Published in final edited form as:

Sci Transl Med. 2015 January 21; 7(271): 271ra8. doi:10.1126/scitranslmed.3010257.

Exogenous and evoked oxytocin restores social behavior in the *Cntnap2* mouse model of autism

Olga Peñagarikano^{1,2}, María T. Lázaro¹, Xiao-Hong Lu³, Aaron Gordon⁴, Hongmei Dong¹, Hoa A. Lam⁵, Elinor Peles⁴, Nigel T. Maidment⁵, Niall P. Murphy⁵, X. William Yang³, Peyman Golshani^{1,6,7}, and Daniel H. Geschwind^{1,2,3,*}

¹Program in Neurogenetics, Department of Neurology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, 90095

²Center for Autism Research and Treatment and Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California at Los Angeles, Los Angeles, CA, 90095

³Center for Neurobehavioral Genetics, Department of Psychiatry and Biobehavioral Sciences, University of California at Los Angeles, Los Angeles, CA, 90095

*To whom correspondence should be addressed: dhg@ucla.edu.

Supplementary Materials

Figure S1. No oxytocin effect in associated behavioral deficits in the *Cntnap2* mouse model.

Figure S2. *Cntnap2* oxytocin co-localization in the PVN.

Figure S3. Total number of neurons in PVN area.

Figure S4. Vasopressin immunoreactivity in the PVN.

Figure S5. Map and sequence of the hM3Dq-mCherry construct.

Table S1. Drugs used for pharmacological testing.

Table S2. Raw data of Figure 1A.

Table S3. Raw data of Figure 1B.

Table S4. Raw data of Figure 1C.

Table S5. Raw data of Figure 1D.

Table S6. Raw data of Figure 1E.

Table S7. Raw data of Figure 2A.

Table S8. Raw data of Figure 2B.

Table S9. Raw data of Figure 3.

Table S10. Raw data of Figure 4.

Table S11. Raw data of Figure 5A.

Table S12. Raw data of Figure 5B.

Table S13. Raw data of Figure 5C.

Table S14. Raw data of Figure 5D.

Table S15. Raw data of Figure 6.

Table S16. Raw data of Figure S1A.

Table S17. Raw data of Figure S1B.

Table S18. Raw data of Figure S1C.

Table S19. Raw data of Figure S1D.

Table S20. Raw data of Figure S3.

Table S21. Raw data of Figure S4.

Authors contribution: O.P. and D.H.G. designed the overall study, O.P. performed histological and behavioral experiments, P.G. designed and M.T.L. performed electrophysiology experiments, X.W.Y. and X.L. designed stereology experiments, N.T.M. and N.P.M. designed and H.A.L. performed RIA experiments, E.P. designed and A.G. performed LacZ staining experiments, H.D. engineered DREADD construct and helped with mouse histological analysis as well as behavioral testing.

Competing interests: the authors declare that they have no competing interests.

Data and Materials Availability: Raw individual data for each experiment can be found in the Supplementary Materials. The map and sequence of the hM3Dq construct used can be found in Figure S5.

⁴Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

⁵Department of Psychiatry and Biobehavioral Sciences, Jane and Terry Semel Institute for Neuroscience and Human Behavior, University of California at Los Angeles, Los Angeles, CA, 90095

⁶Integrative Center for Learning and Memory, University of California at Los Angeles, Los Angeles, CA, 90095

⁷West Los Angeles VA Medical Center, Los Angeles, CA, 90073

Abstract

Mouse models of neuropsychiatric diseases provide a platform for mechanistic understanding and development of new therapies. We previously demonstrated that knockout of the mouse homologue of CNTNAP2, in which mutant forms cause Cortical Dysplasia and Focal Epilepsy syndrome (CDFE), displays many features parallel to the human disorder. Since CDFE has high penetrance for autism spectrum disorder (ASD) we performed an in vivo screen for drugs that treat abnormal social behavior in *Cntnap2* mutant mice and found that acute administration of the neuropeptide oxytocin improved social deficits. We found a decrease in the number of oxytocin immunoreactive neurons in the paraventricular nucleus (PVN) of the hypothalamus in mutant mice and an overall decrease in brain oxytocin levels. Administration of a selective melanocortin receptor 4 agonist, which causes endogenous oxytocin release, also acutely rescued the social deficits, an effect blocked by an oxytocin antagonist. We confirmed that oxytocin neurons mediated the behavioral improvement by activating endogenous oxytocin neurons in the paraventricular hypothalamus with Designer Receptors Exclusively Activated by Designer Drugs (DREADD). Last, we showed that chronic early postnatal treatment with oxytocin led to more lasting behavioral recovery and restored oxytocin immunoreactivity in the PVN. These data demonstrate dysregulation of the oxytocin system in *Cntnap2* knockout mice and suggest that there may be critical developmental windows for optimal treatment.

Introduction

Autism Spectrum Disorder (ASD) comprise a continuum of neurodevelopmental disorders characterized by deficits in social behavior and communication accompanied by restricted interests and repetitive behaviors (1). Other symptoms are frequently associated with ASD such as epilepsy, hyperactivity, and sleep, sensory and gastrointestinal abnormalities (2). Genetic studies have revealed extraordinary heterogeneity in ASD, predicting hundreds of rare risk genes, none accounting for more than 1% of cases (3–5). These findings indicate that the study of rare, but highly penetrant ASD susceptibility genes is likely to be of significant value. Further, among animal models for ASD, mouse models of monogenic forms of autism have the advantage of large effect size; several monogenic mouse models have shown high construct and face validity (6, 7); but few have demonstrated potentially predictive validity for human therapeutics (6, 8).

We have recently characterized a mouse knockout (KO) for the contactin-associated protein-like 2 (*Cntnap2*) gene, which is responsible for Cortical Dysplasia and Focal Epilepsy (CDFE) syndrome, a recessively-inherited disorder that presents with ASD in 70% of the cases (9). Risperidone, an atypical antipsychotic used to treat irritability and other non-core associated behaviors in ASD reduces hyperactivity, motor stereotypies, and perseveration in *Cntnap2* KO mice, while it has no effect on sociability (10), similar to what is observed in human patients. This supports the notion that different pathways lead to the social and repetitive behavioral deficits in ASD and suggests that this mouse model would be useful for testing new pharmacological treatments. For ASD, no treatment has been shown to consistently improve social behavior; currently approved pharmacotherapy targets repetitive behavior and other non-core associated phenotypes.

The neuropeptide oxytocin (OXT) is involved in the modulation of different aspects of social behavior (11, 12). The potential role for OXT in ASD is supported by genetic evidence from multiple sources (13–19), while the power of these studies is limited by small samples, a recent meta-analysis on variation in the OXTR with ASD found significant association with four SNPs (20), although these findings are not genome-wide significant. OXT is synthesized in two main hypothalamic regions, the supraoptic (SON) and paraventricular (PVN) nuclei. OXT expressing neurons in the SON project mainly to the posterior pituitary and are responsible for OXT release into the periphery (21), where OXT has a well-established role in neuroendocrine functions (22). OXT expressing neurons in the PVN, however, also project to several brain regions, including amygdala, hippocampus and frontal cortex (23), where their role in a number of behaviors such as fear, memory and social behavior are receiving increased attention (24, 25). Here, we performed limited in vivo screening for drugs that target social behavior in the *Cntnap2* mouse model.

