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Oxytocin receptor neurons in the adBNST modulate maternal-like behavior in mice

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ABSTRACT

Maternal care plays a pivotal role in the brain and behavioral development of offspring. However, the neural substrates underlying the effects of oxytocin (OT) on maternal-like behavior remain incompletely understood. Using *OTR*-Cre female mice, fiber photometry was utilized to monitor the calcium signals in oxytocin receptor (OTR) neurons. We observed elevated activity of adBNST (anterodorsal bed nucleus of the stria terminalis) OTR neurons during pup-caring behavior, as indicated by increased calcium signals. Chemogenetic activation of adBNST OTR neurons prolonged duration of licking pups, whereas inhibiting these neurons reduced the time of licking pups and prolonged the latency to retrieve pups. Additionally, microinjection of OT into the adBNST increased the time of licking pups, while administration of an oxytocin receptor antagonist (OTA) suppressed pup-caring behavior. Collectively, these findings not only deepen our understanding of the neural basis underlying maternal-like behavior but also provide a potential target for treating or mitigating aberrant maternal care associated with postpartum depression, anxiety or related disorders.

Keywords: Anterodorsal bed nucleus of stria terminalis; Oxytocin receptor; Maternal-like behavior

INTRODUCTION

Newborn offspring are fragile and highly dependent on external support in nearly all mammalian species. Their prospects for survival are heavily reliant on parental care, with mothers playing a particularly pivotal role in providing this essential care and protection (Mei et al., 2023). Given this crucial role, maternal care, encompassing nurturing behaviors provided by biological mothers, plays a pivotal role in ensuring the survival and proper development of offspring (Carcea et al., 2021; Niu et al., 2026). Therefore, to ensure the needs of the newborn offspring are met, a set of robust and stereotyped maternal-like behaviors has evolved, such as approaching, investigating, licking, and retrieving newborn offspring in mice. A wide range of parental behaviors have been

documented across various species and sexes. This diversity has given rise to intriguing questions regarding the mechanisms by which animals recognize their offspring and the ways in which brain circuits orchestrate and modulate maternal behaviors (Dulac et al., 2014).

Oxytocin (OT) is widely recognized as a key hormone that plays a crucial role in initiating and sustaining maternal care behaviors (Yoshihara et al., 2018). OT is predominantly synthesized within the paraventricular nucleus (PVN) and the supraoptic nucleus (SON), and PVN-derived OT plays a pivotal role in the initiation of maternal care behaviors in rats (Munetomo et al., 2016). OT regulates maternal emotional and stress responses, which makes it vital for maternal and infant health (Walter et al., 2021). As an evolutionarily ancient neuropeptide OT also exerts its influence by binding to oxytocin receptors (OTRs) within the brain. This interaction plays a crucial role in modulating a range of social and reproductive behaviors including social recognition, pair bonding, and maternal care that are common across vertebrate species, with humans being no exception (Johnson et al., 2017; Teruyama & Govar, 2024).

The oxytocin receptor (OTR) in the brain is vital for modulating social behaviors such as maternal care and sexual behavior (Mitre et al., 2016). Within the bed nucleus of the stria terminalis (BNST), OTR is expressed in over 40 distinct cell types, spanning both the posterior and anterior subregions of the BNST (Luo et al., 2022). Moreover, the BNST coordinates social behavior through facilitating context-appropriate assessment of social environments, leading to the selection of relevant behavioral responses (Flanigan & Kash, 2022).

BNST is recognized as a critical hub for the regulation of maternal behavior (Bosch et al., 2010). Notably, encountering pups increases the number of c-Fos-positive cells within the

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BNST in virgin females, indicating heightened neuronal activity. However, this increase is not observed in lactating females, suggesting that the neural responsiveness of the BNST to pup stimuli may depend on the reproductive state of the female (Matsushita et al., 2015). Especially, the dorsolateral bed nucleus of the stria terminalis ($BNST_{DL}$) exhibits a high level of OTR expression (Francesconi et al., 2021). And in the anterior BNST subregion examined in prior experiments, Cytochrome P450 Family 26 Subfamily B Member 1 positive cells exhibited robust OTR expression (Luo et al., 2022). However, the precise neural mechanisms through which OTR neurons within the anterodorsal BNST (adBNST) modulate maternal-like behaviors remain to be fully elucidated.

In this study, we used *OTR*-Cre female mice and fiber photometry to monitor calcium signals in OTR neurons of adBNST during maternal-like behavior, comparing their activity across virgin non-retrievers, virgin retrievers, and lactating mice. Subsequently, we examined how chemogenetic manipulation of adBNST OTR neurons and microinjection of OT or an oxytocin receptor antagonist (OTA) into the adBNST affect maternal-like behavior. We hypothesized that OTR neurons within adBNST may regulate maternal-like behavior in adult *OTR*-Cre female mice. These findings deepen our understanding of the neural basis of maternal-like behavior and identify a potential therapeutic target for aberrant maternal care arising from postpartum depression, anxiety or related disorders.

MATERIALS AND METHODS

Subjects

Wild-type C57BL/6 mice and 1-3-day-old pups were purchased from the Animal Center of Shaanxi Normal University. *OTR*-Cre mice (strain 031303) and *Ai9* mice (strain 007909) were purchased from the Jackson Laboratory. All mice were housed in polyethylene cages (35 cm long×20 cm wide×15 cm high) with corn cob bedding. They were provided ad libitum access to standard rodent chow and purified water. The temperature was maintained between 21°C to 23°C in the animal room, with humidity levels ranging from 54% to 56%. The room operated on a 12-hour light/dark cycle. All subjects were 8-week-old female mice. To identify BNST subregions associated with pup-caring behavior, we used *OTR* and *Ai9* double-transgenic virgin mice, which were divided into retrievers and non-retrievers (*n*=3 per group). In the Fiber photometry recordings experiment, the animals were divided into a control group and an experimental group. The control group contained 4 virgin pup-retrieval *OTR*-Cre female mice, while the experimental group was further subdivided into three subgroups: (1) virgin non-retrievers' group : 7 virgin non-retrieve pups *OTR*-Cre female mice; (2) virgin retrievers' group: 7 virgin pup-retrieving *OTR*-Cre female mice; (3) mother group: 6 *OTR*-Cre dams. Chemogenetic manipulation was conducted in two complementary experiments. Based on prior studies (Fang et al., 2018; Mei et al., 2023), virgin female mice were used as experimental subjects because dams exhibited higher mortality rates due to dystocia. The specific animal grouping criteria were as follows. Female *OTR*-Cre mice were selected for screening and subsequent grouping. Prior to the screening process, a wad of absorbent cotton was placed in each mouse's cage to allow the mice to engage in spontaneous nest-building behavior. Subsequently, five pups were positioned at the diagonal corners of the nest. A 10-minute test period was then initiated. If, during this 10-minute interval, the mouse successfully retrieved all five pups back to the nest, it was classified into the "virgin retrievers' group". Conversely, if the mouse failed to retrieve any pups during the same 10-minute period, it was designated as belonging to the "virgin non-retrievers' group". Following the screening, all selected mice underwent viral injections. Specifically, the virgin non-retrievers' group mice were assigned to the chemogenetic activation group. Among them, a subset received injections of chemogenetic activating viruses and served as the experimental group, while another subset received injections of control viruses and functioned as the control group (control: *n*=6; hM3Dq: *n*=9). Similarly, the virgin retrievers group mice were categorized into the inhibition group. Among them, a portion received injections of chemogenetic inhibiting viruses as the experimental group, and the remaining mice were administered control viruses as the control group (control: *n*=9; hM4Di: *n*=10). For pharmacological experiments, we used wild-type virgin female mice. The reason for not selecting dams was that the subsequent implantation of cannulae would likely lead to fatal dystocia. The grouping method, maternal-like behavior testing protocol, and behavioral statistical analysis employed in the pharmacology experiments were identical to those used in the chemogenetics. Among the virgin non-retrievers wild-type female mice, saline and OT were administered sequentially via a cannula (*n*=12). In contrast, among the virgin non-retrievers wild-type female mice, saline and OTA were administered sequentially through the cannula (*n*=9). All experimental protocols received approval from the Institutional Animal Care and Use Committee of Shaanxi Normal University (Protocol #GZK2025-042) and were conducted in strict accordance with China's Guide for the Care and Use of Laboratory Animals.

