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***PAC1R* genotype to phenotype correlations in Autism Spectrum Disorder**

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Abstract

Amygdala dysfunction has been implicated in numerous neurodevelopmental disorders, including autism spectrum disorder (ASD). Previous studies in mice and humans, respectively, have linked *Pac1r/PAC1R* function to social behavior and PTSD-susceptibility. Based on this connection to social and emotional processing and the central role played by the amygdala in ASD, we examined a putative role for *PAC1R* in social deficits in ASD and determined the pattern of gene expression in the developing mouse and human amygdala. We reveal that *Pac1r/PAC1R* is expressed in both mouse and human amygdala from mid-neurogenesis through early postnatal stages, critical time points when altered brain trajectories are hypothesized to unfold in ASD. We further find that parents of autistic children carrying a previously identified PTSD-risk genotype (CC) report greater reciprocal social deficits compared to those carrying the non-risk GC genotype. Additionally, by exploring resting-state functional connectivity differences in a sub-sample of the larger behavioral sample, we find higher functional connectivity between the amygdala and right middle temporal gyrus in individuals with the CC risk genotype. Thus, using multimodal approaches, our data reveal that the amygdala-expressed *PAC1R* gene may be linked to severity of

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Authors' contributions

MG assisted in collection of behavioral and genetic data, performed and imaged ISH, analyzed the results, and drafted the manuscript. ACA carried out behavioral and genotypic data collection. KP, SK, and JD analyzed the SNP status of the genetic samples. AG and CS analyzed and provided the human transcriptome data and helped interpret the results. MJH created and interpreted the interactome and the GO/biological function analysis. CJV and XY carried out behavioral and fMRI data collection and analyzed the data. LK and JC conceived, organized and oversaw the study, analyzed the results, and edited the manuscript. CV, LK, AG and JC revised the manuscript after review. All authors read and approved the final manuscript.

ASD social phenotype and possible alterations in brain connectivity, therefore potentially acting as a modifier of amygdala-related phenotypes.

Lay Summary

In this multimodal study across mouse and human, we examined expression patterns of *Pac1r/PAC1R*, a gene implicated in social behavior, and further explored whether a previously identified human PTSD-linked mutation in *PAC1R* can predict brain connectivity and social deficits in ASD. We find that *PAC1R* is highly expressed in the both the mouse and human amygdala. Furthermore, our human data suggest that *PAC1R* genotype is linked to severity of social deficits and functional amygdala connectivity in ASD.

Keywords

PAC1R; amygdala; autism; genetic modifier; neuroimaging

Introduction

The amygdala, among a number of other brain structures such as the prefrontal cortex and cerebellum, is central for social and emotional processing (LeDoux, 2007; Barak & Feng, 2016). It is widely connected to a large number of brain regions including those that are known to be associated with social processing (e.g., ventromedial prefrontal cortex) as well as those that are generally involved in attentional (e.g., parietal regions) and visual (e.g., primary visual cortex) functions (Roy et al., 2009). A combination of anatomical and imaging studies supports the amygdala, as well as other brain regions of the social-cognitive network, as a key site of pathophysiology in ASD (Baron-Cohen et al., 2000; Mundy, 2018). In addition, amygdala dysfunction has been implicated in numerous other neurodevelopmental and psychological disorders involving socio-emotional impairments (Kennedy & Adolphs, 2012; Mier & Kirsch, 2015).

Although high quality post mortem studies are limited, two well conducted studies have revealed fewer, smaller and more densely compacted neurons in the amygdala of individuals with ASD compared to controls (Kemper & Bauman, 1998; Schumann & Amaral, 2006). Volumetric studies have revealed enlarged amygdalae earlier in childhood in ASD relative to typically-developing children (Schumann, 2004). fMRI studies have revealed decreased or increased activation during social-emotional tasks and aberrant functional connectivity of the amygdala in individuals with ASD compared to typically-developing controls (Baron-Cohen et al., 2000; Green et al., 2016; Guo et al., 2016; Kliemann et al., 2012). Although the source of abnormalities in amygdala processing in ASD is not currently well understood, these studies clearly implicate alteration of amygdala circuitry, structure, and function in ASD.

ASD is a complex genetic disorder, with greater than 90% of cases likely due to mutations in a number of genes that affect brain development (Bozzi et al., 2018; Ramaswami & Geschwind, 2018). While genome-wide association studies (GWAS) have begun to uncover genes that contribute to ASD susceptibility, there are likely other non-causal cohorts of genes that act as modifiers of core ASD behavioral phenotypes (Mullins et al., 2016). Based

on previous studies in mice and humans, we focused on *PAC1R* (also known as *ADCYAP1R1*) as a potential modifier of ASD phenotypes. *PAC1R* encodes a receptor for pituitary adenylate cyclase activating polypeptide (PACAP), a member of the vasoactive intestinal polypeptide (VIP) family. PACAP, via *PAC1R* binding, regulates a number of diverse biological processes including neural development, cellular stress response, glucose metabolism and feeding, promotion of neuroendocrine secretion in pituitary cells, and modulation of the circadian clock (Falluel-Morel et al., 2008; Ghzili et al., 2008; Harmar & Lutz, 1994; Hashimoto, 2006; Mounien et al., 2009; Stroth et al., 2011).

Our interest in *PAC1R* in relation to ASD stems from previous work in both mouse and humans. *Pac1r* mutant mice display altered social behaviors but normal non-social behaviors (Nicot et al., 2004). This finding suggests that *Pac1r* may be involved in neural signaling processes essential for social function. *Pac1r* also appears to have a role in emotional processing as rodents show increased *Pac1r* expression in the amygdala after fear conditioning (Dias et al., 2012). In humans, a single nucleotide polymorphism (SNP) (rs2267735) occurring within a putative estrogen response element (ERE) of *PAC1R* has been linked to PTSD susceptibility in females (Breslau et al., 1998; Kessler et al., 1995). Specifically, females carrying two copies of the C allele at this locus have decreased *PAC1R* expression, which is predictive of PTSD diagnosis and increased severity of symptoms (Ressler et al., 2011). At the functional level, the CC risk genotype in the ERE sequence compromises ER α binding, inhibiting activation of *PAC1R* transcription and resulting in reduced gene expression (Mercer et al., 2016; Ressler et al., 2011). As amygdala dysfunction is a hallmark feature of PTSD (Davis, 1992; Diener et al., 2016; Giustino & Maren, 2015; Shin, 2006), these findings suggest that reduced *PAC1R* expression is linked to a phenotype stemming from disrupted amygdala functioning. Although mutations in *PAC1R* have yet to be found in ASD GWAS screens, the above described findings nevertheless suggest that *PAC1R* may act as a genetic modifier in psychiatric disorders characterized by social deficits, such as ASD. To further investigate this putative link, we employed a multi-modal, integrated approach to test the hypotheses that: 1) *Pac1r/PAC1R* is expressed during amygdala development in both mouse and human and 2) carrying the PSTD-risk genotype in *PAC1R* is predictive of behavioral functional brain alterations in ASD. Our studies reveal: 1) *Pac1r/PAC1R* is expressed during key developmental stages in both mouse and human, and 2) *PAC1R* genotype status is correlated with both social deficits and altered brain connectivity in ASD. Thus, our results suggest that *PAC1R* may act as a genetic modifier in ASD, and if confirmed in larger cohorts, may provide a unique biomarker to stratify individuals with ASD.

