

ORIGINAL ARTICLE

Long-range projections of oxytocin neurons in the marmoset brain

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Abstract

The neurohormone oxytocin (OT) has become a major target for the development of novel therapeutic strategies to treat psychiatric disorders such as autism spectrum disorder because of its integral role in governing many facets of mammalian social behavior. Whereas extensive work in rodents has produced much of our knowledge of OT, we lack basic information about its neurobiology in primates making it difficult to interpret the limited effects that OT manipulations have had in human patients. In fact, previous studies have revealed only limited OT fibers in primate brains. Here, we investigated the OT connectome in marmoset using immunohistochemistry, and mapped OT fibers throughout the brains of adult male and female marmoset monkeys. We found extensive OT projections reaching limbic and cortical areas that are involved in the regulation of social behaviors, such as the amygdala, the medial prefrontal cortex, and the basal ganglia. The pattern of OT fibers observed in marmosets is notably similar to the OT connectomes described in rodents. Our findings here contrast with previous results by demonstrating a broad distribution of OT throughout the marmoset brain. Given the prevalence of this neurohormone in the primate brain, methods developed in rodents to manipulate endogenous OT are likely to be applicable in marmosets.

KEYWORDS

anatomy, marmoset, oxytocin

1 | INTRODUCTION

Oxytocin (OT) is a neurohormone involved in the regulation of many physiological functions in the brain that has a profound effect on a number of behaviors, most notably facets of sociality.¹ Because of the putative role that OT plays in a number of social disorders in humans,² significant efforts have been made to develop novel therapeutic strategies that modulate its regulation. And yet despite our growing understanding of the neurobiological circuits underlying the actions of OT in rodents,^{1,3,4} most clinical trials using OT in human patients have failed to produce conclusive effects.^{5,6} While the reason for this discrepancy is not clear, there are at least two explanations

that may account for the pattern of results. First, some functions of OT system may be different between rodents and humans owing to their evolutionary divergence of primates 100 million years ago. Second, the effects of precise OT manipulations done in rodents with tools such as opto- and chemogenetics or local intracerebral OT injection are not recapitulated by the methods commonly used in human and nonhuman primates, such as intranasal administration, blood measurements, or genetic association studies.^{7–13} While settling these methodological issues will take time, a critical first step toward addressing this gap of knowledge is to compare the anatomy of the OT system between species and determine shared and divergent properties. Unfortunately, to this date, the distributions of OT fibers

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and OT receptor in the primate brain have been only partially studied,^{14–20} thereby precluding our ability to infer the functions of OT neuronal pathways identified in rodents^{21–27} to human and non-human primates.

The limited number of studies seeking to identify OT fibers in the primate brain have yielded somewhat different and conflicting results. Extra hypothalamic OT fibers have not been consistently reported and found mostly around the hypothalamus and brainstem in marmosets¹⁶ and macaques,¹⁴ and only one study reported OT axons in cortical areas.²⁸ The available data thus show a very different picture to the patterns reported in rodents.^{29,30} Here, we performed a whole-brain quantitative analysis of OT fibers in male and female common marmoset monkeys (*Callithrix jacchus*) to bridge the gap in our knowledge of this keystone neurohormone in a primate species by validating and applying a recent OT antibody during immunohistochemical processing. Marmosets have emerged as a powerful neurobiological model of primate brain function, particularly for facets of social brain functions.³¹ This New World primate pair-bonds, cooperatively cares for their young, and engage in a suite of prosocial behaviors including food-sharing, imitation, and social gaze.^{32,33} Moreover, these monkeys are highly voluble, frequently engaging in bouts of vocal exchanges.³⁴ The neurobiological basis of vocal communication in marmosets is the most thoroughly studied of all social brain functions in this primate,^{35–39} and this function may be modulated by OT. Thus, the many similar social characteristics shared between marmosets and humans make this species particularly intriguing to examine the prevalence of OT fibers in the brain of a nonhuman primate.

2 | METHODS

2.1 | Animals

The brains from four adult common marmosets (two males and two females, aged 1 year 11 months, 2 years 4 months, 3 years 1 month, and 7 years 1 month) were used in this study. Animals had to be euthanized for various reasons (two for wasting syndrome, one for implant loss and one for AAV virus investigation). Before euthanasia, all animals were group housed, and experiments were performed in the Cortical Systems and Behavior Laboratory at University of California San Diego (UCSD). All experiments were approved by the UCSD Institutional Animal Care and Use Committee.