Virus

All viruses were obtained from BrainVTA China, including AAV2/9-EF1α-DIO-GCaMP6m (titer: 2.91×10^{12} vg/mL), AAV2/9-EF1α-DIO-EGFP (titer: 2.52×10^{12} vg/mL), AAV2/9-EF1α-DIO-hM4D(Gi)-mCherry (titer: 2.09×10^{12} vg/mL), AAV2/9-EF1α-DIO-hM3D(Gq)-mCherry (titer: 5.62×10^{12} vg/mL) and AAV2/9-EF1α-DIO-mCherry (titer: 5.02×10^{12} vg/mL).

Stereotaxic surgery

Mice were anesthetized with 1%–2% isoflurane and positioned on a stereotaxic rig (R.W.D. Life Science, China). Viruses were administered into the brains using a Hamilton microsyringe. To record the Ca^{2+} signals from adBNST OTR neurons, 300nL of AAV2/9-EF1α-DIO-GCaMP6m (control: AAV2/9-EF1α-DIO-EGFP) was injected unilaterally into the adBNST at coordinates (AP: +0.6 mm, ML: +0.95 mm, DV: –4.75 mm) of *OTR*-Cre female mice. For chemogenetic manipulation experiments, AAV2/9-EF1α-DIO-hM4D(Gi)-mCherry, AAV2/9-EF1α-DIO-hM3D(Gq)-mCherry and AAV2/9-EF1α-DIO-mCherry were stereotaxically injected into the adBNST (AP: +0.6 mm, ML: ±0.95 mm, DV: –4.75 mm) of adult virgin *OTR*-Cre female mice. Infusions were delivered at a constant rate of 100 nL/min via a microsyringe pump (KD Scientific, USA). The needles were left in position for 5 minutes following the infusions.

Fiber photometry

To record the calcium signals of adBNST OTR neurons during maternal-like behavior, one week after virus injection, a 200-μm optical fiber was implanted approximately 100 μm

above the viral injection site and fixed with dental cement. Following a 2-week recovery, the optical fiber was connected to a fiber photometry system (ThinkerTech, China). To avoid bleaching of the sensor, the power of the 470 nm laser at the fiber tip was adjusted to 50 μW. Mice were then placed in a test cage and allowed to move freely for at least 20 minutes to acclimate to the environment. Subsequently, five 1–3-day old pups were introduced into the test cage, positioned diagonally from the nest of the subject. The test was conducted for 10 minutes, with video recordings synchronized to the OTR calcium signals using screen recording software. In the present study, we meticulously annotated the behavioral sequences of the test subjects on a frame-by-frame basis utilizing bespoke software developed in MATLAB2017b. Specifically, the behavior termed “approach pup” was identified when the testing female oriented her face toward and advanced directly toward the pup. The “investigate pup” behavior was characterized by the female’s nose making close contact with any part of the pup’s body. The “lick pup” behavior was defined by the close physical interaction between the female and the pup, accompanied by rhythmic vertical head movements of the female and displacement of the pup. Finally, “retrieve pup” was delineated as the interval from the moment the female lifted the pup using her jaw until the pup was deposited in or around the nest. For every individual trial, we computed the Z-score for each time interval during which the behavior occurred. Each signal underwent photobleaching correction through fitting its decay profile with a double-exponential function. Subsequently, it was normalized to a Z-score. The fiber photometry recording system used in this study is capable of synchronously recording signals excited by a 405 nm light source, and no significant differences were found between the baseline signal values and those after behavioral events. Moreover, we also employed a control virus and observed no impact of motion artifacts on the experimental results.

Chemogenetics

To investigate the effects of chemogenetic manipulation on pup-caring behaviors and neuronal activity in virgin *OTR*-Cre female mice, we conducted a series of experiments. For virgin female mice showing no pup-caring, we injected AAV2/9-EF1α-DIO-mCherry (control) or AAV2/9-EF1α-DIO-hM3D(Gq)-mCherry into the adBNST to investigate effects of chemogenetic activation. For virgin female mice showing pup-caring, we injected AAV2/9-EF1α-DIO-mCherry (control) or AAV2/9-EF1α-DIO-hM4D(Gi)-mCherry into the adBNST to reveal effects of chemogenetic inhibition. After 4 weeks, all subjects were placed in the behavioral testing room for a 1-hour acclimation period. Prior to the commencement of maternal-like behavior testing, we placed a wad of cotton wool into the mouse’s cage, allowing the mice to build nests on their own. Tests for maternal-like behaviors were conducted in the home cage. Each female mouse was first intraperitoneally injected with saline. Following a 30-minute interval, five pups were placed at the opposite corners of the nest, and the test was performed for a duration of 10 minutes. Subsequently, each mouse was intraperitoneally injected with 1 mg/kg clozapine-N-oxide (CNO, BrainVTA, China) after another 30-minute interval, the maternal-like behavior test was repeated. Videos were recorded simultaneously during the maternal-like behavior testing. Subsequently, the videos were analyzed using JWatcher v.1.0 software (http://www.jwatcher.ucla.edu/) to quantify specific maternal-like behaviors. These behavioral parameters were defined as follows: The number of pups retrieved by the test mouse during the 10-minute behavioral test was termed the retrieval rate. The time elapsed from the moment when five pups were placed into the test cage and noticed by the test mouse to the initiation of retrieval of the first pup by the test mouse was referred to as the latency to retrieve the first pup. Similarly, the time interval from the placement of the five pups into the test cage and their detection by the test mouse until the retrieval of the fifth pup by the test mouse was defined as the latency to retrieve the fifth pup. “Licking time” quantified the total duration during a 10-minute maternal-like behavior test in which the test mouse engaged in active licking of pups. This behavior was characterized by stereotyped vertical head movements and often accompanied by pup displacement. For both the control and experimental groups in the chemogenetic activation experiment, if a tested mouse retrieved 5 pups during the 10-minute maternal-like behavior test conducted 30 minutes after an intraperitoneal injection of saline, the mouse was excluded from the study. Similarly, for both control and experimental groups in the chemogenetic inhibition experiment, if a tested mouse failed to retrieve any pups during the 10-minute maternal-like behavior test conducted 30 minutes after an intraperitoneal injection of saline, the mouse was excluded. If the subject did not exhibit any retrieval behavior, the retrieval latency was defined as 600 seconds. Additionally, to examine the activity of OTR neurons after chemogenetic stimulation, the subject was placed in an open field last 5 minutes following a 30-min delay after intraperitoneal CNO administration. These mice were euthanized with an intraperitoneal overdose of pentobarbital sodium 90 minutes later, followed by tissue collection for immunohistochemistry.

Open Field Test (OFT)

Virgin female mice were tested in an open field for 5 minutes. The test mice were placed in the center of the open field test arena (with dimensions of 50 cm long×50 cm wide×25 cm high) and allowed to explore freely. Their behavior was recorded and analyzed using an automated tracking system (Supermaze, China). The total distance traveled and the percentage of time spent in the central area of the arena were quantified.

Microinjection

To investigate the effects of OT and OTA on maternal-like behavior, we pre-screened wild-type virgin female mice to identify those that exhibited pup-caring behaviors. These mice were anesthetized with isoflurane and stereotaxically implanted with bilateral 26-gauge steel guide cannulas (R.W.D. Life Science, China) into the adBNST (brain coordinates: anteroposterior +0.6 mm, mediolateral ±0.95 mm, dorsoventral –4.75 mm). Following surgery, the mice were allowed a 10-day recovery period. Subsequently, we performed microinjections into the adBNST using a 33-gauge needle that extended 0.5 mm below the guide cannula. For drug testing, these mice received an initial injection of 0.9% NaCl, followed by subsequent injections of OT (50-56-6, Bachem, USA) or OTA (L-368899, MCE, USA). In the virgin non-retrieval group, bilateral microinjections of a 1 ng/nL OT were delivered at a volume of 200 nL per side. Furthermore, the virgin retrieval group also received a 1 ng/nL OTA via identical bilateral injections of 200 nL per side. The injections were delivered at a rate of 0.12 μL/min. Maternal-like behaviors were tested 15 minutes after drug injection. Five pups were positioned diagonally opposite corners of the nest,

and the test lasted for 10 minutes. Video recording was conducted simultaneously for all maternal-like behavior tests. The subsequent behavioral analysis was performed using JWatcher v.1.0 software (http://www.jwatcher.ucla.edu/) to quantify specific maternal-like behavior. The same behavioral parameters as in the chemogenetic experiments were quantified. Virgin non-retrievers received intracerebral microinjection of saline. After a 15-min interval, a 10-minute maternal-like behavior test was conducted. Mice that successfully retrieved any pups within the 10-minute test period were excluded. In parallel, virgin retrievers were administered saline via the same intracannular route and subjected to identical behavioral testing conditions. Subjects that failed to retrieve five pups during the 10-minute test were likewise excluded. Histological verification was conducted to confirm the accuracy of cannula placement, and data were excluded if the injection site was located outside the adBNST. After these procedures, the final experimental cohort consisted of 9 virgin retrievers and 12 virgin non-retrievers.

