博士論文

論文題目 **Investigation of neurochemical basis of autism spectrum disorder by analyzing magnetic resonance spectroscopy and functional magnetic resonance imaging data.**

（**MR** スペクトロスコピーと **fMRI** データの解析による自閉症スペクトラム障害の神経化学的基盤の検討）

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0. Abstract

Autism spectrum disorder (ASD) is a developmental disorder characterized by impairment of social communication and social interaction without established pharmacological treatment. Oxytocin, a neuropeptide, is known to influence social behavior among individuals with typically developed (TD) and ASD. ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) is a non-invasive technique to measure metabolite levels in brain *in vivo*. N-acetylaspartate (NAA) is a metabolite that represents neuronal density or activity. This doctoral thesis consists of three studies; meta-analysis, case control study and clinical trial. In meta-analysis of ^{1}H-MRS studies of ASD, I have demonstrated that individuals with ASD show atypical developmental trajectory of NAA in frontal lobe. Then, in case control study, I and co-researchers have recruited 24 men with ASD and 25 men with TD and measured NAA level in the ventromedial prefrontal cortex/anterior cingulate cortex (vmPFC/ACC) to demonstrate that individuals with ASD show atypical aging effect on NAA level. In a double-blind, cross-over, randomized controlled trial where a single dose of oxytocin was administered intranasally to 40 high-functioning men with ASD, I and co-researchers have obtained both ^{1}H-MRS data and functional magnetic resonance imaging (fMRI) data during a psychological task involving autistic behavior. Taken together, oxytocin's influence on NAA level in vmPFC/ACC is related to oxytocin-induced fMRI signal change that is eventually related to oxytocin-induced mitigation of autistic behavior. (216 words)

1. Background

1.1. Symptoms of autism spectrum disorder (ASD)

Autism is a developmental disorder firstly recognized by Kanner (1943)[1] and Asperger (1944).[2] Afterward, Wing and Gould (1979)[3] had defined the triad of impairments: impairments of social communication, social relationships, and imagination. This triad had underlain the international diagnostic criteria, such as International Classification of Diseases (ICD)[4] and Diagnostic and Statistical Manual of Mental Disorders (DSM).[5] For example, the DSM-IV required one or more of the following impairments, such as "social interaction", "communication", and "restricted, repetitive, and stereotyped patterns of behavior, interests, and activities."[5] In the latest international criteria, DSM-5 released in 2013, ASD is characterized by and diagnosed with impairment of social communication and interaction, and repetitive and restricted behavior.[6]

1.2. Diagnosis of ASD

There are some diagnostic tools for ASD, such as the Autism Diagnostic Interview-Revised (ADI-R)[7] and the Autism Diagnostic Observation Schedule (ADOS).[8] Both the ADI-R and ADOS, developed by the Western Psychological Services, are well-validated instruments to make an accurate diagnosis of ASD. The ADI-R is a structured interview, with 93 questions, applied to parents of individuals with probable ASD. The ADI-R has three sub-components, social interaction, communication and language, and restricted and repetitive behaviors. The diagnosis of autism was made when scores in all three behavioral areas exceed the cutoff scores (Cut-off scores for these sub-categories are ten, eight and three, respectively). On the other hand, the ADOS has a set of structured and semi-structured tasks that provide opportunities of social interaction between the examiner and person with probable ASD using books, toys, and dolls.

1.3. Prevalence of ASD

Recently, the prevalence of ASD has consistently increased.[9-11] The increase of prevalence is not supposed to be due to biological reason but increase of recognition of the disorder or change of diagnostic criteria.[12-14] Recent studies have reported that about 1 to 2% of the general population matches the diagnostic criteria of ASD.[15, 16]

1.4. Neural basis of ASD

1.4.1 Atypical developmental trajectory of ASD

According to increase of recognition of ASD and its impact on society, a number of researches have been made to investigate etiology and pathophysiology of ASD. One of the most interest findings about pathophysiology of ASD is atypical developmental trajectory. Concretely, a number of meta-analyses of studies on brain volume or head circumstances have reported that brain volume of individuals with ASD is slightly lower-than-typical at birth, dramatically increases within the first year of life and exceeds typical development (TD), but then gradually plateaus into adulthood.[17, 18] In line with findings from these meta-analyses of studies on brain volume that reported dynamic atypical trajectory during babyhood, recent longitudinal studies have demonstrated atypical developmental curves in brain structure and metabolism in individuals with ASD during this period.[19-21] The atypical brain growth has been demonstrated to occur in various brain areas, but in particular in the frontal lobe, suggesting that the frontal lobe is one of the brain regions associated with pathophysiology of ASD.[17, 22, 23] Further, a number of neuroimaging studies have demonstrated that the prefrontal cortex is critically important in empathy,[24] theory of mind,[25] irony comprehension,[26] social judgment[27] and self referencing.[28, 29] As impairments of these cognitive functions constitute a core feature of ASD, these results suggest that the prefrontal cortex is one potential brain regions that may associate with pathophysiology of ASD.

Beyond a number of studies with children that have demonstrated dynamic change

of brain in infants of ASD, cumulative neuroimaging studies have shown that such atypical developmental trajectory continues even after babyhood.[30] They have demonstrated that the frontal lobe[31-33] and tract involving the frontal lobe,[34] showed atypical aging trajectory in individuals with ASD even during adulthood.

1.5. ^{1}H-MRS

^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) is a non-invasive neuroimaging method that measures specific chemical metabolite levels *in vivo*.[35] Previous studies have utilized ^{1}H-MRS to mainly measure N-acetylaspartate (NAA), a marker of neuronal density, plasticity and regional energy demand; creatine and phosphocreatine (Cre); choline-containing compounds (Cho), a measure primarily reflecting the constituents of cell membranes, a measure of cellular energy metabolism; myo-inositol (mI), a major osmolite precursor for phosphoinositides involved in the second messenger system; glutamine/glutamate (referred as 'Glx', collectively).[36, 37]

1.6. Existing treatments for ASD

Now, individuals, particularly children, with ASD are treated by several kinds of behavioral therapies. For example, the Applied Behavior Analysis (ABA) is one of behavioral therapies applied to individuals with ASD by parents. In the ABA, individuals with ASD are explained how behavior functions and how learning comes about, and rewarded when individuals with ASD have behaved favorable behavior. Using the ABA, parents help individuals with ASD to reduce the autistic behavior and increase social and language skills. The behavioral therapies, including the ABA, have shown their effectiveness on ASD.[38, 39] In contrast, although various existing medications have been applied to psychological/psychiatric symptoms associated with ASD, there is no established pharmacological treatment for the core symptoms of ASD, impairment of social communication and social interaction.[40-42]

1.7. Oxytocin for ASD

A number of recent studies have provided the evidence that oxytocin has an influence on affiliative and social behaviors among individuals with TD,[43-49] although in some circumstances oxytocin may also facilitate aggression, particularly toward out-group people.[50] It is now supposed that the neuropeptide influences social behavior of individuals with ASD.[51-55] Actually, there are some studies that have demonstrated that oxytocin has an impact on autistic behavior,[56-60] and also its neural basis.[61-64] However, it has yet to be elucidated how oxytocin has an influence on neurochemical findings of ^{1}H-MRS.

In the present doctoral thesis, I would like to present a series of studies of ^{1}H-MRS studies with ASD, including a meta-analysis, case control study, and a clinical trial. In the series of studies, firstly, I and co-researchers investigate existing studies of ^{1}H-MRS studies with ASD in order to clarify what is the reason why existing studies have reported inconsistent results. Then, a case control study was conducted to elucidate neurochemical basis of ASD during adulthood which remained unclear in meta-analysis. Finally, I present evidence from a clinical trial where oxytocin was administered to individuals with ASD, obtaining ^{1}H-MRS and functional MRI (fMRI).

2. Meta-analysis

2.1. Introduction for meta-analysis

Atypical aging trajectory at the neural level may constitute underpins of life-long impairment in behavior in individuals with ASD.[65] However, such atypical aging trajectory during adulthood has rarely been investigated on neurochemical aspects of brain.

Based on results from brain structural studies that showed an atypical developmental trajectory, I hypothesized that the degree of abnormalities of neurochemical measured by ^{1}H-MRS may also change according to developmental stages in individuals with ASD. Although one longitudinal ^{1}H-MRS study that focused on lactate level was published in 2012.[66]

Implementing a meta-analysis was the only way to examine age-related change of abnormality measured with ^{1}H-MRS in individuals with ASD when I focused on this theme. In addition, a number of previous studies focused on various brain regions, but they yielded inconsistent results. Thus, performing a meta-analysis might also demonstrate a brain region where individuals with ASD show atypical neurochemical.

Despite such possibility and necessity of conducting a meta-analysis, neither a systematic review nor a meta-analysis of ^{1}H-MRS studies in individuals with ASD has been published previously. The present systematic review and meta-analysis were designed to test the hypothesis that the degree of abnormalities in metabolite levels measured with ^{1}H-MRS would change from childhood to adulthood and also to identify the brain region where individuals with ASD show the greatest abnormality of neurochemical levels.

2.2. Methods

2.2.1. Data sources

To identify studies eligible to a meta-analysis, I and co-researchers conducted systematic screening in the following way.[67] ^{1}H-MRS studies that investigated metabolite levels in the

brains of individuals with ASD and TD were identified through the electrical databases, such as MEDLINE, PsycINFO, EMBASE and Web of Science. The syntax adopted in the systematic screening were "*autism", "autistic", "ASD", "Asperger's", "developmental disorder", "PDD"* and "*mental development"*, which were integrated with "*magnetic resonance spectroscopy"* and "*MRS"*. Titles and abstracts of studies were skimmed to check whether they should be included or not. Reference lists of included studies were also investigated to look for additional studies that should be included.

2.2.2. Selection of study

Then, I and co-researchers have identified the studies to the meta-analysis with the following inclusion criteria.[67] Studies were included if (1) they were brain ^{1}H-MRS studies published by Dec 2010 from inception, (2) they investigated individuals with ASD and compared them with TD, (3) they reported sufficient data to calculate effect sizes, such as means, standard deviations and numbers of participants. There was no language restriction. If they did not report sufficient data to calculate effect size, I contacted the corresponding author and then the last author to obtain them. In cases where neither of them responded, I and co-researchers discarded the study. Two researchers (Yuta Aoki and Hidenori Yamasue) independently conducted a systematic screenings.

2.2.3. Data extraction

In order to conduct the meta-analyses, I defined a standardized mean difference (SMD) as the effect size.[67] The SMD is calculated as the difference between the mean of the experiment group and the mean of the comparison group divided by the pooled standard deviation. In the present meta-analyses, mean levels of NAA, Cre, Cho, mI and Glx in individuals with ASD was subtracted from those in TD in each VOI respectively, and divided by the pooled standard deviation of both groups. Because I assumed effect modification by age on the degrees of differences in metabolite levels in individuals with ASD compared to TD, the

SMDs were separated by the mean age of participants. When the mean age of participants was greater than 20, the study was assigned into the meta-analysis in studies with adulthood.[37, 68-74] In case where a study reporting that age of participants ranged from 3 to 5 years without description of the mean age of participants, the participants were recognized to have a mean age of 4 years.[74] In cases of studies reporting more than two types of levels of metabolites, I and co-researchers determined the priority for extraction as absolute measure then ratio to Cre. Two researchers (Yuta Aoki and Hidenori Yamasue) independently implemented all the data extraction and calculation of effect size to minimize errors. In this study, the PRISMA guidelines was followed.[75]

2.2.4. Identification of brain regions

The scope of my hypothesis is about the developmental trajectory of pathophysiology in the brain in ASD. Thus, I classified the sub-regions into frontal, amygdala-hippocampus region, temporal, parietal, cerebellum and thalamus, based on the similarity of developmental characteristics within each sub-region.[76] In the case of a study reporting levels from two or more areas from one sub-region (e.g., anterior cingulate cortex and dorsolateral prefrontal cortex), they were assigned into the appropriate meta-analysis sub-group (i.e., frontal lobe) as two (or more) independent datasets without any relation to tissue type, such as gray matter, white matter or both. VOIs in the medial temporal lobe that included the hippocampus or amygdala region were included into amygdala-hippocampus region sub-group.[36, 74, 77] VOIs in the intraparietal sulcus (IPS)[71] and temporoparietal junction (TPJ)[71] were categorized as the parietal lobe, while that in the insula[78] was labeled as the temporal lobe. In order to ensure the meta-analysis was sufficiently powered, meta-analysis was implemented in brain regions where there were two or more studies reporting more than three VOIs in total. VOIs in individuals with TD compared with more than two ASD groups were identified[77, 79] and separated into the appropriate number of comparison sub-groups in order to avoid

duplicate-counting.

