# Insular Cortex Mediates Approach and Avoidance Responses to Social Affective Stimuli

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**Social animals detect the affective states of others and utilize this information to orchestrate appropriate social interactions. Social affective behaviors include cooperation, reproductive acts and avoiding sick individuals. In a social affective behavioral test in which experimental adult rats were given the choice to interact with either naïve or distressed conspecifics, the experimental rats demonstrated both approach and avoidant social affective behaviors depending upon the age of the conspecific; experimental adult rats approached the distressed juvenile but avoided the stressed adult. Optogenetic inhibition of the insular cortex, a region implicated in social cognition, disrupted these social affective behaviors. Receptors for the social nonapeptide oxytocin (OT) are found in high density within the insular cortex and oxytocin increased intrinsic excitability and synaptic efficacy in acute insular cortex slices. Insular blockade of oxytocin receptors (OTRs) eliminated the social affective behaviors, while direct administration of OT to insular cortex recapitulated the behaviors typically observed in response to distressed conspecifics. These results implicate the insular cortex as a novel target of OT and a critical circuit component underlying the detection and use of social affective cues to coordinate species-specific social behaviors to others in distress.**

Social animals have an enormous repertoire of behavioral expressions that provide for the transmission of one's affective state to other members of the group[1–3]. Sensory and perceptive systems in the "social brain"[4,5] allow one to appraise these social affective cues and integrate them with past experiences and situational factors to shape subsequent social affective behavioral responses. The amygdala, ventral striatum, prefrontal cortex, anterior cingulate cortex, and insular cortex contribute to emotion, reward, motivation and empathic aspects of social cognition, respectively[6]. The evidence supporting the roles of these nodes in social cognition are drawn from neuroimaging, patient-based and translational studies; however translational studies have not yet assessed the role of insula in empathic like behaviors. When tasked to identify the emotion of another from a facial expression, or to observe another suffer a painful stimulation, a reliable neural correlate is relative increase in the blood oxygen level dependent (BOLD) signal in the insular cortex[7,8]. On the other hand, individuals with autism spectrum disorder (ASD) perform poorly on emotion recognition and empathy tasks and the social affective cues fail to evoke the insular BOLD response[9–11]. Accordingly, there are reports of empathic deficits in insula patient populations [12–14] but the findings do not yet paint a clear picture of insula as necessary [15]. Translational study is needed to better resolve the specific contributions of the insular cortex to socioemotional cognition.

The correlation of insular cortex BOLD response and socioemotional cognition is likely a consequence of insular connectivity and physiology. The insular cortex has olfactory, gustatory, and somatosensory receptive fields[16,17] and is an anatomical locus of sensory integration[17,18]. In addition to emotion recognition, insula is implicated in risky decision-making[19,20], interoception[21,22], anxiety[23,24], salience detection[25,26], danger[27] and safety learning [28–30].

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***A note about the preprint.*** This manuscript was submitted to bioRxiv.org after receiving peer reviews from a well-regarded journal. The manuscript was submitted for peer-review January 18, 2017 and peer reviews were received February 17th. Reviewers raised two very interesting questions: What are the social stimuli that provide for transmission of affect from one rat to another, and what is the role of the insular cortex with regard to these stimuli? Follow-up experiments are ongoing to address these concerns and others raised in the peer review forum. The reviewers found that the methodology, statistical analysis in the original manuscript were generally sound. Therefore, this manuscript is submitted with only aesthetic modifications to facilitate readability.