Results

Oxytocin treatment rescues social behavior in *Cntnap2* knockout mice

No drug has been consistently shown to improve social behavior in either monogenic or polygenic forms of ASD. We performed a limited acute in vivo drug screen using 5 compounds targeting molecular mechanisms putatively related to social behavior in *Cntnap2* KO and WT littermates using average doses selected from the literature (Table S1). Groups consisted of half males and half females juvenile (4–6 weeks old) mice. Preliminary analysis did not detect any sexual dimorphism in the response to treatment, so both sexes were analyzed as a single group and equal numbers of males and females were used in all subsequent testing. Raw individual data for each experiment indicating sex can be found in the Supplementary Materials. Drugs were administered by intraperitoneal (i.p.) injection as an acute single dose, and mice were tested 20–30 minutes after administration with the reciprocal social interaction test. In this test, the time spent in social interaction for a pair of unfamiliar mice, matched for genotype, sex, age and treatment is measured (26). The time reported represents the combination of the behavior of the two animals within a pair. Analysis of the effect of treatment and genotype on social behavior by two-way analysis of variance (ANOVA) revealed a significant main effect of treatment ($F_{5,60}=4.13$, $p=0.0027$) and a genotype/treatment interaction that was close to significance ($F_{5,60}=2.23$, $p=0.0627$).

Pairwise comparisons to the vehicle treated control (Dunnett test) within genotypes revealed that *Cntnap2* mutant mice, but not WT, treated with the neuropeptides oxytocin (OXT) and arginine vasopressin (AVP) showed improvements in juvenile social interaction ($p=0.03$ and $p=0.04$, respectively; Figure 1A). We confirmed this change in social behavior in an independent experiment with the widely used social approach (3 chamber) test performed in young adult mice at 6–8 weeks of age (Figure 1B). In this test, mice are given the choice to interact with either an empty cup or a similar cup containing an unfamiliar stimulus mouse, matched for age and sex. The amount of time interacting with each cup is compared as a measurement of sociability, where normal sociability is defined as spending significantly more time interacting with the cup containing the mouse than the empty cup (27, 28). As shown, vehicle treated WT mice show normal sociability ($p=0.01$), preferring to spend time in the chamber containing the other mouse, which is not changed upon OXT treatment ($p=0.02$). *Cntnap2* mutant mice, which at baseline show no significant preference to interact with the cup containing the mouse versus the empty one ($p=0.13$), which indicates lack of sociability, showed strong preference to interact with the cup containing the mouse when treated with OXT ($p=0.03$) and, a non-significant trend with AVP treatment ($p=0.06$).

Although neuropeptides may cross the blood-brain barrier and reach the central nervous system, intranasal (i.n.) administration is a preferred way to target peptides to the brain without the need of invasive procedures (29). We therefore conducted an independent experiment in which we administered OXT or AVP i.n. and tested *Cntnap2* mutant mice for improvements in social behavior with the juvenile reciprocal social interaction test. Similar to what we found when administered i.p., two-way ANOVA revealed a significant effect of treatment ($F_{2,42}=21.08$, $p<0.0001$) and a treatment/genotype interaction ($F_{2,42}=17.01$, $p<0.0001$). Pairwise comparisons to the control within genotypes showed that when administered i.n., these neuropeptides have a similar or slightly larger effect (Dunnett test; $p<0.0001$ in both cases) on social behavior than when they are administered i.p. to the *Cntnap2* mutant mice (Figure 1C).

OXT and AVP are closely related nonapeptides that share high sequence and structure homology, differing only at residues 3 and 8. While only one receptor exists for OXT in mammals, there are three different receptors for AVP: V1a, V1b and V2, the V1a receptor being the predominant form in brain (11). AVP can bind and activate the OXT receptor. To better characterize the pathway implicated in the improvement in social behavior by both peptides, we preceded the drug treatments with receptor blocking. We treated *Cntnap2* mutant juvenile animals (4–6 weeks old) with either an OXT receptor antagonist, or an AVP-V1a receptor antagonist followed by either OXT or AVP and performed behavioral tests 20 minutes after the agonist dose (Figure 1D). One-way ANOVA revealed a significant effect of treatment on social behavior ($F_{5,41}=22.02$, $p<0.0001$). Post-hoc analysis with Bonferroni correction for multiple comparisons showed that the behavioral improvement seen with OXT was abolished when the OXT receptor was blocked ($p<0.0001$). In contrast, the behavioral effect of AVP was not abolished after AVP-V1a receptor block, but was eliminated by OXT receptor block ($p<0.0001$). Although we cannot eliminate a minor contribution of the AVP system, these data suggest that the observed effect is mainly due to activation of the OXT receptor.

We performed additional behavioral tests to determine whether the improved social behavior seen in these animals upon OXT treatment was accompanied by other behavioral improvements. Other than social deficits, none of the other previously reported behavioral deficits, including hyperactivity, increased repetitive behavior, increased perseveration and hyper-sensitivity to sensory stimuli were rescued by administration of OXT (Figure S1). These data indicate that the behavioral effect of OXT administration seems restricted to the social behavior domain.

One potential limitation of OXT as a treatment for disorders of social cognition is its short half-life, proposed to be around 20 minutes in mammalian brain (30). Therefore, we assessed the time course of the OXT effect on social behavior with the reciprocal social interaction test, observing that the behavioral effect of a single i.n. treatment lasted for about 2h ($p<0.0001$) in *Cntnap2* mice (Figure 1E, one-way ANOVA of the time effect on treatment response $F_{4,35}=23.16$, $p<0.0001$ followed by Bonferroni post-hoc test). This result is in accordance with a recent microdialysis study in which the authors showed that a single i.n. administration of OXT resulted in elevated OXT levels in brain that lasted for about 90 minutes (31). It is also similar to behavioral effects on newborn macaques, in which OXT was correlated with increased social behavior 2h, but not 4h, after administration (32) and in neurotypical human volunteers and clinical studies (33), where behavioral testing is performed approximately 60 minutes after administration.

To extend our pharmacological results and explore the role of the endogenous oxytocin system, we took advantage of an emerging literature demonstrating that OXT neurons express a variety of receptors that can be pharmacologically targeted to stimulate endogenous OXT release, specifically the melanocortin and serotonin receptors. The melanocortin 4 receptor (MC4R) colocalizes with OXT neurons in the PVN (34). Stimulation of these receptors activates PVN OXT neurons in mice (35), induces central but not peripheral OXT release in rats (36) and promotes partner preference in voles (37). We treated WT and mutant mice with a selective MC4R agonist, Ro27-3225 (38), and tested their effects in a reciprocal social interaction test 30 minutes after treatment. Two-way ANOVA detected a genotype/treatment interaction ($F_{1,20}=4.87$, $p=0.04$) and pairwise comparisons to the vehicle treated control (Dunnett test) within genotypes showed that Ro27-3225 led to improvement ($p=0.02$) in social behavior in the *Cntnap2* mouse (Figure 2A). The behavioral effect of this drug was eliminated when the OXT receptor was blocked 15 minutes before administration (Figure 2B, one-way ANOVA $F_{2,18}=14.60$, $p<0.0001$ followed by Bonferroni post-hoc test $p<0.0001$), indicating that the effect is mediated through OXT release.

Cntnap2 knockout mice show reduced number of oxytocin expressing neurons

The improvement in social behavior upon OXT treatment was restricted to *Cntnap2* mutant mice and was not observed in WT littermates (Figure 1A and 1C). This sensitivity to OXT in the mutant mice led us to hypothesize that the OXT system might itself be abnormal in *Cntnap2* KO mice. Expression of *Cntnap2* has been conventionally associated with pyramidal excitatory neurons, a result of its well-established role in anchoring K^+ channels at the nodes of Ranvier in myelinated axons (39). *Cntnap2* mRNA is also expressed in

migrating GABAergic interneurons, where its function is still to be determined (10). Interestingly, in situ hybridization (see Allen Mouse Brain Atlas ISH database) shows that *Cntnap2* is strongly expressed in the hypothalamic PVN, where OXT neurons are clustered, and we found by immunohistochemistry that it is co-expressed with a subset of OXT expressing neurons (Figure S2) in this region, opening a potential role for *Cntnap2* in the development or function of these diencephalic neurons.

