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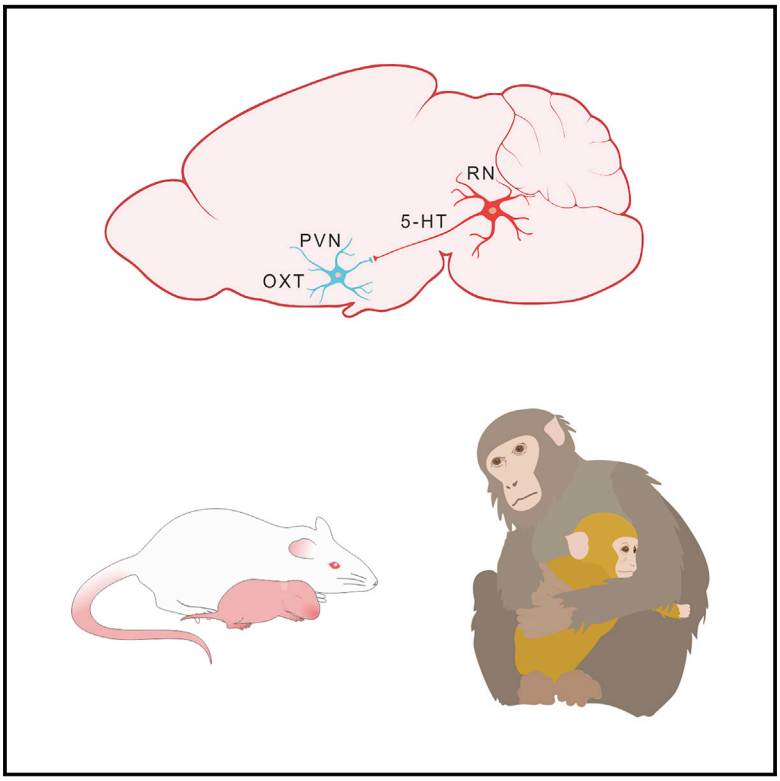
Molecular and cellular mechanisms of the first social relationship: A conserved role of 5-HT from mice to monkeys, upstream of oxytocin

Graphical abstract



Highlights

- *Tph2* gene knockout in mice, rats, or monkeys reduced maternal affiliation by infants
- Serotonergic neurons and oxytocinergic neurons were activated by maternal odors
- Serotonergic neurons in the RN activate oxytocinergic neurons in the PVN
- 5-HT acts upstream of oxytocin in regulating maternal affiliation by infants

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In brief

Maternal affiliation by infants is the first social behavior. Liu et al. have discovered a role for serotonin in this behavior conserved from mice and rats to monkeys. Serotonergic neurons from the raphe nucleus innervates oxytocinergic neurons in the paraventricular nucleus with serotonin acting upstream of oxytocin in maternal affiliation.

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Molecular and cellular mechanisms of the first social relationship: A conserved role of 5-HT from mice to monkeys, upstream of oxytocin

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SUMMARY

Maternal affiliation by infants is the first social behavior of mammalian animals. We report here that elimination of the *Tph2* gene essential for serotonin synthesis in the brain reduced affiliation in mice, rats, and monkeys. Calcium imaging and *c-fos* immunostaining showed maternal odors activation of serotonergic neurons in the raphe nuclei (RNs) and oxytocinergic neurons in the paraventricular nucleus (PVN). Genetic elimination of oxytocin (OXT) or its receptor reduced maternal preference. OXT rescued maternal preference in mouse and monkey infants lacking serotonin. *Tph2* elimination from RN serotonergic neurons innervating PVN reduced maternal preference. Reduced maternal preference after inhibiting serotonergic neurons was rescued by oxytocinergic neuronal activation. Our genetic studies reveal a role for serotonin in affiliation conserved from mice and rats to monkeys, while electrophysiological, pharmacological, chemogenetic, and optogenetic studies uncover OXT downstream of serotonin. We suggest serotonin as the master regulator upstream of neuropeptides in mammalian social behaviors.

INTRODUCTION

For an infant mammalian animal, the first social behavior in the life is to affiliate with or attach to its caregiver, usually the mother.[1–6] Affiliative behavior is beneficial for survival by obtaining nourishment and protection, and influences other behaviors in late life.[7,8] Defective affiliation is detrimental to non-human primates[9,10] and humans.[11] The significance of affiliation is further highlighted by the fact that infants even affiliate with abusive caregivers.[12,13]

Molecular mechanisms of affiliation behavior in infants are not well understood.[14–18] Neuropeptides oxytocin (OXT) and arginine-vasopressin (AVP) are important for social behaviors,[19–23] and they have also been implicated in mouse affiliation as evidenced by their effects on infant ultrasonic vocalizations (USVs) induced by maternal separation.[14,16] However, there were apparent contradictions regarding the functional role(s) of OXT in affiliation of rodents: USVs were found to be reduced in OXT knockout mouse pups,[16] but exogenous OXT application also reportedly reduced USVs in rat pups.[24] Receptors for opioids,[25,26] dopamine,[27] cannabinoids,[28] and neurokinin[29] may be involved in separation induced USVs. Genetic mutations affecting development could influence affiliation behavior.[30–35] Some of these genes are associated with autism in humans, a disorder defined by impairments in social interactions.[36–39] Separation induced USV at room temperature was reduced in mouse pups lacking serotonin,[40] though it has not been distinguished whether this was caused by defective maternal affiliation or temperature sensation.[41–43]

Here, we have generated mutations for the gene tryptophan hydroxylase 2 (*Tph2*) in rodents and non-human primates. It is required for synthesizing 5-hydroxytryptamine (5-HT) in the brain.[44–48] These mutants allowed us to discover a conserved role for 5-HT in affiliation.

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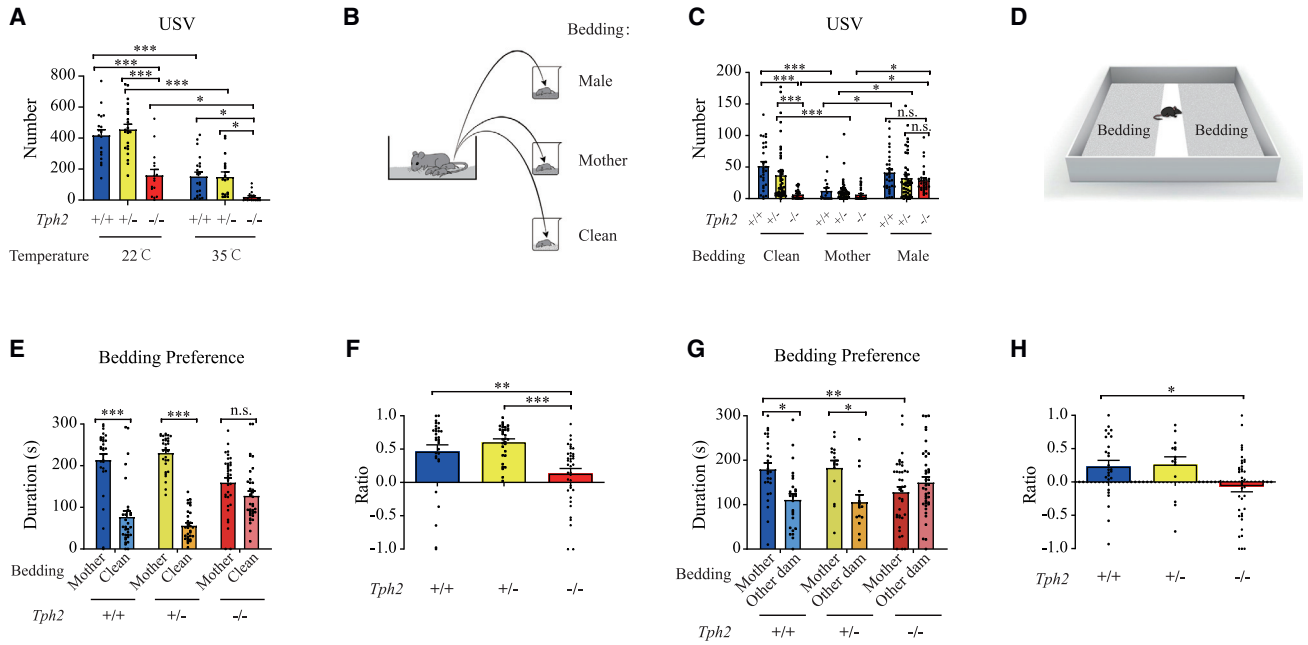

Figure 1. Maternal affiliation by *Tph2* mutant mouse pups

(A) *Tph*$^{-/-}$ mouse pups emitted fewer USVs than their *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ littermates when being separated from their mothers and placed in a clean beaker at room temperature (22°C) (n = 19 for *Tph2*$^{+/+}$, n = 23 for *Tph2*$^{+/-}$, n = 16 for *Tph2*$^{-/-}$) or 35°C (n = 22 for *Tph2*$^{+/+}$, n = 17 for *Tph2*$^{+/-}$, n = 20 for *Tph2*$^{-/-}$). *p < 0.05; **p < 0.01, and ***p < 0.001 (one-way ANOVA and Tukey's post hoc).

(B) A diagram showing that mouse pups were separated from their mothers and placed in a beaker containing bedding with different social odors for (C).

(C) *Tph2*$^{-/-}$ mouse pups (n = 33) emitted fewer USVs than *Tph2*$^{+/+}$ (n = 29) and *Tph2*$^{+/-}$ pups (n = 47) during isolation in clean bedding. When presented with mother bedding, USVs of *Tph2*$^{+/+}$ (n = 17) and *Tph2*$^{+/-}$ pups (n = 47) were decreased to the same level as that of *Tph2*$^{-/-}$ pups (n = 35). Male bedding increased the USVs of *Tph2*$^{+/+}$ (n = 32), *Tph2*$^{+/-}$ (n = 44), and *Tph2*$^{-/-}$ (n = 29) pups, as compared with that of mother bedding. **p < 0.01 and ***p < 0.001 (one-way ANOVA and Tukey's pos thoc).

(D) A diagram for the bedding preference assay for (E)–(H). The left and right parts of the test cage were overlaid with different bedding.

(E) *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ mouse pups spent more time on mother bedding than on clean bedding, while *Tph2*$^{-/-}$ pups spent a similar amount of time on both kinds of beddings. *Tph2*$^{+/+}$, n = 31; *Tph2*$^{+/-}$, n = 31; *Tph2*$^{-/-}$, n = 36. ***p < 0.001; n.s., not significant (Wilcoxon matched-paired t test).

(F) The bedding preference ratio ([time on mother bedding − time on clean bedding]/total time on bedding) was calculated from (E). The ratio of *Tph2*$^{-/-}$ was significantly lower than those of *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$. **p < 0.01 and ***p < 0.001 (one-way ANOVA and Tukey's post hoc).

(G) *Tph2*$^{+/+}$and *Tph2*$^{+/-}$ mouse pups spent more time staying on the bedding of their own mothers than that of other dams, however, *Tph2*$^{-/-}$ pups spent nearly equivalent time on both sides. *Tph2*$^{+/+}$, n = 26; *Tph2*$^{+/-}$, n = 15; *Tph2*$^{-/-}$, n = 39. *p < 0.05, **p < 0.01 (Wilcoxon matched-paired *t* test).

(H) The bedding preference ratio calculated from (G). The ratio of *Tph2*$^{-/-}$ mouse pups was lower than *Tph2*$^{+/+}$ mouse pups (p < 0.05, one-way ANOVA and Tukey's post hoc). Error bars, SEM.

Furthermore, we generated mutants for OXT and its receptor OXTR in rat pups and found deficits in their preference for the odors of their own mothers over the odors of other dams. By combining viral-genetic, chemogenetic, and pharmacological approaches with *in vivo* Ca^{2+} imaging and behavioral analysis, we found that OXT administration could rescue defective maternal preference of *Tph2*$^{-/-}$ pups. Activation of oxytoncinergic neurons in the paraventricular nucleus (PVN) could rescue defective maternal preference of pups resulting from inhibition of serotonergic neurons in the raphe nucleus (RN). Our results indicate that 5-HT is upstream of OXT in affiliative behavior.

RESULTS

Defective affiliation in *Tph2*$^{-/-}$ mouse pups in the absence of defective USVs or defective olfaction

During our behavioral studies of adult mice with the *Tph2*$^{-/-}$ genotype,[48] we noticed defective behavior of infants, which we have followed up with further investigations, in multiple species and with mechanistic dissections.

To alleviate genetically caused differences in maternal behaviors of mothers that secondarily influenced behaviors by infants, all dams had the same genotype *Tph2*$^{+/-}$. Pups derived from these mothers had three different genotypes: *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, or *Tph2*$^{-/-}$. Before using pups for behavioral studies, we used high-performance liquid chromatography (HPLC) to confirm that the levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were significantly lower in *Tph2*$^{-/-}$ mouse pups than those in *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ pups (Figures S1A and S1B), whereas the level of dopamine (DA) was not significantly different among *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, and *Tph2*$^{-/-}$ pups (Figure S1C).

We tested these pups in the standard USV assay: after separation from mother, pups would produce USVs, which serve as signals to attract maternal caring.[49,50] *Tph2*$^{-/-}$ pups emitted fewer USVs than *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ (Figure 1A), when separated from their mothers and placed in a beaker at room

temperature (RT, 22°C). To exclude stimulation from temperature changes, we placed the pups at 35°C, which mimicked the maternal body temperature. *Tph2*$^{-/-}$ mouse pups separated from mothers but placed at 35°C still emitted fewer USVs than *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ (Figure 1A). USVs induced by maternal separation were persistently reduced in *Tph2*$^{-/-}$ pups, from postnatal day (PSD) 4 to 8 (Figure S1D). When exposed to either high (56°C, Figure S1E) or low (4°C, Figure S1F) temperature, USVs were not significantly different between *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ pups, indicating that pups were not defective in USVs induced by noxious stimuli but were defective in USVs induced by maternal separation.

