Target engagement for intranasal oxytocin in Autism Spectrum Disorders, an fMRI dose response study.

Short title: Autism Oxytocin Brain project (AOB)

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Precis/Abstract

Interventions for autism are limited. This study consists of investigating the effects of several doses of acute administration of intranasal oxytocin on brain activity in adults with Autism Spectrum Disorders (ASD). There is increasing evidence for the role of intranasal oxytocin (IN-OT) in enhancing social skills in ASD. Nevertheless, there is still a need of determining target engagement for oxytocin's action on brain and behavior. Here, we are investigating the effects of different doses of IN-OT on the modulation of behavioral outcomes and neural responses in a double blind study in individuals with ASD. The first objective is to study the effects of IN-OT doses on the modulation of brain functional connectivity between key socio-emotional brain regions during rest in ASD. The second objective is to study the effects of IN-OT doses on the BOLD (Blood-Oxygen-Level Dependent) activity of key emotional and perceptual brain networks in response to social cues within a static and within an interactive social environment. We are also investigating the effects of IN-OT doses on the behavioral outcome measures such as visual fixation and emotion recognition. We will compare the neuroimaging and behavioral results of individuals with ASD to control healthy males who will receive intranasal placebo. The results of this study will provide more information on the mechanisms of action of IN-OT and its precision in affecting the brain and behavior.

Introduction and Background:

Summary of the primary hypothesis, purpose, rationale and prior literature.

Autism spectrum conditions (ASD) are a group of neurodevelopmental conditions characterized by impairments in social reciprocity, communication difficulties, and repetitive and stereotyped behaviors. High-functioning ASD subjects show pronounced selective social deficits despite preserved intellectual and linguistic capacities. These individuals display decreased eye-to-eye contact, impairments in orienting attention towards social stimuli (Klin et al., 2002; Nakano et al., 2010), and alterations in responding to social rewards (Izuma et al., 2011). At a neurobiological level, ASD patients do not activate reward circuitry in response to socially rewarding stimuli such as smiling faces and have abnormal brain responses while looking at faces (Schultz et al., 2000). One source of social cognition deficits could be related to lack of motivation to social cues which is characterized by a lack of attention and reward sensitivity to these cues. Another potential source of the social difficulties is the presence of co-occurring alexithymia in ASD (Silani et al., 2008), which is characterized by a reduced emotional awareness and empathy capacities.

One potential approach for treating social deficits in ASD is to target the neural systems involved in socio-emotional processing as a means to enhance social cognition and function. In particular, oxytocin (OT) has emerged as a key modulator of social behavior and is being extensively considered as an intervention to enhance social functioning in ASD (Andari et al., 2010). We believe that OT affects complex social behaviors by modulating fundamental processes, such as the salience of social stimuli and the rewarding value of those social stimuli. OT interacts with the dopamine system in the ventral striatum to facilitate the formation of a social attachment (Young and Wang, 2004). Remarkably, there are dozens of studies suggesting that intranasally (IN) delivered OT has a similar prosocial effect on human behavior (Domes et al., 2007; Heinrichs et al., 2009; Rimmele et al., 2009; Hurlemann et al., 2010). IN-OT increases trust in others, attention to the eye region of faces, and enhances the ability to infer the emotions of others from viewing images of the eyes. fMRI studies have shown that IN-OT is affecting emotional brain regions relevant to saliency, such as the amygdala (Kirsch et al., 2005; Gamer, 2010), and to reward, such as the caudate nucleus, during face perception or during reciprocated cooperation (Rilling et al., 2012).
Andari et al. 2010 used a Cyberball game to demonstrate that IN-OT enhanced the ability of ASD subjects to detect and respond to social reciprocity. The social exchanges between players created two extreme partner profiles (one reciprocating and another not reciprocating). Healthy subjects quickly adjusted their ball tossing to favor the reciprocating player, while ASD subjects did not. Remarkably, IN-OT enabled ASD subjects to detect relevant social information regarding the degree of reciprocation of each partner, to evaluate the significance of partners' actions, and to subsequently toss the ball more frequently to the reciprocating player. IN-OT also increased the patients' visual attention to the face region and in particular to the eye region (Andari et al., 2010). Today, there are more than 19 papers showing positive effects of oxytocin on social cognition and brain function in individuals with ASD. In particular, Auyeung et al. 2015 have replicated our initial work showing that IN-OT enhances gaze behavior. Authors observed that oxytocin administration selectively enhanced gaze to the eyes in autism and control groups. In particular, within the autism group, oxytocin has the most effect on fixation duration in individuals with impaired levels of eye contact at baseline.

Authors showed that IN-OT to young individuals (Guastella et al., 2010) (12-19 years old, 18IU or 24IU) improved performance on the reading the mind in the eyes especially for easy items and for younger participants. Researchers conducted a long term trial in 19 adults with adults (Anagnostou et al., 2012) (24IU twice a day for 6 weeks) and showed no serious adverse effects and some improvements in emotional cognition and quality of life but not in other clinical measures.

Tachibana et al. 2013 (Tachibana et al., 2013) have shown that chronic administration of IN-OT (twice a day for a period of 6 months) to children (11.9 years old on average) with ASD was safe and patients did not show any side effects (blood pressure, serum osmolarity, serum sodium concentration, urine osmolarity, bone metabolism and short term memory).

A recent promising study of Yatawara et al. 2015 (Yatawara et al., 2016) published in Molecular Psychiatry have shown that chronic administration of oxytocin (twice a day for a total of 24IU per day) for 5 weeks to children with autism lead to significant improvements on the primary outcome of caregiver-rated social responsiveness.

At the brain level, several fMRI experiments have been conducted in ASD as well. Our group has shown that IN-OT enhances the BOLD (Blood-Oxygen-Level Dependent) activity of visual areas during the processing of facial stimuli and reduces amygdala activity during interaction with other players. IN-OT also triggered the activity of OFC during social reciprocity and anterior insula during the perception of unfair players in ASD. The group of Hidenori Yamasu (Aoki et al., 2014) in Japan has shown that 24IU administration of oxytocin to 20 males with ASD increased the correct rate in inferring other's social emotions and enhanced the originally diminished brain activity in the right anterior insula while performing this task. Domes et al. 2013 (Domes et al., 2013) have shown that IN-OT (24IU) to 14 individuals with Asperger syndrome increased the activity of right amygdala (as well as for healthy subjects) that was shown initially decreased as compared to a control group during face processing (face and house discrimination task). Gordon et al. 2013 (Gordon et al., 2013) have shown that IN-OT administration to 17 children with autism increased the BOLD activity in the striatum, the nucleus accumbens and superior temporal sulcus during the judgment of social stimuli (eyes).

Watanabe et al. 2014 showed that IN-OT (24IU) (Watanabe et al., 2014) enhanced non-verbal judgments of social stimuli while increasing the brain activity of medial prefrontal cortex in adults with ASD.

Watanabe et al. 2015 (Watanabe et al., 2015) showed that chronic administration of IN-OT for a 6 week interval reduced core symptoms of ASD in social reciprocity (using ADOS criteria). These behavioral improvements were associated with enhanced functional connectivity at rest between the anterior cingulate cortex and dorso-medial prefrontal cortex. Also, the long term administration enhanced patients' capacities during the social judgment task and the activity in the anterior cingulate and medial prefrontal cortex as well as the functional connectivity between these two regions. This resting state increased functional connectivity between ACC and mPFC was found correlated with the oxytocin-induced clinical deficits in ADOS of social reciprocity.

A recent meta-analysis has shown that the oxytocin receptor gene is associated with ASD (in particular the rs7632287, rs237887, rs2268492 and rs2254298 OXR single-nucleotide polymorphisms (LoParo et al., 2016). Also, the team of Smith at Emory showed an association between epigenetic modifications of OXT via DNA methylation, which is an indirect approximation of OXT brain expression (presumably lower OXT DNA methylation is linked to higher OXT

expression), and attachment style, social processing (recognition of emotional expressions) and brain function during socio-cognitive tasks (Haas et al., 2016).
Despite the quick progress in this field, more research is needed in order to study oxytocin's therapeutic potential in alleviating social difficulties in neuropsychiatric disorders.
An important question that has been addressed by Thomas Insel (Insel, 2016) in his latest review concerns target engagement for intranasal oxytocin. Here we would like to address this question by performing a dose response study for intranasal oxytocin and study its effects on functional connectivity of intrinsic activity during resting state and in response to external social cues. This will allow us to better understand the mechanisms of action of this molecule.

- At first, we will study the effects of IN-OT doses on the **resting state functional connectivity** between key socio-emotional brain regions (using fMRI) that are shown to be deficient in ASD. Resting state functional connectivity is a task-independent metric of brain activity that is based on correlations between low-frequency fluctuations of the blood oxygen level-dependent signal between several brain regions. It reflects the strength of a functional connection that is in good agreement with the underlying neuroanatomy but goes beyond simple monosynaptic connections as it provides a system-level understanding of brain function. The power of this technique lies in its relative ease of acquisition and stability within and across scans sessions making it ideal for longitudinal experimental designs, including assessments of different treatments. Task-free acquisition precludes equating levels of performance – or motivation – between individual participants and across comparison groups. Large-scale brain networks are of particular relevance to this study. These networks include the - default mode network (DMN), which is centered on nodes in the mPFC, the midline posterior cingulate cortex (PCC), and the parahippocampal gyrus that has been implicated in mindwandering, planning the future and reflections on the past. The salience network (SN) centered on nodes in the dorsal anterior cingulate cortex and he frontoinsular cortex and sometimes including the ventral striatum that is implicated in the facilitation of attentional orientation to internal or external stimuli. The insular cortex is also involved in interoception and mapping internal bodily and subjective feelings states that are found deficient in Autism and related to a lack of alexithymia. The executive control network (ECN) is centered on nodes in the dorsolateral PFC and the lateral posterior parietal cortex that are associated with attending to and processing exogenous, attentionally-driven executive function. SN and ECN: are task-positive networks, which mean that these regions are identified at rest but are also activated during performance of most cognitive tasks. DMN usually deactivates during task performance. An overactivation or a reduced deactivation of the DMN and reduced ECN activation leads to decrements in task performance for healthy subjects and various neuropsychiatric diseases (Sonuga-Barke and Castellanos, 2007). We are particularly interested in networks that are relevant to oxytocin intake as based on the increased expression of oxytocin receptors in the brain, function of IN-OT on the brain and deficits in ASD.
rsFC measure can provide clinically useful diagnostic, predictive biomarker of oxytocin's effects on the brain and behavior as based on different patterns of dysfunctions, as compared to healthy subjects and based on different patterns of activation. We expect to find a reduction in functional connectivity not only within the different brain networks that were cited above but also in between networks. Based on previous studies, there is a reduced rsFC between mPFC and ACC as well as with PCC. We also expect a reduction in functional connectivity between anterior insula and

ACC and vmPFC and somatosensory areas and a reduction in connectivity between amygdala and insula and vmPFC/ACC as well as ventral striatum.

There are several evidence on the effects of intranasal oxytocin functional connectivity of amygdala. 40 IU normalizes amygdala functional connectivity in PTSD by increasing functional connectivity between amygdala (right CeM subregion) and vmPFC in males (Koch et al., 2016). In autism, authors have found that six-week administration of oxytocin enhanced the connectivity between ACC and dmPFC at rest. At rest, intranasal oxytocin increased functional connectivity between amygdala and ACC (Kovacs and Keri, 2015). IN-OT enhanced rsFC of amygdala and ACC/mPFC in individuals with generalized social anxiety disorder (Dodhia et al., 2014). Acute administration of oxytocin was found to enhance the BOLD activity of several brain regions such as striatum, mPFC, OFC (Gordon et al., 2013).

We will study the effects of several doses of IN-OT (8IU, 24IU and 48IU) on the rsFC between the salience network anterior insula and ACC, vmPFC as well as the amygdala functional connectivity with insula, vmPFC/ACC and ventral striatum (nucleus accumbens, VTA) and to the connectivity between vmPFC and ACC. We will also study the effects of IN-OT on the BOLD activity and functional connectivity of key brain regions that are involved in perception of social cues and reward sensitivity to these cues during social tasks. We will also compare the brain activity at rest after placebo intake in ASD to a group of healthy controls, matched for age, sex, ethnicity and full scale IQ.

Following resting state, participants will perform an emotional face task that consists of looking at morphed faces. We will record eye-movements (via an eye tracker) while participants are looking into faces. Participants will also perform a social ball game (adapted version from our previous project IRB00064623) during which they interact with several other players. Participants will also play a prisoner dilemma game that was previously used by James Rilling's work (investigator at Emory) to look into neural correlates of reciprocal cooperation and defect.