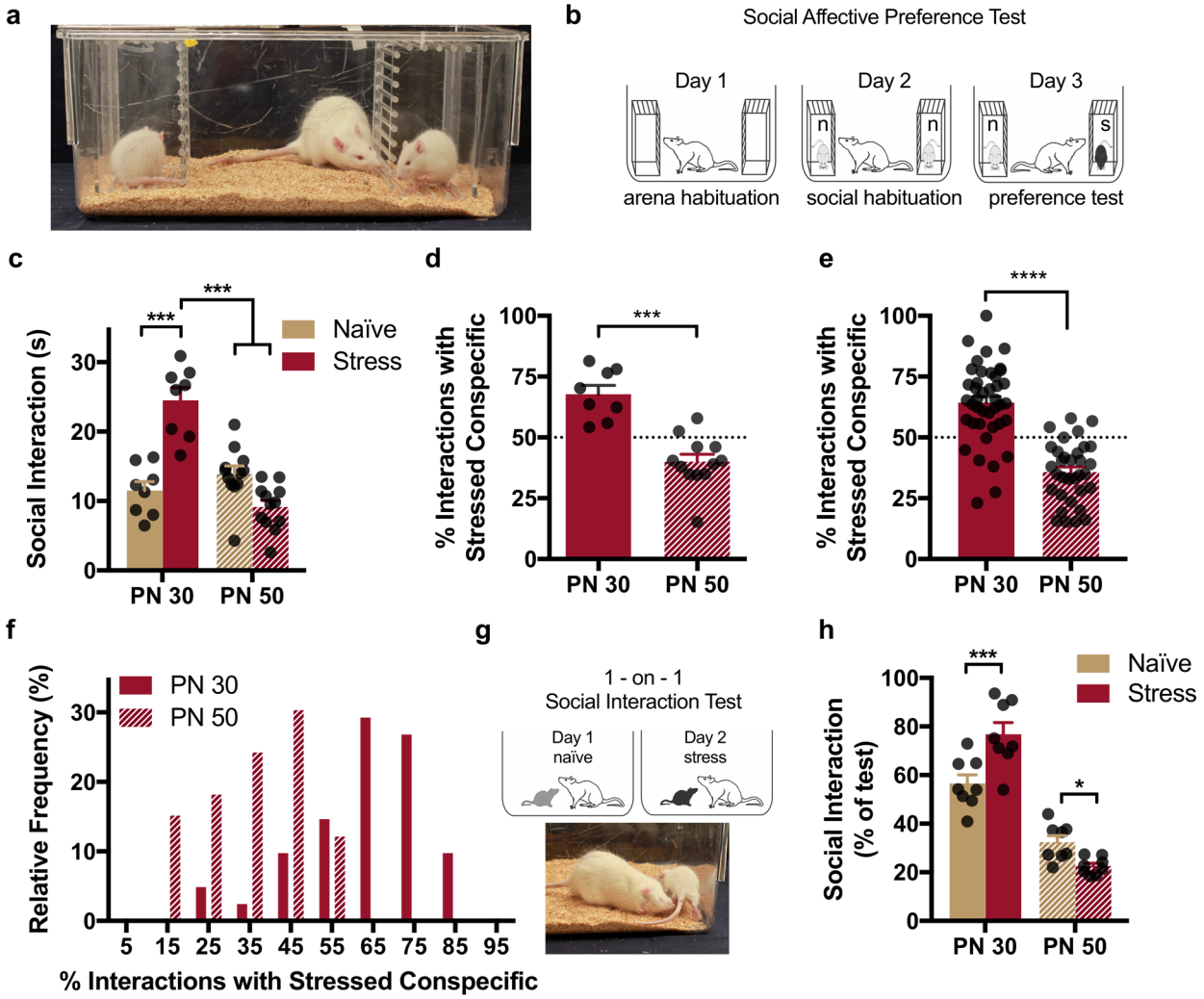


**Figure 1.** Social affective preference (SAP). (A) The SAP arena containing juvenile conspecifics on the left and right with an experimental adult in the center. (B) Diagram of SAP test procedure. Experimental adults were allowed to explore the test arena and conspecific chambers when empty (Day 1), with two experimentally naïve conspecifics (Day 2) and when given a choice to interact with either a naïve or previously shocked conspecific (Day 3). (C) Mean (+ S.E.M. with individual replicates) time spent in social interaction with the naïve or stressed conspecific by age. Although conspecific age did not alter time spent interacting with the naïve conspecific, a bidirectional effect of age was apparent in time spent interacting with the stressed conspecifics. Experimental rats spent more time exploring the stressed PN 30 conspecific compared to the PN 30 naïve, but spent less time exploring the stressed PN 50 conspecific compared to the PN 50 naïve. For analyses of conspecific behavior please refer to Supplementary Figure 1. (D) Mean (+ S.E.M. with individual replicates) data in C expressed as the percent of time spent interacting with the stressed conspecifics relative to the total time spent interacting. Here, experimental adults showed a marked preference (values greater than 50%) for interaction with stressed conspecifics and avoidance (values less than 50%) of stressed adults. (E) Mean (+ S.E.M. with individual replicates) percent preference for interacting with the stressed conspecific pooled from all of the subjects in the experimental control groups included in the current report including vehicle, sham, light OFF control groups of the later experiments. (F) Distribution of percent preference for stress conspecific interaction from all control subjects as in E. Each distribution fits a normal curve and across experiments it is clear that the experimental adult rat reliably prefers to interact with stressed juvenile conspecifics while avoiding stressed adult conspecifics. See text for additional analyses. (G) Diagram of 1-on-1 social interaction test and photo of typical adult-initiated interactions. Experimental adult rats were presented on Day 1 with a naïve conspecific and experimental adult initiated interactions were recorded for 5 min. On the next day, the conspecific received 2 footshocks immediately prior to the test; order of testing was counterbalanced. (H) Mean (+ S.E.M. and individual replicates) time interacting with the naïve or stressed conspecific in a 1-on-1 test shown as percent of test time. Experimental adults spent significantly more time interacting with the stressed PN 30 conspecific but significantly less time with the stressed PN 50 conspecific compared to the respective naïve conspecific targets. *$p < 0.05$, ** $p < 0.0$, *** $p < 0.001$, **** $p < 0.0001$

In the context of social behaviors, it is intriguing that the oxytocin receptor (OTR), a G-protein coupled receptor which activates the $G_{q11}$ signaling cascad [31], is particularly enriched in the insular cortex[32,33] and OT-containing axons terminate in the insula[34]. OT is a critical mediator of reproduction, pair-bonding, and other social behaviors across species[35–37] and action of OT on insular cortex neurons would provide a mechanism for OT to augment insular cortex activity when processing socioemotional stimuli. Indeed, experimenter-administered OT augments behavioral performance and insular cortex response during emotion recognition [38] and relieves some social deficits observed in individuals with ASD while increasing insular activity and connectivity to frontal cortex [39,40]. Furthermore, mutations in the OTR gene predict ASD symptoms [41], insular activation [42] and social cognition [43,44]. In sum, the insular cortex appears critical for social affective behaviors and may be a substrate for pathophysiology in social affective mental illness. However, no mechanistic data exist to link insular cortex activity with the OT system in social cognition.

Translational rodent models seeking to capture aspects of emotion recognition and empathy include emotion contagion [45–47], social buffering [48] and even helping [49,50] (for review of models see [51]). However, interpreting the underlying cognitive mechanisms as empathy is challenging because some paradigms involve repeated conditioning or direct exposure to an aversive stimulus [52–54] making it difficult to rule out self-serving or socially rewarding motives [48,55]. We developed a rat social affective preference (SAP) test in which social affective behaviors were objectively quantified as a preference to approach or avoid interaction with a conspecific that received a mild stressor. The test allowed a direct observation of what de Waal argued to be the most elementary component of empathy: "that one party is affected by another's emotional or arousal state [56] p. 282." Because the experimental rat in the SAP test was not exposed to, nor witness to, the stressor itself, the unconditioned behavior of the experimental subject toward the conspecifics can be interpreted as a response to the affective state of the target which is advantageous in rodent models of empathy-like behavior [48]. Using the SAP test, we report a set of in vivo and in vitro studies that tested whether insular cortex activity and modulation by OT were necessary and sufficient to modulate social affective behaviors in rat.

## RESULTS

### Age and distress of conspecific determine social approach

In the social affective preference (SAP) test an experimentally naïve, adult male rat was presented with a pair of unfamiliar conspecific male stimuli (see Fig. 1 and Supplementary Video 1). To manipulate social affect, one of the conspecifics was exposed to a mild stressor immediately before the test, while the other was untreated. In pilot studies which first used juvenile conspecifics, we were surprised to observe that the experimental adult subjects preferred interaction with stressed juveniles which contrasted with existing literature regarding approach to social danger cues (e.g., [57] and see Discussion). Because social approach behaviors are shaped, in part, by features of the target including age [58], we hypothesized that rats may differentially respond to conspecifics in distress as a function of the target's age. In SAP tests conducted with adult conspecifics, the experimental rat avoided interaction with stressed adults. This bidirectional pattern suggests that the experimental rat integrates information about both age and affect to determine the appropriate social behavior and so all of the experiments included conspecific age as an experimental variable.

To determine if the age or stress state of a conspecific would alter social interaction, 20 experimental adult rats (PN 60-80 days) underwent SAP tests (See Figs. 1A & 1B) in which the choice test involved exposure to a pair of unfamiliar pre-pubertal juveniles (PN 30, n = 8) or a pair of unfamiliar post-pubertal adults (PN 50, n =12) [59]. In each case, one of the unfamiliar conspecifics received a mild stressor in the form of 2 footshocks immediately before placement in the test arena. The design was a 2 by 2 with conspecific Age (PN 30 vs. PN 50) as a between-subjects factor and conspecific Affect (naïve or stress) as a within-subjects factor. Time spent interacting during the 5 min SAP test is presented in Figure 1C. Regarding the influence of age and affect we observed significant main effects of Age, $F(1, 18) = 27.93$, $p < 0.001$ and Affect $F(1, 18) = 9.965$, $p = 0.006$, and an Age by Affect interaction, $F(1, 18) = 46.05$, $p < 0.001$. Post-hoc comparisons between naïve and stress social interaction time revealed a significant increase in social interaction with the stressed juvenile ($p < 0.001$) but a significant decrease in interaction with the stressed adult ($p < 0.05$). To summarize this interaction, we computed a percent preference score by dividing the time spent interacting with the stressed conspecific by the total time spent interacting during the test (Fig. 1D). An independent samples t-test revealed a significant difference in preference, $t(18) = 5.783$, $p < 0.001$. Thus, when faced with a choice to interact with either a naïve or distressed conspecific, we observed a prosocial preference to interact with a stressed juvenile but an asocial avoidance of a stressed adult. Analysis of conspecific behavior revealed that stress caused juvenile

conspecifics to engage in more self-grooming compared to the naïve juvenile conspecific, but no other behaviors differed significantly between stress and naïve in either the juvenile or adult groups (See Supplementary Fig. 1 for additional analyses of conspecific behavior).

The SAP procedure was replicated several times in the latter optogenetic and pharmacology experiments which provided a large sample to evaluate the reliability and generality of these phenomena. Data from SAP tests conducted under either vehicle, Light-Off or sham treatments (n = 41 for PN 30, n = 33 for PN 50) were