Social odors from beddings of mice could change pup USVs: mother odors typically reduce USVs and male odors augment USVs[15,51] (Figure 1B). *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ mouse pups responded to maternal isolation and exposure to clean bedding with high level of USVs, and this effect was significantly reduced in the presence of maternal bedding (Figure 1C). However, when isolated from their mothers and exposed to clean bedding, *Tph2*$^{-/-}$ pups showed very low level of USVs and no further reduction of USVs was observed in the presence of maternal bedding. Although clean bedding could not elicit USVs from *Tph2*$^{-/-}$ pups, odors from unfamiliar adult males significantly increased USVs from *Tph2*$^{-/-}$ pups: USVs elicited by male bedding were not significantly different among *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, and *Tph2*$^{-/-}$ pups (Figure 1C). These results suggest a specific deficit in the preference for maternal odors by *Tph2*$^{-/-}$ pups, in the absence of a general deficit in olfaction.

Pups prefer odors from their mothers[15] when they presented with bedding from their mothers vs. bedding from other dams or vs. clean bedding (Figure 1 D). Both *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ pups spent more time on maternal than clean bedding, whereas *Tph2*$^{-/-}$ pups showed no preference (Figures 1E and 1F). *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ pups preferred bedding from their mothers over that from other dams, whereas *Tph2*$^{-/-}$ pups did not (Figures 1G and 1H).

To further rule out the possibility that defective maternal preference in *Tph2*$^{-/-}$ pups resulted from deficient olfaction, we used two more assays: with either predator odor (fox urine) or non-social odor (peppermint).[48,52,53] *Tph2*$^{-/-}$ pups were not significantly different from *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ pups in avoiding fox urine (Figures S2C and S2D) or peppermint (Figures S2E and S2F). These results, together with pup responses to odors of male adults (Figure 1C), did not support a general deficit in olfaction in *Tph2*$^{-/-}$ pups.

Defective affiliation in *Tph2*$^{-/-}$ rat pups in the absence of defective USVs or defective olfaction

Rats used to be a major model for neurobiology but were overtaken by mice over the last thirty years mainly because of the ease of genetic manipulations in mice. The CRISPR-Cas9 method makes it much easier now to manipulate genes in rats (and other species). We used CRISPR-Cas9 to generate *Tph2*$^{-/-}$ rats. Exon 6 of the *Tph2* gene were completely deleted in rats (Figure S3A). HPLC could not detect 5-HT and 5-HIAA levels in pup brains of *Tph2*$^{-/-}$ rats (Figures S3B and S3C), whereas the level of DA was not affected (Figure S3D).

Maternal potentiation of USVs (an increase in USV frequency induced by a second isolation following a brief contact with their mothers) could be reliably observed in rat pups,[54] but not so robustly in mouse pups[55] (Figure 2A). When compared with their *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ littermates, *Tph2*$^{-/-}$ rat pups showed a significant decrease in USVs during both the first (Figure 2B) and the second separation from their mothers (Figure 2B). The maternal potentiation effect (more USVs after the 2nd separation than those after the 1st separation) was evident in *Tph2*$^{+/+}$ rat pups (Figure 2B), but absent in *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ littermates (Figure 2B). Thus, rat *Tph2*$^{-/-}$ pups showed reduced affiliation to their mothers. Because *Tph2*$^{+/-}$ pups were also defective, a dosage effect in the *Tph2* gene existed, which was also observed in behavioral studies of *Tph2*$^{+/-}$ adults previously.[56]

To test whether *Tph2*$^{-/-}$ pups could still emit USVs, we recorded their USVs in response to noxious stimuli. Similar to *Tph2*$^{+/-}$ pups, rat *Tph2*$^{-/-}$ pups emitted USVs upon heating to 56°C (Figure S3E) or cooling to 4°C (Figure S3F), at a frequency much higher than those emitted after maternal isolation.

Using bedding preference assays similar to that illustrated in Figure 1D, we found that both rat *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ pups showed preference for maternal bedding over clean bedding (Figures 2C and 2D), or over bedding from an unfamiliar dam (Figures 2E and 2F), whereas rat *Tph2*$^{-/-}$ pups showed no preference in either test (Figures 2C–2F).

Unlike those from mice, USVs from rat pups were inhibited by odors of unfamiliar adult males.[57,58] This was not significantly different between *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ rat pups (Figure 2G) and thus did not support a defect in social odor recognition. To further investigate whether olfaction was defective in *Tph2*$^{-/-}$ rat pups, we used avoidance assays for fox urine and peppermint. Rat *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, and *Tph2*$^{-/-}$ pups were not significantly different in their avoidance of fox urine or peppermint (Figures S4C–S4F). Furthermore, an assay for attraction by milk indicated that rat *Tph2*$^{+/+}$, *Tph2*$^{+/-}$, and *Tph2*$^{-/-}$ pups were similarly attracted by milk (Figure S4G).

Taken together, these results indicated that 5-HT was required in rat pups for infant affiliation with their mothers.

Affiliation affected by pharmacological manipulations of 5-HT levels in rat pups

Neither mouse nor rat *Tph2*$^{-/-}$ pups could synthesize 5-HT in the brain from embryogenesis. To rule out an indirect role for 5-HT in affiliation resulting from its role in development, we inhibited 5-HT synthesis in rat pups pharmacologically with p-chlorophenylalanine (pCPA).[59,60] Within 24 h, 200-mg/kg pCPA nearly depleted 5-HT and 5-HIAA in *Tph2*$^{+/-}$ rat pups (Figures S5A and S5B) but not DA (Figure S5C). 200-mg/kg pCPA significantly reduced 5-HT levels of *Tph2*$^{+/+}$ rat pups to ~31%, (Figures S6A and S6B), a higher dosage (600 mg/kg) reduced 5-HT levels of *Tph2*$^{+/+}$ to ~5% (Figures S6A and S6B). 200-mg/kg pCPA could also reduce 5-HT levels of *Tph2*$^{+/-}$ pups to ~5% of that in *Tph2*$^{+/+}$ pups.

When 5-HT levels of *Tph2*$^{+/-}$ (treated with 200mg/kg pCPA) and *Tph2*$^{+/+}$ pups (treated with 600mg/kg pCPA) were reduced to ~5% of the wild-type (WT) level, USVs induced by maternal separation (*Tph2*$^{+/-}$ in Figure 3A, *Tph2*$^{+/+}$ in Figure S6D) was significantly reduced by pCPA treatment. Maternal potentiation

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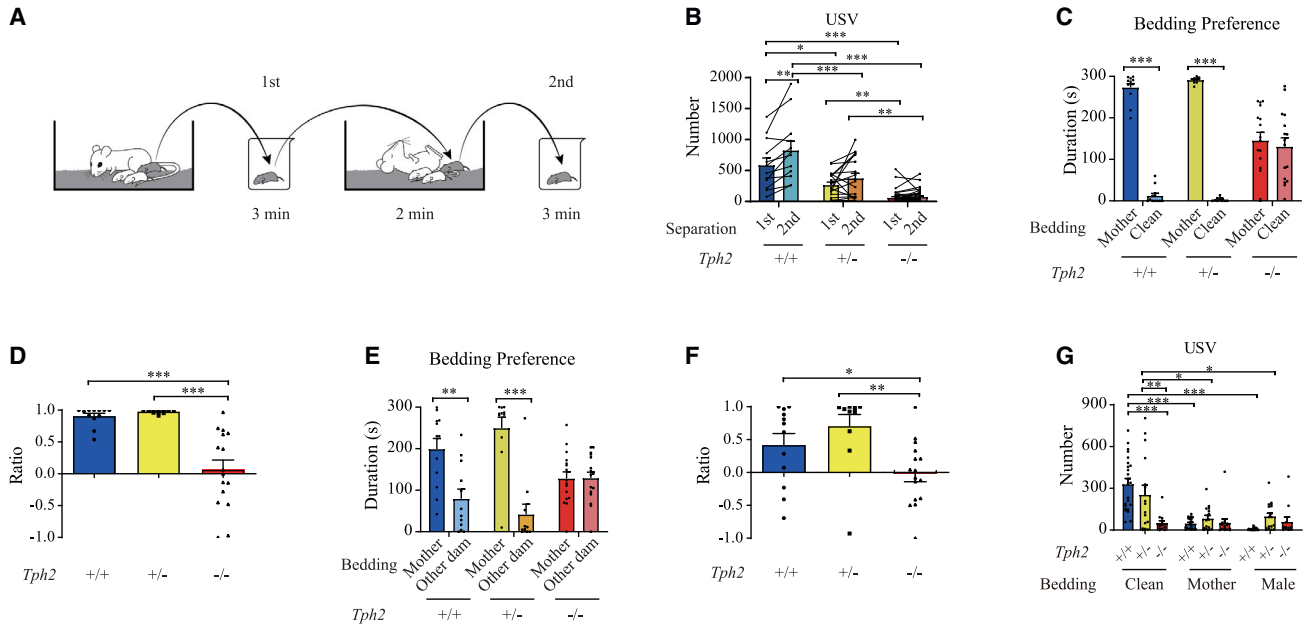

Figure 2. Maternal affiliation by *Tph2* mutant rat pups

(A) A diagram of the maternal separation and potentiation paradigm of rat pups for (B).

(B) When separated from their mothers for the first time (1st), $Tph2^{-/-}$ rat pups (n = 37) emitted fewer USVs than $Tph2^{+/+}$ (n = 12; $p < 0.001$, one-way ANOVA and Tukey's post hoc) and $Tph2^{+/-}$ pups (n = 18; $p < 0.01$, one-way ANOVA and Tukey's post hoc). Reunion with their mothers strongly enhanced USV emissions from $Tph2^{+/+}$ rat pups during second separation ($p < 0.01$, Wilcoxon matched-paired test), but did not enhance that of $Tph2^{-/-}$ rat pups ($p > 0.05$, Wilcoxon matched-paired test).

(C) In bedding preference experiment, $Tph2^{+/+}$ (n = 12) and $Tph2^{+/-}$ (n = 11) rat pups spent much more time on mother bedding than clean bedding, while $Tph2^{-/-}$ rat pups (n = 16) showed no preference. ***$p < 0.001$ (Wilcoxon matched-paired test).

(D) In contrast to high bedding preference ratio (nearly one) of $Tph2^{+/+}$ and $Tph2^{+/-}$ pups, the ratio of $Tph2^{-/-}$ rat pups was reduced to about zero. ***$p < 0.001$ (one-way ANOVA and Tukey's post hoc). (E and F) $Tph2^{+/+}$ (n = 12) and $Tph2^{+/-}$ (n = 11) rat pups preferred mother bedding over other dam's bedding, but $Tph2^{-/-}$ rat pups lost the preference (n = 16).

(G) When isolated on clean bedding, $Tph2^{+/+}$ (n = 23) and $Tph2^{+/-}$ (n = 16) rat pups emitted more USVs than $Tph2^{-/-}$ (n = 14) pups. USVs of $Tph2^{+/+}$ and $Tph2^{+/-}$ pups were markedly reduced when placed on mother bedding (n = 23 for $Tph2^{+/+}$, n = 15 for $Tph2^{+/-}$) or male bedding (n = 24 for $Tph2^{+/+}$, n = 17 for $Tph2^{+/-}$). However, USVs of $Tph2^{-/-}$ pups were not changed by either mother bedding (n = 15) or male bedding (n = 12). *$p < 0.05$; **$p < 0.01$ (one-way ANOVA and Tukey's post hoc). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Wilcoxon matched-paired test) for (F) and one-way ANOVA and Tukey's post hoc in (G)).

of USVs ($Tph2^{+/+}$ in Figure S6D) was also reduced by pCPA treatment. Preference of maternal bedding over clean bedding or bedding from other dams ($Tph2^{+/-}$ in Figures 3B–3E) was significantly suppressed by pCPA treatment. Thus, depletion of 5-HT by pCPA injection mimicked affiliative defects in genetic mutant rats. Moderate reduction of 5-HT in $Tph2^{+/+}$ pups treated with 200-mg/kg pCPA affected only bedding preference (Figures S6E–S6H) but not separation induced USVs and maternal potentiation (Figure S6D), suggesting that maternal bedding preference is more susceptible to 5-HT reduction.

We note that while $Tph2^{-/-}$ pups had lower body weight than $Tph2^{+/+}$ and $Tph2^{+/-}$ pups (Figure S3G), 200-mg/kg pCPA treated $Tph2^{+/+}$ and $Tph2^{+/-}$ pups were not different from saline-treated pups in body weight (Figures S5D and S6I), indicating that body weight changes could not explain changes in affiliation behavior.

To further investigate whether 5-HT in rat pups rather than 5-HT during embryonic development was important for affiliative behavior, we used 5-hyroxytryptophan (5-HTP), a product of tryptophane hydroxylase and a precursor of 5-HT to increase the level of 5-HT post-natally. Our previous study has shown that 5-HTP could restore 5-HT levels in the brain of $Tph2^{-/-}$ mutants.[48] We have now injected 5-HTP (4 mg/kg) into $Tph2^{-/-}$ rat pups 1 h before behavioral tests. We found that their 5-HT levels were restored to $Tph2^{+/+}$ level in the brain (Figure S6J), and their bedding preference for mother over other dams was indeed rescued (Figures 3F and 3G).

Taken together, these findings support that the role of 5-HT in affiliation did not result from indirect roles during development.

Defective affiliation in $Tph2^{-/-}$ monkey infants

The inventions of the transcription activator-like effector nucleases (TALEN) and CRISPR-Cas9 methods for gene editing have revolutionized comparative studies by genetic means.[61,62] We used the TALEN method to delete the *Tph2* gene in the rhesus macaques (*Macaca mulatta*) (Figures 4A and 4B).

