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

Takamitsu Watanabe,1,2,3,* Miho Kuroda,4,* Hitoshi Kuwabara,4 Yuta Aoki,1 Norichika Iwashiro,1 Natsubori Tatsunobu,1 Hitemasu Takao,5 Yasumasa Nippashi,5 Yuki Kawakubo,4 Akira Kunimatsu,5 Kiyoto Kasai1 and Hidenori Yamasue1,6

*These authors contributed equally to this work.

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, randomized, double-blind, placebo-controlled, crossover trial including 20 high-functional adult males with autism spectrum disorder. Data obtained from 18 participants who completed the trial showed that 6-week intranasal administration of oxytocin significantly reduced autism core symptoms specific to social reciprocity, which was clinically evaluated by Autism Diagnostic Observation Scale ($P = 0.034$, $P_{FDR} < 0.05$, 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 ($rbo = -0.60$, $P = 0.011$), which was measured by functional magnetic resonance imaging. Moreover, using the same social-judgement task as 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, $P_{FDR} < 0.05$). Furthermore, despite its longer administration, these effect sizes of the 6-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.

1 Department of Neuropsychiatry, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
2 Department of Physiology, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
3 Institute of Cognitive Neuroscience, University College London, 17 Queen Square, London, WC1N 3AR, UK
4 Department of Child Neuropsychiatry, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
5 Department of Radiology, School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
6 Japan Science and Technology Agency, CREST, 5 Sambanchou, Chiyoda-ku, Tokyo 102-0075, Japan

Correspondence to: H. Yamasuem,
Department of Neuropsychiatry,
Graduate School of Medicine,
Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with over 1% prevalence, and has 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 (Hollander et al., 2007; Andari et al., 2010; Yamasue et al., 2012; Zinik and Meyer-Lindenberg, 2012; Bakermans-Kranenburg and van IJzendoorn, 2013; Gordon et al., 2013; Veening and Olivier, 2013; Aoki et al., 2014, 2015; Domes et al., 2014; Lin et al., 2014; Watanabe et al., 2014a); however, there is no clear evidence that such potentially beneficial responses in laboratories can consequently induce significant effects in individuals with ASD 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 comprising 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 randomized trials of continual administration have not detected clinically valuable effects of oxytocin on ASD with statistical significance. A 6-week trial, which compared 10 oxytocin-treated ASD adults with nine placebo-given 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 5 days (Dadds et al., 2014) or 26/24 children over 8 weeks (Guastella et al., 2015), could not detect any significant changes in 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, hypothesis (ii) was also suggested by a recent study that, using prairie voles, implied that chronic oxytocin treatment could trigger downregulation 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 randomized, double-blind, placebo-controlled, crossover trial. To examine hypothesis (i), we assessed clinical effects of 6-week administration of oxytocin using the Autism Diagnostic Observation Scale (ADOS) (Lord et al., 1989), which is a standard diagnosis tool for ASD but recently has been increasingly adopted as a primary outcome in ASD-related trials (Owley et al., 2001; Aldred et al., 2004; Howlin et al., 2007; Green et al., 2010; 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., 2006; Cox et al., 2012; Lynch et al., 2013; Di Martino et al., 2014; Itahashi et al., 2014; Jung et al., 2014).

To evaluate hypothesis (ii), we used the same psychological task as in our previous single-dose trial (Watanabe et al., 2014a), and directly compared magnitudes of oxytocin’s behavioural and neural effects between 6-week and single-dose interventions. For the precise comparison, we performed this task-based evaluation in virtually the same time schedule as in our previous single-dose trial, and measured the behavioural and neural responses on the last day of each 6-week administration of oxytocin/placebo.

Materials and methods

Study design and participants

This randomized, 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, 6-week interval assessment, and 2-week interval assessment were performed at this main site. Eight participants, who were originally cared for in an outpatient clinic in Showa University Karasuyama Hospital, underwent the recruitment process and 2-week interval assessments in Karasuyama Hospital with the common psychiatrist (H.Y.), who conducted these procedures with 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 5 min, 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 (UMIN0000 07122). Written informed consent was obtained from all the participants.