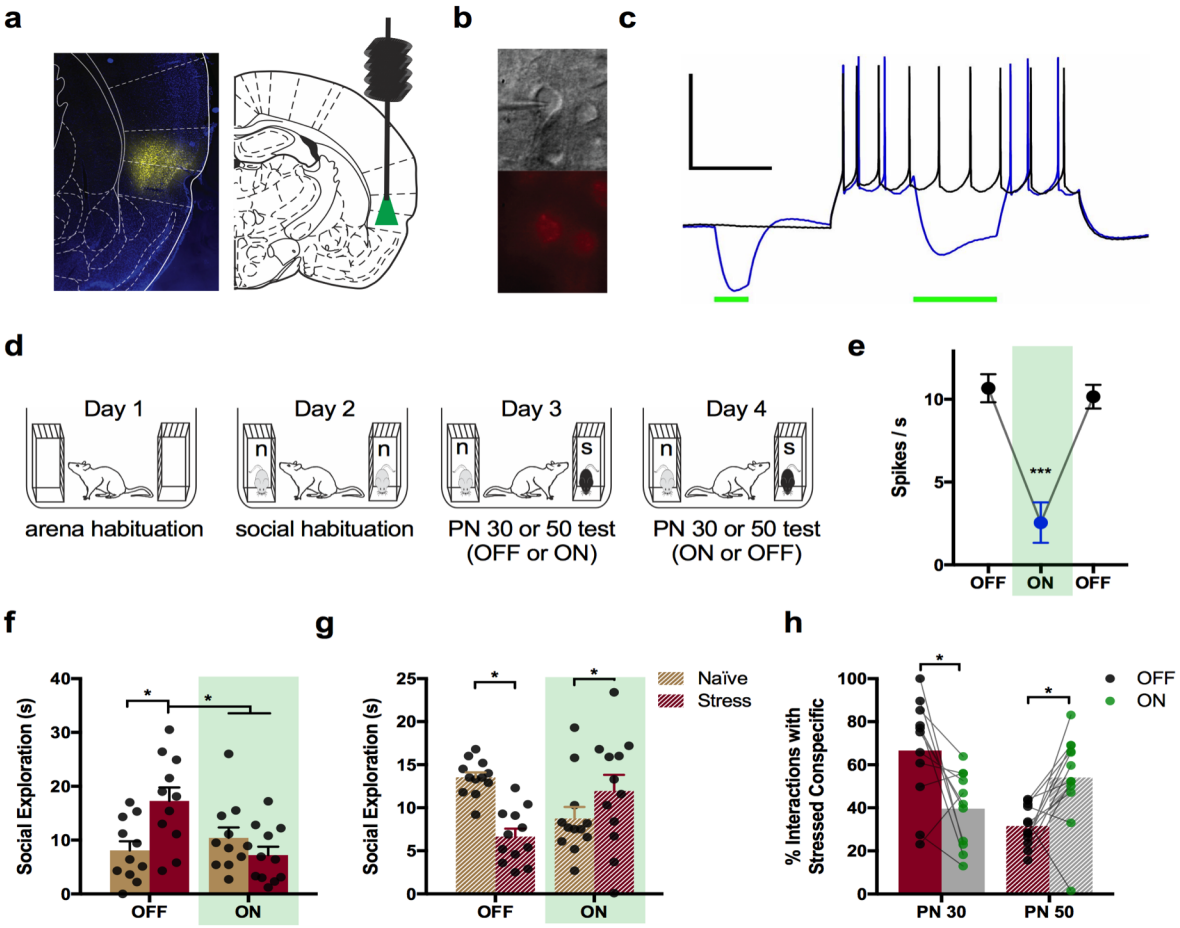


**Figure 2. Optogenetic silencing of insular cortex during SAP tests.** (A) Rats were transfected with a viral construct containing eNpHr3.0 fused to mCherry in the insular cortex and implanted with bilateral fiber optic cannula guides terminating at the dorsal border of the insular cortex. Native (unamplified) mCherry expression in the insular cortex (false colored yellow) from a brain slice adjacent to one containing the cannula tract imaged at 10x. (B) Viral transfer was validated in acute whole cell recordings of mCherry positive neurons in the insular cortex. (C) Application of green light (wavelength = 532nm, 10mW/mm2) through the objective of the electrophysiology microscope induced robust hyperpolarizations which silenced spiking when provided during a train of evoked spikes, scale bar 50mV/250ms. (D) Diagram of SAP tests for optogenetic experiments. On Day 1 rats were habituated to the arena. On Day 2 rats were handled to habituate to connecting fibers and exposed to the arena with naïve conspecific stimuli. On Days 3 and 4, optical fibers were inserted bilaterally and SAP tests were performed under no light (OFF) or continuous green light (ON) conditions; order was counterbalanced. (E) Mean (+/- S.E.M.) spike frequency during sequence of OFF/ON/OFF application as in panel (D), n = 6 cells. (F) Mean (+ S.E.M. and individual replicates) time spent interacting with PN 30 juvenile conspecifics on Days 3 and 4 of the SAP test. In the light OFF condition, the experimental adult spent significantly more time interacting with the stressed conspecific but this pattern was abolished in the light ON condition. (G) Mean (+ S.E.M. and individual replicates) time spent interacting with PN 50 adult conspecifics on Days 3 and 4 of the SAP test. In the light OFF condition, the experimental adult spent significantly less time interacting with the stressed conspecific but this pattern was reversed in the light ON condition. (H) Data from F and G converted to percent preference for interaction with stressed conspecifics. Here a clear age by light interaction is apparent with optogenetic silencing of insular cortex eliminating preference for interaction with the stressed juvenile, and blocking the pattern of avoidance of stressed adult conspecifics. * $p < 0.05$, *** $p < 0.001$.

pooled and converted to percent preference scores (Fig. 1E) and the scores were found to fit a normal distribution, D'Agostino and Pearson normality test, for PN 30: K2 = 2.603, p = 0.27, for PN 50: K2 = 1.57, p = 0.46 (Fig. 1F). One sample t-tests were used to compare PN 30 and PN 50 preference scores to the hypothetical value of 50% (equal time exploring both naïve and stressed conspecifics). As in the initial experiment (Fig. 1D), preference for the stressed PN 30 was apparent, one-sample t(40) = 5.10, p < 0.0001 while preference for the naïve PN 50 was observed, one-sample t(32) = 6.63, p < 0.0001. Accordingly, an independent samples t-test revealed a significant difference between PN 30 and PN 50 percent preference scores, t(72) = 8.407, p < 0.0001. Approximately 83% (34 of 41) of rats tested with PN 30 conspecifics exhibited a preference for the stressed target, while only 15% (5 of 33) of rats tested with PN 50 conspecifics exhibited preference for the stressed target.

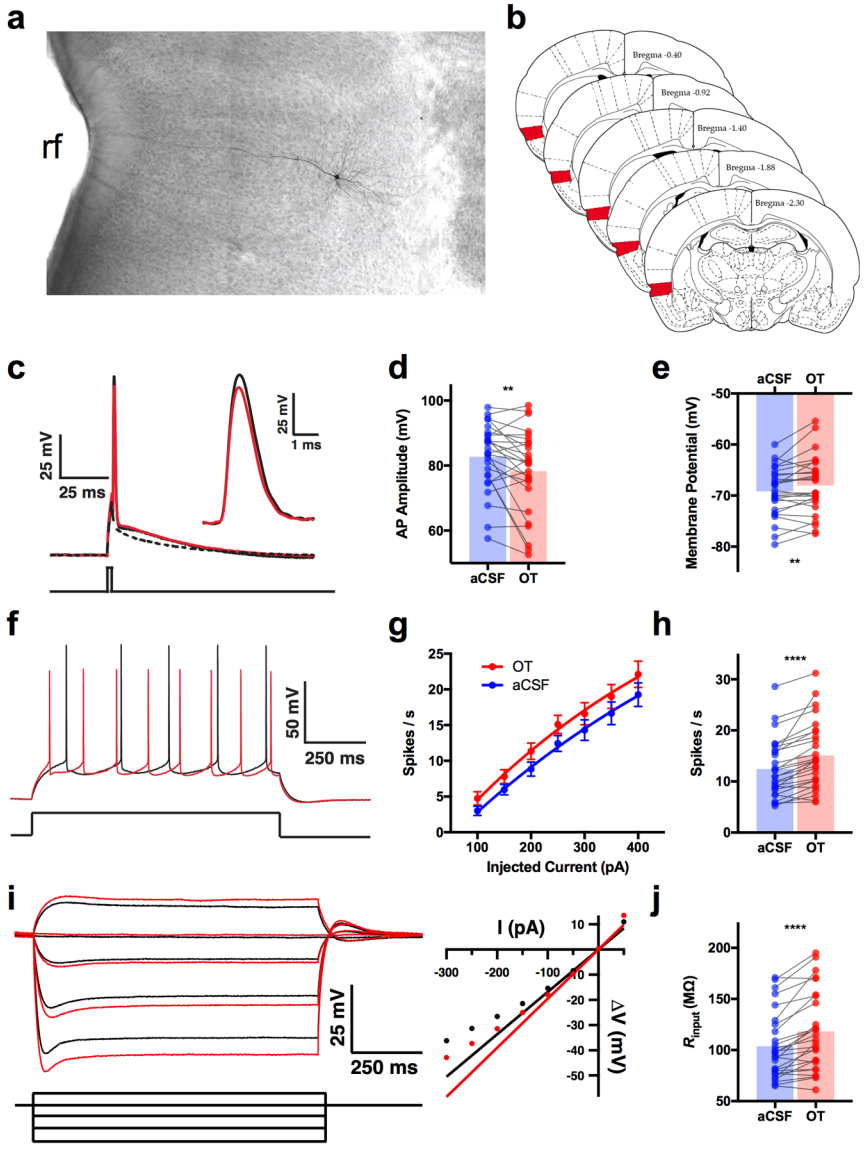


**Figure 3. Intrinsic membrane properties in insular cortex pyramidal neurons are modulated by oxytocin.** (A) Digital photomicrograph of a typical biocytin filled neuron, rf = rhinal fissure. (B) Schematic diagram illustrating (red shading) the region of interest for whole cell recordings. All neurons included in these experiments were found in this region. (C) Typical action potential before (black trace) and after bath application of 500nM OT (red). Inset detail of the AP peak amplitude difference. (D) Mean action potential amplitude; OT significantly reduced amplitude. (E) Mean resting membrane potential. OT significantly depolarized the membrane at rest. (F) Typical train of spikes evoked by 1 s 150pA current injection before (black) and after (red) OT. (G) Mean (+/- S.E.M.) spike frequency evoked by increasing current injections; OT increased the spike frequency (main effect of OT, p = 0.001). (H) Mean action potentials evoked by 1s, 250pA current depolarization; significantly more spikes were evoked after OT. (I) Membrane potentials evoked by subthreshold and hyperpolarizing current injections (left) and typical rectification curve (right) before (black traces) and after (red traces) OT. (J) Mean input resistance ($R_{input}$); OT significantly increased $R_{input}$. Symbols and connecting lines indicate individual replicates. ** p < 0.01, **** p < 0.0001.

