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Exploring the relationship between cortical GABA concentrations, auditory gamma-band responses and development in ASD: Evidence for an altered maturational trajectory in ASD

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Abstract

Lay Abstract—Autism spectrum disorder (ASD) is thought to arise, in part, from an imbalance in the brain's signaling chemicals (neurotransmitters). Multiple studies involving individuals with ASD and relevant animal models show that the concentration of the neurotransmitter, GABA, is decreased in brains of individuals with ASD. Separately, the gamma-band response (Gamma; a key brain response) is thought to be crucially reliant on GABA, and correlates with underlying GABA levels in healthy adults for the motor and visual systems. Additionally, multiple studies have observed reduced Gamma in ASD further supporting a coupling between Gamma and GABA, though the exact association of such responses with GABA remains unknown. To test the association between GABA concentration and the auditory Gamma in both typically developing individuals (TD) and also individuals with ASD, GABA and gamma-band responses were measured (via magnetic resonance spectroscopy (MRS) and magnetoencephalography (MEG) respectively) in 27 TD and 30 individuals with ASD, including both children/adolescents and adults. The effects of autism on the maturation of GABA concentrations, Gamma-band oscillations and their interrelations were examined. Children/adolescents with ASD demonstrated reduced relative cortical GABA concentrations, though typical auditory Gamma. Importantly, children/adolescents with ASD failed to exhibit the typical age-related increases of GABA concentrations and gamma-band coherence, as well as the typical interrelation of these measures. Such suggested altered coupling in childhood/adolescence might result in the decrease of gamma-band coherence observed in the adults with ASD. Therefore, these results suggest that GABAergic intervention in ASD may be most efficacious during childhood/adolescence.

Scientific Abstract—Autism spectrum disorder (ASD) is hypothesized to arise from imbalances between excitatory and inhibitory neurotransmission (E/I imbalance). Studies have

demonstrated E/I imbalance in individuals with ASD and also corresponding rodent models. One neural process thought to be reliant on E/I balance is gamma-band activity (Gamma), with support arising from observed correlations between motor, as well as visual, Gamma and underlying GABA concentrations in healthy adults. Additionally, decreased Gamma has been observed in ASD individuals and relevant animal models, though the direct relationship between Gamma and GABA concentrations in ASD remains unexplored. This study combined magnetoencephalography (MEG) and edited magnetic resonance spectroscopy (MRS) in 27 typically developing individuals (TD) and 30 individuals with ASD. Auditory cortex localized phase-locked Gamma was compared to resting Superior Temporal Gyrus relative cortical GABA concentrations for both children/adolescents and adults. Children/adolescents with ASD exhibited significantly decreased GABA+/Creatine (Cr) levels, though typical Gamma. Additionally, these children/adolescents lacked the typical maturation of GABA+/Cr concentrations and gamma-band coherence. Furthermore, children/adolescents with ASD additionally failed to exhibit the typical GABA+/Cr to gamma-band coherence association. This altered coupling during childhood/adolescence may result in Gamma decreases observed in the adults with ASD. Therefore, individuals with ASD exhibit improper local neuronal circuitry maturation during a childhood/adolescence critical period, when GABA is involved in configuring of such circuit functioning. Provocatively a novel line of treatment is suggested (with a critical time window); by increasing neural GABA levels in children/adolescents with ASD, proper local circuitry maturation may be restored resulting in typical Gamma in adulthood.

Keywords

MEG; gamma-band; GABA; MEGA-PRESS; MRS; auditory; ASD; E/I

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by pervasive social/communication impairments and restricted/repetitive behaviors (American Psychiatric Association, 2013). Current prevalence estimates suggest that 1 in 68 children are diagnosed with ASD (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators & Centers for Disease Control and Prevention (CDC), 2014). For over a decade an imbalance in neural excitation/inhibition (E/I) has been explored as a pathogenic mechanism underlying ASD (Hussman, 2001; Rubenstein & Merzenich, 2003), with support coming from both clinical imaging (Brown, Singel, Hepburn, & Rojas, 2013; Gaetz et al., 2014; Harada et al., 2011; Rojas, Singel, Steinmetz, Hepburn, & Brown, 2014) and post-mortem (Casanova et al., 2006; Fatemi, Folsom, Reutiman, & Thuras, 2009; Fatemi, Reutiman, Folsom, & Thuras, 2009; Fatemi et al., 2002; Zikopoulos & Barbas, 2013) studies. Furthermore, E/I imbalance has been demonstrated in animal models that recapitulate key aspects of the ASD phenotype (Calfa, Li, Rutherford, & Pozzo-Miller, 2015; M J Gandal, Anderson, et al., 2012; Gogolla et al., 2009; Liao, Gandal, Ehrlichman, Siegel, & Carlson, 2012; Lin, Gean, Wang, Chan, & Chen, 2013; Saunders, Gandal, Roberts, & Siegel, 2012).

Recently E/I imbalance has been investigated in ASD using magnetic resonance spectroscopy (MRS), a technique that allows *in vivo* estimation of neurochemical

concentrations. Such experiments have demonstrated alterations in both cortical inhibitory (γ -Aminobutyric acid (GABA)) and excitatory (glutamate) neurotransmitters in individuals with ASD (for review see Rojas, Becker, & Wilson, 2015). While these studies have found ASD-related group differences in *in vivo* concentrations, evidence supporting a mechanistic link to symptomatology remains elusive.

Numerous studies suggest that the gamma-band response (30–80Hz) is critically dependent on E/I signaling (Cardin et al., 2009; Sohal, Zhang, Yizhar, & Deisseroth, 2009; Whittington, Traub, Kopell, Ermentrout, & Buhl, 2000; Yizhar et al., 2011), and is known to be important for basic sensory (e.g. visual and auditory) functions (Ba'ar-Eroglu, Strüder, Schürmann, Stadler, & Ba'ar, 1996; Gray, König, Engel, & Singer, 1989) as well as higher order cognitive processes (Herrmann, Fründ, & Lenz, 2010). The gamma-band response is also clearly perturbed in multiple systems (i.e. visual, auditory, somatosensory and rest) in ASD (Grice et al., 2001; Khan et al., 2015; Orekhova et al., 2007; Wilson, Rojas, Reite, Teale, & Rogers, 2007). Furthermore this neurophysiological ASD-related phenotype is conserved in animal models relevant to ASD, independent of the nature of the specific insult (i.e. environmental versus genetic) (Gandal et al., 2012; Gandal et al., 2010; Saunders et al., 2012).