Immunohistochemistry

Following deep anesthesia with isoflurane, mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). These brains were promptly harvested and subsequently post-fixed in 4% PFA for two days at 4°C. Subsequently, tissues were transferred to a 20% sucrose solution for 24 hours of dehydration, followed by another 24 hours in a 30% sucrose solution. The brains were embedded in optimal cutting temperature compound (OCT) and sectioned into 40μm slices using a cryostat (Leica CM1950, Germany). The brain sections were washed with PBS (3×5 min), treated with 0.5% Triton X-100 for permeabilization (30 min at room temperature), and blocked with normal goat serum (AR0009, Boster, China) for 1 hour at room temperature. Subsequently, they were incubated with primary antibodies: Rabbit anti-OTR (1:300, 23045-1-AP, Proteintech, China) or Rabbit anti-c-Fos (1:1 000, ab190289, Abcam, UK) at 4°C for 48 hours. After the primary antibody incubation, the sections were washed with PBS (3×5 min), incubated with Cy3 conjugated goat anti-rabbit (1:200, 33108ES60, YEASEN, China) or Alexa Fluor 488 conjugated goat anti-rabbit (1:200, 111-545-144, Jackson Immunoresearch, USA) for 2 hours at room temperature. Then, they were rinsed again with PBS (3×5 min), stained with DAPI (AR1177, Boster, China) for 10 minutes, and washed with PBS (3×5 min). Finally, the sections were mounted with an antifade mounting medium.

Image acquisition was performed using a fluorescent microscope and a Nikon camera. Imaging was performed to confirm the viral expression and optic fiber/cannula placements, and to quantify cells positive for c-Fos, OTR, or the viral. To analyze the activity of OTR neurons (co-localization of c-Fos and OTR) among different behaviors and the specificity of viral expression (co-localization of virus and OTR) in the adBNST brain region, brain slices of 40 μm were consecutively collected on two sets of slides. For each mouse, the number of c-Fos or OTR neurons in the adBNST was counted. Three representative sections from anterior to posterior that were anatomically matched in mice were chosen to minimize variability. Manual quantification of immunopositive neurons within the adBNST was analyzed with the results of using anatomical landmarks defined by the Allen Mouse Brain Atlas. Cell counts were averaged across three evenly spaced, non-overlapping sections per mouse.

Statistics

The normality of the data was assessed using the Kolmogorov–Smirnov test. Since some datasets exhibited a normal distribution, parametric tests were subsequently applied for analysis. Paired *t*-tests (two-tailed) were used for fiber photometry (pre vs. post) and pharmacology (Saline vs. OTA). As the data from additional datasets were not normally distributed, non-parametric tests were employed for subsequent analyses. The Wilcoxon signed-rank test was used to compare pharmacological conditions (Saline vs. OT). In fiber photometry experiments, calcium signals associated with different behaviors were analyzed using one-way repeated measures analysis of variance (ANOVA) and same behaviors (approach pups, investigate pups, licking pups) were analyzed using one-way ANOVA. For comparing calcium signals during the same behavior between virgin and mother mice in terms of c-Fos expression, an independent-samples *t*-test was employed. In pharmacology experiments, McNemar's test was also used to analyze number of mice. Two-way repeated-measures ANOVA, McNemar's test and Fisher's exact test were used to analyze the results of the chemogenetical experiments. However, in chemogenetic experiments where the assumptions of the data did not conform to a normal distribution, a generalized linear mixed effect model was employed for statistical analysis. In the open field test, the data on the total distance traveled and the percentage of time spent in the center zone were analyzed using an independent samples *t*-test in mCherry group and hM3Dq/hM4Di group. Statistical significance is defined as $P<0.05$ for the data presented. All values are expressed as the mean±standard error of the mean (SEM).

RESULTS

Identification of BNST subregions mediating pup-caring behavior

To identify BNST subregions critically involved in pup-caring behavior, we first categorized virgin female mice into non-retrieving and retrieving groups based on pup retrieval performance. After a 10-minute test for maternal-like behavior, the mice were perfused transcardially 90 minutes later for c-Fos immunohistochemical analysis (Figure 1A). Quantitative immunohistochemical analysis revealed that the proportion of c-Fos$^+$/OTR$^+$ double-labeled neurons (activated during maternal-like behavior) within the OTR$^+$ neuron population was significantly higher in the adBNST of virgin retrieving mice compared to non-retrieving mice (Figure 1B). There was no significant difference in the proportion of c-Fos$^+$/OTR$^+$ double-labeled neurons (activated during maternal-like behavior) within the OTR$^+$ neuronal population in the BNSTpr (Figure 1C) and alBNST (Figure 1D) between the virgin retrieved group and the virgin non-retrieved group. These findings indicate a critical role for the OTR neurons of adBNST in regulating pup-caring behavior.

Activities of adBNST OTR neurons during the occurrence of maternal-like behavior

We first performed fiber photometry to monitor the activity of adBNST OTR neurons during maternal-like behavior (Figure 2B–D, Supplementary Video S1). To verify that OTR neurons participate during the occurrence of maternal-like behavior, we delivered a Cre-dependent AAV2/9-EF1α-DIO-GCaMP6m or AAV2/9-EF1α-DIO-EGFP (control) into the adBNST and implanted an optic fiber above the same region

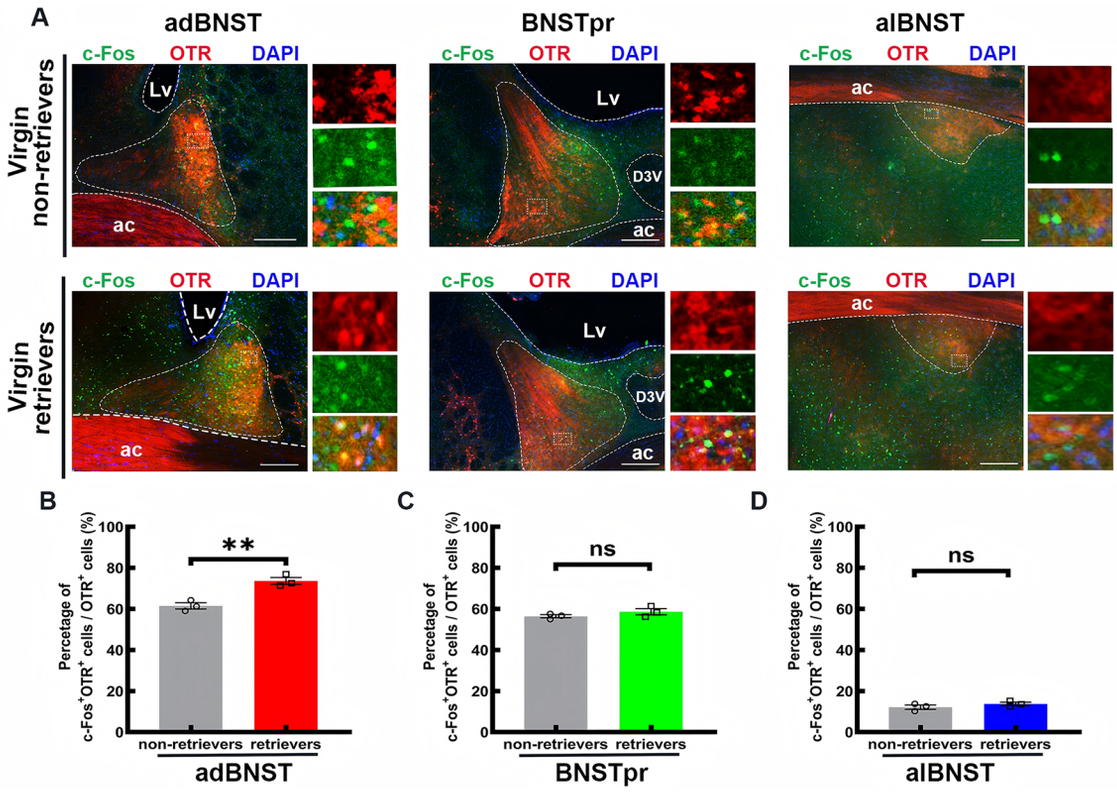

Figure 1 Screening of BNST subregions implicated in pup-caring behaviors in *OTR*: *Ai9* double-positive mice