2.2.5. Meta-analysis

All meta-analyses were conducted using Review Manager 5.1 from the Cochrane Collaboration (http://tech.cochrane.org/Revman). A random effect model was utilized for the current meta-analysis in order to control potential heterogeneity, including implementation of tissue segmentation within VOIs, variation in location of VOI, single- vs. multi-voxel spectroscopy, volume of VOI, and types of metabolites measure. Firstly I have conducted meta-analysis of the whole included studies. Then, because I assumed effect modification in the degrees of differences in metabolite levels in individuals with ASD compared to TD by age, I and co-researchers compared the metabolite levels separately in childhood and adulthood. Conservative threshold was set for significance using Bonferroni corrections, with $P < 0.0022$ in childhood (= 0.05 / 23, number of included metabolites in six regions; Table 1) and $P < 0.0033$ in adulthood (= 0.05 / 15, number of included metabolites in four regions; Table 2)

Table 1. Meta-analyses of metabolites levels comparing children with ASD to TD

Regions	Metabolite	N of participants (ASD vs. TD)	SMD	*P*	I^2	Publication bias
Frontal	NAA	764 vs. 531	-0.35	<0.0001*	42%	0.226
	Cre	561 vs. 362	-0.24	0.01	44%	0.536
	Cho	599 vs. 378	-0.07	0.35	17%	0.851
	mI	314 vs. 138	-0.44	0.008	55%	NA
Amygdala-Hippocampus Region	NAA	245 vs. 115	-0.88	<0.0001*	61%	0.385
	Cre	129 vs. 46	-0.46	0.009	0%	NA
	Cho	245 vs. 115	-0.11	0.59	65%	0.128
	mI	128 vs. 52	0.53	0.22	78%	NA
Parietal	NAA	316 vs. 233	-0.39	0.0006*	31%	0.092
	Cre	178 vs. 106	-0.33	0.08	51%	NA
	Cho	206 vs. 131	-0.07	0.56	0%	NA
Temporal	NAA	252 vs. 186	-0.55	0.001*	62%	NA
	Cre	142 vs. 84	-0.09	0.62	33%	NA
	Cho	142 vs. 84	-0.17	0.55	72%	NA
	mI	142 vs. 84	-0.27	0.22	55%	NA
Cerebellum	NAA	151 vs. 109	-0.35	0.008	0%	NA
	Cre	79 vs. 68	-0.1	0.54	0%	NA
	Cho	151 vs. 109	0.04	0.33	11%	NA
	mI	65 vs. 66	0.29	1.67	80%	NA
Thalamus	NAA	170 vs. 98	-0.58	0.0002*	25%	NA
	Cre	170 vs. 98	-0.38	0.04	48%	NA

Cre	170 vs. 98	-0.38	0.04	48%	NA
Cho	170 vs. 98	-0.44	0.03	54%	NA
mI	126 vs. 58	-0.23	0.25	30%	NA

Abbreviations: ASD: autism spectrum disorder, TD: typically developed, N: number, SMD: standardized mean difference, NAA: N-acetylaspartate, Cre: creatine, Cho: choline containing compounds, mI: myo-Inositol, Glx: glutamate + glutamine, *Statistically significant after Bonferroni-correction

Table 2. Meta-analyses of metabolites levels comparing adults with ASD to TD

Regions	Metabolite	N of participants (ASD vs. TD)	SMD	*P*	I^2	Publication bias
Frontal	NAA	80 vs. 101	0.1	0.7	62%	NA
	Cre	52 vs. 59	0.24	0.23	5%	NA
	Cho	80 vs. 101	0.11	0.6	47%	NA
Amygdala-Hippocampus Region	NAA	62 vs. 56	0.19	0.32	0%	NA
	Cre	62 vs. 56	0.6	0.06	62%	NA
	Cho	62 vs. 56	0.43	0.22	68%	NA
	mI	50 vs. 44	0.39	0.07	0%	NA
Parietal	NAA	99 vs. 102	-0.37	0.01	0%	NA
	Cre	99 vs. 102	-0.15	0.29	0%	NA
	Cho	99 vs. 102	-0.17	0.23	0%	NA
	mI	73 vs. 75	-0.47	0.12	68%	NA
	Glx	73 vs. 75	-0.22	0.18	0%	NA
Cerebellum	NAA	38 vs. 38	-0.7	0.004	0%	NA
	Cre	38 vs. 38	-0.02	0.95	19%	NA
	Cho	38 vs. 38	-0.29	0.21	0%	NA

Abbreviations: ASD: autism spectrum disorder, TD: typically developed, N: number, SMD: standardized mean difference, NAA: N-acetylaspartate, Cre: creatine, Cho: choline containing compounds, mI: myo-Inositol, Glx: glutamate + glutamine, *

2.2.6. Sensitivity analyses

The replicability of significance of results of meta-analysis was further challenged by sensitivity analysis in specified sub-groups discarding studies with potential confounding factor. I and co-researchers recognized the following clinical or methodological factors as potential confounding factors, such as medication, diagnostic tools, comorbid epilepsy, presence of mental retardation, types of MRS measures, field strength of MR scanner, and segmentation within VOIs. The statistical significance level was set at $P < 0.0014$ (= 0.05 / 35 comparisons (corrected for multiple comparison, 7 potential confounds x 5 regions)).

2.2.7. Meta-regression

To examine the hypothesis that the degree of neurochemical abnormalities would change with age, I and co-researchers conducted meta-regression analyses in the combined children-adult group to investigate the relationship between participants' mean age and the SMD for the NAA levels in the frontal lobe, parietal lobe, and amygdala-hippocampus region where the meta-analysis showed significant differences between individuals with ASD and TD in childhood or adulthood. A meta-regression analysis was performed, in case where there was sufficient sample size ($N > 10$).[80] The regression was conducted using SPSS 18.0 (SPSS Inc., Chicago, Illinois). Applying the Bonferroni correction, the statistical significance was set at $P < 0.012$ (= 0.05 / 4 areas).

The studies included in the present meta-analysis have considerable between-study heterogeneities, including pharmacological status, comorbidity of mental retardation, comorbid epilepsy, types of MRS measures (e.g. absolute measure or ratio to Cre), implementation of segmentation within the VOI, and volume of VOI. In order to examine the potential influence of these confounding factors, I and co-researchers

conducted meta-regression analyses for the metabolite measures where the present meta-analyses revealed significant difference between individuals with ASD and TD. The meta-regressions were examined in the childhood and adulthood combined group in order to include a sufficient number of datasets.[80] Conservative threshold for statistical significance was set at $P < 0.05$ so as to strictly evaluate the effect of between-study heterogeneity.

As there is no statistical method to confirm whether a variable is confounding factor or not, it is not possible to conclude that mean age of participants is a unique covariate in metabolite levels. Thus, in order to assess statistical rationale of the hypothesized model, I examined all the possible models that predict effect sizes of NAA level difference in the frontal lobe between individuals with ASD and TD, including other potential covariates, such as TE, TR and size of VOI. I constructed 14 models (four models that contain one covariate, six models for two covariates (${}_4C_2 = 6$), four models for three covariates (${}_4C_3 = 4$) and one model for four covariates). The constructed models were evaluated with multiple measures, including the goodness-of-fit index (GFI), a measure of the overall model fit, the adjusted GFI (AGFI), which is the GFI adjusted for the degrees of freedom used to evaluate the overall model fit, and the Akaike information criteria (AIC). In the case where the GFI and AGFI values are > 0.90, the model is recognized as having good fit. The AIC is a measure that unable us to compare two or more models with a good fit; a smaller value suggests a good fit. I evaluated the fitness of residuals using the root mean square error of approximation (RMSEA). The RMSEA based on the non-centrality parameter. For the RMSEA, values below 0.08 suggest a good fit. Generally, when the model obtains more than two good scores, which includes the RMSEA, the model is recognized as

having a good fit.

2.2.8. Assessing between-study heterogeneity

The I^2 statistics was employed to test between-study heterogeneity. Statistical significance for between-study heterogeneity was set, at $P < 0.10$.[80]

2.2.9. Publication bias

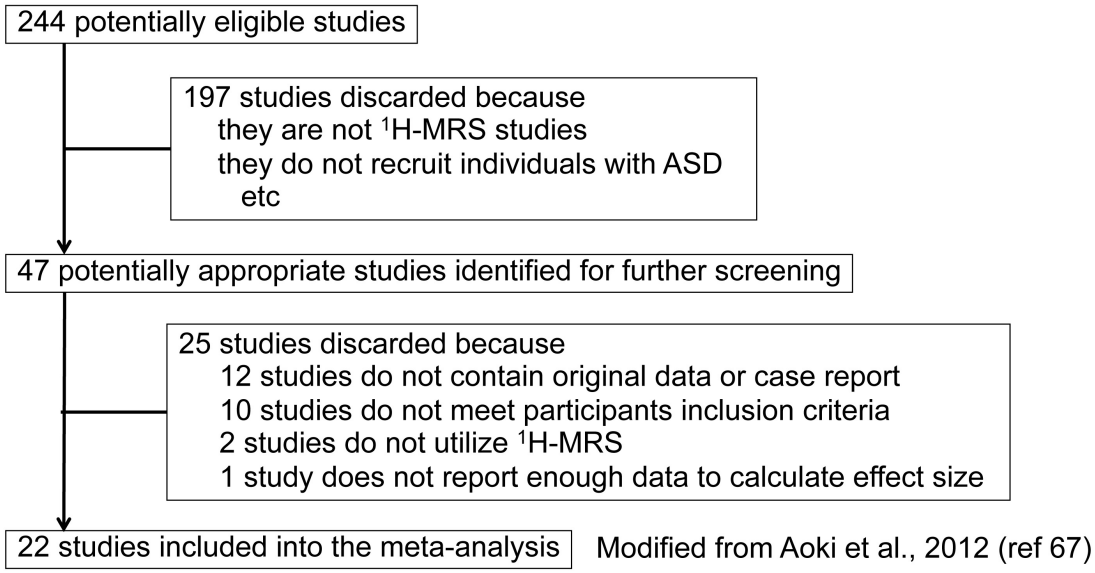
Potential publication bias was evaluated quantitatively by linear regression analysis for each group and each brain region. On the basis of guideline of Cochrane Collaboration, this analysis was performed with datasets of 10 or more.[80]

2.3. Results

2.3.1. Selection of study

The systematic review yielded potentially eligible 244 studies. Among the 244, 47 studies were identified as potential candidates for the meta-analysis. From these 47, nine studies were discarded due to lack of the original data. Twelve studies were discarded because they did not contain original data or they were a case report. Further, ten were excluded because they did not meet participants inclusion criteria. Additionally, two studies were excluded because they did not utilize ^{1}H-MRS. Two were further discarded because they did not report original data. Finally, from the database, one study was excluded from the meta-analysis because they did not provide sufficient data to calculate the SMD. As a result, 22 studies were eligible to meta-analysis (Figure 1).[36, 37, 68-74, 77-79, 81-88]

Figure 1: Process of systematic screening. The syntax has yielded 244 potentially



eligible studies. Among 244 studies, 197 studies were discarded only skimming the abstract. Then, 47 potentially appropriate studies were identified for full-text screening. Twenty-five studies of these 47 were discarded: 12 studies were discarded because they don't contain new or original data, 10 studies were additionally excluded because the don't meet participants inclusion criteria, two studies were not included into the meta-analysis because they don't utilize ^{1}H-MRS, one study was discarded because it doesn't report sufficient data to calculate effect size. Thus, 22 studies were identified to be included in the meta-analysis.

2.3.2. Meta-analysis

Meta-analyses integrating the whole included studies have demonstrated no significant difference in any kinds of metabolite levels in any brain regions between individuals with ASD and TD ($P > 0.05$). During childhood, individuals with ASD demonstrated significantly lower-than-typical NAA levels in all the brain regions except cerebellum included in the meta-analysis ($P < 0.05$, corrected for multiple comparison using Bonferroni method). On the other hand, the analysis showed no significant difference in the other metabolite levels between children with ASD and TD (Table 1).

