



# Oxytocin under opioid antagonism leads to supralinear enhancement of social attention

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**To provide new preclinical evidence toward improving the efficacy of oxytocin (OT) in treating social dysfunction, we tested the benefit of administering OT under simultaneously induced opioid antagonism during dyadic gaze interactions in monkeys. OT coadministered with a  $\mu$ -opioid receptor antagonist, naloxone, invoked a supralinear enhancement of prolonged and selective social attention, producing a stronger effect than the summed effects of each administered separately. These effects were consistently observed when averaging over entire sessions, as well as specifically following events of particular social importance, including mutual eye contact and mutual reward receipt. Furthermore, attention to various facial regions was differentially modulated depending on social context. Using the Allen Institute's transcriptional atlas, we further established the colocalization of  $\mu$ -opioid and  $\kappa$ -opioid receptor genes and OT genes at the OT-releasing sites in the human brain. These data across monkeys and humans support a regulatory relationship between the OT and opioid systems and suggest that administering OT under opioid antagonism may boost the therapeutic efficacy of OT for enhancing social cognition.**

oxytocin | opioid antagonist | naloxone | OXTR | OPRM1

The efficacy of oxytocin (OT) in improving social abilities is under debate, largely due to frequently observed weak effect sizes and problems with replicability (1–3). Clinical trials of OT in autistic patients are ongoing, yet several studies have produced inconclusive results (4–8), demanding improvements to the efficacy and reliability of OT-based therapeutics. One strategy is to take advantage of existing physiological pathways in the brain that regulate OT activity to combinatorially enhance the oxytocinergic effects on social functions. In this regard, a promising candidate is the opioid system.

In addition to the evolutionarily conserved OT system (9), the opioid system has been implicated in regulating social behavior. Excessive opioid activity in the brain has been discussed with respect to the development of early childhood autism (10). Abnormalities in central opioid levels have been observed in some individuals with autism, and clinical trials with predominantly  $\mu$ -opioid blockers, such as naltrexone or naloxone (NAL), have yielded promising results in ameliorating both social and nonsocial deficits (11). Specifically,  $\mu$ -opioid receptors have been studied in relation to reward, emotion, and behavior in the social domain (12) and are strongly expressed in reward-related regions of the primate brain (13). In rhesus macaques, carrying the G allele of the  $\mu$ -opioid receptor gene *OPRM1*, compared with homozygous C alleles, is associated with stronger maternal attachment in infants (14) and more effective prevention of infant separation in mothers (15). Additionally, opioid agonists, such as morphine, decrease physical contact between social partners, whereas NAL administration increases solicitation for social contact, such as grooming and proximity (16–19).

The physiological relationship between the opioid and OT systems has been firmly established (20). Opioids directly inhibit OT secretion in mammals through action in the posterior pituitary

and the hypothalamus (21, 22). Opioids not only inhibit OT release from the axon terminal in the posterior pituitary but also suppress the functional activity of OT neurons via opioid action on their cell bodies in the hypothalamus (23, 24). The chronic administration of morphine, a prototypical  $\mu$ -opioid receptor agonist, also influences both the synthesis and secretion of OT (25). Despite the similar synthesis location of OT and vasopressin in the magnocellular neurons of the hypothalamus (21), opioids selectively regulate release of OT rather than vasopressin (22). Supporting this dissociation, endogenous opioid inhibition triggers central OT, but not vasopressin, release (26), and the high-affinity  $\mu$ -opioid receptor antagonist NAL strongly drives OT, but not vasopressin, release from the posterior pituitary (22). Furthermore, during parturition, endogenous opioids control the release of OT both into blood and the brain in a tightly coordinated manner (26). Enhancing opioidergic tone with morphine severely delays the course of parturition, accompanied by a reduced level of circulating OT, whereas attenuating opioidergic tone with NAL acutely increases the speed of parturition by greatly elevating OT levels, reversing this delay (27–29). Similarly, the inhibition of OT secretion from neural tissues by morphine underlies the mechanism by which morphine disrupts milk ejection in lactation (30, 31).

Exploiting the regulatory relationship between the opioid and OT systems in the brain, we tested here whether administering OT

## Significance

**In the past decade, there has been an increase in studies using oxytocin (OT) for improving social cognition, but results have been inconsistent. In this study, we took advantage of the physiological relationship between the opioid and OT systems and tested the benefit of administering OT under simultaneously induced opioid antagonism during dyadic gaze interactions. Coadministration of OT and opioid blocker leads to supralinear enhancement of prolonged and selective attention to a live partner and increases interactive gaze after critical social events. Furthermore, we provide neurogenetic evidence in the human brain supporting the interaction between specific opioid receptor genes and the genes for OT processing. Our results suggest a new avenue for amplifying the efficacy of OT in clinical populations.**

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Data deposition: The data and codes used in this paper are available and downloadable through GitHub ([https://github.com/changlabneuro/OT\\_NAL](https://github.com/changlabneuro/OT_NAL)).

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in the presence of opioid antagonism could effectively promote social behavior. First, using multiple experimental approaches in nonhuman primates, we tested whether coadministration of OT and NAL leads to supralinear enhancement of attention directed to others compared with the summed effects observed from OT or NAL administered separately, a conservative indicator of the combinatorial benefit. Second, in human brain tissues, we examined the colocalization of the  $\mu$ -opioid,  $\kappa$ -opioid, and  $\delta$ -opioid receptor genes with the OT gene, as well as the OT receptor gene, across 190 brain regions to provide the transcriptional correspondence of different types of opioid receptors with OT-secreting cells and OT receptors.