As stated in our trial registry, one of the aims of the current study was to estimate the power of the 6-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 included 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 included 15 participants with other psychiatric disorders to show that continuing 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
An experienced psychiatrist (H.Y.) 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 2 months of follow-up examinations. Another certified psychiatrist/psychologist confirmed the diagnoses using the Japanese version of Autism Diagnostic Interview-Revised (Lord et al., 1994) (H.K.) and ADOS (Lord et al., 1989) (M.K.). 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) 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 2-week assessment point. On the last day of each 6-week administration, half of the participants underwent clinical assessments, including ADOS, ~15 min after their morning drug inhalation, and MRI-based measurement ~40 min 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 timetable was arranged to ensure the same temporal interval between the current oxytocin administration and assessments as in our previous single-dose trial (Watanabe et al., 2014a).

Randomization and masking of drug administration
The manager of randomization and masking of drug administration randomly assigned the participants to the two groups based on computer-generated randomized 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). The manager completely covered the bottle labels to keep drug types unknown to all 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 end points. ADOS was evaluated by four administrators (H.Y., H.K., Y.K. and M.K.), the first three of whom completed a training course for research use of ADOS and were validated by the other certified administrator (M.K.). To minimize inter-administrator variability, all the ADOS scores were rated by a single certified administrator (M.K.) 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 (M.K.) to compare its sensitivity with that of ADOS.

The secondary outcomes were partly measured as behavioural responses and changes in functional MRI signals in a

### Table 1 Demographic data

<table>
<thead>
<tr>
<th></th>
<th>Oxytocin-initially administered group (n = 9)</th>
<th>Placebo-initially administered group (n = 9)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>35.1 (7.6), 24–42</td>
<td>29.3 (5.9), 24–43</td>
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<tr>
<td>Height, cm</td>
<td>169.7 (3.8)</td>
<td>167.3 (4.9)</td>
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<tr>
<td>Body weight, kg</td>
<td>65.4 (12.6)</td>
<td>66.4 (18.0)</td>
<td>0.89</td>
</tr>
<tr>
<td>SESa</td>
<td>2.7 (0.97)</td>
<td>2.6 (1.0)</td>
<td>0.64</td>
</tr>
<tr>
<td>Parental SESb</td>
<td>2.0 (0.50)</td>
<td>2.4 (0.73)</td>
<td>0.15</td>
</tr>
<tr>
<td>Handedness:</td>
<td>9/0/0</td>
<td>6/1/2</td>
<td></td>
</tr>
<tr>
<td>Right / Mixed / Left IQ6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full IQ</td>
<td>109.3 (9.1)</td>
<td>101.8 (12.6)</td>
<td>0.17</td>
</tr>
<tr>
<td>Verbal IQ</td>
<td>119.6 (8.1)</td>
<td>104.5 (12.0)</td>
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<tr>
<td>Performance IQ</td>
<td>90.9 (12.6)</td>
<td>97.8 (18.9)</td>
<td>0.4</td>
</tr>
<tr>
<td>Autism Diagnostic Interview-Revised</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social</td>
<td>14.0 (6.7)</td>
<td>15.6 (7.2)</td>
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<tr>
<td>Communication</td>
<td>13.0 (4.8)</td>
<td>11.3 (3.5)</td>
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<tr>
<td>Repetitive</td>
<td>4.1 (2.9)</td>
<td>4.6 (2.1)</td>
<td>0.74</td>
</tr>
</tbody>
</table>

SES = socio-economic status.

*aSES assessed using the Hollingshead. Higher scores indicate lower status.

bThe IQs were measured using the Wechsler Adult Intelligence Scale.
priori defined regions of interest 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.

Figure 1 Experiment design. (A) Trial profile. The current exploratory randomized controlled trial first assessed 94 potential individuals, and selected 20 high-functioning adult ASD males. After random assignment to oxytocin-first or placebo-first groups, the 20 participants started the 12-week trial. Each group lost a single participant because they felt worsening of their psychiatric symptoms. The asterisk indicates that another participant in the oxytocin-first group did not complete the functional MRI session on Assessment Day 3, after 6-weeks placebo inhalation. Therefore, clinical effects were assessed based on data from 18 participants (Table 1), whereas effects related to a psychological task were evaluated on data from 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 functional MRI 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 et al., 2014a). First, we measured rsFC between ACC and dmPFC. Second, using the same psychological task as in our previous studies (Watanabe et al., 2012, 2014a, b), we examined the effects on task-related activity of these regions and task-related functional connectivity (psychophysiological 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 non-verbal information were conflicting.
(QOL) (WHOQOL Group, 1995), and resting-state functional connectivity (rsFC) between the regions of interest (Biswal et al., 1995; Raichle et al., 2001; Fair et al., 2008).