To characterize the oxytocinergic system, we analyzed the number of OXT expressing cells in the PVN of the hypothalamus at postnatal day 30 (P30), when the system is fully developed, and found that *Cntnap2* mutant mice have reduced numbers of OXT immunoreactive neurons compared to WT littermates (Figure 3A–B, Student t test, $p=0.03$). To confirm this deficit, we measured OXT levels in whole brain extracts by radioimmunoassay and also found a significant reduction in OXT levels (Figure 3C, Student t test, $p=0.01$). To determine if this reduction was due to a lack of neurons (via death or development), or to a lack of expression of the peptide within neurons, we counted the total number of neurons in the PVN and found no differences in the total number of neurons, nor in the total PVN area analyzed (Figure S3A–B), indicating that it is OXT expression within neurons and not the number of neurons that is reduced. Since OXT and AVP neurons constitute the majority of PVN neurons, we also analyzed the number of AVP expressing cells and found no differences between KO and WT mice (Figure S4).

Early postnatal sub-chronic administration of oxytocin has long lasting behavioral effects

OXT is first detected by immunohistochemistry during early postnatal life in rodents, gradually increasing in abundance from postnatal day 7 (P7) until approximately weaning age (P21), when the system is considered fully developed (40, 41). Consistent with this trajectory, we found many fewer OXT immunoreactive cells at P7 than at P30, and no differences between WT and KO at P7 (Figure 4A–B). The postnatal development of the OXT system suggests that OXT production may be especially vulnerable to early life manipulations. In fact, both environmental (42, 43) and pharmacological (41, 44, 45) manipulations during early postnatal life affect the central OXT system, as well as social behaviors, during adult life. In the prairie vole, a species widely used to study affiliative behavior such as pair bonding, a single i.p. administration of OXT on P1 increases OXT immunoreactivity in the PVN at P21 (41) and strengthens partner preference at P60 (44, 45). To test whether early postnatal OXT treatment would have an effect on social behavior in *Cntnap2* mice, we treated WT and KO littermates with daily i.n. administration of either saline or OXT from P7 to P21 and tested them nine days later, at P30, with a social approach (3 chamber) test. We found that as expected, saline treated *Cntnap2* KO mice showed a lack of normal social interest, displaying no preference for the cup containing a mouse over the empty cup. In contrast, mice receiving daily postnatal OXT administration, showed normal social behavior more than one week after cessation of the treatment (paired Student t test, $p=0.004$). Also, as expected, the normal sociability in WT littermates was unchanged (Figure 5A). We confirmed the behavioral improvement in a second cohort of KO mice using the reciprocal social interaction test (Student t test, $p<0.001$), performed again at P30 (Figure 5B). To test whether this improvement in social behavior correlated with an increase in endogenous OXT expression, we measured OXT levels in whole brain extracts in these

same animals and found that *Cntnap2* KO mice treated with OXT indeed showed a significant increase (Student t test, $p=0.02$) in endogenous OXT levels (Figure 5C) relative to untreated KO mice. In addition, this increase in central OXT was correlated with an increased number of OXT immunoreactive neurons in the PVN of mutant mice (Student t test, $p=0.005$), which were rescued to nearly WT numbers by the postnatal OXT treatment regimen (Figure 5D–E). These data suggest that there may be an early developmental window during which treatment can achieve longer lasting improvements in social behavior.

Stimulation of endogenous oxytocin neurons in the PVN rescues social behavior

To combine the pharmacological evidence for OXT modulation of social behavior with physiological data and directly test the hypothesis that PVN OXT neurons mediate the behavioral effects, we assessed whether activation of endogenous OXT release is sufficient for improving social behavior in *Cntnap2* mutant mice using DREADD (Designer Receptors Exclusively Activated by Designer Drugs) technology (46). We expressed a modified muscarinic acetylcholine receptor tagged with the fluorescent marker mCherry (hM3Dq-mCherry), which is exclusively activated by the otherwise inert ligand clozapine-N-oxide (CNO), in OXT cells in the PVN by means of stereotaxic AAV2 injection. hM3Dq-mCherry expression restricted to OXT cells was achieved by driving the expression with the OXT promoter (Figure 6A–B). Whole cell, current clamp recordings were performed in mCherry expressing PVN neurons to confirm receptor efficiency in depolarizing OXT cells. When added to the bath, CNO (5 μ M) depolarized and markedly increased the firing rate of OXT neurons (Figure 6C). Next, we examined the behavioral effect of hM3Dq-mCherry activation in OXT cells. Following acute in vivo i.p. administration of CNO (5mg/kg), 30 minutes later we could observe normal social interest in the social approach (3 chamber) test in *Cntnap2* mice (paired Student t test, $p=0.001$), while vehicle treated animals showed no preference for the cup containing the mouse. No CNO effect was seen on the normal sociability of WT animals (Figure 6D), consistent with our previous findings using exogenous OXT administration.

Discussion

Effective pharmacological treatments for social deficits in ASD are needed. The OXT system is a key mediator of social behavior in mammals, including humans, in many contexts such as maternal behavior, mother-infant bonding, social memory/recognition and pair bonding (12, 47). Mice with a genetically altered OXT system---such as knockout mice for the OXT gene, the OXT receptor (OXTR) gene or CD38 (cluster of differentiation 38), a gene involved in OXT release---all show social deficits that are restored upon OXT administration (37), and OXT is required for the rewarding properties of social stimuli in mice (48). In addition, OXT administration improves sociability in inbred mouse strains with naturally occurring lower levels of sociability such as BALB/cByJ and C58/J (49). Thus, there is growing exploration of OXT's potential for therapeutic efficacy in ASD with some studies showing a neurobiological evidence of mitigation of some aspects of social deficits in ASD (50, 51). Given the etiological heterogeneity of ASD, a key issue is whether there are actual deficits in this system in some forms of ASD since identifying what patients could potentially benefit the most from OXT treatment is critical (52). Studies addressing

peripheral OXT levels in individuals with ASD have found both lower (53, 54) and higher (55, 56) levels. Interestingly, Green et al. (57) reported lower levels of the peptide but higher levels of its precursor, indicating a potential abnormality in its processing. This apparent lack of consistency is not surprising since dysfunction in the OXT system can happen at different levels. Both the hormone itself (its synthesis, processing, storage and release) and its receptor could be affected in pathological states, therefore, although dysfunction in the OXT system is potentially associated with some forms of ASD, OXT has not yet emerged as a biomarker for disorders involving social cognition. Interestingly Parker et al. (58) have recently shown that both OXT plasma concentration and OXTR polymorphisms affect individual differences in social functioning both in ASD and normal populations.

Here we show in a validated mouse model of a monogenic form of ASD a clear reduction in OXT expression in the PVN of the hypothalamus, which is correlated with reduced OXT levels in brain extracts. Further, we demonstrate through multiple pharmacologic means that acute OXT treatment can rescue the observed social deficits in this mouse model, an effect that lasts about 2 hours, similar to effects observed in a recent study on newborn macaques, in which OXT was correlated with increased social behavior at 2h, but not 4h after administration (32). A similar time course is observed in multiple tasks performed by neurotypical human volunteers and in clinical studies (33), where behavioral testing was performed 60 minutes, or more after administration. This long lasting behavioral effect of a single dose, despite the short half-life of the peptide, has been proposed to be a result of its well characterized self-priming process, in which OXT stimulates its own release in a positive feedback loop, presumably through activation of its own OXTR (30). We also used an alternate pharmacophysiological approach, DREADD, to confirm that activation of PVN OXT neurons is sufficient to rescue this deficit, confirming the role of PVN OXT neurons in mediating the observed changes in social behavior with treatment in the KO mouse. This further suggests that drugs that induce endogenous OXT release would likely improve social behavior in this model. In fact, we observed that stimulation of MC4R, which activates PVN OXT neurons in mice (35) and induces central OXT release (36) also improved social behavior. There are a number of receptors on OXT neurons that could be targeted to induce OXT release (37). The further study of these receptors and the circuits in which they are implicated will help to develop therapeutic approaches based on manipulating this system.