Five pairs of TALENs targeting *Tph2*, whose repeat variable diresidue (RVD)s are shown in Table S1, were assembled by the bead-based method as described previously.[63] Activities of TALENs were examined in HEK293T cells by using the luciferase single-strand annealing (SSA) recombination assay and the Tph2-T2 pair was finally selected because of its efficiency

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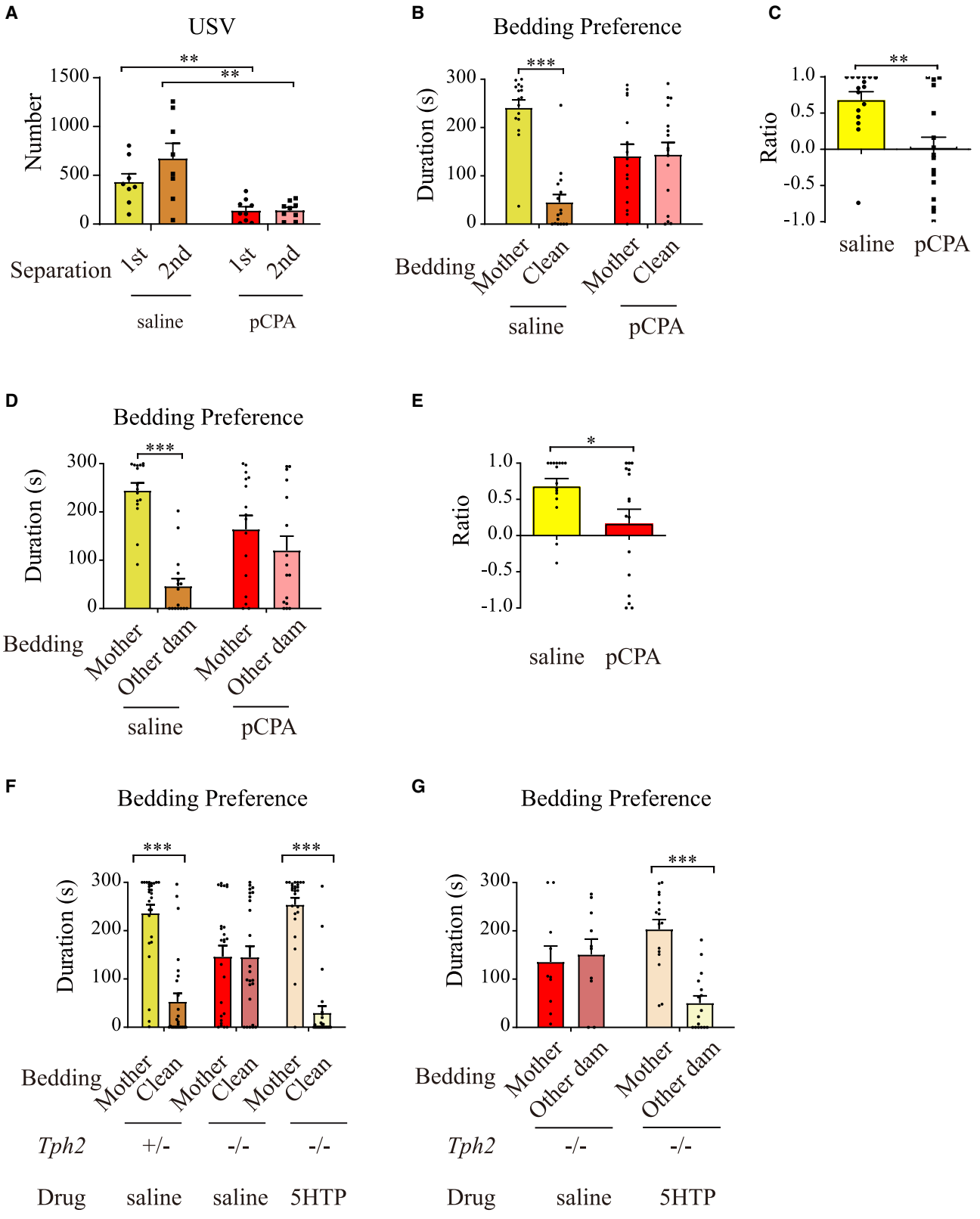

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(Table S2). We implanted one-cell stage zygotes into eighty surrogate female monkeys and obtained a total of nine *Tph2*$^{-/-}$ infant monkeys raised by their mothers. Sequence analysis of DNA obtained from placenta and ear skin showed that *Tph2* gene was disrupted in these *Tph2*$^{-/-}$ monkeys. HPLC analysis confirmed that the 5-HIAA levels in the cerebrospinal fluid (CSF) were significantly reduced in *Tph2*$^{-/-}$ monkeys than those in *Tph2*$^{+/+}$ monkeys (Figure S7A), whereas their DA levels were not significantly different (Figure S7B), further confirming that the *Tph2* gene was inactivated in the brains of *Tph2*$^{-/-}$ monkeys. *Tph2*$^{-/-}$ monkeys were similar to *Tph2*$^{+/+}$ in locomotion and avoidance response (Figures S7C and S7D), and growth as measured by body weight, body length, head circumstance, and tooth number (Figures S8A–S8D). When tested for behavioral responses to human intruders in both the profile period and the stare period,[64] *Tph2*$^{-/-}$ infant monkeys were not significantly different from *Tph2*$^{+/+}$ monkeys in either the duration of eye contact with human intruders or the frequency of vocalization (Figures S9A and S9B).

Analysis of videos of daily behaviors of an infant and its mother in their home cage is widely used in assessing deficits on affiliative behavior of monkey infants.[65–68] We analyzed two behavioral patterns: separation from mothers and ventral contact with mothers in *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ infants. The frequency of ventral contact with mothers was significantly decreased in *Tph2*$^{-/-}$ monkeys, while the frequency of separation was not significantly different between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ monkeys (Figure 4C).

To avoid behavioral interference of the infant by the mother, we designed two experiments for infant-mother interactions with no or restraint maternal initiatives. In the first experiment, the mother was sedated for a short time before she was placed in the same cage as her infant. Infant behavior was recorded by video for 10 min. The total durations of interactions of *Tph2*$^{+/+}$ infants with their mothers (Video S1) were significantly longer than those of *Tph2*$^{-/-}$ infants with their mothers (Video S2) (Figure 4D). Latency to approach the mother was also shorter in the *Tph2*$^{+/+}$ than that in the *Tph2*$^{-/-}$ infants (Figure S9C). In the second experiment, we placed an infant monkey and its mother in two adjoining cages made of wires. The mother immediately approached the side neighboring the infant cage. Compared with *Tph2*$^{+/+}$ infants (Video S3), approachment of the cage holding their mothers was reduced in *Tph2*$^{-/-}$ infants (Video S4): with latency longer (Figure S9D) and duration shorter (Figure 4E) than those in *Tph2*$^{+/+}$ infants.

Different from rodents but similar to humans, monkeys are good at using vision in social recognition. We designed visual preference experiments to detect infant preference between pictures presented on the two opposite sides of a cage and analyzed the amount of time during which each infant spent in front of the pictures (Figure 4F). When given the choice between faces of its own mother and another mother, *Tph2*$^{+/+}$ infant monkeys preferred the mother face, whereas *Tph2*$^{-/-}$ infants showed no preference (Figure 4G). Mother face preference ratio of *Tph2*$^{-/-}$ infants was significantly lower than that of *Tph2*$^{+/+}$ infants (Figure 4H). To examine whether this was due to a general deficit in vision, infants were tested for their preference between an apple and a stone. *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ infant monkeys were not significantly different in preferring an apple over a stone (Figure 4I). It is possible that the difference between an apple and a stone was large, whereas that between two faces is much smaller and thus requires better visual acuity to discriminate. We then tested infant monkeys for their preference between a familiar face and a new face. *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ infants were not significantly different in preferring a new over a familiar face (Figure 4J).

To rule out a role for 5-HT during embryonic development of monkeys, we used two approaches. First, we depleted 5-HT from infant monkeys pharmacologically with *pCPA*. Injection of *pCPA* in WT infant monkeys significantly decreased their approach to awake mothers (Figure S9E). Second, we tried to rescue the defect in *Tph2*$^{-/-}$ infants using 5-HTP. We found that 5-HTP could increase the duration of *Tph2*$^{-/-}$ infants to approach their mother (Figure S9F).

Taken together, results from *Tph2*$^{-/-}$ mutant mouse pups, rat pups, and monkey infants support an essential and conserved role for 5-HT in regulating affiliation of infants with their mothers.

Activation of serotonergic and oxytocinergic neurons by maternal odors

To investigate involvement of serotonergic neurons, we used fiber photometry to record neuronal activities *in vivo*. We injected *AAV-DIO-GCaMP7s*, an adeno-associated viral construct for expressing the calcium^{++} (Ca^{2+}) sensor GCaMP7s[69] under the control of the Cre recombinase, into the RN of newborn *Sert-Cre* mice in which Cre was expressed under the control of the serotonin transporter SERT[70] (Figure 5A1). This allowed GCaMP7s expression in serotonergic neurons, as confirmed by immunoreactivity of GCaMP7s positive neurons for the anti-serotonin antibody (Figures 5A2 and 5A3). An optical fiber was implanted into the RN on P14, and Ca^{2+} signals were recorded on P15 in free-moving mice following 16 h of recovery from surgery (Figure 5A).

Continuous fiber photometry recordings were made every 10 mins when the pups were allowed to freely investigate and sniff beddings (see STAR Methods for details). 470-nm light was the excitation light, with responses normalized by that to

Figure 3. Maternal affiliation by rat pups after pharmacological manipulation of 5-HT synthesis

(A) The mean number of USVs was significantly lower in pCPA (200 mg/kg)-treated *Tph2*$^{+/-}$ rat pups (n = 9) than that of saline group (n = 8) both upon 1st and 2nd separation from mother. **p < 0.01 (one-way ANOVA and Tukey's post hoc).

(B–E) Injection of pCPA abolished bedding preference for mother bedding over clean bedding (B) and (C) or bedding from other dam (D) and (E), a typical behavior phenotype in saline-treated *Tph2*$^{+/-}$ pups. n = 16 for each group. ***p < 0.001 (Wilcoxon matched-paired t test).

(F and G) i.p. injection of 5HTP (4 mg/kg) rescued bedding preference loss of *Tph2*$^{-/-}$ pups. In (F), saline-treated *Tph2*$^{+/-}$ rat pups (n = 27) preferred their mother bedding over clean bedding. In contrast, the *Tph2*$^{-/-}$ littermates showed no preference (n = 25), which was restored by 5-HTP injection (n = 26). ***p < 0.001 (Wilcoxon matched-paired t test). Similar results were obtained in (G) when the pups were presented with mother bedding and another dam's bedding. n = 10 for left two bars and n = 16 for the right two bars, respectively. ***p < 0.001 (Wilcoxon matched-paired t test).

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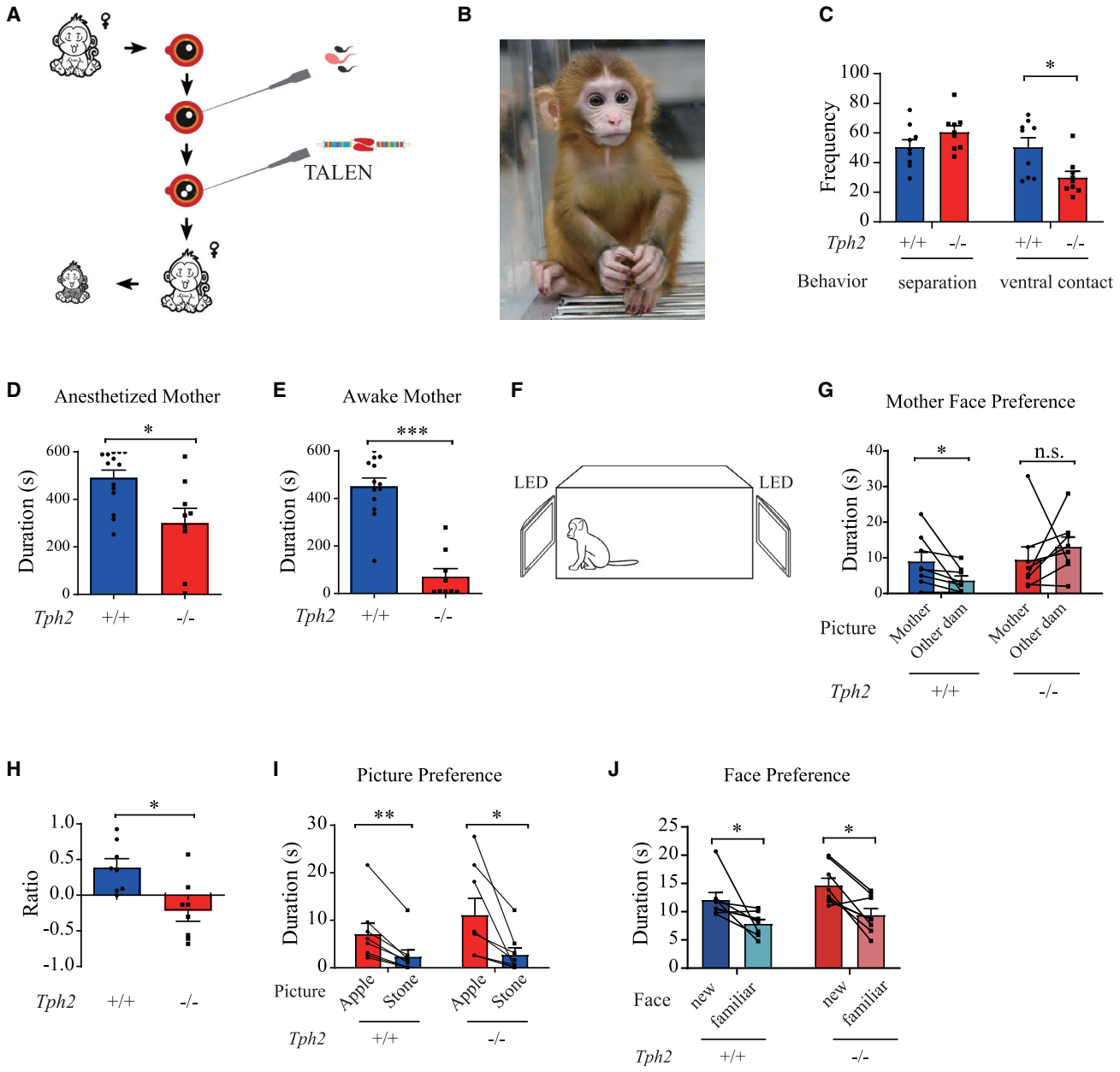

Figure 4. Maternal affiliation by *Tph2*$^{-/-}$ infant rhesus monkeys

(A) A schematic illustration of *Tph2*$^{-/-}$ rhesus monkey generation (see STAR Methods for more details).

(B) A photograph of 3-month-old *Tph2*$^{-/-}$ rhesus monkey.

(C) Daily behavior analysis of infant monkeys living together with their mothers. Ventral contact with mothers was significantly reduced in *Tph2*$^{-/-}$ infant monkeys (n = 9) as compared with *Tph2*$^{+/+}$ infant monkeys (n = 9). *p < 0.05 (Mann-Whitney t test).