A: Representative coronal sections from three mice per group illustrate pup-caring behaviors induced c-Fos immunoreactivity in the anterior dorsal bed nucleus of the stria terminalis (adBNST), posterior bed nucleus of the stria terminalis (BNSTpr), and anterolateral bed nucleus of the stria terminalis (alBNST) in virgin non-retrieving mice (top) and virgin retrieving mice (bottom). Green: c-Fos, Red: OTR, Blue: DAPI. Scale bars (white line): 200 μm. ac: anterior commissure. Lv: lateral ventricle. D3V: dorsal 3rd ventricle. B–D: Percentage of c-Fos$^+$ and OTR$^+$ cells relative to total OTR$^+$ cells in adBNST (B), BNSTpr (C), and alBNST (D) subregions of virgin non-retrievers and retrievers. The data analysis was conducted using an independent-samples *t*-test (two-sided) (B–D). All data is presented as a mean±SEM. ns: no significant difference; **: P<0.01. The detailed statistical analysis is presented in Supplementary Material 1.

of *OTR*-Cre female mice (Figure 2A). The GCaMP6m was efficiently expressed in OTR$^+$ neurons: 96.70% of GCaMP6m$^+$ neurons were OTR$^+$ neurons (Figure 2C). When five 1–3-day-old pups were introduced into the cage at a corner diagonally opposite the nest, we observed that among virgin non-retrievers, virgin retrievers and lactating dams exhibited a marked rise in adBNST OTR neurons Ca^{2+} transients as they approached the pups. The magnitude of this response was significantly larger in both virgin retrievers than in virgin non-retrievers and mothers (Figure 2E, I). During subsequent close investigation and licking of pups, adBNST OTR neuronal activity was significantly elevated among virgin non-retrievers, virgin retrievers, and dams. This response was markedly potentiated in mothers relative to both virgin females that retrieved pups and those that did not (Figure 2F, G, I). Activities of OTR neurons in the adBNST increased in both virgin retrievers and lactating female mice during pup retrieval (Figure 2H, I). Notably, regardless of whether they were virgin non-retrievers, virgin retrievers or dams, these activities of adBNST OTR neurons exhibited the most pronounced increase during the period of licking these pups (Figure 2J). Furthermore, no significant alterations in neural signals were detected in mice expressing the control viral vector (AAV2/9-EF1α-DIO-EGFP) during pup-directed behavior (Supplementary Figure S1A–H). These findings demonstrate that adBNST OTR neuronal activities increase consistently among virgin non-retrievers, virgin retrievers, and dams during maternal-like behavior.

Effects of chemogenetic excitation of adBNST OTR neurons on pup-caring behavior

To further reveal the role of adBNST OTR neurons in the regulation of pup-caring behavior in virgin female mice, we investigated the effect of their chemogenetic activation in virgin female mice without pup-retrieval behavior (Figure 3E). For this, we bilaterally infused AAV2/9-EF1α-DIO-hM3D(Gq)-mCherry or AAV2/9-EF1α-DIO-mCherry (control) into the adBNST of *OTR*-Cre virgin non-retrievers (Figure 3A). Following injection of saline or CNO, we observed changes in maternal-like behaviors (Figure 3B). mCherry expression was predominantly restricted to OTR-expressing neurons, with a colocalization rate above 80.62% as validated by immunohistochemistry (Figure 3C). Immunohistochemical analysis revealed predominant targeting of hM3Dq expression to OTR-expressing neurons, with colocalization exceeding 81%, demonstrating its high specificity and precise targeting of the OTR neuronal population (Figure 3D). Simultaneously, CNO stimulation increased c-Fos expression in brain regions infected by hM3Dq virus, validating the effectiveness of the chemogenetic activation (Figure 3F). We found that chemogenetic activation of adBNST OTR neurons significantly increased the time spent licking pups (Figure 3H), with no significant difference in the latency to retrieve the first and fifth pup (Figure 3G, I, J). We also observed that chemogenetic activation of adBNST OTR neurons had no significant effect on total moving distance or time spent in the center zone. (Supplementary Figure S2B). These results collectively

