

Clinical and neural effects of six-week administration of oxytocin on core symptoms of autism

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Author Contributions

H.Y. designed the study. T.W., M.K., H.K., Y.A., N.I., T.N., H.T., N.Y., Y.K., K.K., and H.Y. were engaged in the data collection and/or clinical assessments and/or recruitment of participants. T.W. and H.Y. analyzed the data, discussed the results, and wrote the paper.

Abstract

Autism spectrum disorder is a prevalent neurodevelopmental disorder with no established pharmacological treatment for its core symptoms. Although previous literature has shown that single-dose administration of oxytocin temporally mitigates autistic social behaviours in experimental settings, it remains in dispute whether such potentially beneficial responses in laboratories can result in clinically positive effects in daily-life situations, which are measurable only in long-term observations of individuals with the developmental disorder undergoing continual oxytocin administration. Here, to address this issue, we performed an exploratory, randomised, double-blind, placebo-controlled, crossover trial employing 20 high-functional adult males with autism spectrum disorder. Data obtained from 18 participants who completed the trial showed that six-week intranasal administration of oxytocin significantly reduced autism core symptoms specific to social reciprocity, which was clinically evaluated by Autism Diagnostic Observation Scale ($P = 0.034$, $P_{\text{FDR}} < 0.05$, Cohen's $d = 0.78$). Critically, the improvement of this clinical score was accompanied by oxytocin-induced enhancement of task-independent resting-state functional connectivity between anterior cingulate cortex and dorso-medial prefrontal cortex ($\rho = -0.60$, $P = 0.011$), which was measured by functional MRI. Moreover, using the same social-judgment task as one used in our previous single-dose oxytocin trial, we confirmed that the current continual administration also significantly mitigated behavioural and neural responses during the task, both of which were originally impaired in autistic individuals (judgement tendency: $P = 0.019$, $d = 0.62$; eye-gaze effect: $P = 0.03$, $d = 0.56$; anterior cingulate activity: $P = 0.00069$, $d = 0.97$; dorso-medial prefrontal activity: $P = 0.0014$, $d = 0.92$; all, $P_{\text{FDR}} < 0.05$). Furthermore, despite its longer administration, these effect sizes of the six-week intervention were not larger than those seen in our previous

single-dose intervention. These findings not only provide the evidence for clinically beneficial effects of continual oxytocin administration on the core social symptoms of autism spectrum disorder with suggesting its underlying biological mechanisms, but also highlight the necessity to seek optimal regimens of continual oxytocin treatment in future studies.

Keywords

Clinical trial, functional MRI, Neuropeptide, Pervasive developmental disorder, resting-state functional connectivity

Abbreviations

ASD, autism spectrum disorder; fMRI, functional MRI; ACC, anterior cingulate cortex; dmPFC, dorso-medial prefrontal cortex; rsFC, resting-state functional connectivity; PPI, psycho-physiological interaction.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with over 1% of prevalence, and has been given no established pharmacological treatment for its core symptoms (Centres for Disease Control and Prevention, 2012). Single-dose oxytocin is currently considered to mitigate its deficits in social cognitions in experimental settings (Andari *et al.*, 2010; Aoki, Watanabe, *et al.*, 2014; Aoki, Yahata, *et al.*, 2014; Bakermans-Kranenburg and van IJzendoorn, 2013; Domes *et al.*, 2014; Gordon *et al.*, 2013; Hollander *et al.*, 2007; Lin *et al.*, 2014; Veening and Olivier, 2013; Watanabe, Abe, *et al.*, 2014; Yamasue *et al.*, 2012; Zink and Meyer-Lindenberg, 2012); however, there is no clear evidence that such potentially beneficial responses in laboratories can consequently induce significant effects in ASD individuals in clinical and daily-life settings after continual intervention, which is impeding practical applications of this neuropeptide.

Although a recent single-armed, open-label trial employing eight ASD males has reported the safety of continual oxytocin administration, and suggested its potentially positive influence on the communication and social-interaction scores of Autism Diagnostic Observation Schedule-Generic and on the caregivers' reports about reciprocal communication quality (Tachibana *et al.*, 2013), previous randomised trials of continual administration have not detected clinically valuable effects of oxytocin on ASD with statistical significance. A six-week trial, which compared oxytocin-treated ten ASD adults with placebo-given nine ASD adults, reported behavioural improvement only in an experimentally-measured ability to detect emotion, but could not find significant benefits on clinically-measured social deficit scores (Anagnostou *et al.*, 2014). Other recent trials, in which oxytocin/placebo were administered to 19/19 ASD children over five days (Dadds *et al.*, 2014) or 26/24 children over eight weeks

(Guastella *et al.*, 2014), could not detect any significant changes in any social behaviours of the children, either.

Here, we assumed that this discrepancy between single-dose and continual administration of oxytocin indicates (i) the necessity to use ASD-specific clinical scoring systems for detecting responses to the continual intervention, and (ii) the possibility that continual oxytocin treatment does not amplify effects of single-dose administration. In fact, the hypothesis (ii) was also suggested by a recent study that, using prairie voles, implied that chronic oxytocin treatment could trigger down-regulation of endogenous oxytocin or its receptor, and reduce acute beneficial effects of the neuropeptide (Bales *et al.*, 2013).

We tested these two hypotheses in the current exploratory randomised, double-blind, placebo-controlled, crossover trial. To examine the hypothesis (i), we assessed clinical effects of six-week administration of oxytocin using Autism Diagnostic Observation Scale (ADOS) (Lord *et al.*, 1989), which is originally a standard diagnosis tool for ASD but recently has been increasingly adopted as a primary outcome in ASD-related trials (Aldred *et al.*, 2004; Green *et al.*, 2010; Howlin *et al.*, 2007; Owley *et al.*, 2001; Wong and Kwan, 2010). In addition, we explored potential biological mechanisms underlying these clinical effects by examining intrinsic functional connectivity in the medial prefrontal cortex (mPFC), which are known to be significantly altered in ASD (Cherkassky *et al.*, 2009; Cox *et al.*, 2012; Di Martino *et al.*, 2014; Itahashi *et al.*, 2014; Jung *et al.*, 2014; Lynch *et al.*, 2013).

To evaluate the hypothesis (ii), we used the same psychological task as one in our previous single-dose trial (Watanabe, Abe, *et al.*, 2014), and directly compared magnitudes of oxytocin's behavioural and neural effects between six-week and single-dose interventions. For the precise comparison, we performed this task-based evaluation in the virtually same time

schedule as in our previous single-dose trial, and measured the behavioural and neural responses on the last day of each six-week administration of oxytocin/placebo.

Methods

Study design and participants

This randomised, double-blind, placebo-controlled, crossover trial was primarily conducted in an outpatient clinic of The University of Tokyo Hospital. The recruitment process, data and drug management, informed-consent collection, confirmation of diagnosis, six-week interval assessment, and two-week interval assessment were performed in this main site. Exceptionally, eight participants, who were originally cared for in an outpatient clinic in Showa University Karasuyama Hospital, underwent the recruitment process and two-week interval assessments in Karasuyama Hospital with the common psychiatrist (HY), who conducted these procedures to the other participants at The University of Tokyo Hospital.

The inclusion criteria comprised ASD diagnosis, gender (male), Full-scale IQ (>80), and age (18–55 years old). The exclusion criteria consisted of any history of allergic responses to oxytocin, seizures, traumatic brain injury with any known cognitive consequences, loss of consciousness for more than 5min, and substance abuse or addiction. Participants with current instability of comorbid psychiatric symptoms and contraindications on MRI scanning were also excluded. The study protocol is registered in University Hospital Medical Information Network Clinical Trials Registry (UMIN000007122). Written informed consent was obtained from all the participants.