Following the scanner, participants will perform some behavioral task and complete some questionnaire in order to study OXT' effects on behavior (such as emotion recognition and alexithymia).

Social reward and social saliency are key phenomena for studying the core deficit of social dysfunction in ASD and for targeting oxytocin manipulation.
We are looking into the effects of IN-OT on brain activity and behavior in a precision medicine fashion. As exploratory analysis, we will look into the different clinical, behavioral and genetic moderators that can moderate the effects of IN-OT on behavior.
<u>The major hypothesis of this project is that intranasal oxytocin affects the activity of brain regions and brain connectivity between areas that are involved in sensory and emotional processing as well as interoceptive networks.</u>

Objectives:

<u>Aim 1: The effects of intranasal oxytocin on brain function at rest in adults with autism (35-43 minutes after spray).</u>

We hypothesize that IN-OT will enhance the functional connectivity at rest in ASD within and between several brain networks that are involved in salience and emotional processes such as insula, amygdala, ACC, mPFC and ventral striatum.

Primary outcome measure:

The primary outcome measure consists of studying the difference in resting state functional connectivity (rsFC) between treatment conditions in socio- emotional brain and perceptual networks in patients with ASD (Fig.1, anterior insula-amygdala-mPFC-OFC-ACC, temporal areas and insular-somatosensory cortices). This stems from the hypothesis that oxytocin affects brain network that is implicated in emotion evaluation, social and affective processing. Seeds will be considered within the **insula region** (we expect to find an increased activity between anterior insula, ACC, amygdala, vmPFC and somatosensory cortex); the different sub-regions of **amygdala** and study its connectivity with vmPFC/OFC, insula/ACC, ventral striatum (VTA, NAcc) and temporal areas. We expect the ventrolateral part of the amygdala to be functionally connected with perception areas such as fusiform, superior temporal sulcus, OFC; medial amygdala with vmPFC and dorsal amygdala with ACC and insula. We will also consider the **NAcc** as a seed region and study its connectivity to other reward regions (caudate, putamen, ACC) and frontal areas, PCC and precuneus (or default network). We will also look into the **vmPFC** as a region of interest and study its functional connectivity to ACC, PCC and subcortical regions. We expect to find differences between doses of IN-OT in terms or rsFC in these key brain regions as well as differences between IN-OT and placebo intake.

Secondary outcome measure:

The secondary outcome measure consists of correlating the effects of treatments (whereas the differences between high and low doses or between high and placebo) on rsFC with clinical and behavioral improvements following IN-OT (as compared to small dose or as compared to placebo) in these individuals with ASD. Clinical improvements will be rated by a clinician as a global clinical improvement as based on a videotaped interview (BB-CGI) post scanner. Behavioral improvements will consist of improvements in natural socio-emotional responses (emotional responses and eye contact) during this clinical interview.

Aim 2: The effects of intranasal oxytocin on the BOLD activity of the social brain network (SBN) in response to social cues (50-60 minutes after IN-OT)

We hypothesize that IN-OT will enhance the brain activity of regions involved in perception and emotional process in response to morphed emotional faces as compared to neutral faces in subjects with ASD.

Primary outcome measure: The primary outcome measure consists of measuring the difference between treatments in terms of BOLD activity in response to emotional social cues (Face perception task (FPT)). We expect to find that a higher dose of IN-OT will enhance BOLD activity in emotional and perceptual regions known to be active during the perception of emotional face, (inferior occipital cortex, fusiform cortex, superior temporal cortex, intra-parietal sulcus, superior

parietal lobule, nucleus accumbens, amygdala and OFC), as compared to a lower dose of IN-OT or to placebo.

Secondary outcome measures: We will study the effects of the difference between treatment conditions in modulating the visual fixation or gaze duration on the face and eye region of faces during this emotional face test in ASD (measured by the eye tracker inside the scanner). Based on the growing evidence in the literature, we expect to find that higher doses will increase the duration of eye gaze on the face region as compared to placebo and other smaller doses of IN-OT.

Aim 3: Effects of intranasal oxytocin on the BOLD activity of SBN in response to an interactive social reward task (60-70 minutes after IN-OT)

We hypothesize that high levels of IN-OT will enhance brain activity and functional connectivity of social brain networks areas involved in perception and emotional processes in response to dynamic social cues as compared to non-social cues. We are also interested in looking into the effects of IN-OT on reducing the activity of amygdala and anterior hippocampus in a context dependent fashion (as based on level of stress). We also believe that the clinical/behavioral and endophenotypes will play a fundamental role in determining OXT's effects on brain activity.

Primary outcome measure: Here, we are looking to differences between treatments conditions on BOLD activity of perceptual and emotional brain regions while participants play a multi-player ball game. We predict that a higher dose of IN-OT will enhance the BOLD activity of reward brain regions (such as OFC, ventral striatum, Nucleus accumbens, ventral tegmental area) and perceptual brain regions (such as inferior occipital, fusiform gyrus, superior temporal sulcus, amygdala, superior parietal lobe) and insight awareness (insula and ACC) for facial expressions as compare to neutral non-social trials, *regions that are expected to be less activated in placebo as compared to healthy subjects*. We also expect increased activity in rewarding brain regions in response to positive social feedback as compared to neutral social feedback. We also expect to find a main effect of IN-OT on reducing the activity of amygdala and hippocampus during the ball-game, in a context dependent manner (which could be related to a reduction of stress and anxiety). Indeed, we expect to find the neutral feedback trials to be more stressful and activating more the amygdala when compared to the positive trials.

Indeed, in contrast to positive feedback, we expect the neutral to be eliciting negative social feedback in this kind of social context.

Secondary outcome measure:

We are looking into differences between treatment conditions on behavioral outcome of social learning paradigm (post scanner) following the ball-game. We are looking into implicit effects of learned emotional associations on reaction time. We expect to find more emotional implicit social biases with IN-OT.

Aim 4: Plasma oxytocin concentration and exposome/metabolome

Here we hypothesize that IN-OT will enhance plasma oxytocin concentration in a dose dependent fashion.

Primary outcome measure: We will investigate the differences between treatment conditions in oxytocin plasma concentration. We expect to find that higher doses of IN-OT will enhance significantly the plasma OXT concentration as compared to lower dose of IN-OT and to placebo.

Secondary outcome measure: We will study the differences between healthy subjects and patients with ASD in terms of baseline oxytocin concentration. We expect significant difference and reduced levels of OXT at baseline in ASD.

different nature of social dysfunctions.

Procedures

Pilot study:

Before we conduct the clinical trial, we will test few volunteers (number maximum 10, friends and colleagues or study staff, men between 18 and 45 years old) on the different tasks of the MRI functional sequences. This will allow us to validate that everything is working (eye tracker installation, presentation of stimuli, synchronization of all data, etc.). We will use the same fMRI screening form as for healthy subjects. It is possible that 2 or 1 pilot will be enough to validate all the MRI parameters.

Inclusion criteria for the pilot study participants:

- Males between 18 and 45

Exclusion criteria for the pilot study participants:

- History of seizures
- Neurological disorder or head trauma
- Presence of a severe medical problem
- Cardiovascular Disease
- Current frequent and untreated asthma
- Current frequent and untreated migraines
- Pacemakers, cochlear implants, surgical clips of metal fragments.

The pilot volunteers will perform all the same tasks inside the MRI scanner as described above. They will not need a debriefing given that they will know that the social tasks are not based on real interactions. Given that the pilot volunteers will be staff members or colleagues, it is possible that they will be informed about the study through direct conversation or through a phone conversation. We will document methods of recruitment and screening. They will complete the MRI screening form and sign appropriate pilot consent form. We will provide all the instructions about the different tasks that they will perform inside the scanner. They will also perform the trainings and quizzes for the tasks that they will conduct inside the MRI scan. We will set up the

camera on the coil of the MRI machine and calibrate their pupil and their eye movements in order ensure quality of recording of eye movements during face processing task. They will also wear the sensor inside the MRI scanner. They will perform the resting state sequence, face perception task and the ball game. All the details of these tasks are explained below.

Clinical Trial

1- Screening and recruitment

All information collected during phone and email correspondences will be documented in an excel sheet on a HIPPA protected location (such as Emory box).

Subjects or and families (healthy subjects and subjects with ASD) will be contacted by email and/or by phone. We are recruiting participants who have previously participated in another study (IRB protocol #64623, where Dr. Andari is an investigator) and who agreed to be contacted for future studies or agreed to participate in this study and who have the basic criteria to be eligible for the study (such as IQ>70 and autism diagnosis as based on ADOS and ADI criteria). Contact information will be collected from protocol 64623. We will contact them to ask if they are still interested and available to participate in this AOB project. Based on the records that we collected from project 64623, we will be able to know which patient with autism have his parents as legal guardian or legal representative.

Recruitment:

We will contact patients and healthy subjects by phone or/and email by following recruitment phone and email scripts. We will introduce ourselves and introduce the study and ask participants if they are still interested in participating in the study. We are likely to call the parents of patients with autism to ask them if their son is interested in participating given that some patients don't like to be in touch by phone and don't respond to emails. In some cases, we need to call parents who have legal guardianship in order to see if they would be available to accompany or willing to mail us a signed consent form so that their son can participate in the study.

In some cases, we will try to do the recruitment, screening and scheduling in the same phone call.

Screening:

The medical screening form (that includes inclusion and exclusion criteria) will be first reviewed over the phone for both healthy subjects and ASD (following an oral consent for screening). In case there is any doubt in the results, the research staff will contact the PI or study physician to make sure that the person can be included in the study. For this initial screening, we do not require study physician signature to enroll a patient in the study. During the research visit, the participant will be asked to review it and sign his name on the form. The legal guardian, if applicable, will be also asked to sign it. The nurse practitioner or the MD need to sign the form also.

The MRI screening form will be also reviewed over the phone with the participant (or with the parent of the patient in case it's difficult for the patient to have the discussion over the

phone) following an oral consent. In case there is a doubt in any of the points, we will ask the MRI specialist to see if we can still include the subject in the study. During the research visit, the participant will review it and sign it and we will give a copy to the MRI technician. The legal guardian, if applicable, will also be asked to sign it.

ASD

Over the phone, the staff member will go through the oral consent for screening for eligibility. Over the phone, we will conduct the medical screening in order to assess eligibility (as based on the inclusion and exclusion criteria of the study). We will introduce the study and answer any questions about the content of the study. We will also go through the MRI screening in order to assess safety and eligibility. We can send them the MRI screening form by email to review. We will ask participants if they have a legal guardianship. We will document in a screening log who conducted the discussion over the phone, whether the person is eligible, whether there are questions for the PI or MD investigators, and the dates of the phone interview. We will follow a screening phone script to contact patients. It is very likely that we need to contact patients several times in order to finish the screening or to come back to patients about eligibility (in case there were questions to the PI).

In some cases, we need to contact the parents of patients to schedule patients' visits given that the parents are managing their son's schedule, or that it's difficult to reach patients (by email or phone) or that it's difficult for patients to understand instructions over the phone or by email. In these cases, we will need to conduct the interview with the parent. We will go through the same phone script for recruitment and screening that includes an oral consent and eligibility criteria and screening assessment (same phone script as above). We will ask parents to inform the patient about all the details of this interview and ask them if they are interested in participating in the study. We will document that the interview was done with the parent and not with the patient in the screening log documentation. When patients show up for their first visit, we will make sure to go over the eligibility and screening forms again. We will follow-up with the parent and patient by email or phone to schedule all the visits and to confirm appointments.

It is also possible that we do the same phone screener with both parents and patients in case there are some doubts about some answers.

In addition to the phone interview, it is likely that we send the parents and/or patients the MRI screening form via email to go over it in order to double check all the criteria. We will use an email recruitment template.

We will respond to emails and phone calls of parents and patients who have questions or need more information about the study.

For adults who have legal guardianship or legal authority representative, parents need to sign the consent form also (in addition to the adult subject's signature). The parent needs to be present during the first visit and is not required to stay for the rest of the visits. However, we are not asking the permission from the legal guardian to conduct the phone screening process. The LAR will be asked to sign the screening form. We will conduct a parent phone call by following a LAR phone

parent script. We will contact the parent by phone and ask them first to verbally confirm if they have the authority to make research decisions (power of attorney, legal guardianship). Once confirmed, we will introduce the study briefly and ask them if they can accompany their sons for the first research visit. We will explain to them that we need their signature on the consent form in order for their son to participate in the study. In this case, the parent and the patient will sign and date the consent form, as well as the screening forms, during the first research visit. During the research visit, the staff member will discuss the consent form with both patient and parent, and will ask them to read it, to ask questions and to sign it when they are ready. The staff member will provide a copy of the consent to the patient and to the parent. There is no need for parents to accompany their sons for all the visits.

