

1 **Wiring diagram of the oxytocin system in the mouse brain**

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17 **Abstract**

18

19 In the brain, oxytocin (OT) neurons make direct connections with discrete regions to regulate
20 social behavior and diverse physiological responses. Obtaining an integrated neuroanatomical
21 understanding of pleiotropic OT functions requires comprehensive wiring diagram of OT
22 neurons. Here, we have created a whole-brain map of distribution and anatomical connections of
23 hypothalamic OT neurons, and their relationship with OT receptor (OTR) expression. We used
24 our brain-wide quantitative mapping at cellular resolution combined with a 2D flatmap to
25 provide an intuitive understanding of the spatial arrangements of OT neurons. Then, we utilized
26 knock-in *Ot-Cre* mice injected with Cre dependent retrograde monosynaptic rabies viruses and
27 anterograde adeno associated virus to interrogate input-output patterns. We find that brain
28 regions with cognitive functions such as the thalamus are reciprocally connected, while areas
29 associated with physiological functions such as the hindbrain receive unidirectional outputs.
30 Lastly, comparison between OT output and OTR expression showed no significant quantitative
31 correlation, suggesting that OT transmission mostly occurs through indirect pathways. In
32 summary, our OT wiring diagram provides structural and quantitative insights of distinct
33 behavioral functions of OT neurons in the brain.

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39 **Significance Statement**

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41 Oxytocin (OT) neurons in the brain play an important role in socio-physiological responses.
42 Impairment of OT signaling has been implicated in many neurodevelopmental disorders. To
43 understand diverse OT functions in the context of discrete neural circuits, it is imperative to
44 understand the anatomical arrangement of OT neurons across the whole brain in significant
45 detail. Here, we have established a comprehensive brain-wide wiring diagram of OT neurons.
46 Our anatomical and connectivity map of OT neurons includes brain-wide cell distribution,
47 synaptic inputs, axonal outputs, and their relationships with the oxytocin receptor expression.
48 This whole brain structural perspective of the OT system provides a foundation for
49 understanding the diversity of neural circuits modulated by OT and will guide future circuit-
50 based OT functional studies.

51 **Introduction**

52 Oxytocin (OT) is a highly conserved neuropeptide, playing key roles in regulating social
53 behavior and other physiological functions (1–4). Impairment in OT signaling has been heavily
54 implicated in many neurodevelopmental disorders including autism (5, 6). Correcting OT
55 signaling is being pursued as a potential therapy to alleviate social behavioral symptoms in many
56 brain disorders (7), but our limited neurobiological understanding of OT signaling in the brain
57 hampers the development of targeted approaches aimed at altering specific OT functions without
58 affecting other biological pathways. A comprehensive anatomical understanding of OT neurons
59 would enable integrated neural circuit specific studies to decipher the neural substrate of distinct
60 OT functions.

61 The majority of OT producing neurons are located in the paraventricular nucleus of the
62 hypothalamus (PVH) and the supraoptic nucleus (SO) while fewer OT neurons reside in the bed
63 nucleus of the stria terminalis (BST), the medial amygdala (MEA), and the accessory nuclei
64 (AC) (8). OT neurons receive input from distinct brain regions [e.g., the thalamus (TH)] to
65 integrate sensory input and internal information to release OT in a context dependent manner in
66 order to modulate specific downstream circuitry (9, 10). The actions of OT are mainly mediated
67 by a single subtype of the OT receptor (OTR) expressed in distinct brain regions as well as
68 peripheral tissues (11–13). In addition to the well-known peripheral release of OT as a hormone
69 via the posterior pituitary, OT neurons send direct projections to discreet brain areas that
70 frequently express OTR, thereby modulating circuit specific functions. For example, OT
71 signaling is linked with the medial prefrontal cortex for social cognition (14, 15), CA2 of the
72 hippocampus for social memory (16, 17), the central amygdala for fear modulations (18, 19), the
73 parabrachial nucleus (PB) for fluid intake (20), and the spinal cord for pain perception (21, 22).
74 Despite these prior studies, we still lack a quantitative and comprehensive wiring diagram of the
75 OT neurons in a standard 3D reference brain. Moreover, quantitatively characterizing the wiring
76 relationships between OT neurons and OTR expression patterns across the whole brain is made
77 difficult due to the need to simultaneously examine microscopic structures (e.g., cell bodies and
78 their axonal projections) in a macroscopic structure, namely the whole brain.

79 Here, we use a high-resolution quantitative brain mapping method in combination with cell type
80 specific transgenic mice and viral tools to establish the wiring diagram of OT neurons in the
81 mouse brain. We devised a 2D hypothalamic flatmap to quantify OT distribution, and analyzed
82 synaptic inputs and long-range output patterns of hypothalamic OT neurons. We find
83 reciprocally and unidirectionally connected brain regions with OT neurons, linked with distinct
84 cognitive and physiological functions, respectively. Moreover, systemic comparisons between
85 the OT projectome and OTR expression reveal potential brain regions with direct synaptic or
86 non-synaptic OT signaling. The imaging data are mapped onto a standard reference brain to
87 facilitate cross-examination (23) and freely available via user-friendly web visualizations at
88 https://kimlab.io/brain-map/ot_wiring/.

