Reconstruction of the Hypothalamic-Neurohypophysial System and Functional Dissection of Magnocellular Oxytocin Neurons in the Brain

Graphical Abstract

Highlights
- A three-dimensional architecture of the HNS with high resolution was reconstructed
- Magnocellular neuroendocrine cells collateral projected to extrahypothalamic areas
- Activation of Magno-OXT neurons promoted social behavior and peripheral OXT release
- Inhibition of Magno-OXT neurons elicited opposite effects

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In Brief
Zhang et al. reconstruct the three-dimensional hypothalamic-neurohypophysial system. They find that some magnocellular neuroendocrine cells simultaneously project to the pituitary and brain, coordinating both peripheral and central actions of oxytocin.
Article

Reconstruction of the Hypothalamo-Neurohypophysial System and Functional Dissection of Magnocellular Oxytocin Neurons in the Brain

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SUMMARY

The hypothalamo-neurohypophysial system (HNS), comprising hypothalamic magnocellular neuroendocrine cells (MNCs) and the neurohypophysis, plays a pivotal role in regulating reproduction and fluid homeostasis by releasing oxytocin and vasopressin into the bloodstream. However, its structure and contribution to the central actions of oxytocin and vasopressin remain incompletely understood. Using viral tracing and whole-brain imaging, we reconstruct the three-dimensional architecture of the HNS and observe collaterals of MNCs within the brain. By dual viral tracing, we further uncover that subsets of MNCs collateralize to multiple extrahypothalamic regions. Selective activation of magnocellular oxytocin neurons promote peripheral oxytocin release and facilitate central oxytocin-mediated social interactions, whereas inhibition of these neurons elicit opposing effects. Our work reveals the previously unrecognized complexity of the HNS and provides structural and functional evidence for MNCs in coordinating both peripheral and central oxytocin-mediated actions, which will shed light on the mechanistic understanding of oxytocin-related psychiatric diseases.

INTRODUCTION

The hypothalamo-neurohypophysial system (HNS), comprising hypothalamic magnocellular neuroendocrine cells (MNCs) and their axonal projections to the posterior pituitary (PPI), is a key gateway for the brain to regulate peripheral function. Primarily residing in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON), MNCs release oxytocin (OXT) and arginine vasopressin (AVP) from their neurohypophysial terminals into the peripheral bloodstream to regulate reproduction and fluid homeostasis (Harris, 1955b; Insel et al., 1997; Leng et al., 2015). Despite the pivotal importance of the HNS in neuroendocrine regulation, knowledge on the structure of the HNS is mainly from histological tract tracing studies conducted decades ago (Buijs et al., 1978; Hou-Yu et al., 1986; Sofroniew and Glassmann, 1981). The deep location of the pituitary and the presence of the fragile neural stalk leave the system frequently overlooked in recent brain-wide connectome studies (Dong, 2008; Oh et al., 2014). To date, the full composition and wiring of the HNS remain incompletely understood.

Accumulating evidence demonstrates that central OXT and AVP are involved in the regulation of various social and stress-related behaviors in the brain (Grinevich and Stoop, 2018; Jurek and Neumann, 2018; Onaka et al., 2012; Ross and Young, 2009). Moreover, defects in the oxytocinergic system are found associated with mental diseases such as autism and anxiety-related disorders (Andari et al., 2010; Hollander et al., 2007; Windle et al., 1997). MNCs are known to send unipolar axons to the neurohypophysis, and peripheral OXT and AVP peptides rarely cross the blood-brain barrier (BBB) (Armstrong and Paxinos, 1995; Armstrong et al., 1980; Leng and Ludwig, 2016; Mens et al., 1983; Swanson and Kuypers, 1980); however, whether and how they contribute to central actions remain controversial.

Other than the PPI-projecting magnocellular OXT (Magno-OXT) neurons (20- to 35-μm soma diameter), the PVN also
contains centrally projecting parvocellular OXT (Parvo-OXT) neurons (10- to 15-μm soma diameter), which were thought to be responsible for the central actions of OXT (Badoer, 1996; Insel et al., 1997; Lee et al., 2009; Swanson and Kuypers, 1980). Parvo-OXT neurons contain much less OXT than Magno-OXT neurons and mainly project to the hindbrain and spinal cord (Swanson and Kuypers, 1980; Swanson and Sawchenko, 1980, 1983); however, whether they participate in the OXT-mediated actions in the forebrain remains elusive (Dolen et al., 2013). Somatodendritic release from Magno-OXT neurons was thought to be the main source of OXT in the brain and was involved in central effects of OXT such as social recognition and maternal behavior (Bergquist and Ludwig, 2008; Ludwig and Leng, 2006; Takayanagi et al., 2017). Given the limited diffusion of locally released peptides to a distant region (Chini et al., 2017), it is less likely that the central actions of OXT observed in multiple extrahypothalamic regions were effects of locally released OXT (Li et al., 2016; Marlin et al., 2015; Nakajima et al., 2014).

Using OXT promoter-driven adeno-associated viruses (AAVs) that specifically infect OXT neurons, recent studies found that OXT neurons extensively project to more than 50 brain regions and that axonal release of OXT neurons contributed to OXT-mediated fear attenuation (Knobloch et al., 2012; Menon et al., 2018). In these studies, however, viruses were injected into the hypothalamus and thus could infect both Magno-OXT and Parvo-OXT neurons (Knobloch et al., 2012). As such, the processes and/or fibers observed within the brain may not necessarily represent projections of Magno-OXT neurons. Instead, different subsets of OXT neurons may independently project to different targets, confounding the origins of OXT in the brain. Mapping the connectivity of MNCs, particularly Magno-OXT neurons in the HNS, is therefore crucial to address these concerns.

As all MNCs project to the PPI (neurohypophysis), we reasoned that retrograde tracing from the PPI would be an appropriate way to selectively label MNCs. Together with newly engineered viral tracers, we may be able to fully delineate the morphology and projections of MNCs. By injecting the green fluorescent protein (GFP)-tagged retrogradely transported virus (AAV-Retro-GFP; hereinafter Retro-GFP) into PPI (Tervo et al., 2016), we selectively labeled the MNC ensemble and reconstructed the three-dimensional (3D) connection map of the rat HNS. We further traced their fiber trajectories at single-cell resolution and uncovered multiple extrahypothalamic collateral projections of MNCs. By dual retro-tracing strategy, we confirmed that subsets of MNCs collateral project to the PPI and different brain regions. Using customized OXT-Cre rats, we revealed that Magno-OXT neurons collateral project to multiple brain areas, including the previously unidentified caudate putamen (CPu). Chemogenetic activation of Magno-OXT neurons not only elevated peripheral OXT release but also drove central OXT-dependent social interactions and locomotion. Conversely, chemogenetic inhibition of Magno-OXT neurons suppressed both peripheral OXT release and social investigation. This study uncovers the full organization of the HNS and provides direct evidence for Magno-OXT neurons in coordinating both central and peripheral actions of OXT.

**RESULTS**

**Retrograde Viral Tracing from the PPI Labeled the MNC Ensemble**

To selectively label the MNCs projecting to the neurohypophysis, we injected the retrogradely transported virus AAV-Retro-EGFP (1 μl, 1.28 · E+13 vg/ml) into the PPI of Sprague-Dawley (SD) rats after optimizing viral dosage (Figures 1A [left panel] and S1A). Two weeks later, intense GFP epifluorescence was observed in the PPI, demonstrating the accuracy of viral injection and infection (Figure 1A, middle panel). Robust GFP signals within the neural stalk reflected the effective retrograde transport of the virus along the axons (Figure 1A, right panel).

GFP+ cells were primarily located from the preoptic to the tuberal areas of the hypothalamus and packed into multiple discrete nuclei, ranging from 0 mm to −3 mm along the anterior-posterior (AP) axis and from −6 mm to −10.5 mm along the dorsal-ventral (DV) axis (Figure 1C) in both male and female rats. Abundant GFP+ cells were distributed in the expected principal magnocellular neurosecretory nucleus (PMN), SON, and PVN (Figure 1B). Moreover, densely packed GFP+ cells were seen in the less defined accessory magnocellular neurosecretory nucleus (AMN) (Fisher et al., 1979; Peterson, 1966; Rhodes et al., 1981), consisting of the anterior commissural nucleus (ACN), the circular nucleus (CiN), the antero perifornical nucleus (AF), the posterior perifornical nucleus (PoF), the nucleus of the medial forebrain bundle (Nmfbb), and the retrochiasmatic nucleus (RCN) (Figures S1B and S1C). Notably, scattered GFP+ cells were also seen in the lateral and preoptic hypothalamic areas, including the bed nucleus of the stria terminalis (BST), medial preoptic nucleus (MPN), medial preoptic area (MPO), and lateral preoptic area (LPO) (Figure S1C), as well as the anteroventral periventricular nucleus (AVPV) and preoptic periventricular nucleus (PVpo) (Figure S1C; data not shown). These areas are hereinafter referred to as the scattered magnocellular neurosecretory system (SMN). Thus, the engineered retrograde viral tracers allowed us to efficiently label the cell ensemble that directly innervates the neurohypophysis from multiple nuclei of the hypothalamus (schematically illustrated in Figures 1C and S1D).