Finally, we demonstrate that there may be an early developmental window that permits achievement of longer lasting effects on symptoms, which suggests that early childhood treatment protocols could be relevant to some patients. Pharmacological manipulation of the OXT system during early postnatal life, affects peptide levels and behavioral outcomes in adult rodents. Hammock and Levitt (59) have recently shown that OXTR binding in mice, similar to other rodents, shows a transient neocortical peak during early postnatal life, the time period of major synaptic wiring and pruning, which could explain why this is a sensitive period for OXT in shaping neuronal circuits which ultimately mediate behavior. In prairie voles, a species widely used to study social behavior in the context of pair bonding, a single postnatal administration of OXT at P1 increases OXT levels in the hypothalamic PVN at weaning (41) and facilitates partner preference and alloparental care in young adulthood (44). Repeated OXT treatments during the first postnatal week increase the number of isolation-induced ultrasonic vocalizations (60), a measure of social vocal communication. In

addition, daily neonatal treatment during this first week of life with a selective MC4R agonist promotes adult partner preference (61). Similarly, in mice, a single postnatal administration of OXT at P1 facilitated alloparental care and social approach in adulthood, while administration of an OXT antagonist produced the opposite effect (62) and sub-chronic OXT treatment in juvenile rats has long lasting effects in social behavior, which is accompanied by increased plasma OXT levels (63). Interestingly, while some of these studies used male only samples (44, 63) others using both sexes found sexual dimorphism in their results, where treatment affected only males (62), only females (41, 60) or both (61). We found that a daily dose of OXT between P7 and P21 yielded effects on social behavior and increased peptide levels in brain extracts when analyzed nine days later at P30 in both sexes, which is likely due to our study of a monogenic, major gene model of ASD. It remains to be determined whether this change is permanent and whether this plasticity is present beyond early childhood, since there might be a critical developmental window when OXT treatment has maximal effect.

Neuropeptides such as OXT contribute to network activity by modulating the effect of classical excitatory and inhibitory neurotransmitters. Owen et al. (64) have shown that OXT stimulates fast spiking parvalbumin interneuron activity, modulating circuit signal-to-noise ratio and improving information processing. Parvalbumin interneurons have a critical role in regulating network activity (65) and their dysfunction has been implicated in ASD. We previously found that the *Cntnap2* mouse model shows deficits in GABAergic interneurons, (10) and OXTR null mice have fewer GABAergic synapses (66). Therefore, it is possible that OXT compensates for GABAergic deficits in this mouse model. Although GABA is inhibitory in adult brain, it is excitatory during fetal and early postnatal periods, due to elevated intracellular chloride concentrations and OXT has been shown to be involved in its perinatal excitatory to inhibitory shift by reducing intracellular chloride levels (67). Tyzio et al. (68) have recently found that this process is altered in the *Fmr1* KO model, a model for Fragile X syndrome, which also shows reduced number of GABAergic interneurons (69); preliminary results in the *Fmr1* KO suggest a reduced OXT immunoreactivity in the PVN (70). Thus, recent studies have begun to investigate intranasal OXT effects in Fragile X syndrome and have found an improvement in eye gaze frequency during a social task, suggesting that OXT could be a potential treatment for social anxiety in this syndrome (71).

Our work opens an avenue for investigation of the precise molecular mechanisms of the effects of *Cntnap2* deletion on OXT neuron development. One possibility is that CNTNAP2, as a cell adhesion molecule, affects OXT neuron function through its well-established role in neuron-glia interaction (39). CNTNAP2 is expressed in OXT neurons, whose development and function depend on dynamic neuron-glia interactions (72). Alternatively, CNTNAP2 could be involved in the structural development of OXT neurons: Anderson et al. (73) have recently shown that CNTNAP2 has a role in neurite outgrowth, which is important for the proper development of neuronal circuits. In addition to studying molecular mechanisms, our model allows exploration of potential critical periods for OXT-induced behavioral effects, as well as potential effects on the seizure phenotype via GABA modulation (65).

Another point that deserves further attention is what aspects of social behavior does OXT improve? This is a limitation of the current study and the majority in the field, as the

standard tests used do not typically distinguish between various forms of social approach or interaction. Further refinement of social testing following OXT or other treatments will be informative, as will the development of more naturalistic social testing. Nevertheless, our results showing OXT deficits in a model of a specific genetic form of ASD, suggests that studying OXT in other defined genetic forms of ASD would be of value. A key goal remains to discern which forms of ASD show a direct or indirect dysregulation of this system, since we suspect that these patients are the most likely to benefit from treatment with OXT. Such data, as well as a better understanding of optimal treatment timing could be used to design more informed clinical trials.

Material and Methods

Study Design

The main goal of this study was to explore the potential of different drugs in restoring abnormal social behavior in the *Cntnap2* mouse model of ASD, which was determined by measuring the time spent in social interaction using two different tests that are most commonly used to assess social behavior in mouse models of ASD. A sample size of at least 6 mice per group was used for all behavioral testing, 3–4 mice per group were used for neuronal counting and 6–8 mice per group were used for biochemical studies. Exact numbers for each experiment are included in the figure legends. All groups consisted of half males and half females and were always matched among compared groups. Data collection was performed as described in the literature and no exclusion criteria were applied. All analyses were performed blinded to genotype and/or treatment. For drug treatments, animals were randomly assigned to treatment. No outliers were taken into account and all collected data was used in statistical analyses.

Mice

Mutant mice lacking the *Cntnap2* gene (*Caspr2* null mice) were obtained from Dr. Elior Peles (39) and backcrossed to the C57BL/6J background for over 12 generations. Mice expressing a tau-LacZ fusion protein under the endogenous *Cntnap2* promoter will be described elsewhere (Gordon et al. in preparation). Experimental *Cntnap2* mutant and WT mice were obtained from heterozygous crossings. The day of birth was designated as postnatal day zero (P0). The three obtained genotypes were housed together with three to four same sex mice per cage. They were kept in 12 hr light/12 hr dark cycle and had ad-lib access to food and water. All procedures involving animals were performed in accordance with the UCLA and the Weizmann animal research committee, and the NIH Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry (IHC)

Mice were deeply anesthetized with Pentasol (sodium pentobarbital, 40 mg/kg body weight) and perfused intracardially first with phosphate-buffered solution (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed, postfixed overnight in the same fixative and cryoprotected by immersion in 30% sucrose in PBS for 3–5 days. Brains were embedded in OCT, sectioned at a thickness of 50 μ m on a cryostat and used for free-floating IHC using standard fluorescence or Avidin Biotin Complex (ABC) methods (Vector Labs).

Incubations with primary antibodies were performed overnight at 4°C and with secondary antibodies for 90 minutes at room temperature. For primary antibodies we used rabbit anti-OXT (1:2000, Immunostar), rabbit anti-AVP (1:2000, Immunostar). Appropriate secondary antibodies were either from the Alexafluor series from Invitrogen for fluorescence or biotinylated antibodies from Vector Labs. Images were acquired with a Zeiss LSM-510 laser-scanning confocal microscope (fluorescence) or an Olympus BX51 microscope (brightfield).

X-GAL Staining

Brains were obtained from 4% PFA perfused mice and were subsequently cryoprotected in 30% sucrose in PBS overnight. Tissues were embedded in OCT (Tissue-Tek) and frozen on dry ice. 14-micron thick coronal sections were prepared using a cryostat. Staining was done with 1mg/ml X-GAL (Sigma) in 20mM Tris pH 7.3, 5mM ferricyanide, 5mM ferrocyanide, 0.01% sodium-deoxycholate, 0.02% NP-40 and 2mM MgCl₂ in PBS overnight at 37°C.