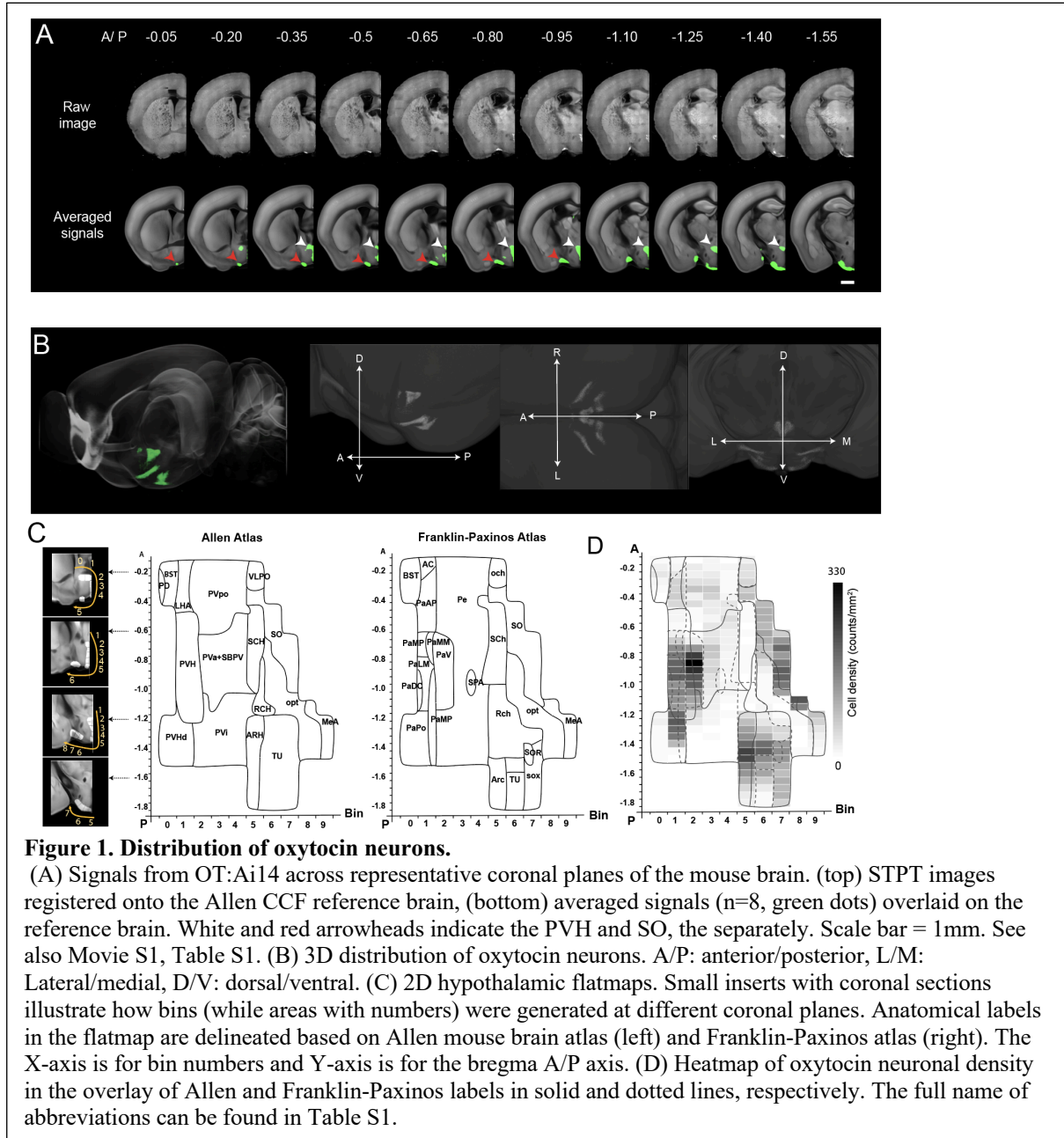
89 Results

90 Quantitative expression of oxytocin neurons in the mouse brain

91 Although the location of OT neurons in the mouse hypothalamus (HY) is relatively well-
92 established (8), data showing quantitative brain-wide OT distribution in complex 3D structures
93 remains elusive. To examine the anatomical distribution of OT neurons across the whole brain,
94 we used heterozygote OT knock-in mice with Cre recombinase (*Ot-Cre*) crossed with Ai14
95 reporter mice (OT: Ai14) (24). We imaged the entire mouse brain at cellular resolution using
96 serial two-photon tomography (STPT) and performed quantitative mapping using previously
97 established computational methods (25) (n=8 brains, 3 males, 3 virgin females, and 2 lactating
98 females, Fig. 1A-B, Movie S1). There was no significant difference between male, virgin female,
99 and lactating female (Table S1), which is consistent with an earlier study (18). The majority of
100 OT neurons (~42%, 792 out of total 1896 cells) were expressed in the PVH regions (PVH,
101 descending division of PVH, anterior, intermediate, and preoptic part of periventricular
102 hypothalamic nucleus, and subparaventricular zone) followed by the SO, the tuberal nucleus
103 (TU), the MEA, and the BST (Table S1). The rest of the OT neurons are expressed in 10
104 different brain regions (Table S1). The Cre-driven reporter system used in the present study
105 permanently labels all Cre positive cells including developmentally transient expression (11). To
106 distinguish neurons actively expressing OT in adult from developmentally labeled cells, we
107 performed immunohistochemistry using an OT antibody in OT: Ai14 mice. We confirmed that
108 almost all OT immuno positive neurons (97%, 1733 out of 1790 cells, n=4) were labeled by
109 tdTomato from OT: Ai14 mice (Fig. S1). In contrast, 76% of tdTomato labeled cells were OT
110 immuno positive (1733 out of 2277 cells) in the PVH. Smaller portions of tdTomato cells in the
111 SO (44%, 654 out of 1508 cells) and the MEA (8%, 31 out of 375 cells) retain active OT
112 expression (Fig. S1). This suggests that OT neurons in different brain regions undergo
113 differential rates of developmental down-regulation in the adult brain (26). To further visualize
114 the spatial expression pattern of OT neurons, we created a 2D hypothalamic flatmap. Evenly
115 spaced bins provide a flattened 2D spatial unit to quantify and to display signals from the 3D
116 brain (Fig. 1C-D). The 2D hypothalamic flatmap was delineated with Allen Common Coordinate
117 Framework (CCF) and Franklin-Paxinos atlas based anatomical labels (23, 27, 28) (Fig 1C). The
118 regional boundaries of the two labeling systems generally agreed with each other in the major
119 OT expressing regions (e.g., the PVH and the SO) despite noticeable discrepancies in the
120 ventricle hypothalamic area (e.g., the TU) (Fig 1C-D) (28). The density heatmap on the
121 hypothalamic flatmap provides an intuitive and quantitative display of the regionally
122 heterogeneous distribution of OT neurons (Fig. 1D). We also provide detailed distribution
123 patterns of the OT neurons within the PVH using a 2D PVH flatmap (Fig. S2). Together, these
124 data provide the quantitative expression pattern of OT neurons throughout the whole brain.

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128 Monosynaptic inputs to oxytocin neurons

129 Previous studies suggest that the PVH area receives input from specific brain areas such as the
 130 dorsomedial hypothalamus area, the medial preoptic area (MPO), the subparafascicular area, and
 131 the posterior intralaminar nuclei to integrate external stimuli and internal state (29–31). To map
 132 brain-wide trans-synaptic inputs in a cell type specific manner, we injected monosynaptic
 133 retrograde rabies viruses into the PVH and the SO of the *Ot-Cre* knock-in mice (32). We used
 134 our qBrain mapping method to quantify input neurons throughout the whole brain (25). To
 135 capture total input to each anatomical area, we combined input signals from multiple
 136 independent injections targeting a specific brain region and overlaid them onto a reference brain

137 while displaying input neurons with pseudo-colored green for the PVH (n=6 brains) and red for
138 the SO (n=4 brains) (Fig. 2A, Movie S2). Our analysis reveals that more than 50 different
139 structures provide inputs to OT neurons (Fig. 2B). Both the PVH and the SO receive major
140 inputs from other hypothalamic brain regions. Overall, PVH OT neurons take inputs from more
141 regions than the SO neurons. Notably, PVH and SO OT neurons have different regional input
142 preferences (Fig. 2B). For example, thalamic regions mainly provide inputs to the PVH neurons
143 (Fig. 2A, blue arrowhead), while the striatum (STR) areas (e.g., nucleus of accumbens) provide
144 more input to SO neurons (Fig. 2A, yellow arrowhead). Moreover, the ventral anterior-lateral
145 complex of the thalamus, piriform cortex, and anterior amygdalar area only provide inputs to the
146 SO neurons. Both the PVH and SO received inputs from the midbrain reticular nucleus (MRN)
147 and the periaqueductal gray (PAG) in the midbrain (MB). The motor-related superior colliculus
148 provides inputs only to the PVH neurons, while the substantia nigra reticular part provides input
149 only to the SO neurons. Notably, PVH OT neurons are strongly connected with the thalamic
150 brain area, which communicates with the cortex and is a key structure for the rapid integration of
151 new learning, working memory, and adaptive decision-making (25), while the SO receives more
152 input from the striatum-like amygdala, which is centered on emotion-based cognitive function
153 (26). This input pattern is overall in agreement with prior reports that utilize traditional
154 retrograde tracers or viral tools (10, 30). The anterior two-thirds of the PVH is known to contain
155 the majority of magnocellular neuroendocrine neurons, while the posterior one-third of the PVH
156 contains the descending preautonomic neurons (8). To investigate potentially segregating input
157 patterns into different PVH subregions, we performed a series of injections with a small volume
158 (150 nL) of the rabies virus into the anterior-posterior PVH area. However, we did not observe a
159 topographically distinct input pattern into different PVH subregions (Fig. S3). Collectively, we
160 conclude that OT neurons in the PVH received stronger and broader inputs than the SO with
161 distinctive regional input preferences (Fig. 2C).