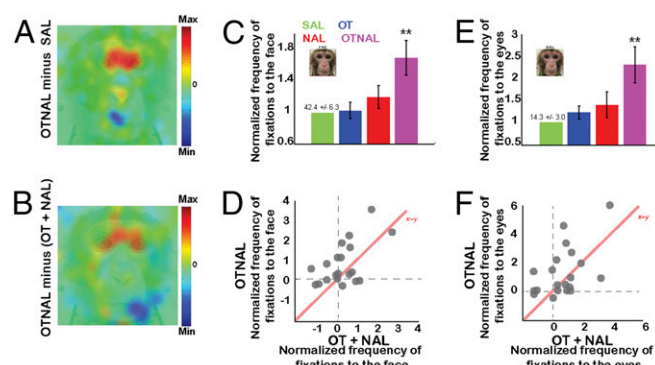
## Results

For testing the behavioral impact of combined delivery of OT and NAL, pairs of monkeys sat directly across from each other while the eye positions from both monkeys were simultaneously and continuously recorded (32) (Fig. S1 A and B). Using a pediatric nebulizer, one of the animals in a given pair received aerosolized drugs intranasally corresponding to one of the four pharmacology conditions: OT [24 international units (IU)], NAL (1 mg), saline (SAL), or the combination of OT (24 IU) and NAL (1 mg) together (OTNAL) (SI Results, NAL Dose-Response Curve and Figs. S1C and S2). Fixation density maps (Fig. 1 A and B) show clear effects of OTNAL that encompass an increase in fixations to the eyes with a relative decrease in fixations to the mouth (Fig. 1A), and those maps suggest a supralinear effect of OTNAL, as shown by an increase in fixations to the eyes after subtracting the effect of OT plus NAL (Fig. 1B). OTNAL was associated with an increased number of fixations to the face of a conspecific compared with the SAL or OT condition [Fig. 1C;  $F(3,76) = 5.14$ ,  $P = 0.003$  for main effect,  $P = 0.005$  for OTNAL over SAL,  $P = 0.073$  for OTNAL over NAL,  $P = 0.007$  for OTNAL over OT, one-way ANOVA with Tukey-Kramer post hoc tests]. To determine whether the effects of OTNAL were supralinear for the conspecific's face, we directly compared the effects of OTNAL with the added effects of OT and NAL alone over SAL control (i.e., a supralinear effect would be indicated by data points reliably falling above the unity line when the OTNAL effects are plotted on the ordinate as a function of the summed effects of OT

and NAL on the abscissa). OTNAL had a larger effect for attention to the face than the added effects of OT and NAL alone [Fig. 1D;  $t(19) = 2.42$ ,  $P = 0.026$ , paired-sample  $t$  test], with the effect size of OTNAL being correlated with the added effect sizes of OT and NAL alone ( $r^2 = 0.33$ ,  $P = 0.008$ ; linear regression). Furthermore, the OTNAL condition was associated with increased frequency of fixations to the eyes of a conspecific compared with the SAL or OT condition [Fig. 1E;  $F(3,76) = 5.87$ ,  $P = 0.004$  for main effect,  $P = 0.004$  for OTNAL over SAL,  $P = 0.069$  for OTNAL over NAL,  $P = 0.022$  for OTNAL over OT], again with a supralinear pattern [Fig. 1F;  $t(19) = 1.94$ ,  $P = 0.067$ ]. This effect size of OTNAL was also correlated with the added effect sizes of OT and NAL alone (for eyes:  $r^2 = 0.29$ ,  $P = 0.014$ ). Critically, both for the face (Fig. S3A) and for the eyes (Fig. S3B), we found no significant modulation of fixation duration for any drug condition relative to SAL, indicating that there was no tradeoff between fixation frequency and fixation duration with respect to our observed effects, and that the effects were specific to increasing fixation frequency across all behavioral sessions to both the face and eyes. Additionally, the supralinear effects of OTNAL cannot be explained by a nonspecific increase in arousal, because the total count of fixations in the OTNAL condition was, in fact, not higher than in the SAL, NAL, or OT conditions (all  $P > 0.05$ , one-way ANOVA with Tukey-Kramer post hoc tests). Finally, in reference to previous literature documenting the effects of OT in social attention (33, 34), OT overall increased fixations to the eyes compared with SAL in this real-life dyadic setting [Fig. 1E;  $t(19) = 1.63$ ,  $P = 0.059$ , paired-sample  $t$  test], although the effect was marginal.

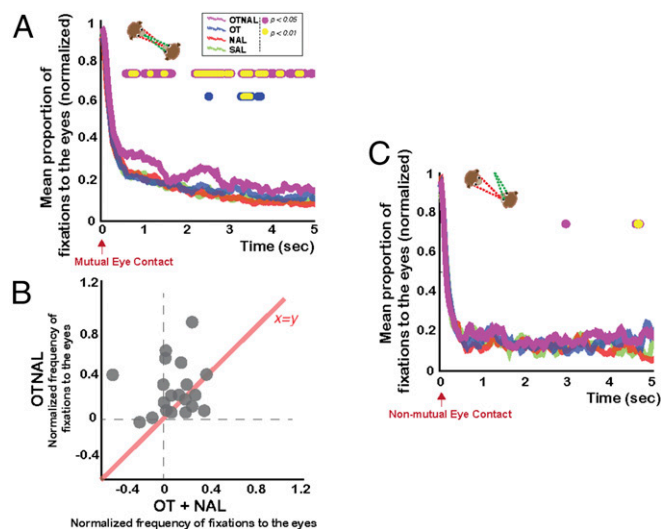
Gaze interactions are by nature dynamic and contingent between interacting agents. Following our pharmacological manipulations, we examined differences in gaze patterns focusing on time periods following mutual eye contact as instances of particular social importance (35–37). Following mutual eye contact, only OTNAL strongly increased attention to the conspecific (Fig. 2A;  $P < 0.01$ , permutation test). In fact, neither OT nor NAL administered alone had a significant effect in increasing attention to the conspecific following mutual eye contact compared with SAL (both  $P > 0.05$ , permutation tests). Notably, when comparing the effects of drugs following mutual eye contact, the resulting gaze patterns were again supralinear [Fig. 2B;  $t(19) = 2.88$ ,  $P = 0.010$ , paired-sample  $t$  test]. We next tested whether the effects of OTNAL were specific to the periods following mutual eye contact as opposed to the periods following looking at the conspecific's eyes when the conspecifics were looking elsewhere than the partner's eyes. Following these instances of nonmutual eye contact, no significant results were observed for any pharmacological manipulation using the identical approach (Fig. 2C; all  $P > 0.05$ , permutation tests), indicating that the differences observed in the OTNAL condition selectively occurred during time periods of heightened social relevance. These effects were also observed to be specific to the eye region of interest (ROI), because gaze position returning to the mouth ROI after mutual eye contact, as well as after mutual gaze to the mouth of the conspecific, was not significantly affected by any pharmacological manipulation (Fig. S4A and B; all  $P > 0.05$ , permutation tests).