(D) When their mothers were anesthetized, *Tph2*$^{-/-}$ infant monkeys (n = 9) spent less time on interacting with mothers than *Tph2*$^{+/+}$ monkeys (n = 14). *p < 0.05 (Mann-Whitney t test).

(E) *Tph2*$^{-/-}$ infant monkeys (n = 9) spent less time on touching the cage side neighboring their awake mothers than that of *Tph2*$^{+/+}$ infant monkeys (n = 13). ***p < 0.001 (Mann-Whitney t test).

(F) A diagram illustrating the picture preference assay for (G)–(J).

(G and H) *Tph2*$^{+/+}$ infant monkeys spent more time on the side with an LED presenting their mother's picture than the opposite side presenting other dam's picture. However, *Tph2*$^{-/-}$ infant monkeys showed no preference. *p < 0.05 (Wilcoxon matched-paired t test).

(I) Both *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ infant monkeys spent more time on the side of the cage with an LED presenting a picture of an apple than that of a stone. *p < 0.05; **p < 0.01 (Wilcoxon matched-paired t test).

(J) Both *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ infant monkeys preferred the side with an LED presenting a picture of a new monkey face rather than that of a familiar monkey face. *p < 0.05, Wilcoxon matched-paired t test. n = 8 monkeys per group for (D)–(J).

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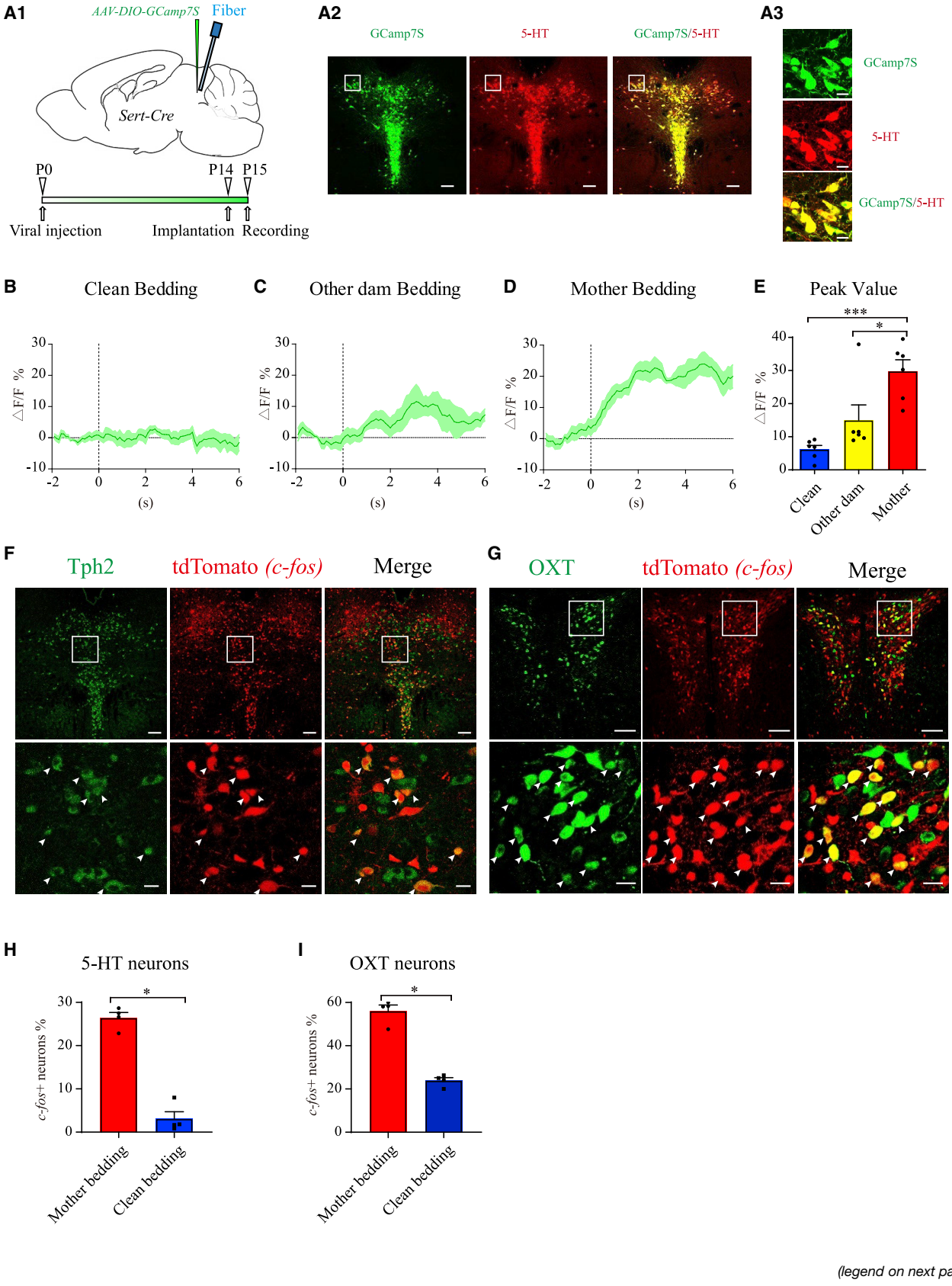

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410-nm control light to correct for movement induced artifacts.[71] Recording traces of Ca^{2+} signals in serotonergic neurons during sniff were extracted offline and aligned to sniff initiation (time 0, Figures 5B–5D). While sniffing of clean bedding correlated with no significant Ca^{2+} change (Figure 5B), and sniffing of other dam's bedding had moderate effect on Ca^{2+} (Figure 5C), sniffing of maternal bedding evoked a large increase of Ca^{2+}, peaking at ~2 s (Figure 5D). Quantification of the peak values of Ca^{2+} signals indicated significantly higher Ca^{2+} in serotonergic neurons correlated with the sniffing of maternal bedding than those of either clean bedding or other dam's bedding (Figure 5E).

The immediate early gene *c-fos* is an indicator of neuronal activities.[72] Injection 4-hydroxytamoxifen (4-OHT) into TRAP2*Ai14 double transgenic mice just before stimulation results in permanent labeling of transiently activated neurons.[73] Using this method, we observed the labeling of the accessory olfactory bulb (AOB) and the olfactory bulb (OB) after exposure to maternal bedding (Figure S10A). More interestingly, maternal bedding elicited *c-fos* labeling in a subset of serotonergic neurons in the RN (Figures 5F and 5H). Similar to our Ca^{2+} recording results, the *c-fos* labeling experiment further supports that serotonergic neurons are activated by maternal odors.

Strong *c-fos* signals were also detected in the PVN (Figure 5G). Immunocytochemistry with anti-OXT antibodies indicated that oxytocinergic neurons were positive for *c-fos* (Figure 5G). *c-fos* and OXT double-positive neurons were significantly more after exposure to maternal bedding than that after exposure to clean bedding (Figure S10B; Figure 5I).

OXT$^{-/-}$ and *OXTR*$^{-/-}$ rat pups defective in maternal preference but not USV components of affiliation

The best comparison of the roles of OXT and OXTR between mice and rats would be to use *OXT* and *OXTR* knockout rat pups. Here, we used CRISPR-Cas9 to generate *OXT*$^{-/-}$ rats and *OXTR*$^{-/-}$ rats (Figures S11A and S11B). Oxytocinergic neurons were lost in *OXT*$^{-/-}$ rats (Figure S11C). The mRNA of *OXTR* was absent from *OXTR*$^{-/-}$ rats (Figure S11D).

Compared with *OXT*$^{+/+}$ or *OXT*$^{+/-}$ littermate controls, *OXT*$^{-/-}$ rat pups were not significantly different in maternal separation induced USVs (Figure 6A). USVs induced by maternal separation were also not significantly different among *OXTR*$^{+/+}$, *OXTR*$^{+/-}$, and *OXTR*$^{-/-}$ rat pups (Figure 6B). These results are different from the results of *OXT*$^{-/-}$ mouse pups emitting fewer USVs than WT mouse pups,[16] indicating that the roles for OXT and OXTR in regulating the USV component of affiliation are not conserved.

When rat pups were tested for discrimination between maternal bedding and clean bedding, *OXT*$^{-/-}$ rat pups were not significantly different from their *OXT*$^{+/+}$ or *OXT*$^{+/-}$ littermates (Figure 6C). *OXTR*$^{-/-}$ rat pups were also not significantly different from their *OXTR*$^{+/+}$ or *OXTR*$^{+/-}$ littermates in this assay (Figure 6D).

When facing the choice between maternal bedding and another dam's bedding, *OXT*$^{+/+}$ and *OXT*$^{+/-}$ littermate controls strongly preferred the bedding of their own mothers (Figure 6E). *OXT*$^{-/-}$ rat pups showed no preference (Figure 6E). *OXTR*$^{-/-}$ rat pups were also significantly different from their *OXTR*$^{+/+}$ and *OXTR*$^{+/-}$ littermates in choosing between the bedding of their mothers and that of other dams (Figure 6F). These results indicate that OXT and OXTR are involved in the maternal preference component of affiliative behavior but not in the USV component of affiliative behavior of rat pups.

Avoidance of peppermint was not significantly different among *OXT*$^{+/+}$, *OXT*$^{+/-}$, and *OXT*$^{-/-}$ rat pups (Figure S11G). Peppermint avoidance was also not significantly different among *OXTR*$^{+/+}$, *OXTR*$^{+/-}$, and *OXTR*$^{-/-}$ rat pups (Figure S11H). These results indicate that OXT or OXTR rats are not generally defective in olfaction.

OXT downstream of 5-HT in the maternal preference component of affiliation

Analysis of mutant animals has revealed one phenotype shared by *Tph2*$^{-/-}$, *OXT*$^{-/-}$, and *OXTR*$^{-/-}$ pups that they were all defective in the choice between maternal bedding and other dams' bedding (Figures 2F, 6C, and 6F). This prompted us to examine whether OXT mediated the role of 5-HT in this component of affiliation.

We delivered OXT into *OXT*$^{-/-}$ rat pups by nasal administration. The defect in the choice between maternal bedding over bedding of other dams in *OXT*$^{-/-}$ rat pups was rescued by nasal application of OXT but not AVP (Figure 7A). The fact that OXT and AVP are close in their peptide sequences supports the specificity of OXT in the choice of maternal over other dams' beddings.

After delivered OXT was delivered into *Tph2*$^{-/-}$ rat pups by nasal administration, the defective choice between maternal

Figure 5. Activation of 5-HT and oxytocinergic neurons by maternal odors

(A) Fiber photometry recordings of Ca^{2+} signals from RN 5-HT neurons. (A_1) Viral injections of *AAV-DIO-GCamp7S* into the RN were performed on P0 *Sert-Cre* mice and optical fiber implantation at P14. Fiber photometry recordings were performed at P15 to allow protein expression of GCamp7S (green) in the RN (A_2). (A_3) High magnification images showing all neurons expressing GCamp7S (green) were 5-HT (red) positive. Scale bar of A_2: 100 and 20 μm in A_3.

(B–D) Ca^{2+} responses of 5-HT neurons when mice were sniffing clean bedding, other dams' bedding or mother bedding. Note that stronger responses were elicited by mother bedding ((D), n = 47 trials) than clean bedding ((B), n = 29 trials) and other dams' bedding ((C), n = 50 trials). Time 0 is aligned to sniff initiation (the vertical dash line). Thick lines indicate the mean and shaded area indicate SEM.

(E) Quantification of the peak of △F/F from (B) to (D). *p < 0.05; ***p < 0.001 (one-way ANOVA and Tukey's post hoc).

(F) After stimulation by maternal odor, a subset of (8/19 in the lower three panels) 5-HT neurons (green, anti-TPH2) in the RN were *c-fos* positive (reflected by TRAP:tdTomato, red). Bottom panels are enlargement of the rectangle area indicated in the upper panels.

(G) A majority (15/24 in lower three panels) of oxytocinergic neurons (green, anti-oxytocin) in the PVN were *c-fos* positive (red) in response to maternal odors. Bottom panels are enlargement of the rectangle area indicated in upper panels. Arrow heads indicate co-localization. Scale bar of (F) and (G): 100 μm in upper panels and 20 μm in lower panels.

(H) Maternal odors (n = 4 mice) elicited higher percentage of 5-HT neurons to be *c-fos* positive than clean bedding (n = 4 mice). *p < 0.05 (Mann-Whitney t test).

(I) Quantification of the percentage of c-fos positive OXT neurons by maternal odors (n = 4 mice) or clean bedding (n = 4 mice). *p < 0.05 (Mann-Whitney t test).