Methods

Investigation of *PAC1R* expression

Animals—C57BL/6J mice obtained from Jackson Laboratories were housed and bred in the temperature and light controlled animal care facilities at Children’s National Medical Center. All animal procedures were approved by Children’s National Medical Center’s Institutional Animal Care and Utilization Committee (IACUC) and conformed to NIH guidelines for animal use.

In-situ hybridization—*In-situ* hybridization (ISH) was performed on C57BL/6J mouse tissue at embryonic (E) days 13.5 and 18.5 and postnatal day (P) 7. Three separate embryos/mice were analyzed at each age group. For E13.5, embryos were dissected and brains post-fixed in 4% paraformaldehyde (PFA) in PBS overnight. For E18.5 and P7, embryos or mice were perfused and brains post-fixed overnight in 4% PFA. After fixation, brains were cryoprotected in 30% sucrose, embedded in OCT, cryostat sectioned at 20 μ m and mounted on charged Superfrost Plus microscope slides. Slides were stored at -80°C until used for ISH. ISH was performed using probes and reagents obtained from RNAscope 2.5 (Advanced Cell Diagnostics, Newark, California, USA). Tissue sections were dehydrated and pre-treated with hydrogen peroxide and protease IV according to RNAscope recommendations. Amplification and detection steps were performed according to the RNAscope protocol using detection kit reagents and the EZ Hybridization oven set to 40°C . ISH signal was detected with a DAB reaction and hematoxylin counterstain was applied. After thorough washing, slides were coverslipped using Cytoseal-60 mounting media. Slides were imaged using an Olympus BX51 inverted light microscope.

Human PAC1R Expression—Human gene-level brain expression data were obtained by datamining publicly available information from the BrainSpan project [www.hbatlas.org], a public (NIH)-private (Allen Brain Atlas) Consortium to map human gene transcriptome expression in the brain. This database contains genome-wide transcriptome data on postmortem developing and adult human brains (Kang et al., 2011). We downloaded *PAC1R* brain expression data from the NCBI GEO database (accession number GSE25219) in the form of \log_2 -transformed signal intensity values (Platform GPL5175, Affymetrix GeneChip Human Exon 1.0 ST Array). Affymetrix uses background probes with matching GC content for background correction for all probes on the array [http://media.affymetrix.com/support/technical/whitepapers/exon_background_correction_whitepaper.pdf]. Median *PAC1R* expression values were plotted using ggplot2 in R for each brain region (neocortex, hippocampus, amygdala, striatum, mediodorsal nucleus of the thalamus, cerebellar cortex) and developmental time period that is outlined in Table 1. Local polynomial regression fitting was used to smooth the scatter plots. Table 2 provides a breakdown of total number of samples for which data were available from BrainSpan.

Interactome and Gene Ontology (GO) analysis—An interactome connecting PAC1R to other proteins was generated with Genomatix Software Suite, Version 3.9 (Munich, Germany). Additional interactors of PAC1R were identified using IPA software (version 2017, QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) (Krämer et al., 2014), GeneMANIA [<http://genemania.org/>] (Warde-Farley et al., 2010), and bibliographic references. We used the web resource AmiGO developed by the GO Consortium for searching and browsing GO terms (AMIGO2, version 1.8, <http://amigo.geneontology.org/amigo>). The Cytoscape plug-in ClueGO (version 1.8) was used to identify a functionally organized network of GO (Bindea et al., 2009). Putative binding sites for promoters located on the SNP were predicted using the specific tool MatInspector from the Genomatix Software Suite.

Genotype to Phenotype Analysis

Participants—129 children (104 males, 25 females) with ASD between 6 and 14 years of age were recruited from the Washington, DC metropolitan area to participate in IRB approved investigations of cognitive and neuroimaging profiles. Participant characteristics and demographics are shown in Table 2A and B. All participants met ASD criteria outlined by the Diagnostic and Statistical Manual of Mental Disorders; Fifth Edition (DSM-5) as assessed by experienced, trained clinicians at the Children’s National Center for Autism Spectrum Disorders (CASD). All participants also met the cutoff for ASD as determined by Lainhart criteria established by the NICHD/NIDCD Collaborative Programs for Excellence in Autism (Lainhart et al., 2006). Exclusion criteria were: any known co-morbid medical conditions, such as Fragile X Syndrome or other genetic disorders that could affect brain and behavioral development, and/or brain trauma/injury.

Behavior Protocol and Analysis—Participants received the Autism Diagnostic Interview-Revised (ADI-R, n=129) and most received the Autism Diagnostic Observation Schedule (ADOS, module 3, n=98) administered by trained, research-reliable clinicians. Each participant also supplied a saliva sample for DNA genotyping. Saliva samples were collected using Oragene collections kits (DNA Genotek Inc., Ottawa, ON, Canada). Written informed consent, and assent where appropriate, were obtained under a Children’s National Health System approved IRB protocol. Data are expressed as mean \pm SEM and are statistically significant at $p < 0.05$. Statistical analysis was performed using the multiple comparisons ANOVA with Bonferroni correction in SPSS (IBM SPSS Software Version 23, Armonk, NY, USA).