Stereological analyses

Stereological analyses in the PVN were conducted as previously described (74) with minor modifications. Briefly, the PVN region defined by OXT or AVP staining was outlined bilaterally on the live computer image using a 5× objective. The Stereo Investigator V10.42.1 software (Micro-BrightField Inc., Wiliston, VT) placed a systematic random sampling grid (70×70µm) within the selected area giving ~10 counting frames per area. A total of 6–7 serial sections with periodicity n=2 from each brain were counted bilaterally with a 40× objective to achieve optimal optical sectioning. For Nissl stained cells, a double staining OXT/Nissl was performed, the PVN area delimited by OXT staining was outlined and Nissl positive cells were counted using a 25×25µm counting frame and a 100× oil immersion objective. The final post-processing thickness of the sections was 11µm on average, so the counting frame height was kept at 9µm for all sections studied to keep a 1µm guard zone. The optical fractionator was used to estimate the total number of cells in the region of interest. The Coefficient of Error (CE, Gundersen m=1) was 0.1 or less for all samples. All counts were made by the same investigator, who was blinded to the genotype/treatment of the mice.

Radioimmunoassay

To measure whole brain OXT levels mice were anesthetized with isoflurane and decapitated. The brain tissue was homogenized at room temperature in 10ml 1N acetic acid using a Kinematika Polytron for 30 seconds at level 5. Samples were centrifuged at 1,000g for 60 minutes at 4°C and 7ml of the resulting homogenate was neutralized with 1.2 ml 5N NaOH, 1.4 ml 1M Na₂HPO₄ and 0.4ml H₂O and centrifuged at 15,000g for 60 minutes at 4°C. Samples were stored at –80°C until measured. The OXT radioimmunoassay (RIA) from Phoenix Pharmaceuticals Inc. was used as follows: the solid phase of the RIA was prepared by adding 50µl of protein A solution dissolved in NaHCO₃ (pH 9) into 96 wells flat bottom microtiter plates (Thermo Fisher) and incubated overnight at 40°C. The protein A solution was dumped and the plate washed 3× with wash buffer (0.15M K₂HPO₄, 0.2mM C₆H₈O₆, 0.1% gelatin, 0.1% tween, pH 7.4). After the washes 25µl of antibody was added to each well and incubated for 4h at room temperature after which the antibody solution was

dumped and the plate washed 3×. The standard (0.25–12.5 fmol) and samples were loaded in the volume of 25µl/well and incubated for 24h at 40°C, 10µl of 5,000 counts per minute (cpm) ¹²⁵I oxytocin was added to each well and incubated overnight at 40°C. The plate was washed 3×, 100µl of Microscint-20 (Perkin Elmer) was added to each well and the plate was counted for 4 minutes using a microplate scintillation counter (Packard).

rAAV construction

A vector expressing a modified muscarinic receptor fused to the fluorescent protein mCherry was obtained from addgene (pAAV-hSyn-DIO-hM3D(Gq)-mCherry, plasmid #44361). To drive the expression of the transgene from the OXT promoter, we first carried out in vitro Cre-recombination to flip and remove the loxP sites in this construct and later exchanged the human synapsin promoter for a mouse OXT promoter consisting of 1kb directly upstream of exon 1 of the OXT gene, which has been shown sufficient to induce cell-type specific expression in OXT cells (75). The final plasmid was sent out to the University of Pennsylvania Vector Core for custom AAV production (serotype 2).

Stereotaxic injection

Stereotaxic injections of 500nl of an rAAV titer of $\sim 10^{10}$ vector genome/ml were performed bilaterally in the PVN of the hypothalamus according to the following coordinates from bregma according to the Allen Brain Atlas: antero-posterior –0.70 mm; dorso-ventral –5.20 mm; lateral+/-0.30 mm. Injections were performed at a rate of 100nl/min with a glass micropipette (50µm tip diameter) using a Nanoliter 2000 microinjection system (WPI) attached to a Micro4 pump (WPI). Behavioral and electrophysiological studies were performed three weeks after injections. A detailed method of stereotaxic injections can be found in Cetin et al. (76).

Electrophysiological Experiments

In vitro slice recordings were performed three weeks after injection of the rAAV into the PVN. Briefly, mice were deeply anaesthetized with isoflurane and beheaded. The brain was removed and set for cutting at a thickness of 300µm on a Leica VT1000S Vibratome, while bathed in an ice-cold sucrose-based dissection solution containing (in mM) 222 sucrose, 11 D-glucose, 26 NaHCO₃, 1 NaH₂PO₄, 3 KCl, 7 MgCl₂, 0.5 CaCl₂, aerated with 95% O₂, 5% CO₂. Slices containing the PVN were then allowed to recover for 30 min at 37°C in artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 D-glucose, 4 sucrose, 2.5 CaCl₂, 2 MgCl₂, aerated with 95% O₂, 5% CO₂ and subsequently transferred to room temperature until time of recording. The recording rig was continually perfused with aCSF, maintained at 32–34°C. Whole-cell recordings of PVN neurons were made in current-clamp mode, using a Multiclamp (Molecular Devices) patch clamp amplifier. mCherry-positive neurons were identified via fluorescence microscopy, under visual guidance of differential interference contrast (DIC) optics with an Olympus BX51 microscope. Patch pipettes of 3–5 MOhms were used, and filled with internal solution containing (in mM) 115 Potassium gluconate, 20 KCl, 10 HEPES, 10 Phosphocreatine, 4 ATP-Mg, and 0.3 GTP. Access resistance was under 25 Ohms and fully compensated in current-clamp mode. For pharmacological stimulation, 5µM CNO (Sigma) was included in

the aCSF bath perfusion. Neuronal responses were acquired and visualized using WinEDR (Strathclyde Electrophysiology Software).

Behavioral Tests

Behavioral tests were performed in the UCLA behavioral test core and analyzed with TopScan (Clever Sys, Inc.) automated system or by two independent experimenters blinded to genotype and treatment.

Open Field

Mice were placed inside a clear Plexiglass arena (27.5cm×27.5cm) for 20 minutes and their general activity (distance traveled and velocity) was recorded. Results were analyzed with TopScan software (Clever Sys, Inc.).

Reciprocal social interaction

Mice were placed in a cage to which they had been previously habituated (for 10 minutes) with an unfamiliar mouse matched in age, genotype and sex for 10 minutes. Both mice in the pair were either treated with the same drug or with vehicle. The time engaged in social interaction (nose-to-nose sniffing, nose-to-anus sniffing, following or crawling on/under each other) for the pair (combining the behavior of both animals) was measured by two independent human observers (26).

Social approach (Three-Chamber) Test

The social approach test was performed as previously described (26). In brief, after 10 minutes of habituation, a mouse was placed in the central chamber of a clear Plexiglas box divided into three interconnected chambers and was given the choice to interact with either an empty wire cup (located in one side chamber) or a similar wire cup with an unfamiliar mouse inside (located in the opposite chamber) which was matched in age and sex. Time interacting with each cup was measured by two independent observers.

Drug Administration

All drugs administered, dose used and method of administration is summarized in Table S1. All drugs were obtained from Sigma-Aldrich except the OXT and AVP receptor antagonists, which were obtained from Tocris Bioscience. Doses were selected as an average found in the literature for these compounds. For the initial drug screening, as well as for the receptor blocking experiments, all drugs were dissolved in 10% DMSO in PBS with the purpose of them all having the same vehicle control. For subsequent experiments, drugs were dissolved in saline. For i.p. administration, drugs were administered in a volume of 10ml/kg mouse weight (250µl for an average 25g mouse). For i.n. administration, a drop of 2.5µl solution was placed in one of the animal's nostrils with a P10 pipette. Once the animal had aspirated the drop into its nasal cavity the process was repeated for the other nostril. Pups (P7-P21) received 2× 1.25µl drop.

