# Oxytocin-Induced Increase in N,N-dimethylglycine and Time-Course of Changes in Oxytocin Efficacy for Autism Social Core Symptoms

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**Research**

# Abstract

**Background:** Oxytocin is expected as a novel therapeutic agent for autism spectrum disorder (ASD) core symptoms. However, previous results on the efficacy of repeated administrations of oxytocin are controversial. Recently, we reported time-course changes in the efficacy of the neuropeptide underlying the controversial effects of repeated administration; however, the underlying mechanisms remained unknown.

**Methods:** The current study explored metabolites representing the molecular mechanisms of oxytocin's efficacy using high-throughput metabolomics analysis on plasma collected before and after 6 week repeated intranasal administration of oxytocin (48 IU/day) or placebo in adult males with ASD ($N$=106) who participated in a multi-center, parallel-group, double-blind, placebo-controlled, randomized controlled trial.

**Results:** Among the 35 metabolites measured, a significant increase in $N,N$-dimethylglycine was detected in the subjects administered oxytocin compared with those given placebo at a medium effect size (False discovery rate (FDR) corrected $P$=0.043, $d$=0.74, $N$=83). Furthermore, subgroup analyses of the participants displaying a prominent time-course change in oxytocin efficacy revealed a significant effect of oxytocin on $N,N$-dimethylglycine levels with a large effect size ($P_{FDR}$=0.004, $d$=1.13, $N$=60). The increase in $N,N$-dimethylglycine was significantly correlated with oxytocin-induced clinical changes, assessed as changes in quantifiable characteristics of autistic facial expression, including both of improvements between baseline and 2 weeks ($P_{FDR}$=0.006, $r$=-0.485, $N$=43) and deteriorations between 2 and 4 weeks ($P_{FDR}$=0.032, $r$=0.415, $N$=37).

**Limitations:** The metabolites changes caused by oxytocin administration were quantified using peripheral blood, and therefore may not directly reflect central nervous system changes.

**Conclusion:** Our findings demonstrate an association of $N,N$-dimethylglycine upregulation with the time-course change in the efficacy of oxytocin on autistic social deficits. Furthermore, the current findings support the involvement of the N-Methyl-D-Aspartate receptor and neural plasticity to the time-course change in oxytocin's efficacy.

**Trial registration:** A multicenter, parallel group, placebo-controlled, double blind, confirmatory trial of intranasal oxytocin in participants with autism spectrum disorders. (The date registered: 30th Oct 2020; UMIN Clinical Trials Registry: https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000017703) (UMIN000015264)

# Background

Intranasal administration of oxytocin is a potential novel treatment for autism spectrum disorder (ASD) core symptoms, which currently have no established therapy (1, 2). Although the beneficial effects of single-dose oxytocin on measures of ASD core symptoms have been consistently reported across studies (3-8), previous studies on the repeated administration of oxytocin have reported inconsistent findings, impeding further development of oxytocin as an approved medication (9). Recently, we found a progressive deterioration in the efficacy of oxytocin (10, 11), and proposed that this phenomenon may account for the reported inconsistencies in the effect of repeated administration. Elucidating the mechanisms underlying the time-course change in the

efficacy of repeated oxytocin administration may help advance the development of oxytocin-based therapy for ASD core symptoms.

Uncovering the interaction of oxytocin with other molecular systems is key to optimizing oxytocin-based therapies, including the identification of co-therapeutic agents (12). We previously reported differential neurochemical effects of the repeated oxytocin administration compared with acute treatment. The repeated administration specifically impacted the glutamatergic system, including the N-Methyl-D-Aspartate (NMDA) receptor (10, 13). In addition, the time course change in the efficacy of repeated oxytocin was detected with our unique dataset employing 2-week longitudinal assessments of objectively quantified measures of ASD social deficits (11). However, to the best of our knowledge, the relationship between the time-course change in efficacy and oxytocin-induced changes in molecular pathways have not yet been examined. In addition, potential links between oxytocin and other molecular systems, other than the glutamatergic system, have not been examined.

In the present study, we explored the interaction between oxytocin and molecular systems by analyzing oxytocin-induced changes with high-throughput metabolomics, which can quantify various metabolites related to the glutamatergic system as well as other molecular systems such as cholinergic or serotonergic systems. Metabolite concentrations were quantified using plasma samples collected from the participants before and after repeated administration of oxytocin or placebo in our previous multi-center, parallel-group, placebo-controlled, double-blind, confirmatory trial of intranasal oxytocin in adult males with high-functioning ASD (11, 14). Furthermore, by utilizing repeatable and quantifiable behavioral outcome measures, we explored the molecular mechanisms underlying the time-course change in oxytocin efficacy on ASD.