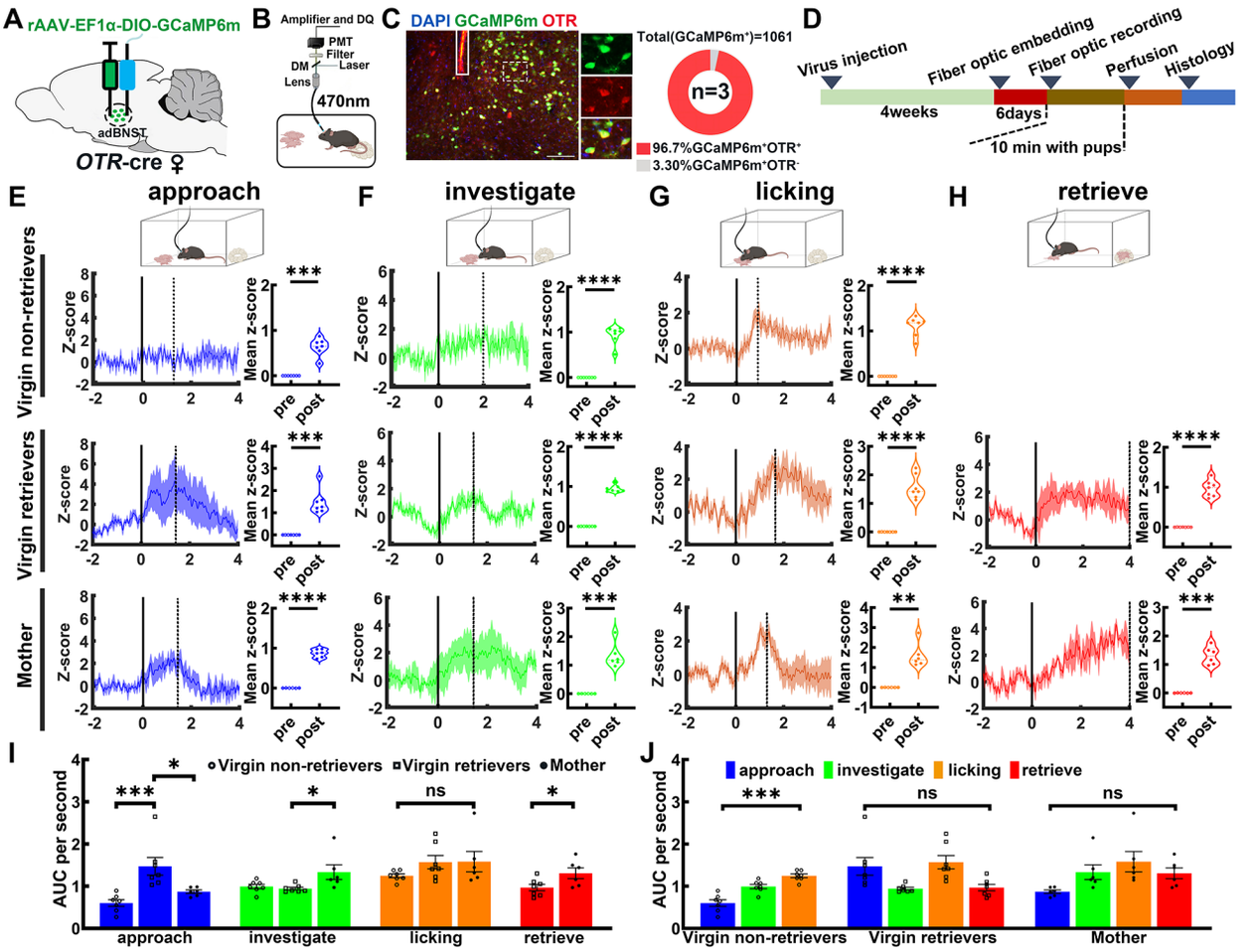

Figure 2 Activities of adBNST OTR neurons during maternal-like behavior

A: Schematic of virus injection and fiber optic implantation. B: Settings of the optical fiber recording apparatus. C: Left: Representative merged images showing OTR staining (red) and GCaMP6m (green) in the adBNST, with DAPI (blue) labeling nuclei. Scale bars (white line): 200 μm. Right: Specificity and efficiency of GCaMP6m expression in OTR neurons of adBNST (*n*=3). D: Experimental timeline. E–H: Schematic diagram of a mouse approaching pups (E), investigating pups (F), licking pups (G) and retrieving pups (H); The schematic illustrations were created using resources from https://app.biorender.com/biorender-templates/figures. Calcium activity in OTR neurons in virgin non-retrievers (*n*=7) (E–G, top), virgin retrievers (*n*=7) (E–H, middle) and dams (*n*=6) (E–H, bottom) during approaching pups (E, left), investigating pups (F, left), licking pups (G, left) and retrieving pups (H, left), compared with mean z-score before and after approaching pups (E, right), investigating pups (F, right), licking pups (G, right) and retrieving pups (H, right). I and J: The average area under the curve (AUC) of the z-score across same pup-directed behaviors among virgin non-retrievers, virgin retrievers, and those who have become mothers (I) and responses of the pup-directed behavior in virgin non-retrievers, virgin retrievers and dams (J). The data analysis was conducted using paired *t*-tests (two-sided) (E–H), One-way analysis of variance (ANOVA) with Bonferroni post-test and independent-samples *t*-test (two-sided) (I) and one-way repeated measures analysis of variance (ANOVA) with Bonferroni post-test (J). All data are presented with a mean±SEM. ns: no significant difference; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$. The detailed statistical analysis is presented in Supplementary Material 1.

demonstrate that chemogenetic activation of adBNST OTR neurons is associated with an increase in the time spent licking pups, without altering their locomotor activity.

Impacts of chemogenetic suppression of adBNST OTR neurons upon pup-caring behavior

Next, we investigated the effects of chemogenetic suppression of adBNST OTR neurons on pup-caring behavior in the virgin retrievers (Figure 4E). We bilaterally micro-infused AAV2/9-EF1α-DIO-hM4D(Gi)-mCherry or AAV2/9-EF1α-DIO-mCherry (control) into the adBNST of the virgin retrievers (Figure 4A). Following injection of saline or CNO, we observed changes in maternal-like behaviors (Figure 4B). Our study revealed that 81.01% of mCherry expression or 83.23% of hM4Di expression was specifically localized within OTR-expressing neurons, demonstrating its high specificity and precise targeting of the OTR neuronal population (Figure 4C, D). Simultaneously, suppression via CNO led to a significant downregulation of c-Fos expression in brain regions infected with the hM4Di virus, validating the efficacy of chemogenetic inhibition (Figure 4F). Chemogenetic silencing of OTR neurons within the adBNST produced a pronounced and selective impairment of maternal responsiveness: it shortened the cumulative time of licking pups (Figure 4H) and progressively prolonged the retrieval latency for both the first and fifth pup (Figure 4G, I, J). Furthermore, chemogenetic inhibition of adBNST OTR neurons produced no statistically significant alterations in either total distance traveled or the percentage of time spent in the center zone (Supplementary Figure S2C). These findings demonstrate that chemogenetic inhibition of adBNST OTR neurons led to the attenuation of pup-caring behavior in the virgin retrievers, without affecting their locomotor activity.

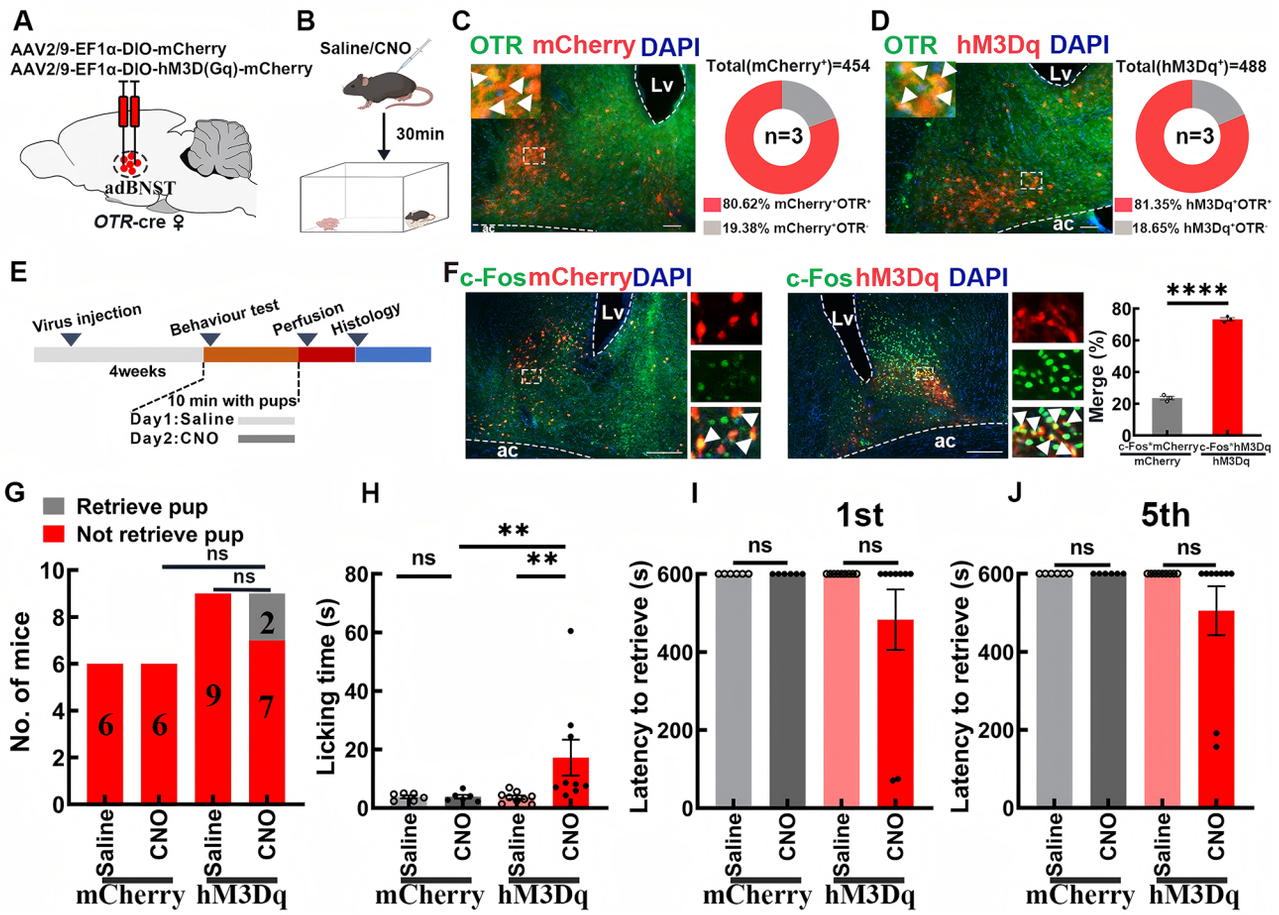

Figure 3 Chemogenetic activation of OTR neurons in the adBNST affects maternal-like behavior