During social interactions, attention can be allocated differentially between interacting individuals depending on the context. For example, a mutually beneficial event is particularly salient to social species living in large groups like macaques and humans, and such a prioritized behavioral relevance may be a foundational component guiding complex social interactions, such as coordinating with one another to obtain mutual benefits (38). Given the supralinear effects of OTNAL following mutual eye contact, we hypothesized similar combinatorial effects of OTNAL following mutual reward receipt by manipulating the receipt of mutual juice rewards during gaze interactions (Fig. S1B). Gaze frequency to the face of the conspecific was only significant in the OTNAL condition (Fig. 3A;  $P < 0.001$ , permutation test). Notably, these effects were again supralinear following mutual reward for OTNAL



**Fig. 1.** Combined delivery of OT and NAL results in a supralinear enhancement of overall attention to the face and eyes of a conspecific. (A) Averaged heat map showing gaze fixations in the OTNAL condition over the SAL condition. (B) Averaged heat map showing fixations in the OTNAL condition over the summed effects of OT and NAL administered independently, demonstrating the supralinear effects. (C) Overall frequency of gaze fixations to the face of a conspecific in the OT (blue), NAL (red), and OTNAL (purple) conditions, normalized by the SAL (green) condition. (D) Effect size of OTNAL compared with the summed effect size of OT and NAL administered independently. (E and F) Overall frequency of gaze fixations to the eyes of a conspecific in the same format as in C and D.  $**P < 0.01$  over SAL condition, one-way ANOVA with Tukey-Kramer post hoc tests for multiple comparisons.





**Fig. 2.** Supralinear enhancements of dynamic gaze interactions following mutual eye contact by OTNAL. (A) Peristimulus time histograms (PSTHs), aligned to mutual eye contact, show the proportion of looking at the eyes following mutual eye contact in SAL (green), OT (blue), NAL (red), and OTNAL (purple) conditions. Horizontal marks indicate 10-ms time bins with significant differences ( $P < 0.05$ , OT over SAL, blue;  $P < 0.05$ , OTNAL over SAL, purple;  $P < 0.01$ , yellow; paired-sample  $t$  tests). (B) Effects size of OTNAL compared with the added effect size of OT and NAL alone for 0.5–3 s after mutual eye contact. (C) PSTH aligned to nonmutual eye contact in the same format as in A.

compared with the summed effects of OT and NAL, with a positive correlation between them [Fig. 3B;  $t(19) = 2.14$ ,  $P = 0.046$ , paired-sample  $t$  test;  $r^2 = 0.25$ ,  $P = 0.023$ , linear regression]. By contrast, when no reward was delivered to either animal, no modulations in gaze behaviors were found across all conditions (Fig. 3C; all  $P > 0.05$ , permutation tests), indicating that OTNAL also supralinearly influenced social gaze dynamics following mutually beneficial events.

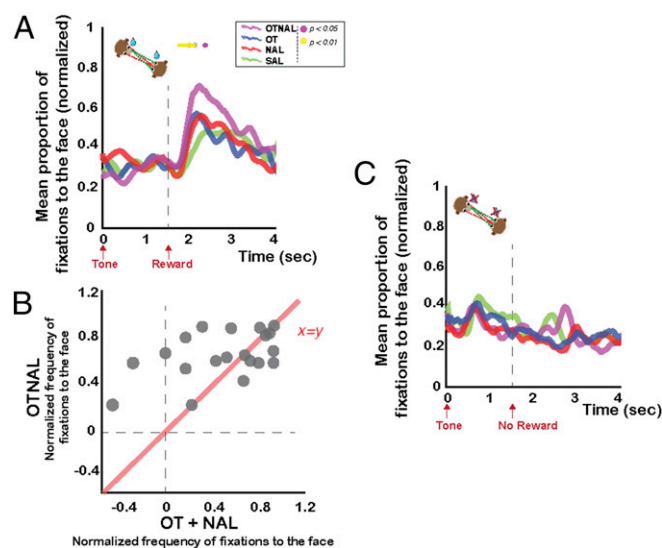
Across mutual eye contact (Fig. 2) and mutual reward receipt (Fig. 3), the supralinear effects of OTNAL were driven by the most socially relevant features within the face of the conspecific, depending on the context of a given interactive event. Although the differences in gaze patterns following mutual eye contact in the OTNAL condition were specifically driven by returning gaze to the eye ROI (Fig. 2A and Fig. S4), the differences following mutual reward were specifically driven by attention to the mouth ROI (Fig. S5). Therefore, the supralinear effects of OTNAL are selective in that they were exerted by the most relevant features within the face of the conspecific depending on the interactive context.

Although the number of males and females tested in our study is underpowered for fully exploring potential sex difference, when we analyzed fixation frequency to the face (Fig. S6A) and eyes (Fig. S6B) over entire behavioral sessions, as well as gaze dynamics to the conspecific following mutual eye contact (Fig. S6C) and mutual reward (Fig. S6D), we found no significant effect of sex. Together, these results show that, within our limited sample size, drug effects are observed indistinguishably across both sexes.

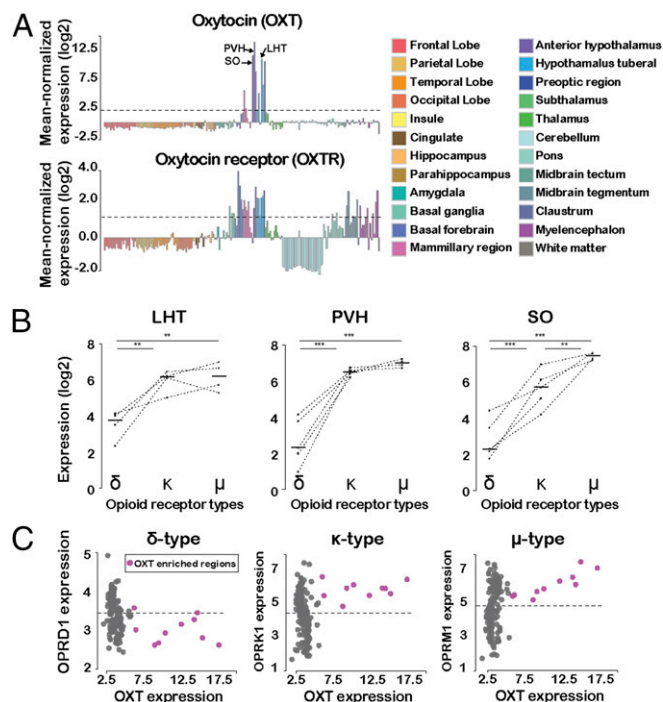
We next identified potential brain regions underlying the facilitatory effects of opioid antagonism on OT in the human brain. We examined colocalization of gene expression patterns for the OT gene (*OXT*); OT receptor gene (*OXTR*); and  $\mu$ -opioid (*OPRM1*),  $\kappa$ -opioid (*OPRK1*), and  $\delta$ -opioid (*OPRD1*) receptor subtypes, using averaged microarray expression data of six postmortem donors from the Allen Human Brain Atlas. Available data included 190 regions containing samples from at least four donors (39). Transcription of the *OXT* was most pronounced in 10 regions

displaying expression  $\geq 1$  SD above the region-wise mean [Fig. 4A; lateral hypothalamic area, tuberal region (LHT); paraventricular nucleus of the hypothalamus (PVH); supraoptic nucleus (SO); dorsomedial hypothalamic nucleus; lateral hypothalamic area, anterior region; lateral hypothalamic area, medial region; ventral hypothalamic area, medial region; perifornical nucleus; posterior hypothalamic area; and preoptic region].