**Retrogradely Traced MNCs Project beyond the BBB and Contain AVP and/or OXT**

MNCs release hormones directly into the bloodstream via their axonal terminals in the neurohypophysis devoid of the BBB. To assess the neuroendocrine property of the virally traced GFP+ cells, we intraperitoneally injected the rats (PPI-viral injected) with Fluoro-Gold (FG), a fluorescent dye that can be taken up by nerve terminals outside the BBB and retrogradely transported to the somas (Figure 1D). Prominent FG-labeled neurons were observed in the SON, PVN, and arcuate nucleus (ARC) that are known to be neurosecretory (Figure S1E; data not shown for ARC). Importantly, all the GFP+ cells in the SON, PVN, and AMN were co-labeled with FG (100% dual labeling). GFP+ cells scattered in the preoptic area (POA) and lateral hypothalamic area (LHA) also exhibited a high percentage (89% and 95%, respectively) of FG dual labeling (Figure 1E), suggesting that retrograde tracing from the PPI faithfully labeled the MNCs...
Figure 1. Retrograde Tracing of MNCs in Multiple Hypothalamic Regions Projecting to the Neurohypophysis

(A) Schematic injection and viral infection of AAV-Retro-CMV-GFP in the posterior pituitary (PPI). Middle and right: representative images of the viral infection in the PPI and the retrograde transport within the pituitary stalk from the PPI.

(B) Representative images of PPI-retrogradely traced (GFP+) cells in SON and PVN.

(C) A diagram summarizing the connections between hypothalamus and neurohypophysis. The hypothalamic nuclei were assembled relative to their rostral/caudal and dorsal/ventral locations and divided into three subpopulations. The orange, red, and yellow circles represent the principal, accessory, and scattered magnocellular neuroendocrine systems (PMN, AMN, and SMN), respectively.

(D) Schematic representation of the Fluoro-Gold (FG) intraperitoneal (i.p.) injection 14 days after Retro-GFP labeling.

(E) Histogram of the percentage of FG+ cells in retrogradely traced cells (GFP+) (n = 3 rats).

(F) Immunostaining and FISH analyses reveal no co-localization between PPI-retrogradely traced GFP+ MNCs (immunostaining) and thyrotropin-releasing hormone (TRH)+ parvocellular neuroendocrine cells (PNCs) in the PVN.

(G) Statistical analysis of the percentage of AVP+ or OXT+ neurons in retrogradely traced cells (GFP+) in different nuclei (n = 5 rats).

(H) Relative ratio of AVP+/GFP+ and OXT+/GFP+ cells within different magnocellular nuclei (n = 5 rats).

(I) A pie chart showing the ratio of AVP+ and OXT+ GFP-positive neurons in all the traced MNCs (n = 5 rats).

AP, anterior-posterior distance from the bregma (in millimeters). Scale bars, 100 μm. Error bars indicate SEM.

See also Figures S1 and S2.
Figure 2. 3D Reconstruction of the HNS Using fMOST

(A) Schematic procedures of the fluorescent micro-optical sectioning tomography (fMOST) imaging. (B and C) 3D projections of the HNS derived from 1,700 fMOST images (thickness of 3,400 μm). A coronal view of the 3D HNS (B) and the multi-color registration of the nuclei (C).

(D) Quantitative analysis of GFP+ cells in different nuclei indicating distinctive subpopulations: PMNs (orange), AMNs (red), and the scattered MNs (yellow) (n = 5 rats). The pie chart indicates the percentage of GFP+ neurons in PMNs, AMNs, and SMNs (n = 5 rats).
innervating the PPI. Given the limited efficiency of viral infection and retrograde transport, FG+ MNCs within the SON, PVN, and AMN were partly labeled by GFP. Approximately 61% of FG+ cells in SON were GFP positive (Figure S1F), which reflects the labeling efficiency of the retrograde viral tracer, as virtually all neurons in the rat SON project to the neurohypophysis (Swanson and Sawchenko, 1983).

PVN contains both MNCs projecting to the PPI and parvocellular neuroendocrine cells (PNCs) projecting to the median eminence (ME) that is closely connected to the pituitary gland. To further examine whether PPI-retrograde tracing specifically labels MNCs but not PNCs in the PVN, we carried out GFP staining and fluorescence in situ hybridization (FISH) analyses using a well-accepted marker for PNCs, thyrotropin-releasing hormone (TRH). No co-localization was observed between GFP- and TRH-positive cells in the PVN, further verifying that PPI-retrograde tracing selectively labels MNCs rather than PNCs (Figure 1F).

MNCs are known to contain AVP/OXT. To examine whether all traced MNCs contain AVP or OXT, we performed co-immunos- staining of GFP with mixed antibodies to AVP and OXT (antibodies were both generated from rabbits). As expected, almost all GFP+ cells in the PMN and AMN were co-labeled with AVP and OXT (Figures 1G and S2A). By contrast, only 80% of GFP+ cells in the LHA expressed AVP or OXT, suggesting that not all cells projecting to the neurohypophysis contain AVP or OXT. Using OXT and AVP antibodies raised from different species, we were able to determine the fraction of OXT or AVP cells in different nuclei. Consistent with previous findings (Hou-Yu et al., 1986; Sofroniew, 1983), we found that ACN contained only OXT neurons, whereas other nuclei contained both AVP and OXT with different percentages (Figures S2B–S2D). All the nuclei, except the Nmfb, contained more OXT neurons than AVP neurons (Figure 1H). Altogether, OXT+ and AVP+ cells accounted for 63% and 37% of retrogradely traced MNCs (Figure 1I).

3D Reconstruction of the HNS by Fluorescent Micro-Optical Sectioning Tomography

Given that retrograde viral tracer clearly labeled the MNC ensemble, we took a further step to construct the HNS. To acquire a full architecture of the HNS, the brains with intact neural stalks and pituitaries were carefully isolated from PPI retro-traced rats and imaged by fluorescent micro-optical sectioning tomography (fMOST) at a resolution of 0.32 × 0.32 × 2 μm³ (schematically illustrated in Figure 2A and Video S1). All samples were scrutinized for GFP signals along the needle track to ensure that only accurately shot and infected samples were used for subsequent 3D reconstruction and analyses (Figures S3A–S3F). An individual dataset containing over 6,600 coronal slices from each brain was generated from qualified samples, and the raw data were registered to the rat brain atlas (Swanson, 2004). Since MNCs were localized between 0 and −3.4 mm along the AP axis, we axially stacked 1,700 re-sampled fMOST coronal images spanning this area to reconstruct the HNS using Imaris software (Figures 2B and 2C). The reconstructed projection gives a 3D overview of the HNS, with MNCs (5,868 cells per rat, on average) packed into more than 8 nuclei in the hypothalamus and axons bundled together to form the arc tracts of Greving (Greving, 1926), subsequently passing through the ME and terminating in the PPI (Figure 2B). Among the multiple nuclei, the SON, abutting the ventral surface of the brain (from 0 to −1.8 mm along the AP axis), contains the most abundant GFP+ cells (1,617 ± 198 cells). Caudal and medial to SON lies the RCN, whereas dorsal to the SON is the Nmfb. Notably, MNCs in the PVN and ACN are arranged in a butterfly-like shape, with the ACN constituting the forewings and the PVN forming the hindwings. The periforrinal MNC aggregates, including both AF and PoF, lie dorsal-lateral to the fornix, whereas CIN lies midway between the PVN and SON (Figures 2B and 2C).

3D measurement of the MNCs reveals that the average volume of GFP+ cells is above 1,300 μm³ (approximately 25 μm in diameter), which meets the criteria for the size of MNCs (20–35 μm; Figures S4A and S4B) (Sofroniew and Glasmann, 1981; Stern and Armstrong, 1998). Qualitatively, about half (53% ± 2.7%) of the GFP+ MNCs were located in the PMN, including SON and PVN, while one third (31% ± 2.3%) and 16% of labeled MNCs were found in the AMN and SMN, respectively (Figure 2D). A heatmap illustrating the number and distribution of GFP+ MNCs in different nuclei is presented on a sagittal plane of the rat brain (Figure 2E).

Collateral Projections of MNCs within the Brain

High-resolution fMOST images allowed us to identify not only the somas but also the fine processes of MNCs (Figure 3A). Within the hypothalamus, the Greving tract of MNC axons was clearly seen (Figure 2B); outside the hypothalamus, GFP+ processes/fibers were also observed in multiple areas. Both the cortical regions, such as the piriform cortex (Piri C) and auditory cortex (Audi C), and the subcortical regions, including the amygdala (AMY), Cpu, lateral septum (LS), and nucleus accumbens (NAc), contained GFP+ fibers (Figures 3B and S4C), with peak density of fibers found in the piriform cortex (Figures 3C and 3D). Of note, GFP+ fibers were undetectable in the reward center, the ventral tegmental area (VTA) (Figure 3B), in accordance with recent findings showing that Magno-OXT neurons do not project to the VTA in mice (Xiao et al., 2017). All the GFP+ areas, except the AMY, are more than 1,000 μm away from the somal location of MNCs in the hypothalamus. As the viruses were injected into the PPI, fibers in the extrahypothalamic regions likely represent collaterals of the retrogradely traced MNCs in the brain.