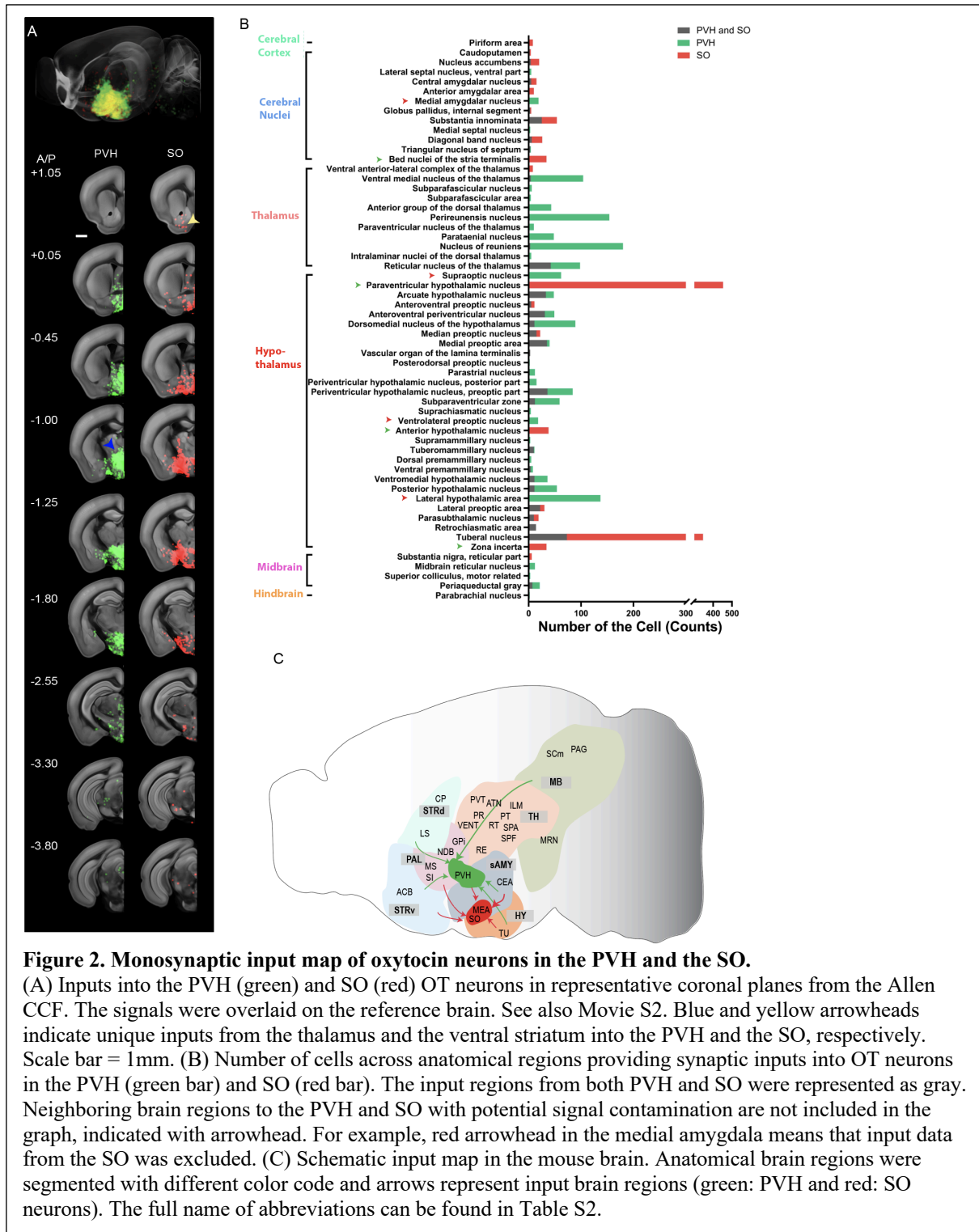


Figure 2. Monosynaptic input map of oxytocin neurons in the PVH and the SO.

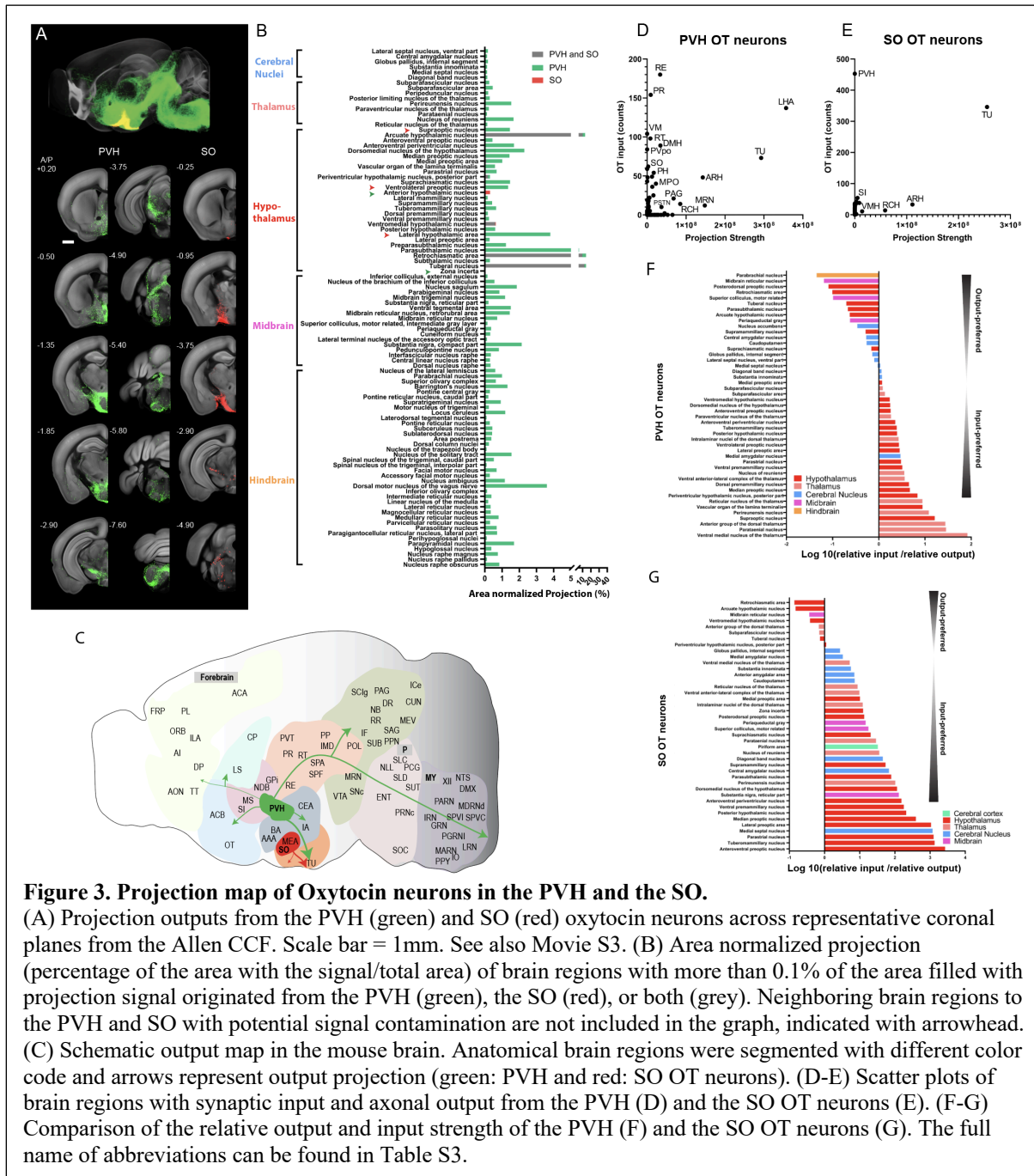
(A) Inputs into the PVH (green) and SO (red) OT neurons in representative coronal planes from the Allen CCF. The signals were overlaid on the reference brain. See also Movie S2. Blue and yellow arrowheads indicate unique inputs from the thalamus and the ventral striatum into the PVH and the SO, respectively. Scale bar = 1mm. (B) Number of cells across anatomical regions providing synaptic inputs into OT neurons in the PVH (green bar) and SO (red bar). The input regions from both PVH and SO were represented as gray. Neighboring brain regions to the PVH and SO with potential signal contamination are not included in the graph, indicated with arrowhead. For example, red arrowhead in the medial amygdala means that input data from the SO was excluded. (C) Schematic input map in the mouse brain. Anatomical brain regions were segmented with different color code and arrows represent input brain regions (green: PVH and red: SO neurons). The full name of abbreviations can be found in Table S2.