# Methods

## Experimental design and participants

In the current study, we analyzed plasma samples collected from participants in our previous multi-center, parallel-group, placebo-controlled, double-blind, confirmatory trial of intranasal oxytocin in adult males with high-functioning ASD. The trial sites were the University of Tokyo Hospital, Nagoya University Hospital, Kanazawa University Hospital, and University of Fukui Hospital in Japan (UMIN000015264) (14). The details of this trial are described elsewhere (11, 14). Briefly, the inclusion criteria of this trial were as follows: (1) 18–54 years of age; (2) male; (3) diagnosis of autistic disorder, Asperger's disorder, or pervasive developmental disorders not otherwise specified (PDD-NOS) based on DSM-IV-TR; (4) score exceeding the cut-off value (i.e. 10) for qualitative abnormalities in social reciprocity on autism diagnostic interview revised (ADIR) (15); and (5) full IQ above 80 and verbal IQ above 85 based on WAIS-Third Edition (WAIS-III) (16). The exclusion criteria were: (1) primary psychiatric diagnosis other than ASD; (2) instable comorbid mental disorders (e.g. instable mood or anxiety disorder); (3) changes in medication or doses of psychotropics within 1 month before randomization; (4) current medication with more than two psychotropics; (5) current pharmacological treatment for comorbid attention-deficit/hyperactivity disorder; (6) history of repeated administrations of oxytocin; (7) history of hyper-sensitivity to oxytocin; (8) history of traumatic brain injury with loss of consciousness for longer than 5 min or seizures; or (9) history of alcohol-related disorders, substance abuse, or addiction. Open to the public recruitment and the processes testing eligibility are explained in detail elsewhere (14).

A total of 106 men with high-functioning ASD were recruited between January 2015 and March 2016. Among these participants, 94 were psychotropic-free other than oxytocin during the all trial period, while 12 continued their medications with psychotropic during the trial period (four antidepressants, four antipsychotics, two mood stabilizers, and two hypnotics). The diagnosis for subtypes of participants with ASD were autistic disorder ($N$ = 83), Asperger's disorder ($N$ = 12), and PDD-NOS ($N$ = 11).

## Intervention

The participants received administrations of oxytocin (48 IU/day) or placebo in the morning and afternoon during 6 weeks (14). The placebo contained all of the inactive ingredients in order to control for any effect of substances other than oxytocin. On the last day of the 6-week administration period, data, including peripheral blood and clinical evaluations including autism diagnostic observation schedule (ADOS) (17), were collected from the participants. These endpoint clinical assessments were started 15 min after the last administration of intranasal oxytocin or placebo. All participants were sufficiently trained with identical instructions for intranasal administration, and the procedure of intranasal administration was evaluated at each 2-week assessment point. A self-report daily record was utilized to record treatment adherence.

## Randomization and masking

Drug administration was randomly assigned the participants to the oxytocin or placebo group in a one-to-one ratio by the manager of randomization and masking based on a computer-generated randomized order. The randomization was stratified based on the trial site and median score of ADIR (<18 or ≥18, defined based on the results from our preliminary trial (18)). Spray bottles with the same visual appearance were utilized to store both active drug and placebo (Victoria Pharmacy, Zurich, Switzerland). The manager covered the labels of spray bottle to keep oxytocin or placebo blind to all the clinicians, assessors, their families, and participants. Registration, allocation, and data management procedures were defined separately (14).

## The main outcome of the current study

The main outcome of the current study was metabolite concentrations in plasma samples collected at baseline, immediately before the first administration of oxytocin or placebo, and at endpoint, 60 min after the last administration of oxytocin or placebo at 6 weeks from baseline. Peripheral blood samples were collected from the participants while they were fasting (>3 hours without any meal and/or nutritious drink). The blood sampling procedure was conducted by experienced physicians. Plasmas were isolated with centrifugation at 1,600 $g$ for 15 min at 4 °C. Then, the plasmas were stored at −80 °C until assay. 450 μL of methanol containing 10 mM each of methionine sulfone and 10-camphorsulfonic acid were added to the plasma samples (100 μL), and mixed well. Then, 500 μL chloroform and 200 μL of Milli-Q deionized water (EMD Millipore, Billerica, MA, USA) were added. The solution was centrifuged at 2,300 $g$ for 5 min at 4 °C. Then, to remove proteins, a 400-μL aliquot of the supernatant was centrifugally filtered with a 5-kDa cutoff filter (Human Metabolome Technologies Inc., Tsuruoka, Japan). The filtrate was centrifugally concentrated in a vacuum evaporator and dissolved in 50 μL of Milli-Q water containing reference compounds before mass spectrometry analyses.

Plasma samples were measured using a capillary electrophoresis system with an Agilent 6210 time-of-flight mass spectrometer (CE-TOFMS, Agilent Technologies, Santa Clara, CA, USA) (19). A customized proprietary software (MathDAMP) was utilized to process raw data files acquired from CE-TOFMS (20). To identify target

metabolites, their mass-to-charge ratio (m/z) values and migration times were matched with the annotation table of the metabolomics library (The Basic Scan metabolomics service of Human Metabolome Technologies Inc.) (21). The relative area was defined by dividing all peak areas with the area of the internal standard. The definition of relative areas allowed avoidance of mass-spectrometry detector sensitivity bias and injection-volume bias across multiple measurements and normalization of the signal intensities. Based on the peak area of internal controls of each metabolite, the absolute quantities of 110 pre-determined major metabolites can be measured with analysis by CE-TOFMS in our system. We used the absolute quantities obtained with CE-TOFMS as metabolite concentrations in plasma samples.