Comparing the relative expression of *OPRM1*, *OPRK1*, and *OPRD1* among the 10 highest *OXT*-enriched regions responsible for endogenous OT release, we found significant main effects of opioid receptor subtype in each region (statistics are given below for three regions, and statistics for seven regions are given in the legend for Fig. S7). In all of these *OXT*-enriched regions, *OPRM1* and *OPRK1* displayed significantly greater expression than *OPRD1* (Fig. 4B and Fig. S7). The effects of opioid receptor subtype were particularly pronounced in the three regions with greatest *OXT* expression: LHT [ $F(1,3) = 54.64$ ,  $P = 0.005$  for main effect,  $P = 0.002$  for  $\mu$  over  $\delta$ ,  $P = 0.002$  for  $\kappa$  over  $\delta$ ,  $P = 0.691$  for  $\mu$  over  $\kappa$ , one-way within-subjects ANOVA with Benjamini–Hochberg corrected post hoc tests], PVH [ $F(1,4) = 39.05$ ,  $P = 0.003$  for main effect,  $P < 0.001$  for  $\mu$  over  $\delta$ ,  $P < 0.001$  for  $\kappa$  over  $\delta$ ,  $P = 0.201$  for  $\mu$  over  $\kappa$ ], and SO [ $F(1,4) = 97.15$ ,  $P < 0.001$  for main effect,  $P < 0.001$  for  $\mu$  over  $\delta$ ,  $P < 0.001$  for  $\kappa$  over  $\delta$ ,  $P = 0.005$  for  $\mu$  over  $\kappa$ ]. Robust above-average expression of *OPRM1* and *OPRK1* genes, but not *OPRD1*, was observed in all 10 *OXT*-enriched regions (Fig. 4C). Notably, the expression levels for *OPRM1* and *OXT* in the 10 *OXT*-enriched regions were correlated [ $r(8) = 0.88$ ,  $P < 0.001$ , Pearson's correlation; Fig. 4C], whereas there were no such correlations for *OPRK1* [ $r(8) = 0.15$ ,  $P = 0.673$ ] and *OPRD1* [ $r(8) = -0.17$ ,  $P = 0.632$ ], insinuating a tighter coupling between *OXT* and *OPRM1*. Moreover, we also reliably observed above-average expression of *OXTR* in the *OXT*-enriched regions (Fig. 4A and Fig. S8), suggesting a regional colocalization of *OXT*, *OXTR*, *OPRM1*, and



**Fig. 3.** Supralinear enhancements of dynamic gaze interactions following mutual reward by OTNAL. (A) PSTH shows the proportion of looking at the face following a tone predicting mutual reward in SAL (green), OT (blue), NAL (red), and OTNAL (purple) conditions. Reward occurred simultaneously for both monkeys 1.5 s after an unpredictable auditory tone. Horizontal marks indicate 10-ms time bins with significant differences ( $P < 0.05$ , OTNAL over SAL, purple;  $P < 0.01$ , yellow; paired-sample  $t$  tests). (B) Effects size of OTNAL versus the summed effect size of OT and NAL alone for 1.5–3.5 s after tone. (C) PSTH aligned to tones that did not result in reward to either monkey in the same format as in A. No 10-ms time bin for any drug condition was significantly higher than SAL (all  $P > 0.05$ , paired-sample  $t$  tests).



**Fig. 4.** Genes encoding  $\mu$ -opioid and  $\kappa$ -opioid, but not  $\delta$ -opioid, receptors display above-average expression in OT-enriched regions. (A) Microarray expression of *OXT* and *OXTR* from 190 regions averaged across six post-mortem human brains from the Allen Human Brain Atlas (AHBA). Ten tissue samples displayed *OXT* expression 1 SD above the mean: LHT, PVH, SO, dorsomedial hypothalamic nucleus (DMH), lateral hypothalamic area, anterior region (LHA), lateral hypothalamic area, medial region (LHM), ventral hypothalamic area, medial region (VHM), perifornical nucleus (PeF), posterior hypothalamic area (PHA), and preoptic region (PrOR). (B)  $\mu$ -Opioid and  $\kappa$ -opioid receptor genes are expressed significantly more than  $\delta$ -opioid receptor genes across the top three *OXT*-enriched regions (data from the remaining seven *OXT*-enriched regions are shown in Fig. S7). Each line connects samples from the same donor. Different numbers of lines are due to nonuniform sampling across the six AHBA donors. The horizontal ticks indicate the mean of different donors. (C) Across all 190 regions,  $\mu$ -opioid and  $\kappa$ -opioid receptors displayed above-average expression within the eight *OXT*-enriched samples. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

*OPRK1*. Taken together, these results indicate the preferential role of  $\mu$ -opioid and  $\kappa$ -opioid receptor subtypes in OT-secreting brain regions.

## Discussion

In this preclinical pharmacological investigation, we report an alternate avenue for amplifying the efficacy of OT in directing selective and prolonged attention to social cues by administering OT under opioid antagonism. The supralinear effects of OTNAL were driven by mutually engaged events (mutual eye contact and mutual reward receipt) and resulted in directing selective attention to socially relevant features (eyes and mouth, respectively). The potential benefit of coadministering OT with NAL is supported by the mechanistic link between the inhibition of opioid processing and increase in OT release from the posterior pituitary (22), as well as the colocalization of the expressed OT receptor and  $\mu$ -opioid and  $\kappa$ -opioid receptor genes at the primary OT-releasing sites in the human brain.