2.2 | Perfusion and tissue processing

Animals were anesthetized with Ketamine and then euthanized with pentobarbital sodium. Phosphate Buffer Saline (PBS) was perfused transcardially until all the blood was flushed out of the body, followed by 200 mL of 4% paraformaldehyde (PFA). The brain was quickly removed and postfixed overnight in 4% PFA, before being stored at 4°C in PBS with 0.025% sodium azide until the tissue was cut using a vibratome in 50 µm thick coronal slices.

2.3 | Immunohistochemistry

Immunohistochemistry was performed on free-floating sections. For each brain, one in six slices (one slice every 250 µm) was processed. Following three washes with PBS, slices were preincubated for 2 h in 1% Triton and 2% NGS on PBS, incubated overnight at room temperature on a plate shaker with 1:5000 oxytocin rabbit antibody (T-4084, Peninsula lab) and/or 1:1000 vasopressin guinea pig antibody (403004, SySy). The day after, slices were washed 3 × 10 min with PBS, incubated in the dark for 2 h with Goat Anti-rabbit IgG, CY3 (111 165 003, Jackson IR) and/or Goat Anti-guinea pig IgG, alexa A488 (706 545 148, Jackson IR), washed again with PBS then mounted on Superfrost glass slides and coverslipped using Vecta-shield mounting medium with DAPI (H-1200-10, Vector laboratories).

For control experiments, 1 µg of exogenous OT peptide (O4375, Sigma Aldrich) or AVP (V0377, Sigma Aldrich) were added to the well during incubation with primary antibody. It should be noted that this exogenous OT peptide is derived from human OT and thus has a different amino acid at the 8th position (New World monkeys have a proline instead of leucine⁴⁰), however, this did not seem to influence our results nor antibody specificity. We also co-stained AVP and OT to ensure the specificity of the OT antibody.

2.4 | Imaging and analysis

Slides were imaged using a slide scanner epifluorescent microscope (Axioscan 7, Zeiss) with 20× NA 0.8 objective and digitalized automatically (Zeiss Zen Lite software). All slices were imaged using the same exposure and LED power. OT fibers were manually counted and attributed to a brain region according to the Paxinos atlas.⁴¹ Fibers were identified as such if their length was >100 µm. Fibers close to each other but clearly discontinuous were counted as two different fibers, although it cannot be ruled out that they were the same fiber going in and out of the slice, or that a section of it was not showing immunoreactivity. A total of 3628 individual fibers were counted across the four brains. Fibers within the hypothalamus were not quantified but qualitatively described because of their high density due to the OT cell bodies being in proximity. Results are shown as percentage obtained by dividing the number of OT fibers found in one brain area by the total number of extra hypothalamic OT fibers. We only integrated an area in our analysis if it was found to contain OT fibers in all four analyzed brains or two of the same sex.

To calculate the average number of fibers per cubic millimeter per region, we extracted each brain area volume from the marmoset brain atlas (brain/MINDS project, BMA 2017 ex vivo brain space 1, <https://dataportal.brainminds.jp/>)⁴² using 3D Slicer software segment statistic module (v5.6.1, www.slicer.org).⁴³ We then divided the number of fibers (multiplied by 6 to account for the fact that we analyzed every six slices) by the volume for each area.

Due to lower tissue quality and processing, the brainstem was not analyzed by counting fibers but by qualitative assessment (see Figure 4).

3 | RESULTS

To provide a whole-brain characterization of OT in the marmoset monkey, we quantified the prevalence of these fibers from the brain slices of four adult marmosets (two male/two female). We first verified the specificity of the OT antibody by performing competition experiments on adjacent slices containing the paraventricular nucleus (PVN) of the hypothalamus. During incubation with the primary OT antibody, we added exogenous OT peptide (1 μ g), exogenous vasopressin (AVP) peptide (1 μ g) or PBS. We found that exogenous OT peptide prevented the OT antibody from labeling OT neurons in the PVN (Figure 1A) and OT fibers in central amygdala (CeA) (Figure 1E)

but that this was not the case when adding exogenous AVP peptide (Figure 1B) or PBS (Figure 1C,F). This validates the specificity of the OT antibody used in this study with marmosets. Furthermore, we also co-stained OT and AVP in the same slice (Figure 1D) and found no co-labeling, confirming that the OT antibody does not cross react with AVP in marmosets.