Although *in vivo* relative cortical GABA has been shown to be decreased in ASD (Gaetz et al., 2014; Harada et al., 2011; Rojas et al., 2014), further elaboration is warranted. While gamma-band neurophysiological correlates of GABA concentrations have been observed in the visual and motor system of healthy adults (Edden, Muthukumaraswamy, Freeman, & Singh, 2009; Gaetz, Edgar, Wang, & Roberts, 2011; Muthukumaraswamy, Edden, Jones, Swettenham, & Singh, 2009) there has been some controversy (Cousijn et al., 2014). Subsequently, a recent EEG study in adults has provided further support for such a GABA to gamma-band response coupling, reporting a positive correlation between GABA level and gamma-band power (Balz et al., 2015). However, no such correlation has been attempted in the auditory system for either typically developing (TD) individuals or individuals with ASD.

This study tests the following three hypotheses: 1) following previous studies, relative GABA concentrations in Superior Temporal Gyrus (STG) (Gaetz et al., 2014; Rojas et al., 2014), as well as phase-locked gamma-band responses (Edgar et al., 2015; Gandal et al., 2010; Rojas et al., 2008; Wilson et al., 2007) will be decreased in ASD; 2) in line with previous studies involving multimodal sensory integration (Balz et al., 2015), *in vivo* relative cortical GABA concentrations will positively correlate to phase-locked gamma-band activity in the auditory system for TD individuals; and 3) since previous studies suggest decreases in both relative cortical GABA levels and phase-locked gamma-band activity (see above), individuals with ASD will exhibit a linear relationship between relative cortical GABA concentrations and phase-locked gamma-band activity.

Examining the last hypothesis in more detail suggests three possible outcomes in ASD: 1) altered E/I balance and gamma-band dysfunction may *not* be correlated, or may at least not be resolvable due to separate sub-populations of ASD individuals, some of whom demonstrate GABA/gamma-band activity correlation, and some of whom do not; 2) altered

E/I balance and gamma-band dysfunction may be present in ASD, yet with altered coupling (regression slope) as compared to typically developing individuals, or 3) altered E/I balance and gamma-band dysfunction may exist in ASD with the same regression slope as in TD (similar coupling), but with both measures commensurately decreased.

This study aims to determine the extent to which *in vivo* relative GABA concentration (via spectrally-edited MRS) predicts gamma-band activity (derived from MEG) in both TD participants and, especially, in participants with ASD. If the neurochemical-neurophysiological relationship is conserved in ASD, such observations provide key interpretational value for diminished gamma-band activity as a “biomarker” (a biologically-based marker) of ASD (Rojas & Wilson, 2014), with anomalous GABA levels providing the etiological basis. Moreover, this observation would suggest a possible mechanism of treatment: restoring E/I neurotransmitter balance may normalize neuronal functioning. This is relevant because several GABA-related pharmacological interventions are emerging for potential treatment of ASD (Berry-Kravis et al., 2012; Erickson et al., 2014; Lemonnier et al., 2012). Similar interventions have been shown effective in murine models of ASD normalizing behavioral phenotypes (e.g. enhance sociability) (Silverman et al., 2015; Tyzio et al., 2014) and restoring neural functioning (Gandal, Sisti, et al., 2012; Tyzio et al., 2014). Identifying a critical window for maximizing the efficacy of such interventions might also shed light on the variable success GABAergic pharmaceuticals have demonstrated anecdotally.

Materials and Methods

Participants

Seventy-four participants (32 TD, 42 ASD), ranging from 6 to 40 years old (age = 18.0 ± 0.9 yrs, mean $\pm$ SEM), were recruited into the current study and completed both MEG and MRS scans. While large, the age range of the recruited population allowed for the critical opportunity to map developmental trajectories. In addition such a large age range ensured that resultant observations represent a phenotype persistent across ages, and not an epiphenomenon of study recruitment criteria. Twenty four participants' MEG data had previously been reported for gamma-band alterations, demonstrating decreased phase-locked gamma-band activity in ASD versus TD (Edgar et al., 2015; Port et al., 2016). These studies examined neither GABA, nor the relation between GABA and gamma-band activity measures. Two TD subjects were prescribed medication (though non-psychotropic). Fifteen individuals with ASD were prescribed medications/took dietary supplements (see Table 1). Tailored recruitment and evaluation strategies were used for children/adolescents vs. adult participants:

Child and Adolescent Recruitment and Inclusion/Exclusion Criteria—This study recruited 38 children/adolescents between the ages of 6 and 14. Following procedures described in Edgar et al. 2015, children/adolescents with ASD (N=27; 11.7 ± 0.36 yrs) were recruited from the Regional Autism Center of The Children's Hospital of Philadelphia (CHOP), from the Adult Autism Spectrum Program in the Department of Psychiatry at the Perelman School of Medicine at the University of Pennsylvania, from local and regional

parent support groups such as ASCEND (Asperger Syndrome Information Alliance for Southeastern Pennsylvania) and local chapters of Autism Society of America. All children/adolescents screened for inclusion in the ASD sample had a prior ASD diagnosis made by an expert clinician, typically a developmental pediatrician in the Regional Autism Center at CHOP. The original diagnosis was made after an extensive clinical interview, documentation of DSM-IV criteria for ASD, and use of various ASD diagnostic tools, such as the Childhood Autism Rating Scale and, in many cases, the Autism Diagnostic Observation Schedule (ADOS; Lord et al. 2000). Subjects with typical development (TD) were recruited through local newspaper advertisements and from pediatric practices of the CHOP primary care network.