Genotyping—DNA was isolated using the Oragene DNA Isolation Protocol, according to manufacturer’s instructions. Screening for the SNP of interest was performed using a TaqMan allelic discrimination assay that employed the 5’ nuclease activity of Taq polymerase to detect a fluorescent reporter signal. Both alleles were detected simultaneously using allele-specific oligonucleotides labeled with different fluorophores, and genotypes were automatically determined by the ratio of the two fluorophores used. Assay ID C_15872945_10 was used to assay SNP rs2267735 (C/G). The PCR for this SNP contained 30 ng of DNA, 900 nM primers, 200 nM probes, and TaqMan Genotyping Universal PCR Master Mix in a final volume of 8 μ L. PCR was performed on a GeneAmp 9700 thermo cycler. The PCR conditions were 10 minutes at 95 $^{\circ}$ C (denaturation), 44 cycles of 15 seconds at 92 $^{\circ}$ C, and 1 minute at an annealing temperature of 60 $^{\circ}$ C. Fluorescence of PCR products was assessed with an ABI 7900HT RT-PCR system, and allele calling was made using SDS software. All instruments and reagents were purchased from ThermoFisher (Waltham, MA). These polymorphisms were tested for allele frequency and cross-referenced to dbSNP [<http://www.ncbi.nlm.nih.gov/projects/SNP/>] for allele calling.

Resting State fMRI Imaging Analysis

Participants—Resting-state functional images were available from 48 of the ASD children recruited, aged 7–13 years old, who had participated in imaging studies at Georgetown University. A final cohort of 20 ASD children (17 males, 3 females) was retained after applying strict criteria for head motion to resting-state fMRI data and other exclusion criteria

stated below. Out of the 20, 9 were *PAC1R* CC genotype and 11 were GC genotype. Demographic information for the final imaging sample (N=20) is presented in Tables 4 and 5. Exclusion criteria included: (1) Full-Scale IQ below 80 as measured by the Wechsler Intelligence Scale for Children (WISC-IV) or Wechsler Abbreviated Scale of Intelligence (WASI); (2) Other neurological diagnosis (e.g., epilepsy) based on parent report; and (3) Contraindications for MRI. Imaging data were acquired according to consenting guidelines of Georgetown University and Children's National Health System IRBs.

Imaging Protocol and Analysis—Resting state functional echo-planar images were acquired on a Siemens Trio 3T (Munich, Germany) with parameters: 3 mm isotropic resolution ($3.0 \times 3.0 \times 2.5+.5$ gap mm), $TR=2000$ ms, $TE=30$ ms, flip angle = 90° , FOV = 192×192 mm. Subjects were asked to stay awake with eyes open during a 5 mins/51secs scan. CONN functional connectivity toolbox was used to perform standard preprocessing and denoising steps, including slice-timing and motion correction, spatial normalization, resampling to 2 mm isotropic, smoothing (8mm kernel), nuisance regression (6 motion parameters, CSF and white matter signal, and high motion volumes, i.e. >3 standard deviations of average signal or >0.5 mm framewise displacement “scrubbed”) and bandpass filtering (0.008–0.09 Hz). Seed regions of interest (ROIs) were created using left and right amygdala masks from the AAL atlas through Wake Forest PickAtlas (Wake Forest University School of Medicine, Winston-Salem, NC). Time series for each of the 2 ROIs were correlated with that of every other voxel in the brain (Pearson's correlation r values were transformed to Z scores), which produced a whole-brain connectivity map for each participant, for left and right amygdala each. We then entered these two connectivity maps in second-level two-way ANOVA to test for the main effect of group for differences between participants with CC (N=9) and GC (N=11) genotypes for any of the ROIs. Results were corrected for voxel-wise multiple comparisons at $p<0.05$ using Monte-Carlo simulation 3dClustSim (2-sided, nearest neighbor = 2; (Cox et al., 2017)) which produced a height threshold of $p<0.005$ with extent threshold $k=56$. Individuals from this imaging cohort with the GG genotype were not analyzed due to low sample size after exclusion for motion artifact.

Results

Pac1r expression

Previous studies revealed that *Pac1r* is expressed throughout the mature mouse brain particularly in the hippocampus and hypothalamus (Otto et al., 1998). However, the developmental pattern of expression within the amygdala has not been assessed. To determine when and where *Pac1r* is expressed during amygdala formation, we examined gene expression patterns by *in situ* hybridization (ISH) at embryonic day (E) 13.5, E18.5 and postnatal day (P) 7, time points that represent early, mid, and later stages of amygdala development (Sokolowski & Corbin, 2012). We found that at E13.5, *Pac1r* mRNA is expressed in the ventral pallidum and the pre-optic area (Figure 1), two major amygdala progenitor zones previously identified by us and others (Carney et al., 2010; Hirata et al., 2009; Waclaw et al., 2010). We further observed high *Pac1r* expression at E18.5 in emerging

amygdala nuclei such as the medial and basolateral amygdala (Figure 2a–d). *Pac1r* mRNA expression remained high and widespread across amygdala nuclei at P7 (Figure 2e).

Next, we sought to examine the expression of *PAC1R* throughout human brain development. To accomplish this we datamined post-mortem human gene expression data from the BrainSpan project [<http://hbatlas.org/>](Kang et al., 2011) to generate gene expression graphs of *PAC1R* throughout the life span from human fetal stages through adulthood. Our brain expression graphs showed high levels of *PAC1R* expression in the amygdala, cerebellar cortex, hippocampus, mediodorsal nucleus of the thalamus, neocortex and striatum commencing at early-mid fetal stages and remaining high throughout childhood and adulthood (Figure 3a, Tables 1 and 2). Specifically, in the amygdala *PAC1R* expression increased throughout fetal development, peaked in expression around birth, and remained stable throughout the remainder of post-natal development. We also found differential expression of *PAC1R* between males and females at numerous stages of development (Figure 3b and Table 2). In the amygdala, expression was higher in males than females during fetal development (Figure 3c). Then, by birth and throughout childhood, *PAC1R* expression was higher in females compared to males, with a leveling off of expression across genders during adulthood.

PAC1R Interactome

To investigate potential interactors of PAC1R protein with other proteins and a potential functional relationship with known ASD susceptibility genes, we generated a protein interactome utilizing Genomatix (Figure 4). The interactome generated indicates that PAC1R is at a hub of different signaling cascades involved in stress, metabolism, and inflammatory responses, among others. Moreover, PAC1R potentially interacts with IL6, MAPK1 and CTNBN1, whose encoding genes have been listed as autism susceptibility genes by the Simons Foundation Autism Research Initiative [SFARI, <https://gene.sfari.org/database/gene-scoring/>]. Additionally, the PAC1R interactome included other proteins whose encoding genes are implicated in ASD with high scores in SFARI Gene (up to category 3) such as: DISC1, NLGN3, PER2 and SHANK1, as revealed by IPA and GeneMANIA analyses (Hattori et al., 2007; Katayama et al., 2009). The enriched biological functions of PAC1R interactome genes associated with ASD were assessed with ClueGO and included nervous system development, dendritic spine and synaptic junction, regulation of synaptic transmission (glutamatergic), gliogenesis, peptide hormone secretion, circadian rhythms, and response to stress. Therefore, mutations in *PAC1R* may affect a variety of these processes, potentially contributing to brain dysfunction in ASD. The intronic rs2267735 variant may not only impair the binding to the estrogen response element as experimentally validated (Mercer et al., 2016), but also other binding sites located in the SNP. Indeed Genomatix identified several susceptible (hypothesized but untested) binding sites within the SNP (Chr7:31095890) for promoters such as cAMP-responsive element binding proteins, v-ERB and RAR-related orphan receptor alpha, Nuclear receptor subfamily 2 factors, Nascent polypeptide associated complex and coactivator alpha, as well as RXR heterodimer binding sites. Thus, these potential interactors provide a rich data source to further investigate functional protein interactions with PAC1R that may modulate ASD phenotypes.