Our initial analysis sought to determine the organization of OT neurons in the marmoset hypothalamus. Consistent with previously described¹⁶ pattern and the Paxinos atlas,⁴¹ we identified the PVN from interaural +10.5 mm, a little anterior to the anterior commissure, to interaural +7.8 mm (Figure 2). The supraoptic nucleus (SON) was likewise located between interaural +11.0 and +8.5 mm. The

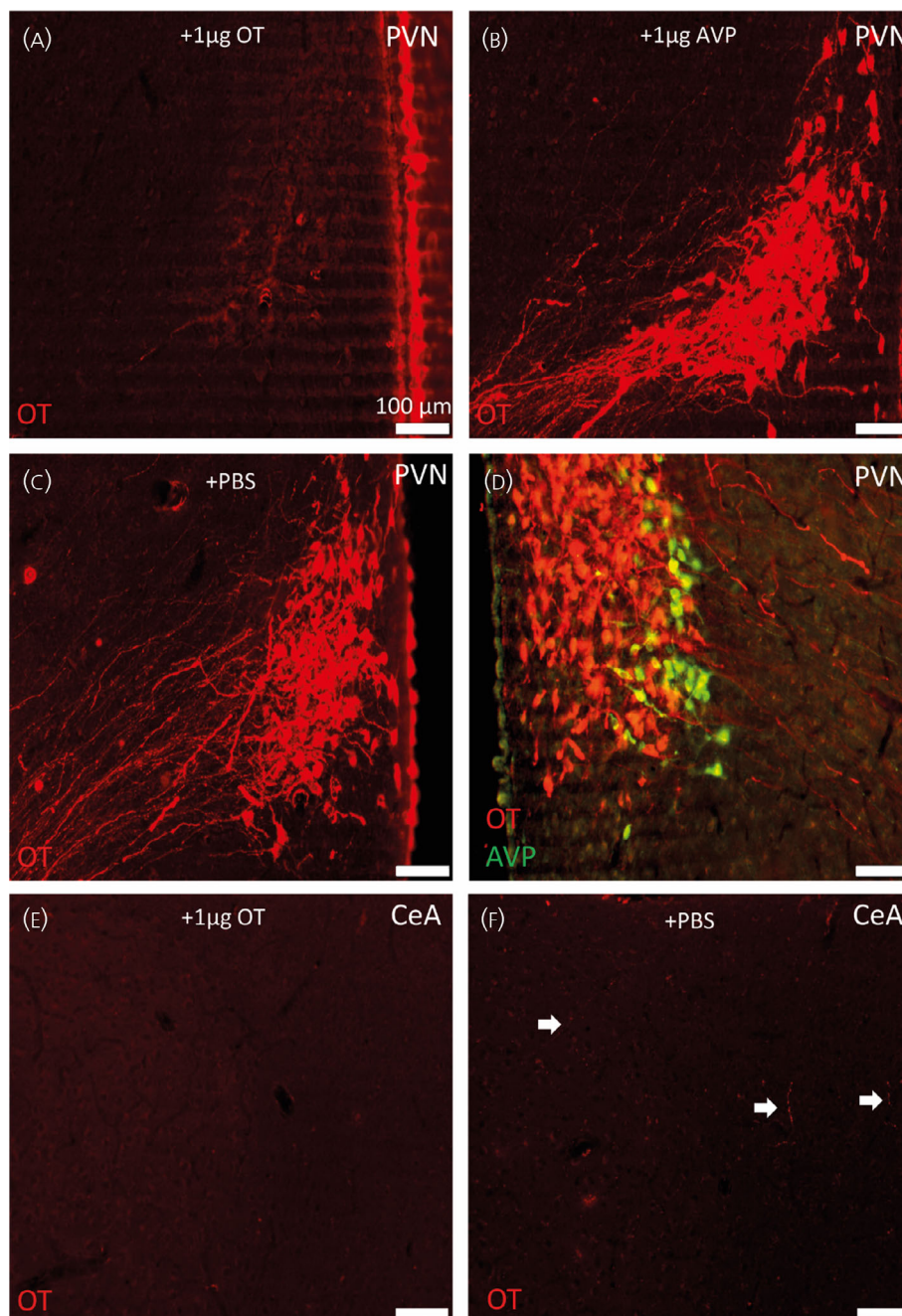