Research participants made two visits to CHOP. During the first visit (2–3 weeks prior to the MEG exam), clinical and diagnostic testing was performed to confirm the referral ASD diagnosis, to administer neuropsychological tests, and to ensure that the TD children/adolescents met study inclusion/exclusion criteria. Assessments were performed by licensed child psychologists with expertise in autism (LB, EK). Given the extensive clinical evaluations upon which original ASD diagnosis was made, an abbreviated diagnostic battery was used to confirm the original diagnosis. Specifically, the ASD diagnosis was confirmed with standard diagnostic tools, including direct observation with the ADOS and parent report on the Social Communication Questionnaire (SCQ; Rutter et al. 2003). Dimensional symptom severity ratings were also obtained by parent report on the Social Responsiveness Scale (SRS; Constantino & Gruber 2012). The Autism Diagnostic Interview-Revised (ADI-R), a parent interview about current and prior ASD symptoms, was utilized to resolve diagnostic discordances between the ADOS and parent rating scales in the rare instances in which such discordances occurred. For final inclusion in the ASD group, children/adolescents were required to exceed established cut-offs on both the ADOS and SCQ, or, in the event of discordance between those measures, on both the ADOS and ADI-R. Children/adolescents 1 point below ADOS cut-offs were included if they exceeded cut-offs on at least two parent questionnaires or the ADI-R. For children/adolescents for whom original diagnosis was not made by an expert clinician according to DSM criteria (e.g., diagnoses made at school), more rigorous standards were applied, with the child/adolescent required to exceed cut-offs on both the ADOS and ADI-R for inclusion in the ASD group. To rule out global cognitive delay, all subjects were required to score at or above the 2nd percentile ($SS > 70$) on the Perceptual Reasoning Index (PRI) of the Wechsler Intelligence Scale for Children-IV (WISC-IV; Wechsler 2003). In all subjects, the WISC-IV Verbal Comprehension Index (VCI) was also obtained.

Eleven TD children/adolescents were recruited (10.6 ± 0.56 yrs). Inclusion criteria for the TD children/adolescents included scoring below the cut-off for ASD on all domains of the ADOS as well on parent questionnaires, and demonstrating performance above the 16th percentile on the Clinical Evaluation of Language Fundamentals—4th edition (CELF-4; Semel & Wiig 2003). Additionally all subjects and families were native English speakers and had no known genetic syndromes or neurological (e.g., cerebral palsy, epilepsy), or sensory (hearing, visual) impairments. The study was approved by the CHOP Institutional Review Board and all participants' families gave written informed consent. As indicated by

institutional policy, where competent to do so, children/adolescents over the age of seven additionally gave verbal assent.

Adult Recruitment and Inclusion/Exclusion Criteria—This sample included 36 young adults between the ages of 18 and 40. Adult participants with ASD ($N=15$; 21.9 ± 1.1 yrs) were recruited from the Adult Autism Spectrum Program in the Department of Psychiatry at the Hospital of the University of Pennsylvania, as well as from cohorts of participants participating in prior MEG studies by the current investigators and prior studies at the Center for Autism Research at CHOP. Adult TD participants ($N=21$, 27.0 ± 1.2 yrs) were recruited through local newspaper advertisements and from participation in prior studies. Diagnostic procedures were similar to those described for the child/adolescent cohort but were modified to meet the constraints of an adult sample. All adults were required to have a prior diagnosis of ASD, made by an expert clinician according to DSM criteria. At the time of study participation, they were required to exceed established cut-offs on the ADOS-2 as well as *either* the SCQ (Lifetime) or SRS-2 Adult-Informant Report (Constantino & Gruber, 2012). Individuals for whom informant report was not available were included in the ASD group if they had a documented prior diagnosis of ASD and exceeded established cut-offs on the ADOS-2 as well as on *both* the SRS-2 Adult-Self Report and Broad Autism Phenotype Questionnaire (BAPQ; Hurley et al. 2007). Individuals 1 point below diagnostic cut-offs on the ADOS-2 were included if they exceeded cut-offs on two informant report questionnaires or on the ADI-R. To rule out global cognitive delay, all subjects were required to score at or above the 2nd percentile ($SS > 70$) on the Perceptual Reasoning Index (PRI) of the Wechsler Abbreviated Intelligence Scale-II (WASI-II; Wechsler 2011)¹. For all subjects, the WASI-II Verbal Comprehension Index (VCI) was also obtained.

Inclusion criteria for the TD adults included scoring below the cut-off for ASD on all domains of the ADOS-2 and below cut-offs on informant and self-report questionnaires, along with performance above the 16th percentile on the CELF-4 (if within age-range for this measure (Semel & Wiig, 2003)), WASI-II Verbal Comprehension Index, and average of the Peabody Picture Vocabulary Test-4 (PPVT-4; Dunn & Dunn 2007) and Expressive Vocabulary Test-2 (EVT-2; Williams 2007). TD adults also had no history of current psychiatric illness, as documented during initial screening and by subsequent self and informant ratings on the Adult Behavior Checklist (ABCL; Achenbach & Rescorla 2003), and they were taking no psychotropic medications. As with the child/adolescent sample, all adult participants were native English speakers, had no known genetic syndromes or neurological or sensory impairments; in addition they had a negative drug/alcohol screen administered prior to both study visits.

Electrophysiological Data Collection

Magnetoencephalography data were obtained using a 275-channel system (VSM MedTech Inc., Coquitlam, BC) in a magnetically shielded room. Prior to data acquisition, three active head-position indicator coils were attached to the subject's scalp at the nasion, left and right-

¹One subject scored a PRI of 59, but was retained based on clinical impression

preauricular points, which provided continuous measurement of head position in relation to the MEG sensors. To minimize fatigue, during the task participants viewed (but did not listen to) a movie projected onto a screen positioned at a comfortable viewing distance.

Electrodes were attached to the left and right clavicles for electrocardiogram (ECG) recordings and to the bipolar oblique (upper and lower left sites) for electro-oculogram recordings (EOG). A band-pass filter (0.03–300Hz) was applied to the EOG, ECG, and MEG signals, and signals were digitized at 1200Hz with third order gradiometer environmental noise reduction applied to the MEG data.

Stimuli

Stimuli consisted of 200, 300, 500 and 1000Hz (300ms duration; 10ms ramps) sinusoidal tones presented using Eprime v1.1 (Psychology Software Tools Inc., Pittsburgh, USA). Tones were presented via a sound pressure transducer and sound conduction tubing to the participant's peripheral auditory canal via ear-tip inserts (ER3A, Etymotic Research, IL, USA). Prior to data acquisition, 1000Hz tones (300ms duration) were presented to determine auditory detection threshold for each ear. Stimulus tones were presented at 45dB sensation level above threshold (45dB SL). Trials consisted of participants passively listening to binaurally presented tones (130 tones/frequency interleaved) with a 1000ms (± 100 ms) inter-trial interval (i.e. epoch duration varied from 1200ms to 1400ms). In total, this protocol took approximately 12 minutes.