To further evaluate the generality of the SAP procedure we conducted a systematic replication of the behavioral phenomenon. The SAP results predict that if an experimental adult was exposed to a distressed juvenile in a one-on-one interaction, that it would engage in more social interaction than if the target had been naïve, or the opposite pattern if the targets were adults. Thus, experimental adult rats were given a series of 2 "one-on-one" social exploration tests (5 min duration, one test per day) with either 1 unfamiliar naïve PN 30 juvenile, or 1 unfamiliar stressed juvenile as the social interaction target (Fig. 1G); a separate set of experimental adults received the same series of tests with PN 50 adult conspecifics. A within-

subjects design was used with test order counter balanced and stress treatment was as above. A two way ANOVA revealed a significant main effect of Age, $F(1, 14)$ 103.10, $p < 0.001$ and significant Age by Affect interaction, $F(1, 14) = 31.34$, $p < 0.0001$. As in the SAP test, experimental adults spent more time interacting with the stressed juvenile ($p < 0.001$) and less time with the stressed adult ($p = 0.043$; Fig. 1H). Because this effect could be influenced by differential conspecific-initiated social interaction, i.e. reciprocal interactions, we also quantified conspecific exploration of the experimental adult; however, there was no difference between naïve-to-adult or stressed-to-adult interactions at either age.

**Optogenetic silencing of insular cortex prevented social affective preference.**

To establish the role of the insular cortex in social affective behaviors we conducted a pilot study in which insular neuronal activity was temporarily inhibited by microinfusion of the $GABA_A$ receptor agonist muscimol prior to SAP tests. Muscimol reversed the pattern of behavior observed in experimentally naïve rats (Supplementary Fig. 2) suggesting that the output of the insular cortex is necessary for normal social affective behavior. To achieve inhibition of insular cortex pyramidal neurons, rats were transfected with halorhodopsin (eNpHr3.0) under the CamKII promoter (AAV5- CamKII- eNpHR3.0 -mCherry) which allowed reversible neuronal silencing [60]. Optogenetic transfections were evaluated by fluorescence microscopy (for representative maps of transfections please see Supplementary Fig. 4) and in vitro whole cell recordings (Fig. 2A-C). Two weeks after transfections, rats underwent SAP tests in a 2 Age (juvenile or adult conspecific) by 2 Affect (naïve or stress) by 2 Light (OFF or light ON) design (Fig. 2D). Green light (ON) or no light (OFF) was delivered to the insula continuously during the SAP test. Rats in the OFF condition exhibited preference to interact with the stressed juvenile and avoid the stressed adult whereas rats in the light ON condition avoided the juvenile (Fig. 2F) and preferred interaction with the adult (Fig. 2G). These observations are supported by a three-way Age by Affect by Light interaction $F(1, 21) = 28.06$, $p < 0.001$; significant two-way interactions were also found for Age and Affect, $p = 0.038$. Pairwise comparisons in the juvenile OFF condition identified an increase in interaction of the stressed juvenile compared to naïve juvenile, $p = 0.002$. Whereas in the adult OFF condition, the opposite was observed; there was a significant decrease in interaction with the stressed adult conspecific compared to the naïve adult conspecific, $p = 0.012$. No significant differences were found in exploration times between naïve and stressed conspecifics in the light ON condition for either juvenile or adult conspecifics, $p = 0.07$ and $p = 0.063$ (approaching significance for the opposite direction of the light OFF condition), respectively. This indicates that optogenetic silencing of the insular cortex prevented the expression of social affective behaviors. Importantly, optical treatment had no effect on rats with sham transfections (Supplementary Fig. 3).

**Oxytocin alters the excitability of the insular cortex.**

The foregoing suggested that exposure to distressed conspecifics triggers a neurobiological response that augments insular cortex function. Given the dense expression of OTR in the insula we hypothesized that OT released during the SAP test could directly modulate insular cortex excitability. To test this hypothesis, we characterized the effect of bath application of OT on the intrinsic excitability of insular cortex pyramidal neurons (Fig. 3) and excitatory synaptic transmission in acute slices (Fig. 4). Active and passive intrinsic properties were quantified, as previously [61], in insular cortex pyramidal neurons before and after bath application of OT in acute slices under 3 doses of OT. A complete list of parameters measured is provided in Table 1 which includes the mean and standard error of the mean (SEM) values obtained in normal aCSF and 10 minutes after bath application of OT (500nM). Significant differences were determined with paired samples t-tests ($df = 26$) and all t-values, and p-values are provided in Table 1.

Figures 3A & 3B depict a typical insular cortex pyramidal neuron and the rostrocaudal extent of insular cortex targeted in this study. All of the analyzed neurons ($N = 27$) were localized in the deep layers of the insular cortex, medial to the rhinal fissure. This is the area identified with dense OTR binding [32,33]. A number of intrinsic properties were altered by application of OT which together suggest an increase in excitability. We observed a depolarization of the resting membrane potential, an increase in input resistance, and a decrease in membrane time constant. These changes would permit the membrane to charge faster and reach the action potential threshold with less excitatory input. Indeed, OT also reduced the minimum current injection required to elicit an action potential (i.e. the rheobase). We also observed a significant reduction in the slow after hyperpolarization (sAHP), quantified as the area (mV x ms) of the hyperpolarization (relative to prestep baseline) immediately following trains containing equal number of action potentials. Together these changes suggest that after OT, insular cortex pyramidal cells might achieve greater spike frequency. Consistent with this prediction, in response to sustained depolarizing currents, we observed an increase in the spike frequency (Fig. 3F-H) which was confirmed as a main effect of drug in a