As stated in our trial registry, one of the aims of the current explanatory study was to

estimate the power of the six-week oxytocin intervention; therefore, from an ethical perspective, the number of participants in this trial was set at the presumably minimal number for sufficient statistical inference (i.e., $N = 20$). In fact, 13–16 ASD participants were employed in previous studies that reported significant behavioural improvements in psychological tests after single-dose oxytocin administration (Andari *et al.*, 2010; Guastella *et al.*, 2010); another study employed 15 participants with other psychiatric disorders to show that continual oxytocin treatment could induce a significant improvement in clinically evaluated psychiatric symptoms (Feifel *et al.*, 2010). Considering these studies, we set the current sample size at 20.

Diagnosis

A well-experienced psychiatrist (HY) made diagnosis of autistic disorder, Asperger's disorder, or pervasive developmental disorder not-otherwise-specified based on the strict criteria of Diagnostic and Statistical Manual-Revision IV-Text Revision with more than two months of follow-up examinations. Another certified psychiatrist/psychologist confirmed the diagnoses using Japanese version of Autism Diagnostic Interview-Revised (Lord *et al.*, 1994) (HK) and ADOS (Lord *et al.*, 1989) (MK). All participants exhibited normal or high intelligence in full scale of Wechsler Adult Intelligence Scale-Revised, Japanese version (Table 1).

Interventions

The participants received oxytocin (24 IU, Syntocinon-Spray; Novartis, Switzerland) in the morning and afternoon over six consecutive weeks (i.e., 48 IU/day) and placebo in the same way with cross-over administration (Fig. 1A). The placebo contained all the inactive ingredients that were included in the oxytocin spray. All the participants trained intranasal self-

administration before the trial initiated, and the manner of self-administration was confirmed at every two-week assessment point. On the last day of each six-week administration, a half of participants underwent clinical assessments, including ADOS, approximately 15min after their morning drug inhalation, and MRI-based measurement approximately 40min after their afternoon inhalation. The other half underwent these clinical and MRI-based evaluations with the order reversed. The examination order was randomly assigned to the participants. This time table was anticipated to ensure the same temporal interval between the current oxytocin administration and assessments as that in our previous single-dose trial (Watanabe, Abe, *et al.*, 2014).

Randomization and masking of drug administration

The manager of randomisation and masking of drug administration randomly assigned the participants to the two groups based on computer-generated randomised order: a group to which oxytocin was initially administered and one to which placebo was initially given. Oxytocin and placebo were stored in spray bottles of the same visual appearance (Victoria Pharmacy, Switzerland). The manager completely covered the bottle labels to keep drug types unknown to all the participants, their families, experimenters, clinicians and assessors including ADOS administrators and assessors.

Outcomes

The primary outcome was changes in ADOS (module 4, for verbally fluent adults; Lord *et al.*, 1989) between baseline and the administration endpoints. ADOS was evaluated by four administrators (HY/HK/YK/MK), the first three of whom completed a training course for research use of ADOS and were validated by the other certified administrator (MK). To

minimize inter-administrator variability, all the ADOS scores were rated by a single certified administrator (MK) using videos. ADOS scores, such as social reciprocity and communication scores, were calculated as the scores in the ADOS diagnostic algorithm. Childhood Autism Rating Scale 2 (CARS2) (Schopler *et al.*, 2010) was also rated as a primary outcome by a trained assessor (MK) to compare its sensitivity with that of ADOS.

The secondary outcomes were partly measured as behavioural responses and changes in functional MRI (fMRI) signals in *a priori* defined regions of interest (ROIs) during a social psychological task (Fig. 1B). Other secondary outcomes comprised Autism Spectrum Quotient (AQ) (Baron-Cohen *et al.*, 2001), Social Responsiveness Scale (SRS) (Constantino *et al.*, 2003), Repetitive Behaviour Scale (RBS) (Lam and Aman, 2007), State and Trait Anxiety Inventory (STAI)-state (Spielberger *et al.*, 1970), Centre for Epidemiologic Studies Depression Scale (CESD) (Radloff, 1977), Quality-of-Life questionnaire (QOL) (WHOQOL Group, 1995), and resting-state functional connectivity (rsFC) between the ROIs (Biswal *et al.*, 1995; Fair *et al.*, 2008; Raichle *et al.*, 2001).

These primary and secondary outcomes were evaluated at the baseline and at six-week administration endpoints, whereas some of the other secondary outcomes—including Clinical Global Impressions (CGI-EI) (Guy, 1976) and Global Assessment of Functioning (GAF) (Aas, 2011), and observational items for safety, such as blood pressures and pulse rate—were evaluated at every two-week assessment points by a psychiatrist (HY).

MRI data acquisition

MRI data were acquired in a 3T MRI scanner (GE Healthcare, UK) in The University of Tokyo Hospital with essentially the same protocol as in our previous trial (Watanabe, Abe, *et al.*,

2014). The quality of MRI data was controlled by daily monitoring over the course of this trial. Trained neuro-radiologists (HT/NY) found no gross anatomical abnormalities in the head of any participant.

For the anatomical co-registration, axial T2-weighted images were recorded (TE = 82.32ms, TR = 4400ms, FOV = 240×240mm², matrix = 256×256, slice thickness = 2.5mm, 62 axial slices). For task-related functional imaging, gradient-echo echo-planar sequences were recorded (TR = 3s, TE = 35ms, FA = 80°, 4×4×4mm³, 42 slices, ventral-to-dorsal interleaved acquisition). For resting-state functional imaging, different gradient-echo echo-planar sequences were adopted (TR = 3s, TE = 30ms, FA = 80°, 3×3×3mm³, 53 slices). The first five functional images in each session were discarded to allow for equilibrium of longitudinal magnetization.

Regions of interest

To compare the effect sizes of continual treatment with those of single-dose administration, we focused on oxytocin's effects on brain activity of two pre-defined ROIs (the anterior cingulate cortex, ACC; dorsal mPFC, dmPFC; Fig. 1B), because the ROIs were the only regions that showed significantly large responses to single-dose oxytocin compared with those to placebo in our previous trial (Watanabe, Abe, *et al.*, 2014). Based on this previous study, ACC and dmPFC were defined as spheres with a radius of 4mm and a centre at [2, 34, 8] and [0, 30, 52], respectively.

Analysis of resting-state fMRI data

Using resting-state fMRI data, we estimated rsFC between ACC and dmPFC. The fMRI data

were recorded while the participants were instructed to vaguely see a fixation point in the centre of the screen. We obtained 10min of the resting-state data (5min \times 2 sessions) for each participant. These fMRI data underwent realignment, slice-timing correction, normalisation to the standard template image (ICBM 152), temporal band-pass filtering (0.01–0.1Hz), and spatial smoothing (full-width half maximum, FWHM, = 8mm). Corrections for head motion (x/y/z/pitch/roll/yaw directions), whole-brain signals, ventricular signals, white matter signals, and the run effect were performed based on GLM using corresponding regressors. We finally computed the rsFC between the two ROIs by calculating a Pearson's correlation coefficient between the time series of the preprocessed fMRI signals from ACC and dmPFC.

Social cognition task

For direct comparison, we used the same psychological task and analysis procedures as in our previous studies (Watanabe *et al.*, 2012; Watanabe, Abe, *et al.*, 2014). In the fMRI scanner, the participants were sequentially presented with 80 monochrome short movies (1.5s) in which one of 20 professional actors spoke different emotional words (verbal information, V) with emotional facial and vocal expressions (nonverbal information, NV); for each movie, the participants were asked to make a judgement whether the actor looked like a friend or foe to them (Fig. 1B). The stimuli consisted of two types of congruent stimulus and two types of incongruent stimulus: the congruent stimuli comprised 'positive NV and V' (NV+V+) or 'negative NV and V' (NV-V-); the incongruent stimuli comprised 'positive NV and negative V' (NV+V-) or 'negative NV and positive V' (NV-V+). After sufficient training with different stimuli, the participants were pseudo-randomly presented with these movies and made friend/foe judgements. This psychological task took approximately 12min (6min \times 2 sessions).

