Fear-enhancing effects of septal oxytocin receptors

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The nonapeptide oxytocin is considered beneficial to mental health due to its anxiolytic, prosocial and anti-stress effects, but evidence for anxiogenic actions of oxytocin in humans has recently emerged. Using region-specific manipulations of the mouse oxytocin receptor (Oxtr) gene (Oxtr), we identified the lateral septum as the brain region mediating fear-enhancing effects of Oxtr. These effects emerge after social defeat and require Oxtr specifically coupled to the extracellular signal-regulated protein kinase pathway.

The established view that oxytocin reduces fear and anxiety1–3 has recently been challenged, as humans given oxytocin intranasally exhibit increased recollection of aversive events4 and startle responses to stressful stimuli5,6. Consistent with these data, oxytocin reactivity is associated with increased postconflict anxiety7.

To understand the neurobiological basis of fear regulation by oxytocin, we focused on Oxtr-mediated signaling in the lateral septum, a brain area with high levels of Oxtr8 and a substantial involvement in stress and fear9. We manipulated the levels of lateral septal Oxtr with two region-specific genetic approaches: for Oxtr knockdown, we injected a replication-defective adenovirus associated virus (rAAV) encoding Cre recombinase in OxtrloxP/loxP mice (Cre OxtrloxP/loxP group); for overexpression of Oxtr, we infused wild-type mice with rAAV vector encoding Oxtr10 (Oxtr wild-type group). Corresponding controls received the rAAV-GFP vector (GFP OxtrloxP/loxP group) or GFP wild-type group. We targeted the vectors to the area of the lateral septum containing a dense population of Oxtr-positive neurons, as previously identified in Venus+/+ reporter mice where the reporter gene Venus is driven by the Oxtr gene promoter (anteroposterior coordinates +0.14 mm to +0.38 mm from bregma; Fig. 1a). Injections of the viral vectors induced significant downregulation (Cre OxtrloxP/loxP, n = 7 mice/group, versus GFP OxtrloxP/loxP, n = 4 mice/group, t9 = 3.49, P < 0.01) or upregulation (Oxtr wild-type group, n = 5 mice/group, versus GFP wild-type group, n = 7 mice/group, t10 = −5.280, P < 0.001) of Oxtr mRNA levels, as determined by independent-sample t-test statistical analyses (Fig. 1b). We next studied the role of Oxtr knockdown or overexpression in context-dependent fear conditioning. We assessed freezing behavior to the context as index of fear one day after pairing the context with a footshock. Oxtr downregulation did not affect fear conditioning (t30 = 0.00, P = 1.00), as revealed by similar freezing levels in OxtrloxP/loxP mice injected with rAAV encoding Cre recombinase (rAAV-Cre; n = 11 mice/group) or rAAV encoding GFP (n = 11 mice/group) (Fig. 1c). Similarly ineffective was Oxtr overexpression, as revealed by indistinguishable freezing in the Oxtr wild-type group and the GFP wild-type group (n = 5 and 7 mice/group, respectively) (t10 = 0.344, P = 0.738; Fig. 1c). We therefore hypothesized that if oxytocin had a role in fear regulation via the lateral septum, this role would be modulatory rather than direct. To test this possibility, we established a mouse model of stress-enhanced fear11 using acute social defeat, a stressor that increases oxytocin release in the septal area12, and preferentially enhanced context-dependent over tone-dependent fear conditioning (Supplementary Fig. 1). We injected wild-type or OxtrloxP/loxP mice with rAAV-Cre and divided them into either nonstressed (separated from the aggressor with a transparent barrier, nonstressed group) or stressed groups (exposed to social defeat; see Supplementary Fig. 2 for experimental design). Six hours after exposing mice to stress, we trained all mice in contextual fear conditioning and tested them 1 d later. One-way ANOVA revealed significant group differences of context fear (F3,28 = 4.034, P < 0.05), with greater freezing of stressed versus nonstressed Cre wild-type mice (P < 0.05, n = 7 mice/group for both). However, this effect was lacking in mice subjected to Oxtr knockdown (social defeat, Cre OxtrloxP/loxP, n = 8 mice/group) that froze similarly to their nonstressed littermates (n = 10; P = 0.759) and significantly less than the wild-type social defeat group (n = 7; P < 0.01) (Fig. 1d). Stress-enhanced fear was also abolished by pharmacological inhibition of Oxtr (Supplementary Fig. 3). In contrast to Oxtr knockdown, Oxtr overexpression exacerbated stress-enhanced fear (social defeat, Oxtr wild-type group, n = 9 mice/group), F2,25 = 13.67, P < 0.001, when compared to the nonstressed Oxtr wild-type group (n = 9 mice/group, P < 0.001) and social defeat–subjected GFP wild-type group (n = 10 mice/group) (Fig. 1e). Unlike Oxtr overexpression, however, administration of oxytocin did not additionally increase freezing in response to social defeat (Supplementary Fig. 3), possibly because endogenous oxytocin saturated available Oxtr and prevented actions of exogenously added peptide. Taken together, these findings demonstrate that Oxtr in the lateral septum mediated the enhancement of fear by social-defeat stress and that Oxtr was necessary but not sufficient to exert a fear-enhancing action.