These primary and secondary outcomes were evaluated at the baseline and 6-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 2-week assessment point by a psychiatrist (H.Y.).

MRI data acquisition

MRI data were acquired in a 3 T MRI scanner (GE Healthcare) in The University of Tokyo Hospital with essentially the same protocol as in our previous trial (Watanabe et al., 2014a). The quality of MRI data was controlled by daily monitoring over the course of this trial. Trained neuro-radiologists (H.T. and N.Y.) found no gross anatomical abnormalities in the head of any participant.

For the anatomical co-registration, axial T2-weighted images were recorded (echo time = 82.32 ms, repetition time = 4400 ms, field of view = 240 × 240 mm², matrix = 256 × 256, slice thickness = 2.5 mm, 62 axial slices). For task-related functional imaging, gradient-echo planar sequences were recorded (repetition time = 3s, echo time = 35 ms, flip angle = 80°, 4 × 4 × 4 mm³, 42 slices, ventral-to-dorsal interleaved acquisition). For resting state functional imaging, different gradient-echo planar sequences were adopted (repetition time = 3s, echo time = 30 ms, flip angle = 80°, 3 × 3 × 3 mm³, 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 predefined regions of interest [the anterior cingulate cortex (ACC), and dorsal medial PFC (dmPFC); Fig. 1B], because the regions of interest were the only regions that showed significantly large responses to single-dose oxytocin compared with those to placebo in our previous trial (Watanabe et al., 2014a). Based on this previous study, ACC and dmPFC were defined as spheres with a radius of 4 mm and a centre at [2, 34, 8] and [0, 30, 52], respectively.

Analysis of resting-state functional MRI data

Using resting state functional MRI data, we estimated rsFC between ACC and dmPFC. The functional MRI data were recorded while the participants were instructed to vaguely see a fixation point in the centre of the screen. We obtained 10 min of the resting state data (5 min × 2 sessions) for each participant. These functional MRI data underwent realignment, slice-timing correction, normalization to the standard template image (ICBM 152), temporal band-pass filtering (0.01–0.1 Hz), and spatial smoothing (full-width at half-maximum = 8 mm). Corrections for head motion (x/y/z/pitch/row/yaw directions), whole-brain signals, ventricular signals, white matter signals, and the run effect were preformed based on GLM using corresponding regressors. We finally computed the rsFC between the two regions of interest by calculating a Pearson’s correlation coefficient between the time series of the preprocessed functional MRI 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, 2014a). In the functional MRI scanner, the participants were sequentially presented with 80 monochrome short movies (1.5s) in which 1 of 20 professional actors spoke different emotional words (verbal information, V), with emotional facial and vocal expressions (non-verbal 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 pseudorandomly presented with these movies and made friend/foe judgements. This psychological task took ~12 min (6 min × 2 sessions).

Analysis of behavioural responses during a psychological task

If a participant made a friend/foe judgement based mainly on non-verbal information, the response was classified to a non-verbal information-based judgment (NVJ). A response that was based mainly on verbal information was labelled as a verbal information-based judgment (VJ). For instance, the ‘friend’ judgement of a stimulus with positive facial and vocal expressions and a negative word was classified as NVJ, because the participant was supposed to emphasize positive non-verbal 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, 2014a, b), 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 the time between the start of the movie stimuli and pressing a button to indicate their judgements.

Eye gaze was tracked during the task using remote infrared-light camera. The data were analysed as in our previous trial (Watanabe et al., 2014a): 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 100 ms. The eye or nose/mouth areas were determined as a common area for all the movies in a relatively liberal manner.