## Other outcome measures of oxytocin efficacy

To examine their relationship to metabolite concentrations, we also included six additional outcomes found to be significant effects of oxytocin in this trial (11, 14) as well as in previous trials (11, 18). The six clinical and behavioral indices of oxytocin efficacy were as follows: (i) ADOS repetitive behavior = changes in the ADOS repetitive score between baseline and 6-week endpoint of oxytocin administration (endpoint − baseline). ADOS is a standard diagnosis tool for ASD but recently has been increasingly adopted as a primary outcome in ASD-related trials (14, 18, 22-26). (ii) Gaze fixation time on socially relevant regions = changes in the percentage of gaze fixation time on the eye region of a talking face presented on a video monitor, between baseline and 6-week endpoint (endpoint − baseline), which were measured with Gazefinder using the standardized and validated method described details in elsewhere (14, 27-29) (JVC KENWOOD Corporation, Yokohama, Japan). (iii, iv, v, & vi) log-$PDF_{mode}$ of neutral facial expression during 0-6, 0-2, 2-4, and 4-6 weeks = changes in the natural logarithm of the mode of the probability density function of neutral facial expression intensity during a semi-structured situation conducting social interaction in "*Cartoons*" an activity of ADOS module 4, quantified with a dedicated software program (30-32)(FaceReader version 6·1, Noldus Information Technology Inc., Wageningen, The Netherlands) in the validated method described details in elsewhere (11, 33). In addition to baseline and the 6-week endpoint, facial expression was assessed every 2 weeks as changes in log-$PDF_{mode}$ of neutral facial expression between each assessment point (i.e., (iii) 6 weeks − baseline, (iv) 2 weeks − baseline, (v) 4 weeks − 2 weeks, and (vi) 6 weeks − 4 weeks). The log-$PDF_{mode}$ for neutral facial expression is considered to reflect variation in facial expression (33).

## Classification of participants according to time-course change in the efficacy of oxytocin

To investigate the mechanism of the time-course change in the efficacy of oxytocin repeated administration, we defined a subgroup from the oxytocin-administered group comprised of participants exhibiting a prominent time-course change. This classification was based on our previous findings on the time course of oxytocin-induced quantitative changes in facial expression in ASD which showed maximum efficacy at 2 weeks and deterioration of efficacy from 2 weeks to 6 weeks (11). Individuals showing reduction of log-$PDF_{mode}$ of neutral facial expression (i.e., improvement in ASD core symptom) from baseline to 2 weeks, and increase of log-$PDF_{mode}$ neutral facial expression (i.e., deterioration in ASD core symptom) from 2 weeks to 6 weeks, were classified as participants exhibiting a time-course change (Figure 2c).

## Statistical analysis

Demographic and clinical information was compared using independent $t$-tests between placebo and oxytocin-administered groups and between the placebo-administered group and the oxytocin-administered group exhibiting the time-course change.

We analyzed the effects of oxytocin on metabolite concentrations using independent $t$-tests for comparing changes in metabolite concentrations during the 6-week administration period between the oxytocin-administered group and the placebo-administered group. Furthermore, because the change in metabolite levels over the 6-week oxytocin administration period could be associated with both clinical improvement and potential attenuation of oxytocin effectiveness, differences in changes in metabolite levels were also examined between the oxytocin-administered group displaying the time-course change in efficacy and the placebo-administered group. The independent $t$-tests were conducted for each metabolite, with absolute quantities successfully measured by CE-TOFMS measurement in at least 80% of all subjects ($\geqq$67 subjects) (34). The Benjamini-Hochberg false discovery rate (FDR) correction for the number of metabolites tested was applied, and FDR-corrected $p$-values of <0.05 were considered statistically significant.

For the oxytocin-administered group, we calculated Pearson's correlation coefficients for 6-week changes in outcomes versus changes in metabolite concentrations (identified as significant differences between the oxytocin and placebo-administered participants). The outcomes used in the correlation analysis were 6-week change in ADOS repetitive behavior, 6-week change in gaze fixation time on socially relevant regions, and log-PDF$_{mode}$ of neutral facial expression change from baseline to 6 weeks. Furthermore, to clarify the relationships between the detected metabolite change and the time-course change in efficacy, changes in log-PDF$_{mode}$ of neutral facial expression between each assessment point (i.e., 2 weeks – baseline, 4 weeks – 2 weeks, and 6 weeks – 4 weeks) were calculated and correlated with changes in metabolites using Pearson's correlation coefficient. The Benjamini-Hochberg FDR correction for the number of outcomes tested was applied to adjust the results, and the statistical significance level was defined as FDR-corrected $p$-values of <0.05. STATA version 14.0 and GraphPad Prism 8.4.1 were employed to conduct all statistical analyses.