Contrastingly, no metabolites demonstrated a significant difference in levels between individuals with ASD and TD during adulthood after correcting for multiple comparisons (Table 2).

Additionally, the Student-Newman-Kuels procedure was adopted to correct multiple comparisons in order to test whether the Bonferroni method is so strict to detect difference in metabolite levels. The analysis with the threshold defined by the Student-Newman-Kuels procedure also did not show any significant difference in metabolite levels between individuals with ASD and TD. Thus, I confirmed that there is effect modification in the degrees of differences in NAA levels of all the brain regions except cerebellum, in individuals with ASD compared to TD by age.

2.3.3. Sensitivity analyses

All the sensitivity analyses conducted in the specified-subgroups with more between-study homogeneity demonstrated significantly lower-than-typical NAA levels in the frontal lobe of children with autism ($P < 0.05$, corrected for multiple comparisons with Bonferroni method). These results showed high replicability of lower-than-typical frontal NAA level during childhood even after accounting for methodological and

participant's heterogeneity, including comorbidity of other neuropsychiatric diseases and medication status, intellectual disability, diagnostic methods, types of MRS measures, implementation of segmentation within VOI and field strength of MR scanner. With regard to the other areas, some sensitivity analyses demonstrated that the significance of lower-than-typical NAA level was not preserved in several subgroups. In the amygdala-hippocampus region, parietal cortex, temporal regions, and thalamus, the significance of lower-than-typical NAA was preserved in the large majority of subgroups, including individuals with ASD without comorbid epilepsy, without medications and acquisition of ^{1}H-MRS in a 1.5-tesla scanner.

2.3.4. Meta-regression

The present meta-regression demonstrated a significant negative effect of mean age of participants on NAA levels in the frontal lobe (P = 0.009) but not in the amygdala-hippocampus region or parietal cortex (Figure 2). With regard to statistical validity of the hypothesized model, the analysis has demonstrated that among 14 potential models, the model that effect sizes were predicted by only age had the smallest RMSEA and relatively small AIC, in addition, had the largest AGFI. In combination with hypothesis that bases biological background, I adopted the model that effect sizes were predicted by only age. The model preserved the significant association even after accounting for clinical and methodological between-study heterogeneity, meta-regression analyses in specified subgroups showed the significance of effects of age on NAA level the frontal lobe. These analyses were conducted in studies with implementation of segmentation within VOIs (P < 0.001), with 1.5-tesla scanner (P = 0.004), with multi-voxel MRS (P = 0.021), without medication (P = 0.006), with participants without comorbid epilepsy (P = 0.001), without intellectual disability (P =

0.032), and without participants whose diagnoses were made without using ADI-R or ADOS ($P = 0.006$).

The meta-regression demonstrated significant effects of the employment of segmentation within VOIs and the type of MRS measures on the NAA levels in amygdala-hippocampus region ($P < 0.05$). However, no potential confounding factors significantly influenced the NAA levels in the frontal and parietal regions.

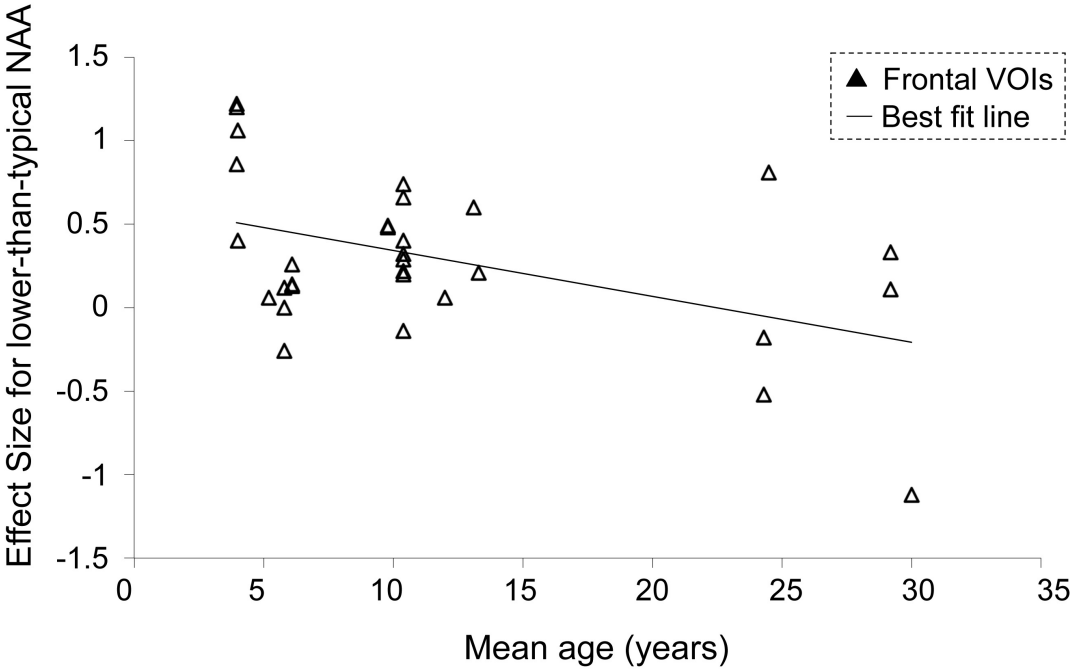
2.3.5. Assessing between-study heterogeneity

No significant heterogeneity was detected in all the metabolites in any regions except in mI levels in amygdala-hippocampus region and cerebellum during childhood ($I^2 = 78\%$ and 80%, respectively) (Table 1 and 2).

2.3.6. Publication bias

The linear regression test demonstrated no significant publication bias in all the metabolites except in the parietal NAA of children ($P < 0.1$; Table 1 and 2).

Figure 2: Relation between effect sizes for lower-than-typical NAA and mean ages of



Modified from Aoki et al., 2012 (ref 67)

study participants. Scatterplots demonstrate relation between effect sizes from each comparison of VOIs and the mean age of individuals with ASD of the study. The line of best fit shows a gradual but substantial decrease of lower-than-typical NAA.

2.4. Discussion of meta-analysis

The present meta-analysis has demonstrated that individuals with ASD showed atypical neurochemical levels and atypical aging trajectory the neurochemical levels, in particular, NAA level in the frontal lobe. Concretely, the NAA level was significantly lower-than-typical during childhood in individuals with ASD and the degree of abnormality decreases with the age advance. The result is concordant with the previous meta-analyses that reported atypical brain developmental trajectory during childhood.[17, 18] Considering the result of previous meta-analyses that head circumference was larger in individuals with ASD than TD and the degree of difference decreases with age advancing during childhood,[17, 18] and NAA may reflect component of neuronal tissue, the present result suggests that the atypically large head circumference is due to non-neuronal tissue.[37]

However, several questions remained un-answered in the present meta-analysis. First, even though there were a number of studies that involved children with ASD, there was not sufficient number of studies that recruited adults with ASD. Thus, it is yet to be elucidated whether there is atypical aging trajectory during adulthood. Second, NAA levels were significantly lower in children with ASD than those with TD during childhood. In contrast, although a meta-analysis has shown no significant difference in NAA level between individuals with ASD and TD during adulthood, a lack of sufficient number of studies with adults with ASD prevent us from concluding the question whether there is a difference in NAA level in the frontal lobe between individuals with ASD and TD.

3. Case control study

3.1. Introduction for case control study

The meta-analysis has demonstrated that degree of lower-than-typical NAA level decreases with age advancing from childhood to adulthood among individuals with ASD in frontal cortex. Namely, individuals with ASD may have tendency that NAA levels among them increases with age in frontal cortex. However, it remains also unclear whether there is atypical aging trajectory during adulthood among adults with ASD. In contrast, among individuals with TD, age-related NAA decrement in the frontal cortex during adulthood has been robustly demonstrated by several cross-sectional ^{1}H-MRS studies and a meta-analysis of ^{1}H-MRS studies.[89-94] The meta-analysis of ^{1}H-MRS studies of individuals with ASD has shown that there was no significant difference in NAA level between individuals with ASD and TD during adulthood. However, as described in discussion of the meta-analysis section, it has been yet to be elucidated whether there is a significant difference in NAA level in the frontal cortex between individuals with ASD and TD, because of a lack of sufficient number of studies recruiting adults with ASD.

Based on these reports and results, I and co-researchers have hypothesized that adults with ASD would also show atypical aging effect of NAA (i.e. lack of age-related decrease of NAA in frontal cortex) in the frontal cortex. As a consequence, an absence of typical decrease may result in no difference or even higher NAA level in frontal cortex among adults with ASD compared with individuals with TD.[67]

To test these hypotheses, 3-tesla ^{1}H-MRS was adopted in order to investigate differences in the frontal NAA levels between non-medicated high-functioning men with ASD and age-, IQ-, and parental socioeconomic background-matched men with

TD. Then, I investigated correlations between the frontal NAA levels and age in individuals with ASD and TD, separately. Further, I compared correlational relationship between NAA levels and age between individuals with ASD and TD.

In prefrontal cortex, many prior studies have demonstrated that ventromedial prefrontal cortex/anterior cingulate cortex (vmPFC/ACC) is associated with a variety of social cognitive components whose impairments were observed in individuals with ASD, including emotion recognition, empathy, and theory of mind.[95-97] In fact, a meta-analysis of a number of neuroimaging studies has shown structural abnormality in the vmPFC/ACC in individuals with ASD.[98] Thus, in the present study, we would focus on vmPFC/ACC as potential neural basis of autistic behavior.

3.2. Methods

3.2.1. Participants

To conduct a case control study, the participants were recruited in the following way. Firstly, twenty-four men (mean age = 29.5, range = 20–44) with a clinical diagnosis of high-functioning ASDs were enrolled from the outpatient clinic of The University of Tokyo Hospital. The individuals with ASD met the following criteria to be included to the study: no psychotropic medication and intelligence quotient (IQ) > 80. The diagnosis of ASD was made on the basis of the international criteria: the DSM-IV. In order to confirm the diagnosis, at least two trained child-adolescent psychiatrists with more than ten years of clinical experience followed up individuals with potential ASD for more than two months. In order to further confirm the diagnoses, the validated Japanese version of ADI-R was adopted to the individuals with probable ASD by another trained child-adolescent psychiatrist.[7, 99] With regard to the participants who did not reach the threshold in the ADI-R social domain, the Childhood Autism Rating Scale

(CARS) was adopted [100] in order to confirm the diagnosis of ASD. All the individuals with ASD were interviewed by a trained psychiatrist to screen whether they have comorbid neuropsychiatric disorders with the Structured Clinical Interview for DSM-IV axis I disorder. Twenty-five age-, IQ-, and parental-socioeconomic status (SES)-matched, men with TD were enrolled as control group. A trained psychiatrist performed an interview participants and screened for the presence or absence of neuropsychiatric disorders through the Structured Clinical Interview for DSM-IV Axis I Disorder, Non-patient Edition.[101] The ethics committee of The University of Tokyo Hospital has approved the present study (P2008047-11X:P2010028-11X). After a full explanation of the study to the participants, written informed consent was obtained from all participants.

The following exclusion criteria were made for both ASD and TD group: current or past neurological comorbidity, a history of electroconvulsive therapy, traumatic brain injury with any known cognitive consequences or loss of consciousness for more than 5 minutes, and substance addiction or abuse. In addition, for the TD group, the following exclusion criterion was set as well: a history of psychiatric disease in the participants themselves or a family history of axis I disorder in their first-degree relatives.

To detect the difference in age-NAA relationship between individuals with ASD and TD, power was estimated based on previous ^{1}H-MRS studies comparing correlation between NAA level in the similar brain region and its functional or behavioral correlates of adults with ASD with those with TD.[69, 72] As these prior studies reported that the effect size for difference between Pearson's correlation coefficient ranged from 0.75 to 1.26, the required total sample sizes, in 80 % power and alpha 0.05,

ranged from 26 to 62. Thus, more than 44 individuals, which is the mean of these two calculated estimated sample sizes, were scanned in the present study.