Genotype to Phenotype Correlations

Previous studies have revealed that a G to C SNP (rs2267735) occurring within a putative ERE in *PACIR* confers increased PTSD risk in females (Mercer et al., 2016). We therefore next examined whether there is a relationship between this SNP and ASD, another disorder with a major amygdala component (Baron-Cohen et al., 2000; Guo et al., 2016; Kemper & Bauman 1998). To accomplish this, we examined the *PACIR* genotype status (CC, GC, GG) within an ASD cohort (Tables 3 and 4; Figure 5) compared to mean raw scores for three subscales of the ADI-R (repetitive behaviors, communication, and reciprocal social interaction). Using a multiple comparisons ANOVA, we found our hypothesized significant difference in mean raw scores for the reciprocal social subscale of the ADI-R between the CC (PTSD risk) and GC (non-risk) groups, with CC individuals scoring higher—indicative of greater clinical difficulty ($F = 3.33$, $p < 0.05$). No significant difference was found between mean raw scores for the risk (CC) and homozygous non-risk (GG) individuals. This may be attributed to our low sample size ($n=26$) for the GG group (Table 4).

To examine whether there is a link between genotype status and brain connectivity, we next conducted resting state fMRI imaging of a subset of individuals that were behaviorally and genetically assessed (Tables 5 and 6). Within the cohort of ASD participants who underwent resting state fMRI imaging, an F test revealed one cluster in posterior right middle temporal gyrus ($k=97$ (776 mm^3), peak at MNI coordinate $x=60, y=-60, z=6$; see Figure 6) where functional connectivity was higher in the CC (PTSD-risk group) than non-risk GC group with the bilateral amygdala. A homologous cluster with the same connectivity pattern was observed in the middle temporal gyrus in the left hemisphere at an uncorrected threshold ($p < 0.005$, $k=44$ (352 mm^2), peak at MNI coordinate $x=-58, y=-70, z=4$). These regions correspond to visual area MT (Tootell et al., 1995), which is involved in processing of visual motion.

Discussion

ASD affects around 1 in 68 children (CDC, 2010) and is characterized by social-communication deficits and repetitive and/or restricted behaviors (American Psychiatric Association, 2013). Although ASD has been recognized for over 70 years as a distinct behavioral disorder (Kanner, 1943), its biological underpinnings remain relatively unknown (Geschwind, 2011). Moreover, ASD diagnosis is solely based on behavioral criteria, which have been modified over the years as the DSM has been revised. However, with the advent of rapid and inexpensive methods of gene sequencing over the past decade, the genetic landscape of ASD is beginning to be mapped (Ramaswami & Geschwind, 2018). Currently, according to SFARI Gene [<https://gene.sfari.org/about-gene-scoring/criteria/>], there are over 850 susceptibility genes, grouped according to level of confidence, that have been identified as potentially contributing to ASD risk. These genes likely act in combination and via complex gene-environment interactions resulting in alterations in brain development that are causal for ASD (Mullins et al., 2016). In addition to these susceptibility genes, there are likely other cohorts of genes that can greatly influence ASD behavioral phenotypes, either acting in a homeostatic manner to ameliorate autistic phenotypes, or acting to enhance core symptoms (Mullins et al., 2016). In this study, we focused our attention on *PACIR* as a gene

that may act as a modifier of social behavioral phenotypes. To accomplish this, we took a multi-modal approach to investigate a putative link between *PAC1R* genotype status, brain connectivity and social behavior in a cohort of children with ASD. We also further investigated gene expression patterns in mouse and human. Our focus on *PAC1R* stems from previous studies in both mice and humans. Adult *Pac1R* knockout mice display markedly abnormal social behaviors in the 3-chambered social task (Nicot, et al., 2004), a gold standard social assay in mice that has provided face validity for animal models of autism (Silverman et al., 2010). This previous finding implicated *Pac1R* as a key mediator of the development and/or function of brain systems that control social processes. Furthermore, studies in humans revealed a link between a SNP (rs2267725) in the *PAC1R* ERE and PTSD susceptibility in females (Mercer et al., 2016; Ressler et al., 2011). Although in our study our subjects were primarily male, the commonality of amygdala circuit dysfunction in both PTSD and ASD provides the justification for our focus on *PAC1R* and warrants continued investigation with larger cohorts of both sexes.

We first wanted to establish whether *Pac1r* is indeed expressed in the developing amygdala, coinciding with time frames critical for brain development and during periods implicated in altered developmental trajectories associated with ASD. We find that *Pac1r* is highly expressed in mouse brain neural progenitor zones previously implicated by others and us for generating neurons of the amygdala (Carney et al., 2010; Hirata et al., 2009; Waclaw et al., 2010). Moreover, at early postnatal stages we find high levels of *Pac1r* expression in emerging mouse amygdala nuclei. Consistent with our findings in the mouse, data mining of the BrainSpan database reveals high levels of *PAC1R* expression in the human amygdala across the lifespan. This datamining further revealed differential *PAC1R* expression in human male and female brains. Amygdala *PAC1R* expression is higher in males prenatally, and shifts to higher expression in females during childhood and early adulthood. Thus, *Pac1r/PAC1R* is expressed in a spatial and temporal manner during critical prenatal stages of amygdala development, and in an apparent sex-dependent manner in humans. Exploration of *Pac1r* sex specific function in mouse amygdala development and social behaviors merits further investigation.