To fully delineate the morphology of MNCs, we traced fiber trajectories of GFP-labeled MNCs and reconstructed the cells at single-cell resolution using Amira software (Figures 3E and S4D–S4G; Video S2). All the 46 traced MNCs send axons
Figure 3. Processes of Retrogradely Labeled MNCs in the Cortical and Subcortical Regions
(A) Representative fMOST images showing the somas and processes of PPi-retrogradely traced cells.
(B) Representative images of GFP+ fibers in extra-hypothalamic areas, including the amygdala (AMY), caudate putamen (CPu), lateral septum (LS), nucleus accumbens (NAc), piriform cortex (Piri C), auditory cortex (Audi C), ventral tegmental area (VTA), and posterior pituitary (PPi).
(C and D) Histogram (C) and heatmap (D) of collateral distribution in cortical and subcortical regions. The density of GFP+ fibers in extra-hypothalamic areas was calculated by the ratio of voxels containing GFP to total voxels in individual nucleus (n = 3 rats).
(E) 3D morphological reconstruction of an MNC in the PVN showing collateral projections to the NAc and ME.
(F) Schematic of collateral labeling of MNCs in the PVN by dual-viral tracing strategy (PPi-retrograde and PVN-anterograde tracing).
(G) Labeled MNCs at the viral injection site in the PVN.
(H) Distribution of GFP-positive fibers and SYP-mRuby-labeled axon terminals in NAc.
AP, anterior-posterior (distance from the Bregma, in millimeters). Scale bars, 100 μm in (B) and (E), 50 μm in (G) and (H), and 10 μm for high-magnification images of terminals in (H). Error bars indicate SEM.
See also Figures S3 and S4, Table S1, and Video S2.
winding through the anterior hypothalamic area/LHA (AHA/LHA), passing by the fornix and the ventromedial hypothalamic nucleus (VMH), to the ME (Figure S4H). Other than ME projection, 7 cells (7/46; 15%) within the PVN and PoF showed bifurcated projections to the NAc, CPu, and lateral globus pallidus (LGP) (Figures 3E and S4I; Table S1; Video S3).

Injection of the retrograde viral tracer expressing Cre recombinase (AAV-Retro-Cre) into the PPi and a Cre-dependent AAV expressing GFP and synaptophysin-conjugated mRuby into the PVN (Figures 3F and 3G) revealed prominent GFP signals and mRuby-positive inflated beaded axon terminals in both the shell and core region of the NAc (Figure 3H), verifying the collateral projections to NAc from MNCs.

To further confirm the collateral projections of MNCs to the extrahypothalamus, we adopted a dual-retrograde tracing strategy by infusing AAV-Retro-Cre into the PPi and AAV-Retro-DIO-mCherry into different extrahypothalamic regions, including the AMY, CPu, and NAc, respectively (Figure 4A). Consistent with the fiber trajectory tracing, mCherry-labeled neurons were broadly observed in multiple magnocellular nuclei, especially in the PVN and PoF (Figures 4B–4J). These data demonstrate that subsets of MNCs send axon collaterals to the PPi and extra-hypothalamus. Immunostaining with OXT antibody found that a majority of these MNCs were Magno-OXT neurons, with 13/17, 17/28, and 22/29 of mCherry-labeled neurons positive for OXT in the AMY, CPu, and NAc (Figure 4K).

Together, these data demonstrate that subpopulations of PPi-projecting MNCs—in particular, Magno-OXT neurons—collaterally projected to multiple extrahypothalamic areas, as schematically summarized in Figure 4L.

**Figure 4. Collateral Projections of MNCs in Extrahypothalamic Regions Revealed by Dual-Retrograde Labeling Strategy**

(A) Schematic of dual-retrograde tracing strategy from the PPi and the extrahypothalamic regions. Fluoro-Gold (FG) was added to the AAV-Retro-DIO-mCherry virus to indicate extrahypothalamic injection sites.

(B–J) Representative images indicating the injection sites (FG+) and retro-labeled MNCs (mCherry+) after viral injections to the AMY (B–D), CPu (E–G), NAc (H–J), or the PPi. Immunostaining showed OXT-positive mCherry+ neurons in the magnocellular nuclei, including the PVN and PoF.

(K) Pie charts indicating the ratio of OXT+ neurons in traced MNCs in different areas (n = 3 rats).

(L) Schematic illustration of an MNC with collateral projections to PPi and extrahypothalamus. AP, anterior-posterior (distance from the bregma, in millimeters). Scale bars, 1,000 μm for coronal sections indicating the injection sites and 50 μm for the other images.

**Magno-OXT Neurons Collaterally Projected to Multiple Brain Regions**

OXT plays an important role in regulating diverse social behaviors, including pair bonding and affiliative behaviors (Burkett et al., 2016; Jurek and Neumann, 2018; Young and Wang, 2004). However, it remains controversial whether Magno-OXT or Parvo-OXT neurons are involved. To address this question, we generated an OXT-Cre rat line, in which a P2A-iCre cassette was inserted after the OXT gene using the CRISPR-Cas9 strategy and verified by Southern blot (Figures S5A–S5C). Co-immunostaining with Cre and OXT-neurophysin (OXT-NP) antibodies on OXT-Cre rat hypothalamic slices demonstrated approximately 92% and 99% co-localization of Cre+ and OXT+ cells in...
Figure 5. Chemogenetic Manipulation of Magno-OXT Neurons Alters OXT Release

(A) Schematic of AAV-Retro-DIO-YFP viral injection in OXT-Cre rats.
(B) Immunostaining of YFP with OXT-NP on viral injected OXT-Cre rat brain slices. Co-localization of YFP with OXT-NP is indicated with white triangles.
(C) Parvocellular neurons in the PVN were negative for YFP.
(D) Histogram of the OXT-NP and YFP co-localization in the PVN and SON (n = 3 rats; 182 cells in the PVN and 257 cells in the SON).
(E) Distribution of YFP-labeled fibers of Magno-OXT neurons in the extrahypothalamic regions, including the NAc, LS, Piri C, and CPu.
(F) Diagram of chemogenetic activation of Magno-OXT neurons and subsequent measurements.
(G) Validation of chemogenetic activation of Magno-OXT neurons as indicated by cFos staining. CNO administration induced cFos expression in Magno-OXT neurons in the PVN and SON, and co-localization of mCherry with cFos is indicated with white triangles.
(H) Histogram of the cFos and mCherry co-localization. 97.4% of the mCherry+ cells were positive for cFos. 64.5% of activated Magno-OXT neurons were mCherry+ in SON (n = 3 rats; 335 cells) and 44.1% in PVN (n = 3 rats; 344 cells).
(I) Elevated OXT levels in the blood after CNO treatment as measured by ELISA (CTRL, 53.97 ± 1.02 pg/mL, n = 3 rats; Dq, 81.04 ± 3.11 pg/mL, n = 4 rats; p = 0.0008, two-tailed t test).
(J) Schematic injection and chemogenetic inhibition of Magno-OXT neurons with AAV-Retro-DIO-hM4Di.

(legend continued on next page)
the SON and PVN. More than 90% OXT-NP+ cells in the hypothalamus were co-labeled by Cre, suggesting that Cre recombinase was specifically expressed in OXT neurons (Figures SS5D and SSE). Injection of the Cre-dependent retrograde viral tracer (AAV-Retro-DIO-EYFP) into the PPi, followed by OXT-NP immunostaining, revealed that more than 95% of virally traced cells in the PVN and SON were positive for OXT-NP, suggesting specific Cre activity in OXT neurons (Figures 5A, 5B, and 5D). In the SON, about two thirds (64.6%) of OXT-NP-positive cells were labeled by YFP, suggesting a labeling efficiency similar to that in SD rats (Figure 5D). Moreover, OXT cells in the AMN were also successfully labeled (Figures SS5F and SS5G). By contrast, none of the Parvo-OXT cells in the PVN were labeled, verifying the specific labeling of Magno-OXT neuronal ensemble by PPI-retrograde tracing (Figure 5C). Consistent with our observations mentioned earlier (Figures 3B and S4C), YFP+ fibers were also found in several brain areas, including the NAc, LS, Piri C, and CPU (Figure 5E). After injection of Cre-dependent retrograde tracer (AAV-Retro-DIO-Flp) into the PPI of the OXT-Cre rats and infusion of Flp-dependent anterograde tracer expressing enhanced yellow fluorescent protein (AAV-fDIO-ChR2-EYFP) into the PVN, YFP-positive fibers were also observed in the AMY, CPU, and NAc (Figures SS5H–SS5J), verifying that subsets of Magno-OXT neurons collateralize project to the extrahypothalamic regions.