# Results

## Demographic information of participants

Detailed flow of participant is shown in Fig. 1. Two participants in the oxytocin group and one in the placebo group did not complete the trial because of withdrawal of consent or discontinuation of administration. Among the remaining 103 participants, after exclusion of subjects failing to be recorded in the ADOS (17) video recordings at any assessment point, 44 subjects in the oxytocin group and 40 subjects in the placebo group remained. One subject in the oxytocin group, not classified as exhibiting attenuation of oxytocin efficacy, was unable to provide a blood sample. In the end, a total of 83 individuals with ASD were analyzed to investigate relationships between the paradoxical attenuation of oxytocin efficacy and metabolite concentration changes (Fig. 1). Twenty of the 44 subjects in the oxytocin-administered group were classified into the time-course change group (Fig 2). This classification of individuals with time-course attenuation was based on our previous findings on the time course of oxytocin-induced quantitative changes in facial expression in ASD which showed maximum efficacy at 2 weeks and deterioration of efficacy from 2 weeks to 6 weeks (11) (Fig. 2c). No significant differences between the oxytocin and placebo-administered participants or between the time-course

change and the placebo groups were detected in background information, except for age between the time-course change group and the placebo group ($p$ = 0.02) (Table 1).

## CE-TOFMS measurement of metabolite concentrations

By CE-TOFMS (19) analysis, which can measure absolute quantities of metabolite concentrations, among the 110 pre-selected metabolites, 50 were detected in the plasma samples. Of these 50 metabolites, 35 were measured successfully in more than 80% of the subjects. We used the concentrations of these 35 metabolites for further analyses. These 35 metabolites were measured in all (i.e., 100%) of the 166 plasma samples.

## Metabolite concentration changes in participants with ASD

We examined the effects of oxytocin treatment on the levels of the 35 metabolites, and found a significant increase in the levels of $N,N$-dimethylglycine (DMG) during the 6-week repeated administration of oxytocin compared with placebo after correction for multiple comparisons ($P_{FDR}$ = 0.043, $d$ = 0.74, $N$ = 83) (Fig. 2d). Although the citric acids level was decreased during the 6-week administration of oxytocin compared with placebo ($P$ = 0.029, $d$ = 0.49, $N$ = 83), the statistical significance was not survived after correction for multiple comparisons ($P_{FDR}$ = 0.51). No significant effects of oxytocin on changes in concentration of the remaining 33 metabolites were found ($P_{FDR}$ > 0.57, Supporting Table 1). Additional analyses confined to psychotropic-free subjects ($N$ = 72) and subjects diagnosed with autistic disorder ($N$ = 62) were conducted, and confirmed that the statistical conclusions were did not changed by considering these potential confounds with excluding subjects with any psychotropic medication ($N$ = 11) or subjects diagnosed with Asperger's disorder or pervasive developmental disorders not otherwise specified (PDD-NOS) ($N$ = 21).

Next, to clarify whether the concentration change was related to clinical improvement or attenuation of efficacy, we examined the effects of oxytocin on metabolite levels in the subgroup of ASD individuals with time-course attenuation in efficacy. This sub-group analysis revealed a significant effect of oxytocin on DMG levels ($P_{FDR}$ = 0.004, $d$ = 1.13, $N$ = 60) (Fig. 2d), but not on the levels of the remaining 34 metabolite levels ($P_{FDR}$ > 0.80, Supporting Table 2). Notably, the effect size of oxytocin on DMG levels was larger in the time-course change subgroup than in the oxytocin-administered group as a whole. Although the age of the time-course change group was significantly older than that of the placebo-administered group, the analyses, controlling age as covariate, did not impact the statistical conclusion (Supporting Table 3). Additional analyses confined to psychotropic-free subjects ($N$ = 54) and subjects diagnosed with autistic disorder ($N$ = 45) also confirmed that the statistical conclusions were preserved.

We further conducted correlational analyses to clarify the relationship between the increased DMG levels and the clinical and behavioral effects of oxytocin. The analyses showed that the increase in DMG was significantly correlated with improvement indexed as change from baseline to 2 weeks in log-PDF$_{mode}$ of neutral facial expression ($P_{FDR}$ = 0.006, $r$ = −0.485, $N$ = 43) (Fig. 3a, Supporting Table 4). Furthermore, the increase in DMG was also significantly related to change from 2 weeks to 4 weeks in log-PDF$_{mode}$ of neutral facial expression in the opposite direction ($P_{FDR}$ = 0.032, $r$ = 0.415, $N$ = 37) (Fig. 3b). In contrast, no significant correlation between the increase in DMG and clinical or behavioral improvements, indexed as changes from baseline to 6 weeks in ADOS repetitive behavior, gaze fixation time on socially relevant regions, and log-PDF$_{mode}$ of neutral facial

expression ($P_{FDR} > 0.65$, Supporting Table 4). In addition, no significant correlation was found between any clinical or behavioral change and change in DMG level in the placebo-administered group ($P_{FDR} > 0.23$). Additional correlational analyses confined to psychotropic-free subjects and subjects diagnosed with autistic disorder also confirmed that the statistical conclusions were preserved.

## Discussion

The current parallel group comparison of metabolites changes between the oxytocin and placebo administered groups revealed a significant increase in plasma DMG levels during the 6-week intranasal oxytocin treatment period. This change was prominent in the participants exhibiting a time-course change in oxytocin efficacy. Furthermore, the increase in DMG was associated with behavioral changes in autistic characteristics of quantified facial expression (i.e. improvements from baseline to 2 weeks and deteriorations from 2 weeks to 4 weeks), although the increase in DMG was not related to improvements in clinical or behavioral outcomes during the 6-week administration period as a whole.