Analysis of behavioural responses during a psychological task

If a participant made a friend/foe judgment mainly based on nonverbal information, the response was classified to a nonverbal-information-based judgment (NVJ). Otherwise, the response that was mainly based on verbal information was labelled as a verbal-information-based judgement (VJ). For instance, the ‘friend’ judgment of a stimulus with positive facial and vocal expressions and a negative word was classified to NVJ, because the participant was supposed to emphasise positive nonverbal information rather than negative verbal information. Note that VJ trials were also defined as a part of responses to incongruent stimuli. As in our previous studies (Watanabe *et al.*, 2012; Watanabe, Abe, *et al.*, 2014; Watanabe, Yahata, *et al.*, 2014), we focused on behavioural and neural responses during NVJ (Fig. 3A). We counted the number of NVJs for each participant for each assessment day, and also calculated the response time for NVJ, which was defined as a time between the start of the movie stimuli and pressing a button for indicating their judgments.

Eye gaze was tracked during the task using remote infrared-light camera. The data were analysed in the same manner as in our previous trial as follows (Watanabe, Abe, *et al.*, 2014): we first smoothed the data with a Gaussian filter, and detected blinking and artefacts; we then calculated the average fixation durations on the eyes or nose/mouth areas relative to the whole screen. The fixations were defined as maintaining a gaze on a target area for at least 100ms. The eye or nose/mouth areas were determined as a common area for all the movies in a relatively liberal manner.

Using the data about these behavioural responses, we calculated the effects of oxytocin and placebo. Each behavioural effect of a given drug was defined as a change between the start

and end of the drug administration (i.e., effect = end value – start value).

Analysis of brain activity during task

In SPM8 (www.fil.ion.ucl.ac.uk/spm/), the task-related fMRI data underwent realignment, correction of slice timing, normalisation to the default template with interpolation to a 2-mm cubic space, spatial smoothing (FWHM = 8mm, Gaussian filter), and high-pass temporal filtering (128s). At a single-participant level, we used a GLM for an event-related fMRI design with eight regressors (the four types of stimulus × the two types of response), and calculated the difference in fMRI signals for each ROI between NVJ and VJ, which was defined as NVJ-specific activity (i.e., NVJ–VJ). We then evaluated effects of oxytocin and placebo, which were defined as differences in brain activity between the start and end of the drug administrations.

Task-related functional connectivity between ACC and dmPFC was estimated by calculating the psycho-physiological interaction (PPI) implemented in SPM8 (Friston *et al.*, 1997). For direct comparison, we focused on NVJ-specific PPI from dmPFC to ACC, which was significantly sensitive to single-dose oxytocin (Watanabe, Abe, *et al.*, 2014). At a single-participant level, this PPI analysis used three regressors: one representing fMRI signals of the seed region (i.e., dmPFC), one representing a psychological factor (i.e., NVJ and VJ), and a PPI factor. At a group level, we evaluated the significance of PPI in a random effects model.

Statistics

We calculated oxytocin's effects on the primary and secondary outcomes using generalised estimating equations with an unstructured correlation and robust standard error estimates. Changes in these outcomes during each six-week administration were treated as the dependent

variables. Main effects and interactions for the pharmacologically different conditions (oxytocin/placebo) and the order of drug administration (first/second) were estimated with a significance level at $P < 0.05$. We analysed the secondary outcomes and observational items that were assessed every two weeks in essentially the same manner with the order of assessments as a factor, instead of the drug order. Changes in rsFC and brain activity in ACC/dmPFC were evaluated based on ROI analysis ($P < 0.05$ in paired t -tests) and voxel-based analysis around the ROIs ($P_{\text{FWE}} < 0.05$ in small volume correction, SVC). The spatial specificity of the ROI analysis was also confirmed in a whole-brain analysis with a statistical threshold at $P_{\text{uncorrected}} < 0.001$ and voxel-size > 20 (Lieberman and Cunningham, 2009; Wandschneider *et al.*, 2014). We performed corrections for multiple comparisons among all the 23 outcomes including the primary and secondary outcomes (i.e., all the scores listed in Tables 2 and 3) by calculating false discovery rates (FDRs) (Benjamini and Hochberg, 2000). The threshold for FDR was set at 0.05.

Results

Between March 2012 and December 2012, 20 high-functioning adult males with ASD were enrolled in this trial, and final assessment at 12th-week endpoint was performed between August 2012 and April 2013. Two participants discontinued this trial by themselves because of feeling deterioration of their psychiatric symptoms: one of them was in oxytocin administration period in oxytocin-first group, whereas the other was in placebo period in placebo-first group (Fig. 1A, Table 1). In addition, due to anxiety attacks in an MRI scanner, another individual could not complete his final fMRI session that was originally planned after six-week placebo

administration. Hence, oxytocin's effects on clinical scores were assessed based on behavioural observations of 18 individuals, whereas its neural effects were evaluated based on fMRI data of 17 participants. No major adverse effects were observed, but four participants reported acute mild nose irritation (two: placebo period, two: oxytocin period), one had diarrhoea during a placebo period, and one felt tired during the entire trial. Fifteen of the 20 individuals participated in our previous single-dose trial that was conducted more than a year before this trial (Watanabe, Abe, *et al.*, 2014).

Clinical effects

We first found that six-week oxytocin administration could improve autistic symptoms related to social reciprocity (ADOS reciprocity: $P = 0.034$, Cohen's $d = 0.78$, a generalised estimating equation, $P_{\text{FDR}} < 0.05$; ADOS communication: $P = 0.78$, Cohen's $d = 0.03$; Fig. 2A) with no significant main effect of administration order (first/second) or no significant interaction between drug type (oxytocin/placebo) and administration order ($P > 0.87$).

This effect size for ADOS reciprocity was preserved even when the effect was separately calculated within oxytocin-first group ($d = 0.86$) and placebo-first group ($d = 0.71$) (Fig. 2B). In addition, even focusing on parallel-group comparison in the first half period of this crossover trial, this effect of oxytocin on ADOS reciprocity preserved its effect size ($d = 0.72$; Fig. 2C). Moreover, a comparable effect size was also seen in psychotropic-free participants after excluding one participant with continual medication of serotonin-norepinephrine reuptake inhibitors for his recurrent major depression ($d = 0.74$). Furthermore, even when focusing on changes within the oxytocin periods and comparing post-period ADOS scores with pre-period scores, we could still observe a significant proportional decrease in

ADOS reciprocity score (%change in ADOS reciprocity: $P = 0.025$, $d = 0.58$; cf. %change in ADOS communication: $P = 0.38$, $d = 0.20$). These results show that, in a social reciprocity domain, six-week oxytocin administration improved an ASD core symptom.

In contrast to the ADOS reciprocity score, neither significant main effects nor interactions were found in other primary and secondary clinical outcomes including ADOS communication score, ADOS repetitive behaviour score, CARS2 total score, SRS, RBS, AQ, QOL, CESD, and STAI ($P > 0.1$; Table 2).

Amongst the secondary outcomes and observational items recorded every two weeks, CGI-EI and pulse rate showed significant interactions between assessment order and type of drug (CGI-EI, $P = 0.02$, $P_{\text{FDR}} < 0.05$; pulse rate, $P < 0.001$, $P_{\text{FDR}} < 0.05$). CGI-EI was also affected by the assessment order ($P = 0.02$; Table 3). These results might reflect larger effects of the assessment order within oxytocin treatment period (CGI-EI and pulse rate, $P < 0.01$.) than within placebo treatment period (CGI-EI, $P = 0.19$; pulse rate, $P = 0.85$).