Selective Manipulation of Magno-OXT Neurons Altered Peripheral OXT Release and Modulated Central OXT-Mediated Social Interactions

To selectively manipulate Magno-OXT neurons, we injected the AAV-Retro-DIO-DREADDs viruses into the PPI of OXT-Cre rats, which express the engineered receptors exclusively activated (hM3D-Gq, Dq) or inhibited (hM4D-Gi, Di) by a designed drug clozapine-N-oxide (CNO) (Armbruster et al., 2007) (Figures 5F and 5J). Consistent with the aforementioned data (Figures 5B and 5C), the viruses selectively labeled Magno- but not Parvo-OXT neurons (Figures 5K and 5L; data not shown for hM3D-Gq). Notably, immunostaining of cFos, an immediate neuronal activation marker, revealed that almost all retro-traced Dq+ Magno-OXT (mCherry+) cells (97.4%) were activated after CNO administration (Figures 5G, 5H, S5K, and S5L). However, none of the Parvo-OXT neurons in the PVN were positive for cFos, suggesting that CNO selectively activated Dq-infected Magno-OXT neurons (Figure SS5M). Conversely, Di-infected Magno-OXT neurons were effectively inhibited by CNO application. Magno-OXT neurons exhibited a typical transient outward rectifying current, known as the hyperpolarizing notch, via ex vivo slice electrophysiology (Figure 5M) (Eliaeva et al., 2016; Luther et al., 2002). CNO administration induced a slight hyperpolarization (about 5 mV) and significantly reduced current-induced action potentials (Figure 5N). Consequently, chemogenetic activation or inhibition of Magno-OXT neurons substantially increased or decreased the peripheral OXT levels by more than 50% after CNO administration (Figures 5I and 5O). Therefore, the retrogradely transported chemogenetic tool provides an effective approach to bi-directionally manipulate the Magno-OXT neuronal ensembles.

To examine whether manipulation of Magno-OXT neurons alone induces central OXT-related behaviors—for instance, social behaviors—male rats injected with Dq or control viruses were placed into an open-field (OF) apparatus to interact with a juvenile stimulus rat (Figure 5A). Compared to the controls, CNO treatment significantly increased the time of Dq+ rats socializing with the stimulus rats (Figures S6B–S6D). In a 3-chamber apparatus, rats showed no preference to a certain chamber during the habituation and exploration stages (Figures 6A and 6C). At the social investigation stage, however, the time for the Dq+ group spent in the stimulus rat-containing chamber was significantly longer than that for the control group (Figures 6B and 6D). Moreover, Dq+ rats spent twice as much time in the social zone (10 cm around the stimulus cage) as the controls and exhibited a robustly elevated social index (duration in the social zone divided by the total duration in two zones) (Figures 6D and 6E), suggesting chamber preference associated with social interactions. In accordance with previous findings showing that OXT conveys social information through olfaction (Oettl et al., 2016; Wesson, 2013), sniff counts were increased 2-fold in Dq+ rats (Figure 6F). Notably, when the stimulus rat was replaced with a toy, Dq+ rats showed no increase of duration or sniff counts in the social zone upon CNO administration (Figures S6F–S6J), suggesting that OXT specifically facilitates social interactions. As peripheral OXT barely passes the BBB (Leng and Ludwig, 2016; Mens et al., 1983), central OXT released from activated Magno-OXT neurons likely mediates these behavioral effects.

To further determine whether Magno-OXT neurons are required in social investigation, we administered CNO twice daily to Di+ rats for 7 days to inhibit Magno-OXT neurons before social tests (Figure 6H). Chronic inhibition of the Magno-OXT neurons reduced the duration of social interactions, accompanied by dramatically decreased sniff counts between the tested rats and stimulus rats (Figures 6I–6K and 6M). Notably, since CNO-treated Di+ rats spent more time in the shuttle chamber than the control group, we found no significant differences in the social index between these groups (Figure 6L). These results suggest that the endogenous activity of Magno-OXT neurons is required for the normal expression of investigation toward social stimuli. Collectively, our data demonstrate that chemogenetic
Figure 6. Chemogenetic Manipulation of Magno-OXT Neurons Regulated Social Investigation
(A) Diagram of viral injection, drug administration, and social investigation tests. AAV-Retro-DIO-hM3Dq-mCherry or AAV-Retro-DIO-mCherry viruses were injected into the PPI of OXT-Cre rats.
(B) Diagram and track plots showing the social investigation test after chemogenetic activation of Magno-OXT neurons in a 3-chamber apparatus.
(C) Duration of CTRL and Dq-injected rats in the left and right chambers at different stages. Activation of Magno-OXT neurons increased the duration in left chamber during the social investigation stage (CTRL: n = 10 rats; social L-chamber, 208.2 s; social R-chamber, 103.6 s; Dq: n = 10 rats; social L-chamber,
manipulation of Magno-OXT bidirectionally modulates the central actions of OXT.

**Activation of Magno-OXT Neurons Promoted Locomotion via OXT Release in the CPu**

In measuring social behaviors, we noticed that CNO-treated Dq+ rats increased travel distances during the habituation stage, in either the OF apparatus or the 3-chamber apparatus, suggesting that activation of Magno-OXT neurons likely enhances locomotor activities (Figures 6G, S6E, and S6K). Given that OXT neurons collaterally project to the CPu/LGP (Figure 5E), a region critical in motor regulation (Grillner et al., 2005), and the OXT receptor (OXTR) is expressed in the CPu/LGP (Figure 7A), we hypothesized that striatum-projecting Magno-OXT neurons might be involved in the regulation of locomotion. As previous studies suggested that OXT may elicit dosage-dependent effects on locomotion (Klenerova et al., 2009; Uvnas-Moberg et al., 1994), we further tested the locomotion of the rats in a specific box (Figures 7B–7D). Measurement of the travel distance, line crossing, and rearing behaviors revealed that CNO-treated, but not saline-treated, Dq+ rats showed elevated horizontal and vertical locomotion, whereas CNO-treated controls showed no changes (Figures 7E–7H). To examine whether the effects were modulated by OXTR-mediated signaling, we infused L-368,899, a commonly used OXTR antagonist, into the CPu and observed that CNO-induced locomotion increase was abolished (Figures 7F–7H), suggesting a role of striatal OXT in the increased locomotion. To further test whether activation of Magno-OXT neuronal terminals induces similar effects, we directly infused CNO into the CPu to trigger release of OXT from local terminals. We did observe increased line crossing; however, the total travel distance and rearing were not significantly different (Figures 7I–7K), likely due to the sparsely distributed collaterals and/or insufficient terminal activation in the region. However, chronic inhibition of Magno-OXT neurons neither altered the total travel distance (Figures 6N and 7L–7N) nor changed the line crossing and rearing during the locomotion test in Dq+ rats (Figures 7O and 7P), suggesting that basal activity of Magno-OXT neurons is not necessary for the locomotor activity.

**DISCUSSION**

Using state-of-the-art techniques in viral tracing and brain-wide imaging, we systematically mapped the connectivity of the HNS and uncovered the anatomical links between multiple hypothalamic MNC nuclei and the neurohypophysis. These data lay the groundwork for fully understanding the architecture and regulation of the neuroendocrine networks in the future. We further reveal that subsets of MNCs collaterally project to both neurohypophysis and multiple brain areas, providing structural evidence for peripheral and central release of neuropeptides from MNCs. In addition, we demonstrate that, manipulation of Magno-OXT neurons alone alters not only peripheral OXT levels but also central OXT release to regulate social interactions and locomotion. Together, these findings suggest that MNCs play important roles in maintaining homeostasis and regulating behavioral responses, and such actions were achieved through coordinated peripheral and central release of peptides.

Earlier attempts to map the HNS with retrograde tracers provided important information on the general structures of the HNS (Fisher et al., 1979; Kelly and Swanson, 1980; Peterson, 1966). However, limited labeling efficiency and imaging resolution prevented a clear visualization of the system (Fisher et al., 1979; Taniguchi et al., 1988). Retrograde viral tracing from the PPi provides a valuable approach to selectively label MNCs, and together with advanced imaging techniques, we fully...

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371.9 s; social R-chamber, 100.3 s). Two-way ANOVA (main effect of zones F(1,36) = 88.64, p < 0.0001; main effect of treatments F(1,36) = 16.10, p = 0.0003; interaction F(1,36) = 17.48, p = 0.0002) with post hoc Bonferroni’s multiple comparisons test.

3.09, p = 0.0001) with post hoc Bonferroni’s multiple comparisons test. (D and E) Activation of Magno-OXT neurons increased the duration in the social zone (D) and the social index (E). Social index was calculated as duration in the left social zone divided by the total duration in both zones (CTRL: n = 10 rats; duration = 176.1 s; index = 0.68; Dq: n = 10 rats; duration = 371.9 s; index = 0.82). Duration analysis, two-way ANOVA (main effect of zones F(1,36) = 112.2, p < 0.0001; main effect of treatments F(1,36) = 27.48, p < 0.0001; interaction F(1,36) = 30.69, p < 0.0001) with post hoc Bonferroni’s multiple comparisons test. Social index analysis, p = 0.0044, two-tailed t test.