If the parent cannot accompany their son to the research visit (onsite at Emory), we will ask the parent (during the phone interview) if we can mail them the consent form and we will ask them for the mailing address. We will discuss the consent form over the phone. We will let them know that they cannot scan and email us back the consent form. They have to mail the signed consent form back to us to the address 1462 Clifton Rd, Rm 269. We will mail a copy of the consent form to the parent (one for them to keep as a copy and one for them to sign and send it back to us by mail) and the screening form with a returned envelop (and a stamp) and ask parents to read and sign. We will make sure to have colorful post-it on the pages where the parent needs to sign, date and time. We will ask them to email us or call us in case they have a question. We will make sure that we receive the signed consent form and screening forms from the parent before patient's first research scheduled visit. If the form was not dated or timed correctly or if the parent forgot to add date and/or time, we will write a note to file regarding this event describing the omission of the date and time. We will go ahead and mail again the form to the parent (with the return envelope and a stamp) and ask them to add the date or/and time. The same applies for signature omission or if the signature is misplaced. We will call them or email them to let them know that we are mailing them the consent because of the omission. Once the consent is signed by the experimenter and the patient, we will send a signed copy to the parent by mail or we send 2 signed copies of the consent with the patient (one for the parent).

We will follow a legal guardian phone script while making this phone interview with the legal guardian. We will document who discussed with the parent and what was the content of the discussion in the screening log document.

All the informed consent process with LAR will be documented in the informed consent process note and will be added to subject's individual folder.

We will respond to email requests and phone calls for information with form recruitment email, including available times, documentation and screening form attached if necessary.

If the patient is eligible for the study, we will request an appointment with the CRN and put an appointment in the calendar. We will also reserve the MRI scanner.

Once the CRN approves, we will notify the patient with a reminder email, we will notify Dr. Haroon or Dr. Cubells and notify IDS.

Healthy subjects screening:

Given that the healthy group of males should match patients with ASD on age, sex and intellectual quotient (IQ), we will first contact patients (from the data base of the previous protocol 64623) and see who is interested and eligible before selecting the matching healthy men controls. We will select healthy participants who already participated in our previous research study (64623). We will use the information collected in project #64623 to match their IQ and age. We will contact participants who agreed to be contacted in the future studies in the consent form of protocol 64623. We will contact healthy subjects by phone and/or email (again from the data base of 64623) to see if they would be interested to participate and whether they are eligible (based on eligibility criteria for healthy subjects and based on safety screening). We will follow a phone script and email for recruitment for healthy subjects. We will respond to email requests for info and include screening form attached.

Given that mid-way study, we have trouble bringing back healthy participants who have participated previously in protocol 64623, we need to recruit new people from general population in Atlanta. We will use flyers to recruit more people. We will post these flyers in different locations around Atlanta area (like in restaurants, college, Marta stations etc). We will need to conduct the IQ test and color blindness prior their main visit to the hospital. Healthy controls should match ASD patients with IQ and race. We are adding race so that samples from both groups stay matched as much as possible.

We have already conducted the total number of patients for this study. The average IQ of ASD patients is 98.39 and the standard deviation is 16.28. The race ratio for ASD is 82% white, 12.5% African American and 6% more than one race,

Accordingly, we are adding a new informed consent and another inclusion criterion for this separate group of healthy subjects (labeled II). The inclusion criteria will include Caucasian race and IQ range between 70 and 115.

The reason that we are adding Caucasian to the inclusion criteria for this new set of healthy subjects is that we have already 2 or 3 contacts from the previous study who are African American and who are interested to participate. This will make approximately 10% of the healthy subject's sample if we were able to recruit 30 total.

If the IQ was higher or lower than this range, we will exclude the participant from participating in the study. For this new set of healthy subjects, they need to come for 2 separate visits. The first visit will consist of performing the IQ (at the hospital or at Clifton 1462) test and consent signature as well as review of eligibility criteria and color blindness test. The second visit will consist of the main clinical visit at the Hospital

If the subject is eligible for the study, we will request an appointment with the CRN and put an appointment in the calendar.

Once the CRN approves, we will notify the subject with a reminder email, we will notify Dr. Haroon or Dr. Cubells and notify IDS.

For both healthy subjects and ASD subjects, in case there is any doubt in the screening form or eligibility of participants, prior scheduling the research visit, the PI will be notified and will review the screening form and if needed consult with study physician or study nurse practitioner. If it is determined that the subject meets inclusion/exclusion criteria, the research staff will schedule the different visits of the subject. On the day of the scheduled visit, the study staff will review the consent form with the subject and give them the opportunity to ask any questions before asking them to sign in person.

Subjects will meet the study staff at the entrance of the Emory Hospital.

Patients will participate in 5 visits total and these visits can be separated by a one-week interval approximately (± 2 to 3 days). We have to be little flexible in the schedule given that we are dealing with complicated scheduling system (scheduling CRN units at the hospital, MRI scanner at BITC, nurse practitioner or physician, patients' availability).

2- Visit1-pre clinical (around 3 hours)

All the visits for healthy subjects and ASD subjects will occur at the Emory Hospital. With the help of ACTSI facility, we will use one of their external outside CRN unit rooms to test participants on computer tasks, make interviews, collect saliva samples, filling questionnaires and getting snacks. For all the medical and health assessments (physical exam, vital signs, blood withdraw, spray intake) will occur inside the CRN outpatient services. For the MRI scan, we will conduct it at BITC scanner. All post-scanner questionnaires will be conducted in this external room provided by ACTSI inside the hospital.

ASD subjects

Upon arrival, staff will review the screening form previously filled as well as eligibility criteria and the MRI screening form out for correctness and affirm that no changes in their health or medical history have occurred. If any changes have occurred, the study staff will consult with the PI or the study physician, if necessary. We will review the consent form with the subject. We will also assess his understanding of the consent by asking specific questions that are part of an assessment of capacity form. The experimenter will ask the questions to the patient and will take notes of his responses. If the patient did not answer correctly to all questions, we will review the consent again and ask the questions again to ensure a full understanding of the consent. Once the patient understands the consent, we will proceed to the signature. This assessment of capacity form will be only administered to patients with ASD and not to healthy subjects.

The nurse practitioner or the study physician will conduct a brief history and physical. The nurse practitioner will go through the inclusion and exclusion criteria again to ensure that the patient is ready to participate in the subsequent clinical visit. This health assessment will be reviewed again one more time during one of the subsequent clinical visits.

Participants will fill a family history form related to themselves and to their family history .

We will collect saliva samples (3 DNA Genotek kits) for genotyping analysis and methylation analysis.

The patient will complete several questionnaires.

Given that there is lot of questionnaires to complete, we can ask participants if they prefer to complete them at home. Participants can complete the questionnaires and bring them back during the following visits.

Participants will also perform a social learning test during which the participants match faces or words as fast as possible. This test is an adaptation of the implicit association task during which we study implicit associations.

Participants will be trained on the different behavioral tasks that they will be performing during the MRI scanner: the face perception task and the social games. There will be specific instructions and a practice session on the computer screen for each of these tasks. Participants will complete a quiz for the face perception task and for the ball-game to double check the comprehension of the task. If there are errors in responses, the experimenter will repeat the instructions or, if necessary, present some training sessions on the screen and repeat the questions that participants did not answer correctly, until he has all the answers correct.

At the end of this visit, participants will go through a brief clinical standardized interview (Bio-behavioral clinical global interview BB-CGI) with the experimenter during which the experimenter will ask questions to the subject and record their interactions.

This clinical test will be repeated at the end of each of the following 4 MRI visits in order to evaluate the progress or come up with a score of socio-emotional, verbal, physiological and clinical index of improvement.

We will take a photo from this video to keep the photo identification in the folder of the patient.

At the end of the interview, the study staff will take the patient to the BITC MRI scanner at the Emory Hospital for a practice/habituation session. The participant will lay down in the scanner (same way as described below during the clinical visits), get familiarized with the coil and the tunnel, with the response box (that he will use to answer during games), with the noise, and to with some examples of the tasks that he will perform during the actual clinical visits. We will run an fMRI sequence for few minutes (that might include a resting state, face test, ball-game). At the end of the MRI training/habituation, we will ask participants few questions about his experience. The participant will be also asked about any adverse events. In case of mild anxiety, the experimenter will make sure to give the participant time to rest and ask him if he would like to continue habituation.

Participants will also have a look at the CRN unit.

Given the complexity of scheduling MRI scanner, the CRN unit and the nurse practitioner, the tasks above do not need to be performed in a particular order. Even the health assessment can be performed at any time given that the goal of the health assessment is to make sure that the subject is healthy before its inclusion in the subsequent 4 clinical visits.

Of note, the results of the health assessment do not determine whether the subject can or cannot participate in this first non-clinical visit.

Given that the MRI scanner is always functioning, we will make sure to collect MRI screening forms from subjects before approaching the magnet.

	Screening And health asessment	Consent	DNA	Questionnaires	Computer tasks	Clinical interview	Training on behavioral tasks and MRI + questionnaire
Prior visits	X						
Pre-MRI visit	X	X	xxx	X	X	X	X

Table 1: Pre-clinical Visit 1 for subjects with ASD

3- Second visit: clinical visit for ASD (total duration= around 3 to 4 hours)

Subjects will meet the study staff at the Emory University Hospital. The different behavioral questionnaires, trainings and physical exams will be performed inside the hospital (in a separate room of the CRN unit). All the vitals as well as the nasal spray delivery and blood samples and DNA saliva samples will be performed inside the CRN unit in the presence of a nurse.

Given that the staff researcher needs to synchronize between MRI scan appointment, CRN unit, IDS and hormone timeline, we will be flexible with the order of administering questionnaires, training and practice sessions to participants (dependent on breaks and fatigue of participants).

Inside the scanner, participants will perform these 2 tasks:

Task number 1: Face perception task (FPT): participants will be given a tutorial and a short trial practice session to familiarize them with the procedure of the task. The Subjects will also complete a simple quiz to insure the comprehension of the task. If the subject incorrectly answers one or more question, the researcher or coordinator will explain why the answer was wrong and will not proceed with the experiment until convinced that the subject understands the game. During this test, we will use an eye tracker (from the SMI company) to track participants' eye movements inside the scanner.

Task number 2: Multi-player emotion-based ball game (MP-EBG): participants will be given instructions and a short trial practice session to familiarize them with the procedure of the game. The Subjects will also complete a simple quiz to insure the comprehension of the task. If the subject incorrectly answers one or more question, the researcher or coordinator will explain why the answer was wrong and will not proceed with the experiment until convinced that the subject understands the game.

Subjects will be asked to refrain from use of caffeinated drinks or tobacco for at least 12 hours prior the blood pressure and heart rate measurement.

- **Consent and training:** We will first review the screening and the consent form. Participants will also perform the training/practice and quizzes on the face perception task and the ball-game that will be performed inside the scanner. Participants will also be asked to complete simple questionnaire (such as the face rating questionnaire). Questions about the mood will be again asked after spray intake.

- **Vital signs:** Prior to hormone administration, temperature, heart rate and blood pressure will be measured and recorded by the CRN nurses. Weight and height will be also measured. If heart rate is above 100bmp, or blood pressure is above 140/90, vitals will be re-measured. If levels remain elevated after 3 successive recordings in both limbs (total of 6 recordings) measured at least 5 minutes apart, the study physician will be notified and asked to advise on how to treat the subject and whether it is safe to continue with the protocol. Subjects may still be included at the discretion of the study physician. This is done in order to proceed with baseline vital signs that are within post-hormone administration guidelines. Researcher will ask if the subject is experiencing any adverse events at this time, to provide a baseline before hormone administration.

- **Blood draw:** A registered nurse from the CRN will collect 10 ml of blood by venipuncture for subsequent baseline measurement of plasma oxytocin. Blood will be collected in a lavendar-top EDTA-treated tube. Samples will be immediately centrifuged at 4C by the CRN core laboratory and the plasma and blood cells will be stored at -80 until assay at the CRN unit.