Structural MRI and MRS Methods

After MEG, MRI and MRS data were acquired on a 3T Siemens Verio™ scanner using a 32-channel receive only RF head coil. First an axial oriented 3D MP-RAGE anatomic scan was obtained for each subject (field of view = 256×256×192 mm and matrix = 256×256×192 to yield 1 mm isotropic voxel resolution. The MEGAPRESS spectral editing sequence (Mescher et al., 1998), with TE = 68ms, TR = 1500ms and 128 pairs of interleaved spectra (acquisition time ~7 minutes) was utilized to obtain single voxel (4cm × 3cm × 2cm) MRS. Pilot observations and recent published studies (Gaetz et al., 2014) indicated that this approach provided a reasonable compromise between SNR of GABA estimation vs. exposure to degradation over acquisition time due to motion and potential static field drift². The MRS voxel was first aligned to the left mid-temporal lobe with the long aspect (4 cm) of the cuboid positioned such that the top of the voxel contained the STG. Medial–lateral adjustments were then made to abut (but not include) the left lateral ventricle.

MRS Analysis and Quantification

GABA measurements using the conventional MEGAPRESS sequence are known to be contaminated by co-edited macromolecules. Thus, our estimates are reported using the conventional GABA+ (where the “+” indicates the unknown macromolecular contribution) notation. A 5Hz Lorentzian line-broadening filter was applied prior to jMRUI fitting. Then for the un-edited runs, Hankel Lanczos Singular Value Decomposition (HLSVD) was used

²Note separate measures of field drift of a cold magnet undergoing a 7 minute MEGAPRESS suggests a field drift on this magnet ~2-3Hz, and so is considered a minimal impact on blurring or efficiency

to quantify creatine (Cr) and N-acetylaspartate (NAA). HLSVD fits used up to 15 components to minimize residual signal in the 1–4ppm region. For the subtraction-edited spectra, GABA+ was modeled using an identical technique (up to 15 components, one component modeling the entire GABA+ signal at 3ppm, with no other component overlapping the GABA component region). GABA+ estimates were normalized to Cr estimates from the same ROI to account for inter-session/subject sensitivity variations.

Tissue Segmentation and Quantification

To account for possible confounding effects of tissue composition on MRS quantitation, tissue composition (i.e. gray matter, white matter, and cerebral spinal fluid) of the MRS voxel was calculated from the subject's MP-RAGE. The automated tissue segmentation tool, FAST (Zhang et al., 2001) was utilized to yield three partial volume images, each describing the fraction of tissue type present. Tissue composition fraction was included as a covariate in analyses below.

Gamma-band Data Analysis

Gamma-band responses were analyzed using the MatLab (Mathworks, Natick, MA) open-source toolbox Fieldtrip (Oostenveld et al., 2011), using identical procedures as (Port et al., 2016). Using the procedures outlined in Fieldtrip (FieldtripWiki, 2015a, 2015b), independent component analysis (ICA) identified heartbeat and eye artifacts (blinks and saccades), and these artifact components were removed from the stimulus-epoched data (± 500 ms around trigger). Trials with jump and muscle artifact were rejected using Fieldtrip's Z-score based method. Lastly, to account for differences in head motion during the MEG scan, if any scalp fiducial moved more than 10mm from its average session position within a trial, the trial was rejected. For each group, over 93% (483 trials) of trials remained ($TD = 95.6 \pm 0.5\%$, $ASD = 93.9 \pm 0.7\%$; $p < 0.05$), yielding sufficient data for a stable response. Despite statistical significance, these slight group differences were considered mitigated in the light of overall high success rates.

Subject-specific single-shell head models were created from individual MP-RAGE (sMRI) scans. To coregister MEG and sMRI data, three anatomical landmarks (nasion and right and left preauricular) as well as an additional 200+ points on the scalp and face were digitized for each participant using the Probe Position Identification (PPI) System (Polhemus, Colchester, VT), and a transformation matrix that involved rotation/translation between the MEG and sMRI coordinate systems was obtained via least-squares matching of the PPI points to the scalp/face surface. This head model was then fitted to the mean head position (in MEG coordinate space) determined by the active fiducials. Separately, the participant's MRI was normalized to an age-matched average brain template (children/adolescents - (Fonov et al., 2011); adults - ICBM152 average brain) using non-linear warping. Further analyses focused on the left-hemisphere because multiple independent hypotheses exist that suggest left-hemisphere auditory cortex's role in processing of speech-specific stimuli (for further discussion see Tervaniemi & Hugdahl, 2003), particularly germane to the language deficits commonly associated with ASD. Additionally, this allowed direct comparison to GABA levels measured from these same left temporal lobe areas. Left Heschl's Gyrus was

identified from the Harvard-Oxford Cortical Atlas, and these coordinates were non-linearly transformed to individual subject space for subsequent beamformer analysis.

A linearly-constrained minimum variance (LCMV) beamformer was computed for each participant's left Heschl's Gyrus, discarding the contralateral hemisphere's sensors to reduce inter-hemispheric signal cancellation from correlated activity (Herdman et al., 2003). Heschl's Gyrus virtual electrodes (VE) were then computed using an orientation optimized for post-stimulus gamma-band activity (i.e. orientation calculated by using principal component analysis on the 0–270ms post-stimulus window with a band-pass filter of 30–58Hz so as to avoid line artifact contamination). Broadband resultant VE time courses were then time-frequency transformed (Morlet Wavelets; 3–100Hz; cycles ranging from 3 (low) to 6 (high); both with 1Hz bins). For each participant, baseline corrected evoked power (relative change) and inter-trial coherence (ITC) were calculated. Fieldtrip's permutation testing was then utilized to identify a spectrotemporal ROI of increased (over baseline) Gamma activity. Monte-Carlo permutation testing, with cluster correction for multiple comparisons, was used to compare the post-stimulus window (0 to 200ms) to an equal pre-stimulus window (–300 to –100ms) across all participants. Statistically significant (corrected $P < 0.05$) spectrotemporal gamma (>30Hz) ROIs (Figure 1) were used to mask individual subject's time-frequency plots, for which average evoked-power and ITC were evaluated on a subject-wise basis.

Statistical Analyses

ANOVAs were utilized to assess group differences for the metrics of interest. For all analyses Age was a covariate if permitted by the corresponding lack of significant group differences in age. An ANOVA with fixed effects of Diagnosis, additionally covaried for Age (when permitted) as well as Gray Matter fraction was used in the GABA+/Cr analysis. For gamma-band measures (evoked power and ITC), ANOVAs with a fixed effect of Diagnosis and covaried for Age (when appropriate) were applied.