(F) Increased sniff counts of the Dq group after intraperitoneal administration of CNO (CTRL, n = 10 rats; sniff count, 33.5; Dq, n = 10 rats; sniff count, 64.0; p < 0.0001, two-tailed t test).

(G) Increased total travel distance upon activation of Magno-OXT neurons during the habituation stage (CTRL, n = 10 rats; Dq, n = 10 rats; p = 0.0002, two-tailed t test.). (H) Diagram of viral injection, drug administration, and social investigation tests. AAV-Retro-DIO-hM4Di-mCherry or AAV-Retro-DIO-mCherry viruses were injected into the PPI of OXT-Cre rats.

(i) Diagram and track plots of the social investigation test after chronic inhibition of Magno-OXT neurons in a 3-chamber apparatus. (J) Duration of CTRL and Di-injected rats in the left and right chambers at different stages. Inhibition of Magno-OXT neurons reduced the duration in the left chamber during the social investigation stage (CTRL: n = 7 rats; social L-chamber, 228.9 s; social R-chamber, 69.1 s; Di+CNO: n = 6 rats; social L-chamber, 134.3 s; social R-chamber, 43.6 s). Two-way ANOVA (main effect of zones F(1,22) = 36.13, p < 0.0001; main effect of treatments F(1,22) = 8.306, p = 0.0087; interaction F(1,22) = 2.742, p = 0.1120) with post hoc Bonferroni’s multiple comparisons test.

(K and L) Inhibition of Magno-OXT neurons decreased the duration in the social zone in the left chamber (K) but not the social index (L) (CTRL: n = 7 rats; duration = 199.0 s; index = 0.75; Di+CNO: n = 6 rats; duration = 104.9 s; index = 0.72). Duration analysis, two-way ANOVA (main effect of zones F(1,22) = 25.00, p < 0.0001; main effect of treatments F(1,22) = 8.09, p = 0.0094; interaction F(1,22) = 2.930, p = 0.1010) with post hoc Bonferroni’s multiple comparisons test. Social index analysis, p = 0.5609, two-tailed t test.

(M) Chronical intraperitoneal administration of CNO reduced the sniff counts of the Di-pos group (CTRL: n = 7 rats; sniff = 60.1; Di+CNO: n = 6 rats; sniff = 26.7; p = 0.0010, two-tailed t test).

(N) Total travel distance between the control and experimental groups during the habituation stage (CTRL: n = 7 rats; distance = 13,709 mm; Di+CNO: n = 6 rats; distance = 11,983 mm; p = 0.0516, two-tailed t test).

*p < 0.05, **p < 0.01 and ***p < 0.001. Error bars indicate SEM. See also Figure S6.
Figure 7. Chemogenetic Activation of Magno-OXT Neurons Enhanced Locomotion

(A) Left panel: a schematic of striatal slice. Middle panel: YFP+ fibers in the CPu and LGP of retrogradely traced Magno-OXT neurons. Right panel: expression of OXT receptors in CPu by single-molecule FISH.

(B–D) Diagram of drug administration and locomotion tests. AAV-Retro-DIO-hM3Dq-mCherry or AAV-Retro-DIO-mCherry viruses were injected into the PPI of OXT-Cre rat, and microinfusion cannulas were implanted in bilateral CPu (B). The cannula tract is indicated with a dotted line (C). Drugs were administrated following the habituation and basal stages. Locomotion was tested 20 min after administration of CNO/saline in the presence or absence of OXTR antagonist L-368,899 (D).

(E) Representative track plots of the locomotion activity of animals after different treatments.

(F–H) Increased locomotion after CNO-induced activation of Magno-OXT neurons. Total travel distance (F), line-crossing (G), and rearing (H). OXTR antagonist abolished the locomotion-promoting effects (ns = 10, 10, 10, and 4 rats for CTRL, Dq+CNO, Dq+CNO+Antagonist, and Dq+Saline groups, respectively). Data in (F) and (G) were normalized to the basal stage. Distance analysis, F(3,29) = 20.59, p < 0.0001, one-way ANOVA with post hoc Turkey’s test; Line-crossing analysis, F(3,29) = 14.67, p < 0.0001, one-way ANOVA with post hoc Turkey’s test; Rearing analysis, two-way ANOVA (main effect of stages F(1,58) = 86.36, p < 0.0001; main effect of treatments F(3,58) = 2.419, p = 0.0753; interaction F(3,58) = 1.463, p = 0.2341) with post hoc Bonferroni’s multiple comparisons test.

(legend continued on next page)
reconstructed the architecture of the HNS with high resolution for the first time. The reconstructed 3D atlas uncovers the complexity of the HNS and expands the composition of the HNS, which has not been recognized previously. It should be noted that the predominantly studied PVN and SON only harbor approximately a half of the MNCS, and functions of the rest of the MNCS remain largely unknown. It would be interesting to investigate whether MNCS in the AMN and SMN play complementary or opposite roles to those in PMN. On the other hand, the reconstructed atlas is unable to specifically delineate the distribution and projections of Magno-AVP or Magno-OXT neurons. Given the functional importance and segregation of AVP and OXT in the HNS (Heinrichs et al., 2009; Meyer-Lindenberg et al., 2011; Neumann and Landgraf, 2012; Stoop, 2012), it would be important to selectively map the connectomes of Magno-OXT and Magno-AVP neurons in the brain.

MNCs have been regarded as the output, or “motor,” neurons that send unipolar axons and secret neuropeptides into the bloodstream (Buïjs and Van Eden, 2000; Harris, 1955a; Swanson, 2012; Watts, 2005). Using single-cell fiber tracing, we found that a fraction of MNCS collateral projects to both the neurohypophysis and the extrahypothalamic regions. These data suggest that, in addition to maintaining homeostasis by peripheral hormone secretion, MNCS also coordinate related behaviors by central peptide release. For example, the full engagement of Magno-OXT neurons during parturition and lactation, which reaches maximal OXT release into the bloodstream, may also promote central release through the collaterals to enhance maternal behaviors and mother-infant affiliation (Kendrick, 2000; Young and Wang, 2004). To support this notion, a study using dual-retrograde labeling observed that OXT neurons in the PVN collateral project to the NAc in prairie voles, likely promoting pair bonding effects (Ross et al., 2009). A recent study also showed that Magno-OXT neurons in the SON collateral project to the septum to attenuate conditioned fear (Menon et al., 2018). Meanwhile, a limitation of the present study is that single-cell fiber tracing was carried out in a small number of MNCS due to the high density of traced cells. Future studies will use the newly developed dual-AAV suites to sparsely label MNCS in different nuclei to further map their projections in full spectrum (Lin et al., 2018).

The wide distributions of MNCS in a variety of hypothalamic nuclei raised a challenge in labeling and manipulating the neuronal ensemble. Knobloch et al. (2012) carried out multiple injections to label the OXT neurons and uncovered extensive projections of OXT neurons to the brain. However, these results may have obscured the contribution of Parvo-OXT neurons, as both Magno-OXT and Parvo-OXT neurons can be infected. Using rabies viruses tracing, the authors observed collateral projections of Magno-OXT neurons to the AMY (Knobloch et al., 2012). We further clarified that Magno-OXT neurons collateral project to multiple extrahypothalamic regions, including the AMY, CPu, and NAc by 3D reconstruction and dual-retrograde tracing (Figures 4B–4J, 5E, and S5J). These findings may help to explain the roles of MNC-derived central OXT in regulating social recognition, learning and memory, and social rewards (Choe et al., 2015; Marlin et al., 2015). Intriguingly, although OXT has been shown to gate social rewards at both the NAc and VTA (Dölen et al., 2013; Hung et al., 2017), we found that MNCS and/or Magno-OXT neurons specifically project to the NAc but not the VTA, consistent with a recent study demonstrating that Parvo-OXT neurons innervate the VTA and regulate the activity of VTA neurons in mice (Xiao et al., 2017).