T Maze Spontaneous Alternation

Mice were placed on the base of a T maze and were given the choice to explore either the right or left arm of the maze for ten consecutive trials. Entry into an arm was registered once mice stepped with all four paws into the arm. At that moment, the gate to that arm was closed and the animal allowed to explore the arm for 5 seconds.

Grooming

Mice were individually caged and the time spent grooming during a 10 minute period after a 10 minute acclimation was measured.

Hot plate test

Mice were placed on a heated plate (52.5°C) and the latency to paw withdrawal (up to 15 seconds) was measured.

Statistical Analyses

Results are graphically presented as a box and whiskers plot. The whiskers extend from the minimum to the maximum value and the box extends from the 25th to the 75th percentile. The median (50th percentile) is shown by a line and the mean by a plus sign. One-way or two-way ANOVA followed by Bonferroni post-hoc tests in the case of multiple pair-wise comparisons or Dunnett test in the case of planned comparisons to a control as well as paired and unpaired 2 tail Student t test were used as indicated in each Figure Legend. F values, p values and modified significance values after Bonferroni correction are presented in the Figure Legends. Data presentation and statistical analysis were performed with GraphPad software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the UCLA behavioral testing core and its supervisor, Dr. Jesse D. Cushman, for assistance with behavioral testing. We also thank Dr. Lynn Talton for diagrams of behavioral tests. **Funding:** This work was supported by grants NIH/NIMH R01 MH081754-02R to D.H.G.; NIH/ NS0220 to E.P.; NIH ACE Center 1P50-HD055784-01 to D.H.G. (Project II), Network grant 5R01-MH081754-04 to D.H.G., Simons Foundation Autism Research Initiative to E.P. and D.H.G., and Autism Speaks grant in translational research 7657 to O.P. X.W.Y. is supported by the NIH/NINDS grants R01 NS049501 and R01 NS074312, and the Brain Disorder Award from McKnight Foundation.

References

1. APA. Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Washington, DC: American Psychiatric Publishing; 2013. American Psychiatric Association
2. Geschwind DH. Advances in autism. Annual review of medicine. 2009; 60:367.
3. Iossifov I, et al. The contribution of de novo coding mutations to autism spectrum disorder. Nature. 2014 Oct 29.
4. De Rubeis S, et al. Synaptic, transcriptional and chromatin genes disrupted in autism. Nature. 2014 Oct 29.

5. Berg JM, Geschwind DH. Autism genetics: searching for specificity and convergence. *Genome biology*. 2012; 13:247. [PubMed: 22849751]
6. Won H, Mah W, Kim E. Autism spectrum disorder causes, mechanisms, and treatments: focus on neuronal synapses. *Frontiers in molecular neuroscience*. 2013; 6:19. [PubMed: 23935565]
7. Ey E, Leblond CS, Bourgeron T. Behavioral profiles of mouse models for autism spectrum disorders. *Autism research : official journal of the International Society for Autism Research*. 2011 Feb.4:5. [PubMed: 21328568]
8. Crawley JN. Translational animal models of autism and neurodevelopmental disorders. *Dialogues in clinical neuroscience*. 2012 Sep.14:293. [PubMed: 23226954]
9. Strauss KA. *The New England journal of medicine*. 2006; 354:1370–1377. [PubMed: 16571880]
10. Penagarikano O, et al. Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell*. 2011 Sep 30.147:235. [PubMed: 21962519]
11. Insel TR. The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. *Neuron*. 2010 Mar 25.65:768. [PubMed: 20346754]
12. Ross HE, Young LJ. Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior. *Frontiers in neuroendocrinology*. 2009 Oct.30:534. [PubMed: 19481567]
13. Wu S, et al. Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population. *Biological psychiatry*. 2005 Jul 1.58:74. [PubMed: 15992526]
14. Ebstein RP, et al. Arginine vasopressin and oxytocin modulate human social behavior. *Annals of the New York Academy of Sciences*. 2009 Jun.1167:87. [PubMed: 19580556]
15. Lerer E, et al. Association between the oxytocin receptor (OXTR) gene and autism: relationship to Vineland Adaptive Behavior Scales and cognition. *Molecular psychiatry*. 2008 Oct.13:980. [PubMed: 17893705]
16. Lerer E, et al. Low CD38 expression in lymphoblastoid cells and haplotypes are both associated with autism in a family-based study. *Autism research : official journal of the International Society for Autism Research*. 2010 Dec.3:293. [PubMed: 21182206]
17. Munesue T, et al. Two genetic variants of CD38 in subjects with autism spectrum disorder and controls. *Neuroscience research*. 2010 Jun.67:181. [PubMed: 20435366]
18. Wermter AK, et al. Evidence for the involvement of genetic variation in the oxytocin receptor gene (OXTR) in the etiology of autistic disorders on high-functioning level. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*. 2010 Mar 5.153B:629.
19. Skuse DH, et al. Common polymorphism in the oxytocin receptor gene (OXTR) is associated with human social recognition skills. *Proceedings of the National Academy of Sciences of the United States of America*. 2014 Feb 4.111:1987. [PubMed: 24367110]
20. LoParo D, Waldman ID. The oxytocin receptor gene (OXTR) is associated with autism spectrum disorder: a meta-analysis. *Molecular psychiatry*. 2014 Aug 5.
21. Brownstein MJ, Russell JT, Gainer H. Synthesis, transport, and release of posterior pituitary hormones. *Science*. 1980 Jan 25.207:373. [PubMed: 6153132]
22. Kiss A, Mikkelsen JD. Oxytocin--anatomy and functional assignments: a minireview. *Endocrine regulations*. 2005 Sep.39:97. [PubMed: 16468232]
23. Knobloch HS, et al. Evoked axonal oxytocin release in the central amygdala attenuates fear response. *Neuron*. 2012 Feb 9.73:553. [PubMed: 22325206]
24. Stoop R. Neuromodulation by oxytocin and vasopressin. *Neuron*. 2012 Oct 4.76:142. [PubMed: 23040812]
25. Lee HJ, Macbeth AH, Pagani JH, Young WS 3rd. Oxytocin: the great facilitator of life. *Progress in neurobiology*. 2009 Jun.88:127. [PubMed: 19482229]
26. Silverman JL, Yang M, Lord C, Crawley JN. Behavioural phenotyping assays for mouse models of autism. *Nature reviews. Neuroscience*. 2010 Jul.11:490.
27. Silverman JL, et al. Negative allosteric modulation of the mGluR5 receptor reduces repetitive behaviors and rescues social deficits in mouse models of autism. *Science translational medicine*. 2012 Apr 25.4:131ra51.