Here, we found a significant increase in DMG induced by oxytocin administration in the participants with ASD. DMG, the *N,N*-dimethylated derivative of glycine, is an important intermediate in the amino acid metabolism from choline to glycine. DMG can modulate NMDA receptors (NMDAR), as sarcosine (monomethylglycine) and glycine act as NMDAR co-agonists by occupation of glutamate binding site in the NMDAR (35, 36). A putative functional partial agonist for glycine-site of NMDAR produces psychotropic effects (37). A previous study showed that NMDAR is critical to develop and rescue of ASD-like phenotypes observed in Shank2-mutant mice, and that, by modulating NMDAR, metabotropic glutamate receptor 5 can be a novel treatment target for ASD (38). DMG derivatives also have pharmacological activities in the central nervous system, and decrease oxidative stress (39), improve immune responses (40), and exhibit anticonvulsant activity in animal models (41). Psychotropic effects of DMG have also been reported by an animal study as having an antidepressant-like effect with reduction in ketamine-induced psychotomimetic behaviors (42). A few studies, including randomized controlled trials, have reported lower level of plasma DMG (43) and clinical effects of administrations of DMG (44-47) in individuals with ASD, although the results are controversial. Our current study provides the first clinical evidence for a relationship between changes in DMG and oxytocin treatment in subjects with ASD. Together with previous animal studies showing interactions between central oxytocin and NMDAR such as central oxytocin release stimulated by NMDAR glycine site agonists (10, 48, 49), the current study supports the potential combination therapy of DMG or a NMDAR modulator and oxytocin for ASD.

The increase in DMG was most prominent in the ASD participants exhibiting a time-course change in oxytocin efficacy. In addition, although the increase in DMG was not related to clinical or behavioral improvements during the 6-week administration period as a whole, the increase was associated with improvements from baseline to 2 weeks and also with deterioration from 2 weeks to 4 weeks, assessed as behavioral changes in quantified characteristics of autistic facial expression. Collectively, our findings show that upregulation of DMG is associated with time-course changes in the efficacy of oxytocin for ASD. Together with previous animal studies on the relationship between oxytocin efficacy and NMDAR-dependent neural plasticity (10, 50, 51), our present clinical study supports a contribution of NMDAR and neural plasticity to the time-course change such as improvement and subsequent deterioration in oxytocin efficacy.

Previous animal studies support a relationship between oxytocin and neural plasticity via glutamatergic transmission—oxytocin enhances excitatory synaptic transmission (52) and facilitates long-term potentiation (50). Our recent human clinical trial and animal study (10) further supports a relationship between NMDAR and oxytocin: repeated administration of oxytocin downregulates medial prefrontal glutamatergic metabolites (i.e. N-acetylaspartate and glutamate-glutamine), measured with $^1$H-magnetic resonance spectroscopy, compared with acute oxytocin (13). The decreases in these metabolite levels were negatively and specifically correlated with oxytocin-induced improvements of medial prefrontal function. Furthermore, we showed that repeated administration of oxytocin decreased expression of the transcript for NMDA receptor type 2B in the medial prefrontal region, in contrast to acute oxytocin, in wild-type mice (10). The current study further shows a link between changes in NMDA and time-course change in the efficacy of repeated administration of oxytocin in individuals with ASD.

## Limitations

There are several potential limitations to the current study. First, the participants in this study were all Japanese, adult, males with high-functioning ASD. Therefore, although the uniformity in demographic backgrounds enhanced the ability to detect metabolomics changes in the current study, the current findings should carefully be generalized to other clinical or non-clinical populations. Second, the metabolites changes caused by oxytocin administration were quantified using peripheral blood, and therefore may not reflect central nervous system changes. Further study is needed to clarify the interaction between oxytocin and molecular systems in the central nervous system.

## Conclusions

In conclusion, the present high-throughput metabolomic analysis of plasma from a large-scale multi-center randomized controlled trial provides clinical evidence for an association between oxytocin-related increase in DMG and time-course changes in the efficacy of oxytocin for ASD social core symptoms. The results further support a contribution of NMDAR and neural plasticity to the time-course change. Our findings should help optimize oxytocin-based therapy as well as combinatorial therapy of DMG or a NMDAR modulator and oxytocin for ASD.

## Abbreviations

ASD: Autism spectrum disorder; FDR: False discovery rate; NMDA: N-Methyl-D-Aspartate; NMDAR: N-Methyl-D-Aspartate receptors; PDD-NOS: Pervasive developmental disorders not otherwise specified; ADIR: Autism diagnostic interview revised; WAIS-III: WAIS-Third Edition; ADOS: Autism diagnostic observation schedule; CE-TOFMS: Capillary electrophoresis system with an Agilent 6210 time-of-flight mass spectrometer; DMG: N,N-dimethylglycine

## Declerations

### Ethics approval and consent to participate

All experimental procedures complied with the Declaration of Helsinki and were approved by the institutional review board of Hamamatsu University School of Medicine, The University of Tokyo, Nagoya University, Kanazawa University, and University of Fukui. Participants gave written informed consent before participating in the study.