We further demonstrate that retrograde tracing from the PPi followed by chemogenetic stimulation is an efficient approach to selectively manipulate the activities of Magno-OXT neurons without affecting Parvo-OXT neurons. Activation of Magno-OXT neurons alone promotes peripheral release of OXT and enhances central OXT-mediated social interactions, whereas inhibition of these neurons produced opposite effects, demonstrating that Magno-OXT neurons have both central and peripheral actions. Whereas the vast majority of studies have mainly focused on OXT neurons (including both Magno-OXT and Parvo-OXT neurons) in the PVN (Choe et al., 2015; Marlin et al., 2015; Oettl et al., 2016; Resendez et al., 2020; Xiao et al., 2017), this study made a first attempt to clarify functions of individual OXT neuronal subgroups and demonstrated that Magno-OXT neurons contribute to the central actions of OXT. Although injection of CNO at the CPu only led to moderately increased locomotion, local infusion of OXTR antagonist significantly attenuated the locomotion increase. Together with previous observations that OXT knockout mice showed a decreased tendency in travel distances in a social approach test (Crawley et al., 2007), these findings suggest that OXT in the CPu enhances locomotion. We postulated that this increased locomotion may contribute to active exploration to facilitate social interaction (Blume et al., 2008). It is possible that the activation of Magno-OXT neurons promotes central OXT release, which drives the animals to be more active and exploratory to facilitate social interactions (Caldaglioni et al., 2014; Oettl et al., 2016).
HNS serves as the Rosetta stone in the regulation of neuroendocrine networks. Our work provides important references for further illumination of the hard-wiring circuits and functions of MNCs. The extensive collaterals of Magno-OXT neurons and the effects elicited upon their activation suggest that they directly modulate neural circuits related to social behaviors. The present work will also provide new insights and clues to better understand the mechanisms underlying a wide range of mental disorders, such as depression, autism, and anxiety, in which dysfunctions of the oxytocinergic transmission have been implicated.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.10.032.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

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### Chemicals, Peptides, and Recombinant Proteins

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### Critical Commercial Assays

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## RESOURCE AVAILABILITY

### Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Zhihua Gao (zhihuagao@zju.edu.cn).

### Materials Availability
This study did not generate new unique reagents, except for the OXT-Cre rat which is commercially available from Biocytogen (Nanjing, China).

### Data and Code Availability
Continuous 2D images showing the whole view of the hypothalamo-neurohypophysial system, coronal sections spanning the needle track and three-dimensional presentation of reconstructed neurons registered to the reference rat brain space can be accessed at http://atlas.brainsmatics.org/a/zhang2008.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Animals
All animal experiments were approved by the Zhejiang University Animal Facilities and followed the guidelines. Oxytocin-Cre (OXT-Cre) rats were generated by Biocytogen (Nanjing, China) on the Sprague Dawley (SD) genetic background. Both wild-type (WT) and OXT-Cre SD rats were maintained in the Lab Animal Center at Zhejiang University and group housed on a 12-hour light cycle (lights off at 7:00 PM) at 25°C. Detailed information regarding animals used in the current study are shown in the Table S2.

To produce the OXT-Cre rats, a targeting construct was created after the exon 3 of the *oxytocin* gene. To avoid abolishing OXT function, the P2A-iCre cassette was introduced between the protein coding sequences of exon 3 and 3’UTR. A 1488 bp 5’ homologous arm and a 1401 bp 3’ homologous arm were added to the upstream and downstream of iCre, respectively. The Cas9/sgRNA and iCre constructs were electroporated into the rat zygotes. The genotypes of offsprings were validated by Southern blot and genotyping PCR. OXT-Cre rats were backcrossed to the SD rats for at least five generations before behavioral analyses.

## METHOD DETAILS

### Stereotaxic injection
Male rats (WT or OXT-Cre rats, as indicated) aged 12–16 weeks (260–300 g) were used for injection. The rats were anesthetized with 1% pentobarbital and placed on a heating pad to maintain the body temperature at 37°C. Under sterile surgical conditions, the rats were fixed in a stereotaxic instrument (David Kopf Instruments) with its eyes protected with ointment. Via a 10 mm midline scalp incision, the needle was lowered to the target locations based on the stereotaxic atlas (Paxinos and Watson, 2006; Swanson, 2004), through a small craniotomy made with a dental drill in the brain skull. Virus was infused using a 2.5 μl Hamilton syringe driven by...
a pressure microinjector (KD Scientific) at a slow rate over at least 10 min. To allow diffusion and reduce backflow, the syringe was maintained at the target position for 10 min at the end of injection, then uplifted 50 μm and maintained for another 3 min before complete withdrawal. The incision site was sutured after application of antibiotics. The rats were monitored for 24 h to ensure full recovery before being sent back for normal housing.

Given the deep location of the posterior pituitary, it is necessary to avoid potential contamination around the needle track during viral infusion. We took the following precautions to avoid needle tract contamination: First, after viral loading, the outer wall of the Hamilton syringe was carefully and thoroughly wiped with wet cotton swabs from top to bottom to remove the residual virus at the tip. Second, before lowering the syringe into the brain, gently withdraw the pistol of the syringe to allow an extra 20 nl air staying at the needle tip. This helps to maintain a small negative pressure that prevents the viral outflow during needle injection through the brain. After full expression of the virus, samples were carefully screened to avoid viral overflow to the adjacent tissue surrounding the needle track and injection sites. Those with fluorescent signals in the ME, ARC or regions around the needle track were discarded and excluded from subsequent analyses.

For retrograde tracing, 1 μl rAAV2/2-Retro-CMV_bGlI-EGFP-WPRE-pA was injected into the posterior lobe of the pituitary (PPI) (AP, −5.80 mm; ML, +0.05 mm; DV, −10.20 mm) of SD rats at 100 nl/min. To achieve low density labeling of neurons for single-cell tracing and morphology reconstruction, reduced viral dose (100 nl) was adopted. Virus-injected rats were sacrificed or further processed after 2-3 weeks.

For collateral projection analysis using dual viral strategy, 1 μl rAAV2/2-Retro-hSyn-Cre-WPRE-pA was injected into the PPI of SD rats at 100 nl/min, followed by 200 nl AAV2/9-hSyn-DIO-mGFP-2A-Synaptophysin-mRuby injection 1 week later into the PVN (AP, −0.72 mm; ML, +0.60 mm; DV, −7.20 mm) at 20 nl/min. Rats were sacrificed 4 weeks after the final injection.

To further examine the collateral projections of the MNcS, dual-retrograde viral tracing strategy was adopted. 1 μl rAAV2/2-Retro-hSyn-Cre-WPRE-pA was injected into the PPI of SD rats at 100 nl/min, followed by injection of 300-400nl rAAV2/2-Retro-hSyn-DIO-mCherry-WPRE-pA mixed with 0.1% FG into the AMY (AP, −2.76 mm; ML, +4.20 mm; DV, −7.80 mm), CPU (AP, −0.60 mm; ML, +3.60 mm; DV, −5.60 mm) or NAc (AP, +1.56 mm; ML, +1.00 mm; DV, −7.40 mm) at 30 nl/min 1 week later. The addition of FG to the virus helps to indicate the injections sites in the brain. Rats were sacrificed 3 weeks after the final injection.

To label cells projecting beyond the BBB, rats injected with rAAV2/2-Retro-CMV_bGlI-EGFP-WPRE-pA received an i.p. injection of 4% FG in 0.9% NaCl (30 mg per kg body weight) and sacrificed 10 days later.

To retrogradely trace the magnocellular OXT neurons, 1 μl rAAV2/2-Retro-hEF1α-DIO-EYFP-WPRE-pA was injected into the PPI of OXT-Cre rats at 100 nl/min and allowed to be expressed for another 2 weeks.

To trace the axonal projections of PVN magnocellular OXT neurons in the brain, 1 μl Cre-dependent rAAV2/2-Retro-CAG-FLEX-Flopo-WPRE-pA was injected into the PPI at 100 nl/min, followed by 400 nl rAAV2/2-Retro-hEF1α-fDIO-ChR2-EYFP-WPRE-pA injection 1 week later into the PVN (AP, −0.72 mm; ML, +0.60 mm; DV, −7.20 mm) of OXT-Cre rats at 40 nl/min. Rats were sacrificed 4 weeks after the final injection.

For chemogenetic activation and inhibition, 1 μl rAAV2/2-Retro-hSyn-DIO-hM3D(Gq)-mCherry-WPRE-pA, rAAV2/2-Retro-hSyn-DIO-hM4D(Gi)-mCherry-WPRE-pA or control virus rAAV2/2-Retro-hSyn-DIO-mCherry-WPRE-pA was injected into the (PPI) at 100 nl/min. 2 weeks post injection, cannulas were implanted bilaterally at CPu (AP, −0.60 mm; ML, ± 3.60 mm; DV, −5.40 mm). The rats were allowed to recover for 1 week before handling.

**Behavioral tests**

All behavioral tests were performed with male OXT-Cre rats aged 15-20 weeks weighing 280-320 g during the dark cycle within a noise cancellation room maintained at 25°C. The rats were fed in the same room for 7 days to accclimate to the environment after surgery. For all experiments, experimenters were blinded to genotypes or experimental manipulation. All the apparatuses and cages were sequentially wiped with 70% ethanol and ddH2O then air-dried between stages.

At the end of behavioral tests, 1 ml blood was collected for ELISA from the left heart ventricle of deeply anesthetized rats 60 min post drug administration. After blood collection, rats were perfused with 4% paraformaldehyde followed by post hoc analysis to confirm the injection sites and cannula locations.