As the adult study participants differed significantly with respect to age at recruitment for the TD to ASD comparison (TD=27.2±1.3yrs, ASD=21.8±1.2yrs, $p < 0.05$) additional analyses decimated the adult study populations to age-match the diagnostic group. This resulted in a smaller sample of adults who did not significantly differ with respect to age (TD=22.0±0.4yrs, ASD=21.2±0.4yrs, $p > 0.05$). Subsequently, the ANOVAs were rerun with Age as a covariate to ensure equivalent statistical modeling of both sub-populations.

To further determine if gray matter contributed significantly to GABA+/Cr group differences, a hierarchical linear regression was utilized, with Diagnosis and Age entered into the first block, and then Gray Matter fraction in the second. Additionally robust regressions (MatLab function: *robustfit*) were performed between age and GABA+/Cr as well as gamma-band activity metrics. Furthermore, the direct association of GABA+/Cr and gamma-band activity metrics was tested with similar robust regressions. Such regressions were performed for both diagnoses separately, and after collapsing across diagnosis. Additionally, after the TD child/adolescent specific association of GABA+/Cr and gamma-band ITC was observed, further (more stringent) correlations were performed using the *robustcorrtool* toolbox (Pernet, Wilcox, & Rousselet, 2013) to ensure that this observation

was not due to potential outliers not sufficiently dealt with by a robust regression. A skipped Pearson's correlation (Wilcox, 2004) (using outlier detection based on the box-plot rule) and subsequent bootstrapping of correlation coefficients was implemented using *robustcorrtool*.

Results

Demographics

Seventy-four participants were recruited into the study and completed both MEG and MRS experiments. Of these, 17 participants (~20%) were removed due to poor MEG/MRS scan quality (5 TD, 12 ASD). As such, fifty-seven participants remained in the final data analyses (27 TD, 30 ASD), of whom 6 were females (5 TD, 1 ASD). Groups did not differ on gender (Chi-squared: $p>0.05$). Considering children/adolescents and adults pooled together, groups differed significantly in age (TD=21.2±1.8yrs, ASD=16.4±1.1yrs, $p<0.05$), with the TD cohort being older on average. To examine the effect of age within the diagnostic groups, both TD and ASD cohorts were split into children/adolescents (<18yrs old) and adults (≥18yrs old). The child/adolescent-aged participants (10 TD children/adolescents, 16 ASD children/adolescents) did not significantly differ on age (TD=10.9±0.5yrs, ASD=11.6±0.5yrs, $p>0.05$). The adult participants (17 TD adults, 14 ASD adults) did significantly differ with respect to age (TD=27.2±1.3yrs, ASD=21.8±1.2yrs, $p<0.05$). To negate potential confounds arising from the difference in age, the adult study population was *a-posteriori* decimated (reducing TD, as well as increasing ASD, average age) to age match samples at the expense of sample size. This results in 5 TD and 6 ASD adults who did not significantly differ with respect to age (TD=22.0±0.4yrs, ASD=21.2±0.4yrs, $p>0.05$).

ASD Participants Demonstrate Reduced Gamma-Band Coherence (ITC)

Gamma-band responses were visible at the diagnostic group level for both TD and ASD cohorts (Figure 1A & 1B). A main effect of Diagnosis, $F(1,55)=4.94$, $p<0.05$, demonstrated gamma-band coherence (ITC) was significantly decreased in participants with ASD compared to TD (TD=0.067±0.003ITC, ASD=0.058±0.003ITC, Figure 2B). A similar, though non-significant, main effect of Diagnosis ($F(1,55)=1.65$, $p>0.10$) suggested reduced gamma-band evoked *power* in ASD versus TD (TD=2.32±0.41 relative change from baseline, ASD=1.60±0.38 relative change from baseline, Figure 2C). Thus, gamma-band ITC was observed to be more sensitive to group differences than gamma-band evoked power, largely attributable to lower measurement variance.

ASD Participants Demonstrate Decreased GABA+/Cr

A significant main effect of Diagnosis was observed, $F(1,54)=7.83$, $p<0.01$, demonstrating that the ASD group exhibited less relative cortical GABA than the TD group (TD=0.31±0.01GABA+/Cr, ASD=0.28±0.01GABA+/Cr, Figure 2A). Notably, a significant main effect of voxel Gray Matter Fraction was *not* observed, ($F(1,44)=1.02$, $p>0.1$) between groups, and furthermore Gray Matter Fraction did not account for significant variance above that accounted for by Age and Diagnosis (R^2 change=0.027, $p>0.1$).

The Typical Relation of Relative Cortical GABA to Gamma-Band ITC is not Apparent in ASD

To address the potential influence of outliers on subsequent regression analyses, robust regressions were utilized. Across the whole study population, gamma-band ITC was significantly correlated with relative cortical GABA+ (intercept $\beta=0.025$, slope $\beta=0.126$, $t(55)=2.73$, $p<0.01$; Figure 3C). This relationship was marginally significant in the TD group (intercept $\beta=0.025$, slope $\beta=0.133$, $t(25)=1.92$, $p<0.1$; Figure 3C), and not significant for the ASD cohort (intercept $\beta=0.036$, slope $\beta=0.078$, $t(28)=1.03$, $p>0.10$; Figure 3C). No relationship between GABA+/Cr and evoked power were observed either across the whole study population, or within diagnostic groups ($p>0.1$). To ensure that the association between STG GABA+/Cr and auditory gamma-band coherence extended to the whole age-range of TD subjects, further analyses investigated the effect of age on this relationship.