A: Schematic diagram depicting the viral experimental strategy. B: Schematic of intraperitoneal injection of Saline or CNO followed by behavioral testing 30 min later. The schematic illustrations were created using resources from https://app.biorender.com/biorender-templates/figures. C, D: Left: Representative histological images of mCherry (red) (C) or hM3Dq (red) (D) expression and OTR staining (green), enlarged view of the boxed area is on the top left corner. The white triangle marks the region that is enlarged in the overlay panel. Blue, DAPI. Scale bars (white line): 50 μm. Lv: lateral ventricle. ac: anterior commissure. Right: Statistics of the specificity of mCherry or hM3Dq expression in three *OTR*-Cre mice; more than 80% of mCherry-positive neurons or 81% of hM3Dq-positive neurons overlapped with OTR-positive neuron. E: Timeline of the experiment. F: Left: c-Fos (green) expression overlapping with mCherry (red) or hM3Dq (red) after CNO injection, enlarged view of the boxed area is on the right corner. The white triangle marks the region that is enlarged in the overlay panel. Blue, DAPI. Scale bars (white line): 200 μm. Right: CNO induced more c-Fos expression in hM3Dq which indicated the effectiveness of hM3Dq (n=3). Data were analyzed using an independent-samples t-test (two-sided). G–J: Number of *OTR*-Cre virgin non-retrievers exhibiting pup retrieval or non-retrieval behavior (G), the time of licking pups (H), the latency to retrieve the first (I) and fifth (J) pup after injection with saline or CNO in the mCherry (n=6) and hM3Dq (n=9) groups. Statistical analysis was performed using Fisher's exact tests for comparing behavior outcomes (retrieve vs. not retrieve) between mCherry and hM3Dq groups with the same CNO treatment and McNemar's test for comparisons between Saline and CNO trials in a group (G). Data were analyzed with paired t-tests (two-sided) and independent-samples t-tests (two-sided) (H–J). All data are presented as mean±SEM. ns: no significant difference; **: P<0.01; ****: P<0.0001. The detailed statistical analysis is presented in Supplementary Material 1.

Effects of microinjection of OT into the adBNST on pup-caring behavior

To investigate the role of OT within the adBNST in maternal-like behavior, bilateral cannulae were implanted into the adBNST of wild-type virgin non-retrievers (Figure 5A, B). Following a recovery period of ten days, mice underwent microinjections of Saline or OT through the implanted cannulae. Subsequently, a maternal-like behavior test was conducted (Figure 5C). Microinjection of OT prolonged the time of licking pups in mice (Figure 5E). However, it did not affect pup retrieval behavior and no significant differences were observed in the latency to retrieve the first and fifth pup (Figure 5D, F, G). These results indicate that bilateral OT injection into the adBNST increases the time of licking pups in the virgin non-retrievers.

Effects of microinjection of OTA into the adBNST on pup-caring behavior

To investigate the effects of OTA within the adBNST on pup-caring behavior, bilateral cannulae were implanted into the adBNST of wild-type virgin retrievers (Figure 6A, B). Following a recovery period of ten days, mice underwent microinjections of Saline or OTA through the implanted cannulae. Subsequently, a 10-minute quantitative assessment of pup-caring behavior was conducted (Figure 6C). Following OTA microinjection, 5 out of 9 mice failed to retrieve pups (Figure 6D) and there was a significant decrease in the time of licking pups in mice (Figure 6E) and a prolongation in the latency to retrieve the first (Figure 6F) and fifth (Figure 6G) pup. These results demonstrate that bilateral injection of OTA in the adBNST attenuates pup-caring behavior in the virgin

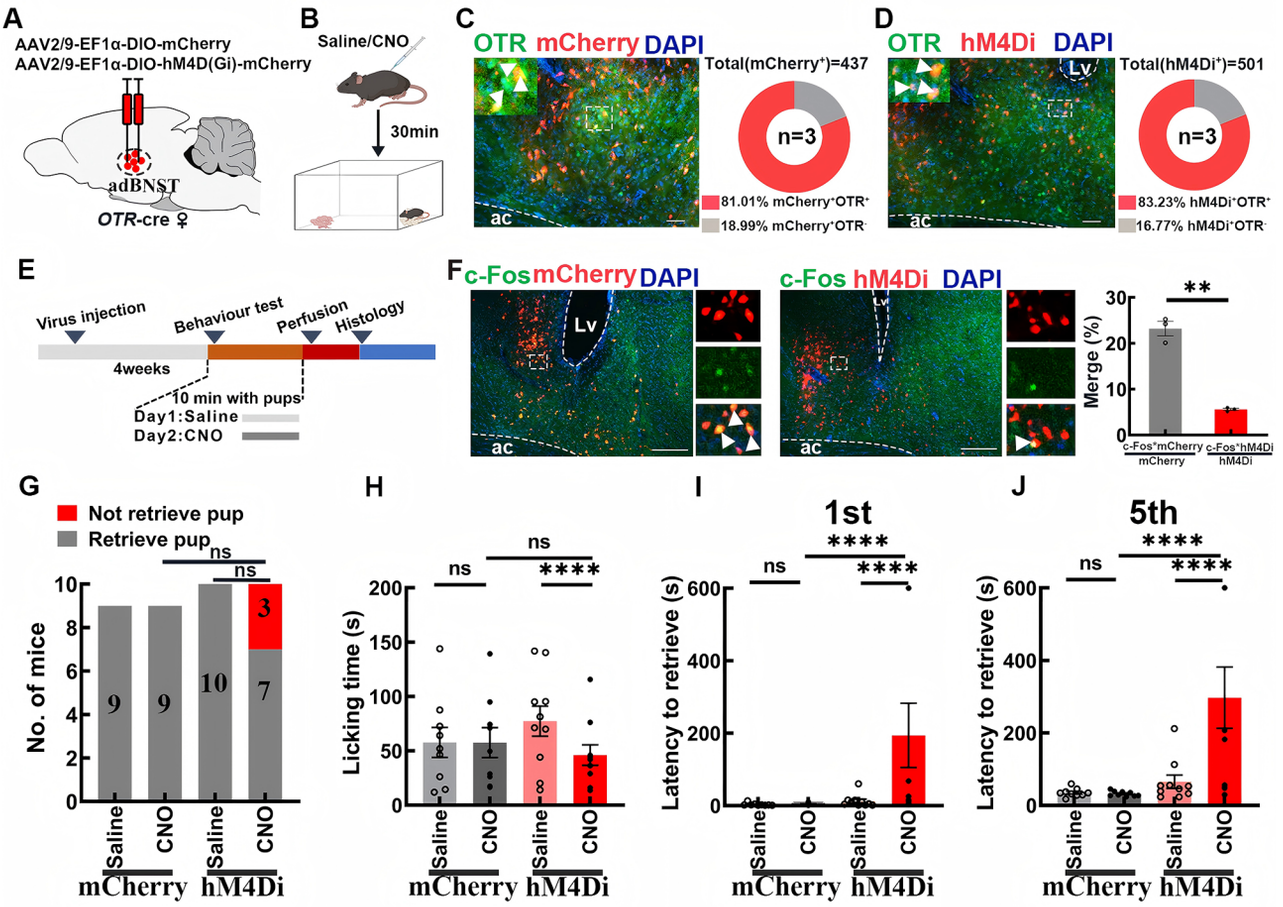

Figure 4 Chemogenetic silencing of OTR neurons in the adBNST affects pup-caring behavior

A: Schematic diagram depicting the viral experimental strategy. B: Schematic of intraperitoneal injection of Saline or CNO followed by behavioral testing 30 min later. The schematic illustrations were created using resources from https://app.biorender.com/biorender-templates/figures. C, D: Left: Representative histological images of mCherry (red) (C) or hM4Di (red) (D) expression and OTR staining (green), enlarged view of the boxed area is on the top left corner. The white triangle marks the region that is enlarged in the overlay panel. Blue, DAPI. Scale bars (white line): 50 μm. Lv: lateral ventricle. ac: anterior commissure. Right: Statistics of the specificity of mCherry or hM4Di expression in three *OTR*-Cre mice; more than 81% of mCherry-positive neurons or 83% of hM4Di-positive neurons overlapped with OTR-positive neuron. E: Timeline of the experiment. F: Left: c-Fos (green) expression overlapping with mCherry (red) or hM4Di (red) after CNO injection, enlarged view of the boxed area is on the right corner. The white triangle marks the region that is enlarged in the overlay panel. Blue, DAPI. Scale bars (white line): 200 μm. Right: CNO induced less c-Fos expression in hM4Di which indicated the effectiveness of hM4Di (n=3). Data were analyzed using an independent-samples t-test (two-sided). G–J: Number of *OTR*-Cre virgin retrievers exhibiting pup retrieval or non-retrieval behavior (G), the time of licking pups (H), the latency to retrieve the first (I) and fifth (J) pup after injection with saline or CNO in the mCherry (n=9) and hM4Di (n=10) groups. Statistical analysis was performed using Fisher's exact tests for comparing behavior outcomes (retrieve vs. not retrieve) between mCherry and hM4Di groups with the same CNO treatment and McNemar's test for comparisons between Saline and CNO trials in a group (G). Data were analyzed with two way repeated-measures ANOVA (H) and a generalized linear mixed effect model (GLMM) (I, J). All data are presented as mean±SEM. ns: no significant difference; **: P<0.01; ****: P<0.0001. The detailed statistical analysis is presented in Supplementary Material 1.

retrievers.