Previous studies investigating genetic links to PTSD revealed that a G to C SNP (rs2267735) within the ERE of an intron of the *PAC1R* gene is predictive of PTSD severity in females (Mercer et al., 2016; Ressler et al., 2011). Estrogen binding to ER α on the ERE of the *PAC1R* is disrupted, causing a reduction in transcription and decreased *PAC1R* expression. This intronic variant might not only impair binding to the ERE (Mercer et al., 2016) but may also modify binding sites for the promoters identified by Genomatix reported here. As these promoters may affect *PAC1R* function they should be further investigated. Furthermore, several genes within the *PAC1R* interactome are found in the SFARI ASD risk gene list, including *IL6*, *MAPK1*, *CTNNB1*, *DISC1*, *NLGN3*, *PER2* and *SHANK1*. These data together suggest that *PAC1R* is closely tied to amygdala function in stress and fear response pathways, which are disrupted in PTSD. Furthermore, both PTSD and ASD involve impairment of amygdala function and social related symptoms, albeit likely via distinct etiologies (American Psychiatric Association, 2013; Davis, 1992; Diener et al., 2016; Giustino & Maren, 2015; Shin, 2006).

Beyond studies of gene expression, we examined how the G to C SNP (rs2267735) may be related to ASD social symptoms and brain connectivity. Despite only being able to assess relatively small cohorts, we nevertheless find a significant correlation between the PTSD-linked CC genotype and raw score on the reciprocal social subscale of the ADI-R. Individuals with ASD carrying the homozygous CC genotype displayed a higher score, indicating greater social deficit than those carrying the heterozygous genotype (GC, n=66). No significant difference was found between the risk genotype (CC, n=35) and the wildtype genotype (GG, n=26), which may reflect limited power based on the small sample size. Additionally, exploratory analysis of fMRI resting state imaging data from a small subset of the behavioral sample provides evidence in support of genetic differences in functional connectivity of the amygdala. Individuals carrying the risk genotype (CC) had higher resting-state functional connectivity between the amygdala and right posterior temporal gyrus relative to carriers of the non-risk genotype (GC). *PACIR* genetic differences in connectivity of the amygdala with a region involved in visual motion processing may influence how children with ASD perceive socially meaningful information. Indeed, motion perception is altered in ASD (Schauder et al., 2017) and individual differences may relate to *PACIR* genetic differences in connectivity to the amygdala, a structure critical for emotional processing. However, it is important to note that our behavioral and connectivity findings represent a small sample of individuals with ASD, and while these findings are highly intriguing, it will be critical to extend these studies with larger cohorts and to explore potential sex differences in the role of *PACIR* on behavioral phenotype and connectivity. It will also be critical to examine measurements of other ASD-related behavioral deficits, for example anxiety. As anxiety disorders are commonly comorbid with ASD and increased anxiety is a feature of PTSD, investigating the link between anxiety and *PACIR* allele status may reveal further links between genotype and behavior. Despite these limitations, our intriguing findings form a strong basis for future studies to examine this relationship in larger cohorts and in females with ASD and suggest that *PACIR* may be a novel biomarker for predicting social and brain connectivity alterations in this amygdala related disorder.

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List of Abbreviations

ASD	Autism Spectrum Disorders
ADI-R	Autism Diagnostic Interview-Revised
ADOS	Autism Diagnostic Observation Schedule
AMY	amygdala
ANOVA	analysis of variance

CBC	cerebellar cortex
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders; Fourth Edition
ERE	estrogen response element
HIP	hippocampus
IACUC	Institutional Animal Care and Utilization Committee
NCBI GEO	National Center for Biotechnology Information Gene Expression Omnibus
NCX	neocortex
MD	mediodorsal nucleus of the thalamus
MT	middle temporal gyrus visual area
PACAP	Pituitary adenylate cyclase activating polypeptide
PAC1R	pituitary adenylate cyclase-activating polypeptide type 1 receptor
PCW	post conceptual weeks
SNP	single nucleotide polymorphism
STR	striatum
VIP	vasoactive intestinal polypeptide
Y	years

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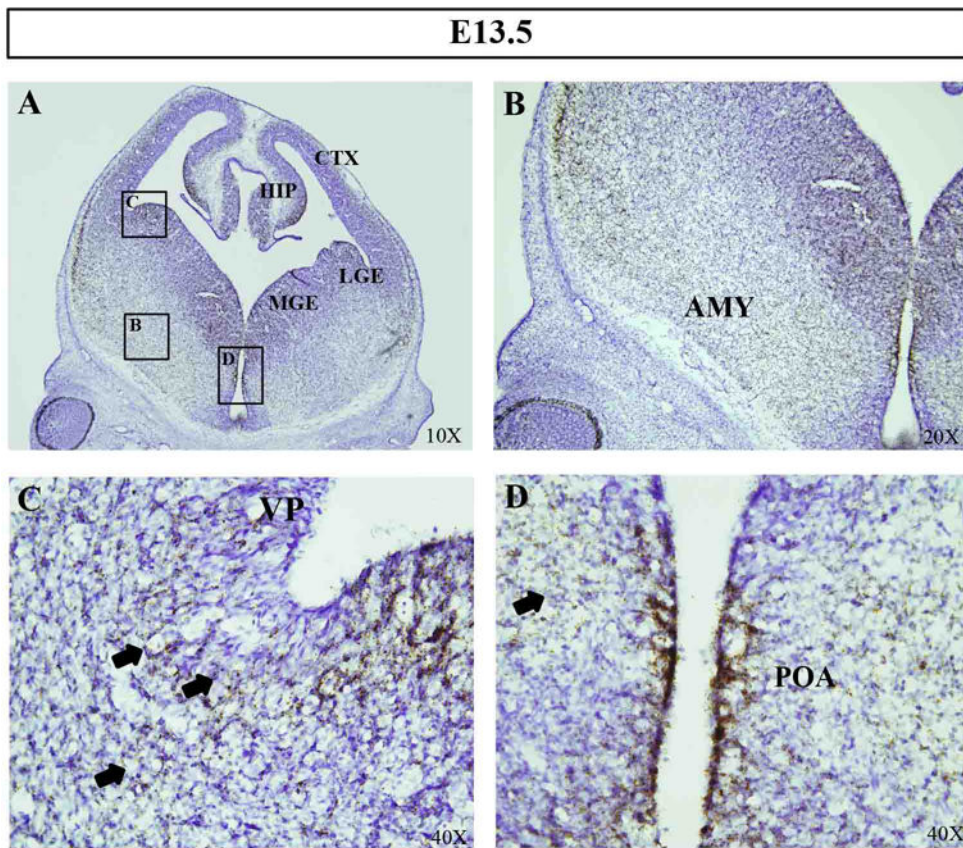


Figure 1. ISH analyses at E13.5 shows *Pac1r* mRNA expression in known amygdala progenitor zones (A, boxed regions). Higher magnification image shows numerous *Pac1r*⁺ cells (brown) in the emerging amygdala (B) and the VP (C, arrows), a source of progenitors destined to give rise to excitatory output neurons in the BLA. *Pac1r*⁺ cells (arrow) are also observed in the POA (D), a source of progenitors destined to give rise to inhibitory output neurons in the MeA. Abbreviations: AMY, Amygdala; BLA, Basolateral Amygdala; CeA, Central Amygdala; CTX, Cortex; HIP, Hippocampus; MeA, Medial Amygdala; LGE, Lateral Ganglionic Eminence; MGE, Medial Ganglionic Eminence; POA, Pre-Optic Area; VP, Ventral Pallium.