Effects on intrinsic functional connectivity

This improvement of social reciprocity deficits was accompanied by enhancement of intrinsic functional coordination between pre-defined ACC and dmPFC (Fig. 1B). Six-week oxytocin intervention significantly increased rsFC between ACC and dmPFC than placebo administration ($t_{16} = 3.5$, $P = 0.00029$, $d = 1.1$ in a paired t -test, $P_{\text{FDR}} < 0.05$; Fig. 2D), independently of the administration order (oxytocin-first group, $t_{16} = 3.6$, $P = 0.002$, $d = 1.2$; placebo-first group, $t_{16} = 3.0$, $P = 0.008$, $d = 1.0$). The spatial specificity of this effect was confirmed by a whole-brain analysis ($t = 3.6$, $P_{\text{uncorrected}} < 0.001$ and voxel-size > 20 ; Fig. 2E).

Moreover, this rsFC increase was strongly correlated with the oxytocin-induced

decrease in ADOS reciprocity score ($\rho = -0.60$, $P = 0.011$; Fig. 2F). Such a correlation suggests that neurobiological changes in mPFC underlie the mitigation of the social reciprocity deficits, and provides further validation to the observed clinical effect of oxytocin.

Behavioural and neural effects during a psychological task

We then compared effect sizes of this six-week regimen with those of our previous single-dose intervention (Watanabe, Abe, *et al.*, 2014).

First, we confirmed that the current six-week intervention reproduced qualitatively the same behavioural and neural effects as those observed in our previous single-dose trial (Watanabe, Abe, *et al.*, 2014): although effects on response time for NVJ were moderate ($t_{16} = 1.9$, $P = 0.07$, $d = 0.50$; Fig. 3C), oxytocin-induced increases in the number of NVJ and fixation time on eye areas were significant (NVJ number, $t_{16} = 2.6$, $P = 0.019$, $d = 0.62$, Fig 3B; fixation time, $t_{16} = 2.3$, $P = 0.03$, $d = 0.56$, Fig. 3D; both, $P_{FDR} < 0.05$).

In addition to these behavioural effects, oxytocin significantly increased originally-impaired NVJ-specific activity in ACC and dmPFC (ACC, $t_{16} = 4.2$, $P = 0.00069$, $d = 0.97$; dmPFC, $t_{16} = 3.8$, $P = 0.0014$, $d = 0.92$ in paired t -tests; $P_{FWE} < 0.05$ in SVC; Fig. 4A), which was confirmed by a whole-brain analysis ($t > 3.5$, $P_{uncorrected} < 0.001$, voxel size > 20 ; Fig. 4B). Oxytocin also increased the task-related functional connectivity (PPI) from dmPFC to ACC ($t_{16} = 3.5$, $P = 0.0028$, $d = 0.83$ in a paired t -test, $P_{FWE} < 0.05$ in SVC; Fig. 4C) with a significant spatial specificity ($t = 3.8$, $P_{uncorrected} < 0.001$, voxel size > 20 ; Fig. 4D).

Furthermore, as in the single-dose trial, these neural effects were predictive of the oxytocin-induced increase in the number of NVJ (ACC, $r = 0.62$, $P = 0.01$; dmPFC, $r = 0.69$, $P = 0.002$; dmPFC–ACC, $r = 0.55$, $P = 0.02$; Fig. 4E). As a whole, these behavioural and neural

responses to the six-week oxytocin administration are qualitatively comparable to those to the previous single-dose intervention (Watanabe, Abe, *et al.*, 2014).

Comparison of effect size between single-dose and six-week regimens

Quantitatively, however, despite its length, the current six-week regimen did not magnify the effects of oxytocin seen in the single-dose administration (Fig. 4F). Based on Cohen's classification (Cohen, 1992), almost all the behavioural and neural effects of this six-week intervention had the same levels of effect size as those observed in the single-dose trial (Watanabe, Abe, *et al.*, 2014): effects on the number of NVJ and response time for NVJ were categorized into medium in both regimens (single-dose/six-week: NVJ number, $d = 0.55/0.62$; response time for NVJ, $d = 0.63/0.50$); neural effects were large in both (single-dose/six-week: ACC activity, $d = 1.1/0.97$; dmPFC activity, $d = 1.1/0.92$; task-related functional connectivity, $d = 0.78/0.83$). Only eye-gaze effect was weaker in the six-week administration than in the single-dose treatment (single-dose/six-week: $d = 0.83/0.56$, Cohen's category: large/medium).

Relationship between clinical and task-related effects

These neural effects measured in the psychological task also provided further validation to oxytocin's clinical effects: the decrease in ADOS reciprocity score was correlated with the increase in NVJ-specific activity of ACC and dmPFC (ACC: $\rho = -0.67$, $P = 0.0032$, Fig. 5A; dmPFC: $\rho = -0.60$, $P = 0.011$, Fig. 5B) and the enhancement of the task-related functional connectivity from dmPFC to ACC ($\rho = -0.61$, $P = 0.010$; Fig. 5C). These associations suggest that oxytocin's clinical effects on ASD social reciprocity deficits may be supported by biological modulations in mPFC.

Discussion

These findings suggest that the current six-week intranasal administration of oxytocin could clinically mitigate an ASD core symptom about social reciprocity with enhancement of brain activity and functional coordination in mPFC. Moreover, we showed that the current six-week administration also significantly improved behavioural responses and medial prefrontal activity during the task, although the continual intervention did not amplify effects seen in the single-dose trial.

Such reciprocity-specific effects of oxytocin are consistent with previous findings about effects of single-dose oxytocin on neurotypical individuals (Shamay-Tsoory *et al.*, 2009; van IJzendoorn and Bakermans-Kranenburg, 2012) and ASD individuals (Hollander *et al.*, 2007). Although it did not always positively affect neurotypical individuals (Bartz *et al.*, 2011; Bethlehem *et al.*, 2014; De Dreu *et al.*, 2010; 2011; Shalvi and De Dreu, 2014; Shamay-Tsoory *et al.*, 2009; Stallen *et al.*, 2012), single-dose oxytocin was found to enhance social cognition such as reciprocal cooperation and in-group trust, which indicates qualitative robustness of the current observations.

The current observations are based on an exploratory trial; thus, the relatively small number of participants is a potential limitation. In addition, although the oxytocin-induced changes were statistically significant and the proportional changes seem to be large (Table 2), raw figures of such clinical score changes appear to be relatively small. Therefore, it may be somewhat difficult to conclude clinically beneficial effects of continual oxytocin regimens merely based on the current results. However, despite this small sample size and seemingly

small raw values of the effects, all the observed significant effects had an effect size of >0.5 , and were classified into medium or large effects (Cohen, 1992). In addition, the improvement of ADOS reciprocity score was significantly correlated with multiple neural effects of oxytocin (Figs. 2F and 5). Moreover, the decrease in ADOS reciprocity score was weakly correlated with the improvement of QOL (ADOS reciprocity change v QOL change, $r = -0.21$). Furthermore, the effect size of the oxytocin-induced improvement of QOL score was relatively large ($d = 0.45$; Table 2). Therefore, these relatively large effect sizes and correlations with biological changes and other clinical scores are considered to validate the findings about the clinical benefits of continual oxytocin administration. Future studies with a larger sample size may detect significant improvement of other clinical scores.

This study used ADOS, a semi-structured, trained clinician administered, and video-taped assessment, as its primary outcome, because this ASD-specific assessment system is considered to be more reproducible than other subjective and self-reporting scoring systems. Although effectiveness of this diagnosis-oriented scale as a primary endpoint should be tested in more large-scale future trials, the current significant ADOS-based results provide face validation to this selection of clinical outcomes.