- **Spray intake:** Patients with ASD will receive intranasal administration of either syntocinon oxytocin spray 8IU or 24IU or 48IU or placebo. Given that it is a double blind study, we will make sure that all participants receive the same amount of puffs (12 total puffs) across all visits across all doses and placebo. Nurse will instruct the patient to clear the nose from obvious obstruction before administration. The nurse will prime the bottle by spraying in the air before administration. The nurse instructs the subject to sit with the head upright with little tilting to the back. The patient can close one nostril with one finger while the nurse is administering the spray to the other nostril. The nurse will ask the patient to sniff and breathe in gently directly after each delivery and block the opposing nostril. The insertion depth of the spray should be greater than 1.5 cm into the nostril. Ideal is around 1 cm. The nurse will hold the bottle on angle > 45 degrees from tip of spray bottle to horizontal plane. The nurse will make sure that the correct amount was entered and that there is no drainage from the nose. We are adding an appendix on chemistry and design at the end of this protocol to provide more details on the drug design. The placebo solution will contain all the inactive ingredients of the OXT solution except for the active oxytocin neuropeptide. The placebo and the Syntocinon spray will be purchased from a pharmacy in Switzerland. Both the experimenter and the subject will be blind to the solution they are receiving. Nurses will be trained on the instructions for the application of the nasal spray. They need to make sure that they depress the lever until the subject feels the mist of spray in the nostril and then let them breathe in deeply through the nose and afterwards to place

the applicator in the other nostril and repeat the process with some few seconds interval. Subjects will be asked to answer some questions on whether he could discriminate the different doses of oxytocin or oxytocin from placebo. Participants will be asked on whether they think they took placebo or oxytocin following spray intake. A stopwatch will be started at the middle of the administration of the spray so that the resting state fMRI starts exactly at 40 minutes later.

Healthy subjects will only receive 12 puffs of placebo spray. They will not receive Syntocinon spray. However, they believe that they might be taking placebo or Syntocinon.

- **Blood draw:** 5 minutes after spray intake, a registered nurse from the CRN will collect 10 ml of blood by venipuncture for subsequent baseline measurement of plasma oxytocin. Blood will be collected in a lavendar-top EDTA-treated tube. Samples will be immediately centrifuged at 4C by the CRN core laboratory and the plasma and blood cells will be stored at -80 until assay.

- **Vital signs:** 15 minutes after spray intake, temperature, heart rate and blood pressure will be re-evaluated by the CRN nurse to check for any side effects of IN-OT. There are several evidence of safety and absence of side effects from chronic and acute intake of IN-OT. Thus, we do not expect any side effects from the hormone oxytocin intake. However, we will ask subjects if they feel any side effects. If no side effects are reported, but temperature has increased or decreased more than 2 degrees, heart rate is above 100 bpm, or blood pressure is above 140/90, the vitals will be re-measured. If levels remain elevated after 3 successive recordings in both limbs (total of 6 recordings) measured at least 5 minutes apart, the study physician will be notified and asked to advise on how to treat the subject and whether it is safe to continue with the protocol. Subjects may still be included at the discretion of the study physician. If subjects were to experience a serious adverse event, such as a heart attack or an anaphylactic reaction, a code will be called and, if necessary, the subject will be taken to the Emory ER. Dr. Haroon or/and Dr. Cubells and the CRN nurses will be notified by the experimenter. Dr. Haroon or Dr. Cubells will call to admit the subject to the ER. As we already mentioned, it is not likely that oxytocin has any side effects based on the growing evidence in the literature. In addition to vital signs, we will ask participants if they feel any side effects.

- **MRI scanner (one-hour duration):** Subjects will next be escorted to the Biomedical Imaging Technology Center in Emory University Hospital from the CRN, accompanied by study staff. Subjects will next be positioned in the MRI scanner (Siemens, 3T). Subjects will lay motionless inside. Padded head restraint will minimize head movement during scanning. Each scanning session will begin with a scan to localize the position of the brain within the magnet. A vitamin E capsule will be taped to the subjects left temple as a localizer for the images collected. Participants will be given a button box which they will use to perform all the tasks. We can also give them a blanket if they are cold. We will explain to participants how to use the box for each of the tasks. Before starting the tasks below, we will run localizer sequence.

- **At first, (40 minutes after spray intake)** participants will be asked to rest and not think of something specific for 8 minutes. We will be recording brain activity during resting state (no task involved).
- **Second,** (**FPT or face perception task**) participants will be told that faces will be presented at the screen and that these faces will be separated by a fixation cross that can be blue or red. They are asked to press a key whenever they observe a blue cross. During this task, participants' eye movements will be recorded by an eye tracker that will be implemented inside the MRI scanner. Participants will not be told about the eye tracker given that we don't want to bias their attention. Participants will be wearing the watch sensor. Participants will be debriefed about the presence of the eye tracker at the end of the study.
- **Third, (MP-EBG or multi-player's emotion-based ball game)** participants will play several runs of the ball game with several players. We might have a discussion that will be shown at the screen with another coordinator to convince participants that they are playing with other players that are staying in another room. Participants were already instructed about the details of the game.
- **Anatomical Image Acquisition:** Field map and T1 will be acquired (this can be acquired in the middle in between games or at the end of the session).
- **Post MRI scan:** Subjects will be escorted to the office next to the scanner and will be asked to rest and then complete some tasks and questionnaires. It is possible that we offer participants some snacks or lunch. Participants will complete:

    - **Face Rating questionnaire (post MRI):** participants are asked to rate (on a scale 1 to 7) faces.
    - **A social learning test (SLT):** participants are asked to match faces and words as fast as they can.
    - **Emotion Matching test (EMT):** participants are asked to identify the emotion that is displayed on a face by matching the emotional face that appears on the left to one of the faces that appears to the right (different faces but same emotion).
    - **Toronto Alexithymia scale**: participants are asked to complete a simple questionnaire.
    - **Mood questionnaire (PANAS scale)**.
    - **BB-CGI (clinical interview):** the same interview that is performed during the pre-MRI visit will be performed during this visit in order to collect a clinical score of improvement as well as physiological, verbal and socio-emotional improvements.

4- Second, Third and Fourth visit of scan (ASD subjects) (total duration= around 3 to 4 hours each): The exact same procedures will be performed during these other 3 visits. These 3 additional visits will be conducted only in ASD subjects. Also, participants will receive different content of spray (either different dose of oxytocin or placebo).

Of note, the health assessment will be reviewed one more time (in addition to the assessment during the first pre-clinical visit) by the nurse practitioner or the study physician during one of the 4 clinical visits, if the original health assessment expires (more than 30 days).

	Screening and consent review/training	Vital signs 1	Blood samples Pre	Hormone Spray (PL, 8, 24, 48 IN-OT)	Blood samples post	Vital signs 2	MRI scan	Post MRI scan
Visit 1	X	X	X	X	X	X	Rest+ 3 tasks	Quest+ 2 tasks + clinical interview
Visit 2	X	X	X	X	X	X	Rest+ 3 tasks	Quest+ 2 tasks + clinical interview
Visit 3	X	X	X	X	X	X	Rest+ 3 tasks	Quest+ 2 tasks + clinical interview
Visit 4	X	X	X	X	X	X	Rest+ 3 tasks	Quest+ 2 tasks + clinical interview

Table 2: Clinical trial visits for subjects with ASD

Visits will be separated by a one-week interval approximately (± 2 to 3 days). We have to be flexible with the scheduling given that we are facing challenges while scheduling all the units and personals. Every patient will receive the 3 doses of IN-OT (8IU, 24IU, 48IU) and placebo in a double blind, placebo controlled trial.

It is also possible that some patients will feel tired and will not be able to go through all the tests inside the MRI scanner. In this case, we will stop as based on participants' level of fatigue. We will try to have similar number of tests across 4 clinical visits.

I would like to mention here that given the complexity of this study, we expect that some of the tasks/procedures will be omitted or not completed during each of the 5 visits. We do not expect that all patients perform all the tasks described above (inside or outside the MRI scanner). We will try our best to test participants on all these tasks above but we could face some challenges and decide not to complete them. For instance, we will be based on the fatigue level of patients and patients' feedback to make these decisions. The level of fatigue varies from one participant to another.

Healthy subjects

Healthy subjects will participate only in one visit (around 4 hours) during which they will:

- Revision of the screening forms which includes eligibility criteria and MRI screening form by the PI or study coordinator. The PI will update the physician at regular meetings about the inclusion of these subjects. If there is any doubt about inclusion criteria, the PI will ask physician for advice. However, there is no need for the signature of the physician or the nurse practitioner on the eligibility criteria given that we are including healthy subjects and we are administering only placebo spray. The nurse practitioner or the study physician will not conduct a health assessment.
- Read and sign the informed consent (no assessment of capacity is necessary).
- Complete some questionnaires: Symptom Checklist SCL-90, the interpersonal Reactivity Index, emotion rating of faces
- Complete a computer test of social learning test
- Complete a training on the face perception task and social games (as described above) and will perform quizzes
- Subjects will refrain from use of caffeinated drinks or tobacco for at least 12 hours the testing.
- Blood draw as described above 5 minutes after the spray
- Vital signs collection directly after the blood draw (no need to wait for an exact 15 minutes)
- Spray intake as described above. Subjects will think that they might be taking oxytocin or placebo. The experimenter is aware that the subject is receiving placebo. The nurses who are administering the drug are not necessary aware of the content of the drug.
- Second blood draw 5 minutes after spray intake
- Vital signs collected
- MRI scan: Subjects will also complete the resting state, the FPT task and the ball-game inside the MRI scanner. Ideally, subjects will also start around 40 minutes after the spray intake. The timing will be more flexible given that subjects are not receiving drug.
- Participants will complete face rating questionnaire, the SLT and the EMT as well as the Toronto Alexithymia and mood questionnaire.

We will not collect physiological data on healthy subjects as we are not conducting an interview. We might be able to collect some data while they are inside the MRI scanner.

	Screening and consent, questionnaires, practice on behavioral tasks, computer tasks	Vital signs 1	Blood samples Pre	Spray (PI)	Blood samples post	Vital signs 2	MRI scan	Post MRI scan
Visit 1	X	X	X	X	X	X	Rest+ 3 tasks	Quest+ 2 tasks

The new group of healthy subjects that we are recruiting from general population to match patients with autism will have to do two visits.

The first visit will consist of revision of informed consent and screening tests (that includes an IQ test and a color blindness test).

The second visit will be the same as above.

Risks/discomforts and potential benefits

DNA: A breach in confidentiality, resulting from inadvertent disclosure of genetic information to parties outside the research group, has the potential to negatively affect participant's insurability, employability or reproduction plans, and could have a negative impact on family relationships and/or could result in paternity suits or stigmatization.

DNA methylation: DNA methylation data is not informative for individual identification.

Intranasal OT administration (Syntocinon): In rare cases, nausea, vomiting, headache, irregularities of the pulse, skin rashes and allergic reactions in combination with shortage of breath, blood pressure decreases or circulatory collapse are reported as adverse effects of regular and repeated administration of Syntocinon. However, it is important to note that no serious adverse side effects have been reported following the administration of one single dose in any of the previously published studies using intranasal oxytocin.

Today, there are several studies with single doses and chronic oxytocin administration to individuals with ASD. Aoki et al. 2014 administered 24IU to 40 ASD with an average age of 29.6 and reported no adverse side effects. Auyeung et al. 2015 administered 24IU to individuals with ASD (34 years old on average) and found no serious side effects. They reported runny nose (in 3 cases with oxytocin vs 5 cases with placebo), tiredness (2 cases of oxytocin vs 3 cases with placebo), sore throat (1 case with oxytocin vs 0 case with placebo), shakiness (0 with oxytocin vs 1 case with placebo). Healthy subjects who received placebo spray reported runny nose, tiredness and mild nausea. Guastella et al. 2010 showed no difference in the number of reported side effects between oxytocin and placebo conditions in ASD (18IU or 24IU). Under oxytocin, they felt tired, relaxed and sweating. Under placebo, they felt tired, relaxed, headaches and coughing.

In a chronic administration (2months administration of 8IU, 6IU and 24IU) to ASD subjects between the ages of 10 and 14, Tachibana et al. 2013 conducted a safety study. They monitored blood pressure, urine osmolality, urine sodium concentration and serum osmolarity, serum sodium concentration and plasma OXT concentration, serum bone specific alkaline phosphatase and short memory and found no side effects. Hence, studies in the literature show that oxytocin administration is safe, especially in our study context. We do not expect serious adverse events related to oxytocin and do not expect significant differences in adverse effects between oxytocin doses and placebo.

Blood draws: The needle stick for the blood draw may cause momentary pain or discomfort. In rare cases, bruising at the site of the draw may occur. In rare cases, the subject may feel light-headed or faint as a result of the blood draw.