Because the opioid system involves at least three principal receptor types ( $\mu$ -opioid,  $\kappa$ -opioid, and  $\delta$ -opioid receptors), determining which specific receptor subtypes mediate the effect of endogenous opioids on the OT system is of interest. Bicknell and Leng (40) examined the effects of a range of opioid agonists and

antagonists with different relative selectivity toward opioid receptor subclasses on the secretion of OT from the isolated posterior pituitary. The authors reported that NAL, the relatively  $\mu$ -selective antagonist (41) used in our study, enhanced OT secretion by up to 90%. Douglas et al. (42) showed that in late pregnant rats, endogenous opioids inhibit OT neurons through  $\mu$ -opioid receptors rather than  $\kappa$ -opioid receptors. Norbinaltorphimine, a specific  $\kappa$ -opioid receptor antagonist, did not increase neuronal activity or affect OT release in the SO, whereas NAL rapidly increased OT secretion (43, 44) and increased neuronal activity in the SO (42). Our analysis of human brain tissue indicates the colocalization of  $\mu$ -opioid and  $\kappa$ -opioid receptor genes, but not  $\delta$ -opioid receptor genes, and OT genes at the OT-releasing sites in the brain, including the SO, PVH, and LHT, but not in other cortical and subcortical regions. Based on NAL's much higher affinity for  $\mu$ -type than for  $\kappa$ -type (greater than eightfold) and  $\delta$ -type (>60-fold) receptors (45), the supralinear results of OTNAL here are likely driven most strongly by the OT-secreting cells expressing the  $\mu$ -opioid receptor subtypes, as suggested from posterior pituitary as well as lactation and parturition studies (30, 31). This finding is consistent with the notion that OTNAL is amplifying the known OT effects on social attention associated with systemic OT manipulations, in which the functional specificity is likely to be localized to OT-sensitive brain regions following an enhancement of the oxytonergic tone of the brain. Taken together, these findings suggest that the effects of NAL are likely mediated by inhibition of  $\mu$ -opioid and possibly also  $\kappa$ -opioid receptors, but not  $\delta$ -opioid receptors.

We hypothesize that the supralinear effects of OTNAL results from elevations of endogenous OT toward a threshold that allows the brain to engage robustly in social functions. The supralinear effects of OTNAL may be due to simultaneous engagement of feedforward endogenous OT release from neurons with OT receptors (46), as well as the removal of opioid-mediated inhibition of OT release in OT neurons (27), resulting in overall higher endogenous OT levels that reach a threshold needed for social functions. Observational studies in patients with autism have shown that administration of an opioid antagonist could decrease self-injurious behavior, stereotypies, hyperactivity, and withdrawal, although increasing verbal production, social communication, proximity seeking, and eye contact (47–50). However, in the past decade, there has been a striking reduction in research using opioid antagonists as a means to ameliorate social deficits, whereas there has been a marked increase in studies testing the benefits of OT on social attention, although with inconclusive results. Our results indicate that combined administration of exogenous OT and NAL may improve the efficacy of pharmacological therapies to enhance social functions by leveraging endogenous OT release.

## Materials and Methods

All procedures were approved by the Yale Institutional Animal Care and Use Committee and were in compliance with the Public Health Service's *Guide for the Care and Use of Laboratory Animals* (51).

### Behavioral Tasks.

**Live gaze interaction task.** Two animals sat in front of each other with no task constraints while the eye positions of both animals were recorded simultaneously and continuously (Fig. S1A). Horizontal and vertical eye positions were sampled at 1,000 Hz using two infrared eye monitor camera systems (Eyelink; SR Research). The two monkeys were positioned 62 cm apart from one another, with the top of each monkey's head at 76 cm from the floor. Before starting to record gaze behavior, each animal underwent a systematic calibration procedure (details are provided in *SI Materials and Methods, Behavioral Tasks* and ref. 32). During the calibration and until the beginning of each session, the two animals had no visual access to each other, with a screen fully separating the view of both animals. The screen was lifted at the start of each session, marking the beginning of the live gaze interaction task. Each session lasted 3 min, for a total of eight sessions each day, with a 3-min break between sessions with no visual access. One of the two animals in a given pair was administered a drug via nebulizer (details are provided in *SI Materials and Methods, Subjects*) corresponding to one of the four pharmacology conditions: OT (24 IU), NAL (1 mg), SAL,



or the combination of OT (24 IU) and NAL (1 mg) together (OTNAL). Behavioral testing began 30 min after each treatment and lasted for about 45 min. A total of five animals (three males and two females) with between three and five distinct conspecifics (total of 20 pairs) participated in all four pharmacology conditions. We collected 640 sessions in total: 32 sessions for each drug condition per subject.

**Mutual reward task.** Animals were placed in the same configuration as in the live gaze interaction task. In this task, two speakers (each speaker located by the chair of each monkey) and two juice tubes, one for each monkey, were added to the setup. An auditory tone was played at random time intervals (between 4 and 16 s). Following the tone, simultaneous juice rewards to both monkeys (0.5 mL) or no rewards to both monkeys could occur at equal probabilities after 1.5 s (Fig. S1B). Before and between these sessions, an occluder was placed between the animals. Similar to the live gaze interaction task, the eye movements of both animals were completely unconstrained, allowing for examination of naturalistic gaze patterns, and one of the two animals in a given pair was administered one of the four drugs via a nebulizer. Each session lasted 5 min, for a total of four sessions each day, with a 3-min break between sessions with no visual access. A total of five animals (three males and two females) with between three and five distinct conspecifics (total of 20 pairs) participated in all four drug conditions. We collected 320 sessions in total: 16 sessions for each drug condition per subject. The mutual reward task was always performed after the live gaze interaction task, ~75 min after administration of each drug, and lasted for about 30 min.

### Statistical Analysis.

**Fixation analyses over entire sessions.** Fixations to the defined eye regions were first obtained for each monkey, as were the fixations to the face and total fixations occurring in the setup for the SAL, NAL, OT, and OTNAL drug conditions. Fixations to the eye region and overall face were normalized in the NAL, OT, and OTNAL conditions by the SAL baseline within pairs, and pairs were then averaged within each drug condition (Fig. 1). These values were compared with a one-way ANOVA (with condition as a factor) and subsequent multiple comparison tests (Tukey–Kramer post hoc tests). For measures of overall attention to the eyes and face of a conspecific, supralinearity was determined by comparing the effects of OTNAL with the summed effects of OT and NAL alone with a paired-sample *t* test. Correlation between these two measures was also evaluated via linear regression, with significance determined by an *F* test for the model.