## Availability of data and materials

The data underlying the findings of this study are available from the corresponding author (H.Y.) on request from investigators providing a methodologically sound proposal and whose proposed use of the data has been approved by an independent review committee identified for this purpose. Maintenance of the identified data set in the participants of clinical trials will be ended 5 years following article publication, but the deidentified data will be maintained indefinitely. The data are not publicly available due to them containing information that could compromise research participant privacy or consent.

## Consent for publication

Not applicable.

## Funding

## Competing interests

The funding agency had no role in the design and conduct of the study; the collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication. There are no conflicts of interest.

## Acknowledgments

## Author Contributions

HY designed the study. YK, HK, and HY interpreted the results. YK and HK carried out statistical analyses. YK, HK, TO, TM, SB, MaKo, WY, YE, YK, CM, KK, NO, HK, and HY organized and carried out subject recruitment and biological material collection, whereas MiKu, and HY carried out clinical assessment. YK, HK, and HY drafted the manuscript, and all authors contributed to the final version of the paper

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# Tables

| | Placebo-administered group | | Oxytocin-administered group | | Oxytocin-vs Placebo-group | Time-course change group | | Time-course change vs Placebo-group |
|---|---|---|---|---|---|---|---|---|
| | (N = 40) | | (N = 43) | | | (N = 20) | | |
| | Mean | SD | Mean | SD | P value[c] | Mean | SD | P value[c] |
| Age (range) | 26.4 (18-46) | 6.9 | 28.2 (18-48) | 7.4 | 0.26 | 30.9 (22-47) | 6.6 | 0.02 |
| Height, cm | 172.2 | 5.7 | 170.5 | 6.4 | 0.21 | 170.5 | 6.1 | 0.30 |
| Body weight, kg | 66.7 | 11.5 | 67.3 | 11.5 | 0.80 | 69.5 | 11.2 | 0.37 |
| SES[a] | 2.8 | 1.2 | 3.2 | 1.0 | 0.08 | 3.3 | 1.1 | 0.12 |
| Parental SES[a] | 2.2 | 0.6 | 2.2 | 0.6 | 0.66 | 2.1 | 0.6 | 0.75 |
| Handedness: Right / Left | 37 / 3 | | 41 / 2 | | | 20 / 0 | | |
| IQ[b] | | | | | | | | |
| Full IQ | 110.3 | 14.1 | 105.2 | 13.7 | 0.10 | 106.4 | 14.6 | 0.31 |
| Verbal IQ | 116.5 | 14.3 | 110.7 | 13.2 | 0.06 | 110.8 | 13.4 | 0.14 |
| Performance IQ | 100.2 | 14.7 | 96.5 | 15.4 | 0.27 | 98.1 | 16.3 | 0.61 |
| ADIR | | | | | | | | |
| Social | 21.9 | 5.2 | 21.2 | 5.0 | 0.57 | 21.7 | 4.4 | 0.91 |
| Communication | 16.3 | 3.4 | 15.6 | 3.9 | 0.36 | 15.8 | 3.6 | 0.56 |
| Repetitive | 5.9 | 2.3 | 5.3 | 2.6 | 0.36 | 5.3 | 2.6 | 0.41 |

Table 1. Demographic background and clinical characteristics of the participants

[a]SES assessed using the Hollingshead scale. Higher scores indicate lower status.

[b]Intelligence quotients were measured using the Wechsler Adult Intelligence Scale.

[c]P-values were calculated using the independent t-test.

Abbreviations: SES, socio-economic status; IQ, intelligence quotient; SD, standard deviation; ADIR, Autism Diagnostic Interview-Revised