3.2.2. Questionnaire measures

Handedness was determined on the basis of the Edinburgh Handedness Inventory,[102] with a laterality index of > 0.5 used as the cut-off for right-handedness. Individuals whose laterality index score ranged from -0.5 to 0.5 were recognized to be mixed-handedness. The IQ of the individuals with TD was estimated with the Japanese version of the National Adult Reading Test.[101, 103] Although the National Adult Reading Test can represent the full-scale IQ among individuals with TD, it could matter for individuals with ASD due to their well-known imbalanced intellectual abilities. Therefore, for individuals with ASD, the Wechsler Adult Intelligence Scale Revised Japanese version was adopted in order to evaluate full-scale IQ.[104] Participants' own and their parents' SES were calculated with the Hollingshead scale.[105]

3.2.3. MRI acquisition

MRI data were obtained using a 3-tesla scanner (GE Signa HDxt, Waukesha, WI, USA). All the participants of both ASD and TD groups were scanned during the same period, between January 2010 and November 2011. In this period, there was no upgrade of MRI scanner or software. An 8-channel brain phased array coil was used in order to obtain both structural MRI and ^{1}H-MRS. Firstly, a sagittal localizer scan was obtained, and then the axial T2 weighted images (echo time (TE) = 82.32 ms, repetition time (TR) = 4400 ms, field of view (FOV) = 240×240 mm, matrix = 256×256, slice thickness = 2.5 mm, number of axial slices = 62) in order to localize the VOI. Three-dimensional fast spoiled gradient recalled acquisition with steady state (3D-FSPGR) (TE = 1.94ms, TR = 6.80 ms, FOV = 240×240 mm, matrix = 256×256, flip angle = 20°, slice thickness

= 1.0 mm, number of axial slices = 176) was obtained for tissue segmentation correction. Trained neuroradiologists assessed the structural MRI scans and found no gross abnormalities in any of the participants.

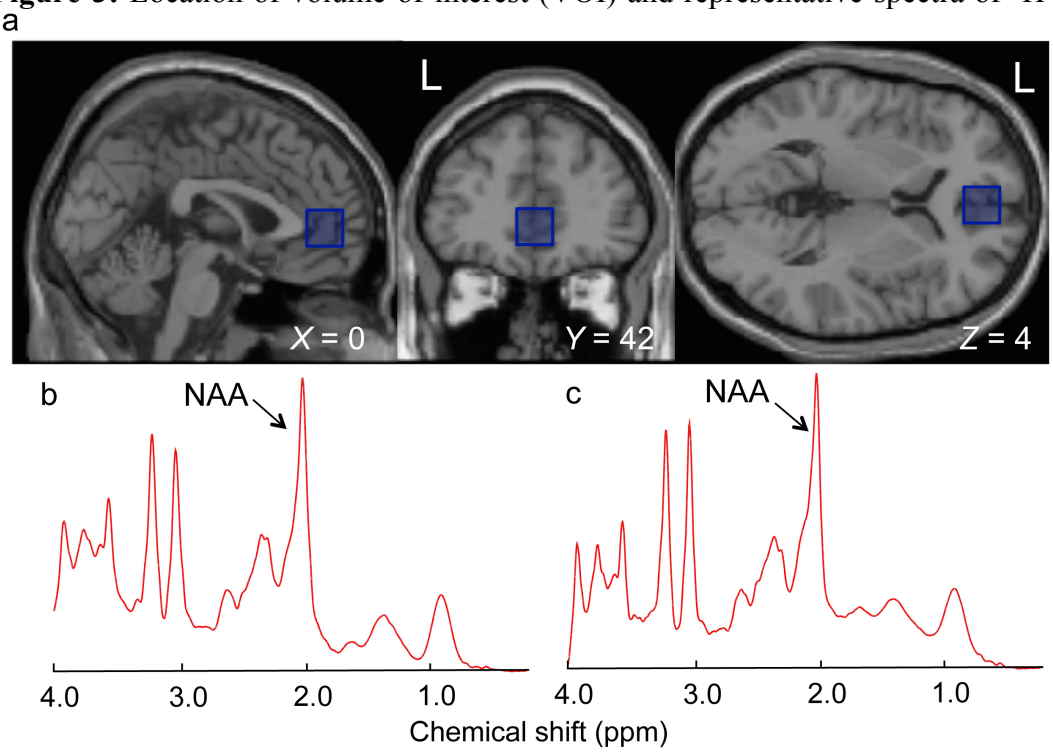
3.2.4. ^{1}H-MRS acquisition

The stimulated echo acquisition mode (STEAM) was adopted (TR = 3000 ms, TE = 15 ms, mixing time = 13.7 msec, 128 water-suppressed and 8 water-unsuppressed averages) to obtain ^{1}H-MRS. The VOI (20 mm × 20 mm × 20 mm) was located closest to the most anterior part of the genu of the corpus callosum with the center of the VOI, containing mainly the gray matter of the ventromedial prefrontal cortex (vmPFC)/anterior cingulate cortex (ACC) bilaterally (Figure 3, using the mid-sagittal slice based on the T2 weighted image).

3.2.5. Spectrum quantification

All spectra were quantified using an LCModel (ver. 6.1-4F, Stephen Provencher, Oakville, ON, Canada). The raw data of spectra were put into an LCMgui. Using LCMgui, spectrum processing was conducted automatically. On the basis of the comparison of *in vitro* spectra with its measurements analyzed with the LCModel basis set, the raw values for 17 metabolites, including NAA, N-acetylaspartylglutamate (NAAG), alanine, g-aminobutyric acid, aspartate, choline, Cre, glutamate, glutamine, glutathione, glycerophosphocholine, glycine, mI, scyllo-inositol, lactate, phosphocholine, taurine, were estimated from in vivo spectra. Of these 17 metabolites, the present study focused on the following five major metabolites, such as NAA, Cre, Glx, mI, and Cho (glycerophosphocholine plus phosphocholine). Examples of spectra of individuals with ASD and TD are shown in Figure 3b and 3c.

Figure 3: Location of volume-of-interest (VOI) and representative spectra of ^{1}H-MRS



Modified from Aoki et al., 2012 (ref 110)

of individuals with ASD and TD. (a) A T2-weighted brain image in orthogonal slices in in individuals with TD. The square indicates the VOI, 20 mm cube, in ventromedial prefrontal cortex/anterior cingulate cortex. (b and c) Representative of ^{1}H-MRS spectra of (b) individuals with ASD or (c) individuals with TD as fit by LCModel.

3.2.6. Spectrum quality

Metabolite spectra with low quality were discarded from the analysis. Concretely, the spectra with %SD > 20% or full-width-at-half-maximum (FWHM) < 0.16 ppm, or signal-to-noise ratio (SNR) > 3 were excluded. All of the five major metabolites levels of all the participants satisfied the inclusion criteria for spectrum quality.

3.2.7. Tissue segmentation

The 3D-FSPGR images were used in order to calculate the volumes of each tissue types (gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF)) using new segmentation tool of SPM8 (www.fil.ion.ucl.ac.uk/spm). Using SPM8, T2 weighted and 3D-FSPGR images were co-registered. Then, the volume of GM, WM, and CSF were calculated. To obtain tissue-contamination-corrected metabolite levels, each metabolite raw value was corrected for the ratio of CSF volume to the VOI using the following formula: Corrected-level=Uncorrected-level/(1-C), where C was the percent of CSF content to the VOI.[106]

3.2.8. Statistical method

SPSS 18.0 (SPSS Inc., Chicago, Illinois) was used for all statistical analyses. Demographic variables, such as age, self-SES, parental-SES, handedness, and IQ, volumes of each tissue component in the VOI (volumes of GM, WM, and CSF), and indices that reflect ^{1}H-MRS quality (i.e. %SD, FWHM and SN ratio) were compared using independent two-tailed t-tests between individuals with ASD and TD. To strictly assess the potential effect of confounding factors or ^{1}H-MRS quality, threshold for statistical significance was set at $P < 0.05$ without correcting multiple comparisons.

With regard to the group comparison of metabolite levels, I and

co-researchers adopted multivariate analyses of covariance (MANCOVA), treating levels of each metabolite as a dependent variable (NAA, Cre, Cho, mI, and Glx), and diagnosis (ASD vs. TD) as a main factor. Because potential effect of difference in ratio of the CSF components have already been accounted for, GM and WM components were treated as covariates of nuisance in the MANCOVAs in order to control the significant difference in the GM within VOI in the present study and well-known difference in WM water content between individuals with ASD and TD.[107] Threshold for statistical significance was set at $P < 0.05$. As I have *a priori* hypothesis that focused on NAA level in individuals with ASD and the metabolite level is deviated from that in TD in adulthood, multiple comparisons was not corrected.

Using Pearson's correlation analysis, associations between NAA level and age were analyzed in individuals with ASD and TD groups, respectively. Then, potential difference in correlations between individuals with ASD and TD was investigated using the Fisher's r-to-z transformation. The level of statistical significance was set at $P < 0.05$.

3.3. Results

3.3.1. Group difference in demographic characteristics

The analyses showed there were no significant differences in age, parental-SES, and IQ between individuals with ASD and TD, although individuals with ASD had significantly lower self-SES than individuals with TD. Individuals with ASD had significantly higher GM ($P = 0.006$) and lower CSF ($P = 0.003$) ratio to the VOI (Table 3).

3.3.2. Diagnostic differences in spectral quality and metabolite level

The quality of the spectra satisfied our inclusion criteria, with a mean (SD) signal noise

ratio (SNR) reported by the LCModel at 9.96 (2.85) and 11.12 (2.67) in individuals with ASD and TD, respectively. FWHMs measured by LCModel in individuals with ASD and TD were 0.075 (0.003) and 0.064 (0.019), respectively. %SDs recorded by the LCModel in individuals with ASD and TD were 5.08 (2.23) and 5.08 (1.79) in NAA 4.04 (0.93) and 4.67 (1.20) in Cre, 4.08 (1.22) and 4.04 (0.75) in Cho, 5.84 (1.65) and 6.46 (1.61) in mI, and 7.40 (2.04) and 7.46 (1.56) in Glx, respectively. Independent t-tests showed that there were no significant differences in SNR (P = 0.147) and FWHM (P = 0.107) between individuals with ASD and TD. On the other hand, independent t-test showed no significant difference in %SD of mI (P = 0.192), NAA (P = 0.995), Cho (P = 0.896) and Glx (P = 0.911). Conversely, there was a significant difference in %SD of Cre between individuals with ASD and TD (P = 0.047).

The MANCOVAs accounting for the effect of structural differences between individuals with ASD and TD demonstrated that the medial prefrontal NAA level was significantly higher in individuals with ASD than in TD (F = 4.832, P = 0.033). There was no significant difference in the other major metabolite levels (Table 4).

3.3.3. Diagnostic differences in relation between age and NAA levels

The Pearson's correlation analysis has demonstrated a significant negative correlation between age and NAA levels in individuals with TD (r = -0.618, R^2 = 0.383, *slope* = -0.113, *intercept* = 11.41, *SE* = 0.91, P = 0.001). In contrast, there was no significant correlation between age and NAA levels among individuals with ASD (r = 0.258, R^2 = 0.0067, *slope* = 0.064, *intercept* = 6.48, *SE* = 1.69, P = 0.223). The Fisher's r-to-z transformation has demonstrated that these correlations were significantly different between individuals with ASD and TD (Z = -3.23, P = 0.001), suggesting that the typical relation between age and NAA levels was absent in individuals with ASD

(Figure 4).

3.4. Discussion of case control study

I and coauthor have shown that men with ASD have shown atypical aging effect on NAA level in vmPFC/ACC. Namely, although men with TD have demonstrated significant age-NAA relation that NAA level in vmPFC/ACC decreases with age, men with ASD did not show any significant aging effect of NAA level in vmPFC/ACC. After accounting for structural difference between individuals with ASD and TD, individuals with ASD have shown higher-than-typical NAA level in the brain region.

Although I and co-researchers have demonstrated neurochemical abnormality in vmPFC/ACC among individuals with ASD, it remains unknown whether the NAA level in the vmPFC/ACC region is influenced by intervention. And if the level is influenced by the intervention, is there behavioral change that relate to the neurochemical change?