The different effects of oxytocin on different clinical scores (Table 2) may suggest some properties of clinical scores that could increase detectability of oxytocin's effects. First, the differences between effects on ADOS sub-domains and those on CARS2 Total suggest that oxytocin's behavioural effects may be more detectable in domain-specific core symptoms than general scales. In addition, the differences between ADOS, CES-D, and STAI states imply that effects on core symptoms may be generally larger than those on comorbid symptoms. Moreover, comparisons between ADOS, AQ, SRS, and QOL scales indicate that objective

scoring systems (i.e., ADOS and SRS) might have larger detectability than self-reporting methods (i.e., QOL and AQ). Although inter-individual difference may deteriorate the sensitivity (e.g., one psychologist evaluates ADOS scoring; 15 different parents score SRS and RBS), evaluations by trained examiners (i.e., ADOS) may also increase sensitivity to oxytocin's effects. Finally, considering low detectability of unstructured scales such as CARS2, CGI, and GAF (Table 3), semi-structured scoring systems (e.g., ADOS) may be another factor to enhance the sensitivity.

The current results may have a significant influence on future biological studies and clinical trials on oxytocin's effects on ASD. In theory, it is not necessarily the case that continual oxytocin administration induces the same effects seen in single-dose trials. In fact, recent clinical trials have reported the difficulty in detecting statistically significant effects of oxytocin in continual intervention (Anagnostou *et al.*, 2014, Dadds *et al.*, 2014, Guastella *et al.*, 2014), which has raised a possibility that continual regimes induce negative feedback or down-regulation and indicated the necessity to search for the optimal time interval between oxytocin administrations to avoid the activation of such negative feedback systems and the decline of oxytocin's therapeutic effects. In contrast, the current findings appear to suggest that such adverse effects of continual intervention are smaller than expected. In addition, the similarity of the effect size between the current continual administration and previous single-dose trial seems to indicate another possibility that oxytocin's effects on ASD depend on the dose not on the length of administration. If the current observations are applicable to the general ASD population, future studies would need to search for the optimal dose of oxytocin rather than the administration interval. Collectively, therefore, the current findings appear to indicate the necessity to re-consider the effects of long-term oxytocin treatment and its underlying

neurobiological mechanisms, and may consequently influence the direction of future clinical trials and biological studies on oxytocin's effects on ASD.

Although future studies with a larger sample size are necessary to confirm the current clinical effect, this study suggests that long-term intranasal administration of oxytocin could ameliorate clinically relevant, ASD core social symptoms with robust neural effects.

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Conflict of Interest

The authors declare no conflict of interests.

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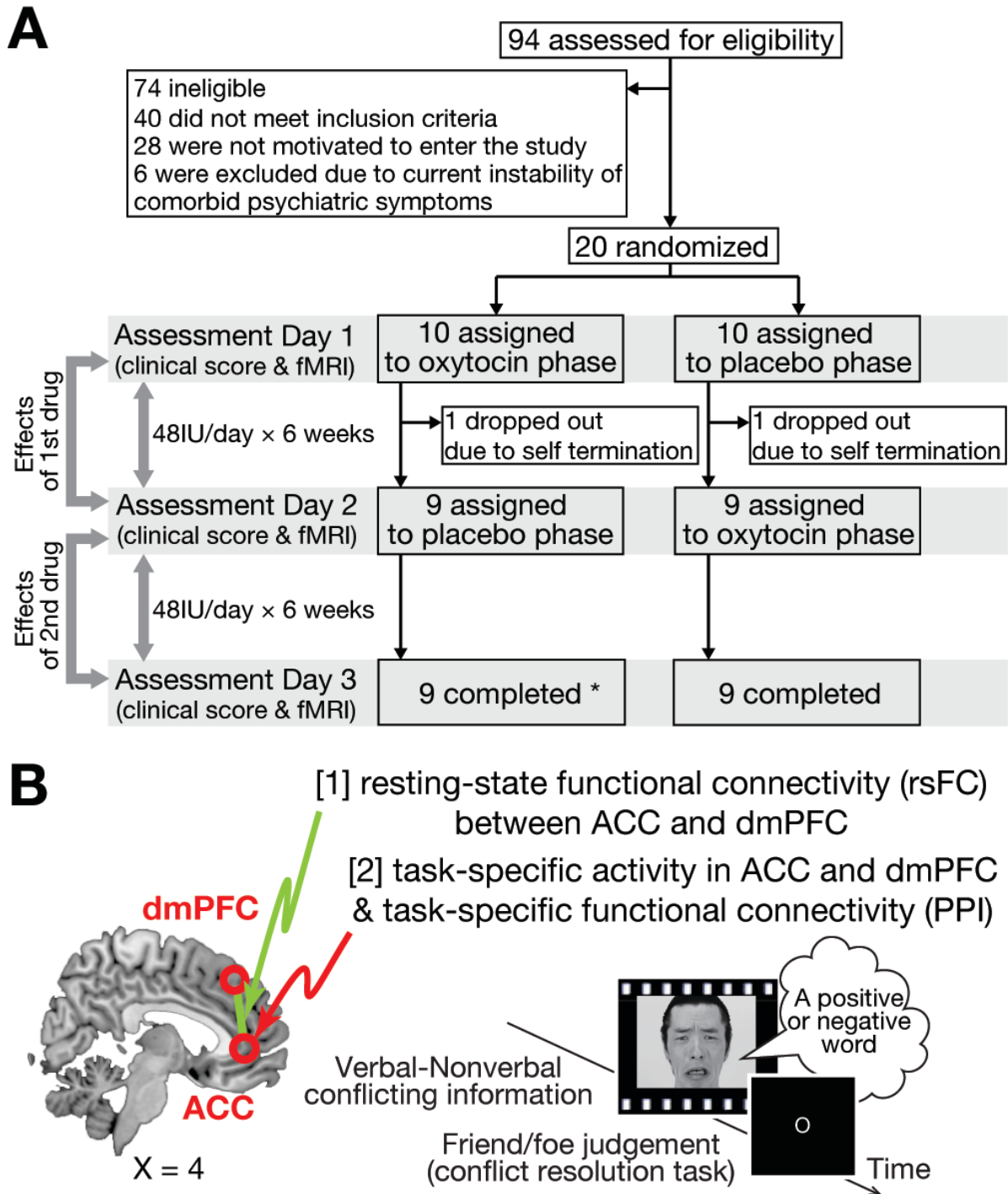
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Figure legends

Fig. 1



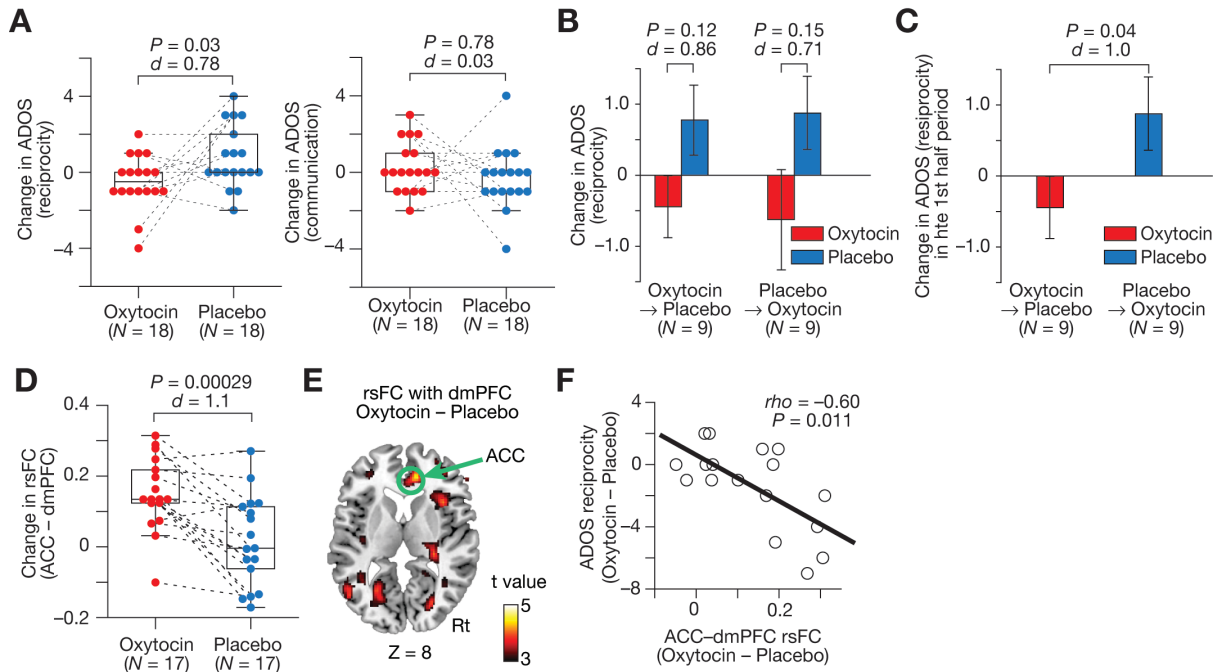
Experiment design.