MRI: Potential risk to subjects without contraindications includes mild anxiety, claustrophobic reaction, and movement of ferromagnetic objects. Certain MRI scanning procedures can produce loud (>100 dB) noises, so ear protection will be provided by the use of sound-reduction earplugs. Beyond these risks, there are no known side effects of MRI to subjects without contraindications.

Participants might feel fatigued from the several hours of testing.

Procedures for minimizing risks:

Genetic testing: We recognize the sensitive nature of genetic information and the need to maintain strict confidentiality procedures for data that may ultimately be available to the wider scientific community. To minimize the risk of inadvertent disclosure of research information to outside parties, all information obtained from participants will be kept strictly confidential. No individual genetic results will be given to participants or their relatives. DNA used in this study will be assigned a code and the link to further identifying information concerning the individual will be available only to the PI. Release of data from NIMH to the wider scientific community will be in accordance with confidentiality procedures that have been established by the NIMH Genetics Initiative. We will adhere to the guiding principle that study identifiers cannot be connected with personal identifiers at any state of the data collection. Personal identifiers (i.e. name, address, email contact details) will be maintained on a secure server that follows HIPAA regulations. We will be transferring blood cells, plasma samples to other laboratory within Emory and outside Emory (after getting a permission of a medical transfer agreement). We will make sure to send de-identified coded samples. The same applies for saliva samples.

We would like to add more specific details about the processing of plasma samples. We are transferring some of the plasma samples of healthy subjects and patients with ASD from the CRN unit to Shota Nishitani who is at Emory University. He will be analyzing the levels of oxytocin in plasma. The plasma samples are coded and do not contain names. However, there are some identifiers that are present on the samples: such as date of submission and other. We added Shota to the staff personnel.

We are likely to give him saliva samples too to measure DNA methylation of the oxytocin receptor gene.

Intranasal OT administration: With a single dose of Syntocinon, no adverse side effects of the substance are to be expected. To further minimize risk, subjects will receive OXT at Emory Hospital (CRN unit at ACTSI). To monitor potential side effects of OXT administration, temperature, heart rate and blood pressure will be measured before hormone administration and 15 minutes after hormone administration. Experimenter will again ask if the subject is experiencing any of the following symptoms: chest pain, difficulty breathing, itching. If the subject

reports any of these symptoms, the study physician or study nurse practitioner will be contacted and asked to advise on how to treat the subject and whether it is safe to continue with the protocol. If necessary, a code will be called. If no side effects are reported, but temperature has increased or decreased more than 2 degrees, heart rate is above 100 bpm, or blood pressure is above 140/90, the subject will be asked to sit quietly for 10 minutes after which vital signs will be re-measured. If vital signs are within the normal range, and no side effects are reported, they will proceed through the experiment. If not, the study physician or study nurse practitioner will be contacted and asked to advise on how to treat the subject and whether it is safe to continue with the protocol. If subjects were to experience a serious adverse event, such as a heart attack or an anaphylactic reaction, a code med will be called and, if necessary, the subject will be taken to the Emory ER. Dr. Haroon or Dr. Cubells and the CRN nurses will be notified by the project coordinator. Dr. Haroon or Dr. Cubells will call to admit the subject to the ER.

Blood Draw: Subjects will be monitored for signs that they may faint such as clammy skin, dizziness, confusion, irregular breathing, and decreased blood pressure. If these signs are observed, subjects will be asked to lie down with their legs elevated and blood pressure will be monitored until symptoms subside. If a subject loses consciousness and requires medical attention while in the Anthropology Building, Emory First Responders will be contacted (911). If a subject loses consciousness and requires medical attention while at the Biomedical Imaging Technology Center in Emory University Hospital, study staff will instruct the MRI technician to call a hospital code for medical care.

MRI: Risks will be minimized in the following manner: 1) Subjects will be screened for contraindications as described above. In particular, subjects who have a history of moderate or severe claustrophobia may be excluded at the discretion of the PI. Subjects with a history or possible history of intra-ocular, intracranial, intrathorax, or intra-abdominal metal or cardiac pacemakers will be excluded. 2) Anxiety will be minimized by thoroughly explaining the procedures and the nature of the magnetic resonance scanner to subjects prior to study. During the scanning procedures, the subjects are continually monitored visually and auditorily for any potential problems, and subjects are assured that they can and will be removed from the scanner at any time if problems should arise or they indicate that they are experiencing discomfort. As noted above, subjects will be screened twice prior to study, once at the time of initial contact, and a second time when they arrive at the MRI facility prior to study. All of the data collected for these studies will be kept strictly confidential. Under no circumstances will individually identifiable data be released to anyone without the written consent of the subject. MRI copies of the brain anatomy will be provided to subjects.

Risk-Benefit Ratio:

Subjects participating the study will receive a monetary compensation to compensate for their time and effort. The results of this study can provide more knowledge about the role of intranasal oxytocin in autism and about the neural correlates of its action. It will also provide knowledge about the heterogeneity in oxytocin effects that could be related to different intrinsic factors (such as DNA methylation, clinical classification, genetic polymorphisms, environmental factors, metabolites and immune factors). It will also provide more information on the biological basis of social functioning in ASD. It is hoped that such knowledge will ultimately lead to a better

understanding of psychiatric disorders involving social behavioral deficits. These potential benefits outweigh the minimal risk this research poses to subjects.

Type of information collected

1- We are collecting behavioral data from questionnaires and computerized social games and tasks. Responses to questionnaires, accuracy during games or tasks, reaction time will be collected and responses.
2- Video-taping participants and getting a photo during the clinical interview BB-CGI (see above).
3- Clinical diagnosis of severity of symptoms based on the BB-CGI that will rated by a psychiatrist.
4- Based on the video-taping, staff members will code behavioral and emotional expressions and verbal aptitudes of participants and synchrony with the experimenter.
5- Physiological measures from the sensor watch that will consist of temperature, electrodermal activity (or skin conductance), heart rate variability.
6- MRI scans of anatomy that will be kept for future
7- FMRI data (functional BOLD signal) in response to cues and in response to rest
8- Vital signs (BP, Temp, RR, HR), weight, height
9- General information form: the questions regarding participants are: medication, difficulties, current occupation, activities, social network, participation in behavioral therapy or group activities to stimulating social aptitudes, specific interest and knowledge, specific talents). The questions regarding subject's family will consist of: number of siblings, gender of siblings, education/occupation/social aptitudes of siblings, parents and grandparents, health-related questions, presence of any psychiatric, autism-related disorders.
10- Eye tracking data: eye movements of subjects while they are laying down in the scanner performing a task. We will quantify subjects' visual duration and fixation on regions of interest of the stimuli that are presented on the screen.
11- Physical that is performed by the NP.

Specimens

3 Saliva samples will be collected for DNA analysis. We will use the Oragen DNA kits. Some samples might be used to quantify DNA methylation of OXTR and OXT gene or OXTR polymorphisms, MET gene. The remaining samples will be kept safe for potential future use. We will not collect saliva samples from healthy subjects.

8 blood samples (EDTA purple tubes of 10 ml each) will be collected per subject for all 4 MRI visits (4 blood samples will be collected before intranasal administration and 4 blood samples will be collected after oxytocin administration for all visits). Samples were kept chilled in ice and centrifuged within 1 h from collection at 1600 g for 15 min at 4 °C. Plasma was removed and initially frozen at −20 °C, then stored at −80 °C. Plasma samples and blood cells will be extracted and stored at −80 °C until the assays were performed. The centrifuging will be performed at

ACTSI and the samples will be stored at CRN until we are ready to transport some of the samples for the different laboratory to analyze it.

We intend to send some aliquots of plasma from each of these 8 samples to public health to quantify oxytocin concentration in the plasma and to quantify the exposome and metabolome (chemicals/pesticides).

Plasma will be analyzed for cytokines levels.

Blood cells from 4 blood samples (prior to OXT administration) will be sent to analyze the DNA methylation and/or to analyze OXTR polymorphism analysis.

The rest of blood cells and plasma will be kept safe at CRN unit for future analysis.

All these specimens are collected from healthy subjects and from patients with autism.

Randomization and blinding

Clinical trial for ASD subjects: The trial will be a double-blind, placebo-controlled, several doses of oxytocin, within subject design study. Patients will undergo 4 clinical visits (in addition to a first pre-clinical visit) during which they receive 12 puffs (6 in each nostril) of 8IU total, 24IU and 48IU oxytocin (syntocinon) and placebo (assigned randomly). The placebo will contain the same ingredients as syntocinon, except the active molecule oxytocin. This is essential for making sure that the patient does not recognize the type of treatment he is receiving. Hence, both patients and the experimenter will be blind to the type of the treatment administered.

The pharmacy IDS of Emory will be in charge of the randomization sequence and one of the staff members of the team (who is not involved in testing procedures) will be de-blinded.

We will break the blind after testing 25 patients with ASD to check the results half-way for a mid-point analysis. The mid-point analysis will focus on measures where effect sizes are anticipated to be large enough that the risk of false positives is minimal. We are expecting larger effect size on some measures and therefore we break the blind for those measures to be analyzed and published. Additional data on these measures will continue to be collected and we will attempt to publish the combined analysis at the end to avoid publication bias.

However, the data collection will continue until a sample size equal to 50. Given that we are facing challenges with ASD recruitment and that the study requires the participation in 5 long visits, it is possible that the study will stop at N=30 or 40, in case the PI was not able to recruit more ASD. We also expect dropouts after recruitment. We believe that we will be able to test 30 patients with ASD.

It is possible that this project will be funded by other grant funds which will allow adding more participants (more 50 ASD). In this case, the PI will submit an amendment accordingly.

Healthy subjects: There will be only one clinical visit for healthy subjects and they will all receive placebo spray (12 puffs, 6 per nostril). The study will be conducted in a single-blind design. The PI is aware that the subject is receiving placebo. However, the participant will be told that he might

receive oxytocin or placebo. This is important in order to control for the placebo effect on behavior and brain activity.

Storage:

Saliva DNA samples will be stored in a locked place

Blood samples will be centrifuged into plasma and blood cells at the CRN unit and stored at -80˚C at CRN unit until the PI is ready to transfer the tubes to laboratories for analysis. The PI will make sure that he has all the transfer material documents in place and all the procedures detailed in the IRB protocol before sending the samples for laboratories. All the samples that will be transferred to other locations for specific analysis will be de-identified and coded and will not have any personal identifiers related to participants' identity. Only the PI will have access to the identifiers and link between codes and participants' identity. Physical material such as files and folders (that includes identifiable information) will be stored in a secure and locked place. All the electronic excel data that are linked to identifiers will be saved on the psychiatry server (which is HIPAA compliant). Collaborators who will conduct the analysis cited above will collect the results in an excel sheet but will include only coded de-identified information. They will send the PI the excel files with the results via Emory email and only the PI will have access to the links between identifiers and the coded results.

Our collaborators at public health have communicated with us the details of the storage of the plasma samples for oxytocin measurement and metabolites/exposome/pesticides measurements:

Data files obtained from metabolic profiling will be stored on a secure, offsite RAID that was specifically designated for use by the Clinical Biomarkers Laboratory. Feature tables will be stored on the network hard drive available to Biomarkers personnel associated with the project only. All analyses of the result files will be logged, and include type of analysis, statistical parameters, date of analysis, investigator and summary of results. All method operational parameters will be printed and stored with raw analysis logs, and any alterations to instrumental configuration will be noted in a log book kept for that specific instrument. Laboratory notebooks will be maintained by all project personnel, with all entries made in ink. Due to the nature of the research completed by the Clinical Biomarkers Lab, all technical infrastructures required for data management are in place and can be adapted for use for this project.

Future use:

All the samples remaining will be stored at the CRN unit/Emory hospital until further notice. The PI will decide when to destroy the samples. All the data and materials of this project (including samples, specimens, behavioral results, brain function, physiology and other) will be used only by the PI (Elissar Andari) or with her permission for future analysis, future studies etc. If the PI (Elissar Andari) leaves Emory, she would like to have access to all the samples and data for future studies. Any future study/collaboration at Emory or other locations using any data or materials

that were collected under this protocol (genetics, clinical, behavior, brain, etc) is possible only after permission of Elissar Andari.

Participant selection:

50 ASD between the ages of 18 and 45 will participate in this study. 30 healthy controls who are matched by IQ, race, ethnicity and age will also participate in this study. All participants are males.

Healthy subjects will only receive placebo spray. Healthy subjects will be told that they might receive placebo or oxytocin spray (one of the 3 doses). Healthy subjects will participate in one visit during which they complete questionnaires, behavioral tasks, clinical interview and training on the different tasks that they will be doing inside the MRI scanner. They will get a physical, vital signs, blood draws, placebo spray, MRI scan (including all the tasks that are described above) and behavioral tasks after the MRI scan.