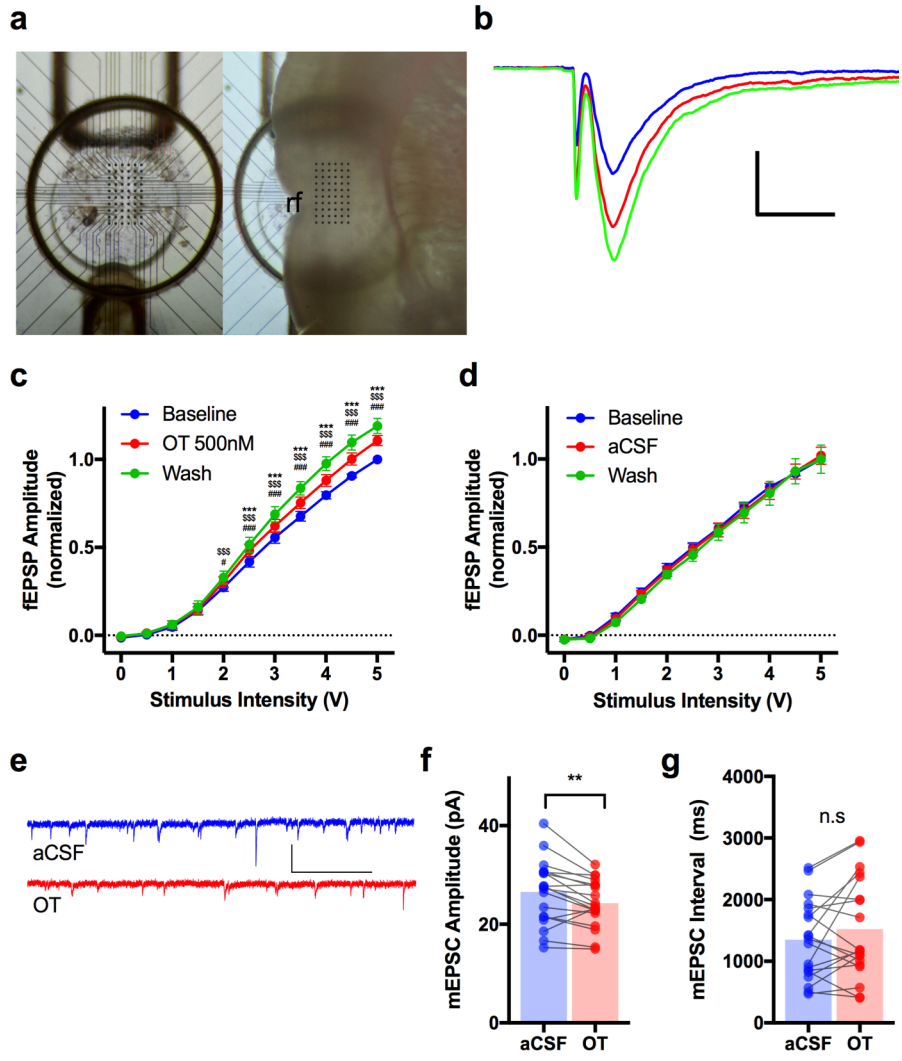


**Figure 4. Oxytocin modulates excitatory synaptic transmission in the insular cortex.** (A) Top view of 60 channel perforated MEA (left) for acute extracellular recordings of the insular cortex. Right depicts location of insular cortex slice during recording, rf = rhinal fissure. (B) Typical fEPSPs evoked by biphasic extracellular stimulation at baseline (blue) during application of 500nM OT (red) and after washout (green). Scale bar 500V/ms. (C) Input/output curve for fEPSPs (Mean +/- S.E.M.) normalized to the peak amplitude observed in response to 5V stimulation under baseline conditions. OT significantly increased EPSP amplitude beginning at 2V with further enhancement during the washout. # $p < 0.05$ OT vs. Baseline, ### $p < 0.001$ OT vs. Baseline, $$$ $p < 0.001$ Wash vs. Baseline, *** $p < 0.001$ Wash vs. OT. (D) Curves as in (C). Without application of OT EPSPs remain stable across the duration of the experiment. (E) Representative voltage clamp recordings of mEPSCs recorded before (aCSF) and after OT. (F) Mean mEPSC amplitude before and after OT; OT significantly reduced amplitude, ** $p < 0.01$. (G) Mean mEPSC interval; no effect of OT was apparent.

repeated measures 2 way ANOVA, $F(1, 279) = 10.42$, $p = 0.001$. There was also the expected effect of current, $F(6, 279) = 40.01$, $p < 0.001$. Interestingly, after OT, action potentials were significantly smaller (Fig. 3C-D) with decreased amplitude, rise rate, decay rate and longer duration (half-width), which may indicate that the increase in firing frequency could come at a cost of reduced neurotransmitter release.

To put the effect of OT on intrinsic physiology into the context of an intact insular cortex circuit, we next investigated the effect of OT application on evoked synaptic efficacy. Input/output curves were computed in acute insular cortex slices on a perforated 60 channel multiple electrode array (Fig. 4A). A clear leftward shift was apparent when OT was applied with significantly larger fEPSP amplitude compared to baseline at stimuli from 2 to 5V. This effect grew larger during the washout period (Fig. 4C), consistent with a Gq mechanism of modulation. These observations are supported by significant main effect of Stimulus $F(10, 90) = 598.20$, $p < 0.0001$, of Drug, $F(2, 18) = 11.99$, $p < 0.001$, and Drug by Stimulus interaction, $F(20, 180) = 11.34$, $p < 0.0001$. Post hoc comparisons were made at each stimulus intensity and revealed significant increases in fEPSP amplitude during OT and washout compared to baseline beginning at 2.5V (ps < 0.01), with washout levels differing from OT beginning at 3V (ps < 0.001, see Fig. 4 for additional comparisons). In the aCSF control slices (Fig. 4D), synaptic responses were stable across all phases of the experiment and only a main effect of Stimulus intensity was observed, $F(10, 80) = 385.90$, $p < 0.0001$. Thus, application of OT augmented evoked excitatory synaptic transmission.

An increase in intrinsic excitability might also be reflected in a change in the miniature and/or spontaneous release of excitatory neurotransmitter in the slice. We made continuous whole cell, voltage clamp recordings of deep layer insular cortex pyramidal neurons with either tetrodotoxin (n = 7) or without (n = 12) before and after application of OT (500nM) to quantify miniature and spontaneous excitatory postsynaptic currents (EPSCs), respectively. However, in our recording conditions very few large events, indicative of spontaneous glutamate release, were observed so all events were assumed to be miniature EPSCs (mEPSCs) and the data were pooled (N = 19). OT reduced mEPSC amplitude, paired $t(18) = 3.29$, $p = 0.004$ but had no effect on mEPSC frequency, paired $t(18) = 1.42$, $p = 0.17$. This finding likely reflects a small modulation of presynaptic vesicle release machinery by OT, but not an effect of OT on the spontaneous circuit activity per se. Taken together, the electrophysiology suggests that OT rendered insular cortex pyramidal neurons more responsive to excitatory inputs.

**Insular cortex oxytocin receptors are necessary for both prosocial and asocial responses to distressed conspecifics.**

We next determined whether insular cortex OTRs contribute to the prosocial or asocial behaviors in the SAP test. To this end, 20 rats were implanted with cannula guides to the insular cortex. After recovery, rats were exposed to the SAP procedure with preference tests on days 3 and 4 in a repeated measures design (Fig. 5A). Here, the experimental design was a 2 by 2 by 2 with conspecific Age (PN 30 vs. PN 50) as a between-subjects factor and Drug (Vehicle or OTR antagonist (OTRa) 20ng/side as in [62]) and conspecific Affect as within-subjects factors. Microinjections were made 15 min before SAP tests on days 3 and 4, with drug order counter balanced such that one half of the experimental subjects received vehicle on day 3 and OTRa on day 4 and vice versa. The conspecific stimuli were always unfamiliar; no effect of test order was apparent. Two subjects in the PN 50 condition with misplaced cannula were excluded; the resulting groups had PN 30, n = 10 and PN 50, n = 9 (for cannula placement see Supplementary Fig. 6).

Time spent interacting with the naïve and stressed conspecifics is shown in Figure 5B and time spent interacting with the stressed conspecifics is shown as a percent of total interactions in Figure 5C. In the vehicle condition we replicated the pattern of preference for stressed juveniles and asocial avoidance of stressed adults, without any effect of OTRa on time spent in interaction per se. Importantly, intra-insular cortex OTRa administration reversed these patterns. There was a significant Age by Drug by Affect interaction, $F(1, 16) = 20.44$, $p < 0.001$. Planned pairwise comparisons on the vehicle treated rats replicated the significant increase in time spent investigating the stressed PN 30 conspecific, ($p = 0.03$) and decrease in time spent investigating the stressed PN 50 conspecific ($p = 0.007$). Application of OTRa completely reversed behavior, with significantly more time interacting with the naïve PN 30 conspecific, $p = 0.032$, and less time with the naïve PN 50 conspecific, $p = 0.003$. A two way ANOVA on the percent preference scores revealed a significant Age by Drug interaction, $F(1, 17) = 20.16$, $p < 0.001$ and post hoc comparisons revealed significant, but opposing effects of OTRa in both PN 30 ($p = 0.028$) and PN 50 ($p = 0.002$) groups, respectively. Thus, blockade of insular cortex OTRs prevented the typical approach and avoidance behavior observed in response to stressed conspecifics in the SAP test.