Analysis of Child/Adolescent Participants

The main effect of Diagnosis was not significant ($p>0.1$) for either gamma-band activity measures (evoked power and ITC), though both were qualitatively decreased in ASD versus TD (Coherence: TD= 0.066 ± 0.006 ITC, ASD Coherence= 0.058 ± 0.004 ITC, $F(1,23)=1.35$, $p>0.1$; Evoked Power: TD= 2.32 ± 0.88 relative change from baseline, ASD= 1.82 ± 0.69 relative change from baseline, $F(1,23)=0.20$ $p>0.1$; Figures 2B,2C). GABA+/Cr concentrations demonstrated a significant main effect of Diagnosis, $F(1,22)=8.89$, $p<0.01$, with ASD exhibiting lower concentrations than TD (TD= 0.33 ± 0.01 , ASD= 0.28 ± 0.01 , Figure 2A). TD children/adolescents exhibited significant/near-significant maturational increases in GABA+/Cr (intercept $\beta=0.0398$, slope $\beta=0.0261$, $t(8)=2.83$, $p<0.05$) and gamma-band coherence (intercept $\beta=-0.0282$, slope $\beta=0.0084$, $t(8)=2.23$, $p<0.1$) respectively. Children/adolescents with ASD demonstrated no such maturation of GABA+/Cr or gamma-band coherence ($p>0.1$).

Robust regressions examined the relationship between GABA+/Cr and gamma-band activity metrics for each diagnostic cohort in the child/adolescent-aged study population. GABA+/Cr was significantly positively associated with gamma-band ITC in TD (intercept $\beta=-0.0298$, slope $\beta=0.294$, $t(8)=3.42$, $p<0.01$; Figure 3A). The *robustcorrtol* toolbox (Pernet et al., 2013) was subsequently used to ensure the validity of such a relationship, since such an observation may be due to potential outliers not sufficiently dealt with by a robust regression. A skipped Pearson's correlation and subsequent bootstrapping of regression beta coefficients determined the relationship of GABA+/Cr to gamma-band ITC in TD children/adolescents to be significant ($r=0.900$, $t(7)=5.86$, confidence intervals= $0.066-0.976$; Figure 4). No association (using either robust regression or skipped Pearson's correlation) of GABA+/Cr to ITC was evident for the ASD children/adolescents. Additionally, the relationship of gamma-band evoked power and GABA+/Cr was non-significant in for children/adolescents regardless of diagnosis.

Analysis of Adults Participants

A main effect of Diagnosis was observed for gamma-band ITC, $F(1,29)=5.25$, $p<0.05$, with ASD demonstrating less coherence than TD (TD= 0.069 ± 0.004 ITC, ASD= 0.057 ± 0.004 ITC;

Figure 2B). No such main effect was apparent for evoked power ($TD=2.28\pm0.37$ relative change from baseline, $ASD=1.41\pm0.41$ relative change from baseline, $F(1,29)=2.49$ $p>0.1$; Figure 2C). Additionally, a marginally significant main effect of diagnosis was observed for GABA+/Cr, $F(1,29)=3.45$, $p<0.10$, with ASD demonstrating decreased GABA+/Cr ($TD=0.30\pm0.01$ GABA+/Cr, $ASD=0.27\pm0.01$ GABA+/Cr; Figure 2A). Unlike the TD children/adolescents in this study, adults (either TD or ASD) did not demonstrate age-dependence of gamma-band coherence or GABA+/Cr. Additionally, neither TD nor ASD adults exhibited a significant relationship between either gamma-band activity metric (evoked power or ITC) and GABA+/Cr concentrations ($p>0.1$; Figure 3B).

To control for potential confounds arising from the significant age difference between TD and ASD, the adult participants were decimated to ensure age-matched samples. Such decimation resulted in 5 TD and 6 ASD individuals, which was considered too small and statistically underpowered to find robust significance effects. Instead, the data is presented purely to demonstrate qualitatively similar findings as the complete adult population findings. A near significant main effect of Diagnosis was determined for gamma-band ITC, $F(1,8)=3.79$, $p<0.10$, with again ASD demonstrating reduced coherence ($TD = 0.067\pm0.005$ ITC, $ASD = 0.053\pm0.005$ ITC). Though again qualitatively decreased, gamma-band evoked power did not demonstrate a significant main effect of Diagnosis ($p>0.10$) (Evoked Power: $TD=2.11\pm0.54$ relative change from baseline, $ASD=1.11\pm0.49$ relative change from baseline). No main effect of Diagnosis was observed for GABA+/Cr concentrations ($TD=0.29\pm0.02$ GABA+/Cr, $ASD=0.29\pm0.02$ GABA+/Cr; $p>.10$). Furthermore, no maturational effects on either GABA+/Cr nor gamma-band coherence were observed for either TD or ASD individuals ($p>0.1$). Additionally, robust relationships between GABA+/Cr and gamma-band activity metrics were not significant for either decimated TD or ASD adult cohorts ($p>0.1$).

Discussion

The brain is known to communicate using excitatory and inhibitory neurotransmission, and numerous studies have suggested that the brain's gamma-band response is critically reliant on such E/I balance (Cardin et al., 2009; Sohal et al., 2009; Whittington et al., 2000; Yizhar et al., 2011). Gamma-band activity is thought to be involved with numerous brain functions ranging from basic sensory (e.g. visual, auditory) (Balar-Eroglu et al., 1996; Gray et al., 1989) functions to higher order cognitive processes (Herrmann et al., 2010). Of relevance to this study, the gamma-band response has been repeatedly observed to be perturbed in ASD (Grice et al., 2001; Orekhova et al., 2007; Wilson et al., 2007). Several independent studies have demonstrated decreased cortical GABA in ASD (Gaetz et al., 2014; Harada et al., 2011; Rojas et al., 2014), thus supporting the notion that gamma-band activity is reliant on E/I balance. Moreover, gamma-band neurophysiological activity has been shown to correlate to corresponding GABA concentrations in both the visual and motor system of healthy adults (Gaetz et al., 2011; Muthukumaraswamy et al., 2009), though this relationship is not fully established (Cousijn et al., 2014). In addition, Balz and colleagues (2015) recently observed a correlation between gamma-band power and GABA levels for visual-audio sensory integration in the human brain. However, the analogous correlation had yet to be

observed for the auditory system for either typically developing (TD) children/adolescents or individuals with ASD.

This study examined both the gamma-band response to simple auditory tones as well as the corresponding STG GABA+/Cr levels in individuals with ASD and TD controls. On the whole group level, the ASD cohort demonstrated both reduced relative GABA+ concentration and gamma-band ITC deficits compared to TD. While the finding that relative cortical GABA is significantly reduced in ASD as compared to TD is supported by previous findings (Gaetz et al., 2014; Rojas et al., 2014), the relative consequence of such a specific GABA deficiency has not been addressed. Additionally, the findings of reduced gamma-band ITC are also supported by previous studies (Edgar et al., 2015; Rojas et al., 2008). The direct relation of these metrics has not been examined though until now. Across diagnoses, a significant positive relationship was observed between the concentrations of relative cortical GABA and gamma-band coherence. Further detailed examination revealed, that this association was suggested in the TD, but not ASD cohort.