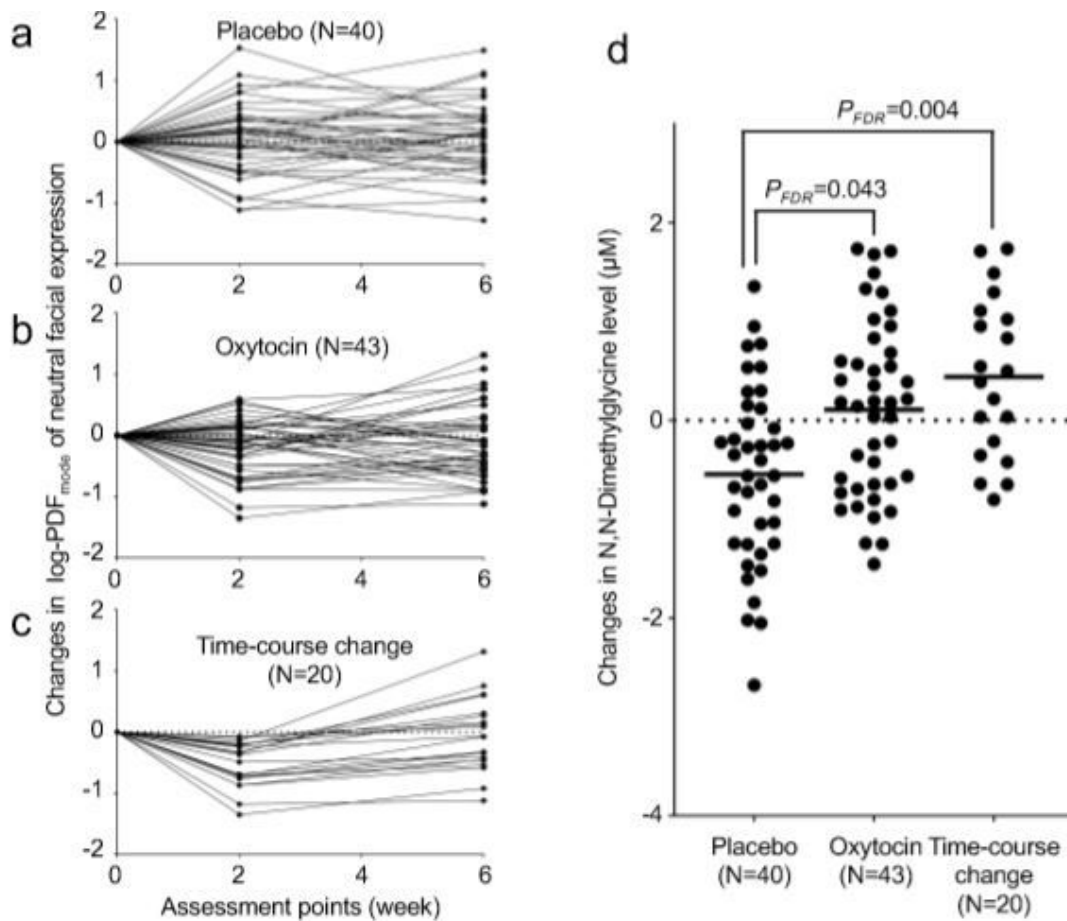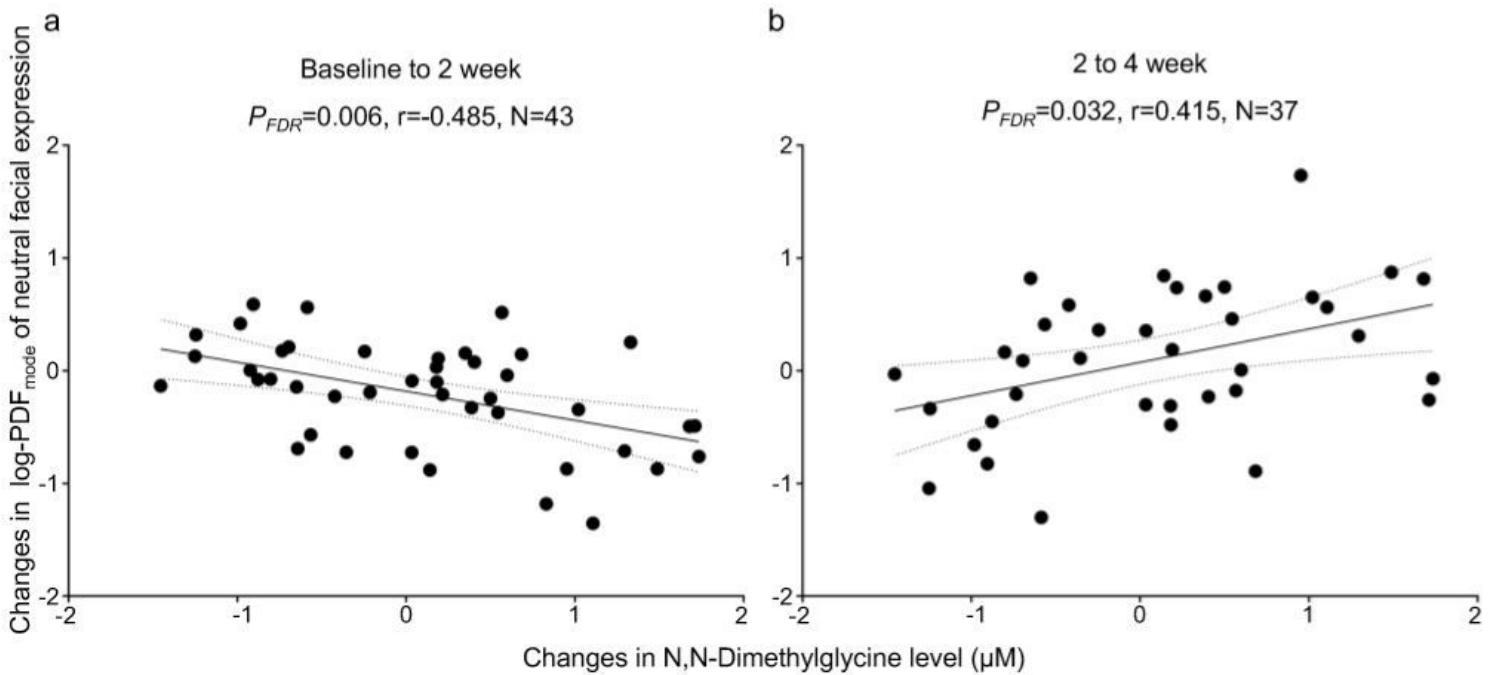# Figures
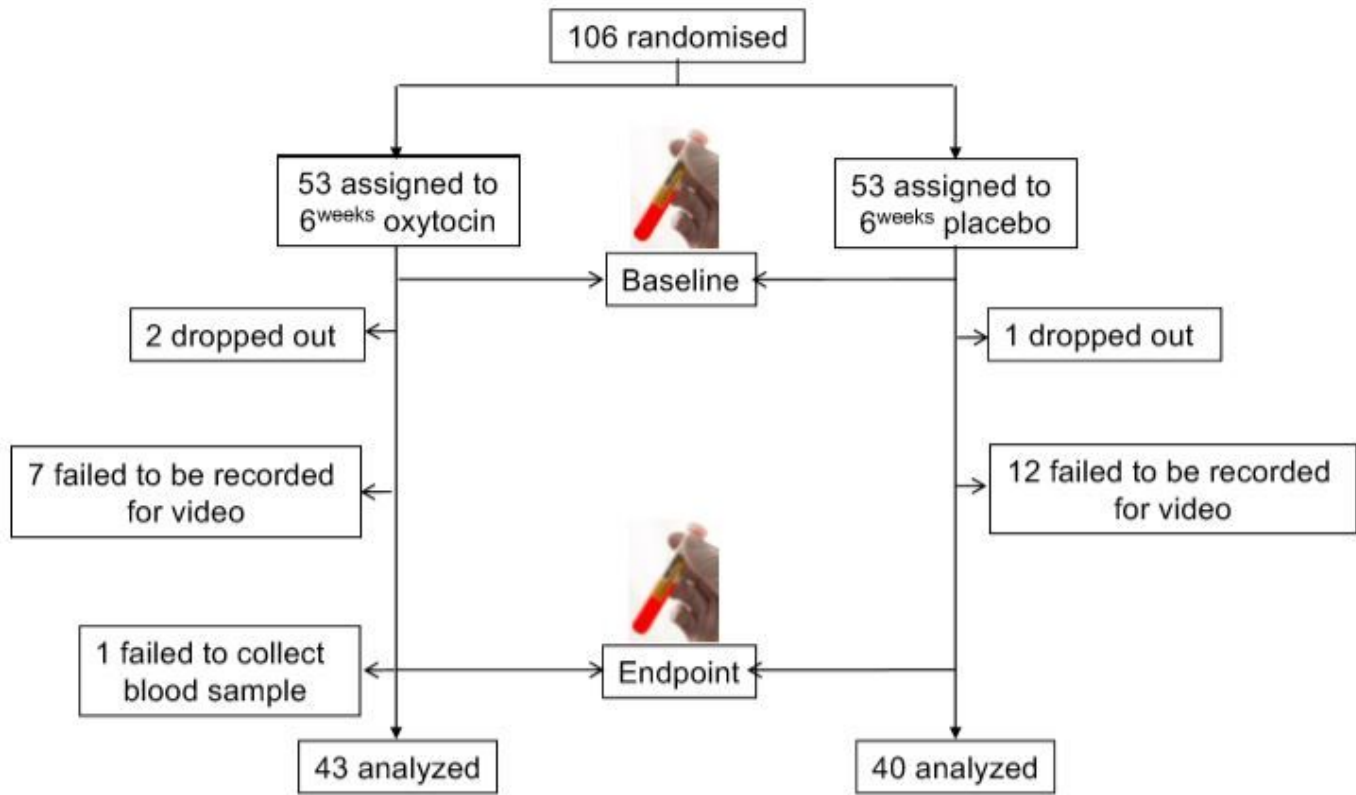
**Figure 1**