We next sought to replicate the effect of OTRa using the one-on-one version of the social affective behavior test (Fig. 5D). As above, bilateral microinjection cannula were placed in the insular cortex in 16 rats. Rats were randomly assigned to either PN 30 juvenile or PN 50 adult conspecific treatments (between-subjects) and received vehicle and OTRa injections (within-subjects) in a 2 by 2 design; drug order was counter balanced by day as above. All rats received 2 social exploration tests with an unfamiliar, stressed conspecific preceded 15 min by either vehicle or OTRa (10ng/side) microinjections. One rat in the adult group had misplaced cannula and the resulting groups had PN 30, n = 8 and PN 50, n = 7. Time spent interacting with the stressed PN 30 conspecifics was reduced by OTRa, but time interacting with stressed PN 50 conspecifics was increased in the one-on-one tests (Fig. 5E). ANOVA revealed a significant main effect of Age, $F(1, 13) = 28.66$, $p < 0.001$, and a significant Age by Drug interaction, $F(1, 13) = 32.56$, $p < 0.001$. The main effect of age indicates more interaction time with the stressed juveniles than the stressed adults which replicates the pattern to approach stressed juveniles but avoid stressed adults that was apparent in the SAP test (as in Fig. 1H). To explore the significant interaction, comparisons were made between vehicle and OTRa conditions within the PN 30 and PN 50 treatments. OTRa reduced time spent interacting with the stressed juvenile ($p < 0.001$) but increased interaction of the stressed adult conspecifics ($p = 0.009$).

To control for possible effects of insula OTRa administration on social behavior per se, a separate set of 16 rats received insula cannula implants and either OTRa or vehicle injections prior to one-on-one interactions between experimentally naive adults and juveniles. In this control experiment, no effect of OTRa

was apparent: Mean(S.E.M) time interacting in seconds after Vehicle = 60.27(5.42) and after OTRa = 67.23(7.28), paired t(15) = 1.186, p = 0.25. This is consistent with our prior work in which we reported no effect of excitotoxic lesion [28] or pharmacological inhibition [29] on adult to naïve juvenile social interactions.

**Insular cortex oxytocin administration is sufficient to recapitulate social affective behaviors toward naïve conspecifics.**

Together, the OTRa experiments demonstrate that modulation of the insular cortex by OTRs is necessary for these species-specific approach and avoidance responses to distressed conspecifics. We next determined whether intra-insular OT administration was sufficient to increase adult interaction with juveniles in the absence of an affective manipulation. Insular cannula implants were made in 32 rats and after recovery each received 2 one-on-one social interaction tests (3 min duration) with either unfamiliar naïve PN 30 or PN 50 conspecifics. A within-subjects design was used such that each rat received vehicle injections prior to one test and OT (250 pg/side; equivalent to 500nM) before the other. Injections were made 15 min before testing and drug/vehicle order was counterbalanced. One rat from each age group did not receive injections and was removed from analysis resulting in ns = 15. Time spent interacting with the naïve PN 30 conspecifics increased after OT, but time with naïve PN 50 decreased after OT treatment (Fig. 5F). A two way ANOVA revealed a significant Age by OT interaction, F(1, 28) = 30.08, p < 0.0001 and post hoc comparisons identify significant but opposing effects of OT on social interaction time for both PN 30 (p < 0.001) and PN 50 (p = 0.002) groups. Thus, intra insular administration of OT was sufficient to promote interaction with a naïve juvenile but the same treatment reduced interaction with a naïve

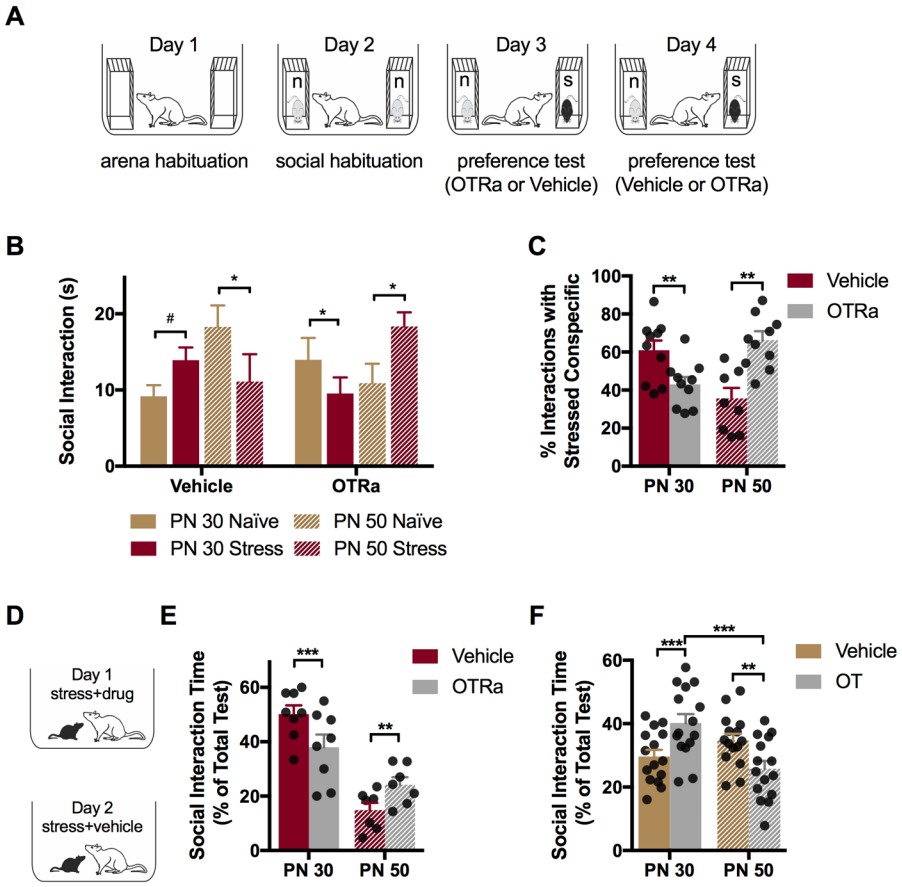


**Figure 5. Social affective behaviors require insular cortex oxytocin.** (A) Diagram of experimental design. (B) Mean (+ SEM) time spent exploring either a naïve conspecific or a stressed conspecific in the SAP test with either PN 30 or PN 50 conspecifics after intra-insula infusion of a selective OTR antagonist (OTRa, 20ng/side). Vehicle treated experimental adult rats spent more time interacting with the stressed PN 30 juvenile conspecifics and less time with the stressed PN 50 adult conspecifics. These trends were blocked and reversed by infusion of OTRa. (C) Data in (B) expressed as percent preference for interaction with the stressed conspecific (Mean + S.E.M. with individual replicates). OTRa significantly reduced preference for the stressed PN 30 while increasing time spent with the stressed PN 50 conspecific. (D) Diagram of 1-on-1 social interaction tests with stressed conspecifics and pretreatment with either vehicle or OTRa, order counterbalanced. (E) Mean (+ S.E.M. with individual replicates, normalized as percent of test) time spent interacting with the stressed conspecific in a 1-on-1 test. OTRa significantly reduced time interacting with the stressed PN 30 conspecific, but increased time interacting with the stressed PN 50 conspecific. (F) Mean (+ S.E.M. with individual replicates) time spent interacting with a naïve conspecific after intra insular cortex OT (250pg/side) or vehicle administration in a 1-on-1 social interaction. OT caused a significant increase in social interaction with naïve PN 30 juveniles but a significant decrease in interaction with naïve PN 50 adults. *p < 0.05, **p < 0.01, ***p < 0.001, # p < 0.05(one-tailed).

adult, suggesting that the effect of insular cortex OT is dependent upon conspecific age. To summarize the behavioral pharmacology studies, experimental adult rats preferred to interact with distressed juveniles but avoided interaction with distressed adult conspecifics. The insular cortex OTR was necessary for these behaviors and OT administration was sufficient to reproduce the phenomena with naïve conspecifics.

## DISCUSSION

Using a combination of social behavior assays, optogenetics, pharmacology and in vitro slice electrophysiology we uncovered a critical role for OT as a modulator of insular cortex excitability and necessary component of a social affect neural system. The novel SAP test allowed for the simultaneous study of approach and avoidance behaviors that depended upon the age and emotional state of the conspecific target. These bidirectional effects may reflect a species-specific adaptation in which social distress signals are perceived and responded to as danger cues when generated by an adult, but as prosocial cues when generated by juveniles. The in vivo pharmacological experiments suggest that the determination of the appropriate social affective behavior, i.e. to approach or avoid, requires the insular cortex OTR. The results suggest that exposure to a stressed conspecific evokes OT release within the insular cortex which, via modulation of insular cortex output neuron excitability, orchestrates species-specific approach or avoidance behaviors. The findings provide new insight into the neural basis of elementary empathic processes and, consistent with human neuroimaging work, warrant consideration of the insular cortex as a critical node within the social brain network.