**Dynamic gaze analyses.** To measure gaze dynamics following mutual eye contact, we identified mutual eye contact events in which both animals initiated eye contact within a window measuring a  $7.7^\circ \times 3.8^\circ$  visual angle. A 5-s window after each instance of mutual eye contact was examined to identify when and how long the monkey looked back into the eyes of the conspecific. Similarly, a 4-s window was taken after each tone that eventually predicted mutual reward. These 5-s and 4-s windows were chosen empirically based on the observation that looking behaviors return to a rough baseline, with no significant differences between drug groups observed after these periods (Figs. 2 and 3). We used 10-ms bins and created binary datasets to characterize whether the animal was looking at the face of the conspecific within each bin. If a given animal looked back into the face of a conspecific at any time within a given 10-ms bin, that bin was given a value of 1. Otherwise, that bin was given a value of 0. Total amount of return viewing was summed over all instances of mutual eye contact on a given day of testing, normalized by the bin with the highest instance of returning gaze, and then averaged across pairs to compare quantitatively across drug conditions.

To analyze differences between drug groups, we first performed *t* tests to compare values at all bins within the 5-s periods after mutual eye contact. Windows of activity were defined in which gaze behavior between the two groups diverged (500–3,000 ms for mutual eye contact and 1,500–3,500 ms for mutual reward). Significant differences of drug conditions over the SAL baseline within these windows were determined with a permutation test by shuffling the data 1,000 times and randomly assigning the data from each monkey an identity in one of the groups (SAL baseline or a given drug group) being compared in each condition. The data were then averaged and compared, with a discrete value being obtained as the maximum difference between groups in some bin within the defined window. The permuted

values were then sequentially ordered for determining threshold values for significance. To determine whether our findings were specific to periods following interactive events, we aligned to nonmutual eye contact and tones after which reward was not administered to either monkey. Analyses identical to those analyses mentioned above were applied.

To test whether discrete areas of the face were driving these differences in each condition, we redefined the ROIs for determining whether or not animals looked back at the conspecific. For mutual eye contact, we conducted two control analyses. The first control analysis retained mutual eye contact events, but instead measured attention to the mouth of the conspecific following eye contact. The second control analysis determined attention to the mouth of the conspecific following simultaneous gaze to the mouth ROI. The mouth region was defined with a window measuring  $5.3^\circ \times 8.0^\circ$  visual angle. We then repeated the same analyses as mentioned above for these controls. For mutual reward receipt, we examined the eye and mouth individually to determine what smaller ROIs were driving overall attention to the face. After defining these two new ROIs, analyses identical to those analyses mentioned above were again applied.

When comparing the effects of OT and NAL alone with the OTNAL combination condition, supralinearity was determined by first quantifying the effect sizes for OT, NAL, and OTNAL over the SAL baseline for mutual eye contact and mutual reward events. This quantification analysis was accomplished by searching in our 500- to 3,000-ms window of activity for mutual eye contact and in our 1,500- to 3,500-ms window of activity for mutual reward for the time bin in which OTNAL had a maximal effect over SAL. This effect of OTNAL was then compared with the added maximal effects of the separate OT and NAL conditions over SAL with a paired-sample *t* test to determine significance. Correlation between these two measures was also evaluated via linear regression, with significance determined by the *F* test for the model.

**Human neurogenetics analysis.** Six postmortem human brains from the Allen Human Brain Atlas (39) were analyzed and downloaded after updated normalization procedures implemented by the Allen Institute in March 2013. For the mRNA dataset, the data can be downloaded directly from the Allen Brain Atlas ([brain-map.org](http://brain-map.org)). Each individual donor's dataset includes normalized microarray expression data ([help.brain-map.org/display/humanbrain/documentation](http://help.brain-map.org/display/humanbrain/documentation)). Microarray probes without an Entrez ID were not examined. Probe selection was conducted according to the analysis of Miller et al. (52) to analyze probes that most reliably align to RNA-sequencing values, resulting in 20,736 unique gene probes. To prevent biased expression values due to sparse sampling across the six donors, only regions that were sampled in at least four donors were examined. The resulting 190 unique regions and their ontological assignment to each category in Fig. 4 and Figs. S7 and S8 are documented in Table S1. If more than one sample was present in a given region in a given donor, the microarray expression values were averaged.

To identify regions that most express *OXT*, microarray data for each of the 190 regions were averaged across subjects. Regions falling 1 SD above the *z*-transformed mean were identified as “*OXT*-enriched” (Fig. 4 and Figs. S7 and S8). Differences in expression of the  $\mu$ -opioid (*OPRM1*),  $\kappa$ -opioid (*OPRK1*), and  $\sigma$ -opioid (*OPRD1*) receptor subtypes within each *OXT*-enriched region were then examined using a within-subject one-way ANOVA (with opioid receptor subtype as a factor) and subsequent multiple comparison tests (false discovery rate Benjamini–Hochberg-corrected). Finally, we examined whether *OPRM1*, *OPRK1*, and *OPRD1*, as well as *OXR*, displayed above-average expression within *OXT*-enriched regions by plotting their region-wise microarray expression values against the microarray expression values of *OXT*.

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- Bartz J, et al. (2011) Oxytocin can hinder trust and cooperation in borderline personality disorder. *Soc Cogn Affect Neurosci* 6:556–563.
- Walum H, Waldman ID, Young LJ (2016) Statistical and methodological considerations for the interpretation of intranasal oxytocin studies. *Biol Psychiatry* 79:251–257.
- Leng G, Ludwig M (2016) Intranasal oxytocin: Myths and delusions. *Biol Psychiatry* 79:243–250.
- Anagnostou E, et al. (2014) Intranasal oxytocin in the treatment of autism spectrum disorders: A review of literature and early safety and efficacy data in youth. *Brain Res* 1580:188–198.
- Yatawara CJ, Einfeld SL, Hickie IB, Davenport TA, Guastella AJ (2016) The effect of oxytocin nasal spray on social interaction deficits observed in young children with autism: A randomized clinical crossover trial. *Mol Psychiatry* 21:1225–1231.
- Anagnostou E, et al. (2012) Intranasal oxytocin versus placebo in the treatment of adults with autism spectrum disorders: A randomized controlled trial. *Mol Autism* 3:16.
- Preckel K, Kanske P, Singer T, Paulus FM, Krach S (2016) Clinical trial of modulatory effects of oxytocin treatment on higher-order social cognition in autism spectrum disorder: A randomized, placebo-controlled, double-blind and crossover trial. *BMC Psychiatry* 16:329.