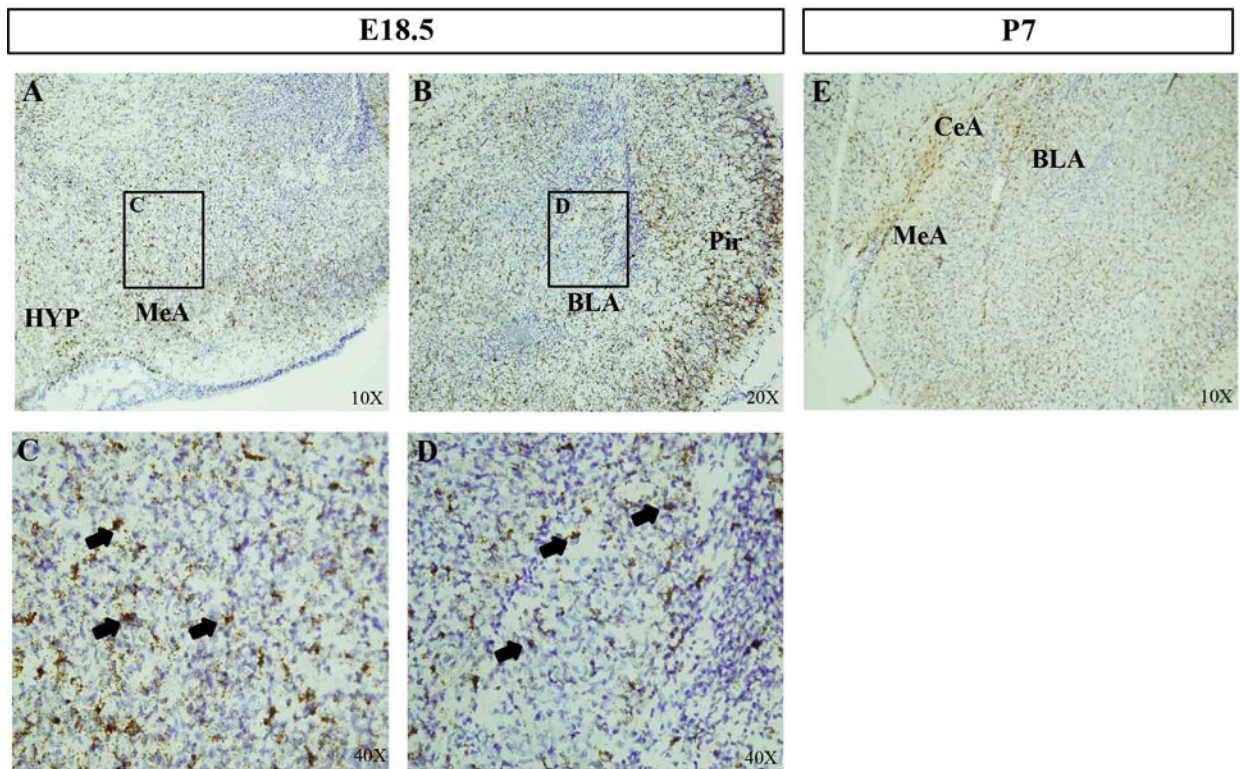


Figure 2. ISH analyses at E18.5 shows high levels of *Pac1r* mRNA in developing amygdala nuclei (A, B). Higher magnification of the MeA (C) and BLA (D) show numerous *Pac1r*+ cells (arrows). (E) At post-natal day 7 many *Pac1r*+ cells are observed in multiple amygdala sub-nuclei. Abbreviations: BLA, basolateral amygdala; CeA, central nucleus of the amygdala; MeA, medial amygdala; HYP, hypothalamus; Pir, piriform cortex

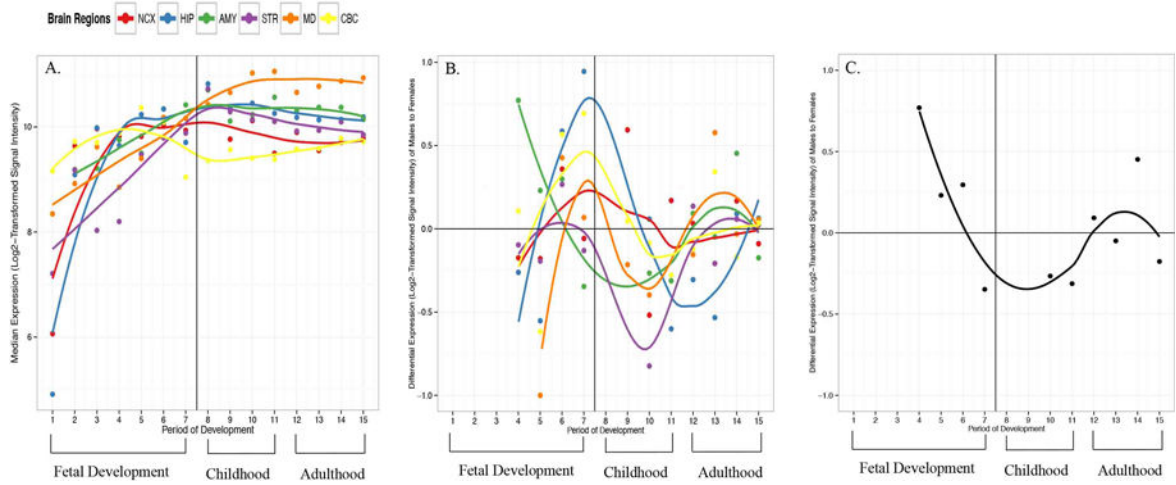


Figure 3.