164 OT neurons can secrete OT through axonal and dendritic processes in coordinated or
165 independent compartment-specific manner (33, 34). To visualize the entire central projection
166 patterns of OT neurons, we injected Cre-dependent adeno associated virus 2 (AAV2-CAG-Flex-
167 EGFP) in multiple subregions of the PVH and the SO of *Ot-Cre* knock-in mice. Long-range
168 projection signals from multiple injections were registered onto a reference brain and merged to
169 represent efferent output from each anatomical area (Fig 3A, Movie S3). First, we observed that
170 OT neurons in the PVH project to the HY, the TH, the STR, and the pallidum (PAL), as well as
171 the posterior region including the MB and the hindbrain (HB) (Fig 3A-B). In contrast, the SO OT
172 neurons project to very limited areas (e.g., pons) in the brain (Fig 3A-B). Quantitatively, OT
173 neurons in the PVH project to over 99 brain regions including sparse projection to forebrain
174 areas (N = 3 males and 3 females, Fig 3B, Table S3). OT neurons in the SO project mainly to
175 hypothalamic regions and sparsely to posterior brain regions including the PB and the superior
176 olivary complex (SOC) (Fig. 3A-B and Table S3). To examine potential projection topology
177 within the PVH, we targeted PVH subregions by injecting a small volume (40~150 nL) of the
178 AAV virus injection along different A/P locations. We did not observe a strong regional
179 projectome difference within the PVH (Fig. S4).

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181 **Comparison of input-output connection of oxytocin neurons**

182 Next, we systematically compared brain regions connected with OT neurons in the PVH and the
183 SO either unidirectionally or bidirectionally (Fig. 3D-E). In the PVH, selected hypothalamic
184 areas (e.g. the TU, the lateral hypothalamic area, and the arcuate hypothalamic nucleus) and the
185 MB (e.g. the PAG) show reciprocal input-output connection. Moreover, the thalamic area (e.g.
186 the nucleus of reuniens, the parataenial nucleus, the ventral medial nucleus of the thalamus, and
187 the reticular nucleus of the thalamus) has strong input to the PVH OT neurons while the MRN
188 shows a stronger output pattern (Fig. 3D). In the SO, OT neurons have dominant input from the
189 PVH and reciprocal connection with the TU (Fig. 3E). To further understand the input-output
190 relationship quantitatively, we calculated relative input-output strength within the brain regions
191 bidirectionally connected with OT neurons and plotted a log scale of relative input-output ratio
192 (Fig. 3F-G). For example, log-ratio '0' means that the relative strength of input and the output to
193 that specific region is similar. In PVH OT neurons, many thalamic and hypothalamic regions
194 show input preference and cerebral nuclei have balanced input-output log-ratios while MB
195 regions have output preference (Fig. 3F). In contrast, the SO OT neurons show stronger input
196 preference largely due to their limited projection areas (Fig. 3G). These input-output patterns
197 indicate that after integrating cognitive and physiological information from relatively closer
198 regions (e.g., the TH, the HY), PVH OT neurons broadly project to other brain regions from the
199 forebrain to the brain stem to exert fast and site-specific neuronal modulation (Fig. 3F). In
200 contrast, OT neurons in the SO mainly receive emotion-based cognitive information from
201 cerebral nuclei (e.g., the PAL) and social-sexual information from hypothalamic area while
202 mainly providing limited output to the HY and posterior pituitary to regulate metabolism and
203 autonomic body functions (Fig. 3G).



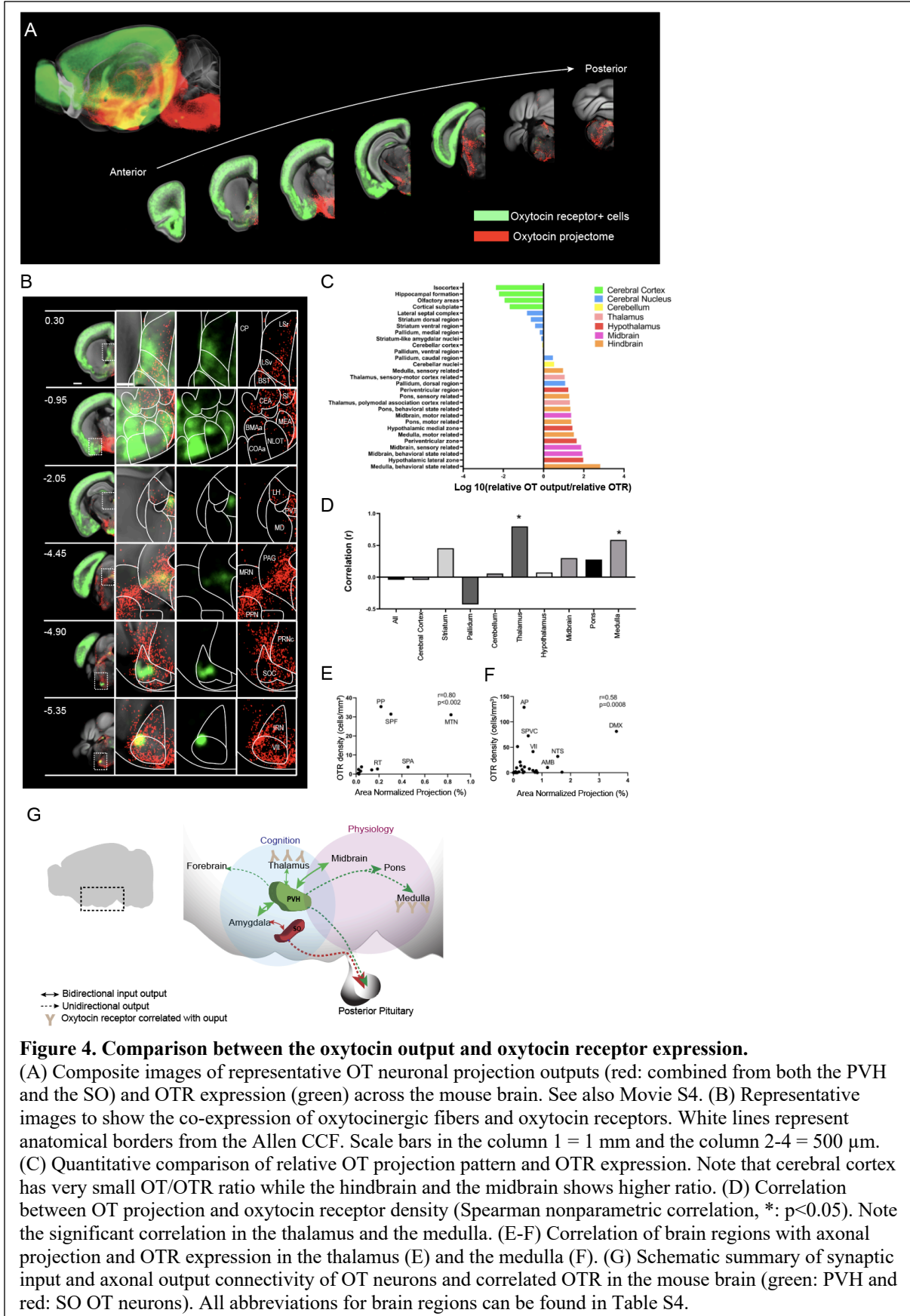
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205 **Comparison of oxytocin output and oxytocin receptor expression**