ASD subjects will participate in a pre-clinical visit and will perform all the tasks and procedures that are described above. They will also participate in 4 clinical visits that will include treatment intake (syntocinon spray 8IU, 24IU, 48IU and placebo), blood draws, vital signs, MRI scan, behavioral tasks and questionnaires (see below the procedure for more details). It is a placebo-controlled randomized clinical trial, double-blind and within subject design.

Subjects will have normal or corrected-to-normal (with contact lenses) visual acuity of 20/40. MRI-compatible glasses are available for those who wear glasses rather than contact lenses. A medical history will be obtained and reviewed by the study physician or study nurse practitioner, including information about prescription and non-prescription medications.

Inclusion/exclusion criteria for ASD subjects:

Given that the inclusion criteria list and the screening will be first conducted during the first pre-clinical visit (by E. Andari or staff and not by nurse practitioner), the experimenter will contact physician Dr. Cubells or Dr. Haroon or the nurse practitioner in case there were some doubts.

The nurse practitioner will conduct this screening again during the first clinical visit to make sure that the entire subject meets the criteria of inclusion and exclusion.

Inclusion:

- Sex males (we are excluding women for this present study given the difficulty to find the same number of women with ASD. Autism is found in men 4 times more than in women as based on CDC epidemiological studies). Also, only male will be included to minimize possible side effects of oxytocin administration that can be related to uterine contractions and lactation in females and to minimize confounding factors that can arise from hormonal interactions with estrogen in women. We are including male sex which means that a gender male person who was born as a woman is not considered part of the inclusion criteria.

- Age: between 18 and 45 years old.
- Have an ASD diagnosis based on the ADOS and ADI criteria, gold standards of research-based autism diagnosis.
- Not color blind (some of the behavioral tasks inside the scanner require detection of color)
- IQ>70
- Normal or corrected-to-normal vision

Exclusion:

- Recent occurrence of seizures (past 5 years) (lot of patients with autism have seizures when they were young and these seizures never re-reappear and do not provide a risk for subjects in this study).
- Brain damage or head trauma (can be included at discretion of PI and sponsor)
- Cardiovascular disease
- Presence of a severe medical problem
- Severe mental retardation
- Alcoholism or substance abuse
- Asthma (can be included at the discretion of study physician/nurse practitioner if episodes are infrequent and no active problems at time of the study)
- Migraine headaches (at the discretion of the nurse practitioner or the study physician)
- Claustrophobia (at discretion of study physician/designee/PI)
- Pacemakers, cochlear implants, surgical clips or metal fragments

In the general form, we will take note of any comorbid psychiatric disorder, current medications, current medical problems such as diabetes, migraines and take these factors as covariates in our analysis. We will also take a note of the date of last event and the frequency. We want to try to include as many patients with ASD as possible to represent at best the general population with high functioning autism.

Those who are on medication, we do not exclude them from the study but ask them to continue their treatment as normal.

Inclusion/exclusion criteria for healthy subjects:

Inclusion:

- Sex males between 18 and 45 years old (given that they should be controls for ASD subjects)
- Matched to ASD group
- Not color blind
- IQ > 70

Exclusion:

- History of seizures (past 5 years)
- Neurological disorder

- Current psychiatric disorder
- Previous psychiatric disorder (can be included at discretion of PI). If the data of the last episode is more than one year ago then the PI will include him. If the date of the last episode dates to less than a year, at discretion of PI).
- Head trauma (can be included at discretion of PI)
- Alcoholism or substance abuse
- Cardiovascular disease
- Asthma (can be included at the discretion of study physician/nurse practitioner if episodes are infrequent and no active problems at time of the study)
- Migraine (at the discretion of the nurse practitioner or the study physician)
- Claustrophobia (at discretion of study physician/designee/PI)
- Presence of a severe medical problem
- Severe mental retardation
- Pacemakers, cochlear implants, surgical clips or metal fragments

Given that midway of the study, we had trouble recruiting healthy subjects that are matched with ASD, we asked to add another group of heatlhy subjects with more selective criteria of inclusion.

We are adding Caucasian as a criterion of inclusion and IQ within the range of 70 and 115.

Of note, given that healthy subjects will not be evaluated by a NP or study physician, the PI and the coordinator will rely on the self-report of these healthy subjects. They will sign the inclusion criteria document.

Subjects will be studied only if they pass the Society of Magnetic Resonance Imaging standardized MRI screening protocol (exclusions for ferrous metal in any part of body, such as pacemakers, cochlear implants, surgical clips or metal fragments, serious medical conditions). Subjects with claustrophobia will be excluded at the discretion of the PI based on consultation with the subject. Subjects with claustrophobia will be carefully monitored and reminded that they are free to withdraw from the study at any time. Subjects who report a history of asthma or migraine headaches will be included at the discretion of the study physician or study nurse practitioner only if episodes are infrequent; there are no active problems at the time of the study. Subjects with a history of head trauma or psychiatric illness will be included at the discretion of the PI. Any cognitive consequence of head injury could be taken into account during the decision.

Subjects will be asked to refrain from use of caffeinated drinks or tobacco for at least 12 hours prior the blood pressure and heart rate measurement.

Given that this project is the continuity of the previous IRB protocol 64623, we will contact healthy subjects and patients with autism that were already recruited in this previous protocol. We are planning to contact those who gave us approval to be contacted (in the consent form). We will go through the screening process (see above for more details) over the phone with the patient and family member if necessary and make sure he is interested and he is eligible for the study. The study coordinator will then schedule them for the first pre-clinical visit.

If a subject withdraws from the study, we will prepare a note to file (and put it in the regulatory binder) and report to the IRB during the continuing review.

We expect to recruit at least 30 patients with autism and 20 healthy controls. We expect a drop off of patients with ASD given that they need to come for 5 visits.

Informed consent process

- Consent forms will be obtained in person from adult participants. All participants, including patients with autism and healthy subjects are adults and capable of understanding the consent form. However, some of our patients with autism have legal guardianship or legal authority representative by one of the parents because of money expenses and other. For these cases, we will ask for a signature from the parent as well as from the adult subject.
- We will ensure that compensation is in line with effort and time required for the study. We will ensure comprehension of the informed consent information by allowing time for questions, verbally summarizing the material with the subject instead of just having them read it and asking subjects to answer a few questions to gauge understanding.

Communication of results: given that we are including MRI scans, we will be asking a medical doctor to look at them to double check any un-anticipated or incidental findings (such as tumors or apparent lesions). We will communicate incidental findings with the participant in person (after contacting him by email), only if the medical doctor decides that the MRI scan should be reviewed by an expert neurologist with a medical MRI to double check some doubts. We will be reporting only apparent incidents that could be life-threatening to the subject. We are not equipped to share with the participant's minor or more subtle problems given that we are conducting a research MRI scan and not a medical MRI. Participants will be provided a copy of the MRI anatomy scan. General results of the study can be communicated with participants after publication.

Compensation: Patients with autism will receive a total of $200 for their participation in 5 research visits ($40 per visit). Healthy subjects will receive a total of $80 for their research visit.

Statistical analysis:

Power analysis (using G power 3.1 software) shows that a sample size of 50 subjects with ASD receiving 4 different treatments is needed to reliably detect (95% power) effect sizes small or larger (Cohen's d=0.2) on one measure. In this study, we expect to find small to medium size effects of oxytocin inhalation on behavioral measures (based on previous meta-analysis of Bakermans-Kranenburg and Van I Jzendoorn. 2013). Authors found a combined effect size of d=0.32 (95% confidence interval: 0.18-0.47). As mentioned above, we are planning to break the blind with N=25 ASD subjects for a mid-way analysis, which is sufficient to detect effect sizes between medium and small (Cohen's d=0.3). We expect larger effect sizes on measures of brain

activity (BOLD activity and functional connectivity). Therefore, these measures will be mainly analyzed during the mid-way analysis for potential publications. Of note, we are also planning to publish the combined analysis with the total number of subjects on the measures that were published mid-way to avoid any publication bias.

For healthy subjects, we need at least 30 subjects receiving on treatment to reliably detect (95% power) the differences between social and non-social conditions or emotional faces versus neutral faces in the aims above with an effect size between small and medium (Cohen's d=0.35).

When comparing healthy subjects to patients with autism (placebo administration only), the above numbers are sufficient to reliably detect small effect sizes between groups (Cohen's d=0.2) for one measure.

The PI will submit an annual report to the IRB and address whether the study is safe and whether it is appropriate to continue. This report will include compliance, review of eligibility, treatment review, summary of responses, survival, adverse events and safety monitoring rules.

Given that we do not expect serious adverse events and we are investigating the effects of this hormone on brain activity and behavior, we do not expect an early stopping.

There will be analysis at various stages of the trial. We might need to analyze the behavioral and physiological data for data monitoring committee, in any or to write reports to NIH about the progress. There will be analysis of primary outcome measures of the different aims (cited above) during mid-way analysis (when we break the blind half way at N=25 of ASD). The analysis will also be conducted at the final stage of the trial.

Brain imaging procedures using MRI, fMRI will be conducted at the Biomedical Imaging Technology Center (BITC) at Emory. Anatomical Image Acquisition: participants will be positioned supine in the MRI scanner (Siemens, Magnetom Prisma, Trio 3T) with instructions to lie motionless. Padded head restraint will minimize head movement during scanning sessions, each of which will begin with a 15 second scout scan to localize the position of the brain within the magnet. This will be followed by T1-weighted MPRAGE scan (TR= 2300 ms, TE = 4 ms, matrix = 256x256, FOV=256, slice thickness = 1.00 mm, gap = 0 mm).

For aim 1, *the primary outcome measure* consists of investigating the effects of different treatments (3 doses of oxytocin and one dose of placebo) on brain functional connectivity at rest in patients with ASD, using fMRI. During resting state functional image acquisition (8 minutes), participants will be asked to remain awake and not moving. Image acquisition will be obtained using a multi-band slice accelerated gradient echo-planar imaging (EPI) pulse sequence 179, 180 with the following parameters: TR = 1000 ms, TE = 24 ms, matrix = 74 x 74, FOV = 220 mm, slice thickness = 2.0 mm, 64 axial slices, to reduce magnetic susceptibility artifact in the OFC region.

The aim of this analysis is to determine whether there is target engagement for intranasal oxytocin by demonstrating differences between doses of IN-OT on BOLD rsFC or whether there is a simple oxytocin effect. We define target engagement OXT effect (TEE-OXT) as any differential effect between a dose of OXT from another dose of OXT on brain or behavior. We define oxytocin effect

(OXT-Effect) as any differential effect between oxytocin (any dose) and placebo administration in ASD.

Initial preprocessing steps for single-subject resting-state fMRI data will be conducted with FSL software or SPM with the help and training of Dr. Gopinath. FMRI data will undergo standard preprocessing steps: realignment, slice-timing correction, volume registration, spatial normalization to MNI152 template, spatial smoothing with 5 mm FWHM Gaussian filter and temporal band-pass filtering (0.01-0.1 HZ). Corrections for head motion, whole-brain signals, ventricular signals, white matter signals. We will define specific regions of interest that includes the social brain network that we discussed in the sections above (MPFC, OFC, PCC, precuneus, insula, ACC, ventral striatum, amygdala, temporal areas, SMA etc). We will compare the functional connectivity between and within the different social brain networks (including the default, the salience network and temporal network) between the different treatment conditions. This analysis will be performed based on the GLM using corresponding regressors. We will also compute the rsFC between the different regions of interest by calculating a Pearson's correlation coefficient between the time series of the preprocessed functional MRI signals. In order to study target engagement effect on within network correlations, the resulting of the cross-correlation maps will be Fisher transformed to z-scores and fed into a 2-factor (4 treatment condition X 3 networks (default, salience, temporal); covariate: visit order) ANOVA. In order to study target engagement effect on between network correlations, the resulting of the cross-correlation maps will be Fisher transformed to z-scores and fed into a repeated Anova (4 treatment condition X 3 networks (Salience+Default; Salience+Temporal; Default+Temporal); covariate: visit order) ANOVA. In order to determine simple oxytocin effect, T-contrast maps will determine the differences in rsFC between placebo and each of the different doses of oxytocin. Multiple comparison correction will be conducted. The specificity of the results will also be conformed in a whole brain analysis. Given that baseline rs-FC is not acquired, and in order to account for non-specific fluctuations, we will use a two-stage GLM procedure during which we account for within-subject variability (across the different visits). Time regressor will be added to the model as a covariate to control for external factors that could influence rs-FC other than the treatment.