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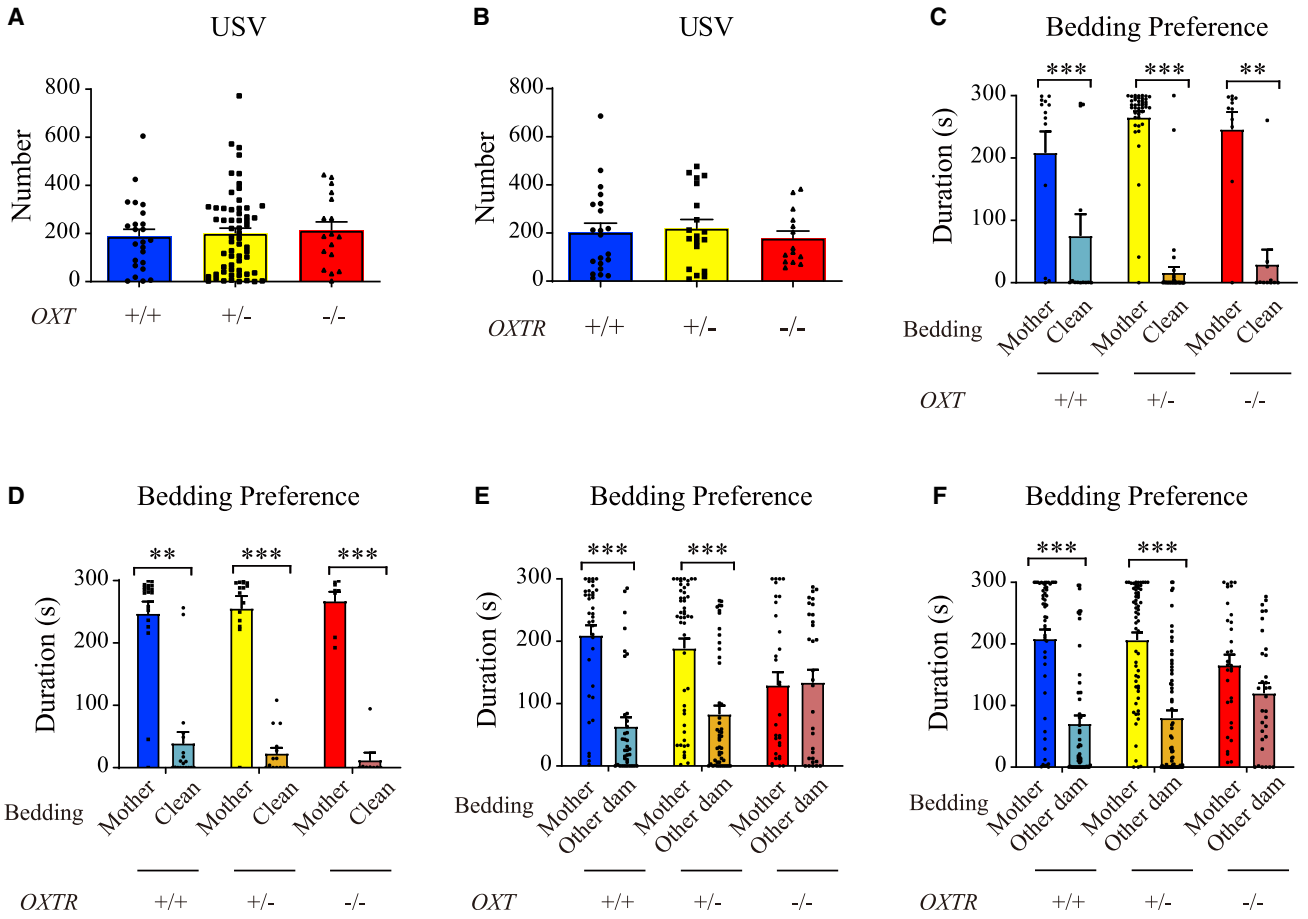

Figure 6. Maternal affiliation by rat pups with or without oxytocin or oxytocin receptor

(A) When separated from their mothers, $OXT^{-/-}$ rat pups (n = 17) emitted USVs at a level similar to $OXT^{+/+}$ (n = 24) and $OXT^{+/-}$ (n = 60) rat pups (one-way ANOVA and Tukey's post hoc).

(B) Separation induced USVs were similar among in with $OXTR^{+/+}$ (n = 21), $OXTR^{+/-}$ (n = 19), and $OXTR^{-/-}$ (n = 14) rat pups. One-way ANOVA and Tukey's post hoc.

(C) Similar to their littermates, $OXT^{-/-}$ rat pups preferred maternal bedding over clean bedding (n = 13 for $OXT^{+/+}$, n = 43 for $OXT^{+/-}$, n = 11 for $OXT^{-/-}$). **p < 0.01; ***p < 0.001 (Wilcoxon matched-pairs t test). $OXT^{+/+}$ (n = 36) and $OXT^{+/-}$ (n = 48) rat pups showed strong preference to their mothers' bedding over other dams' bedding, whereas $OXT^{-/-}$ rat pups (n = 31) had no preference. ***p < 0.001 (Wilcoxon matched-pairs t test).

(D) $OXTR^{-/-}$ rat pups (n = 8) and their littermates preferred maternal bedding over clean bedding (n = 19 for $OXTR^{+/+}$, n = 15 for $OXTR^{+/-}$). **p < 0.01; ***p < 0.001; Wilcoxon matched-pairs t test.

(E) $OXT^{+/+}$ (n = 36) and $OXT^{+/-}$ (n = 48) rat pups showed strong preference to their mothers' bedding over other dams' bedding, while $OXT^{-/-}$ rat pups (n = 31) had no preference. ***p < 0.001 (Wilcoxon matched-pairs t test).

(F) $OXTR^{+/+}$ (n = 52) and $OXTR^{+/-}$ (n = 62) rat pups strongly preferred their mothers' bedding over other dams' bedding. $OXTR^{-/-}$ rat pups (n = 32) showed no preference. *p < 0.05; **p < 0.001 (Wilcoxon matched-pairs t test).

bedding over bedding of other dams in $Tph2^{-/-}$ rat pups was rescued (Figure 7B). The same defect in $OXT^{-/-}$ rat pups could not be rescued by injection of 5-HTP (Figure 7C), the intermediate for 5-HT synthesis, which could rescue defective affiliation in $Tph2^{-/-}$ rat pups (Figures 3F and 3G). The non-reciprocal effects of OXT rescue of $Tph2^{-/-}$ phenotype but no 5-HTP rescue of $OXT^{-/-}$ phenotype support that OXT is downstream of 5-HT in regulating the choice of maternal odors over other dams' odors.

OXT administration could not rescue the defective USVs induced by maternal separation in $Tph2^{-/-}$ rat pups (Figure 7D), which was consistent with our finding of no changes in USVs in $OXT^{-/-}$ or $OXTR^{-/-}$ rat pups (Figures 6A and 6D).

To further investigate OXT functioning in primates, we use nasal spray to deliver OXT into infant monkeys whose 5-HT was pharmacologically depleted by pCPA. OXT application increased the duration of infant monkey spent with their mothers (Figure 7E). These results indicated that the OXT played a conserved role in regulating infant maternal preference from mice to primates.

Chemogenetic inhibition of dorsal raphe 5-HT neurons eliminated mother affiliation

We applied the chemogenetic strategy, by using designer receptors exclusively activated by designer drugs (DREADDS),[74] to

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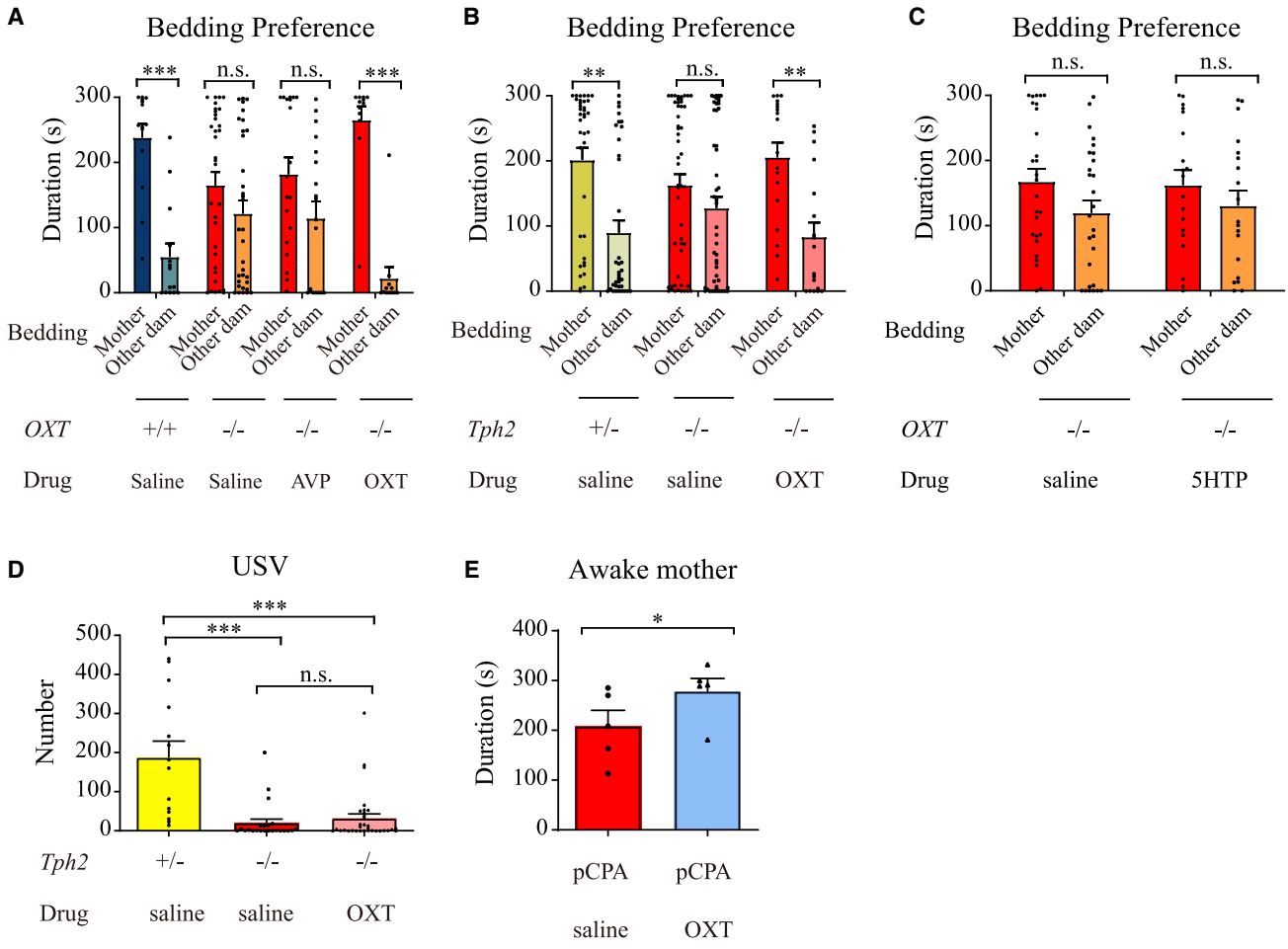

Figure 7. The relationship of 5-HT and oxytocin in regulating affiliation

(A) Unlike $OXT^{+/+}$ (n = 15) rat pups, saline-treated $OXT^{-/-}$ (n = 33) rat pups showed no preference for maternal bedding over other dams' bedding. Nasally applied OXT (50 μg per rat) (n = 12), but not AVP (20 μg per rat) (n = 18), rescued the preference defect of $OXT^{-/-}$ rat pups. **p < 0.001; n.s., not significant; (Wilcoxon matched-pairs t test).

(B) Oxytocin rescued the maternal odor preference of $Tph2^{-/-}$ (n = 18) rat pups. n = 37 for $Tph2^{+/-}$ and n = 50 for $Tph2^{-/-}$ in saline group.

(C) $OXT^{-/-}$ rat pups injected with saline (n = 27) or 5HTP (n = 19) showed no maternal preference.

(D) When separated from their own mothers, saline-treated $Tph2^{-/-}$ (n = 23) rat pups had fewer USVs than $Tph2^{+/-}$ (n = 14) rat pups. Nasal delivery of OXT (0.1 mg per rat) did not significantly change USVs of $Tph2^{-/-}$ (n = 34) rat pups. ***p < 0.001 (one-way ANOVA and Tukey's post hoc).

(E) Nasal spray of OXT (0.2 mg per monkey) increased maternal approach by infant monkeys from which 5-HT was depleted by pCPA. n = 5 for saline treatment and n = 5 for OXT treatment (Wilcoxon matched-paired t test).

inhibit the activity of serotonergic neurons. hM4Di, a Gi-coupled inhibitory DREADD receptor, inhibits neuronal activities. Injection of *AAV-DIO-hM4Di-P2A-mScarlet* into the RN of *Sert-Cre* mouse pups led to Cre-dependent expression of hM4Di in serotonergic neurons (Figure S12A). Electrophysiological recordings in dorsal serotoninergic neuron (DRN) slices revealed that these serotonergic neurons were silenced by hM4Di ligand clozapine-N-oxide (CNO) (Figure S12B).[74] To silence serotonergic neurons *in vivo*, CNO was administered in *AAV-DIO-hM4Di-P2A-mScarlet* injected *Sert-Cre* mice. Pups with serotonergic neurons silenced by chemogenetics were defective in maternal preference (Figure 8A), whereas none of the other (fairly extensive) controls (viral injection alone, CNO alone, *Sert-Cre* alone, or combinations of any two) showed the phenotype (Figure 8A).

5-HT neurons in the RN are composed of DRNs and medial (MRN) serotoninergic neurons (Figure S12C), both projecting to PVN.[75] To delineate which population of serotoninergic neurons regulate mother affiliation, we injected *AAV-DIO-hM4Di-P2A-mScarlet* in the DRN or the MRN separately in *Sert-Cre*$^{+}$ pups (Figures S12D and S12E). Chemogenetic silencing indicated that 5-HT neurons in the DRN were mainly responsible for the maternal odor preference regulation (Figure S12F).

Role of PVN-projecting serotonergic RN neurons in mother affiliation

The RN is known to project to the PVN.[76–79] We carried out a series of experiments to study the functional importance of serotonergic projection from the RN in the PVN.