206 The actions of OT are transduced by a single subtype of the OTR, a seven-transmembrane
 207 protein (1). Previous studies suggested a mismatch between presynaptic OT projection and
 208 postsynaptic OTR expression (3, 34), yet quantitative brain-wide comparison has not been
 209 conducted. Previously, we validated that OTR-Venus mice represent the endogenous OTR
 210 expression faithfully (11). We imaged a cohort of adult OTR-Venus mice using serial two-
 211 photon tomography and mapped OTR expression in the whole adult brain (n=14, green in Fig.

212 4A). Then, we compared the combined projection pattern of *Ot-Cre* mice in the PVH and the SO
213 (Fig. 3A-C) and OTR-Venus (+) cell density across the entire brain via image registration onto
214 the reference brain (Fig. 4, Table S4, Movie S4). Overall, the OTR (pseudo colored as green)
215 showed high expression in the cortical area with minimal OT projection, while many midbrain
216 and hindbrain regions (e.g., nucleus of the lateral lemniscus) have strong OT projection (pseudo
217 colored as red) with little OTR expression (Fig 4A-C; Movie S4). Correlation analysis between
218 OT and OTR expression across the whole brain did not result in any significant correlation (Fig
219 4D). Nevertheless, a few brain areas contained both OT projection and OTR expression
220 including the lateral septal nucleus, the central amygdalar nucleus (CEA), the paraventricular
221 nucleus of the thalamus (PVT), the PAG, the SOC, and the facial motor nucleus (Fig. 4B).
222 Moreover, a significant OT-OTR correlation was observed within the thalamic and medullary
223 areas. (Figs. 4D-4F). Thus, our results highlight quantitative and spatial discrepancies between
224 OT and OTR in the mouse brain, suggesting largely indirect OT transmission in the brain.
225

226



227 Discussion

228 The wiring diagram of the brain is a structural foundation to decipher neural circuits underlying
229 brain function. Here, we present a comprehensive anatomical and connectivity map of the
230 hypothalamic OT neurons and their relationship with postsynaptic OTR expression in the whole
231 mouse brain. We find that OT neurons broadly project to brain regions from the forebrain to the
232 brain stem, while the majority of inputs to OT neurons comes from the thalamus and the
233 hypothalamus. Moreover, we confirm the quantitative mismatch between OT projection and
234 OTR distribution in the brain.

235 OT neurons are mostly located in hypothalamic nuclei with a complex 3D shape (8). To examine
236 OT expression intuitively and quantitatively, we devised a 2D flatmap for OT containing
237 hypothalamic regions from an Allen CCF based reference brain while incorporating anatomical
238 labels from the Allen Institute and Franklin-Paxinos (23, 27). This approach allows for the
239 interpretation of OT anatomical location from two independently created and commonly used
240 atlases (28) and provides an alternative coordinate system to understand anatomical connectivity.
241 Our transgenic labeling approach using OT:Ai14 mice revealed that OT neurons in PVH largely
242 maintain their expression throughout adulthood whereas other brain regions (e.g., SO, MEA)
243 showed significant down-regulation of OT expression. Considering the significant role OT
244 signaling plays in shaping the brain development including neural connectivity (35), this
245 transient OT expression in non-PVH areas may provide extra OT signaling to promote early
246 brain development.

247 Our OT input-output wiring diagram provides brain-wide insights about connectivity-based brain
248 function, which can be divided into two groups (Fig. 4G). The first group contains the brain
249 regions with unidirectional OT output. This pattern is mainly observed in the midbrain, pons, and
250 medulla which are known to be associated with physiological function including body
251 metabolism, sleep, and motor sensory control. For example, the nucleus of the solitary tract
252 (NTS) in the medulla is a part of the neural circuit for food intake and energy expenditure (36).
253 OT neurons in the PVH directly communicate with the NTS to modulate visceral afferent
254 transmission (37). The second group contains brain regions with reciprocal input-output
255 connections with OT neurons including the hypothalamus, thalamus, and striatum that regulate
256 cognitive brain functions such as sexual behavior, memory, attention, fear, reward, and pain. For
257 instance, reciprocally connected regions in the hypothalamic area are highly related to the
258 parental and sexual behaviors such as the MPO (38). Although OT neurons can be further
259 divided into magno- and parvo-cellular neurons, a recent study in rats showed that both cell types
260 in the PVH receive overall similar inputs despite small differences (10). Moreover, we did not
261 observe topographically distinct subregions within the PVH based on input-output maps. This is
262 consistent with a previous retrograde mapping study from the spinal cord, showing that spinal
263 cord projecting OT neurons are distributed across the entire A/P axis (8).

264 Our data also provides a brain-wide perspective on the spatial relationships between OT central
265 projections and oxytocin receptor expression (Fig. 4G). Although the spatial discrepancy of OT-
266 OTR was noted before, recent studies showed that most OTR expressing areas contain at least
267 sparse OT projection (12, 18). Nonetheless, our analysis showed that there is overall no
268 significant quantitative correlation between OT and OTR across entire brain regions. For
269 example, the cerebral cortex area contains abundant OTR with little to no OT axons. However,
270 OT can still mediate sensory stimuli in the cortex to modify mouse behavior (39). Previous

271 studies suggest that OTR neurons in the isocortex may receive OT signals indirectly from
272 ventricular pathways via cerebral spinal fluid with delayed and long-lasting effects (40, 41). In
273 addition, OT axonal ends in the CEA exert a direct effect on fear suppression with delayed
274 electrophysiological responses (~seconds) in the CEA, suggesting that OT is being released in a
275 non-synaptic way (18, 42). In contrast, we also found few areas with high levels of both OTR
276 and OT projection such as the PVT which is involved in maternal behavior (43) and the
277 ventrolateral periaqueductal gray that is implicated in for social defeat stress (44). OT signaling
278 in these areas is likely to occur as the spatially and temporally precise synaptic transmission.
279 Another noteworthy OT-OTR discrepancy is brain regions with abundant OT projection without
280 OTR expression such as sensory related hindbrain areas. Although OTR is a main OT receptor,
281 OT can bind to another receptor to exert its effect. For example, OT can elicit TRPV1 activity in
282 the spinal cord to modulate nociception (45). Non-canonical pathways like this are an under
283 explored mechanism of OT modulation in the central nervous system that requires further study.

284 In summary, our study provides an anatomical foundation to understand diverse functions based
285 on OT neurons in the brain. We deposit all high-resolution imaging data in publicly accessible
286 databases and our website to facilitate data mining. We envision that this OT wiring diagram
287 with quantitative expression data will guide future studies to understand circuit-based
288 mechanisms of OT function and its changes in various brain disorders such as autism.