Gene-level brain expression graphs were generated with data from the *BrainSpan* project (Kang et al., 2011) collected from post-mortem human brain samples. The x-axis shows periods of development with the dark vertical line signifying birth (developmental time points are defined in Table 1). Along the y-axis are values of median expression (Log₂-Transformed Signal Intensity) for which values ≥ 6 signify high levels of expression in at least one post-mortem brain sample. A. Expression of *PAC1R* is observed in multiple brain regions starting at stage 1 of fetal development. B. Differential expression of *PAC1R* is observed between males and females in multiple brain regions with median values above 0.0 denoting higher expression in males and values below 0.0 denoting higher expression in females. C. Differential expression of *PAC1R* between males and females only in the amygdala. Abbreviations: AMY, amygdala; CBC, cerebellar cortex; HIP, hippocampus; MD, mediodorsal nucleus of the thalamus; NCX, neocortex; STR, striatum.

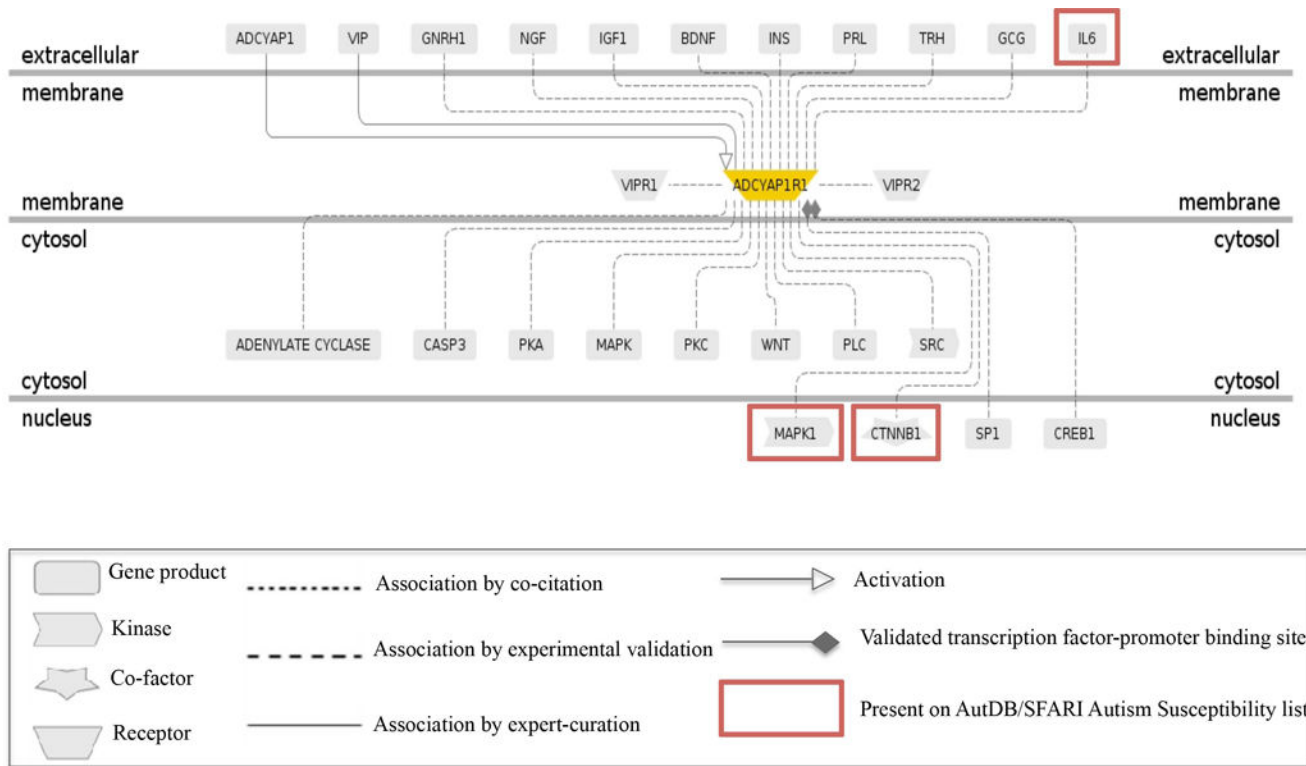


Figure 4. *PAC1R* is at a hub of different signaling cascades involved in stress, metabolism, and inflammatory responses, among others. Plot and legend adapted from Genomatix (v.s. 3.9).

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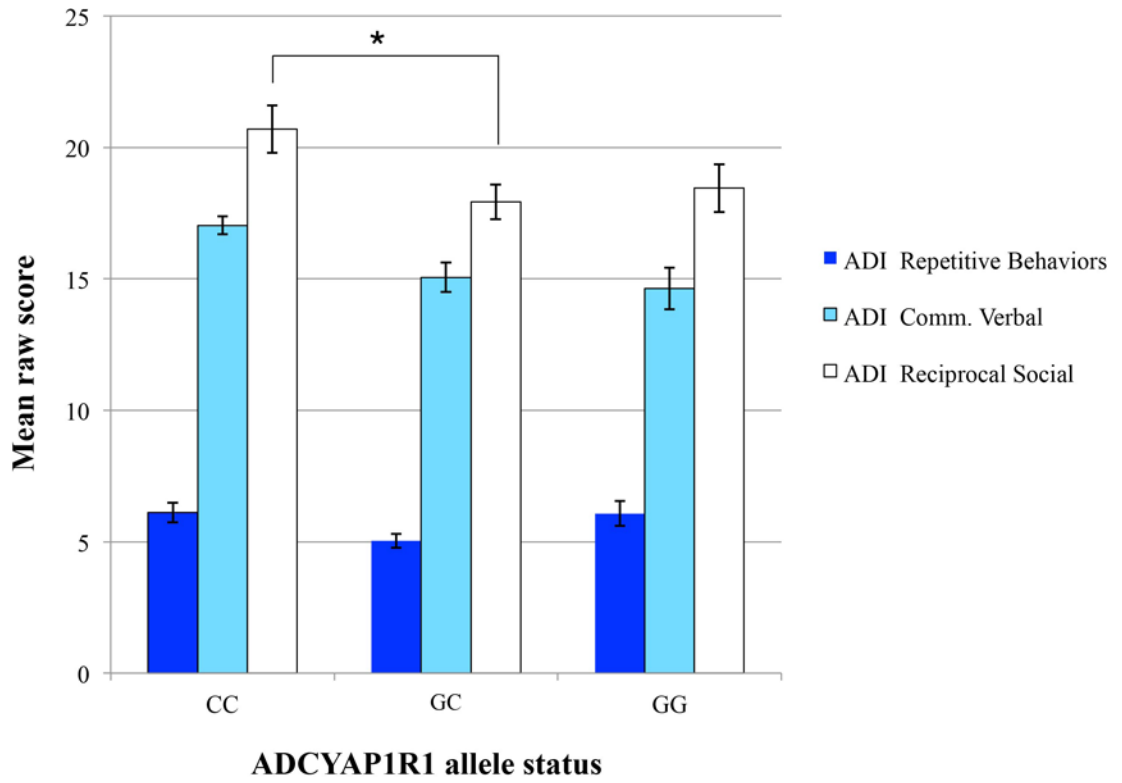


Figure 5. ADI behavioral scores by *PAC1R* SNP allele status. A significant correlation exists between Reciprocal Social scores and allele status. Bars show standard error, $n = 129$, $p < 0.05$.