Table 3. Demographic characteristics of the participants

	Individuals with ASD (N = 24)		Individuals with TD (N = 25)		T-test	
Variables	Mean	SD	Mean	SD	*t*	*P*
Age (Range)	29.5 (20-44)	6.9	29.4 (20-41)	6.2	0.10	0.923
Self-SES*	2.8	1.1	1.6	0.5	5.13	<0.001
Parental SES*	2.3	0.7	2.2	0.4	1.08	0.284
Handedness: Right / Mixed / Left	19／3／2		25／0／0		Chi square	0.032
IQ						
FIQ	104.2	11.6	108.5	7.5	1.53	0.134
VIQ	111.3	14.0				
PIQ	91.3	14.6				
HFA** / Asperger / PDD-NOS	24／1／0					
ADI-R						
Social	15.0	5.9				
Communication	12.4	3.3				
Repetitive	4.7	2.3				
GMV within VOI	5.2	0.3	4.9	0.4	2.87	0.006
WMV within VOI	0.6	0.3	0.6	0.3	0.25	0.801
CSF within VOI	2.2	0.3	2.5	0.3	3.14	0.003

*Socioeconomic status, assessed using the Hollingshead index.

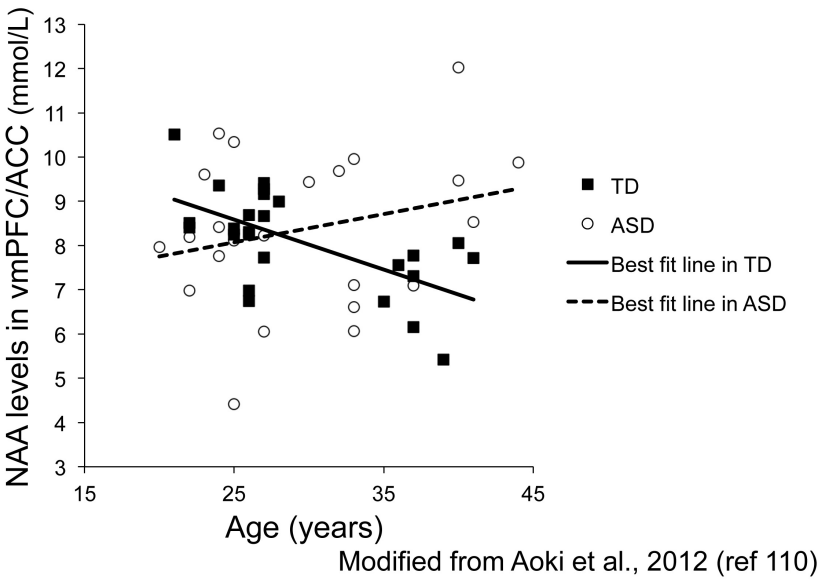
Abbreviations: ASD: autism spectrum disorder, TD: typically developed, SES: socioeconomic status, IQ: intelligence quotient, FIQ: full-scale IQ, VIQ: verbal IQ, PIQ: performance IQ, HFA: high functioning autism, ADI-R: Autism Diagnostic Interview-Revised, PDD-NOS: pervasive developmental disorder not otherwise specified, GMV: gray matter volume, WMV: white matter volume, CSF: cerebrospinal fluid, VOI: volume of interest

Table 4. Comparison of metabolite levels between individuals with ASD and TD

		MANCOVAs	
Metabolites	df	*F*	*P*
NAA	47	4.83	0.033
Cre	47	0.33	0.570
Cho	47	0.39	0.534
mI	47	0.39	0.534
Glx	47	1.82	0.184

Abbreviation: ASD: autism spectrum disorder, TD: typically developed, df: degree of freedom, NAA: N-acetylaspartate, Cre: creatine, Cho: choline containing compounds, mI: myo-Inositol, Glx: glutamate + glutamine

Figure 4: Relation between age and NAA level in individuals with ASD and TD. Scatterplots depict correlations between NAA levels in ventromedial prefrontal cortex/anterior cingulate cortex and age among individuals with ASD and TD. Fisher's r-to-z transformation has demonstrated that there was a significant difference in correlation coefficients between individuals with ASD and TD, (Z = -3.23, P = 0.001).



4. Clinical trial

4.1. Introduction for clinical trial

In line with other previous studies that reported structural atypical developmental trajectory of individuals with ASD, I and co-researchers have demonstrated that individuals with ASD show atypical developmental trajectory of NAA level in frontal cortex using meta-analytical method.[67] Then, I and co-researchers have conducted a case control study that has shown that men with ASD show higher-than-typical NAA level in the vmPFC/ACC.[108] However, it remains unknown whether the abnormality in NAA level can be influenced by therapeutic intervention. The Department of Neuropsychiatry The University of Tokyo launched a clinical trial where oxytocin was administered to individuals with ASD. In this clinical trial, fMRI and ^{1}H-MRS were obtained. I became a member of the experimenters team of the clinical trial and addressed the question of how oxytocin may work in the brain by analyzing the ^{1}H-MRS, fMRI and behavior during fMRI scan. I participated to scan the participants, providing instruction about fMRI task and ^{1}H-MRS scan. I have conducted preprocessing, analysis of data and interpretation of the result of one task of fMRI data.[109] I have performed quality assessment of ^{1}H-MRS data, analysis of ^{1}H-MRS data and association between fMRI data and ^{1}H-MRS data in addition to interpretation of the results. It should be noted that theme of this doctoral thesis is not whether oxytocin is effective to symptoms of ASD or not, but investigating the model how oxytocin may act in brain in association with behavior by analyzing the data obtained by fMRI and ^{1}H-MRS during the clinical trial.[110]

A number of previous studies have shown that oxytocin influences affiliated and social behaviors among individuals with TD and also ASD[43, 44, 46-49, 51-55, 57-61, 63, 111,

[112] [113-116] In fact, as another outcome of fMRI in the clinical trial, it was recently reported that intranasal oxytocin induced increment of the frequency of judgments based on non-verbal communication information via incrementing originally-diminished brain activity.[64] This triggered a long and active discussion about administration of oxytocin to individuals with ASD in our trial. From ethical perspective, the most important issue is whether administration of oxytocin to individuals with ASD may influence their basic inclinations that underlie their agreement to join the clinical trial, because oxytocin is supposed to increase trust.[117] If oxytocin changes basic inclinations of participants that underlie agreements to join the clinical trial, it is not possible to whether the continued consent to join the clinical trial was based on participants' natural inclinations. Namely, participants may keep on agreeing to join the clinical trial because of the pharmacological effect of oxytocin. This is a problem that is not essentially solved. However, we tried to address this problem by implementing the following two points. Firstly, full explanation including that administration of oxytocin might influence the decision-making was provided, before administration of oxytocin to participants and their caregivers (mainly their mothers). Secondly, participants and their caregivers were informed that caregivers also could decide to leave the clinical trial whenever they want. In this clinical trial, participants' caregivers also came to The University of Tokyo to complete ADI-R. So it was possible for participants' caregivers to request to stop joining the clinical trial directly to experimenters. As participants' caregivers do not receive oxytocin, their inclinations are not influenced by oxytocin. Thus, giving such opportunity to care givers may partially solve the problem. Another problem is potential adverse effect of administration of oxytocin. Oxytocin induces uterine contraction and acts at the mammary glands. Thus,

to avoid these physiological functions of oxytocin in human, only males were recruited. In addition, as oxytocin may have influence on development and function of reproductive organs in puberty,[118] only adult participants (whose age is 18 or higher) were recruited. Then, as oxytocin is approved as a labor-inducing drug (intravenous) and as a lactation-inducing drug (intranasal),[119] a search was conducted to survey what had actually happened when oxytocin was administered to human through an appended paper, researches, Food and Drug Administration web-site and gray literature. It was confirmed that there was no common adverse effect except rarely happening non-specific symptoms such as shock, confusion, convulsions, difficulty in breathing, dizziness, irregular heartbeat, and headache. Further, one meta-analysis of randomized controlled trials has demonstrated that there was no significant difference in the prevalence of the adverse effect between oxytocin and placebo.[120] On the other hand, because structures of oxytocin and vasopressin are similar to each other, it is supposed that oxytocin may also increase blood pressure and/or heart rate.[121] Then, it was discussed whether there is a concern which is specific to individuals with ASD. Although there were some previous studies that administered oxytocin to individuals with ASD,[56-59] potential risk or benefit of administration of oxytocin that are specific to individuals with ASD were further assessed. From the perspective of pathophysiology of ASD, some studies have reported abnormality in oxytocin receptor gene (Later reviewed in [122]) others reported that plasma oxytocin level among individuals with ASD was similar to or lower than that among individuals with TD (eg. [123, 124]). In addition, an animal study showed that reported CD38, which is involved in secretion of oxytocin, knockout mice showed recovery of social behavior deficit after subcutaneous injection of oxytocin.[44] These studies have indirectly supported the prediction that some of

individuals with ASD have deficit in secretion of oxytocin and external administration may recover the deficit and rationalized administration of oxytocin. It has been concluded that potential benefit in future may overwhelm the risk of administration of oxytocin in individuals with ASD.

Then, it has been discussed which administration method of oxytocin should be taken. As oxytocin is digested in the guts, it is administered intravenously or intranasally. Some previous studies have administered oxytocin to individuals with ASD intravenously[56, 57] to find that participants in one study have shown drowsiness, anxiety, depression, headache, tingling, backache, trembling, restlessness, stomach cramps, enuresis, although the study did not demonstrate statistically significant difference in prevalence of these side effects between oxytocin and placebo. In contrast, studies that administered oxytocin intranasally did not report adverse effect.[59, 125] It has yet to be elucidated how intranasally administered oxytocin influences behavior. There are at least three possible mechanisms. First, intranasally administered oxytocin directly reaches brain through blood-brain barrier. Second, intranasally administered oxytocin induces increase of oxytocin level in the brain through indirect peripheral mechanism. Third, intranasally administered oxytocin doesn't reach brain nor change oxytocin level in the brain but indirectly influence behavior through peripheral mechanism.[126] There was an animal study that showed increase of oxytocin level in the CSF after intranasal administration of oxytocin,[127] which suggest the prior two possibilities are more likely than the last one. Although it was not possible to conclude whether intranasal oxytocin directly reaches brain or indirectly increase oxytocin level in the CSF, it seems quite likely that intranasal oxytocin influences behavior via brain. Thus, administrating oxytocin intravenously was not adopted but intranasal administration of the peptide was

adopted. As per the dosage of oxytocin, it was also argued how much oxytocin we should administer. The meta-analysis that integrated the risk of previous studies that administered oxytocin intranasally delivered in doses of 18 to 40 IU showed that there was no significant risk of oxytocin compared with placebo (Reviewed in [128]). One puff of spray contains 4IU of oxytocin. In order to reduce the possibility of blocked nose, it is preferable to administer oxytocin from both nostrils. Thus, one pair of administrations of oxytocin contains 8IU. In this context, three puffs for each nostril were adopted, which is the minimum dosage between 18 and 40IU. The amount of oxytocin, 24IU is equal to the dosage studies with similar design have administered.[117, 129 59, 130-132]

In addition to potential that the vmPFC/ACC is associated with pathophysiology of ASD (described above), prior studies have shown that the brain region is the area where oxytocin may act.[61, 116, 133, 134] Thus, it was reasonably hypothesized that the oxytocin's influence on NAA levels in the vmPFC/ACC underlies our recently reported fMRI signal changes in the same brain region and the associated improvement of autistic behavior. To test this hypothesis, I and co-researchers measured the NAA levels during the same clinical trial in the vmPFC/ACC immediately after the fMRI implementation in both the oxytocin and placebo sessions with interval of 1-week. I and co-researchers investigated the relation between the oxytocin's influence on NAA levels and the oxytocin-induced changes in the task-dependent fMRI signal. Then, we conducted a path analysis to clarify the relation between the oxytocin administration and NAA levels, the fMRI signal change, and the observed behavioral changes. Finally, I and co-researchers have compared NAA level in the vmPFC/ACC between oxytocin and placebo sessions to examine influence of oxytocin on NAA level.

4.2. Methods

4.2.1. Trial design

This is a placebo-controlled double-blind crossover clinical trial where individuals with ASD were administered a single-dose of oxytocin or placebo intranasally between an interval of 1 week. Thus, although participants were divided into two groups depending on order of oxytocin/placebo, all of the participants underwent both oxytocin and placebo sessions.