Manipulations of Oxtr did not alter the behavioral measures of social defeat or exploratory activity to the context (Supplementary Fig. 4). We therefore hypothesized that, rather than affecting the experience of social defeat, Oxtr exhibited a delayed effect by enhancing aversive social memory13,14. We measured the approach

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Coupling of lateral septal Oxtr to SD NS injected with rAAV-Oxtr or rAAV-GFP. For fear in SD WT mice injected with rAAV-Oxtr when compared to SD WT mice injected with rAAV-GFP, # OxtrloxP/loxP neurons (dashed lines demonstrate injection sites that correspond to septal region rich in Oxtr). We targeted the viral injections to anteroposterior coordinates corresponding to the highest number of Oxtr-positive controls, * compared to NS WT mice injected with rAAV-Oxtr, *** compared to NS WT mice injected with rAAV-Oxtr or rAAV-Oxtr in wild-type (WT) mice. **P < 0.01, ***P < 0.001 (independent-sample t-test, compared to corresponding rAAV-GFP controls). (c) Freezing by OxtrloxP/loxP mice injected with rAAV-Cre and rAAV-GFP (control), and by WT mice injected with rAAV-Oxtr or rAAV-GFP. (d) Freezing in nonstressed (NS) and stressed (social defeat; SD) groups of WT and OxtrloxP/loxP mice injected with rAAV-Cre. For freezing in WT mice injected with rAAV-Cre compared to NS WT controls, *P < 0.05; NS OxtrloxP/loxP, *P < 0.05; and SD OxtrloxP/loxP, #P < 0.01 (Fisher’s LSD post hoc test). (e) Freezing in NS and SD groups of WT mice injected with rAAV-Oxtr or rAAV-GFP. For fear in SD WT mice injected with rAAV-Oxtr when compared to SD WT mice injected with rAAV-GFP, *P < 0.05; and compared to NS WT mice injected with rAAV-Oxtr. ***P < 0.01; for SD WT mice injected with rAAV-GFP compared to NS WT mice injected with rAAV-GFP, *P < 0.05 (Fisher’s LSD post hoc test). (f) Approach index of NS WT and SD WT, SD OxtrloxP/loxP injected with rAAV-Cre, or SD WT injected with rAAV-Oxtr. For the approach index of SD WT, *P < 0.05, and of SD WT mice injected with rAAV-Oxtr, **P < 0.01 when compared to NS WT mice (Fisher’s LSD post hoc test). Data are expressed as mean ± s.e.m.

Figure 1 Oxtr mediates the enhancement of fear by social-defeat stress. (a) GFP immunostaining (anti-GFP) shows the localization of Oxtr-positive neurons in the lateral septum of Venus+/- ‘Oxtr reporter’ mice (top; LSD, lateral septum dorsal; LSI, lateral septum intermediate; LSV, lateral septum ventral; cc, corpus callosum); Cre immunostaining (anti-Cre) in wild-type mice injected with rAAV-Cre (bottom right), and X-gal cytochemical staining of β-galactosidase in B6.129S4-Gt(ROSA) reporter mice injected with rAAV-Cre (Cre-ROSA; bottom left). Bregma 0.14 µm. Oxtr mediates the enhancement of fear by social-defeat stress. (b) Quantification of pErk-1/2 in Venus-positive neurons of Venus Oxtr reporter mice 6 h after social defeat (‘SD6’ group) or in naive controls. Percentage of pErk-1/2 is significantly greater in Venus-positive neurons of heterozygous Venus+/- mice that underwent SD when compared to heterozygous Venus+/- naive mice (**P < 0.01) or SD6 homozygous Venus+/- mice that lack the Oxtr gene (**P < 0.01). (c) Freezing in mice treated with U0126 or vehicle after social defeat (SD) or nonstressed (NS) condition; *P < 0.05 versus vehicle control; †P < 0.05 versus U0126 NS; #P < 0.05 versus U0126 SD (one-way ANOVA followed by Fisher’s LSD post hoc comparisons).