289

290

291 **Material and Method**

292 Animals

293 All animal care and experimental procedures are approved by the Penn State University
294 Institutional Animal Care Use Committee (IACUC). *Ot-Cre* mice (24) were originally produced
295 in the Gloria B. Choi lab at the Massachusetts Institute of Technology and imported to the Penn
296 State University (Kim Lab). To generate OT: Ai14 mice, *Ot-Cre* mice were crossed with Ai14
297 mice, expressing tdTomato following Cre-mediated recombination (Jax: 007914, C57Bl/6 J
298 background). Mice received food and water ad libitum and were housed under constant
299 temperature and light conditions (12 hrs light and 12 hrs dark cycle).

300

301 Stereotaxic surgery and virus injections

302 *Ot-Cre* mice (8-11 weeks old, males and females) were anesthetized with isoflurane (controlled
303 with Somnosuite, Kent Scientific) and mounted on a stereotaxic instrument (Angle Two, Leica)
304 with a heating pad placed underneath. All injections were performed with pulled micropipettes
305 (VWR, Cat# 53432-706). Through the small opening of the micropipette, virus was delivered at
306 a rate of 75-100 nL per minute. The speed and volume of injection were monitored along with
307 the calibration marks on the micropipette (1 mm =100 nL). To target the PVH, coordinates are
308 anteroposterior (AP) from the Bregma: -0.58 mm; mediolateral (ML): 0.27 mm; dorsoventral
309 (DV): -4.75 mm. Anterior PVH and posterior PVH injection coordinates are -0.35 mm (AP), 0.3
310 mm (ML), and -4.5 mm (DV) and -0.94 mm (AP), 0.39 mm (ML), and -4.55 mm (DV),
311 respectively. Coordinates for the SO are -0.66 mm (AP), 1.3 mm (ML), and -5.8 mm (DV). For
312 anterograde tracing, 50-500 nL of AAV2-CAG-Flex-EGFP virus (titer 3.7×10^{12} vg/ml,
313 purchased from UNC vector core) was injected into the PVH (500 nL for maximum coverage,
314 50-150 nL for PVH subregion) and 150 nL of the virus was injected into the SO. Mice were
315 euthanized three weeks later with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) mixture. For
316 monosynaptic retrograde labeling, 50-500 nL of rAAV1-svnp-DIO-STPEPB (titer 3.9×10^{12}
317 cg/ml, purchased from UNC vector core, a gift from Ian Wickersham (46), Addgene plasmid #
318 52473 ; <http://n2t.net/addgene:52473> ; RRID:Addgene_52473) was injected into the PVH,
319 followed by the same quantity of EnvA G-deleted Rabies-mcherry virus (titer: 8.12×10^8
320 transduction unit (TU) /ml, purchased from the Salk Institute Viral Vector Core, a gift from
321 Edward Callaway (47), Addgene plasmid # 32636 ; <http://n2t.net/addgene:32636> ;
322 RRID:Addgene_32636) three weeks later into the same location. Mice were euthanized 7-8 days
323 later with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) mixture.

324

325 STPT imaging and related data analysis

326 Transgenic or virus injected mice were transcardially perfused with 4% paraformaldehyde (PFA)
327 in 0.1M phosphate buffer (PB, pH 7.4) after 0.9% saline. Brains were dissected out and post-
328 fixed in 4% PFA overnight at 4°C. Fixed brains were stored in 0.05 M phosphate buffer at 4°C
329 until imaged. To image the entire brain, a serial two-photon tomography (TissueCyte 1000;
330 Tissuevision) was used as previously described (25, 48). Briefly, the brain was embedded in 4%
331 oxidized agarose and cross-linked with 0.2% sodium borohydride solution. The brain was
332 imaged as 12 x 16 x 280 tiles with 1 x 1 μm^2 x,y pixel resolution in every 50 μm z-section. We
333 used 910 nm wavelength for two-photon excitation to excite both green (e.g., eGFP) and red

334 signals (e.g., tdTomato). Signals were separated with 560 nm dichroic mirror and two band path
335 filters (607/70-25 for red and 520/35- 25 for green). Imaging tiles in each channel were stitched
336 with custom-built software (25).

337 For quantitative projection data analysis, we used our previously published pipeline (49). Briefly,
338 both signal and background channels were z-normalized. Then, the background channel images
339 were subtracted from the signal channel images to increase signal-to-noise ratio. Then, projection
340 signals were converted to a binary map by applying an optimized threshold (8x standard
341 deviation) to detect signals while minimizing noise from background autofluorescence. Then,
342 binarized signals in each pixel were counted in 20 x 20 (x,y) pixel unit (voxel) and the value was
343 assigned the corresponding voxel across the brain, which is defined as “projection strength”.
344 Thus, range of the projection strength in a given voxel is between 0 and 400. Projection strength
345 of each area is calculated by summing up all projection strength within an anatomically defined
346 area. Autofluorescence of brains was used to register each brain to the Allen CCF using Elastix
347 (50), then, the projection signals were transformed to the reference brain. “Area normalized
348 projection” represents normalized occupancy of projection signals in the ROI by dividing the
349 projection strength with a total number of voxels in each ROI. For example, if total voxel count
350 for one ROI was 20,000 and our projection strength showed 2,000 in the ROI, it will be
351 $(2,000/20,000)*100 = 10\%$. For cell counting analysis, we used a machine-learning algorithm to
352 detect fluorescently labeled cells (25). The cell density in 2D (counts/mm²) was calculated by
353 dividing cell number with ROI area. 2D counting numbers were also converted into the 3D
354 counting using our previously calculated 3D conversion factor (1.4 for tdTomato) (25). To
355 measure the volume of anatomical ROI, the reference Allen CCF was reversely registered onto
356 individual brains using the Elastix. “Cell density (counts/mm³)” was calculated by dividing
357 detected cell numbers in 3D with the anatomical ROI volume. The cell counting analysis was
358 applied to OT: Ai14 and OTR-Venus cell distribution and inputs to the OT neurons.

359 To compare relative quantity of OT input-output (Fig. 3F-G), bidirectionally connected areas
360 were first selected. Within the selected areas, relative cell density or output data in each region
361 was calculated by dividing each data by summed density or output data from all chosen areas,
362 respectively. Then, \log_{10} (relative input/relative output) was used to quantify input or output
363 preference of each area. The same approach was used to calculate relative abundance between
364 OT output and OTR expression in Fig. 4C.

365 To create a representative connectivity map for each anatomical area, we microinjected OT
366 neurons with a large quantity of virus into subregions within an anatomically defined area. Then,
367 the maximum signal of several (4-6) brains from each ROI registered in the reference was used
368 for final analysis to cover entire target anatomical areas for OT input and output data in Figures
369 2-4, and Movies S2-S4.

370 To determine the correlation between OT area normalized projection and OTR density, we first
371 tested for the normality of the data using the D’Agostino-Pearson normality test. Based on the
372 normality test result, we performed Spearman nonparametric correlation test. GraphPad Prism 8
373 was used for all statistical analysis and graphs.

374

375 2D hypothalamic and PVH Flatmap

376 To generate the hypothalamic flatmap, we adapted the previously used method (25) and applied
377 it to the hypothalamic region. First, we created a binary image in the hypothalamic area based on
378 the oxytocin expression. Second, a zero line was placed to generate evenly spaced bins along the
379 dorsal to the ventral direction of the PVH and laterally extended to include TU and MEA at
380 different coronal plains. To capture signals on the flatmap, bins were registered into the reference
381 brain and the cell number in each bin was quantified as described before in the STPT data
382 analyses section. Lastly, the mean number of the OT neurons in 8 OT: Ai14 brains were plotted
383 in each flatmap using a custom-build matlab code. For the PVH flatmap, we followed the same
384 procedure to generate a hypothalamic flatmap except for bin generation. Instead of delineating
385 bins in a binary image, we assigned bin numbers in the PVH subregion of Franklin-Paxinos atlas
386 (27) along the dorsal to the ventral direction.