For chronic inhibition, Clozapine N-oxide (CNO) (in 500 μl 0.9% saline) was intraperitoneally injected at 1.5 mg per kg body weight twice daily with 12 h intervals for 7 days. For acute manipulation, 500 μl CNO in 0.9% saline was intraperitoneally injected 20 min before behavioral test at 1 mg per kg body weight. For CPU microinfusion, the infusion catheter was designed to be 200 μm longer than the guiding cannula and connected to a 2.5 μl Hamilton syringe via a polyethylene tube. 1000 nl ACSF or drugs were infused into bilateral CPu (500 nl each side) with a manual microinfusion pump (RWD, 68606) over 3 min. The catheter was kept in the CPu for another 1 min to maintain complete diffusion. The dosage we used was listed as below. CNO was diluted in ACSF to make a 5 μM working solution. L-368,899 was dissolved in ACSF to make a 2 μg/ml working solution.

Movements were recorded using a Sony camera connected to the ANY-maze system. The duration spent exploring different areas was automatically acquired by ANY-maze. The sniffing was defined as valid when the noses of subject and object rats touched or oriented toward each other within 2 cm, the counts were manually calculated by two experimenters unaware of the treatments. The rearing was defined as the rat standing with the hindlimbs and also manually calculated.
Locomotion test
A black box of 33 cm in width and 45 cm in length was used to assess the locomotion activity of rats. A grid consisting of 3 × 3 cm square was applied to trace the line-crossings and distance during movement. The subject rat was first allowed to habituate in the box for 5 min and the basal locomotion level of the animal was measured for 5 min right after the habituation. The animal was then put back to the homecage, where drugs were given via intraperitoneal injection or CPu microinfusion. 20 minutes after the injection, the subject rat was placed back to the black box and locomotor activity was measured for 5 min. The total travel distance and line-crossings were calculated by ANY-maze to indicate the horizontal locomotion. The rearing was manually counted to indicate the vertical locomotion.

Open field social test
The open field apparatus was 100 × 100 × 40 cm (width × length × height).

The subject rat was exposed to the empty apparatus for 10 min. Then a transparent Plexiglas cylinder of 20 cm in diameter was placed at one corner of the apparatus. The rat was allowed to explore for 10 min to acclimate to the environment. Lastly, a virgin male SD rat was introduced into the cylinder cage and the vicinity (10 cm) of the cage was defined as social zone. The subject rat was allowed to explore for another 10 min. The total travel distance and duration in the social zone were calculated by ANY-maze.

3-chamber social test
The T-shaped three-chamber apparatus was composed of two 27 × 40 × 40 cm (width × length × height) side chambers and one 15 × 40 × 40 cm (width × length × height) shuttle chamber connecting side ones. Two identical cages of 15 cm in diameter were used as stimulus cages and the vicinity (10 cm) of the cages was defined as social zone. For the first stage (habituation), the subject rat was placed in the shuttle chamber with head orienting the right chamber and allowed to explore the apparatus freely for 5 min to acclimate. Then, for the second stage (exploration), two stimulus cages were placed at the corner of each side chamber. The rat was allowed to explore the apparatus containing empty cages for 10 min. For the third stage (novelty), we introduced a toy into the cage of the left chamber while leaving the other cage empty to test the effect on novel object exploration. The subject rat was allowed to freely explore the apparatus for another 10 min.

A parallel paradigm was adopted from the above to test the effect on social investigation. The habituation and exploration stages were repeated. At the third stage (sociability), a virgin male SD rat was introduced into the stimulus cage in the left chamber and a different toy in the other cage. The total travel distance and duration in the social zone were calculated by ANY-maze. The sniff counts were manually calculated based on the video. The social index is calculated as \( \frac{\text{Duration}_{\text{L}}}{(\text{Duration}_{\text{L}}+\text{Duration}_{\text{R}})} \), an index of the preference to the left social zone.

ELISA
Blood was temporarily stored in iced tubes pre-coated with heparin, protease inhibitor cocktail and Ethylenediaminetetraacetic acid (EDTA). After immediate centrifugation at 2000 g at 4° C for 2 min, the supernatants (serum) were transferred, aliquoted and stored at −80° C until use. Serum oxytocin levels were measured using a commercially available oxytocin ELISA kit combined with Sep-Pak C18 column (Waters) extraction to avoid interference from other components in the blood. Briefly, the 200 mg C18 columns were equilibrated with 1 ml acetonitrile, followed by 20 ml 0.1% trifluoroacetic acid (TFA). 200 μl serum was mixed with an equal volume of 0.1% TFA and centrifuged at 16000 g at 4° C for 15 min. The supernatants were applied to the column and washed with 20 ml 0.1% TFA. Samples were eluted from the column using 1 ml 95% acetonitrile/5% of 0.1% TFA and evaporated with Eppendorf Concentrator plus Complete System (Eppendorf).

Samples were reconstituted with 20 μl Assay Buffer from the ELISA kit and analyzed according to the product manual. Briefly, the oxytocin standard samples were prepared via gradient dilution and corresponding diluents were loaded into appropriate wells. Testing samples were added to different wells followed by Conjugate and Antibody incubation at 4° C overnight. After washing in Wash Solution 3 times, pNpp Substrates were added to the wells and incubated at room temperature for 1 h, followed by the addition of Stop Solution. The plate was immediately read at 405 nm (correction between 570 nm and 590 nm) with SpectraMax M5 Microplate Readers (Molecular Devices) and the oxytocin contents were calculated based on the standard curve and the optical density of the samples.

Slice preparation and ex vivo electrophysiology
The OXT-Cre rats injected with AAV-Retro-DIO-hM4Di-mCherry were euthanized. The brain was quickly removed and placed in an ice-cold N-Methyl-D-glucamine (NMDG)-containing cutting solution (93 mM NMDG, 93 mM HCl, 2.5 mM KCl, 1.2 mM NaH2PO4, 30 mM NaHCO3, 25 mM Glucose, 10 mM MgSO4, 0.5 mM CaCl2, 20 mM HEPES, 5 mM Sodium ascorbate, 3 mM Sodium pyruvate, 2 mM Thiourea and pH 7.3-7.4, 300-310 mM). 300 μm coronal brain slices containing the SON were cut using a VT1200 S Vibratome (Leica). Slices were subsequently recovered in the cutting solution saturated with 95% O2 and 5% CO2 at 37°C for 10 min, transferred and incubated in the recording solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1.3 mM MgCl2, 1.3 mM Na2HPO4, 25 mM NaHCO3, 10 mM Glucose and pH 7.4, 310-320 mM) at room temperature for at least 1 h.
SON-containing slices were superfused and submerged in continuously flowing oxygenated recording solution at a rate of 2 ml/min in a perfusion chamber attached to the fixed stage of an upright BX51WI microscope (Olympus) at room temperature. Neurons were visualized under a 20× water immersion lens and identified with differential interference contrast optics (DIC) and mCherry epifluorescence. Electrophysiological recordings were made with the MultiClamp 700B Microelectrode Amplifier (Axon Instruments, Molecular Devices Cellular Neurosciences). Patch electrodes were made by pulling 3-000-203-G/X glass capillaries (Drummond Scientific Company) on a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument). The pipette resistance was typically 3-5 MΩ after filling with the internal solution (126 mM K-gluconate, 10 mM KCl, 2 mM MgSO₄, 0.1 mM BAPTA, 10 mM HEPES, 4 mM ATP, 0.3 mM GTP, 10 mM Phosphocreatine and pH 7.3, 290 mOsm). All electrophysiological recordings were carried out by an investigator blind to the experimental conditions.

Whole-cell current-clamp was made from the soma labeled with mCherry to verify the typical hyperpolarizing notch. The membrane potentials were recorded with a series of gradually increasing depolarizing current injections with 25 pA current steps from −100 pA to +200 pA. To validate the inhibition of CNO on hM4Di-expressing Magno-OXT neurons activity, mCherry-positive neurons in the SON were patched and recorded with 25 pA current injections in the SON were patched and recorded with 25 pA current injections. The responses were recorded for 10 min as the baseline and lasted for 3 min before washing out. All data were acquired with the Digidata1440A Low-noise Data Acquisition System (Axon Instruments, Molecular Devices Cellular Neurosciences), low-pass filtered at 3 kHz, digitized at 10 kHz and analyzed using pClamp10 software (Molecular Devices).

**Histology and imaging**

**Immunofluorescence analysis**

Animals were perfused with normal saline and 4% paraformaldehyde (PFA) sequentially. The dissected brains and pituitary glands were post-fixed in 4% PFA, followed by dehydration in 30% sucrose. Brains were embedded in Tissue-Tek O.C.T. Compound (O.C.T.) and cryostat sectioned into 30 μm thick slices by a CM1950 Microtome (Leica), adhered to superfrrost slides. Antigen retrieval of brain slices was carried out as described previously (Gao et al., 2012). The brain slices were permeabilized in 0.5% Triton X-100 in Tris-buffered saline, blocked with 100 mM glycine and 5% bovine serum albumin (BSA) containing 5% normal donkey serum. Tissue sections were subsequently incubated with diluted primary and secondary antibodies as indicated, nuclei stained with 6-diamidino-2-phenylindole (DAPI), and slides mounted with antifade reagents. Images were acquired using the Olympus FV1200 confocal microscope.