Figure 2 Coupling of lateral septal Oxtr to Erk-1/2 signaling. (a) Immunofluorescence with antibodies to GFP and to pErk-1/2 (top), pCREB (middle) or pPKC (bottom) in the lateral septum of heterozygous Venus+/- Oxtr ‘reporter’ mice. Asterisks, colocalization; arrows, Venus-expressing neurons; and arrowheads, protein kinase-expressing neurons. (b) Quantification of pErk-1/2 in Venus-positive neurons of Venus Oxtr reporter mice 6 h after social defeat (‘SD6’ group) or in naive controls. Percentage of pErk-1/2 is significantly greater in Venus-positive neurons of heterozygous Venus+/- mice that underwent SD when compared to heterozygous Venus+/- naive mice (**P < 0.01) or SD6 homozygous Venus+/- mice that lack the Oxtr gene (**P < 0.01). (c) Freezing in mice treated with U0126 or vehicle after social defeat (SD) or nonstressed (NS) condition; *P < 0.05 versus vehicle control; †P < 0.05 versus U0126 NS; #P < 0.05 versus U0126 SD (one-way ANOVA followed by Fisher’s LSD post hoc comparisons).

behavior of defeated mice toward the aggressive resident 6 h after stress. Nonstressed control mice (n = 11 mice/group) approached the resident (approach score 262 ± 62 or 36% of total time; Fig. 1f), consistent with a lack of lasting memory of their interaction.15 Conversely, wild-type mice (n = 7 mice/group) and mice overexpressing Oxtr exposed to social defeat (n = 10 mice/group) approached significantly less, Fs,2,32 = 3.582, P < 0.05 (Fig. 1f), indicating prolonged social memory of the aggressor. We did not observe such memory in Oxtr knockdown mice (n = 8 mice/group) whose approach index did not differ from that of the nonstressed group.
(P = 0.0828). These findings showed that the potentiation of fear by lateral septal OXTR might be due, at least in part, to the maintenance of social memory after defeat, rather than general enhancement of fear or anxiety.

The adverse effects of stress on emotional behavior have been linked to distinct signaling pathways. To identify the protein kinases activated in OXTR-bearing neurons, we used reporter mice with one or both OXTR alleles replaced with Venus, which codes for a variant of GFP. Social defeat did not affect the number of Venus-expressing cells (Supplementary Fig. 5), suggesting that the activity of the OXTR promoter did not change in response to stress. However, the amount of phosphorylated extracellular signal-regulated kinase-1/2 (pErk-1/2) was significantly greater in OXTR-bearing neurons of the heterozygous, Venus<sup>+/−</sup> mice (n = 4 septi/group) 6 h after social defeat compared to the amount in naive mice (F<sub>2,12</sub> = 13.329, P < 0.01; n = 5 septi/group) (Fig. 2a,b), an effect that was robustly abolished by OXTR knockout in homozygous Venus<sup>−/−</sup> neurons (n = 6 septi/group). Notably, Venus-expressing cells were completely devoid of phosphorylated protein kinase C (pPKC) or phospho-cAMP response element binding protein (pCREB) activity (Fig. 2a), demonstrating highly specific coupling of lateral septal OXTR to the Erk-1/2 signaling pathway. In this way, OXTR signaling in the lateral septum differs from other tissues, where coupling to PKC has been established.