387

388 Immunohistochemistry, microscopic image, and cell counting

389 For immunohistochemistry, fixed brains were either embedded in 3% agarose or frozen after
390 sinking in 30% sucrose in 0.2 M Phosphate buffer. Embedded or frozen brains were then cut on a
391 vibratome (Leica vt1000s) or a microtome (Leica SM2010 R) at 50 μm thickness. Sections were
392 stored at -20°C in a cryoprotectant solution (30% sucrose and 30% glycerol in 0.1 M PB) until
393 immunostaining. For oxytocin staining, sections were washed three times in 1x PBS. After 1
394 hour incubation in blocking solution (10% donkey serum and 0.1 % Triton X-100), slices were
395 incubated with oxytocin primary antibody (ImmunoStar Cat# 20068, RRID:AB_572258, 1:1000)
396 in blocking solution for overnight at 4°C . Sections were then washed three times with 1x PBS
397 and further incubated in secondary antibodies (Thermo Fisher Scientific Cat# A-21206,
398 RRID:AB_2535792, 1:500) for 1 hour at room temperature. After washing three times, slices
399 were mounted onto slides and coverslipped with vectashield mounting media (Vector
400 laboratories, H-1500-10). For microscopic imaging, a BZ-X700 fluorescence microscope
401 (Keyence) and a confocal microscope (Zeiss 510) were used. A low magnification objective lens
402 (4x) was used to image with a large enough view to define brain anterior-posterior location from
403 bregma and higher magnification objective lenses (10x ~ 40x) were used to image sections
404 depending on the cell density. Images were delineated manually based on the Franklin-Paxinos
405 atlas and fluorescently tagged cells were manually quantified using the cell counter plug-in in
406 FIJI (ImageJ, NIH).

407

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414 Penn State College of Medicine.

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418 **Contributions**

419 Conceptualization, Y.K.; Data Collection and analysis, S.S., ; Data Collection, S.M., K.N., M.C.,
420 Computer Coding, Y.W.; Web visualization, D.J.V., K.C.; Manuscript preparation: S.S., Y.K
421 with help from the other authors.

422

423

424 **Competing Interests**

425 The authors declare no competing interests.

426

427

428 **Data Sharing Plan**

429 High-resolution serial two-photon tomography images can be found at [https://kimlab.io/brain-](https://kimlab.io/brain-map/ot_wiring/)
430 [map/ot_wiring/](https://kimlab.io/brain-map/ot_wiring/)

431 Flatmaps and custom-built codes are available at <https://kimlab.io/> and can be used without any
432 restriction.

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552 Supplementary Information

553

554 **Wiring diagram of the oxytocin system in the mouse brain**

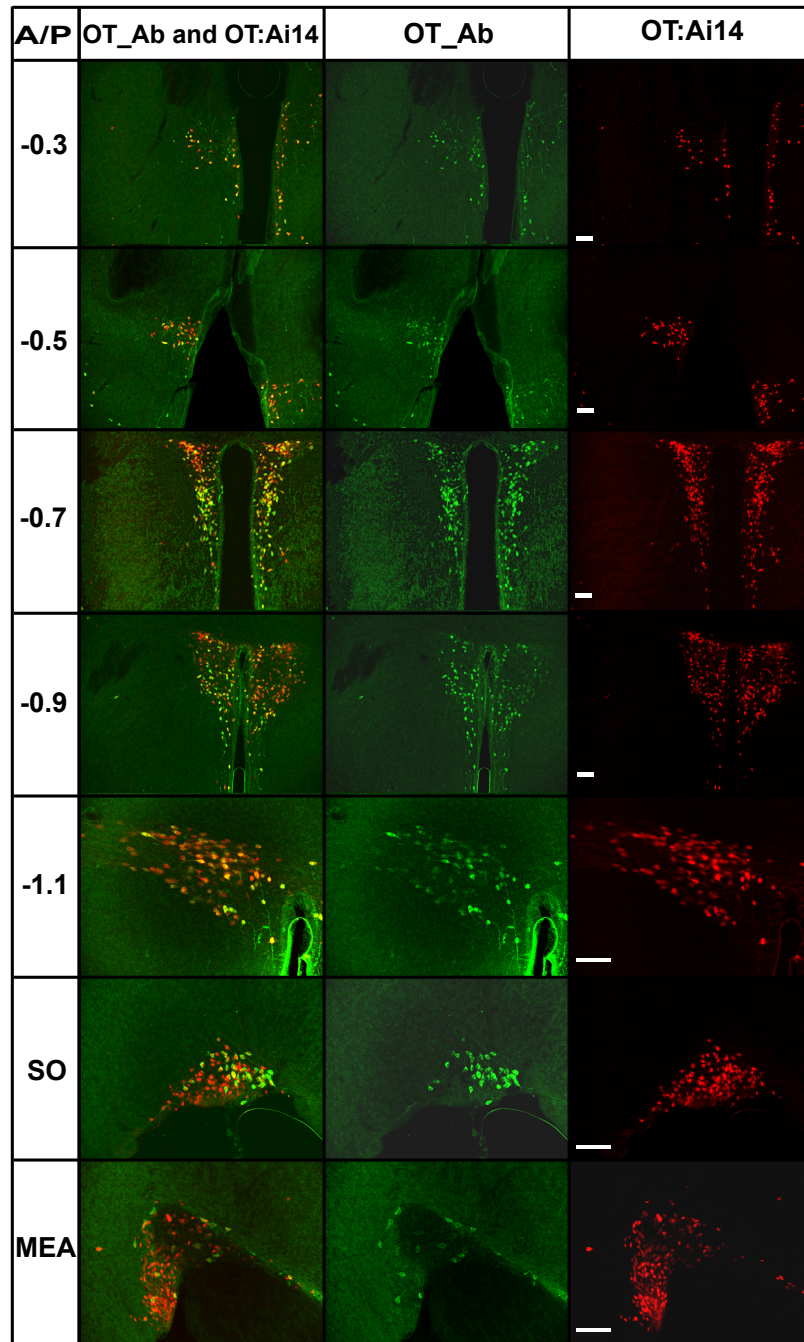
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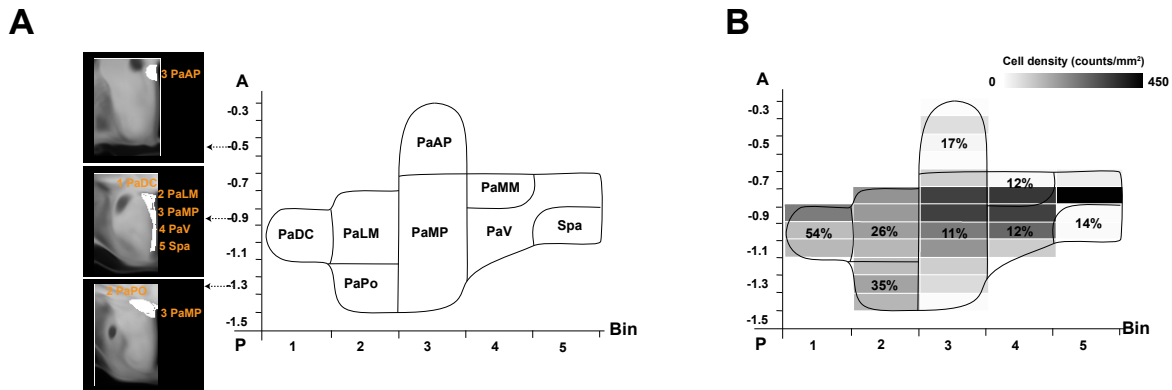
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Figure S1. Fluorescent images across 5 levels of the PVH, SO, and MEA. Genetically expressed oxytocin neurons (OT: Ai14) are red and oxytocin immuno staining cellular are labeled with green fluorescent marker are green. Scale bar = 50 μ m.