**Fluorescent in situ hybridization**

Total RNA was extracted from the rat hypothalamic tissue and then reverse-transcribed. TRH DNA templates were amplified using primers: 5'-GGTGCTGCTTAGACTCCTG-3’ and 5’-TCTGGCCTTACTCCAGA-3’. Anti-sense RNA riboprobes were transcribed with T7 RNA polymerase (Promega) and digoxigenin (DIG)-labeled nucleotides (DIG RNA Labeling Mix, Roche). Fluorescent in situ hybridization was carried out according to previously described procedure (Wei et al., 2018). Briefly, rats were perfused with cold DEPC-treated 0.1 M PBS and 4% PFA. Brains were dissected and post-fixed in 4% PFA overnight, then embedded in 3% agar/DEPC-PBS and vibratome-sliced into 30 μm sections. After washing and acetylation, brain slices were prehybridized in the prehybridization mix, followed by hybridization in the hybridization mix containing different probes at 65°C overnight. After hybridization, slices were extensively washed in TBS-T and transferred to 2% agarose/TAE gel for electrophoresis at 60 V for 2 h to remove the unbound probes. Slices were subsequently rinsed in TBS-T and incubated in Anti-DIG-AP Fab Fragments (1:2000, Roche), goat anti-GFP antibody (1:300) and rabbit anti-OXT/AVP antibody (1:1000/1:2000) at 4°C overnight. Slices were washed and incubated in fluorescence-conjugated secondary antibodies at RT for 2 h, followed by the detection buffer and DAPI staining and mounted. **Single molecule fluorescence in situ hybridization (smFISH)**

smFISH was performed to examine the expression levels of OXT receptors (OXTRs) in the CPu. Rats were perfused with normal saline and 4% PFA. Brains were dissected and post-fixed in 4% PFA overnight, followed by dehydration in 30% sucrose. Brains were embedded in O.C.T. and cryostat sectioned into 20 μm thick slices by a CM1950 Microtome, adhered to superfrost slides and frozen at −80°C until use. smFISH was conducted according to the manufacturer’s instructions in the RNAscope Fluorescent Multiplex Assay Manual (Advanced Cell Diagnostics). Briefly, the brain slices were pretreated with hydrogen peroxide, followed by antigen retrieval and proteinase III incubation. After washes with double distilled water, slices were incubated with probes for Oxtr (Advanced Cell Diagnostics) for 2 h at 40°C. After incubation, slices were rinsed twice with wash buffer followed by Amplification steps I, II and III. The fluorescent signals were visualized with TSA Plus Fluorescein System (Perkin Elmer) fluorophores and nuclei were stained with DAPI, followed by mounting with antifade reagents.

**3-D reconstruction with fMOST (fluorescence micro-optical sectioning tomography)**

**Tissue preparation for fMOST**

All histological procedures were performed as described previously (Gong et al., 2016; Yang et al., 2013). Rats were anesthetized and perfused with 0.01 M PBS (Sigma-Aldrich Inc., St. Louis) and PBS containing 4% paraformaldehyde (PFA). Brains were carefully dissected and post-fixed in 4% PFA for 24 h. Fixed brains were transferred to 0.01 M PBS and incubated at 4°C overnight and dehydrated in graded ethanol solutions. Then, individual brain was impregnated with Glycol Methacrylate (GMA, Ted Pella Inc.) and embedded in a vacuum oven.
Whole-brain imaging
The whole-brain imaging process was based on a homemade Brain-wide Position System (BPS) (Gong et al., 2016), with a simultaneous propidium iodide (PI) staining to label the cytoarchitectonic landmarks and a brain-wide fluorescence micro-optical sectioning tomography (fMOST) via structured illumination. For whole-brain imaging, each sample was immobilized in a water bath on a three-dimensional nano-precision translation stage. The brain sample was sectioned coronally at an interval of 2 μm in an antero-posterior (AP) direction to achieve the axial scan, counterstained in PI, followed by fluorescence image acquisition via mosaic scan. The combination of mosaic x-y-scan and axial z-scan produced continuous dataset at a voxel resolution of 0.32 × 0.32 × 2 μm³, which allowed 3D reconstruction (Video S1). For individual rat brain, approximately 8000 coronal slices were sectioned from an intact adult rat brain, with the original data size of 30 TB.

Image pre-processing
The original images were collected and saved in TIFF formats with high fidelity. Image preprocessing including mosaic stitching and illumination correction were performed on dual-channel images (PI and GFP). Briefly, the mosaics of each coronal section were stitched to obtain entire section images. Lateral and axial corrections were performed by calculating mean intensity along each direction and quantifying the average gray-scale values, respectively. Image preprocessing was implemented in C++, parallelly-optimized with the Intel MPI Library (v3.2.2.006). Images were executed on a computing server and saved at 8-bit depth in LZW-compression TIF format or transformed to TData format (Li et al., 2017).

Registration and reconstruction of the hypothalamo-neurohypophysial system
The reconstructions of all the projection neurons were completed interactively in Amira 3D/4D+ visualization and analysis software (Thermo Fisher Scientific, USA). 10 × 10 × 32 μm³ resampled datasets were loaded into the Amira segmentation module for surface generation. For the absence of 3D high-definition SD rat brain atlas, we adopted Swanson’s atlas to register the brain regions or nuclei. And the boundaries of magnocellular aggregates were defined according to Swanson’s atlas and PI staining cytoarchitectonic landmarks.

Cell-counting and soma volume analysis
Each GFP-positive region was extracted into single file from raw 3D datasets for further analysis. GFP-positive cells were semi-automatically counted and manually verified in Imaris. The percentage of GFP⁺ cells within individual nucleus/region was calculated and normalized to the total number of GFP⁺ cells. The soma volume was automatically acquired with Imaris based on the same parameters. The anatomical boundaries and hallmarks were identified based on the Brain Maps III: Structure of the Rat Brain (Swanson, 2004).

Long-distance neuron reconstruction
A TData plugin was used to convert the high-resolution, whole-brain datasets into individual data blocks for subsequent processing in Amira (Li et al., 2017; Lin et al., 2018). The dendrites and/or axons in each sub-volume were traced interactively using the Filament Editor module. The contrast and thickness were adjusted to extract the fluorescent signals from the background. The same parameters were set to data blocks of one sample to maintain the reproducibility. Segments with high signal-to-noise ratio (SNR) were traced automatically on a 2D plane. Semi-automatic tracing strategy was applied to those with low SNR. The initial nodes of fibers were determined by experienced annotators and Amira semi-automatically calculated the path between nodes. The orientation consistency, fluorescence intensity and structural features were taken into consideration to determine the connection between related nodes missing a certain segment. To validate the tracing, the skeleton including nodes and fibers was merged to the raw data. Only data blocks occupied by the target soma or passed by the fibers were presented. All the results were further checked by an independent annotator. Detailed procedures regarding single-cell fiber tracing were shown in the supplemental movie (Video S2).

Registration of neuron reconstructions to the reference rat brain space
We used the BrainsMapi method (Ni et al., 2020) to accomplish the registration of different datasets to the reference rat brain space (Waxholm Space Atlas of the Sprague Dawley Rat Brain) (Papp et al., 2014). First, a set of regional features were extracted from the whole brain. Then, the linear and nonlinear registration methods were used to map and warp these features, and the deformation parameters are obtained. Finally, these parameters were used to register the reconstructed neurons (Video S3).

Fiber density calculation
Fiber density calculation was performed for specific brain regions including the auditory cortex, BLA, CEA, CPu, LGP, LS, motor cortex, Nac, piriform cortex and VTA, with left and right halves calculated separately. The entire images were resampled to the resolution of 0.96 × 0.96 × 2 μm³ and imported to Amira for segmentation. The voxels with gray value larger than 15 were detected as GFP signals. Fiber density was calculated as the percentage of signal voxels in segment voxels.
QUANTIFICATION AND STATISTICAL ANALYSIS

All the measurements were exported to the GraphPad Prism 8 or SigmaPlot 12 and analyzed by Student’s t test, one-way ANOVA or two-way ANOVA according to the forms of the data. Nonparametric tests were used if the data did not match assumed Gaussian distribution. Data were presented as Mean ± SEM, with statistical significance taken as *p < 0.05, **p < 0.01 and ***p < 0.001.

The statistical details of experiments were attached to corresponding legends. Briefly, for histology analysis, Student’s t test was used, n value was from n = 3 to n = 5 (rats) or from n = 30 to n = 100 (cells). For behavioral test analysis, Student’s t test, one-way ANOVA and two-way ANOVA were used, as appropriate. The group size ranged from n = 4 to n = 10. Since no statistically significant differences were found across the control groups (mCherry⁻-saline/CNO, Dq⁻/Di⁻-CNO and Dq⁺/Di⁺-saline), data from these groups were combined in the chemogenetic activation experiment. As for the chemogenetic inhibition experiment, the Di⁻-CNO group was directly used as the control.