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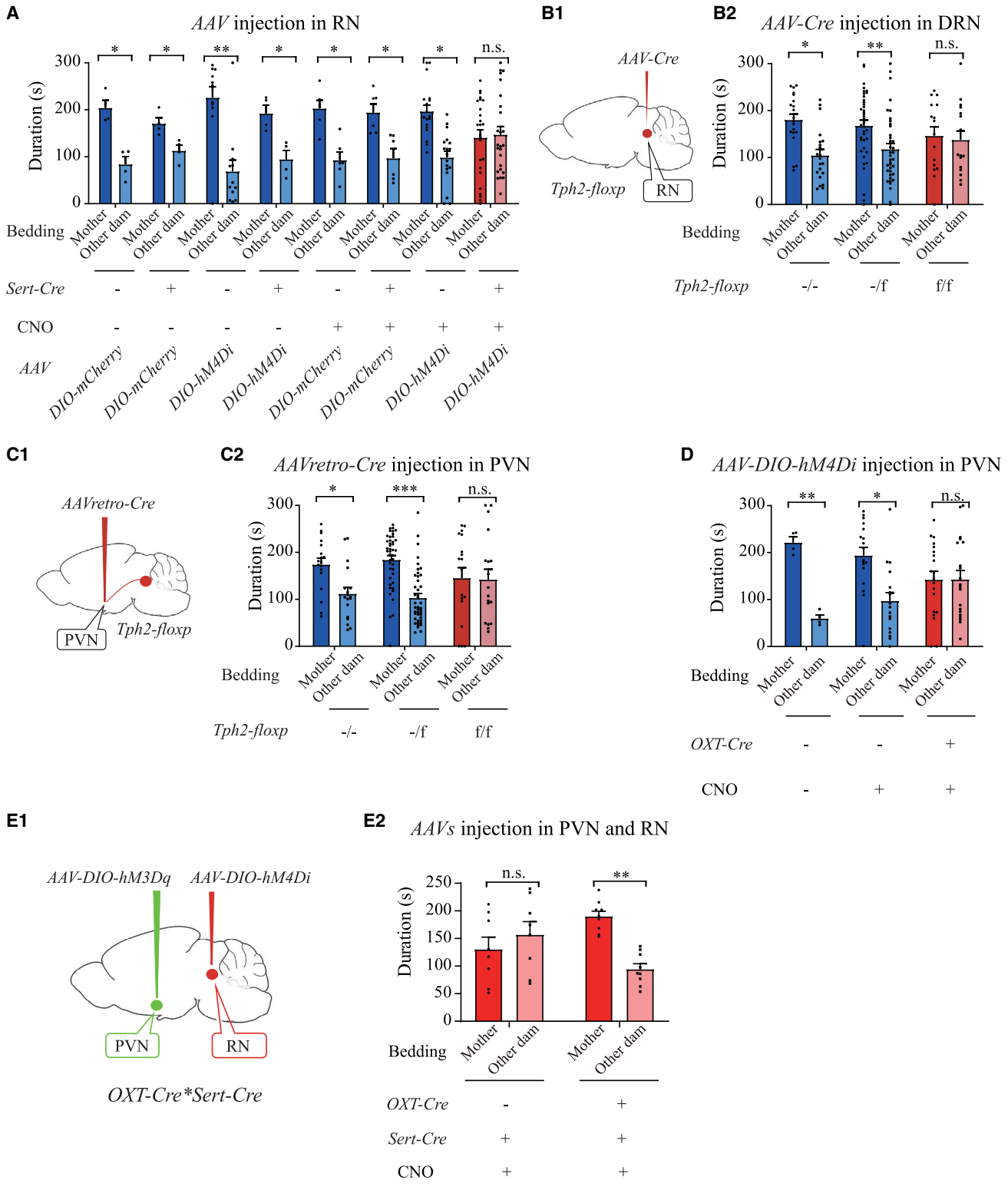

Figure 8. Oxytocinergic neurons downstream of serotonergic neurons in regulating affiliation

(A) As indicated by red bars, chemogenetic silencing (CNO+) of serotonergic neurons (*Sert-Cre*$^{+}$*; AAV-DIO-hM4Di* +) in the RN caused the loss of preference for mothers' bedding. 7 groups of blue bars served as controls, and *AAV-DIO-mCherry* as the control virus. n = 4, 4, 12, 4, 6, 6, 18, and 28 for each group. *p < 0.05; **p < 0.01 (Wilcoxon matched-pairs t test).

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We first examined whether specific depletion of 5-HT in RN serotonergic neurons could affect affiliation. *AAV-Cre* was injected to the RN of *Tph2-floxp* newborn mice (Figure 8B1) before behavioral experiments on P15. *AAV-Cre* mediated deletion of *Tph2* gene in *Tph2-floxp*$^{f/f}$ pups resulted in 5HT depletion in RN (Figure S13A). *Tph2-floxp*$^{f/f}$ pups injected with *AAV-Cre* lost their preference of maternal bedding over other dams' bedding, whereas those retaining both copies of the *Tph2* gene (*Tph2-floxp*$^{-/-}$) or one copy of *Tph2* (*Tph2-floxp*$^{-/f}$) showed significant maternal preference (Figure 8B2). These results confirmed the involvement of RN serotonergic neurons in affiliation.

To investigate the role of RN serotonergic neurons projecting to the PVN in affiliation, we crossed the *Tph2-floxp* mice with the *Ai14* mice (a Cre-Dependent Tdtomato Reporter Line) before injecting a retrovirus,[80] *AAVretro-Cre*, into the PVN of *Tph2-floxp*$^{f/f}$*$Ai14^{+}$ mouse pups (Figure 8C1; Figure S13B). The retrovirus infected the terminals of serotonergic neurons in the PVN and was retrogradely transported to cell bodies in the RN where they deleted floxp-flanked *Tph2* gene (Figures S13D and S13E). *Tph2* deletion in the RN of *Tph2-floxp*$^{f/f}$*;$Ai14^{+}$ mice by *AAVretro-Cre* injected into the PVN resulted in a loss of preference of maternal bedding over other dams' bedding (Figure 8C2), whereas pups retaining both copies of the *Tph2* gene (*Tph2-floxp*$^{-/-}$) or one copy of *Tph2* (*Tph2-floxp*$^{-/f}$) showed significant maternal preference (Figure 8C2). There are about 128 ± 29 5-HT neurons in DRN per mice and 205 ± 18 5-HT neurons in MRN per mice were infected by the *AAVretro-Cre* in PVN.

Additionally, we used optogenetics to stimulate the 5-HT fiber. *AAV-DIO-hM4Di-P2A-mScarlet* and *AAV-DIO-ChR2-mcherry* were injected together into the RN of *Sert-Cre*$^{+}$ pups to drive specific expression of *hM4Di* and *ChR2* in serotonergic neurons (Figures S14A–S14C). Light could stimulate 5-HT neurons even with CNO (Figures S14D1 and S14D2). Optogenetic activation of 5-HT fibers in PVN could rescue the behavior effect of somatic chemogenetic inhibition of DRN 5HT neurons (Figure S14E).

These results indicated that PVN-projecting serotonergic neurons in the RN were involved in the maternal preference component of affiliation.

Oxytocinergic neurons downstream of serotonergic neurons in the maternal preference component of affiliation

To further delineate serotoninergic neurons projection to PVN oxtocinergic neurons, we used a Cre-dependent rabies virus, which mediated monosynaptic retrograde tracing in *OXT-Cre* mice. We found that OXT neurons in the PVN received direct inputs from 5-HT neurons in the RN (Figures S15A–S15C). We carried out three sets of experiments to study the functional importance of serotonergic projection from the RN to oxytocinergic neurons in the PVN.

Chemogenetic silencing of oxytocinergic neurons in the PVN resulted in defective maternal preference (Figure 8D), which was significantly different from any of the controls (injection of *AAV-DIO-hM4Di* virus into pups without *OXT-Cre*, no CNO treatment, or CNO treatment of pups without *OXT-Cre*) (Figure 8D).

We examined whether activation of oxytocinergic neurons could rescue the maternal preference defect caused by silencing of serotonergic neurons (Figure 8E1) via chemogenetic method. hM3Dq, a Gq-coupled exhitatory DREADD receptor, increased neuronal activities (Figure S15D). *AAV-DIO-hM4Di* and *AAV-DIO-hM3Dq-EGFP* (Figure S15E) were injected to the RN and the PVN, respectively, of pups carrying either *Sert-Cre or* both *Sert-Cre* and *OXT-Cre*. Application of CNO inactivated serotonergic neurons injected with *AAV-DIO-hM4Di* in the RN and activated oxytocinergic neurons injected with *AAV-DIO-hM3Dq-EGFP* in the PVN (Figure 8E1). We found that activation of oxytocinergic neurons in the PVN rescued the maternal preference defect caused by silencing of serotonergic neurons in the RN (Figure 8E2). Furthermore, chemogenetic activation of 5-HT neurons in the DRN could not rescue behavior effects of inhibiting oxytocin neurons in the PVN (Figure S15F).

5-HT activation of its receptors[81–83] could either be excitory or inhibitory.[84,85] First, we applied 5-HT on the slice and found that the activity of OXT neurons was increased, which returned to baseline upon wash (Figures S16A–S16C). Second, we specifically expressed ChR2 in 5-HT neurons and labeled OXT neurons by mCherry (Figures S16D–S16F). When we optogenetically stimulated serotonergic neuronal terminals in the PVN, OXT neurons were activated (Figure S16G), indicating that 5-HT from the DRN activates OXT neurons in the PVN.

Taken together, these results support that oxytocinergic neurons act downstream of serotonergic neurons in regulating maternal affiliation by infants.

DISCUSSION

Our results have demonstrated a role for 5-HT in infant affiliation behavior, which is a crucial social behavior in mammals. This is the first report of a molecule shown to play a behavioral role conserved from mice, rats to monkeys by genetic targeting.

(B_1) *AAV-Cre* injection in RN. (B_2) Unlike *Tph2-floxp*$^{-/-}$ (n = 21) and *Tph2-floxp*$^{-/f}$ mice (n = 39), *Tph2-floxp*$^{f/f}$ mice (n = 16) injected with *AAV-Cre* in the RN showed no preference of mothers' bedding over other dams' bedding. *p < 0.05; n.s., (Wilcoxon matched-pairs t test).

(C_1) a schematic illustration of bilateral injections of *AAV$_{retro}$-Cre* into the PVN of *Tph2-floxp* mice.

(C_2) Conditional knockout of *Tph2* from RN neurons projecting to the PVN following viral injection in *Tph2-floxp*$^{f/f}$ mice (n = 18) resulted in the loss of bedding preference, as compared with that in *Tph2-floxp*$^{-/-}$ (n = 18) and *Tph2-floxp*$^{-/f}$ mice (n = 43). *p < 0.05; ***p < 0.001; n.s., not significant (Wilcoxon matched-pairs t test).

(D) Chemogenetic silencing (CNO +) of oxytocinergic neurons (*OXT-Cre*$^{+}$) through *AAV-DIO-hM4Di* injection in the PVN reduced the preference for mothers' bedding (shown in red bars, as compared with control blue bars). n = 6, 18, and 20 for each group. *p < 0.05; **p < 0.01 (Wilcoxon matched-pairs t test).

(E) Chemogenetic silencing of serotonergic neurons in the RN and activation of oxytocinergic neurons in the PVN by *AAV-DIO-hM4Di* and *AAV-DIO-hM3Dq*, respectively. (E_1) a schematic illustration of viral injections. (E_2) Silencing (CNO +) serotonergic neurons in the RN (*Ser-Cre* $^{+}$), and leaving the activity of oxytoncinergic neurons unchanged (*OXT-Cre*$^{-}$), led to the loss of preference for mothers' bedding in *Ser-Cre*$^{+}$ **OXT-Cre*$^{-}$ mice (n = 8). Chemogenetic activation of oxytocinergic neurons on the basis of slicing 5HT neurons in *Ser-Cre*$^{+}$ **OXT-Cre*$^{+}$ mice, restored preference to mother bedding of mouse pups (n = 9). **p < 0.01; n.s., not significant (Wilcoxon matched-pairs t test).

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Furthermore, we have combined genetics, pharmacology, optical imaging, immunocytochemistry, and chemogenetics to support that OXT, OXTR, and oxytocinergic neurons in the PVN are electrophysiologically and functionally downstream of 5-HT and serotonergic neurons in the RN, revealing a part of the circuit mediating 5-HT regulation of affiliation behavior. We speculate that 5-HT may serve as a key regulator of OXT and arginine-vasopressin (AVP) neurons in social behaviors, and thus 5-HT may play more roles in social behaviors than previously suspected.

The roles of 5-HT in affiliation are specific. They are not secondary to vocalization or olfactory problems in *Tph2*$^{-/-}$ mutant mice and rats (Figures 1C and 2C; Figures S1E, S1F, S2C–S2F, S3E, S3F, and S4C–S4F) or visual problems in *Tph2*$^{-/-}$ monkeys (Figures 4I and 4J). They are also not developmental because the affiliation phenotype could be created in *Tph2*$^{+/+}$ pups by pharmacological inhibition of 5-HT synthesis (Figures 3A–3E; Figures S6D–S6H) and the affiliation phenotype in *Tph2*$^{-/-}$ pups could be rescued by 5-HTP injection (Figures 3F and 3G). A previous paper reported that USVs by infants were increased,[86] but it did not specifically study maternal affiliation by infants and did not distinguish between USVs elicited by temperature changes vs. those elicited by maternal deprivation.

The roles of 5-HT in affiliation are conserved between mice and rats in all assays: USVs induced by maternal separation, preference of maternal over clean bedding, and preference of maternal over other dams' bedding (Figures 1 and 2).

The roles of OXT and OXTR in affiliation behavior are more limited and apparently not all conserved between mice and rats. *OXT* and *OXTR* knockout mice have been generated previously.[18,87–89] *OXT*$^{-/-}$ mouse pups emitted fewer USVs than WT mouse pups.[16] However, it was apparently confusing because injection of OXT into rat pups was previously reported to reduce USVs.[24] We have generated *OXT*$^{-/-}$ and *OXTR*$^{-/-}$ rat pups but found that they are not defective in USV emission (Figures 6A and 6D) or the choice between maternal and clean beddings (Figures 6B and 6E), but they are only defective in the choice between maternal bedding and other dams' bedding (Figures 6C and 6F). Thus, OXT and OXTR are involved in some but not all components of affiliative behavior in rats.

Ca^{2+} imaging (Figure 5D) and *c-fos* immunocytochemistry (Figures 5F and 5H) have shown the activation of serotonergic neurons in the RN by maternal odors. *c-fos* immunocytochemistry also shows activation of oxytocinergic neurons in the PVN by maternal odors (Figures 5G and 5I). OXT administration could rescue the maternal preference phenotype of *Tph2*$^{-/-}$ pups, but 5-HTP could not rescue the same phenotype in *OXT*$^{-/-}$ pups, supporting that OXT is downstream of 5-HT.

Serotonergic neurons in the DRN have been known to project to the VTAT for reward and the BNST for anxiety.[90] Our work provides a circuit for regulating the social behavior. Viral-genetic deletion of 5-HT from serotonergic neurons in the RN projecting to the PVN resulted in defective maternal preference (Figure 8B). Optogenetic activation of serotonergic terminals in the PVN rescued the behavior phenotype caused by inhibition of 5-HT neurons in the RN (Figure S14), indicating that RN serotonergic neurons projecting to the PVN are required for affiliative behavior. The maternal preference defect caused by chemogenetic inhibition of RN serotonergic neurons could be rescued by chemogenetic activation of PVN oxytocinergic neurons (Figure 8E), further supporting that RN serotonergic neurons function upstream of PVN oxytocinergic neurons in regulating maternal preference. Furthermore, the rabies virus tracing proved the OXT neurons in PVN received direct input from 5-HT neurons in RN. In addition to the brain,[44–48] *Tph2* was also expressed in serotonergic enteric neurons.[91,92] Our virally mediated *Tph2* knockout experiments in the mouse brain established the importance of *Tph2* in the brain for 5-HT regulation of affiliation behavior.