For a more comprehensive analysis, the two cohorts were separately split into children/adolescents and adults. Findings demonstrated significant reductions in GABA+/Cr, along with qualitatively reductions for both gamma-band metrics in children/adolescents with ASD versus their TD age-matched counterparts. Additionally, GABA+/Cr was significantly positively associated to gamma-band ITC in TD children/adolescents, but not children/adolescents with ASD.

In adults, gamma-band coherence was significantly reduced in the ASD cohort, which continued to be suggested after *a-posteriori* age-matching decimation. Of note, in either set of analyses (all adults or age-matched adults), the GABA+/Cr to gamma-band ITC association was non-significant.

These results suggest that with typical childhood/adolescence development, a “healthy” concentration of GABA allows for the proper maturation of local circuit function that ultimately results in neurotypical gamma-band coherence in adulthood. We speculate that this typical maturation of local circuit function is indexed by the observed GABA / gamma-band coherence coupling in TD children/adolescents. In adulthood, gamma-band coherence levels plateau, more or less independent of GABA levels. Individuals with ASD, however, demonstrate reduced relative cortical GABA concentrations as well as an altered maturational trajectory for GABA levels in childhood/adolescence. Reduced development of local circuit activity in ASD may manifest as a lack of coupling between relative cortical GABA and gamma-band coherence in childhood/adolescence, and additionally in adulthood as a continued relative reduction in gamma-band coherence. The hypothesis of E/I imbalance during *critical periods* leading to improper circuit development in ASD is supported by both clinical and preclinical literature (LeBlanc & Fagiolini, 2011).

In this study, auditory gamma-band *evoked power* deficits in ASD failed to reach significance. While the differential significance (ITC significant, evoked power not significant) for the group differences in phase-locked gamma-band activity metrics is contrary to previous studies (Edgar et al., 2015; Rojas et al., 2008), non-significant trends for

reduced evoked power in ASD have also been reported previously (Gandal et al., 2010). The lack of significance of the decrease in evoked power may be due to the use of a larger age range in the current study or other undetermined population effects. Additionally, previous studies have averaged over hemispheres to increase signal to noise (Gandal et al., 2010; Port et al., 2016), whereas the current study only reported the left hemisphere responses to compare directly with *in vivo* left STG GABA estimates. Moreover, it may simply be that *gamma power* is less sensitive than *gamma coherence* for subtle disturbances in neural circuitry architecture and function.

A limitation of the current study is the relatively low sample sizes. By dividing the study population by age (child/adolescent versus adult) as well as by diagnosis (TD versus ASD), groups were specific yet possibly underpowered/not generalizable (sample sizes were 10 TD children/adolescents, 16 ASD children/adolescents, 17 TD adults and 14 ASD adults). Further studies are needed to see if the current findings replicate in larger samples.

Another limitation of the current study, and of other previous MRS ASD studies is, the relative inaccessibility of a simultaneous measure of the excitatory neurotransmitter glutamate. This arises partly from technical resolution challenges (often leading to a proxy measure of Glx, comprising both glutamate and glutamine (Rojas et al., 2015)) but more importantly from the existence of glutamate in the brain in both a neurotransmission and metabolic role. Glutamate is involved in both synaptic transmission and metabolic processing, shuttling between neurons and astrocytes as part of the Glutamate–Glutamine cycle (for review see Hertz 2013). Of note though, a recent study demonstrated increased glutamate concentrations in ASD (Brown et al., 2013), also consistent with increased E/I ratio. GABA, with its function as the main inhibitory neurotransmitter, also has a role in the TCA cycle. The exact role of GABA is hypothesized to be dependent on the isoform of glutamate decarboxylase (GAD) that produced the molecule (GAD65 is associated with neurotransmission; GAD67 is associated with the GABA shunt) (Martin & Rimvall, 1993). This clear separation of GAD65/67 derived GABA in neurotransmission has been called into question though (Soghomonian & Martin, 1998). Nevertheless, current MRS methods are not able to resolve the metabolite versus neurotransmitter pools of either glutamate or GABA, because the voxel size required to achieve adequate signal in reasonable time encompasses all neuronal compartments (e.g. synaptic, white matter & somatic compartments) as well as glial cells. This concern should temper interpretations. Nonetheless establishing any association between GABA and gamma-band activity would add support for the hypothesis that gamma-band activity may serve as a functional index of E/I balance.

The present data support the hypothesis that decreases in relative cortical GABA+ during development are of functional consequence. Previous studies have shown a significant relationship of relative cortical GABA to gamma-band activity in adult TD controls (Balz et al., 2015; Edden et al., 2009; Gaetz et al., 2011; Muthukumaraswamy et al., 2009). Though the previous reports of a significant correlation in TD *adults* is contrary to the current findings, these studies 1) utilized *peak frequency* and not ITC as their gamma-band metrics and 2) investigated a gamma-band response largely arising from *non-phase-locked* activity. There is controversy about such findings in the field (Cousijn et al., 2014), though

differences may be related to hardware or analytic methodological differences. Further replication of such findings and continued *in vivo* methods development will resolve this issue. Our results support the hypothesis that relative cortical GABA concentrations derived from MRS may relate to the E/I balance in healthy participants.

Importantly, and in contradistinction to much of the previous literature, the results from this study suggest a *critical developmental window* in which disruptions in GABAergic signaling may predict local circuit perturbations in later in life. While speculative, it may suggest that only those amenable to recovery of GABAergic signaling during a critical childhood/adolescence period may find GABA-based interventions effective. For example, if the GABAergic system in a child/adolescent with ASD is less effective at local circuit function, an increase in GABAergic tone may recover proper circuit maturation. This is not to imply that ASD arises from solely improper GABAergic modulation of local circuit function, but to the extent that it does, a critical window for modulation may exist.