4.2.2. Participants and diagnoses

Among the 323 individuals with probable ASD who visited The University of Tokyo Hospital or Showa University Karasuyama Hospital between November 1, 2009 and April 30, 2011, 40 individuals with ASD were recruited based on their firm diagnosis, age (≥20 years), FIQ (>80), and written consent. The protocol for diagnoses in the current study was the same as that utilized in our previous studies.[27, 64, 108] An experienced psychiatrist carefully made diagnoses of ASD based on the strict criteria of the DSM IV-Text Revision[5] after more than 2 months of follow-up examinations and observations. Another certified psychiatrist confirmed the diagnoses with the Japanese version of the ADI-R.[7, 99] The ASD diagnoses of eight individuals who did not meet the threshold in the ADI-R social domain were confirmed through an evaluation with the ADOS by another certified psychologist (Miho Kuroda).[8] All of eight individuals was diagnosed as having autism based on the ADOS communication + social interaction scores (range: 11–20, where 10 is the minimum threshold for autism). All ASD participants had confirmed to have normal or higher intellectual ability by the full scale of the Wechsler Adult Intelligence Scale-Revised, Japanese version.[103] The SES was also evaluated using the Hollingshead Index;[105] higher scores indicate lower status. The handedness of the participants was determined using the Edinburgh Handedness

Inventory in the same fashion as the case control study demonstrated above,[102, 108] Intelligence quotients were evaluated with the Wechsler Adult Intelligence Scale.[104] All the experiments were completed in The University of Tokyo Hospital.

4.2.3. Intervention

The participants were administered oxytocin or placebo 40 min before MR scan. As we have conducted the ^{1}H-MRS after the fMRI, the interval between the intranasal administration of oxytocin and the start of the ^{1}H-MRS scan ranged from 69-123 min (mean ± SD: 87.5 ± 10.3) Because previous studies have demonstrated increased plasma oxytocin levels 30-150 min after intranasal administration,[135] it is rationally expected that the plasma oxytocin levels were elevated during the ^{1}H-MRS scan.

4.2.4. Outcome

The outcome was set as difference in metabolite levels measured by ^{1}H-MRS between oxytocin and placebo sessions, and their relations to fMRI signals changes between two sessions.

4.2.4.1. Structural MRI acquisition

The same procedure was adopted to obtain structural MRI as the previous case control study.[108] Trained neuroradiologists assessed the structural MRI and found no gross abnormalities in any of the participants.

4.2.4.2. ^{1}H-MRS acquisition

The same method was adopted for ^{1}H-MRS acquisition as the previous case control study.[108] The VOI was located in the same way as the case control study.[108]

4.2.4.3. Spectrum quantification

The same way was taken for spectrum quantification as the previous case control study.[108]

4.2.4.4. Spectrum quality

The similar way was taken for assessing spectrum quality as the previous case control study.[108] However, as the recent studies tend to adopt severer inclusion criteria for spectrum quality, inclusion criteria of the recent studies that have been published in high impact journal were followed. Concretely, the following severer inclusion criteria were adopted for the present clinical trial: %SD ≤ 13%, FWHM ≤ 0.13 ppm and SNR ≥ 5.[136, 137] On the basis of the severer inclusion criteria, two participants were discarded from the analyses. In addition, the Glx data from four participants were not included into the analysis because of low spectra quality.

4.2.4.5. Tissue segmentation

The tissue segmentation within the VOI was conducted in the same way as the previous case control study.[108]

4.2.4.6. fMRI task/stimuli

Results of fMRI in the present clinical trial have been published previously.[64] Thus, the details of fMRI task and stimuli are available elsewhere. Briefly, the stimuli consisted of 80 original black-and-white movies with 1,500 ms. In each movie, one of 20 professional actors/actresses (10 men and 10 women) spoke a different emotional word with an emotional face expression and verbal prosody. Eighty words with emotional valence and arousal were chosen from the list of Affective Norms for English Words (40 positive valence words and 40 negative valence words).[138] They were used as the verbal information (V). For non-verbal information (NV), the actors/actresses created positive or negative facial expressions and prosody, with concurrently speaking each word. The facial expressions and verbal prosody share emotional directionality in common.[27, 64] As a result, there were four types of stimuli: a positive facial expression

and prosody paired with a negative word (i.e., NV+V−), and a positive facial expression and prosody paired with a positive word (NV+V+), a negative facial expression and prosody paired with a negative word (NV−V−), a negative facial expression and prosody paired with a positive word (NV−V+). The 20 videos were shown for each category to participants. “NV−V−” and “NV+V+” were defined as congruent stimuli, whereas “NV−V+” and “NV+V−” as incongruent stimuli. The participants were instructed to judge the actors/actresses are “friend or foe” in each movie. Based on the type of information that prominently influenced judgments, the responses to the incongruent stimuli were categorized into non-verbal-information-based judgments (NVJs) and verbal-information-based judgments (VJs). For instance, a judgment of foe responding to a “NV−V+” stimulus was recognized as an NVJ, and a judgment of foe responding to a “NV+V−” stimulus was recognized as a VJ. The difference in the number of NVJs between oxytocin and placebo sessions was calculated and utilized as an index of oxytocin-related behavioral change.

4.2.4.7. fMRI scanning

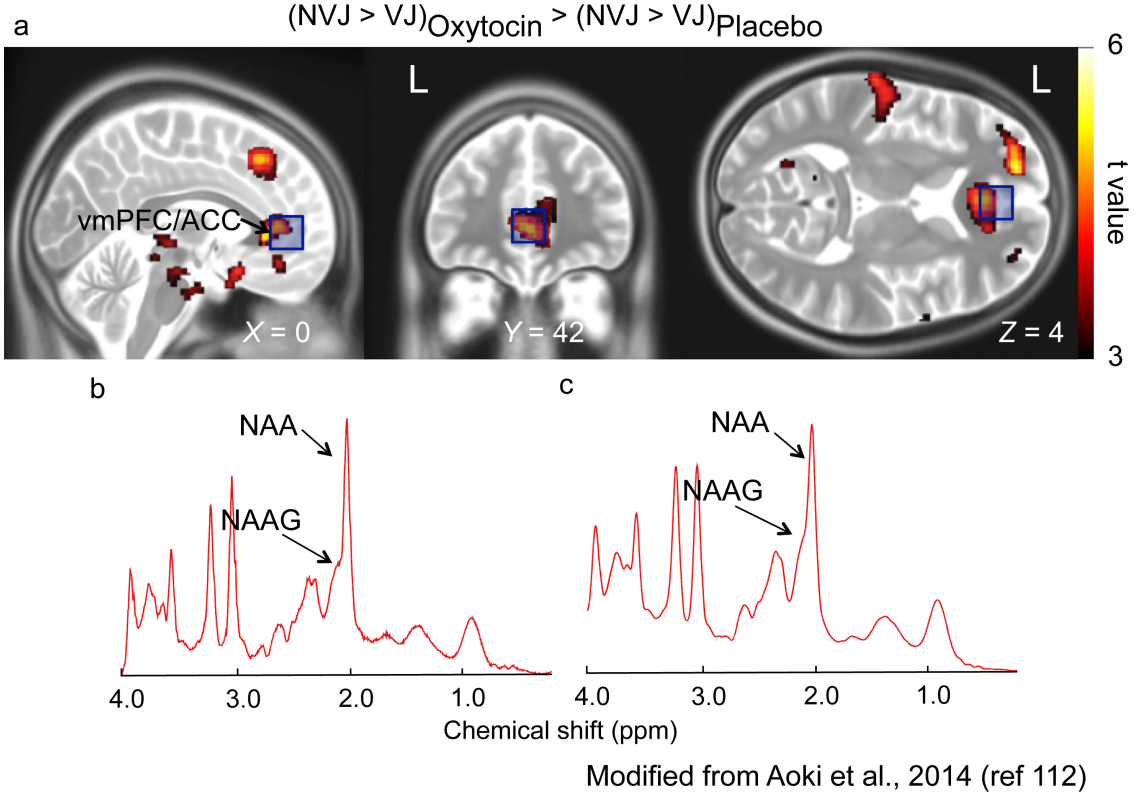
Details of fMRI scan procedure are available elsewhere.[64] Briefly, gradient-echo echo-planar sequences were adopted for functional imaging (TR = 3s, TE = 35 ms, FA = 80°, 4 × 4 × 4 mm3, 42 slices, ventral to dorsal interleaved acquisition). The first five functional images of each run were not used in the analysis in order to ensure steady-state longitudinal magnetization.

4.2.4.8. Extraction of fMRI signals from the ^{1}H-MRS VOI and mPFC regions

As it is important to investigate metabolite/fMRI signal relationships in the same

anatomical region, the average fMRI signal change of the ^{1}H-MRS VOI, cubic with 20 mm × 20 mm × 20 mm (the VOI center: x=0, y=42, z=4, Montreal Neurological Institute coordinates) was calculated. By comparing the average fMRI signal between the oxytocin and placebo sessions using a paired t-test, the oxytocin's effect on the fMRI signal change of the ^{1}H-MRS VOI was investigated. Then, the potential relationship between the oxytocin-related fMRI signal change in the ^{1}H-MRS VOI and the change in socio-communication behavior were also examined using Pearson's correlation analysis (i.e., judgment of others' hostility mainly based on non-verbal communicative cues, such as facial expression and prosody, rather than the meaning of word). In addition, the average fMRI signal change was extracted from the two brain regions where recently published fMRI data in the current clinical trial exhibited significant effects of oxytocin on fMRI signal (these fMRI signal changes were related to the mitigation of autistic socio-communication behavior).[64] These two areas are the vmPFC/ACC (x=2, y=34, z=8) and dorsomedial prefrontal cortex (dmPFC) (x=0, y=30, z=52). The vmPFC/ACC area partially overlapped with the ^{1}H-MRS VOI, whereas dmPFC is apart from the VOI (Figure 5).

Figure 5: Anatomical details between the ^{1}H-MRS VOI and oxytocin-related fMRI signal change. (a) Brain regions that demonstrated a significant effect of oxytocin on the fMRI signal related to socio-communication behavior (non-verbal communication information based judgment (NVJ)-specific activity > verbal communication information-based judgment (VJ)) (i.e., the ventromedial prefrontal/anterior cingulate cortices (vmPFC/ACC) and the dorsomedial prefrontal cortex (dmPFC), $P < 0.001$, uncorrected for the purpose of presentation) are overlaid on orthogonal slices. Blue squares represent the ^{1}H-MRS VOI (20 mm cube). Participants' representative spectrum of (b) oxytocin and (c) placebo sessions as fit by the LCModel.



4.2.5. Sample size

As the ^{1}H-MRS a secondary outcome of the clinical trial and fMRI was the main outcome, sample size of the clinical trial was calculated according to the fMRI experiment.[64] Among 40 participants who underwent an fMRI scan, 7 participants were discarded from the analysis due to clinical and technical problems, namely behavior recording during the fMRI task (two people), the current use of a psychotropic medication (two people), or frequent atypical responses to congruent stimuli (three people). As a result, recent fMRI report from the present clinical trial primarily analyzed the fMRI data of the remaining 33 non-medicated individuals with ASD.[64]

When the present clinical trial was designed, it was assumed difficult to recruit a sufficient number of individuals with ASD who do not take any psychotropic medication. Therefore, although non-medicated individuals with ASD were recruited with a preference over individuals with medication, individuals with ASD who take psychotropic medication were also recruited. As a consequence, contrary to expectations, a large number of individuals with ASD without medication were recruited. To minimize the potential confounding effects of medication, the data collected from medication-free individuals were mainly analyzed.

4.2.6. Randomization

A randomization and masking manager assigned participant to a group in which placebo was firstly administered or a group in which oxytocin was firstly administered in a computer-generated random fashion.

4.2.7. Blinding

In order to keep blindness in the participants and other research members, the manager completely covered the label of the nasal sprays. Thus, both the experimenters and the

participants were not able to identify the administered drug. The placebo contained all inactive ingredients other than oxytocin, in order to avoid subjective effects of substances other than those induced by the neuropeptide. The participants abstained from food and drink except water for 2 hours before the experiment and from exercise, caffeine, and alcohol for 24 hours before the experiment.