DISCUSSION

The results showed that the activity of adBNST OTR neurons increased during the emergence of maternal-like behavior among virgin non-retrievers, virgin retrievers, and lactating mice. Chemogenetic activation of adBNST OTR neurons only increased the time of licking pups without affecting the latency to retrieve pups in virgin non-retrievers. Whereas chemogenetic inhibition of adBNST OTR neurons, in contrast, decreased the time of licking pups and increased the latency to retrieve pups in virgin retrievers. Additionally, we demonstrated that bilateral microinjections of OT only augmented the time of licking pups, while bilateral microinjections of OTA into the adBNST reduced the time of licking pups and enhanced the latency to retrieve pups. The present study revealed that adBNST OTR neurons modulate maternal-like behavior.

OT is essential for social behaviors (Wang et al., 2024). OT, a pivotal neuropeptide and vital molecular signal in the maternal brain, mediates physiological adaptations and nurturing behaviors essential for maternal care (Valtcheva et al., 2023). Our study revealed that OTR-positive neurons in the adBNST exhibited increased activity in virgin non-retrievers, virgin female mice with pup-caring behavior, and lactating mice during different aspects of maternal-like behaviors, such as approaching, investigating, retrieving, and licking pups.

Next, chemogenetic activation of OTR neurons in the adBNST only increased the time of licking pups, without affecting the latency to retrieve pups. The results suggest that

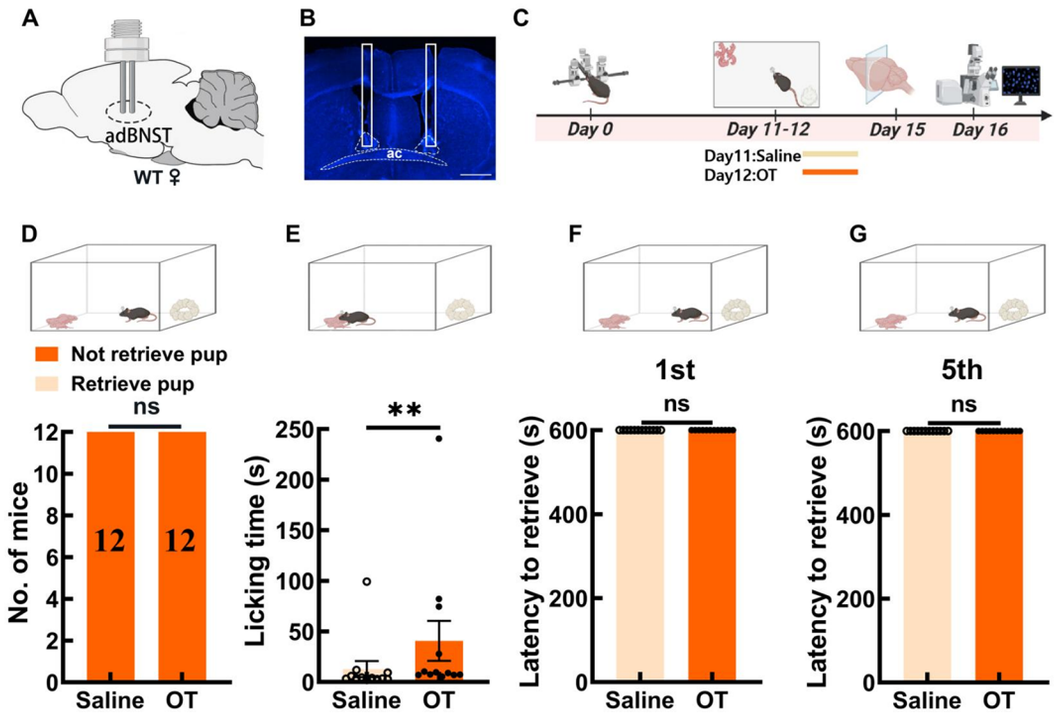

Figure 5 Effects of adBNST OT administration on pup-caring behavior

A: Schematic of bilateral cannula implantation. B: Representative immunofluorescence image showing bilateral cannula tracks in the adBNST (marked by white lines). ac: anterior commissure. Scale bar: 1 mm. C: Timeline of the experiment. D–G: Schematic of pup-retrieval failure (D, top), licking time of pups (E, top), latency of the first pup retrieval (F, top) and the fifth pup retrieval (G, top) in virgin non-retrievers. The schematic illustrations were created using resources from https://app.biorender.com/biorender-templates/figures. Number of wild-type virgin female mice exhibiting pup retrieval or non-retrieval behavior (D, bottom), the time of licking pups (E, bottom), the latency to retrieve the first (F, bottom) and fifth (G, bottom) pup after injection with saline or OT (n=12). Statistical analyses were performed using McNemar's test for comparisons between Saline and OT trials (D) and Wilcoxon signed-rank test (E–G). The data are presented as mean±SEM. ns: no significant difference. **: P<0.01. The detailed statistical analysis is presented in Supplementary Material 1.

licking pup behavior and pup retrieval behavior may be mediated by distinct neural pathways. The PVN-adBNST OT pathway regulates the motor component of the behavior, while PVN-Central Amygdala (CeA) OT projection controls the emotional process of rescue-like behavior (Zhang et al., 2025). The activation of $BNST_{DL}$ OTR neurons led to a decrease in anxious arousal during the fear-potentiated startle response and an enhancement of exploratory behavior in the elevated-plus maze (Francesconi et al., 2025). In contrast, the present study found that chemogenetic inhibition of OTR neurons in the adBNST reduced the time of licking pups and increased the latency to retrieve pups. OTR in the BNST, similar to those in the amygdala, are instrumental in transforming the perception of pertinent environmental signals processed within these brain areas into nurturing and caregiving behaviors (Leng et al., 2008; Meyer-Lindenberg et al., 2011). The BNST serves as a crucial element within the OT signal neural circuits that underpin parental care (Grinevich et al., 2016). Animal studies have demonstrated that the BNST plays a pivotal role in proactive parental caregiving behaviors initiated by the parent, such as retrieving offspring, while playing a less significant role in more passive caregiving actions, such as feeding (Numan, 2006). The present study further identified that adBNST OTR neurons regulate maternal-like behavior.