28. Smith SE, et al. Increased gene dosage of Ube3a results in autism traits and decreased glutamate synaptic transmission in mice. *Science translational medicine*. 2011 Oct 5.3:103ra97.
29. Chapman CD, et al. Intranasal treatment of central nervous system dysfunction in humans. *Pharmaceutical research*. 2013 Oct.30:2475. [PubMed: 23135822]
30. Ludwig M, Leng G. Dendritic peptide release and peptide-dependent behaviours. *Nature reviews. Neuroscience*. 2006 Feb.7:126.
31. Neumann ID, Maloumy R, Beiderbeck DI, Lukas M, Landgraf R. Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. *Psychoneuroendocrinology*. 2013 Oct.38:1985. [PubMed: 23579082]
32. Simpson EA, et al. Inhaled oxytocin increases positive social behaviors in newborn macaques. *Proceedings of the National Academy of Sciences of the United States of America*. 2014 May 13.111:6922. [PubMed: 24778211]
33. Striepens N, Kendrick KM, Maier W, Hurlemann R. Prosocial effects of oxytocin and clinical evidence for its therapeutic potential. *Frontiers in neuroendocrinology*. 2011 Oct.32:426. [PubMed: 21802441]
34. Siljee JE, et al. Melanocortin 4 receptor distribution in the human hypothalamus. *European journal of endocrinology / European Federation of Endocrine Societies*. 2013 Mar.168:361. [PubMed: 23211571]
35. Kublaoui BM, Gemelli T, Tolson KP, Wang Y, Zinn AR. Oxytocin deficiency mediates hyperphagic obesity of Sim1 haploinsufficient mice. *Molecular endocrinology*. 2008 Jul.22:1723. [PubMed: 18451093]
36. Sabatier N. alpha-Melanocyte-stimulating hormone and oxytocin: a peptide signalling cascade in the hypothalamus. *Journal of neuroendocrinology*. 2006 Sep.18:703. [PubMed: 16879169]
37. Modi ME, Young LJ. The oxytocin system in drug discovery for autism: animal models and novel therapeutic strategies. *Hormones and behavior*. 2012 Mar.61:340. [PubMed: 22206823]
38. Benoit SC, et al. A novel selective melanocortin-4 receptor agonist reduces food intake in rats and mice without producing aversive consequences. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2000 May 1.20:3442. [PubMed: 10777807]
39. Poliak S, et al. Juxtaparanodal clustering of Shaker-like K⁺ channels in myelinated axons depends on Caspr2 and TAG-1. *The Journal of cell biology*. 2003 Sep 15.162:1149. [PubMed: 12963709]
40. Lipari EF, et al. The hypothalamic magnocellular neurosecretory system in developing rats. *European journal of histochemistry : EJH*. 2001; 45:163. [PubMed: 11512637]
41. Yamamoto Y, et al. Neonatal manipulations of oxytocin alter expression of oxytocin and vasopressin immunoreactive cells in the paraventricular nucleus of the hypothalamus in a gender-specific manner. *Neuroscience*. 2004; 125:947. [PubMed: 15120854]
42. Zheng JJ, et al. Oxytocin mediates early experience-dependent cross-modal plasticity in the sensory cortices. *Nature neuroscience*. 2014 Mar.17:391.
43. Ahern TH, Young LJ. The impact of early life family structure on adult social attachment, alloparental behavior, and the neuropeptide systems regulating affiliative behaviors in the monogamous prairie vole (*Microtus ochrogaster*). *Frontiers in behavioral neuroscience*. 2009; 3:17. [PubMed: 19753327]
44. Bales KL, Carter CS. Developmental exposure to oxytocin facilitates partner preferences in male prairie voles (*Microtus ochrogaster*). *Behavioral neuroscience*. 2003 Aug.117:854. [PubMed: 12931969]
45. Ross HE, et al. Characterization of the oxytocin system regulating affiliative behavior in female prairie voles. *Neuroscience*. 2009 Sep 15.162:892. [PubMed: 19482070]
46. Lee HM, Giguere PM, Roth BL. DREADDs: novel tools for drug discovery and development. *Drug discovery today*. 2014 Apr.19:469. [PubMed: 24184433]
47. McCall C, Singer T. The animal and human neuroendocrinology of social cognition, motivation and behavior. *Nature neuroscience*. 2012 May.15:681.
48. Dolen G, Darvishzadeh A, Huang KW, Malenka RC. Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature*. 2013 Sep 12.501:179. [PubMed: 24025838]
49. Teng BL, et al. Prosocial effects of oxytocin in two mouse models of autism spectrum disorders. *Neuropharmacology*. 2013 Sep.72:187. [PubMed: 23643748]

50. Aoki Y, et al. Oxytocin improves behavioural and neural deficits in inferring others' social emotions in autism. *Brain : a journal of neurology*. 2014 Nov.137:3073. [PubMed: 25149412]
51. Watanabe T, et al. Mitigation of sociocommunicational deficits of autism through oxytocin-induced recovery of medial prefrontal activity: a randomized trial. *JAMA psychiatry*. 2014 Feb. 71:166. [PubMed: 24352377]
52. Cochran DM, Fallon D, Hill M, Frazier JA. The role of oxytocin in psychiatric disorders: a review of biological and therapeutic research findings. *Harvard review of psychiatry*. 2013 Sep-Oct; 21:219. [PubMed: 24651556]
53. Modahl C, et al. Plasma oxytocin levels in autistic children. *Biological psychiatry*. 1998 Feb 15.43:270. [PubMed: 9513736]
54. Andari E, et al. Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. *Proceedings of the National Academy of Sciences of the United States of America*. 2010 Mar 2.107:4389. [PubMed: 20160081]
55. Jansen LM, et al. Autonomic and neuroendocrine responses to a psychosocial stressor in adults with autistic spectrum disorder. *Journal of autism and developmental disorders*. 2006 Oct.36:891. [PubMed: 16865550]
56. Jacobson JD, et al. Evidence for alterations in stimulatory G proteins and oxytocin levels in children with autism. *Psychoneuroendocrinology*. 2014 Feb.40:159. [PubMed: 24485488]
57. Green L, et al. Oxytocin and autistic disorder: alterations in peptide forms. *Biological psychiatry*. 2001 Oct 15.50:609. [PubMed: 11690596]
58. Parker KJ, et al. Plasma oxytocin concentrations and OXTR polymorphisms predict social impairments in children with and without autism spectrum disorder. *Proceedings of the National Academy of Sciences of the United States of America*. 2014 Aug 19.111:12258. [PubMed: 25092315]
59. Hammock EA, Levitt P. Oxytocin receptor ligand binding in embryonic tissue and postnatal brain development of the C57BL/6J mouse. *Frontiers in behavioral neuroscience*. 2013; 7:195. [PubMed: 24376405]
60. Kramer KM, Choe C, Carter CS, Cushing BS. Developmental effects of oxytocin on neural activation and neuropeptide release in response to social stimuli. *Hormones and behavior*. 2006 Feb.49:206. [PubMed: 16112115]
61. Barrett CE, et al. Neonatal melanocortin receptor agonist treatment reduces play fighting and promotes adult attachment in prairie voles in a sex-dependent manner. *Neuropharmacology*. 2014 Oct.85:357. [PubMed: 24923239]
62. Mogi K, Ooyama R, Nagasawa M, Kikusui T. Effects of neonatal oxytocin manipulation on development of social behaviors in mice. *Physiology & behavior*. 2014 May 21.133C:68. [PubMed: 24857720]
63. Suraev AS, et al. Adolescent exposure to oxytocin, but not the selective oxytocin receptor agonist TGOT, increases social behavior and plasma oxytocin in adulthood. *Hormones and behavior*. 2014 May.65:488. [PubMed: 24631584]
64. Owen SF, et al. Oxytocin enhances hippocampal spike transmission by modulating fast-spiking interneurons. *Nature*. 2013 Aug 22.500:458. [PubMed: 23913275]
65. Sohal VS, Zhang F, Yizhar O, Deisseroth K. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature*. 2009 Jun 4.459:698. [PubMed: 19396159]
66. Sala M, et al. Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. *Biological psychiatry*. 2011 May 1.69:875. [PubMed: 21306704]
67. Tyzio R, et al. Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery. *Science*. 2006 Dec 15.314:1788. [PubMed: 17170309]
68. Tyzio R, et al. Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. *Science*. 2014 Feb 7.343:675. [PubMed: 24503856]
69. Selby L, Zhang C, Sun QQ. Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein. *Neuroscience letters*. 2007 Feb 2.412:227. [PubMed: 17197085]

70. Francis SM, et al. Oxytocin and vasopressin systems in genetic syndromes and neurodevelopmental disorders. *Brain research*. 2014 Jan 22.
71. Hall SS, Lightbody AA, McCarthy BE, Parker KJ, Reiss AL. Effects of intranasal oxytocin on social anxiety in males with fragile X syndrome. *Psychoneuroendocrinology*. 2012 Apr.37:509. [PubMed: 21862226]
72. Theodosis DT, Schachner M, Neumann ID. Oxytocin neuron activation in NCAM-deficient mice: anatomical and functional consequences. *The European journal of neuroscience*. 2004 Dec. 20:3270. [PubMed: 15610159]
73. Anderson GR, et al. Candidate autism gene screen identifies critical role for cell-adhesion molecule CASPR2 in dendritic arborization and spine development. *Proceedings of the National Academy of Sciences of the United States of America*. 2012 Oct 30.109:18120. [PubMed: 23074245]
74. Tolson KP, et al. Postnatal Sim1 deficiency causes hyperphagic obesity and reduced Mc4r and oxytocin expression. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2010 Mar 10.30:3803. [PubMed: 20220015]
75. Fields RL, Ponzio TA, Kawasaki M, Gainer H. Cell-type specific oxytocin gene expression from AAV delivered promoter deletion constructs into the rat supraoptic nucleus in vivo. *PloS one*. 2012; 7:e32085. [PubMed: 22363799]
76. Cetin A, Komai S, Eliava M, Seeburg PH, Osten P. Stereotaxic gene delivery in the rodent brain. *Nature protocols*. 2006; 1:3166.