4.2.8. Statistical analysis

All statistical analyses were conducted with the Statistical Package for Social Science (SPSS) Version 21.0 and Amos Version 21.0 (SPSS Inc., Chicago, IL, USA). To investigate the potential differences in the tissue composition within the VOIs, the spectrum quality, and the metabolite levels between oxytocin and placebo sessions, paired t-tests were performed.

4.2.8.1 Regression analyses between ^{1}H-MRS and fMRI signals

Single linear regression analyses were performed to investigate the association between metabolite level differences between the oxytocin and placebo sessions and the extracted fMRI signal change in the ^{1}H-MRS VOI and two mPFC regions identified by the fMRI analysis. As I and co-researchers had an *a priori* hypothesis that differences in NAA levels between oxytocin and placebo sessions underlie the fMRI signal changes extracted from the ^{1}H-MRS VOI in the vmPFC/ACC, the statistical threshold was set at $P < 0.05$ for the ^{1}H-MRS VOI and at $P < 0.025$ (=0.05/2, corrected for multiple comparisons, number of brain regions) for the other two mPFC regions.

I and co-researchers also examined the effects of NAA level differences between oxytocin and placebo sessions on the behavioral changes during the fMRI task, which was significantly associated with the fMRI signal change in the ^{1}H-MRS VOI (see Results section of "4.2.2. Relation between the influence of oxytocin on NAA

levels and oxytocin-induced fMRI signal changes") using a single linear regression analysis by regressing out the fMRI signal change in the ^{1}H-MRS VOI. The statistical threshold was set at $P < 0.05$.

Further, to examine the specificity of NAA among the other four major metabolites, the relation between the oxytocin-related change in the fMRI signal of the ^{1}H-MRS VOI and the influence of oxytocin on the other four metabolite levels were investigated. The statistical threshold was set at $P < 0.0125$ (=0.05 / 4, corrected for multiple comparisons, number of metabolites).

4.2.8.2. Assessing the effects of potential confounding factors on the relation between ^{1}H-MRS and fMRI signals

Differences in the time interval between the fMRI and ^{1}H-MRS scans may affect the relationship between differences in NAA levels between oxytocin and placebo sessions and oxytocin-related fMRI signal changes. Therefore, I and co-researchers measured the interval between the fMRI and ^{1}H-MRS scans within each session. Then, I and co-researchers calculated the difference in the intervals between oxytocin and placebo sessions. This variable was used to account for the potential effect of differences in the timing schedule in the clinical trials.

I and co-researchers assessed the potential effects of the order of drug administration by conducting two regression analyses in the two independent groups ("oxytocin-placebo" and "placebo-oxytocin" groups). Then, using Fisher's r-to-z transformation, I and co-researchers assessed the difference in the regression coefficient between the two groups. To test whether the NAA-fMRI signal association is preserved after accounting for the effects of drug administration order, I and co-researchers have performed the following analysis. I and co-researchers conducted a single linear

regression analysis between differences in NAA levels between oxytocin and placebo and oxytocin-related fMRI signal changes treating the oxytocin/placebo administration order as a covariate of nuisance.

Additionally, I and co-researchers investigated whether the NAA-fMRI signal relation was preserved even when I and co-researchers include participants discarded from the main analysis due to usage of psychotropic medications or unusual behavior during the fMRI task. Concretely, because of strict inclusion criteria, I and co-researchers mainly analyzed the NAA-fMRI signal relation in 31 individuals with ASD. However, I and co-researchers collected data of both fMRI signals and metabolite levels in 37 individuals with ASD (as I and co-researchers have described above, six individuals were not included into the main analysis). In order to investigate the replicability of the significance of relation between the oxytocin's influence on the NAA levels and the oxytocin-related fMRI signal changes, I and co-researchers repeatedly performed linear regression analyses including some of the six individuals discarded from the main analysis. Totally, there were 63 combinations to add one or more of the six discarded individuals, i.e. one pattern to add all six people, six patterns to add one or five people, 15 patterns to add two or four people, and 20 patterns to add three people.

4.2.8.3. Path analysis

I and co-researchers performed a path analysis to clarify the multiple relationships between the oxytocin's influence on NAA levels, the oxytocin-related changes in fMRI signals, and the oxytocin's effect on socio-communication behavior during fMRI task, with a standard maximum likelihood estimation. Pathways between these three variables were suggested. Then, a path coefficient of each pathway was calculated. The

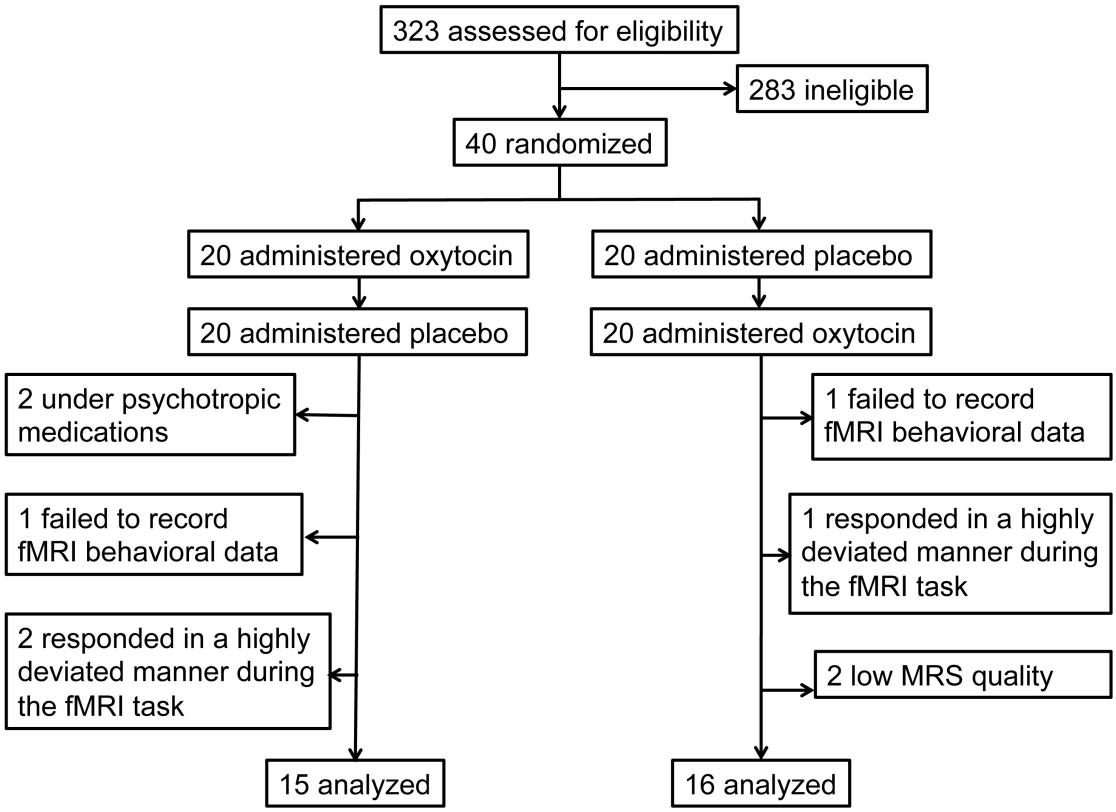
constructed models were evaluated with multiple measures shown above (in the section of **2.2.7. Meta-regression**), including GFI, AGFI, AIC and RMSEA.

4.3. Results

4.3.1. Participants flow and number analyzed

In the present ^{1}H-MRS study, additionally two individuals with ASD were discarded due to a failure to record the ^{1}H-MRS data (one person) and a low quality of ^{1}H-MRS data (one person). Consequently, the present study firstly analyzed the ^{1}H-MRS data combined with fMRI data obtained from the remaining 31 non-medicated individuals with ASD (age, 28.8 ± 6.0 years, mean ± SD) (Table 5 & Figure 6).

Figure 6: Participants flow. Among the 323 individuals with probable ASD who visited



The University of Tokyo Hospital or Showa University Karasuyama Hospital, 40 individuals with ASD were recruited in the present study. The 40 individuals were divided into two 20-individual groups depending on the pharmacological condition of the first session in a pseudo-random order. People in the group administered oxytocin first received placebo in the next session and vice-versa. Five participants from the group first administered oxytocin were discarded from the main analysis of the present study because two participants were under psychotropic medications, one participant failed to record fMRI behavioral data, and two participants responded in a highly different manner in the fMRI task. Four participants were discarded from the group administered placebo first because one participant failed to record fMRI behavioral data, one participant responded in a highly different manner in the fMRI task, and two participants demonstrated low MRS quality. Totally, 15 of the 20 participants in the group administered oxytocin first and 16 participants in the group administered placebo first were analyzed in the main analysis.

Table 5. Demographic characteristics of the participants analyzed

Variables	Individuals with ASD (N = 31)	
	Mean	SD
Age (Range)	28.8 (20-44)	6.0
Self-SES	2.9	1.2
Parental SES	2.3	0.7
Handedness: Right / Mixed / Left	26 / 2 / 3	
IQ		
FIQ	105.2	10.5
VIQ	110.5	12.7
PIQ	94.2	16.7
Autism spectrum disorder subtype	HFA: 30, Asperger disorder: 1	
ADI-R		
Social	14.4	6.7
Communication	11.7	4.1
Repetitive	4.2	2.1

Abbreviations: ASD: autism spectrum disorder, SES: socio-economic status, IQ: intelligence quotient, FIQ: full-scale IQ; VIQ: verbal IQ; PIQ: performance IQ, HFA: high functioning autism: ADI-R: Autism Diagnostic Interview-Revised

4.3.2. Relation between the influence of oxytocin on NAA levels and oxytocin-induced fMRI signal changes

A paired t-test did not demonstrate a significant difference in the quality spectra or tissue composition between the oxytocin and placebo sessions ($P > 0.088$). There were no statistically significant differences in the NAA levels between the oxytocin and placebo conditions ($t_{30} = 1.315$, $P = 0.198$; Table 6). However, a linear regression analysis has shown a significant relation between the oxytocin's influence on NAA levels and the oxytocin-related changes in the fMRI signal of the ^{1}H-MRS VOI ($R = 0.540$, *slope* = 0.306, *intercept* = 0.847, *SE* = 0.723, $R^2 = 0.327$, $P = 0.002$, N = 31; Figure 7a). Notably, the NAA-fMRI signal relation was preserved when NAA+NAAG, an alternative marker of NAA and its derivatives, was adopted instead of NAA alone ($R = 0.439$, *slope* = 0.247, *intercept* = 0.801, *SE* = 0.772, $R^2 = 0.214$, $P = 0.013$, N = 31).

A significant NAA-fMRI signal relation was also preserved in the vmPFC/ACC region ($R = 0.425$, *slope* = 0.377, *intercept* = 1.076, *SE* = 1.218, $R^2 = 0.201$, $P = 0.017$, N = 31; Figure. 7b), but was not observed in the dmPFC after correcting for multiple comparisons ($R = 0.384$, $P = 0.033$, N = 31; Figure 6c). It should be noted that the location of the ^{1}H-MRS VOI was not identical to that of the cluster of activation in the vmPFC/ACC detected in our recently published fMRI data from the present clinical trial.[64] However, I and co-researchers also detected oxytocin's effects on the fMRI signal in the same manner in the ^{1}H-MRS VOI (i.e., significantly increased fMRI signal, $t_{30} = 4.875$, $P < 0.001$, and a significant positive correlational relation between increment of fMRI signal and increased usage of judgments based on non-verbal communicative cues, $R = 0.853$, $P < 0.001$, N = 31).

An additional regression analysis that accounted for the difference in the

fMRI-^{1}H-MRS scan interval between the oxytocin and placebo sessions has also demonstrated significant NAA-fMRI signal relation (R = 0.550, P = 0.006, N = 31). This result suggests that the relation does not rely on the time interval between the fMRI and ^{1}H-MRS scans. In addition, there was no significant difference in the regression coefficient between the group to whom I and co-researchers administered oxytocin first and the group to whom we administered placebo first (R = 0.237 and R = 0.737, respectively, t = 1.325, P = 0.196), suggesting that there was no significant carry-over effect. Additionally, the regression analysis that accounted for differences in the administration order has also preserved significance of the NAA-fMRI signal relation (R = 0.618, P = 0.001, N = 31), suggesting that the relation does not depend on the drug administration order. These findings support the notion that potential neuronal changes during the 1-week interval are not necessary to observe the NAA-fMRI signal relation.