Our *retroAAV* experiments observed more serotonergic neurons in the MRN projecting to the PVN than those in the DRN. Some 5-HT neurons in the MRN, as well as some in the DRN, have direct connections with OXT neurons in the PVN. However, the chemogenetic results indicate that the 5-HT neurons in the DRN is responsible for regulating maternal odor preference. The functional role of the 5-HT neurons in the MRN projecting to the PVN remains to be further investigated.

An obvious candidate as another molecule downstream of 5-HT in social behaviors is AVP. Separation induced USV was reduced in mice mutant for the 1b receptor of AVP (AVP1b).[14] AVP1b receptor mutant mouse pups were not defective in maternal bedding preference.[14] One possibility is that AVP mediates the role of 5-HT in USVs induced by maternal separation while OXT mediates the role of 5-HT in preference for maternal odors.

Our genetic studies in monkeys provide strong evidence that 5-HT plays a role in primate infants. Behaviors of monkeys are closer to humans than rodents.[93] Both analysis of daily life and assays of visually based mother preference indicate that the *Tph2*$^{-/-}$ infant monkeys were defective in preference for their own mothers, while *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ monkey infants were not significantly different in distinguishing familiar and unfamiliar faces. These results indicate that 5-HT plays a conserved role in infant affiliation.

In additional to rodent studies, pharmacological experiments in infant monkeys have demonstrated that OXT works downstream of 5-HT in regulating maternal preference in non-human primates (Figure 7E).

In humans, infants interact with the caregiver.[6] 5-HT has been implicated in regulating infant attention to emotional cues[94] and genetically associated with autism spectrum disorder (ASD).[95] Our work should stimulate further research on the role(s) of 5-HT in human infants.

We note here that, because maternal affiliation with one's mother is usually the first social behavior of an animal, its defect may affect other behaviors in later life including those during adulthood. It is of paramount importance to study molecular basis of infant behaviors than adult behaviors, both for the intrinsic value of studying infant behaviors and for ruling out that effects on later behaviors were secondary to those on infant behaviors.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.neuron.2023.02.010.

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AUTHOR CONTRIBUTIONS

Y.R. and Y.L. directed this study. Experimental design, data acquisition, and interpretation: Y.L., L.S., T.L., J.L., Y.C., C.S., C.Y., X.B., Y.N., C.Z., J.X., and Y.R.; manuscript writing: Y.R. and Y.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-Tph2	Sigma-Aldrich	Cat#NB100-74555
Rabbit polyclonal anti-5-HT	ImmunoStar	Cat#20080
Rabbit polyclonal anti-OXT	Millipore	Cat#ABN911
Bacterial and virus strains		
AAV-CMV_bGI-Cre-eGFP	Shanghai Taitool Bioscience Co	Cat#S0263-9
AAVretro Plus-hSyn-Cre-WPRE-pA	Shanghai Taitool Bioscience Co	Cat#S0230-2RP
AAV-hEF1a-DIO-mcherry-WPRE-pA	Shanghai Taitool Bioscience Co	Cat#S0197-9
AAV- hSyn-DIO-hM3D(Gq)-eGFP-WPRE-pA	Shanghai Taitool Bioscience Co	Cat#S0260-9
AAV- hEF1a-DIO-hChR2(H134R)-mCherry-WPRE-pA	Shanghai Taitool Bioscience Co	Cat#S0170-9
AAV- EF1a-DIO-hM4Di-P2A-mScarlet-WPRE-PA	CIBR	N/A
AAV- EF1a-DIO-GCaMP7s	CIBR	N/A
AAV-EF1a-DIO-H2B-EGFP-T2A-WPRE-hGH PolyA	BrainVTA Technology Co. Ltd., China	Cat#PT-0021
AAV-EF1a-DIO-RVG-WPRE-hGH PolyA	BrainVTA Technology Co. Ltd., China	Cat#PT-0023
RV-CVS-ENVA-△G-mcherry	BrainVTA Technology Co. Ltd., China	N/A
Chemicals, peptides, and recombinant proteins		
p-chlorophenylalanine methyl ester hydrochloride	Sigma-Aldrich	Cat#C3635
5-HTP	Sigma-Aldrich	Cat#H9772
Oxytocin acetate salt hydrate	Sigma-Aldrich	Cat#O6379
CNO	Sigma-Aldrich	Cat#C0832
4-Hydroxytamoxifen	Sigma-Aldrich	Cat#H6278
Critical commercial assays		
RNA simple total RNA kit	TIANGEN	Cat#DP419
RevertAid First Strand cDNA Synthesis Kit	Thermo	Cat#K1621
Deposited data		
Face database of non-human primates	visiome	http://visiome.neuroinf.jp/primface
Experimental models: Organisms/strains		
Mouse: Tph2$^{-/-}$: B6	Liu et al.[48]	N/A
Mouse: Tph2-$^{fl/fl}$: B6-Tph2tm1Zfc/J	The Jackson Laboratory	RRID:IMSR_JAX:027590
Mouse: Sert-Cre: B6-Slc6a4tm1(cre)Xz/J	The Jackson Laboratory	RRID:IMSR_JAX:014554
Mouse: OXT-Cre: B6-Oxttm1.1(cre)Dolsn/J	The Jackson Laboratory	RRID:IMSR_JAX:024234
Mouse: TRAP2: B6-Fostm2.1(icre/ERT2)Luo/J	The Jackson Laboratory	RRID:IMSR_JAX:030323
Mouse: Ai14: B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J	The Jackson Laboratory	RRID:IMSR_JAX:007914
Rat: OXT$^{-/-}$	This paper	N/A
Rat: OXTR$^{-/-}$	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rat: Tph2$^{-/-}$	This paper	N/A
Monkey: Tph2$^{-/-}$	This paper	N/A
Oligonucleotides		
Rat: Tph2-/- Genotyping-Forward: GACCATATGTCTTGTCACGTTTC	This paper	N/A
Rat: Tph2-/- Genotyping-Reverse: CTTCTCGGGTGTCCTTTGGGGCAG	This paper	N/A
Rat: OXT-/- Genotyping-Forward: CAAGAGACCTGCTGTGACCA	This paper	N/A
Rat: OXT-/- Genotyping-Reverse: CGCTGTGCACAATCCATATC	This paper	N/A
Rat: OXTR-/- Genotyping-Forward: GTGGTTCATCGCAAGCCTCCTC	This paper	N/A
Rat: OXTR-/- Genotyping-Reverse: GAGTCTCAGATTCTGCCAGATCTTG	This paper	N/A
Rat: OXTR-/- RT-PCR-Forward: CTGCTGTGTCGTCTGGTCAA	This paper	N/A
Rat: OXTR-/- RT-PCR-Reverse: CCAGCAATCGAAGACTCCGT	This paper	N/A
Software and algorithms		
MATLAB	MathWorks	https://www.mathworks.com/
GraphPad Prism (version 8.0.2)	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Avisoft-SASLab Pro	Avisoft Bioacoustics	https://www.avisoft.com/sound-analysis/
Avisoft-RECORDER USGH	Avisoft Bioacoustics	https://www.avisoft.com/downloads/
inper Data Process	Inper	https://inper.com/shop/product/15#attr=455

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yi Rao (yrao@pku.edu.cn).

Materials availability

Animals generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Stocks

Tph2$^{-/-}$ mice had been described previously.[48] The *Tph2*$^{-/-}$ line was maintained by crossing *Tph2*$^{+/-}$ heterozygotes. Littermates include *Tph2*$^{+/+}$, *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ mice. *Tph2-floxp* mice (JAX 027590) were also maintained by crossing heterozygotes, but *Sert-Cre* (JAX 014554), *OXT-Cre* (JAX 024234), *TRAP2* (JAX 030323) and *Ai14* tdTomato Cre reporter mice (JAX 007914) were maintained by crossing heterozygotes with the wild type. The genotyping primers for *Sert-Cre* mice were: GAGCTCTCAGTCTTGTCTCCA, GAGT GTGGCGCTTCATCC, AGGCAAATTTTGGTGTACGG. The genotyping primers for *OXT-Cre* mice were: ACACCGGCCTTATTCCAAG,

TTTGCAGCTCAGAACACTGAC, AGCCTGCTGGACTGTTTTTG. All mice were backcrossed to C57B/6 for at least 10 generations before use. Mice were maintained on a 12 hour light:12 hour dark cycle. Food and water were provided ad libitum. RT was 22±1°C and the humidity was 40-60%. Pregnant females were examined daily for newborn pups and 6 offsprings from each colony were maintained for behavior assays. Both male and female pups were used for behavior tests. All tests were carried out before weaning, and the time for each tests were described in the method details.

Generation of *Tph2*$^{-/-}$, *OXT*$^{-/-}$ and *OXTR*$^{-/-}$ rat

All these three genes were mutated in SD rats using the CRISPR method. For *Tph2* gene, three guide RNAs (gRNAs) were designed to disrupt the 6th exon of *Tph2*. Cas9 mRNA and three gRNAs were injected into 120 fertilized eggs of SD rats. The oosperms were implanted to surrogate females and 31 offsprings were obtained. Tail samples were used for sequencing. A male rat lacking the entire exon 6 was chosen as the founder. Primers for genotyping were: GACCATATGTCTTGTCACGTTTC, CTTCTCGGGTGTCCTT TGGGGCAG. The procedure of generating *OXT*$^{-/-}$ and *OXTR*$^{-/-}$ rat were similar. For *OXT* gene, three guide RNAs (gRNAs) were designed to disrupt the 1st exon of *OXT*. A rat lacking 86 base pairs in 1st exon of *OXT* gene, which caused frame shift mutation, was chosen as the founder. For *OXTR* gene, two guide RNAs (gRNAs) were designed to disrupt the 1st exon of *OXTR*. A rat lacking 117 bp to 367 bp from ATG start codon, was chosen as the founder. All rats were maintained on a 12 hour light:12 hour dark cycle. Food and water were provided ad libitum. RT was 22±1°C and the humidity was 40-60%. Pregnant females were examined daily for newborn pups and 12 offsprings from each colony were maintained for behavior assays. Both sex were used for behavior test. All tests were carried out before weaning, and the time for each tests were described in the method details.

Generation of *Tph2*$^{-/-}$ rhesus macaques by TALENs

All animals were housed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility at Kunming Biomed International (KBI). All animal procedures were approved by the Institutional Animal Care and Use Committee. Healthy female monkeys with regular menstrual cycles were used as oocyte donors for superovulation by intramuscular injection with rhFSH (Recombinant Human Follitropin, Merck Serono) for 8 consecutive days followed by rhCG (Recombinant Human Chorionic Gonadotropin, Merck Serono) on day 9. Oocytes were collected by laparoscopic follicular aspiration 32-35 hours after rhCG treatment. Target gene TALENs were injected into the cytoplasm of 80 one-cell embryos fertilized by intracytoplasmic sperm injection (ICSI). Embryos developed till the 2-cell to blasterocyst stage were transferred to surrogate females. Surrogates at synchronized reproductive cycles were identified through their hormone profiles. The earliest pregnancy was diagnosed by ultrasonography about 20-30 days after transfer. Both pregnancy and number of fetuses were confirmed by fetal cardiac activity and the presence of a yolk sac as detected by ultrasonography.[96]

Monkey housing

After birth, infant monkeys were raised by, and living with, their mothers. Animals were housed in a controlled environment. The light cycle is 12L:12D cycle (lights on at 08:00 a.m.). The temperature was set to 22±1°C and humidity was set to 50%±5%. Both male and female infant monkeys were used for behavior tests. All tests were carried out before 6 month old.

All animal procedures were approved by the Institutional Animal Care and Use Committee.

METHOD DETAILS

Monoamine measurement

Levels of 5-HT and its metabolites in mouse, rat and monkey samples were separated by high performance liquid chromatography (HPLC) and measured by an electrochemical detector. Mouse and rat pups were euthanized before perfusion by saline to remove blood from their brains for further dissection. Monoamines were extracted by perchloric acid from homogenates of whole brains. Samples were injected into RP-HPLC (Waters). DA, 5-HIAA and 5-HT were measured by an electrochemical detector. Their concentrations were calculated by Empower 3 software (Waters) based on standard samples. For infant monkeys, the cerebrospinal fluid (CSF) was injected into RP-HPLC (Antec). DA, 5-HIAA and 5-HT were measured by an electrochemical detector. Their concentrations were calculated by DataApex Clarity based on standard samples.

Maternal separation induced vocalization

The test was carried out with mouse pups at postnatal day 7. Mouse pups were removed individually from their home cages and placed in a clean beaker with room temperature or 35°C. USV from mouse pups was recorded by a recorder (Avisoft Ultrasound Gate) for 5 minutes. Recorded data was analyzed with SASLab (Avisoft) by experimenters in a double blind manner. Sounds over the frequency range of 30 Hz-110 kHz was analyzed.

Maternal potentiation

At postnatal day 10, rat pups were removed individually from their home cages and placed into in a clean beaker with temperature of 35°C. Their USV was recorded using a recorder (Avisoft Ultrasound Gate) for 3 minutes. Rat pups were placed back into the home cage near its anesthetized mother for 2 min, before being placed into the beaker again and recorded for next 3 min. USV recorded in these two periods were analyzed in a double-blind manner.

USV upon exposure to different beddings

Mouse pups were tested on PSD 7 and rat pups on PSD 10 in this assay. Social odors came from the same species as the tested pups. Three beakers were covered with clean bedding, bedding from the home cage of a pup or bedding from the cage of an unfamiliar mother. Pups were individually placed in one of three beaker and their USV was recorded. USV in each beaker were analyzed in a double-blind manner.

USV induced by heating and cooling

Mouse (of PSD 7) and rat (of PSD 10) pups were tested in this assay. Pups were individually placed in a chamber with at 56°C (heating) or in a beaker immersed in cold water at ~4°C (cooling) for 30 seconds. USV was recorded and analyzed in a double-blind manner.