Across mammalian species, social transmission of environmental threat occurs via alarm signals. In rat, alarm signals include chemosignals [63,64], vocalizations [65], and other behavioral expressions [66]. Chemosignals themselves are sufficient to evoke anxiety and avoidance behaviors in the absence of social interaction [67]. Rats tend to avoid 22kHz alarm vocalizations [68], bedding soiled by stressed conspecifics [69,70], and even images of adult conspecifics in distress [71]. The subjects in these studies were adult male rodents and so our pattern of avoidance of stressed PN 50 conspecifics is consistent and reflects an adaptive response to avert risk. It is challenging to speculate on the motivation for approaching the juvenile in distress, but the difference in behavior could be the result of unique social signals generated by the juvenile or how they were perceived by the adult. For example, although juveniles emit 22kHz alarm vocalizations [65], they do not emit stress-induced chemosignals [72]. In human social behavior, features of the target stimulus, including age, are critical determinants to whether or not another will approach, help or avoid another in distress [58]. Thus, it is conceivable that inconspicuous behavioral signals or facial expressions (which were not possible to quantify in the present design), which differ in response to stress in juvenile versus adult rats, shape the behavior of the experimental rat. In our analysis of conspecific behavior, stress only altered the amount of time spent self grooming in juveniles. More work is required to understand the communication between juveniles and adults but it is likely that the decision to approach or avoid a distressed conspecific is a consequence of the interaction of multiple innate, bottom-up sensory and some top-down executive processes that converge on the insular cortex.

Like social behavior itself, the effects of systemic OT administration upon social behavior are sensitive to situational, interpersonal and personal factors with OT sometimes producing prosocial effects, and at other times anti-social effects [73]. Relative to other cortical regions, the high degree of OTR found in the insular cortex [32,33] provides a waypoint for modulation by OT in the orchestration of social behaviors. We speculate that OT differentially modulates the insular cortex efferents to the amygdala, ventral striatum, and ventromedial prefrontal cortex [74,75], which are the proximal mediators of the emotional, rewarding, and empathic aspects of social behavior, respectively [4,6,76,77]. By integrating social, situational and other factors the insular cortex contributes to social decision making. Correlational support for this view was reported in male prairie voles, in which insular OTR binding density predicted reproductive success [78]; a behavioral endpoint that depends upon integration of social affect such as a partner's sexual status with other contextual or recollected stimuli.

A wide range of OT effects on neurophysiology and putative mechanisms in neurons are known, suggesting both cellular and regional adaptations for this signaling molecule [79,80] that could increase the salience of social information by modulation of neuronal excitability. OT altered several intrinsic properties, namely, reduction in AP amplitude, increase in $R_{input}$, increase in input/output relationships, and reduction in sAHP; modulation of the latter three predict that OT could boost the output of insular cortex pyramidal neurons in response to synaptic inputs. Indeed, OT has been shown to increase spike output [79,81,82] and facilitate synaptic long-term potentiation [83–85]. OT application led to a marked increase in evoked insular fEPSCs which is likely a consequence of the increase in input sensitivity observed in the intrinsic measures. Intrinsic properties are a product of neuronal morphology and the type and location of membrane bound ion channels [86]. Our data

suggest that changes in intrinsic physiology could underlie shifts in synaptic efficacy and excitatory/inhibitory balance found in brain regions where OT is critical for myriad social behaviors including auditory cortex [87], hippocampus [82], nucleus accumbens [88], and piriform cortex [89].

Abnormalities in empathy and emotion recognition are central to numerous psychiatric conditions including ASD, Fragile X Mental Retardation, depression, psychopathy, schizophrenia, borderline personality disorder and alexithymia [90–94]. Deficits in OT and insular cortex activity and connectivity are well documented correlates of symptom severity for many of these conditions [41,95,96] and our results suggest that disruption of insula function causes deficits in empathy like behavior. The SAP test presented here provides a simple paradigm for further research into the neuroanatomical and physiological systems that operate in concert with the insular cortex and OT to transduce social information into cortical computations which inform social behaviors.

## ONLINE METHODS

**Rats:** Male Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Rats were allowed a minimum of 7 days to acclimate to the vivarium after arrival and housed in groups of 2-3 with free access to food and water on a 12 h light/dark cycle. Behavioral procedures were conducted within the first 4 h of the light phase. All reagents and chemicals were purchased from Fisher Scientific, Tocris or Sigma unless otherwise noted. All procedures were conducted in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals and were approved by the Boston College Institutional Animal Care and Use Committee.

**Social Affective Preference (SAP) Test**: The SAP test allowed quantification of social interactions initiated by an adult test rat when presented simultaneously with two unfamiliar conspecific stimuli. To begin the test, the adult test subject was placed into a clear plastic cage (50 x 40 x 20 cm, L x W x H) with a wire lid. Pairs of stimuli rats were either juvenile (PN 30 +/- 2 days old) or adult (PN 50 +/- 2 days old) and were placed inside one of two clear acrylic plastic enclosures (18 x 21 x 10 cm, L x W x H) on either end of the arena. Interaction between the experimental and stimuli rats was permitted on one side of the enclosure, which consisted of clear acrylic rods, spaced 1 cm center-to-center (see photo, Fig. 1) and as in [97]. To habituate subjects to the procedure on days 1 and 2 the adult was placed in the arena for 60 min and then empty enclosures (Day 1) or enclosures containing experimentally naïve, unfamiliar stimuli (Day 2) were added for 5 min. To assess social affective preference, on Day 3 two unfamiliar stimuli were added, but one of the stimuli rats was exposed to 2 footshocks (1mA, 5 sec duration, 60 sec inter-shock-interval, Precision Regulated Animal Shocker, Coulbourn Instruments, Whitehall, PA) immediately prior to the 5 min test to induce a stressed affective state. Shock occurred in a separate room and shock parameters were selected because they were sufficient to produce a conditioned fear in our laboratory (data not shown). The 5 min test length was selected after pilot studies in which we observed a reliable decrease in social behavior after the first 5 min of test. In experiments involving optogenetics or intracerebral injections, a within-subjects design was employed such that each adult test subject was exposed to both vehicle and experimental treatments in SAP tests on consecutive days. A trained observer quantified the time spent in social interaction with each of the stimuli. Social interaction consisted of nose-nose and nose-body sniffing, and reaching into the enclosure to contact the stimulus rat. Digital video recordings were made of each test for later determination of inter-rater-reliability by a second observer completely blind to the experimental manipulations and hypotheses. Across the experiments included in this report we observed very high inter-rater reliability, $r(80) = 0.966$, $r^2 = 0.93$, $p < 0.0001$. Although conceived independently, this paradigm has a number of features in common with the method recently reported with voles [98].

**One-on-One Social Exploration Tests**: As in [29] each experimental subject was placed into a plastic cage with shaved wood bedding and a wire lid 60 min before the test. To begin the test a juvenile or adult was introduced to the cage for 5 min and exploratory behaviors (sniffing, pinning, and allogrooming) initiated by the adult test subject were timed by an observer blind to treatment. Juvenile and adult stimuli rats were used for multiple tests but were never used more than once for the same adult test rat. Each experimental adult was given tests on consecutive days once with an unfamiliar naïve conspecific and once with an unfamiliar stressed conspecific (2 foot shocks, exactly as above); test order was counterbalanced.

**Insular Cortex Cannula Placement and Microinjection**: Under inhaled isoflurane anesthesia (2-5% v/v in $O_2$), cannula (26g, Plastics One, Roanoke, VA) were inserted bilaterally into the insular cortex (from Bregma: AP: -1.8mm, ML: +/-6.5mm, DV: -6.2mm from skull surface) and fixed in place with acrylic cement and stainless steel screws. Rat were administered the analgesic meloxicam (1mg/kg, Eloxiject, Henry Schein)

and antibiotic penicillin G (12,000 Units, Combi-pen 48, Henry Schein) after surgery and allowed between 7-10 days recovery prior to experimentation. The OTR antagonist (OTRa) desGly-$NH_2$-$d(CH_2)_5$[$Tyr(Me)^2$,$Thr^4$]OVT [99] and OT were dissolved in sterile 0.9% saline vehicle. All injections were 0.5µL per side and infused at a rate of 1µL/min with an additional minute for diffusion. At the conclusion of the experiment, rats were overdosed with tribromoethanol (Sigma) and brains were dissected and sectioned at 40 µm to verify the microinjector tip location using cresyl violet stain and comparison to stereotaxic atlas [100]. Rats with occluded injectors or having cannula located outside of the insular cortex were excluded from all analyses (See Supplementary Fig. 6).