A. Trial profile. The current exploratory randomised controlled trial first assessed 94 potential individuals, and selected 20 high-functioning adult ASD males (see Methods).

After random assignment to oxytocin-first or placebo-first groups, the 20 participants started the 12-week trial. In both groups, one participant quit the trial by himself because they felt worsening of their psychiatric symptoms. The asterisk indicates that another participant in oxytocin-first group did not complete his fMRI session on Assessment Day 3 after six-week placebo inhalation. Therefore, clinical effects were assessed based on data from the 18 participants (Table 1), whereas effects related to a psychological task were evaluated on data from the 17 individuals. Effects of each intervention were defined as differences between before and after the administration.

B. Evaluation of oxytocin's neural effects. Oxytocin's neural effects were evaluated using fMRI signal of ACC and dmPFC, because the activity of these regions was originally impaired in ASD individuals (Watanabe *et al.*, 2012) and was sensitive to single-dose oxytocin (Watanabe, Abe, *et al.*, 2014). First, we measured rsFC between ACC and dmPFC. Second, using the same psychological task as one used in our previous studies (Watanabe *et al.*, 2012; Watanabe, Abe, *et al.*, 2014; Watanabe, Yahata, *et al.*, 2014), we examined effects on task-related activity of these regions and task-related functional connectivity (psycho-physiological interaction, PPI) from dmPFC to ACC. In the psychological task, participants were asked to make friend/foe judgements based on short videos whose verbal and nonverbal information were conflicting to each other.

Fig. 2



Effects on clinical scores and rsFC.

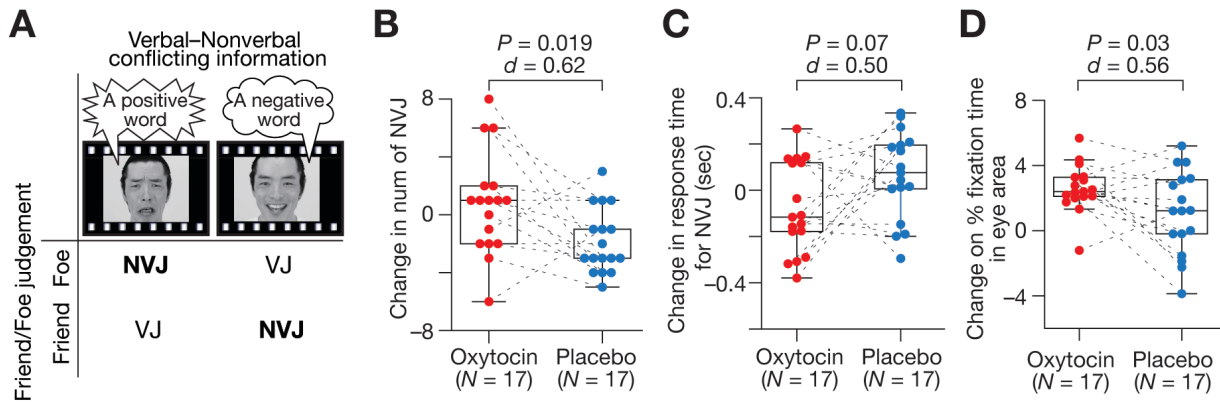
A, B, and C. Effects on clinical score. (A) Unlike ADOS communication score, ADOS social reciprocity scores were significantly reduced by six-week oxytocin administration. The boxes and bars show box-whisker plots. (B) Although P values did not reach a threshold, the large size of the effect (Cohen's d) on ADOS reciprocity score was essentially preserved even when we separately analysed the effects in oxytocin-first and placebo-first groups. Error bars: s.e.m. (C) This effect was also seen in parallel-group comparison of drugs' effects on ADOS reciprocity score. Even when focusing on the first half period of the trial, we could observe a comparable size of oxytocin's effect on ADOS reciprocity score. Error bars: s.e.m.

D and E. Effects on rsFC. (D) Oxytocin also significantly increased rsFC between ACC and dmPFC. (E) The spatial specificity of the effect on rsFC was confirmed by a whole-brain analysis searching for brain regions with a large increase in rsFC with dmPFC. The circle

represents approximate location of pre-defined ACC.

F. Correlation between improvement of clinical scores and enhancement of rsFC. The oxytocin-induced change in ADOS reciprocity score was significantly correlated with that in rsFC between ACC and dmPFC. Each circle represents each participant.

Fig. 3

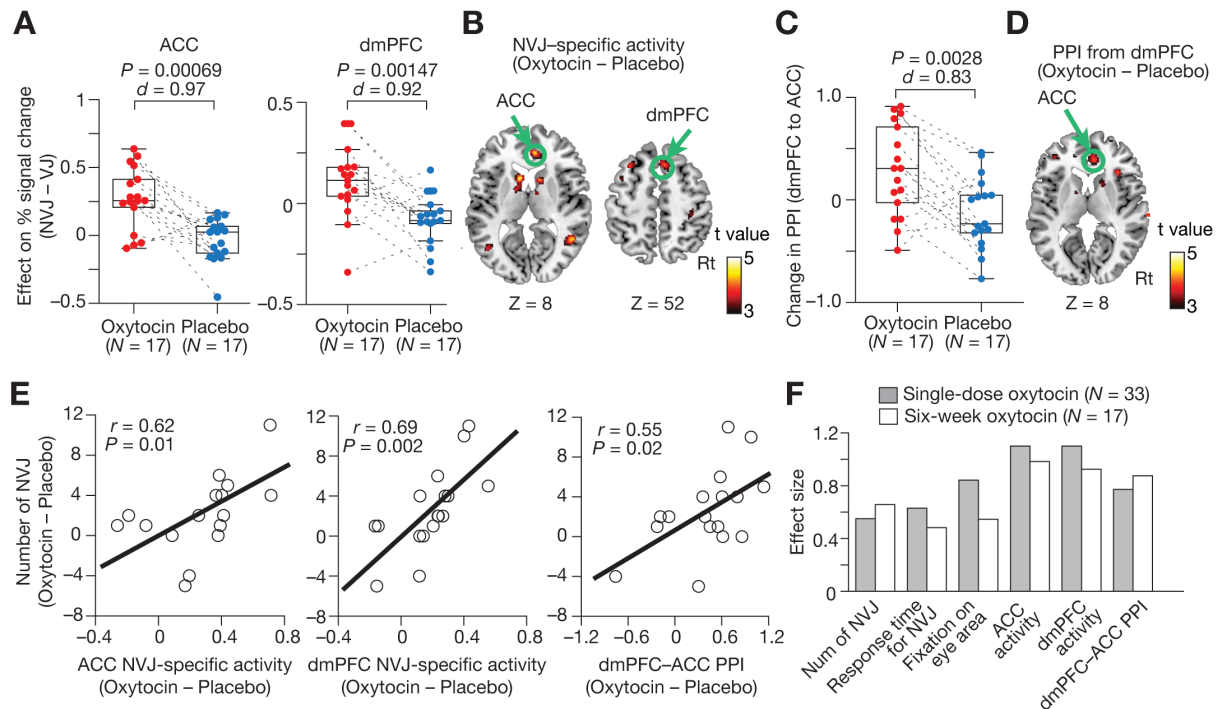


Behavioural responses during a psychological task.