The secondary outcome measure consists of correlating the effects of treatments on rsFC with the clinical improvements following treatments and post scan in ASD. The variables consist of BB-CGI clinical score (that will be rated blindly by a physician) and a socio-emotional behavioral score (rated by PI or staff blindly) during the BB-CGI. These two variables will be analyzed separately as they are dependent (based on the same test) but answer two different questions. We will conduct correlation analysis (Spearman or Pearson) between TEE-OXT or OXT-effect on rsFC and behavioral and clinical improvements. Behavioral variables will include: emotional responses, gestures, eye contact with experimenter and synchrony).

For aim 2: *The primary outcome measure of the second aim* consists of investigating the differences between the effects of the different treatments on BOLD activity of socio-emotional brain regions in response to social cues (Face perception task) in ASD. ROI analysis will include regions such as inferior occipital cortex, fusiform gyrus, superior temporal cortex, intra-parietal sulcus, superior parietal lobule, nucleus accumbens, amygdala and OFC). In this analysis, we have two variables that we will contrast (BOLD signal in response to emotional faces versus

neutral faces) and 4 treatment conditions within subject design (8IU, 24IU, 48IU and placebo) with covariates (order of visits to control for familiarity effects given that the treatment conditions are completely randomized). The presence of TEE-OXT will consist of finding differences between different treatment conditions of OXT and not only between OXT and placebo. We will also compare each of these OXT treatments with placebo condition separately to study OXT-effect.

The secondary outcome measure will consist of studying the effects of different treatments on a behavioral measure that is recorded during the MRI scan of the FPT: visual fixation duration on facial stimuli. Here, we have one variable (duration of visual fixation or eye gaze on emotional and neutral faces) and 4 treatment conditions. We will analyze the data with ANOVA repeated measures for determining TEE-OXT and t-tests for simple OXT-effects.

For aim 3: *The primary outcome measure* of the third outcome measure consist of studying the different effects of treatments on BOLD activity of key brain regions (OFC, ventral striatum, inferior occipital cortex, fusiform gyrus, amygdala, superior parietal lobe, insula and ACC) while playing a social interactive ball-game in ASD. During this ball-game, we will analyze the BOLD brain activity during social output videos that will display either neutral or positive face expressions. We expect more rewarding brain regions to be more activated during the process of positive social feedback with oxytocin treatment. In order to determine TEE-OXT, we will conduct repeated ANOVA analysis with variables (BOLD activity in response to positive and neutral) and treatments (8, 24, 48, placebo). We will also investigate the general effect of TEE-OXT on the BOLD activity of socio-emotional brain regions in response to both positive and neutral faces with the variable (BOLD activity in response to all faces) and treatments. We will also investigate the differences between oxytocin treatment and placebo. We expect at least the doses of 24IU and 48IU to be different from placebo. Covariates such as visit order will be entered in the GLM model as a regressor to control for habituation effects.

The secondary outcome measure consists of studying the effects of treatments on behavioral outcome measure (post scanner on social learning). In this behavioral task, we will analyze the accuracy and reaction time in recognizing faces as familiar. We will conduct ANOVA and t-tests to compare the performance in these 2 variables (accuracy and reaction time) between the 4 treatment conditions.

For aim 4: *The primary outcome measure* of the fourth outcome measure consists of investigating the differences between treatments on the modulation of brain activity of reward brain regions in response to punishment and helpful behavior. In the GLM model, we will conduct an ANOVA or t-tests with 4 treatment conditions and the BOLD signal in NAcc, OFC, VTA and ACC.

For aim 5:

The primary outcome measure consists of investigating the differences between treatment conditions in oxytocin plasma concentration in ASD. Samples of post-administration of IN-OT will be analyzed. We will conduct ANOVA repeated measures on the different OXT concentration in 4 treatment conditions.

The secondary outcome measure consists of studying the differences between baseline oxytocin concentration between healthy subjects and ASD. T-tests will be conducted to determine the difference between two groups.

In this protocol, we have thought of several exploratory or secondary aims that include to compare brain function and functional connectivity between healthy controls and patients with ASD in response to resting state, in response to social cues and in response to interactive social games. We will also investigate the differences in behavioral and physiological responses between ASD and healthy subjects. We will also investigate differences between these two groups (during placebo administration) in baseline plasma OXT concentration, metabolome and exposome and cytokines levels.

In our exploratory analysis, we suggested to study the different factors that could explain heterogeneity in ASD such as clinical dissociation (different kinds of social deficits), behavioral and genetic and epigenetic contribution. We will conduct separate correlation analysis for each of these aims with the severity of ASD and brain function differences with controls. It is also possible to conduct factor analysis to study which behavioral variable for instance best predict ASD severity in clinical and brain function. The same applies for the best predictors of target engagement. We believe that not all patients with ASD will benefit from IN-OT. Hence, we will study the genetic factors, epigenetic factors, clinical classifications and behavioral patterns in determining TEE-OXT or OXT-effects.

It is possible that we publish separately target engagement results (comparison between doses of OXT) and oxytocin effects (24 and 48 as compared to placebo) on the same tasks that are conducted inside the MRI scanner.

Withdrawals, missing data and ineligible patients will be stated. Data will be controlled and double checked by the PI before publishing papers. Outliers will be investigated with statistical tools. Post-analysis will be checked for quality and checked for confounder factors, covariates. Additional precision on the type of analysis (behavioral, physiological and brain) will be submitted as amendments once the PI and staff members start working on the analysis of the data of the project.

Data and Safety Monitoring Plan

Pre-clinical visit (visit 1) Participants will perform behavioral tests, fill questionnaires, provide saliva DNA samples and conduct training (see above for more details). The training might occur at the Emory hospital or at the Laboratory of Darwinian Neuroscience (as part of the collaboration with Dr. James Rilling, PhD) that is located at the anthropology building at Emory University. Participants with ASD might be able to lay down inside the scanner for training purposes to habituate to the context.

We do not expect any serious adverse event and if any adverse event happened during the first visit. We expect several adverse events:

1- Tiredness

2- Moderate anxiety or fear reaction in response to lying down in the MRI scanner for ASD.

Participants will be asked how they feel. However, the PI or the experimenter will make sure to take note of these potential adverse events or any unexpected adverse events. All unexpected adverse events related to the first visit will be reported promptly to the IRB and DSMB. All serious adverse events related or not to the first visit will be reported promptly to the IRB, DSMB and to the sponsor. Serious adverse event (based on FDA documentation) includes: life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct a normal life functions, or a congenital anomaly/birth defect). Unexpected adverse event is an adverse event that is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed or is not consistent with the risk information described in the protocol.

Severity grading scale for adverse events will be based on this following chart (FDA-based):

- **Grade 1**: mild: asymptomatic or mild symptom; clinical observations only; no intervention indicated
- **Grade 2**: Moderate: minimal, local or noninvasive intervention indicated, have some effect on daily activity
- **Grade 3**: Severe or medically significant that affects daily activity but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling.

We do not consider any event as related to the study unless if the participant was on-site at Emory with the experimenter. All events that occurred before participants' arrival and after participants' departure will be considered unrelated.

Clinical visits (4 visits): Each participant will participate in 4 clinical visits at the CRN unit at the Emory Hospital and at BTCI MRI scanner. All the rules below apply for healthy subjects and for patients with ASD.

Based on prior literature, we have a list of expected adverse events that could be linked to oxytocin inhalation or placebo inhalation. This list includes any random symptom that was mentioned in trials previously conducted on single administration of oxytocin and placebo. They are considered random because some of them occurred during placebo spray intake, some of them were not found to be significantly different between placebo and oxytocin, and some of them are mentioned in the medical brochure. The list of expected adverse events includes:

1- Nausea or mild nausea
2- Vomiting or tendencies to vomit
3- Headaches
4- Irregularities of the pulse (tachycardia, bradycardia, arrhythmia, very rare hypertension)
5- Skin rashes and allergic reactions
6- Shortage of breath
7- Blood pressure decrease
8- Circulatory collapse
9- Runny nose

10- Tiredness
11- Sore throat
12- Shakiness
13- Sweating
14- Coughing

Participants will not be given this list and asked explicitly about it. Instead, the experimenter will ask participants how they are feeling following spray administration.

Participants will be also asked upon arrival on the 2nd, 3rd, 4th and 5th visit if there were any changes in their health.

In this clinical trial, we do not expect serious adverse events, and given that the subject is receiving 3 doses and placebo across 4 different visits (within the same study), we would like to define the terminology of reportable events and a plan to notify the IRB of reportable events:

Relatedness: the PI will consider that any event (serious, unanticipated, death, adverse event, not serious) is related to the treatment only if the event occurred internally during the clinical visit after spray intake (4 clinical visits at CRN unit at Emory Hospital and BITCI MRI scanner at the Emory Hospital). Thus, an event is considered as a related adverse event or a related unanticipated event if it occurred during the few hours of testing (up to 4 hours) and will not be considered related to the treatment once the subject leaves the hospital. The PI will take note of all related expected adverse events (list above) in a log document and report them periodically to the sponsor and the IRB during the continuing review. The PI will report promptly any unanticipated problem (not expected, related, and provides a risk to the participant) to the IRB and to the sponsor. The PI will also report promptly any serious adverse event that is related to the spray intake (that occurred on site visits of CRN unit) to the IRB and the sponsor. The PI will also report to the IRB periodically if any serious adverse event not related to the spray but during the period of the study that occurred off site Emory or before spray intake or after the participant left the hospital and between study visits (please see the definition below of unrelated events). The PI prefers to report promptly any unrelated or related serious adverse events to the IRB and to the sponsor with a precision of relatedness to avoid any problems or misunderstanding. The PI will also report serious adverse events to the sponsor (Larry Young). The serious adverse events include: death, life threatening experience, hospitalization, disability or permanent damage, medical or surgical intervention, emergency seizures.

Written IND safety reports will be submitted to the FDA by the IND sponsor, for serious, unexpected suspected adverse reactions within 15 calendar days of learning of its occurrence. If the event is fatal or is deemed to be life threatening, the report will be made within 7 calendar days. The IND sponsor will also make an assessment of whether the event constitutes an unanticipated problem posing risks to subjects or others (UP). This assessment will be provided to the Emory University IRB, which, in turn will make a final

determination. If the Emory IRB determines an event is a UP it will notify the appropriate regulatory agencies and institutional officials.

Unrelated events: If an event happened while the subject is coming to the first visit (before spray intake) or after the subject left the hospital (after spray but not at visit sites) or in between the 4 visits (not at visit sites) and that the subject told the experimenter about it, the event will be considered as an unexpected adverse event but unrelated to the treatment. Some patients might have comorbid anxiety traits or hyperactivity traits or being on drugs (other than oxytocin) and so sometimes these symptoms could underlie the medical baseline condition and are considered not related.

Expected **Adverse events** (please see the list above)	**Unanticipated** **Problems** (related, unexpected and risk to subjects)	**Protocol deviations and non-compliance**	**Serious adverse events** (deaths) related and unrelated
Reportable to IRB during continuing review board	Promptly reportable to IRB and DSMB	Promptly reportable to IRB	Promptly reportable to IRB and DSMB with specificity of relatedness
Reported to the sponsor at regular intervals	Promptly reportable to the sponsor	Reported to the sponsor at regular intervals	Promptly reportable to the sponsor
Not reportable to the FDA	Not reportable to the FDA, if not serious	Not reportable to the FDA	Promptly reportable to the FDA by the sponsor

Table 5.
Reportable events for the IRB (regarding the clinical visits).

The sponsor does not require reporting above and beyond IRB requirements.

The PI will review the overall data safety periodically. The PI is actually actively involved in testing participants and will make sure that all adverse events are written down and logged into adverse events templates after testing. Every week, the PI will review the regulatory binder to make sure that all the adverse events and procedures are done accordingly to the protocol. If there is any deviation or any unexpected adverse event or serious adverse event, the PI will report promptly all that to the IRB. The PI will make sure to review the literature on intranasal oxytocin (single doses) periodically and search for any adverse events. If there is any addition in the literature that the PI heard about or read about, she will inform the IRB promptly.

Regarding the plan for site monitoring for data quality and protocol compliance, the PI will review the data after each subject visit and the research coordinator will monitor for protocol compliance and data quality. In addition, we will perform self-monitoring (twice a year) using the EU-Self-monitoring Tool. The PI will meet at regular intervals (once per month) with the sponsor (L. Young) to update him about the study and the adverse events. The sponsor will be responsible for ongoing safety evaluation and will inform the PI of new safety information. The PI will inform the sub-investigators and the IRB if she is provided with new safety information. The sponsor will inform the FDA.