**Optogenetics**: Adult male rats underwent stereotaxic surgery to be implanted with bilateral guide cannula designed to fit a 200um optical fiber (Plastics One). After the cannula was secured, 250nL of a viral vector containing the neuronal silencing halorhodopsin eNpHr3.0 under the CamKIIα promoter (AAV5-CamKIIα-eNpHR3.0-mcherry; Tye et al., 2011) or a sham virus (AAV5-CamKIIα-YFP) was microinjected at a depth 1mm below the termination of the guide cannula at a rate of 50nL/min and allowed 5 min for diffusion. During testing, a multimodal fiber optic wire (200um core, 0.39NA, Model FT200EMT, Thorlabs) extending 1mm below the cannula tip was affixed to the stylet via a screw top ferrule (Plastics One) and connected to a laser (GL523T3-100, Shanghai Laser & Optics Century). Throughout the length of a social test green light (523nm) at a power of ~10-15mW/$mm^2$ was administered to maintain insular inhibition for the light ON conditions. During the light OFF condition, rats underwent the social test while connected to the laser but no light was administered. Functional photo inhibition was verified in whole cell recordings of mCherry positive insular cortex pyramidal neurons in acute brain slices before, during, after green light administration ~15mW/mm2) delivered through the objective of the electrophysiology microscope. The extent of transfections was determined by imaging mCherry expression with widefield fluorescent microscopy (Zeiss AxioImager Z2). Locations of transfections are provided in Supplementary Figures 5 and 6.

**Electrophysiology Solutions and Drugs:** All chemicals were purchased from Fisher Scientific, Sigma-Aldrich or Tocris. Standard artificial cerebrospinal fluid (aCSF) and recording solutions were used [101]. aCSF recording composition was (in mM) NaCl 125, KCl 2.5, $NaHCO_3$ 25, $NaH_2PO_4$ 1.25, $MgCl_2$ 1, $CaCl_2$ 2 and Glucose 10; pH = 7.40; 310 mOsm; aCSF cutting solution was: Sucrose 75, NaCl 87, KCl 2.5, $NaHCO_3$ 25, $NaH_2PO_4$ 1.25, $MgCl_2$ 7, $CaCl_2$ 0.5, Glucose 25 and Kynureinic acid 1; pH=7.40, 312 mOsm. The internal recording solution consisted of (in mM) $K^+$-Gluconate: 115, KCl 20, HEPES 10, Mg-ATP 2, Na-GTP 0.3, and Na-Phosphocreatine 10. pH = 7.30; 278 mOsm with 0.1% biocytin. Kynurenic acid 1 mM and SR-95531 2 µM were always added to the recording aCSF to block synaptic transmission for intrinsic recordings.

**Insular cortex slices**: Adult rats were anesthetized with isofluorane, intracardially perfused with chilled (4°C), oxygenated aCSF cutting solution and quickly decapitated. 300 µm coronal slices including the insular cortex were taken using a vibratome (VT-1000S, Leica Microsystems, Nussloch, Germany). The slices were placed in oxygenated aCSF cutting solution (95% $O^2$ and 5% $CO^2$) at 37°C for 30 min and then at room temperature for a minimum of 30 min before slices were used for electrophysiological recordings.

**Electrophysiology**: Whole-cell current-clamp recordings were obtained at 30 ± 2°C. Patch-clamp electrodes were pulled (P-1000, Sutter Instruments, CA) from 1.5 mm outer diameter borosilicate glass (Sutter Instruments, CA) and filled with intracellular solution. Electrode resistance was 3–5 MΩ in the bath and recordings were only included if the series resistance remained less than 30 MΩ with less than 10% change from baseline throughout the experiment. Slices were visualized using a 40x (0.75 NA) water immersion objective under infrared differential interference contrast imaging on an upright microscope (AxioExaminer D1, Zeiss, Germany). All recordings were obtained with an Axon 700B amplifier and pClamp 10 (Molecular Devices), using appropriate bridge balance and electrode-capacitance compensation. After achieving a whole-cell configuration, baseline recordings were made in aCSF until 10 minutes of stable baseline were observed, at which point 500 nM oxytocin citrate was added to the bath. The dose of 500 nM was selected after a pilot study using a range of doses from 50nM to 1M, the largest dose reported [88]. Because OT has high affinity for the Vasopressin 1A receptor (V1a), experiments typically isolate effects of OT to the OTR by using synthetic OTR agonists or a cocktail of OT and V1a antagonists. These steps were not taken here because although V1a receptor mRNA has been reported throughout cortex [102] V1a receptor binding is not evident in adult male rat insula [103]. Analyses were performed using custom software written for Igor Pro (Wavemetrics Inc., Lake Oswego, OR).

Active properties were quantified from single spikes by holding the neuron at -67 mV, and 2.5 ms current pulses were injected to elicit a single AP. Passive properties were measured by holding the membrane potential at -67 mV and injecting 1 s current pulses through the patch electrode. The amplitudes of the current injections were between -300 pA and +400 pA in 50 pA steps. All traces in which APs were elicited were used to generate input-output curves as the total number of APs per second plotted against the injected current. EPSCs were made in the whole cell configuration with the same internal solution and aCSF with tetrodotoxin (1uM) added to some of the recordings to isolate miniature EPSCs. Recordings were made for 10 min prior to OT (10 min) and then for 10 min after OT. EPSC frequency and amplitude were determined with the mini analysis program (Synaptosoft). After recording, the slice was fixed in 4% paraformaldehyde and biocytin was visualized using the ABC method and NovaRed (Vector labs, Burlingame, CA). Only neurons with a pyramidal morphology and soma in deep layers of insular cortex were included for analysis.

Evoked EPSPs were recorded on a 6 x 10 perforated multiple electrode array (Model: MCSMEA-S4-GR, Multichannel Systems) with integrated acquisition hardware (Model: MCSUSB60) and analyzed with MC_Rack Software (Version 3.9). Slices were placed on the array and adhered by suction of the perfusion through the perforated substrate. Bath solutions were as above and perfused through the slice from above. A stimulating electrode was selected in the deep layers of insular cortex, and field excitatory postsynaptic potentials (fEPSPs) were recorded after stimulation (0 to 5V, biphasic 220us, 500mV increments) before, during application of 500nM OT, and after (Wash). Each step in the I/O curve was repeated 3 times (20s inter-stimulus-interval) and each family of steps was replicated 3 times in each phase of the experiment. fEPSPs from channels displaying clear synaptic responses (as in Fig. 4B) and in the vicinity of the stimulating electrode were normalized to the individual channel's maximum response to 5V stimulation at baseline; channels from the same slice were averaged for group analysis.

**Statistical Analysis**: Sample sizes were initially determined based on prior work using social interaction [28,29] and intrinsic physiology [61]. To compare differences between mean scores of social interaction and electrophysiological endpoints we used t-tests and analysis of variance (ANOVA). When possible, individual replicate data are provided in the figures. In most experiments, there were within-subjects variables, which were treated as such in the analysis (paired samples t-test or repeated measures ANOVA). Main effects and interactions were deemed significant when $p < 0.05$ and were followed by either Tukey HSD, or Holmes-Sidak post hoc tests, to maintain an experiment-wise risk of type I errors at $a = 0.05$. Statistical analyses were conducted in Prism 7.0a (GraphPad Software) and SPSS Statistics 24 (IBM).

**Author Contributions**

Conceptualization, J.P.C., J.A.V., M.M.R.; Methodology, M.M.R., J.A.V., and J.P.C.; Investigation; J.A.V., M.M.R., K.B.G., A.P., M.M., and J.P.C.; Writing – Original Draft, J.A.V., M.M.R., and J.P.C.; Writing -- Revision & Editing, J.P.C.; Funding Acquisition, J.P.C.

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