A. Task paradigm. In this social cognition task, friend/foe judgements participants made were classified into verbal-information-based (VJ) or nonverbal-information-based judgments (NVJ). For direct comparison of effect size with our single-dose trial (Watanabe, Abe, *et al.*, 2014), we focused on behavioural and brain responses related to NVJ.

B, C, and D. Behavioural effects. Consistently with effects of single-dose intervention, six-week oxytocin significantly increased the number of NVJ (B) and fixation time on eye areas (D). The effect on response time for NVJ was moderate (C).

Fig. 4



Neural responses during psychological tasks

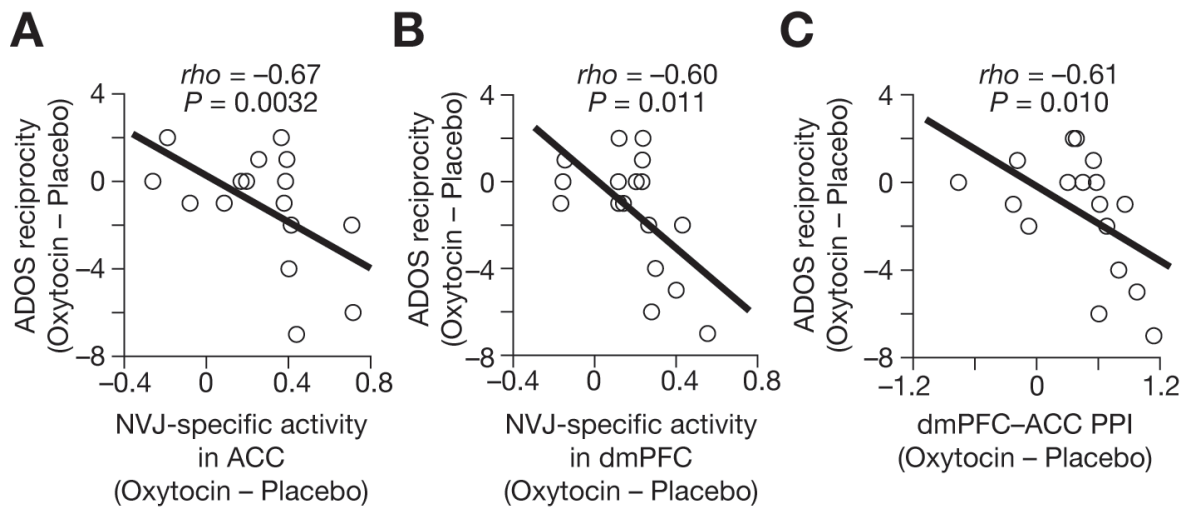
A and B. Effects on brain activity. (A) Oxytocin significantly increased NVJ-specific activity in ACC and dmPFC. (B) The locational specificity of these effects were validated by a whole-brain analysis. Circles represent approximate location of the pre-defined regions.

C and D. Effects on task-related functional connectivity. (C) Six-week administration also increased task-related functional connectivity (i.e., NVJ-specific PPI) from dmPFC to ACC. (D) A whole-brain PPI analysis with dmPFC a seed provided locational validation.

E. Correlations between oxytocin's behavioural and neural effects. In the psychological task, the behavioural response was correlated with the neural responses. Each circle represents each participant.

F. Comparison of effect size. Despite its continual administration, the effects of the current six-week intervention were not larger than those seen in our single-dose trial using the same psychological task (Watanabe, Abe, *et al.*, 2014).

Fig. 5



Correlations between improvement of clinical score and enhancement of brain activity.

The improvement of ASD social reciprocity score was not only correlated with an increase in ACC-dmPFC rsFC (Fig. 2F), but also accompanied by enhancement of brain activity measured during the psychological task, such as the increases in ACC and dmPFC activity (A and B) and in task-related functional connectivity (PPI) from dmPFC to ACC (C).

Table 1. Demographic Table

	Oxytocin-initially administered group (<i>N</i> = 9) Mean (SD)	Placebo-initially administered group (<i>N</i> = 9) Mean (SD)	<i>P</i> value
Age (range)	35.1(7.6), 24–42	29.3 (5.9), 24–43	0.09
Height, cm	169.7 (3.8)	167.3 (4.9)	0.27
Body weight, kg	65.4 (12.6)	66.4 (18.0)	0.89
SES *	2.7 (0.97)	2.6 (1.0)	0.64
Parental SES *	2.0 (0.50)	2.4 (0.73)	0.15
Handedness: Right/Mixed/Left	9/0/0	6/1/2	
IQ **			
Full IQ	109.3 (9.1)	101.8 (12.6)	0.17
Verbal IQ	119.6 (8.1)	104.5 (12.0)	0.01
Performance IQ	90.9 (12.6)	97.8 (18.9)	0.4
Autism Diagnostic Interview-Revised			
Social	14.0 (6.7)	15.6 (7.2)	0.67
Communication	13.0 (4.8)	11.3 (3.5)	0.44
Repetitive	4.1 (2.9)	4.6 (2.1)	0.74

* SES assessed using the Hollingshead. Higher scores indicate lower status. ** The intelligence quotients were measured using the Wechsler Adult Intelligence Scale. SES: socio-economic status, IQ: intelligence quotient.

Table 2. Oxytocin’s effects on main and secondary outcomes measured every six weeks.