Participants flow in the current study.

**Figure 2**

Effects of intranasal oxytocin on changes in metabolite concentrations and time-course change in effects of oxytocin. (a–c) Individual changes from baseline in the natural logarithm of the mode of the probability density function (log-PDFmode) of neutral facial expression intensity. Plots show changes from baseline of log-PDFmode of neutral facial expression intensity in participants administered placebo (a) or oxytocin (b). Among the participants administered with oxytocin, individuals showing reduction of log-PDFmode of neutral facial expression from baseline to 2 weeks, and increase of log-PDFmode neutral facial expression from 2 weeks to 6 weeks, were classified as participants exhibiting a time-course change (c). (d) Plots show changes in plasma DMG levels during the 6-week administration of oxytocin or placebo. Bars indicate mean concentration change in each group.

**Figure 3**

Relationship between oxytocin-related changes in metabolite concentration and time-course change in behavioral effect of oxytocin on autistic facial expression. Oxytocin-related increase in DMG level showed significant correlations with both the decrease in autistic facial expression, indexed as log-PDFmode of neutral facial expression, from baseline to 2 weeks (a: PFDR = 0.006, r = −0.485, N = 43) and the increase from 2 weeks to 4 weeks (b: PFDR = 0.032, r = 0.415, N = 37) in participants with autism spectrum disorder. One participant in baseline to 2 weeks and 7 participants in 2 to 4 weeks were excluded because of recording failure, defocused video images, or poor facial recognition rate at least one assessment point among these. Regression lines (solid) and 95% confidence band (dashed) were fitted using simple linear regression.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- YamasueSupportingTable2.xlsx
- YamasueSupportingTable1.xlsx