Moreover, additional analyses demonstrated that the relationship between NAA and fMRI signal was observed even when the participants who exhibited unusual behavior during the fMRI tasks or were on medication were included (P < 0.014). Additionally, associations between oxytocin's influence on other metabolite levels and oxytocin-related fMRI signal change were not observed, such as Cre (Figure 7d), Cho (Figure. 7e), mI (Figure. 7f) and Glx (Figure. 7g) (P > 0.172).

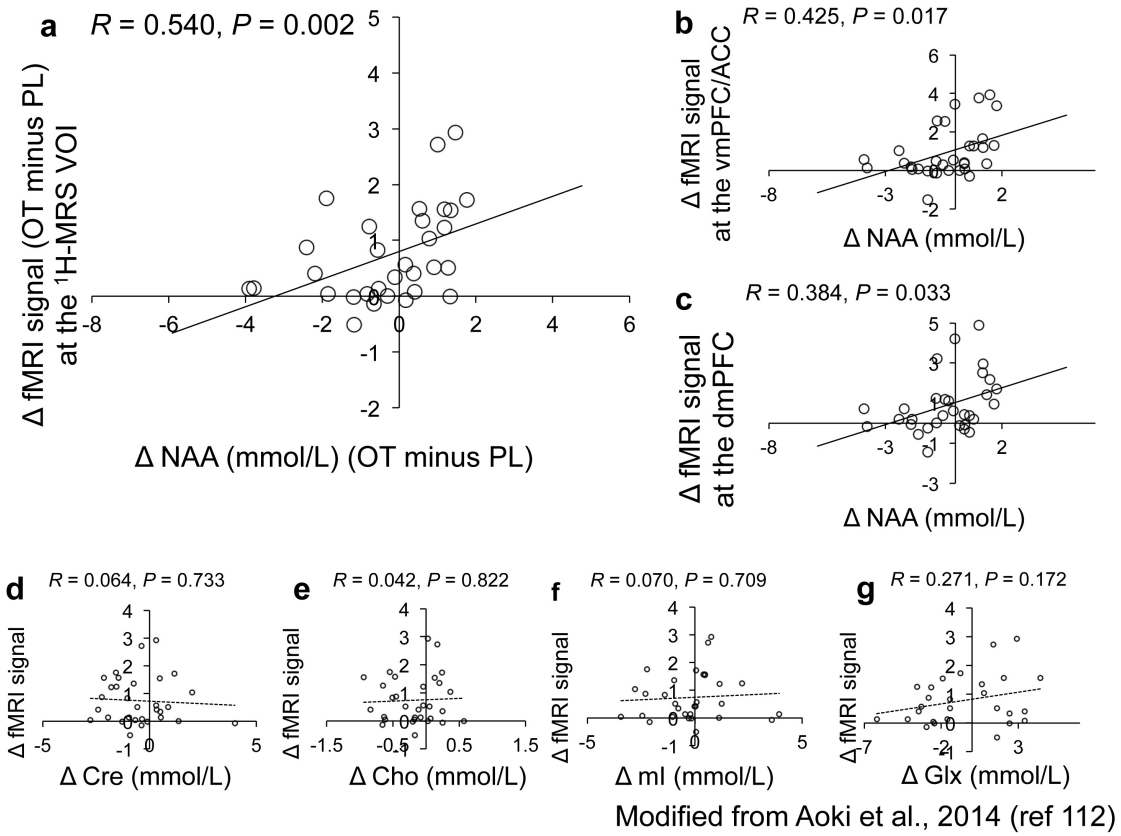
Table 6. Comparisons of metabolite levels, tissue compositions, and blood pressure/heart rate between the oxytocin and placebo sessions

	Oxytocin (N = 31)		Placebo (N = 31)		Paired t-test	
	Mean	SD	Mean	SD	*t*	*P*
Metabolite levels						
NAA	7.77	1.24	8.12	1.39	1.32	0.20
Cre	7.79	1.06	8.22	1.35	1.66	0.11
Cho	2.50	0.37	2.67	0.45	2.49	0.02
mI	6.18	1.09	6.35	1.33	0.58	0.57
Glx*	11.66	2.16	12.18	2.32	0.96	0.35
Tissue compositions within VOI						
GMV ratio within VOI	5.18	0.28	5.16	0.34	0.43	0.67
WMV ratio within VOI	0.61	0.30	0.63	0.32	0.58	0.57
CSF ratio within VOI	2.21	0.37	2.21	0.37	0.01	0.99
Blood pressure (BP) and heart rate (HR)						
Systolic BP (mmHg)	123.3	15.0	116.6	12.0	2.52	0.02
Diastolic BP (mmHg)	81.9	8.4	77.7	9.6	2.23	0.03
HR (beat/minute)	70.6	10.3	67.9	9.6	1.73	0.09

Abbreviations: NAA: N-acetylaspartate, Cre: creatine, Cho: choline containing compounds, mI: myo-Inositol, Glx: glutamate + glutamine, GMV: gray matter volume, WMV: white matter volume, CSF: cerebrospinal fluid

*The degree of freedom for Glx was 27 after excluding low-quality spectra.

Figure 7: Relation between the oxytocin-related differences in ^{1}H-MRS levels and the



changes in fMRI signal. Scatterplots demonstrate the relation between the oxytocin-related NAA differences (NAA levels at oxytocin (OT) sessions minus NAA levels at placebo (PL) sessions) and the fMRI signal changes during the task in (a) the VOI, (b) the vmPFC/ACC, and (c) the dmPFC. No significant relationship was detected between the changes in the fMRI signal and the differences in (d) creatine (Cre), (e) choline-containing compounds (Cho), (f) myo-Inositol (mI) or (g) glutamine and glutamate (Glx) levels.

4.3.3. Statistical confirmation of the hypothesized model

A correlation analysis has shown that the oxytocin's influence on NAA levels was significantly associated with an increment of frequency of judgments on the basis of non-verbal information (R = 0.454, P = 0.010, N = 31). However, no significant association between these parameters was preserved when I and co-researchers regressed out the fMRI signal change (R = 0.124, P = 0.515), suggesting that there was no direct relation between the oxytocin's influence on NAA levels and oxytocin-related increment of the frequency of judgments on the basis of non-verbal information. Based on the result that indicates that relation between oxytocin's influence on NAA level and oxytocin-related behavioral change was partial correlation, I and co-researchers hypothesized that differences in NAA levels underpin the fMRI changes that eventually related to behavioral changes. It has been shown that the hypothesized model had satisfactory indices of the goodness-of-fit using a path analysis (GFI = 0.990, AGFI = 0.940, and RMSEA < 0.001; Table 7). Moreover, among the possible models that based on the notion that the oxytocin-related neural changes (i.e., metabolite level and/or fMRI signal) underpin behavioral change,[116] the hypothesized model had the smallest value of AIC = 10.462. These results indicate that among possible models the hypothesized model is statistically most relevant (Table 7, Figure 8).

4.3.4. Harms

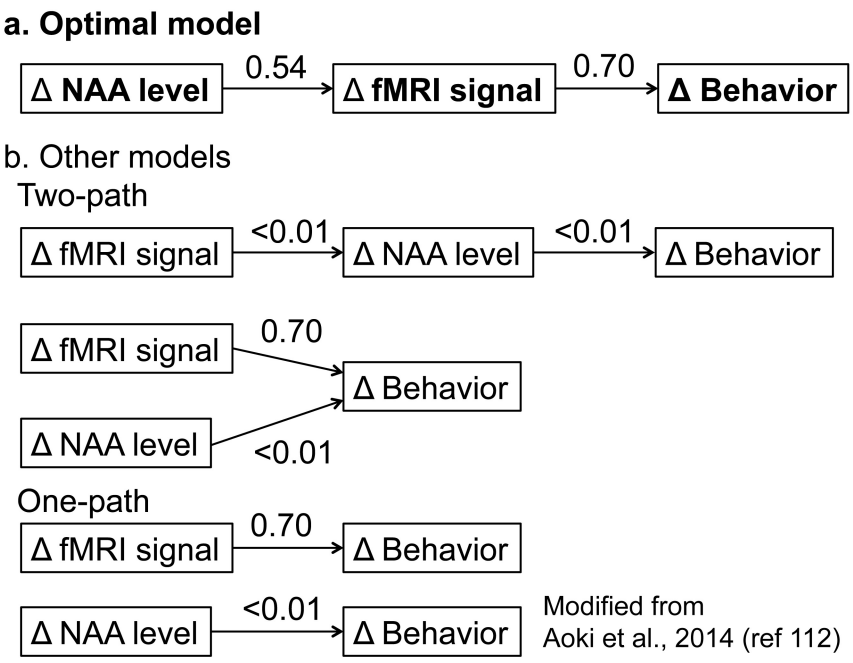
No severe adverse effect was recorded in the present clinical trial.

Table 7. Results of the path analysis

Model	RMSEA	GFI	AGFI	AIC
Two-path model				
NAA→fMRI signal→Behavior*	<0.001	0.990	0.940	10.462
fMRI signal→NAA→Behavior	0.660	0.800	-0.198	24.054
NAA and fMRI signal→Behavior	0.558	0.837	0.024	20.333
One-path model				
NAA→Behavior	0.383	0.832	0.497	18.794
fMRI signal→Behavior	0.611	0.729	0.188	32.387

Abbreviations: GFI: goodness of fit index, AGFI: adjusted goodness of fit index, AIC: Akaike information criterion, RMSEA: root mean square error of approximation. *Optimal model.

Figure 8: Path analytical confirmation of the hypothesized model. (a) A hypothesized model of the relation between NAA level differences, fMRI signal changes, and behavioral changes during the task. Path coefficients are demonstrated above each arrow. (b) The model shown is statistically more likely compared with all other possible models based on the hypothesis that oxytocin-related behavioral changes are induced by neural changes.



4.4. Discussion of clinical trial

In this clinical trial, we have demonstrated that NAA in the vmPFC/ACC is associated with fMRI signal that eventually relates to autistic behavior. The present analysis provided insight that how oxytocin may influence in brain by analyzing ^{1}H-MRS and association between ^{1}H-MRS and fMRI data. However, it is not possible to apply the present results to clinical usage of oxytocin to ASD.

5. Discussion

In a series of studies, firstly I and co-researchers have conducted a systematic review and meta-analysis of studies with ^{1}H-MRS and identified that altered NAA in frontal lobe is main atypical neurochemical among individuals with ASD. In addition, I and co-researchers have demonstrated that individuals with ASD show atypical developmental trajectory that individuals with ASD showed lower-than-typical NAA level in the frontal lobe and the degree of abnormality was largest during childhood and gradually decreased with age.[67] Such atypical developmental trajectory was the reason why the existing studies have reported inconsistent results about NAA levels in individuals with ASD. In addition, the result suggests that atypically large brain volume during childhood is not due to neuronal tissue. Because of lack of sufficient number of studies that enrolled adults with ASD, it was not possible to conclude whether adults with ASD show atypical trajectory or atypical NAA level. To answer these questions raised by the results of meta-analysis, I and co-researchers have measured NAA level in the vmPFC/ACC of 24 men with ASD and 25 men with TD to find that there is atypical aging effect on NAA level during adulthood in ASD. In addition, individuals with ASD showed higher-than-typical NAA level in the vmPFC/ACC after controlling effect of

structural difference.[108] These results suggest that characteristics of neurochemical levels in individuals with ASD during adulthood are similar to those of TD during childhood. Based on the relation with results of fMRI study in the clinical trial where a single dose of oxytocin was administered to 40 men with ASD, I and co-researchers have demonstrated that influence of oxytocin on NAA level in the vmPFC/ACC underlies oxytocin-related fMRI signal change in the same brain region that eventually related to mitigation of autistic behavior during fMRI psychological task. It should be noted that the aim of the present doctoral thesis was not addressing potential of oxytocin as a brand new therapeutic strategy for ASD but examining how oxytocin may act in the brain in association with behavior by analyzing ^{1}H-MRS, fMRI and behavior data during fMRI scan. Thus, it is not possible to put the present results to practical use.

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