Based on the literature and the growing evidence of safety of intranasal oxytocin administration and that prior data suggest that the intervention does not have potential to induce significant risks or toxicity to subjects, we do not think that a DSM or DSMB is necessary. However, the PI has a plan for reporting adverse events and any deviations promptly or periodically based on IRB policies and regulations. We have discussed these reportable evens in the section above and table 5.

When the data collection stops, the DSMB and self-monitoring will stop too given that there are no more data that are being collected.

For data analysis, we will be working closely with Dr. Jennifer Stevens and her technician Tom Ely here at Emory to process the neuroimaging data.

Human subjects' data to be collected includes 1) DNA from saliva samples for genetic analysis, 2) hormone levels assayed from blood plasma, DNA genotyping from blood cells, immune analysis from plasma, exposome and metabolome analysis from plasma. 3) behavioral responses during computerized tests, during MRI scans, during clinical interviews, questionnaires and forms 3) functional and anatomical brain images, 4) physiological responses such as EDA and HRV, 5) Vital signs. Subject data will be identified by ID number, with only the PI of this study (E.A.) having access to the list linking subject names and ID #s. This list will be saved on the Emory server of the department of Psychiatry that is highly secured and protected by my emory login and password. All data will be collected specifically for the purpose of this research project.

Before the end of each year prior to the IND annual report oxytocin, a literature review on single-dose intranasally administered oxytocin studies will be done to assess any new safety information pertaining to the administration of this hormone. The results of this review will be kept in the

regulatory binder, and any discovery of additional adverse effects of oxytocin or any changes relevant to the safety of the subjects will be reported to the IRB and the FDA. Additionally, protocol changes would be made to account for these safety issues based on study physician recommendations. The dates of this review will be kept in the outlook calendar of the project coordinator.

In addition to the above plan, we would like to add that the clinical aspects of the study will be conducted under the auspices of the Department of Psychiatry, which has a well-established procedure to deal with adverse events resulting from our planned studies. All study participants will be evaluated by Dr. Haroon or Dr. Cubells or the nurse practitioner before taking the treatment spray. The clinical aspects of the study procedures and assessments will be performed in the Atlanta Clinical and Translational Science Institute (ACTSI) – which is a dedicated research unit housed inside the Emory University Medical Center in the same building which houses Emory Intensive Care Unit, Pulmonary and Critical Care Service and Emory Emergency Room. The ACTSI is staffed by fully trained nurses who are experienced in providing support to NIH-funded studies in all medical specialties. The ACTSI management is highly qualified, consisting of Dr. Thomas Ziegler, who is a Professor of Internal Medicine and Endocrinology.

The adverse events will be also reviewed by the PI and clinical investigators and also will be reviewed by the Emory Data Safety Monitoring Board (DSMB). All adverse events will be reported annually to the Emory DSMB.

The co-chair of the DSMB is Boady Dunlop, MD and other clinical researchers who serve as the external DSMB for investigator-initiated clinical trials conducted by Emory researchers in the Department of Psychiatry and Behavioral Sciences.

At least four weeks prior to DSMB meeting, the research coordinator/study staff will prepare a report to be reviewed during that meeting. The report will include the number of participants who signed consent for the study and were randomized, the number of post-randomization dropouts, reasons for these dropouts, and safety concerns, adverse events, etc. An up-to-date consent form will be provided, as well as a summary of measures taken to protect confidentiality (data storage, use of coded ID numbers, etc). The PI will also prepare a report summarizing any new data/evidence that might alter the risk/benefit ratio for participating in the study (such as newly published studies, etc). The PI will report any serious and unexpected adverse events in timely fashion to the Emory DSMB and Emory IRB. Actions taken by the IRB in response to adverse event reports will be immediately reported to the Emory DSMB.

Confidentiality:

All information obtained from participants will be kept strictly confidential. No individual genetic results will be given to participants or their relatives. DNA as well as other measures (brain scans, saliva samples, blood samples) will be assigned a code and the link to further identifying information concerning the individual will be available only to E. Andari. The PI will provide codes to the majority of the measures collected on the computer and other devices. The materials that have the linkage information and personal information about the subject (folders) will be kept

safely in locked offices. All electronic files that have the linkage (such as charts) will be saved on a HIPAA compliant server of Emory University.

The identifiable information will be destroyed once the E. Andari decides that the data is no longer needed.

We are likely to submit the data to NIH/ NDAR and Rdoc to help contribute to autism research. NDAR usually send us guides and we need to fill names and identifiers but NIH does not receive any identifiable information.

After each scan, intranasal spray bottles containing drug or placebo residual will be kept in a locked filing cabinet until leftover amounts in intranasal spray bottles are recorded. Then, the intranasal spray bottles will be de-identified, and then the bottles and leftover drug amounts will be incinerated per recommendation of the Investigational Drug Service (IDS). A log will be kept in the regulatory binder to keep a current record of this process.

Amendment 5:

We are submitting an amendment 5 (July 2019) to ask permission from patients with autism and from healthy subjects to provide authorization to submit their deidentified data to NDA or National database for Autism Research. These data can include anything that has been collected from subjects during this protocol: behavioral, clinical, imaging and genetics.

In the same amendment, we are asking patients with autism to give E.A. permission to use video-taped interviews and or/photos publically (during conferences, talks) for educational and research purposes. The goal of presenting these videos is to present the different characteristics of autism deficits and also what patients area capable of. The goal is also to present potential effects of drug (oxytocin), if any, on social interaction and improvements in eye gaze, emotional expressions and other types of behavior.

This will help researchers, general public and students to get a better idea of what is autism, its phenotypical heterogeneity and deficits as well as aptitudes. These video-taped interviews consisted of interviews that were conducted during the AOB project between the patient and E.A. They include face/body and voice of the patient. These interviews consisted of discussions about different topics related to social cognition, empathy and emotions. These video-taped interviews were collected during each of patients' 5 visits.

Showing these clips will allow researchers to have a much better idea of the different phenotypical in patients and the nature of heterogeneity.

In order to get permission, we will adopt these above steps"

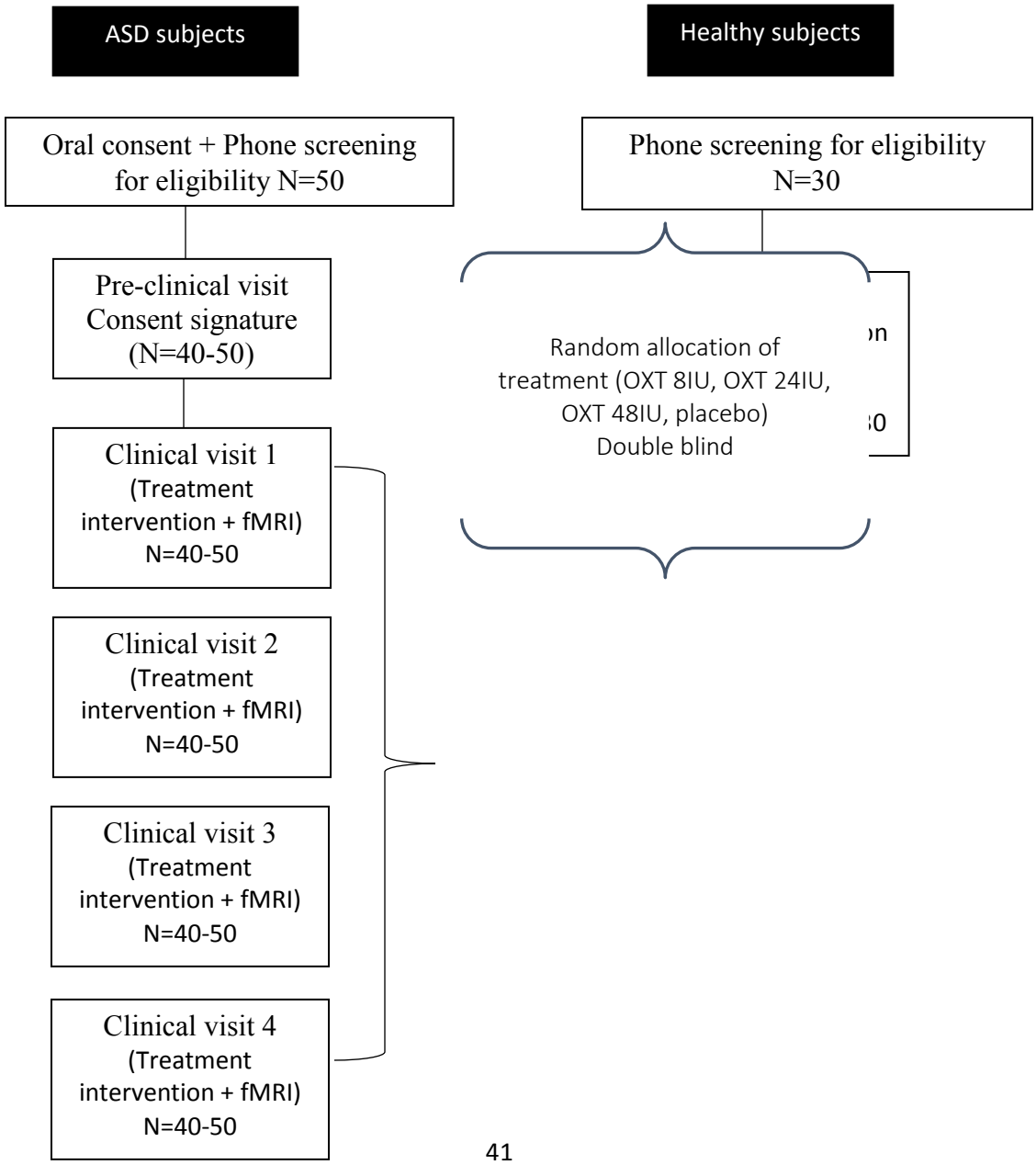
1- Contact patients or/and parents by phone and provide them with the general idea of the content of the consent and that I will be sending it to them by secure email or by mail.
2- Sending patients and/or parents the informed consent by secure email or/and mail for signature. For the mail option, we will mail a copy of the consent to the patient/parent (one

for them to keep as a copy and one for them to sign and send it back to us by mail) with a returned envelop (and a stamp) and ask parents/patients to read and sign.

3- Expect to receive the consent scanned by email from subjects or to receive the original by mail. They will mail us the consent to the address of Yerkes 954 Gatewood road, Atlanta GA 30329. They can also fax it to 404 727 8070 (administration of Dr. Young).

For those who have legal-guardian, both parents and patients will co-sign on the consent.

Appendix A



Appendix B

Procedures	Screening by phone/ Eligibility	(Visit 1)	(Visit 2)	(Visit 3)	(Visit 4)	(Visit 5)
Inclusion/Exclusion criteria/Screening form	X **X**	X **X**				
Informed Consent	X **X**	X **X**				
Family history form		**X**				
DNA saliva collection (3)		**X**				
Questionnaires*		X **X**				
Behavioral tasks**		X **X**				
Practice session 1***		X **X**				
Bio-behavioral clinical global interview (BB-CGI)		**X**				
Practice session 2****		**X**				
Revision of tasks and consent			**X**	**X**	**X**	**X**
Simple questionnaires (rating faces)		X	**X**	**X**	**X**	**X**
Physical and history assessment (by NP)		**X**				
Vital signs		X	**X**	**X**	**X**	**X**
Blood draw 10ml		X	**X**	**X**	**X**	**X**
Spray intake (treatment: OXT 8IU or OXT 24IU or OXT 48IU or PL)		X (placebo only)	**X**	**X**	**X**	**X**
Blood draw 10ml (5 minutes after spray)		X	**X**	**X**	**X**	**X**

Vital signs		X	**X**	**X**	**X**	**X**
MRI scan: resting state (40 min after spray)		X	**X**	**X**	**X**	**X**
MRI scan: Face perception task		X	**X**	**X**	**X**	**X**
MRI scan: Ball game 1		X	**X**	**X**	**X**	**X**
MRI scan: Anatomy		X	**X**	**X**	**X**	**X**
Post scan: Face questionnaire						
Post scan: Social learning		X	**X**	**X**	**X**	**X**
Post scan: Emotion Matching test		X	**X**	**X**	**X**	**X**
Post scan: Toronto Alexithymia scale		X	**X**	**X**	**X**	**X**
Post scan: Mood questionnaire						
BB-CGI (clinical interview)			**X**	**X**	**X**	**X**
Debriefing		X				**X**

X: Healthy subjects

X: ASD subjects

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