8. Guastella AJ, et al. (2015) The effects of a course of intranasal oxytocin on social behaviors in youth diagnosed with autism spectrum disorders: A randomized controlled trial. *J Child Psychol Psychiatry* 56:444–452.
9. Carter CS (2014) Oxytocin pathways and the evolution of human behavior. *Annu Rev Psychol* 65:17–39.
10. Panksepp J (1979) A neurochemical theory of autism. *Trends Neurosci* 2:174–177.
11. Roy A, Roy M, Deb S, Unwin G, Roy A (2015) Are opioid antagonists effective in attenuating the core symptoms of autism spectrum conditions in children: A systematic review. *J Intellect Disabil Res* 59:293–306.
12. Resendez SL, et al. (2013)  $\mu$ -Opioid receptors within subregions of the striatum mediate pair bond formation through parallel yet distinct reward mechanisms. *J Neurosci* 33:9140–9149.
13. Ragen BJ, Freeman SM, Laredo SA, Mendoza SP, Bales KL (2015)  $\mu$  and  $\kappa$  opioid receptor distribution in the monogamous titi monkey (*Callicebus cupreus*): Implications for social behavior and endocrine functioning. *Neuroscience* 290:421–434.
14. Barr CS, et al. (2008) Variation at the mu-opioid receptor gene (OPRM1) influences attachment behavior in infant primates. *Proc Natl Acad Sci USA* 105:5277–5281.
15. Higham JP, et al. (2011) Mu-opioid receptor (OPRM1) variation, oxytocin levels and maternal attachment in free-ranging rhesus macaques *Macaca mulatta*. *Behav Neurosci* 125:131–136.
16. Kalin NH, Shelton SE, Barksdale CM (1988) Opiate modulation of separation-induced distress in non-human primates. *Brain Res* 440:285–292.
17. Fabre-Nys C, Meller RE, Keverne EB (1982) Opiate antagonists stimulate affiliative behaviour in monkeys. *Pharmacol Biochem Behav* 16:653–659.
18. Martel FL, Nevison CM, Simpson MJ, Keverne EB (1995) Effects of opioid receptor blockade on the social behavior of rhesus monkeys living in large family groups. *Dev Psychobiol* 28:71–84.
19. Keverne EB, Martensz ND, Tuite B (1989) Beta-endorphin concentrations in cerebrospinal fluid of monkeys are influenced by grooming relationships. *Psychoneuroendocrinology* 14: 155–161.
20. Vuong C, Van Uum SH, O'Dell LE, Lutfy K, Friedman TC (2010) The effects of opioids and opioid analogs on animal and human endocrine systems. *Endocr Rev* 31:98–132.
21. Vandesande F, Dierickx K (1975) Identification of the vasopressin producing and of the oxytocin producing neurons in the hypothalamic magnocellular neurosecretory system of the rat. *Cell Tissue Res* 164:153–162.
22. Bicknell R, Leng G (1982) Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis. *Nature* 298:161–162.
23. Pumford KM, Leng G, Russell JA (1991) Morphine actions on supraoptic oxytocin neurones in anaesthetized rats: Tolerance after i.c.v. morphine infusion. *J Physiol* 440: 437–454.
24. Bicknell RJ, Ingram CD, Leng G (1983) Oxytocin release is inhibited by opiates from the neural lobe, not those from the intermediate lobe. *Neurosci Lett* 43:227–230.
25. You ZD, Li JH, Song CY, Wang CH, Lu CL (2000) Chronic morphine treatment inhibits oxytocin synthesis in rats. *Neuroreport* 11:3113–3116.
26. Neumann I, Russell JA, Wolff B, Landgraf R (1991) Naloxone increases the release of oxytocin, but not vasopressin, within limbic brain areas of conscious parturient rats: A push-pull perfusion study. *Neuroendocrinology* 54:545–551.
27. Bicknell RJ, et al. (1988) Hypothalamic opioid mechanisms controlling oxytocin neurones during parturition. *Brain Res Bull* 20:743–749.
28. Leng G, et al. (1988) Endogenous opioid actions and effects of environmental disturbance on parturition and oxytocin secretion in rats. *J Reprod Fertil* 84:345–356.
29. Bicknell RJ, Leng G, Lincoln DW, Russell JA (1988) Naloxone excites oxytocin neurones in the supraoptic nucleus of lactating rats after chronic morphine treatment. *J Physiol* 396:297–317.
30. Clarke G, Wood P, Merrick L, Lincoln D (1979) Opiate inhibition of peptide release from the neurohumoral terminals of hypothalamic neurons. *Nature* 282:746–748.
31. Rayner VC, Robinson IC, Russell JA (1988) Chronic intracerebroventricular morphine and lactation in rats: Dependence and tolerance in relation to oxytocin neurones. *J Physiol* 396:319–347.
32. Dal Monte O, Piva M, Morris JA, Chang SW (2016) Live interaction distinctively shapes social gaze dynamics in rhesus macaques. *J Neurophysiol* 116:1626–1643.
33. Chang SW, Platt ML (2014) Oxytocin and social cognition in rhesus macaques: Implications for understanding and treating human psychopathology. *Brain Res* 1580: 57–68.
34. Guastella AJ, MacLeod C (2012) A critical review of the influence of oxytocin nasal spray on social cognition in humans: Evidence and future directions. *Horm Behav* 61: 410–418.
35. Wilms M, et al. (2010) It's in your eyes—using gaze-contingent stimuli to create truly interactive paradigms for social cognitive and affective neuroscience. *Soc Cogn Affect Neurosci* 5:98–107.
36. Schilbach L (2015) Eye to eye, face to face and brain to brain: Novel approaches to study the behavioral dynamics and neural mechanisms of social interactions. *Curr Opin Behav Sci* 3:130–135.
37. Emery NJ (2000) The eyes have it: The neuroethology, function and evolution of social gaze. *Neurosci Biobehav Rev* 24:581–604.
38. Mitani JC, Call J, Kappeler PM, Palombit RA, Silk JB (2012) *The Evolution of Primate Societies* (Univ of Chicago Press, Chicago).
39. Hawrylycz MJ, et al. (2012) An anatomically comprehensive atlas of the adult human brain transcriptome. *Nature* 489:391–399.
40. Bicknell RJ, Leng G (1982) Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis. *Nature* 298:161–162.