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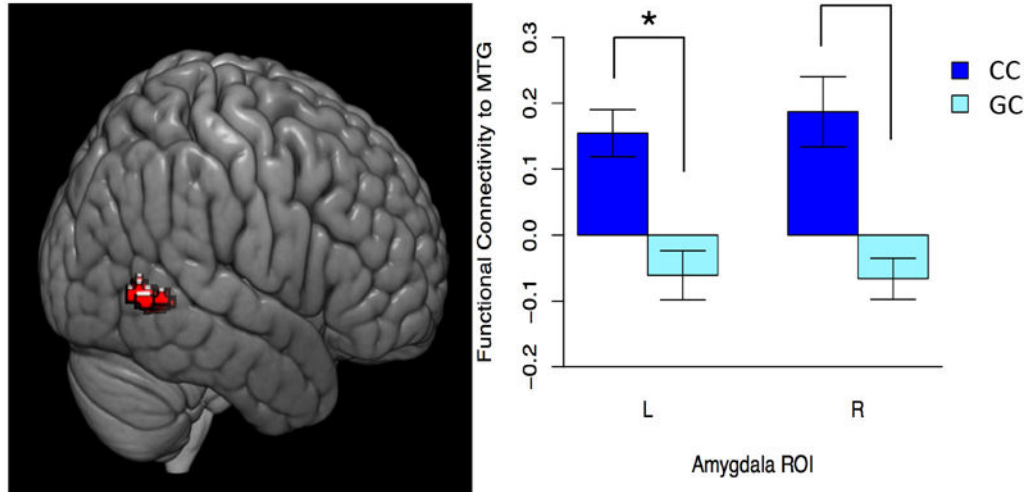


Figure 6. F test reveals one cluster in right middle temporal gyrus where functional connectivity to the bilateral amygdala was significantly higher in the CC group compared to the GC group. Bars show standard error, $n = 20$, $p < 0.005$.

Table 1

Gene Expression Developmental Time Periods.

	Developmental Period	Age
1	Embryonic	4–8 PCW
2	Early fetal	8–10 PCW
3	Early fetal	10–13 PCW
4	Early-mid fetal	13–16 PCW
5	Early-mid fetal	16–19 PCW
6	Late-mid fetal	19–24 PCW
7	Late fetal	24–38 PCW
8	Neonatal and early infancy	0–6 M
9	Late infancy	6–12 M
10	Early childhood	1–6 Y
11	Middle and late childhood	6–12 Y
12	Adolescence	12–20 Y
13	Young adulthood	20–40 Y
14	Middle adulthood	40–60 Y
15	Late adulthood	60+ Y

Defined developmental periods of human brain expression graphs corresponding to Table 1, modified from Kang et al., 2011
 Abbreviations M, postnatal months; PCW, post-conceptual weeks; Y, years.

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Table 2:

Brainspan Data Per Period of Development

Period of Development	Total Samples (Fig. 3A)	# Males	# Females	# Samples for Fig. 3B	# Samples for Fig. 3C
1	2	2	0	0	0
2	2	2	0	0	0
3	3	0	3	0	0
4	3	2	1	3	3
5	4	3	1	4	4
6	7	2	5	7	7
7	3	1	2	3	3
8	3	3	0	0	0
9	3	1	2	3	0
10	4	2	2	4	4
11	3	2	1	3	3
12	4	2	2	4	4
13	9	5	4	9	9
14	4	3	1	4	4
15	3	1	2	3	3
Totals	57	31	26	47	44

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Table 3

Characteristics and Demographics

Participant Characteristics	<i>N</i>	<i>M</i>	<i>SD</i>	<i>Range</i>
Chronological age (years)	129	10	2	6–14
ADI Reciprocal Social	128	18.7	5.37	7–30
ADI Communication Verbal	126	15.4	4.35	6–24
ADI Repetitive Behavior	129	5.5	2.31	0–12
ADOS Communication	95	3.4	1.99	0–9
ADOS Social Interaction	95	8.2	3.24	1–14
ADOS Social Communication	95	11.6	4.9	0–22
Full Scale IQ	108	105.4	20.6	62–149

Participant Demographics	
<i>Race</i>	<i>N</i>
African American	17
Asian	7
Caucasian	99
Other	7
Missing	2
Total	132

<i>Ethnicity</i>	<i>N</i>
Hispanic or Latino	11
Non-Hispanic or Latino	115
Missing	6
Total	132

<i>Gender</i>	<i>N</i>
Male	104
Female	25

Table 4

ASD Cohort Demographics.

Race	CC	GC	GG
African American	7	7	1
Asian	2	5	0
White	25	49	24
Other	1	5	1
Total	35	66	26
Ethnicity	CC	GC	GG
Hispanic/Latino	1	8	2
Not Hispanic/Latino	32	57	23
Total	33	65	25

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Table 5

Imaging Participant Characteristics.

	<i>N</i>	<i>M</i>	<i>SD</i>	<i>Range</i>
Chronological age	20	11.2	1.44	8–14
ADI Reciprocal Social	19	20.8	5.88	10–29
ADI Communication Verbal	19	16.1	4.94	6–24
ADI Repetitive Behavior	19	5.1	2.05	1–9
ADOS Communication	19	3.2	1.61	1–7
ADOS Social Interaction	19	7.5	2.85	2–13
ADOS Social Communication	19	10.7	4.15	3–18
Full Scale IQ	20	118.9	16.4	94–149

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Table 6.

Imaging cohort details

A fMRI Cohort Gender Groups.		
	<i>N</i>	<i>Percent</i>
Male	17	85
Female	3	15
Total	20	100
B fMRI Cohort Allele Status Groups.		
	<i>N</i>	<i>Percent</i>
CC	9	45
GC	11	55
Total	20	100
C fMRI Cohort Demographics.		
Race	CC	GC
African American	2	0
Asian	1	1
White	6	8
Other	0	1
Total	9	10
Ethnicity	CC	GC
Not Hispanic/Latino	7	10
Total	7	10

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