The clinical trials of arbaclofen (Berry-Kravis et al., 2012), and other GABA-related treatments have not proven effective in a broad study population. What remains unknown is the neuro-electrophysiological characteristics (i.e. gamma-band activity response) of both responders and non-responders in these clinical trials. While highly speculative, it may be the case that for those who improve clinically in response to GABAergic modulation, gamma-band activity may also be normalized. Such an observation would allow gamma-band activity to be used a treatment marker, reflecting at least drug activity (i.e. target engagement) for an enriched study population. Additionally, a stratification marker for potential GABAergic treatment efficacy could be operationalized by an individual's ratio of ITC to GABA+/Cr as a metric of "functional to neurochemical coupling". Those whom exhibit a "functional to neurochemical coupling" within a certain range (i.e. 95% confidence interval, Figure 3) of typically developing values may be able to be recovered with GABAergic treatments as the neurophysiological mechanism linking GABA to local circuit maturation may still be intact.

It still remains to be established if diminished gamma-band activity or even altered GABA concentration is causal for the *behavioral* alterations in ASD, or a closely linked proxy for relevant neural bases. Indeed, though we have interpreted GABA's role as a key for proper local circuit development, this is not to suggest a "master" or "up-stream" pathogenic mechanism might not control the proper development of both GABA and local circuitry though on different timescales. In such a case, local circuit function may not be reliant on GABAergic tone, but rather an independent albeit slower process that convergences up-stream of GABA development. Further studies are needed to confirm both the use of these measures as biomarkers appropriate for stratification for treatment, and their causal relationship to behavioral symptomatology.

The current study focused solely on the auditory system, however previous findings have demonstrated relative cortical GABA to be differentially altered depending on which cortical/subcortical structure was examined (Gaetz et al., 2014; Harada et al., 2011). What remains undetermined is if the developmental relationship between underlying

neurochemistry and oscillatory activity remains and is of the same magnitude in other brain systems.

To conclude, this study observed correlations between MEG measures of gamma-band oscillatory activity and regionally co-localized estimates of GABA concentrations within the auditory system in TD children/adolescents, but not adults. However, no coupling was observed for individuals with ASD, neither child/adolescent nor adult. Further work is needed to test the hypothesis that a “critical period dependent coupling of GABAergic and electrophysiological functioning” is of relevance to the treatment of individuals with ASD.

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Figure 1. Both TD and ASD individuals demonstrated robust and quantifiable results Group average evoked power (A) and ITC (B) in response to auditory stimuli demonstrate quantifiable results for both TD (left) and ASD (right). Individuals with ASD exhibit less phase-locked gamma-band coherence, but not evoked power, in response to the auditory stimuli. Outline is the permutation test derived region of significant post-stimulus gamma-band activity (C) Exemplar MRS spectra for TD (left) and ASD (right) show clear and defined GABA+ peaks (gray bar overlay).

Figure 2. Individuals with ASD exhibit less gamma-band coherence and relative cortical GABA in auditory cortex

(A) Relative cortical GABA+ in auditory cortex is reduced in ASD (red) as compared to TD (blue) for both all ages pooled together (left) and the child/adolescent-aged participants (middle). A trend toward decreased GABA+/Cr in ASD versus TD in the adult-aged study population is also observed (right). (B) A significant decrease in gamma-band coherence in response to auditory stimuli is observed for ASD compared to TD for both all ages pooled together and also the adult study population. (C) A similar, though only qualitative decrease is observed for gamma-band evoked power in ASD as compared to TD. # $p < 0.10$ * $p < 0.05$ ** $p < 0.01$

Figure 3. Auditory gamma-band coherence and relative cortical GABA in auditory cortex are associated across the whole study population, and specifically for TD children/adolescents (A) TD (blue), but not ASD (red), children/adolescents exhibit a significant positive association of GABA+/Cr to gamma-band ITC. (B) Neither TD, nor ASD, adults exhibit an association of GABA+/Cr to gamma-band coherence. Straight blue line shows robust regressions for TD sub-populations. Curved blue lines are the 95% confidence intervals. Note that during childhood/adolescence (A) an approximately equal number of ASD children/adolescents lie within, and outside the 95% confident interval for TD, suggest possible sub-groups within the ASD population. (C) The whole study population exhibits a positive association of GABA+/Cr to gamma-band coherence (black line). This is also suggested for TD (blue line), but not ASD (red line) cohorts.

Figure 4. The association of GABA+/Cr to gamma-band coherence observed in TD children/adolescents observed via robust regressions survives more stringent statistical analyses
Using the *robustcorrtool* toolbox (Pernet et al., 2013), the observed association of GABA+/Cr to gamma-band coherence in TD children/adolescents was investigated. Left, the Pearson's correlation of GABA+/Cr to gamma-band coherence after rejection of outliers using the box plot rule (purple – non outlier data, red – outlier data, red line – linear regression, red shade – confidence intervals). Right, results of the bootstrapping of the data shown on the left (red lines – confidence intervals. Pearson's corr h reference to whether the null hypothesis should be rejected (1 = reject, 0 = keep).

Table 1

Medications and supplements taken by participants. Subjects listed by Diagnosis (Dx).

DX #	Medications
ASD 1	Abilify
ASD 2	Buproion HCL, Concerta ER, Clonidine HCL
ASD 3	Concerta, Metadate, Focalin
ASD 4	Concerta, Doxycycline hyclate
ASD 5	Flovent, Albuterol
ASD 6	Focalin
ASD 7	Focalin, Prozac, Melatonin
ASD 8	Lamictal, Xanax, Zyprexa
ASD 9	Metadate, Abilify
ASD 10	None
ASD 11	None
ASD 12	None
ASD 13	None
ASD 14	None
ASD 15	None
ASD 16	None
ASD 17	None
ASD 18	None
ASD 19	None
ASD 20	None
ASD 21	None
ASD 22	None
ASD 23	Strattera, Fluoxetine
ASD 24	Tenex, Focalin, Strattera, Abilify
ASD 25	Zoloft
ASD 26	None
ASD 27	Intuniv
ASD 28	Concerta, Wellbutrin
ASD 29	None
ASD 30	Zoloft
TD 1	None
TD 2	None
TD 3	None
TD 4	None
TD 5	None
TD 6	None
TD 7	None

DX #	Medications
TD 8	None
TD 9	None
TD 10	None
TD 11	None
TD 12	None
TD 13	None
TD 14	None
TD 15	None
TD 16	None
TD 17	Omeprazole, Ketoconazole (topical cream)
TD 18	None
TD 19	None
TD 20	None
TD 21	Zyrtec, Flovent, Albuteral
TD 22	None
TD 23	None
TD 24	None
TD 25	None
TD 26	None
TD 27	None