Figure 1. Oxytocin administration rescues social behavior in the *Cntnap2* mouse model
(A) Reciprocal social interaction test for WT (left) and KO (right) drug or vehicle treated mice by i.p. injection. The time spent engaged in social interaction for each pair of mice is shown (n=6 pairs, 3M-3F, per genotype/condition). Two-way ANOVA $F_{11,60}=2.89$, $p=0.004$, treatment effect $F_{5,60}=4.13$, $p=0.0027$, treatment/genotype interaction $F_{5,60}=2.23$, $p=0.0627$, followed by Dunnett pairwise comparisons to controls $WT_{vehicle}-KO_{vehicle}$ $p=0.03$, $KO_{vehicle}-KO_{OXT}$ $p=0.03$, $KO_{vehicle}-KO_{AVP}$ $p=0.04$. **(B)** Social approach (3 chamber) test. Time spent interacting with an empty cup or a cup with a stranger mouse

inside is shown for each genotype when treated i.p. with vehicle, OXT or AVP (n=8–10, 4/5M-4/5F, mice per genotype/condition). Paired Student t test comparing 'mouse' to 'empty' as a measure of sociability within each group. WT_{vehicle} p=0.01, WT_{OXT} p=0.02, KO_{OXT} =0.03 **(C)** Reciprocal social interaction test when OXT and AVP are administered intranasally (n=8, 4M-4F, pairs per genotype/condition). Two-way ANOVA $F_{5,42}=15.88$, $p<0.0001$, treatment effect $F_{2,42}=21.08$, $p<0.0001$, genotype/treatment interaction $F_{2,42}=17.01$, $p=0.0001$ followed by Dunnett pairwise comparisons to controls WT_{vehicle} - KO_{vehicle} $p<0.0001$, KO_{vehicle} - KO_{OXT} $p<0.0001$, KO_{vehicle} - KO_{AVP} $p<0.0001$. **(D)** Reciprocal social interaction test (n=8 pairs of mice, 4M-4F, per genotype/condition). One-way ANOVA $F_{5,41}=22.02$, $p<0.0001$ followed by Bonferroni post-hoc test, modified significance level $p=0.003$, all significant comparisons $p<0.0001$. **(E)** Reciprocal social interaction test showing time course of a single acute dose of intranasal OXT administration (n=8 pairs of mice, 4M-4F, per genotype/condition). One-way ANOVA $F_{4,35}=23.16$, $p<0.0001$, followed by Bonferroni post-hoc test, modified significance level $p=0.005$, all significant comparisons $p<0.0001$. M=male, F=female. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.

Figure 2. Pharmacological stimulation of oxytocin release improves social behavior in the *Cntnap2* model

(A) Reciprocal social interaction test in vehicle or drug treated animals (n=6 pairs of mice, 3M-3F, per genotype/condition). Two-way ANOVA genotype/treatment interaction $F_{1,20}=4.87$, $p=0.04$, followed by Dunnett pairwise comparisons to controls, $WT_{vehicle}-KO_{vehicle}$ $p=0.04$, $KO_{vehicle}-KO_{MC4Rag}$ $p=0.02$. (B) Reciprocal social interaction test in *Cntnap2* KO (n=7 pairs of mice, 4M-3F, per condition). One way ANOVA $F_{2,18}=14.6$ treatment effect $p=0.0001$ followed by Bonferroni post-hoc test, modified significance level $p=0.017$, vehicle-agonist $p=0.002$, agonist-antagonist $p<0.0001$. M=male, F=female. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.

Figure 3. *Cntnap2* mutant mice show reduced central OXT levels at P30

(A) Representative images of OXT immunoreactivity in different antero-posterior levels of the PVN in *Cntnap2* KO and WT controls at P30. Scale bar: 100 μ m. (B) Stereological counts of OXT positive cells in the PVN region of both genotypes at P30 (n=4 mice, 2M-2F, per genotype). Student t-test, p=0.03. (C) Quantification of OXT levels in whole brain extracts by radioimmunoassay at P30 (n=10 mice, 5M-5F, per genotype). Student t-test, p=0.01. M=male, F=female. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.

Figure 4. Oxytocin immunoreactivity in *Cntnap2* mutants at P7 is normal

(A) Representative images of OXT immunoreactivity at different antero-posterior levels of the PVN in *Cntnap2* KO and WT controls at P7. Scale bar=100 μ m. **(B)** Stereological counts of OXT positive cells in the PVN region of both genotypes at P7 (n=4 mice, 2M-2F, per genotype). No differences were found with Student t test (p=0.53). M=male, F=female. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.

Figure 5. Early postnatal oxytocin treatment restores peptide levels and improves social behavior at P30

(A) Social approach (3 chamber) test shown for WT and KO mice at P30 treated early with vehicle or OXT (n=10–14 mice/group). Paired Student t test comparing 'mouse' to 'empty' within each group as a measure of sociability, WT_{saline} p=0.006 (n=10, 4M-6F), WT_{OXT} p=0.006 (n=14, 8M-6F), KO_{saline} p=0.3 (n=12, 6M-6F), KO_{OXT} p=0.004 (n=13, 6M-7F). (B) Reciprocal social interaction test shown in pairs of KO mice at P30 treated early with saline or with OXT (n=7 and 8 pairs of mice, 4M-3/4F, respectively). Student t test, p<0.001

(C) Quantification, as detected by radioimmunoassay, of OXT levels in whole brain extracts for KO mice treated with saline or with OXT at P30 (n=6, 3M-3F; n=8, 4M-4F, respectively). Student t test, p=0.02. (D) Stereological quantification of the number of OXT immunoreactive cells in *Cntnap2* KO mice treated with either saline or OXT at P30 (n=4 mice, 2M-2F, per condition). Student t test, p=0.005. (E) Representative images of OXT immunoreactivity in the PVN of saline or OXT treated KO animals at P30. Scale bar: 100µm.). M=male, F=female. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Evoked oxytocin release improves social behavior in the *Cntnap2* mouse

(A) Schematic representation of the construct used to express designer receptors in OXT cells (B) mCherry fluorescence in the PVN of AAV2 injected mice colocalizes with OXT immunoreactivity (C) Representative trace of whole cell, current clamp recordings from an mCherry labeled oxytocin neuron. CNO (5 μ M) was perfused in the bath for 250 seconds, which resulted in consistent depolarization of membrane potential and an increase in action potential firing (D) Social approach (3 chamber) test in saline or CNO (5mg/kg) treated KO and WT mice (n=6 male mice per genotype/condition). Paired Student t test comparing 'mouse' to 'empty' within each group as a measure of sociability, WT_{saline} p<0.001, WT_{CNO} p=0.03, KO_{saline} p=0.13, KO_{CNO} p=0.001. Box-plots represent the median plus the 25th and 75th percentiles. Whiskers represent the minimum and maximum values. The mean is represented as a plus (+) sign.