The role of septal Erk-1/2 signaling as a mediator of OXTR effects on fear was supported by the findings that inhibition of Erk-1/2 activity downregulated, abolished the enhancement of fear in the social defeat group (F<sub>3,24</sub> = 3.569, P < 0.05; social defeat and vehicle (n = 5 mice/group) versus social defeat and U0126 (n = 7 mice/group), P < 0.05) without affecting fear conditioning in the nonstressed group (nonstressed and U0126 (n = 8 mice/group) versus nonstressed and vehicle (n = 8 mice/group), P = 0.8283; Fig. 2c).

Our results demonstrate that social defeat robustly activated the lateral septal OXTR-Erk pathway and thereby enhanced contextual fear conditioning. This mechanism may underlie the unconditioned anxiety and fear-enhancing effects of oxytocin in humans and mice, as opposed to anxiolytic actions mediated by the central amygdala. We therefore propose that rather than displaying a unidirectional influence on anxiety, the oxytocin system has a modulatory role, possibly by changing the salience or emotional valence of social and nonsocial contexts. This could improve the cognitive tuning of emotional processes and thus provide superior behavioral adaptation.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Y.F.G. performed all of the experiments and data analyses, Y.F.G. and J.R. designed the studies and wrote the paper, K.S., H.M. and K.N. developed the rAAV-OXTR and rAAV-GFP viral vectors, K.N. provided the OXTR<sup>−/−</sup> and Venus reporter mice, N.C.T. developed the model of social defeat, V.J. helped with the quantitative PCR analyses, and A.L.G. bred and genotyped the mice.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Wild-type mice, C57BL/6N mice (Harlan), OxtrloxP/loxP mice14 backcrossed for nine generations with C57BL/6N mice, and mice with Venus reporter driven by the Oxtr promoter16 backcrossed for nine generations with C57BL/6N mice (Harlan) were used in the experiments. B6.129S4-Gt(ROSA) mice were provided by W. Tourtellotte. The mice were bred in the Center for comparative medicine facility of Northwestern University. One week before experiments, at the age of 8 weeks, the mice were transferred to our satellite facility and individually housed in cages (Scanbur) under standard conditions (22 °C, 40–60% humidity, 12 h–12 h light-dark cycle, water and food ad libitum). Experiments were performed with 9-week-old male mice during the light cycle. All studies were approved by the Animal Care and Use Committee at Northwestern University and are in compliance with US National Institute of Health standards.

Surgery. Double-guided cannula (Plastic One) were implanted into the lateral septum of mice as described previously21. Mice were anesthetized with 1.2% tribromoethanol (Avertin) and implanted with bilateral 26-gauge cannulae using a stereotaxic apparatus. Lateral septum was targeted using coordinates: anteroposterior +0.2 mm, mediolateral ± 0.5 mm, dorsoventral −3.0 mm (ref. 22). The mice were allowed to recover for 1 week before injection of peptides or viruses.

Injections. Oxytocin (Sigma), (1-D(CH2)5,Tyr(ME)2,Thr4,Tyr-NH2(9)) ornithine vasotocin (OTA, Bachem) and MEK inhibitor (U0126, Sigma) were injected over 30 s, 0.2 µl per side. The viral vectors rAAV-Cre (driven by a synapsin promoter and obtained from P. Osten), rAAV-GFP (driven by a synapsin promoter, University of Pennsylvania Viral Vector Core), rAAV-Oxtr (driven by a CMV promoter12) and rAAV-CreloxP (driven by a CMV promoter12), were infused over 2 min (0.1 µl/min) intraorally in mice under a light isoflurane anesthesia. All viruses had a titer of 1–2 × 1012 particles/ml. Mice were allowed to recover for 1 week before behavioral tests.

Immunohistochemistry and immunofluorescence. Immunohistochemistry with antibodies to Cre protein (1:2,000; Covance, MMS-106P). For β-galactosidase cytochemical staining, sections were postfixed in 2% paraformaldehyde (PFA), 0.2% glutaraldehyde, 5 mM EGTA and 0.01% NP-40 in PBS with Mg at 4 °C and reacted for 6–12 h at 37 °C in reaction buffer (1 mg/ml X-gal, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide). After reaction, the tissues were postfixed and dehydrated in methanol and cleared in 2:1 benzyl benzoate/benzyl alcohol as described24. For all other stainings, brains were fixed with 4% PFA, dehydrated in 30% sucrose and stained using the Vectastain system (Vector, BA-9200 for mouse and BA-1000 for rabbit primary antibodies), as described previously21. Immunostaining with anti-Cre was visualized with d-amino benzidine. Immunostaining with mouse anti-GFP (1:6,000; Abcam, AB1218) was visualized with fluoresceinisothiocyanate, whereas rabbit anti-pErk-1/2 (1:1,600; Cell Signaling, 4370), anti-pCREB (1:8,000; Cell Signaling, 9198) and anti-pPKC (1:400; Cell Signaling, Cat #9371) were visualized with rhodamine. The antibodies were previously validated using peptide preadsorption or knockout approaches25,26.