41. Lutz-Bucher B, Koch B (1980) Evidence for a direct inhibitory effect of morphine on the secretion of posterior pituitary hormones. *Eur J Pharmacol* 66:375–378.
42. Douglas AJ, et al. (1995) Central endogenous opioid inhibition of supraoptic oxytocin neurons in pregnant rats. *J Neurosci* 15:5049–5057.
43. Hartman RD, Rosella-Dampman LM, Emmert SE, Summy-Long JY (1986) Inhibition of release of neurohypophysial hormones by endogenous opioid peptides in pregnant and parturient rats. *Brain Res* 382:352–359.
44. Douglas AJ, Dye S, Leng G, Russell JA, Bicknell RJ (1993) Endogenous opioid regulation of oxytocin secretion through pregnancy in the rat. *J Neuroendocrinol* 5: 307–314.
45. Codd EE, Shank RP, Schupsky JJ, Raffa RB (1995) Serotonin and norepinephrine uptake inhibiting activity of centrally acting analgesics: Structural determinants and role in antinociception. *J Pharmacol Exp Ther* 274:1263–1270.
46. Bakermans-Kranenburg MJ, van IJzendoorn MH (2013) Sniffing around oxytocin: review and meta-analyses of trials in healthy and clinical groups with implications for pharmacotherapy. *Transl Psychiatry* 3:e258.
47. Bouvard MP, et al. (1995) Low-dose naltrexone effects on plasma chemistries and clinical symptoms in autism: A double-blind, placebo-controlled study. *Psychiatry Res* 58:191–201.
48. Campbell M, et al. (1993) Naltrexone in autistic children: Behavioral symptoms and attentional learning. *J Am Acad Child Adolesc Psychiatry* 32:1283–1291.
49. Leboyer M, et al. (1992) Brief report: A double-blind study of naltrexone in infantile autism. *J Autism Dev Disord* 22:309–319.
50. Williams PG, Allard A, Sears L, Dalrymple N, Bloom AS (2001) Brief report: Case reports on naltrexone use in children with autism: Controlled observations regarding benefits and practical issues of medication management. *J Autism Dev Disord* 31:103–108.
51. National Research Council (2011) *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington, DC), 8th Ed.
52. Miller JA, et al. (2014) Improving reliability and absolute quantification of human brain microarray data by filtering and scaling probes using RNA-Seq. *BMC Genomics* 15:154.
53. Neumann ID, Maloumy B, Beiderbeck DI, Lukas M, Landgraf R (2013) Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. *Psychoneuroendocrinology* 38:1985–1993.
54. Chang SW, Barter JW, Ebitz RB, Watson KK, Platt ML (2012) Inhaled oxytocin amplifies both vicarious reinforcement and self reinforcement in rhesus macaques (*Macaca mulatta*). *Proc Natl Acad Sci USA* 109:959–964.
55. Dal Monte O, Noble PL, Turchi J, Cummins A, Averbach BB (2014) CSF and blood oxytocin concentration changes following intranasal delivery in macaque. *PLoS One* 9:e103677.
56. Brainard DH (1997) The psychophysics toolbox. *Spat Vis* 10:433–436.
57. Wermeling DP (2013) A response to the opioid overdose epidemic: Naloxone nasal spray. *Drug Deliv Transl Res* 3:63–74.
58. Wolfe TR, Bernstone T (2004) Intranasal drug delivery: An alternative to intravenous administration in selected emergency cases. *J Emerg Nurs* 30:141–147.
59. Sabzghabae AM, Eizadi-Mood N, Yaraghi A, Zandifar S (2014) Naloxone therapy in opioid overdose patients: Intranasal or intravenous? A randomized clinical trial. *Arch Med Sci* 10:309–314.
60. Hussain A, Kimura R, Chong-Heng H, Kashiwara T (1984) Nasal absorption of naloxone and buprenorphine in rats. *Int J Pharm* 21:233–237.
61. Middleton LS, Nuzzo PA, Lofwall MR, Moody DE, Walsh SL (2011) The pharmacodynamic and pharmacokinetic profile of intranasal crushed buprenorphine and buprenorphine/naloxone tablets in opioid abusers. *Addiction* 106:1460–1473.
62. Kerr D, Dietze P, Kelly AM (2008) Intranasal naloxone for the treatment of suspected heroin overdose. *Addiction* 103:379–386.
63. Wardle MC, Bershad AK, de Wit H (2016) Naltrexone alters the processing of social and emotional stimuli in healthy adults. *Soc Neurosci* 11:579–591.
64. Dal Monte O, Noble PL, Costa VD, Averbach BB (2014) Oxytocin enhances attention to the eye region in rhesus monkeys. *Front Neurosci* 8:41.
65. Landman R, Sharma J, Sur M, Desimone R (2014) Effect of distracting faces on visual selective attention in the monkey. *Proc Natl Acad Sci USA* 111:18037–42.
66. Gamer M, Zurovski B, Büchel C (2010) Different amygdala subregions mediate valence-related and attentional effects of oxytocin in humans. *Proc Natl Acad Sci USA* 107:9400–9405.
67. Gamer M, Büchel C (2012) Oxytocin specifically enhances valence-dependent parasympathetic responses. *Psychoneuroendocrinology* 37:87–93.
68. MacDonald E, et al. (2011) A review of safety, side-effects and subjective reactions to intranasal oxytocin in human research. *Psychoneuroendocrinology* 36:1114–1126.
69. Campbell M, et al. (1990) Naltrexone in autistic children: A double-blind and placebo-controlled study. *Psychopharmacol Bull* 26:130–135.
70. Campbell M, et al. (1989) Naltrexone in autistic children: An acute open dose range tolerance trial. *J Am Acad Child Adolesc Psychiatry* 28:200–206.
71. Domes G, et al. (2013) Effects of intranasal oxytocin on the neural basis of face processing in autism spectrum disorder. *Biol Psychiatry* 74:164–171.
72. Merlin MA, et al. (2010) Intranasal naloxone delivery is an alternative to intravenous naloxone for opioid overdoses. *Am J Emerg Med* 28:296–303.
73. Walløe AY, et al. (2013) Opioid overdose prevention with intranasal naloxone among people who take methadone. *J Subst Abuse Treat* 44:241–247.
74. Doe-Simkins M, Walløe AY, Epstein A, Moyer P (2009) Saved by the nose: Bystander-administered intranasal naloxone hydrochloride for opioid overdose. *Am J Public Health* 99:788–791.