove all of Git protein from the *Drosophila* brain. Also, the *GIT1* variant, even if homozygous, causes only a slight reduction in *GIT1* expression. Importantly, the two-fold higher spontaneous locomotor activity in an open-field test in *Git1* knockout mice [Won et al., 2011] was only observed in the homozygous condition with no detectable protein. Mice with a heterozygous deletion showed normal locomotor activity, in agreement with our findings in flies and humans.

At the cellular level, the effect of *GIT1* knockdown has been demonstrated in different model systems. We showed in human blood samples, that rs550818 affects *GIT1* gene expression. Interestingly, the cellular effects in the *Git1* knockout mouse model of Won and others seemed to be cell specific, as specifically inhibitory synaptic transmission was decreased [Won et al., 2011]. Won and colleagues suggested that the resulting increase in neuronal excitability might contribute to the development of ADHD-like phenotypes. Although we demonstrated that genetic variation in the *GIT1* locus is not associated with ADHD in humans, we cannot rule out any other effects of the *GIT1* locus on different behavioral characteristics. The observed effect of *Git1* deficiency in mice on fear learning and adaptation to new environments, might be interesting starting points for future studies in humans.

The present findings should be viewed in light of several strengths and limitations. The main strengths of our study are its comprehensive approach on multiple levels and the use of the largest and well powered ADHD meta-analysis data set currently available. Moreover, we did not only test association for a single SNP, but also investigated the *combined* effect of *all* SNPs within the *GIT1* locus available in our data sets. We also studied the role of the *GIT1* locus in various neuropsychological measures and investigated potential effects of *GIT1* on brain morphology in humans, in patients as well as a large population sample. Next to the association analyses, we also assessed the functional role of SNP rs550818 by mRNA expression analysis. For our functional analyses we used a novel and validated fly model for ADHD-related hyperactivity, which has been shown to be very useful in characterizing effects of ADHD candidate genes on synapse morphology and locomotor behavior [van der Voet et al., 2015]. A clear weakness of our study was the limited size of our patient sample for the neuropsychological analyses, which might have been underpowered to reliably detect genetic effects in a relevant range of explained phenotypic variance. Additionally, the association of rs550818 with ADHD was originally identified in a childhood sample [Won et al., 2011], whereas our association analyses for neuropsychological and brain-related traits were performed in adult participants. This can be criticized as we know that differential genotype-phenotype association can exist at different ages and that genetic and neurocognitive mechanisms underlying ADHD may change throughout life [Greven et al., 2011; Larsson et al., 2011; Thissen et al., 2015]. To overcome these limitations, it would be recommendable to also test for association with the number of ADHD symptoms in larger samples (of children) with ADHD. Furthermore, this study focused only on common genetic single nucleotide variants (SNVs), although it is known that these cannot completely explain the heritability of ADHD [Gratten et al., 2014]. Therefore, rare genetic variation within the *GIT1* locus, be it single nucleotide or structural variants, might still play a role in ADHD. However, we already showed that an alteration of *GIT1* mRNA expression does – if not complete – not affect behavior. Even when *Git* is knocked down strongly in neurons, no behavioral changes in the model system were observed. Thus, we think it is unlikely that rare genetic variants within the *GIT1* locus will contribute to ADHD. Lastly, the gene-based testing methods we used did not provide us with effect size measures, which can help to better interpret the results of association findings.

In summary, our findings do not provide evidence for an impact of the *GIT1* locus on ADHD risk or the variation of ADHD-related traits in humans. Although rs550818 is associated with the variation of *GIT1* expression in blood, this does not appear to be a risk factor for ADHD. Therefore, *GIT1* is not supported as a candidate gene for this psychopathology, despite its reproduced and newly identified functional roles in neuronal morphology. Our study stresses the need for multi-level approaches in the study of genetic risk factors influencing the neurobiological mechanisms underlying ADHD etiology.

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CONFLICT OF INTEREST

J. Buitelaar has been in the past 3 years a consultant to/member of advisory board of/and/or speaker for Janssen Cilag BV, Eli Lilly, Shire, Novartis, Roche, and Servier. He is not an employee and not a stock shareholder of any of these companies. He has no other financial or material support, including expert testimony, patents, royalties.

In the past year, S. Faraone received income, travel expenses and/ or research support from and/or has been on an Advisory Board for Pfizer, Ironshore, Shire, Akili Interactive Labs, CogCubed, Alcobra, VAYA Pharma, Neurovance, Impax, NeuroLifeSciences and research support from the National Institutes of Health (NIH). With his institution, he has US patent US20130217707 A1 for the use of sodium-hydrogen exchange inhibitors in the treatment of ADHD. In previous years, he received consulting fees or was on Advisory Boards or participated in continuing medical education programs sponsored by: Shire, Alcobra, Otsuka, McNeil, Janssen, Novartis, Pfizer and Eli Lilly. S. Faraone receives royalties from books published by Guilford Press: Straight Talk about Your Child's Mental Health, Oxford University Press: Schizophrenia: The Facts and Elsevier, ADHD: Non-Pharmacologic Treatments. All other authors report no conflict of interest.

ETHICAL STANDARDS

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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