Quantification of immunostaining signals was performed as described previously27. Slices were mounted in Vectashield (Vector) and observed with a confocal laser scanning microscope (Olympus Fluoview FV10i) for double-labeling at ×40. Septal sections were counted for double-labeling at the anteroposterior coordinates22 ±0.14 mm to +0.38 mm from bregma and the number of kinase-positive neurons expressed as percent of total Venus-positive neurons per septum. One septum (the highest number of Venus-positive neurons) per mouse per group was used to calculate group means.

DNA extraction and quantitative PCR. Mice were injected with methylene blue to visualize the viral injection site and then killed by cervical dislocation. Brains were dissected and the lateral septum was collected using a brain matrix. Tissue was homogenized in lysis buffer with β-mercaptoethanol and frozen in liquid nitrogen. RNA was extracted using miRCURY total RNA isolation kit (Exiqon), reverse-transcribed and subjected to real-time PCR using SYBR Green master mix (Applied Biosystems) and primers for Oxtr or mouse hypoxanthine phosphoribosyl transferase 1 (Hprt1) as an internal control. For Oxtr the forward primer was 5′-GGG GCG TCT GGC ACG TCA AT-3′ and the reverse primer was 5′-AGG AGG CGG TGC ACG AGT T-3′. For Hprt1 the forward primer was 5′-GGG GTC ACC TCA TCG CTG TC-3′ and the reverse primer was 5′-TCT CCA CCA ATA ACT TTG TCC-3′. Oxtr expression in OxtrloxP/loxP mice injected with rAAV-Cre or wild-type mice injected with rAAV-Oxtr was normalized to that of Hprt1 and shown relative to that in the rAAV-GFP control.

Protein extraction and immunohiblot. Individual septal were collected into the tip of the cannula from nonstressed mice injected with vehicle, or mice injected with vehicle or U0126 5 h after stress. Septi were lysed in a modified radioimmunoprecipitation (RIPA) buffer, incubated 15 min on ice and centrifuged for 15 min, 15,000g, 4 °C. RIPA buffer contained 50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Boehringer Manheim), 1 mM Na3VO4 and 1 mM NaF. After determining the protein concentration for each lysate (Bio–Rad protein assay), input samples (10 µg protein/µl) were reduced in loading buffer with DTT and boiled for 5 min, then subjected to SDS–PAGE (25 µg/well) and blotted to PVDF membranes (Millipore). Using the SnapID system (Millipore), membranes were saturated with 1-block (Tropix), incubated with mouse anti-pErk-1/2 (Sigma, 1:16,000, M8159) or rabbit Erk-1/2 (Santa Cruz, 1:5,000, K-23) and corresponding secondary antibodies (goat anti-rabbit 1:10,000, Applied Biosystems, T2191). For detection, we used alkaline phosphatase chemiluminescence.

Statistical analyses. We used about 5–8 mice per experimental group for behavioral and 4–8 biological samples/group for immunohistochemical, qPCR and immunohiblot assays. The numbers for each biochemical assay are biological replicates. The behavioral and molecular studies were replicated at 2–4 times (on average 3 times). These sample sizes gave us statistical power of 80% to detect stress or drug effects with the behavioral and biochemical data. Group sizes were balanced to ensure equal variance between the tested groups, and this was verified with Levene’s weighted F test computed by the SPSS package for all comparisons. All data showed normal distribution and were analyzed by two-tailed Student's
t-test for two groups or one-way ANOVA for three or more groups with treatment as a main factor. Post-hoc comparisons were performed using Fisher’s LSD test. Each key study was replicated at least twice.

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