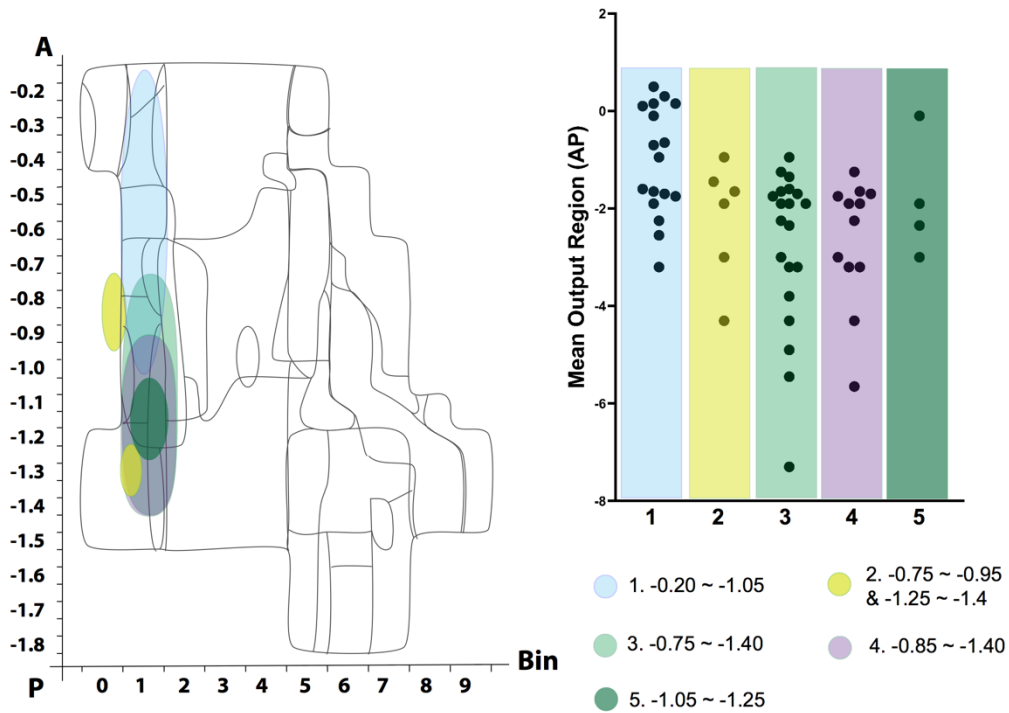


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569 Figure S2. (A) 2D PVH flatmap. Small inserts with coronal sections illustrate the binning
570 system. Bin numbers were assigned on the subregion of PVH at different coronal planes in
571 Franklin-Paxinos labels. (B) Heatmap of oxytocin neuronal density in the 2D PVH flatmap. The
572 number in each brain region represents the percentage of developmentally expressed neurons.
573 The X-axis is for bin numbers and Y-axis is for the bregma A/P axis.

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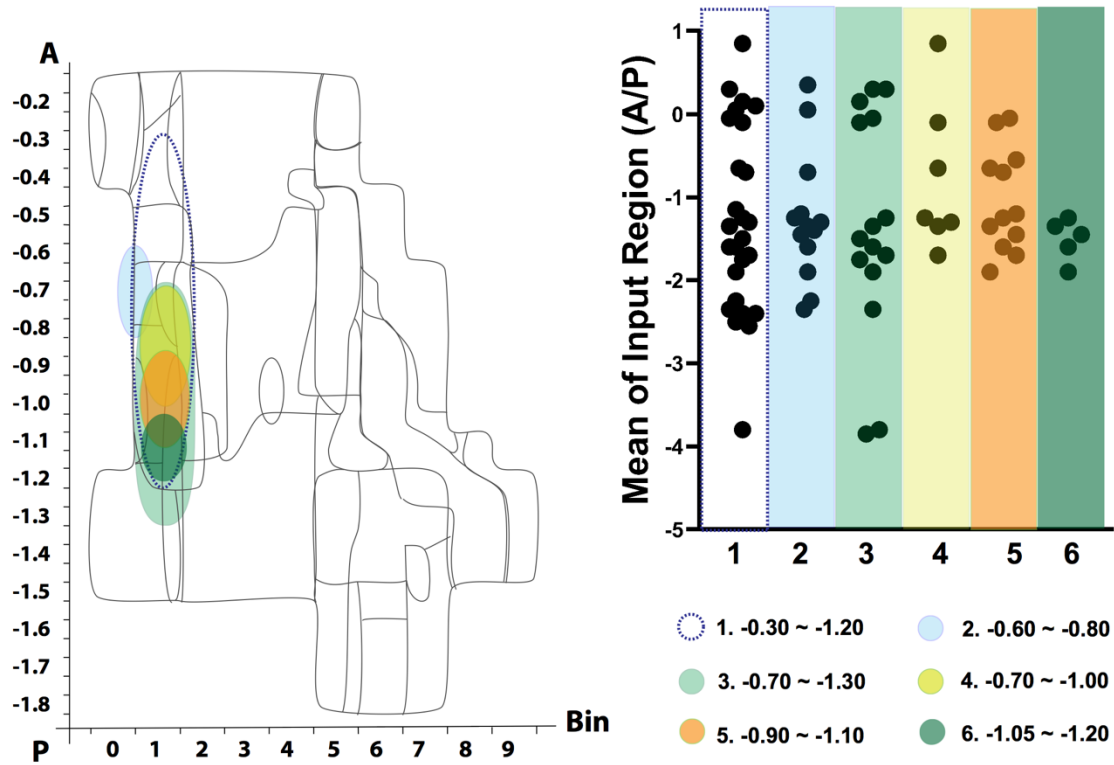
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579 Fig S3. PVH subregion infection. Left: infection coverage on the 2D hypothalamic flatmap.

580 Right: scatter plot between the virus injection coverage and the mean bregma A/P value of input

581 brain regions to the PVH.

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Fig S4. PVH subregional topology. Left: infection area of five different injection on the 2D hypothalamic flatmap. Right: scatter plot of the mean Bregma A/P value of output brain regions.

589 **Supplementary Tables**

590

591 Table S1. Oxytocin neuronal distribution in the mouse brain

592

593 Table S2. Number of cells in brain areas providing monosynaptic input to oxytocin neurons

594

595 Table S3. Projection percentage of brain regions with oxytocin neuronal projection

596

597 Table S4. Comparison between oxytocin projection and oxytocin receptor expression

598

599

600 **Supplementary Movies**

601

602 Movie S1. Oxytocin neuronal expression in the whole brain

603

604 Movie S2. Monosynaptic input to oxytocin neurons in the PVH and the SO

605

606 Movie S3. Brain-wide projection of oxytocin neurons in the PVH and the SO

607

608 Movie S4. Comparison between oxytocin receptor expression and projection of hypothalamic
609 oxytocin neurons.

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611