Using these behavioural response data, 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 functional MRI data underwent realignment, correction of slice timing, normalization to the default template with interpolation to a 2-mm cubic space, spatial smoothing (full-width at half-maximum = 8 mm, Gaussian filter), and high-pass temporal filtering (128 s). At a single-participant level, we used a General Linear Model for an event-related functional MRI design with eight regressors (the four types of stimulus × the two types of response), and calculated the difference in functional MRI signals for each region of interest 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 et al., 2014a). At a single-participant level, this PPI analysis used three regressors: one representing functional MRI 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 generalized estimating equations with an unstructured correlation and robust standard error estimates. Changes in these outcomes during each 6-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 2 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 region of interest analysis (\( P < 0.05 \) in paired \( t \)-tests) and voxel-based analysis around the regions of interest (\( P_{\text{FWE}} < 0.05 \) in small volume correction, SVC). The spatial specificity of the region of interest 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 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 the 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 was in oxytocin administration period in oxytocin-first group, the other was in placebo period in placebo-first group (Fig. 1A and Table 1). Because of anxiety attacks in the MRI scanner, another individual could not complete the final functional MRI session that was originally planned after 6-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 functional MRI 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, which was conducted more than a year previously (Watanabe et al., 2014a).

Clinical effects

We first found that 6-week oxytocin administration could improve autistic symptoms related to social reciprocity (ADOS reciprocity: \( P = 0.034 \), Cohen’s \( d = 0.78 \), a generalized 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, 6-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 2 weeks, CGI-EI and pulse rate showed significant interactions between assessment order and type of drug (CGI-EI, P = 0.02, FDR < 0.05; pulse rate, P < 0.001, 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 the oxytocin treatment period (CGI-EI and pulse rate, P < 0.01) than within the 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_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 ([oxytocin-first group, t(16) = 3.3, P = 0.001, voxel-size > 20; Fig. 2E]. Moreover, this rsFC increase was strongly correlated with the oxytocin-induced decrease in ADOS reciprocity score (r = −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.
First, we confirmed that the current 6-week intervention reproduced qualitatively the same behavioural and neural effects as those observed in our previous single-dose trial (Watanabe et al., 2014a): 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; Fig. 4A].

The diagram illustrates the behavioural and neural responses to oxytocin administration. Figure 3A shows the task paradigm, where responses were classified into verbal-information-based (VJ) or non-verbal-information-based judgements (NVJ). Figure 3B-D depict the changes in the number of NVJ, response time for NVJ, and fixation time on eye areas, respectively, with oxytocin and placebo interventions. Figure 4A-B exhibit the effects on brain activity, specifically increased NVJ-specific activity in ACC and dmPFC. Figure 4C-D validate the locational specificity of these effects through whole-brain analysis and task-related functional connectivity. Figure 4E-F correlate oxytocin’s behavioural and neural effects, and compare effect sizes between single-dose and 6-week interventions.
P_{FWE} < 0.05 in SVC; Fig. 4A], which was confirmed by a whole-brain analysis \((t > 3.5, P_{uncorrected} < 0.001, \text{voxel size} > 20; \text{Fig. 4B})\). 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 \((t = 3.8, P_{uncorrected} < 0.001, \text{voxel size} > 20; \text{Fig. 4D})\).

Furthermore, as in the single-dose trial, these neural effects were predictive of the oxytocin-induced increase in the number of NVJ \((\text{ACC}, r = 0.62, P = 0.01; \text{dmPFC}, r = 0.69, P = 0.002; \text{dmPFC–ACC}, r = 0.55, P = 0.02; \text{Fig. 4E})\). As a whole, these behavioural and neural responses to the 6-week oxytocin administration are qualitatively comparable to those to the previous single-dose intervention (Watanabe et al., 2014a).

### 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 \((\text{ACC}: \rho = -0.67, P = 0.0032, \text{Fig. 5A}; \text{dmPFC}: \rho = -0.60, P = 0.011, \text{Fig. 5B})\) and the enhancement of the task-related functional connectivity from dmPFC to ACC \((\rho = -0.61, P = 0.010; \text{Fig. 5C})\). 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 6-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 6-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 regarding 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 (Shamay-Tsoory et al., 2009; De Dreu et al., 2010, 2011; Bartz et al., 2011; Stallen et al., 2012; Bethlehem et al., 2014; Shalvi and De Dreu, 2014), 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 versus QOL change, $r = -0.21$). 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-recorded 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 subdomains 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 interindividual 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., 2015), which has raised the possibility that continual regimes induce negative feedback or downregulation 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 reconsider 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 symptoms with robust neural effects.

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