Six week interval items		Baseline	6th week	12th week	OT-induced change	PL-induced change	P value	Cohen's d	
Main outcomes	ADOS: reciprocity	OT-in (n=9)	8.3 (2.1)	7.9 (2.6)	8.7 (2.2)	-8.8 (15.2) %	12.2 (24.1) %	0.03†	0.78
		PL-in (n=9)	7.8 (1.4)	8.6 (2.3)	8.0 (2.1)				
	ADOS: communication	OT-in (n=9)	3.8 (1.0)	3.8 (1.2)	4.0 (1.2)	9.3 (43.7) %	4.7 (41.6) %	0.78	0.03
		PL-in (n=9)	3.7 (1.9)	3.3 (1.1)	3.7 (1.6)				
	ADOS: repetitive	OT-in (n=9)	0.3 (0.5)	0.3 (0.7)	0.1 (0.3)	2.5 (34.3) %	-13.9 (38.0) %	0.26	0.24
		PL-in (n=9)	0.4 (0.5)	0.3 (0.5)	0.3 (0.5)				
	CARS: total	OT-in (n=9)	30.6 (2.8)	31.1 (3.2)	30.7 (2.7)	0.41 (1.6) %	-0.10 (2.4) %	0.55	0.23
		PL-in (n=9)	31.0 (3.1)	30.9 (3.5)	30.8 (3.4)				
Secondary outcomes	SRS	OT-in (n=6)	86.7 (20.7)	80.0 (23.3)	77.1 (27.8)	-4.9 (17.1) %	-5.5 (16.5) %	0.39	0.32
		PL-in (n=9)	73.2 (27.4)	71.4 (24.7)	66.2 (21.5)				
	RBS	OT-in (n=6)	14.2 (10.9)	13.7 (12.5)	11.8 (11.2)	2.6 (64.8) %	-32.5 (61.8) %	0.86	0.14
		PL-in (n=9)	12.2 (9.3)	10.9 (8.4)	7.2 (5.3)				
	rsFC: ACC-dmPFC	OT-in (n=9)	0.18 (0.09)	0.32 (0.09)	0.18 (0.04)	0.15 (0.10)	-0.01 (0.13)	<0.001†	1.1
		PL-in (n=8)	0.17 (0.06)	0.16 (0.08)	0.31 (0.06)				
	NVJ number	OT-in (n=9)	24.7 (4.4)	25.0 (3.5)	23.9 (3.5)	1.9 (9.6) %	-4.5 (8.3) %	0.019†	0.62
		PL-in (n=8)	27.3 (4.9)	26.2 (3.1)	26.5 (3.0)				
	NVJ response time	OT-in (n=9)	2.6 (0.16)	2.2 (0.16)	2.4 (0.26)	-9.9 (6.9) %	-2.5 (11) %	0.09	0.46
		PL-in (n=8)	2.4 (0.22)	2.4 (0.22)	2.2 (0.12)				
	Fixation time for eye area	OT-in (n=9)	25.5 (3.1)	29.4 (3.7)	25.8 (2.9)	11.8 (6.7) %	4.5 (1.9) %	0.03†	0.53
		PL-in (n=8)	27.9 (2.7)	29.9 (3.9)	33.0 (5.6)				
	NVJ-specific activity: ACC	OT-in (n=9)	0.10 (0.07)	0.37 (0.08)	0.27 (0.11)	0.27 (0.22)	-0.033 (0.16)	<0.001†	0.97
		PL-in (n=8)	0.08(0.07)	0.12(0.12)	0.38 (0.09)				
	NVJ-specific activity: dmPFC	OT-in (n=9)	0.17 (0.1)	0.28 (0.09)	0.19 (0.08)	0.10 (0.17)	-0.083 (0.12)	0.0014†	0.92
		PL-in (n=9)	0.14 (0.07)	0.16 (0.06)	0.27(0.08)				
	Task-related func. connectivity	OT-in (n=9)	0.11 (0.12)	0.39 (0.11)	0.24 (0.09)	0.28 (0.44)	-0.13 (0.34)	0.002†	0.83
		PL-in (n=8)	0.18 (0.13)	0.09 (0.06)	0.35 (0.15)				
	AQ score	OT-in (n=9)	36.8 (6.5)	34.0 (8.3)	33.2 (9.4)	-2.2 (10.0) %	-0.5 (9.0) %	0.77	0.1
		PL-in (n=9)	34.5 (4.7)	34.2 (5.9)	35.3 (5.4)				
QOL	OT-in (n=9)	3.25 (0.65)	3.28 (0.57)	3.20 (0.65)	3.5 (7.2) %	0.4 (9.8) %	0.1	0.45	
	PL-in (n=9)	2.68 (0.82)	2.66 (0.96)	2.79 (0.91)					
STAI state	OT-in (n=9)	44.1 (13.4)	46.4 (13.1)	46.3 (13.8)	5.9 (36.4) %	-3.1 (19.0) %	0.9	0.04	
	PL-in (n=9)	50.7 (17.1)	50.6 (17.1)	47.1 (17.8)					
CES-D	OT-in (n=9)	16.1 (11.1)	16.0 (10.8)	14.8 (8.8)	-0.088 (25.3) %	-4.9 (35.6) %	0.48	0.23	
	PL-in (n=9)	22.2 (11.9)	20.7 (15.2)	20.2 (16.6)					

Note that figures in “Baseline”, “6th week”, and “12th week” columns represent raw values, whereas, except fMRI-oriented outcomes, those in OT-/PL-induced changes denote the proportion of the change values to the values before the administrations (mean ± std). OT: Oxytocin; PL: Placebo; OT-/PL-in: OT-/PL-initially; ADOS: Autism Diagnostic Observation Scale; CARS: Childhood Autism Rating Scale; SRS: Social Responsiveness Scale; RBS: Repetitive Behaviour Scale; NVJ: Non-verbal information-based judgment; AQ: Autism spectrum quotient; QOL: Quality-of-Life questionnaire; STAI: State and Trait Anxiety Inventory; CES-D: Centre for Epidemiologic Studies Depression Scale; ACC: Anterior cingulate cortex; dmPFC: dorso-medial prefrontal cortex; rsFC: resting-state functional connectivity. † indicates $P_{FDR} < 0.05$.

Table 3. Oxytocin’s effects on outcomes that were measured every two weeks

		Baseline	Period 1			Period 2			P value			
			2nd week	4th week	6th week	8th week	10th week	12th week	Time x Treatment	Treatment	Time	
Secondary outcomes	CGI-EI	OT-in (n=9)	NA	10.8 (2.1)	9.9 (2.7)	12.1 (1.8)	11.7 (2.0)	10.3 (2.8)	12.6 (1.3)	0.02*†	0.76	0.02
		PL-in (n=9)	NA	10.9 (2.0)	12.2 (1.9)	12.1 (1.8)	12.5 (1.3)	11.2 (2.1)	10.8 (2.1)			
	CGI-SI	OT-in (n=9)	4.2 (1.0)	4.1 (0.9)	4.0 (0.9)	3.9 (0.6)	4.0 (0.9)	4.0 (0.7)	4.0 (0.7)	0.5	0.13	0.52
		PL-in (n=9)	4.1 (0.8)	4.0 (0.7)	4.0 (0.7)	4.0 (0.9)	4.0 (0.7)	4.0 (0.9)	4.0 (0.9)			
	GAF	OT-in (n=9)	47.0 (5.4)	48.2 (5.3)	49.4 (5.2)	47.9 (6.2)	50.7 (5.0)	48.7 (6.5)	47.9 (7.2)	0.33	0.56	<0.001
		PL-in (n=9)	46.2 (5.9)	46.2 (6.7)	46.6 (6.9)	49.9 (5.0)	47.7 (6.8)	50.9 (4.9)	51.1 (4.9)			
Observational items	SBP	OT-in (n=9)	115.6 (13.7)	117.3 (12.3)	114.1 (15.3)	117.0 (10.7)	118.7 (13.4)	116.7 (11.2)	114.7 (12.1)	0.08	0.09	0.96
		PL-in (n=9)	105.4 (7.7)	117.7 (16.1)	119.3 (10.1)	116.8 (13.8)	110.4 (18.8)	116.4 (12.6)	116.9 (14.4)			
	DBP	OT-in (n=9)	65.6 (13.6)	71.6 (13.7)	73.8 (7.8)	61.6 (6.7)	72.6 (14.0)	70.2 (10.9)	67.4 (7.5)	0.18	0.36	0.001
		PL-in (n=9)	58.4 (7.5)	66.2 (14.0)	69.0 (9.2)	66.2 (12.4)	65.1 (11.7)	71.1 (10.5)	66.1 (12.9)			
	PR	OT-in (n=9)	72.1 (9.5)	79.0 (10.6)	76.1 (8.1)	72.4 (10.8)	75.4 (6.9)	74.8 (12.5)	70.2 (11.7)	< 0.001***†	0.84	0.24
		PL-in (n=9)	65.2 (7.6)	73.8 (14.7)	73.8 (10.9)	68.6 (9.6)	79.0 (15.7)	78.8 (10.7)	74.7 (9.2)			

CGI: Clinical Global Impressions; GAF: Global Assessment of Functioning; SBP: Systolic blood pressure; DBP: diastolic blood pressure; PR: Pulse rate. * The interaction indicates greater time effect in oxytocin treatment ($P < 0.001$) than in placebo treatment ($P = 0.19$). ** The interaction indicates greater time effect in oxytocin treatment ($P < 0.001$) than in placebo treatment ($P = 0.85$). † indicates $P_{FDR} < 0.05$.