Further experiments showed that bilateral microinjections of OT into the adBNST only increased the time of licking pups, without affecting the latency to retrieve pups. These findings paralleled those observed following chemogenetic activation of OTR neurons in the adBNST. Similar to our findings, bilateral microinjection of OT into the BNST (10 and 20 ng/nucleus) elicited no significant change in retrieval activity (Consiglio et al., 2005). Electrophysiological techniques revealed that, in lactating female rats, OT elevates the spontaneous firing frequency within a specific subpopulation of neurons in the BNST (Ingram et al., 1990; Ingram & Moos, 1992; Wilson et al., 2005). This is similar to the effects of activating OTR neurons. This result may be due to the fact that simple receptor activation alone may not fully replicate the specific co-release of neuropeptides and neurotransmitters that occurs with altered neuronal activity (Jurek & Neumann, 2018). Additionally, the activation of downstream regulatory feedback mechanisms may require the coordination of Esr1 signaling in the medial preoptic area or dopamine in the ventral tegmental area (Fang et al., 2018). AVP enhances the intrinsic excitability of type I and III $BNST_{DL}$ neurons through an OTR-dependent mechanism, and this enhancement is independent of independent of V1aR or V1bR signaling (Francesconi et al., 2025). However, some other studies have reported different results. This result is inconsistent with the finding that the infusion of OT into the BNSTam (anteromedial bed nucleus of the stria terminalis) of stress-naïve mice heightened their social vigilance while diminishing social approach behavior (Duque-Wilckens et al., 2020), and intraperitoneal injection of carbetocin—a biased OTR-Gq agonist—into california mice also reduced their social approach behavior (Luo et al., 2022). The possible reasons for these discrepancies may lie in differences in species (C57 mice vs. california mice), exact injection site (anterodorsal BNST vs. anteromedial BNST), behavioral paradigm

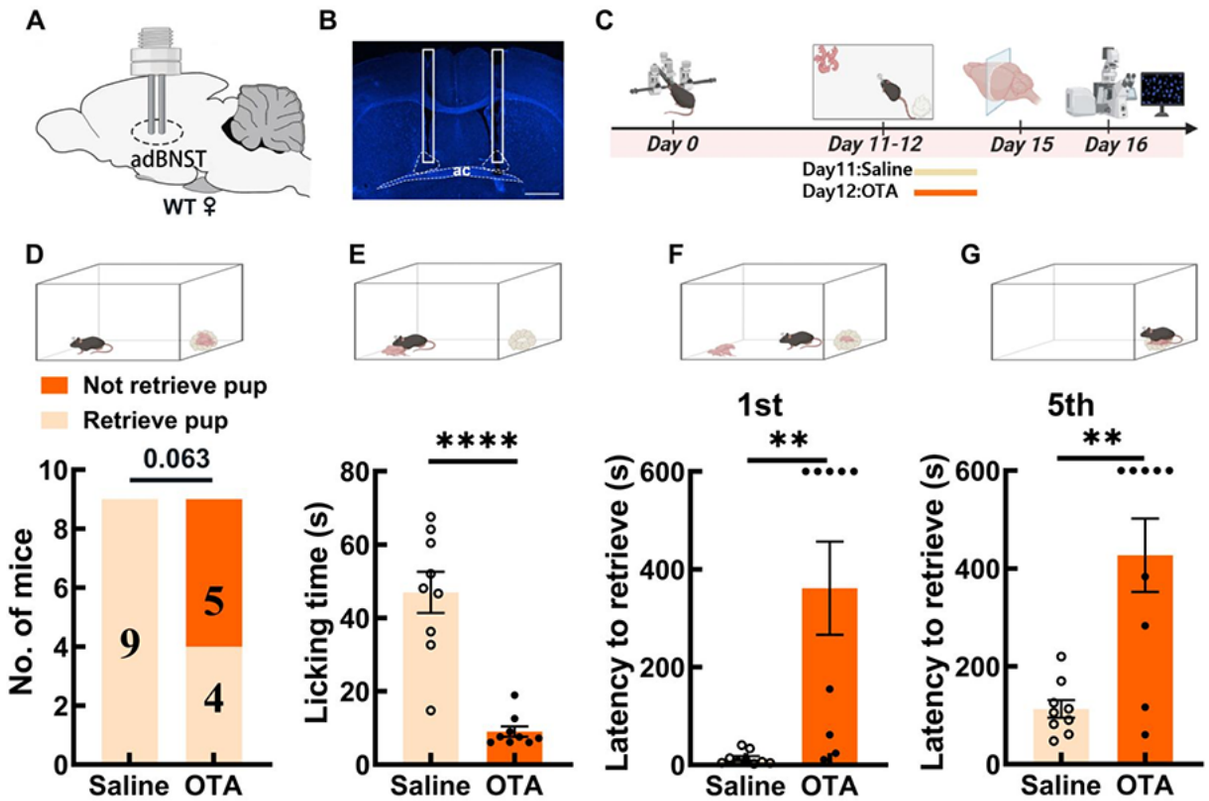

Figure 6 Effects of adBNST OTA administration on pup-caring behavior

A: Schematic illustration depicting the bilateral cannula implantation procedure. B: Representative immunofluorescence image highlighting bilateral cannula tracks within the adBNST (marked by white lines). ac: anterior commissure. Scale bar: 1 mm. C: Timeline of the experiment. D–G: Schematic of pup-retrieval failure (D, top), licking time of pups (E, top), latency of the first pup retrieval (F, top) and the fifth pup retrieval (G, top) in virgin retrievers. The schematic illustrations were created using resources from https://app.biorender.com/biorender-templates/figures. Number of wild-type virgin female mice exhibiting pup retrieval or non-retrieval behavior (D, bottom), the time of licking pups (E, bottom), the latency to retrieve the first (F, bottom) and fifth (G, bottom) pup after injection with saline or OTA (n=9). Statistical analyses were conducted using McNemar's test for comparisons between Saline and OTA trials (D) and paired t-tests (two-sided) (E–G). The data are presented as mean±SEM. **: P<0.01; ****: P<0.0001. The detailed statistical analysis is presented in Supplementary Material 1.

(maternal-like behavior vs. social interaction), or dose of injection (200 ng vs. 1 ng). We acknowledge the complex mechanisms underlying OT's functional regulation through OTR within the BNST. OT is capable of exerting both activating and inhibitory effects on BNST OTR neurons. L-368 899, an antagonist targeting the OTR, effectively inhibits the activation of Gq, Gi, and β-arrestin pathways induced by OT in the BNST (Duque-Wilckens et al., 2018). Conversely, bilateral injection of OTA reduced the time of licking pups and increased the latency to retrieve pups. This is consistent with the results of chemogenetic inhibition of OTR neurons. As we all know, the BNST is essential for the regulation of anxiety-related behaviors (Wang et al., 2020). A previous study has shown that oxytocin receptors (OTRs) in the BNST contribute to the acquisition of cued fear as measured by fear-potentiated startle (Martinon et al., 2019). While the BNST is dispensable for the generation of rapid-onset, short-duration defensive behaviors elicited by discrete, immediate threats, it plays a critical mediating role in slower-onset, longer-lasting adaptive responses. These BNST-dependent responses are typically associated with sustained threat exposure and often persist beyond the termination of the threatening stimulus, reflecting its functional specialization in processing prolonged or ambiguous threat signals (Duvarci et al., 2009). Therefore, chemogenetic or pharmacological manipulation of the adBNST is likely to affect both anxiety-like and maternal-like behavior. In the future, we will investigate how OTR in the adBNST simultaneously influences maternal-like behavior and anxiety-like behavior.

Our findings provide important clues for understanding the finely tuned regulatory mechanisms of the OT system in maternal behavior. As a key brain region within this system, the activity patterns and functional modulation of OTR neurons in the adBNST are likely to have profound implications for the establishment and maintenance of mother-infant interactions. However, several limitations should be considered. First, we defined the latency to pup retrieval as 600 seconds for mice that failed to retrieve any pups. This definition approach indeed establishes an "artificial ceiling" for mice that did not complete pup retrieval. Although such a data-processing method is common in behavioral experiments, it may diminish our ability to detect true statistical differences. This is especially the case when a subset of animals does not exhibit retrieval behavior throughout the entire testing period. Second, a limitation lies in the lack of analysis regarding the role of downstream pathways of adBNST OTR neurons in maternal-like behavior. Future research should further explore dynamic changes of the OT system under different physiological and environmental conditions. These findings provide novel perspectives and robust data support for neurobiological research on the OT system. Furthermore, they may offer potential therapeutic targets for relevant disorders, such as aberrant maternal care resulting from postpartum depression and anxiety disorders.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

P.T.G., Z.X.H. and F.D.T. participated in study design. P.T.G. conducted most experiments and wrote the original draft. Y.Z., Y.T.B., L.Z.Z participated in fiber photometry recording experiments. K.R.X. and Y.R L. participated in chemogenetic manipulation experiments. W.L. and Z.Y.W. participated in behavioral experiments and data collection and analysis. All authors read and approved the final version of the manuscript.

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