Bedding preference

Mouse (of PSD 15) and rat (of PSD 11) pups were tested in this assay. The chamber for bedding preference test is an black Plexiglas box (30cm×25cm×20 cm) with a metal grating of the same size as the bottom area. At the beginning of a test, two kinds of beddings were evenly placed on either side of the box under the grating, with a 2 cm wide blank zone of no bedding. Pups were individually placed in the middle blank zone and its locomotion were recorded by a camera on the top of the box. The total duration of a pup spent in each area was analyzed in a double-blind manner. The preference ratio was calculated as the percentage of time the pup spent on one bedding divided by the total time on both beddings.

5-HT depletion by pCPA treatment

SD rat pups was randomly divided into a moderate dosage pCPA (200 mg/kg) group, or a higher dosage pCPA (600 mg/kg) group, or a saline-treatment group. *Tph2*$^{+/-}$ rat pups were treated with either 200 mg/kg pCPA or saline.

pCPA was dissolved in saline injected intraperitonially (10 ml/kg, i.p.) at 24 hours before behavioral testing. The control groups received saline. Pups lived with their own mothers after the injections.

Wild type infant monkeys (of 3 to 5 months old) were injected with saline (*i.p.*) for 3-day before behavioral tests. One week later, these infant monkeys received pCPA (100 mg/kg, i.p.) for 3 days before behavioral tests.

RT-PCR of *OXTR* in *OXTR*$^{-/-}$ rat

The total mRNA from the brains of *OXTR*$^{+/+}$ and *OXTR*$^{-/-}$ rat was extracted by RNA simple total RNA kit (TIANGEN) and then reversed into cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo). The cDNA of *OXTR* was detected by PCR. The PCR primers for *OXTR* were: CTGCTGTGTCGTCTGGTCAA, CCAGCAATCGAAGACTCCGT. The PCR primers for *GAPDH* were: AGAACATCATC CCTGCATCC, CACATTGGGGGTAGGAACAC.

Analysis of infant-mother interactions of rhesus macaques in their daily life

Daily behaviors of each pair of infant monkeys (of 6 to 7 months) and its mother in their home cages were recorded using a digital video camera for a length of 23 min twice per day (one in the morning and one in the afternoon). 132 frames (with 10-second intervals) of each video were analyzed. The number of ''separation'' (no physical contact between the infant and the mother) and ''ventral contact'' (infant being belly to belly with its mother) among 132 frames was determined. For each mother-infant pair, 12 video were analyzed and the mean frequency of each pattern was used as individual frequencies.

Infant approach to sedated mother

Infant monkeys were tested when they were 3 to 5 months old. A wire cage was used for this test. The size of cage was 120×60×80 cm (l×w×h). Similar to a previous report,[97] mother monkey was anaesthetized by 10 mg/kg of ketamine and 1.5 mg/kg of xylazine. The mother monkey was placed in one corner of the cage. Her infant was introduced into the cage. A video camera recorded infant behaviors for 10 minutes. Videos were analyzed later for the latency and duration of infant interaction of direct body contact with its mother.

Infant approach to awake mother

A mother monkey was placed in its home cage. Her infant monkey (of 3 to 5 months old) was introduced into the neighboring cage. They could see, smell and hear, but could not touch each other. A video camera was used to record infant behavior for 10 minutes. Videos were analyzed later for the latency and duration of the infant to stay close the mesh wire closed to mother cage (defined as infant on the separating wire or within 5 cm of the separating wire).

Picture choice

A cage made of acrylic was used, with a size of 120×60×80 cm (l×w×h). Two LED screens controlled by a computer were placed at the opposite side of the cage. Before the picture choice test, an infant monkey was placed in the cage for 20 minutes each day for a week for habituation, without being shown any pictures. On the test day, the infant monkey was placed in the test cage for 10 minutes before pictures were presented on the LED screens. After pictures were present on the LED screens, a video camera recorded infant behavior for one minute. Videos were analyzed later for the duration of the infant staying within 5 cm of each screen.

Face recognition

Pictures of faces from twenty rhesus monkeys, downloaded from the visiome website (http://visiome.neuroinf.jp/primface), were divided into two groups randomly. Pictures from 10 monkeys were presented on an LED screen to an infant monkey from 9:00 am to 4:30 pm each day for 5 consecutive days and treated as the familiar face pictures on the test day. Pictures of another 10 monkey faces were used as the new pictures on the test day.

Human intruder test

This test was performed with infant monkeys of 6 months old. At the beginning of a test, an infant was individually placed in a new cage in a new room for 10 min habituation. Then a human intruder (female, unfamiliar to the infant) entered the room and sat at 0.3 m away from the cage with her face toward the monkey for 2 min (“profile period”). The intruder left the room leaving the infant alone for the next 2 min. The intruder re-entered the room, sitting at the same place keeping continuous direct eye contact with the monkey for 2 min (“stare period”) before exiting. Infant behavior in these three periods was recorded by a video camera behind the intruder. Videos were analyzed in a double-blind manner and behavioral parameters included frequency of vocalization and duration of eye contact with the human intruder in both “profile period” and “stare period”.

Locomotion

A sound proof room (l×w: 240×200 cm) was used for the locomotion test. The rectangle ground was divided into squares (40×40 cm) using straight lines. The test monkey was place in the room and a video camera fixed on the ceiling recorded its behavior in 5 min. Videos were analyzed by the computer for how many squares the monkey has crossed.

Active avoidance test

It evaluated monkey’s fear response to loud noises and was described by others.[97] Briefly, two observation chambers (80×80×80 cm) were connected by a tunnel (60×40×20 cm). A high-pitch loudspeaker was placed in each chamber. The test monkey was placed in one chamber for 15 min per day for 5 days, to be familiar with the chambers and learning how to move between the chambers. On the test day, an infant was introduced into a chamber and allowed 10 min before a loud noise (120 dB) was presented in the chamber where the monkey was. A video camera recorded the monkey’s response. The latency of the monkey to escape to another chamber was analyzed.

Pharmacological administration

Oxytocin acetate salt hydrate (sigma, O6379) was dissolved in saline and the final concentration was 12.5 mg/ml. 5-HTP (sigma, H9772) and CNO (sigma, C0832) were also dissolved in saline.

For mouse and rat pups, 4μl oxytocin solution was applied nasally[98] to rat pups 1hr before the behavior test. 5-HTP (4 mg/kg) were injected intraperitoneally 1hr before behavior test. CNO (2 mg/kg i.p.) was administrated 1hr before behavior test.

For infant monkeys, OXT (200 μg per monkey) was delivered by nebulization (PARI TurboBOY, Germany) and 5-HTP (3mg/kg) were injected intraperitoneally 1hr before behavior test.

Stereotaxic surgeries

Mouse pups were injected AAV on the day of birth. Mouse pups were anesthetized with 1.5% isoflurane and placed in a stereotaxic apparatus designed for young mice (RWD, China). Injection was carried out by Nanoliter 2000 microinjector (WPI, USA). For better survival rate, the head skin of the neonatal mice was not removed. Because the skin and head bone of newborn mice is soft and thin, they can be penetrated by beveled glass pipettes used for the AAV injection. Blood vessels under the skull, the sinus and the transverse sinus, were landmarks to guide surgery. The intersection point of the sinus and the transverse sinus was defined as zero point. For RN injection, pipette tips were placed 0.1 mm post the zero point with a depth of 3.2 mm from head surface. For DRN injection, the injection site was 0.1mm post zero-point, 2.4 mm depth. For MRN injection, the injection site was zero-point, 3.8 mm depth. For bilateral PVN injection, the injection site was 2.45 mm anterior the zero-point, 0.1 mm both left and right, 3.95 mm depth. Injection was slow (40 nl/min). After the injection, the glass pipette was left in place for 10 additional mins before being withdrawn slowly. Neonatal mice recovered on a heater before being returned to their mother cages.

For rabies tracing, a mixture of *AAV-EF1a-DIO-H2B-EGFP-T2A-WPRE-hGH polyA* and *AAV-EF1a-DIO-RVG-WPRE-hGH polyA* was injected into the PVN. Two weeks later, *RV-CVS-ENVA-△G-mcherry* was injected into the PVN.

Viruses

Viruses were injected at P0 in mouse pups (indicated as virus, location, volumn). *AAV-CMV_bGI-Cre-eGFP* (Shanghai Taitool Bioscience Co), RN, 400 nanoliters (nl). *AAVretro Plus-hSyn-Cre-WPRE-pA* (Shanghai Taitool Bioscience Co) PVN, 20 nl. *AAV-hEF1a-DIO-mcherry-WPRE-pA* (Shanghai Taitool Bioscience Co) RN, 500 nl. *AAV- EF1a-DIO-hM4Di-P2A-mScarlet-WPRE-PA* (CIBR) RN 500 nl; DRN 100 nl; MRN 60 nl; PVN, 350 nl. *AAV- hSyn-DIO-hM3D(Gq)-eGFP-WPRE-pA* (Shanghai Taitool Bioscience Co), PVN, 350 nl. *AAV- EF1a-DIO-GCaMP7s* (CIBR), RN, 500 nl. *AAV-EF1a-DIO-H2B-EGFP-T2A-WPRE-hGH polyA*, *AAV-EF1a-DIO-RVG-WPRE-hGH polyA* (BrainVTA Technology Co. Ltd., China), PVN, 300 nl. *RV-CVS-ENVA-△G-mcherry* (BrainVTA Technology Co. Ltd., China), PVN, 200 nl. *AAV-DIO-ChR2-mcherry* (Shanghai Taitool Bioscience Co), DRN 150 nl

Histology

Animals were perfused transcardially with saline and then with 4% paraformaldehyde (PFA). Brains were dissected and fixed in 4% PFA for 12 hours in 4°C, then placed in 30% sucrose for cryoprotection. Brains were sectioned coronally (30 μm thick) with a cryostat (Leica). Slides were washed in PBS and blocking by 2% BSA in PBS with 0.3% Triton X-100. Slides were then incubated with a primary antibody (Tph2, Sigma; 5-HT, ImmunoStar; OXT, Merck) at 4°C for 24 hours. After being washed by PBS, a secondary antibody was applied for 1 hours at room temperature. PBS-washed sections were mounted in coverslips with 60% glycerol mounting medium.

TRAP induction

4-OHT (Sigma, H6278) were prepared as previously reported.[73] 15 day old *TRAP2;Ai14* double transgenic mouse pups were isolated from their mothers and placed in a new cage for 4 hr. Mouse pups were injected with 4-OHT (50 mg/kg i.p.) and placed in a fresh baker separately, which contained clean bedding or mother bedding for 10 hr. Mouse pups were returned to their mother cage. Two weeks later, mice were dissected and tdTomato labelled neurons were examined.

Fiber photometry

On P14, mice were anesthetized by 1.5% isoflurane and placed in a stereotaxic apparatus which was designed for young mice (RWD, China). For RN recording, skin was cut open and a small craniotomy was made 5 mm posterior. An optical fibre (200 μm O.D., 0.37 NA) in ceramic ferrule was inserted to RN in the hole with 15 angle from caudal to rostral (dorsal-ventral depth =2.5mm). The cannula was fixed to skull using dental cement. After surgery, mice recovered on a heater and then was placed in another cage with soft food and water, avoiding the mother's biting of the cannula in the head.

One night later, GCaMP recording was carried out (inper, China). Fiber photometry was performed using 410 nm and 470 nm LEDs. The light path was coupled to a 0.37-NA, 200-mm optical fiber patch cord, which was then coupled to the fiber implant in a mouse. Sampling frequency was set to 20 Hz. The mouse was placed in a test cage for habituation for 10 mins with an empty wire mesh box, which was taken out when the habituation is finished. In each 10 min test phase, a new wire mesh box filled with stimulating bedding was placed in the test cage to let the mouse to sniff freely. A successful sniffing event was defined as the mouse put its nose to sniff the box for at least 6 s. The interval of each separate sniff should be more than 10 s. There was 10 mins for resting between each test. Clean bedding, mother bedding or other dam bedding were used alone in each separate test. Clean bedding was given at the first test. Mother bedding and other dam bedding were given in the following test in random order. For data analysis, the 470-nm signals were corrected by 410-nm signals in the inper software. We derived the values of fluorescence change (△ F/F) by calculating (F-F0)/F0, where F0 is the baseline fluorescence signal averaged over a 1.5-s-long control time window, which was set 0.5 s preceding the sniff initiation.

Optogenetic stimulation

The process of the optical fiber implantation was similar to that for fiber photometry. A ceramic ferrule with an optical fiber (200 μm O.D., 0.37 NA) was implanted on both PVN areas (bregma -0.36 mm, lateral ±1.3 mm, dura -4.79 mm) at P14, and behavior tests were carried out at P15. The output of the laser in optical stimulation system (inper. China) were measured and adjusted to 10 mW. Light stimulation (20 Hz, 10 mW) were applied when the behavior test carried on.

Whole-cell electrophysiology of acute brain slices

Acute brain slices (300 μm) were prepared as described before.[99,100] Slices were maintained in a storage chamber containing aCSF (125 mM NaCl, 5 mM KCl, 10 mM glucose, 2 mM NaH_2PO_4, 2.6 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 26mM $NaHCO_3$, pH7.4, mOsM 300–310) at room temperature. Whole-cell recordings were performed using EPC10 Patch Clamp Amplifier (HEKA, Lambrecht, Germany). Patch pipettes (3–5MΩ) were filled with internal solution consisting of 145 mM KCl, 5 mM NaCl, 10 mM HEPES, 5 mM EGTA, 4 mM Mg ATP, and 0.3 mM Na2GTP, with the pH adjusted to 7.2, Osm 305. The current was clamped at 0 pA for current-clamp recordings. For current-clamp recordings, action potential firing rates were elicited by applying 5 s of light pulses (473 nm) with no current injection. For voltage-clamp recording, EPSCs were evoked by applying 100 ms of light pulses (473 nm).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data are presented as the mean ± SEM, superimposed with individual data. Sample sizes for each experiment are indicated in the corresponding figure legends. Two-tailed unpaired Student's *t*-tests were used to analyze differences between two groups of samples and paired *t*-tests were used for analyzing one group respond to two different stimulation. One-way ANOVA with Tukey's multiple comparisons correction was performed for comparison between multiple groups. All analysis were carried out by GraphPad Prism software.