FIGURE 1 Oxytocin (OT) staining of a slice containing paraventricular nucleus (PVN) or central amygdala (CeA) incubated with 1 μ g of exogenous OT peptide (A and E), 1 μ g of exogenous AVP peptide (B) or saline (C and F). All images were taken with the same acquisition parameters (light intensity, gain, exposure, color brightness, and balance). (D) Co staining for OT (in red) and AVP (in green) in the same slice. Arrows indicate fibers.

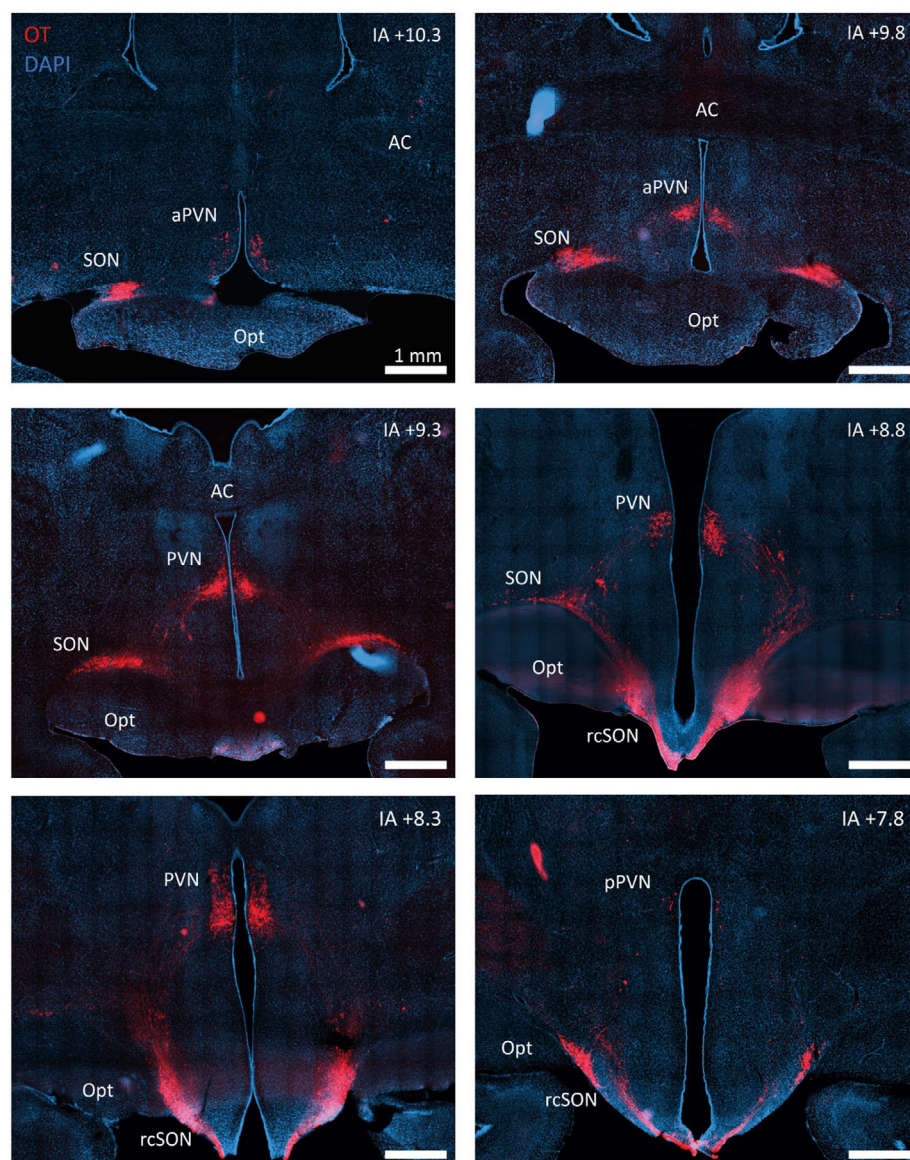


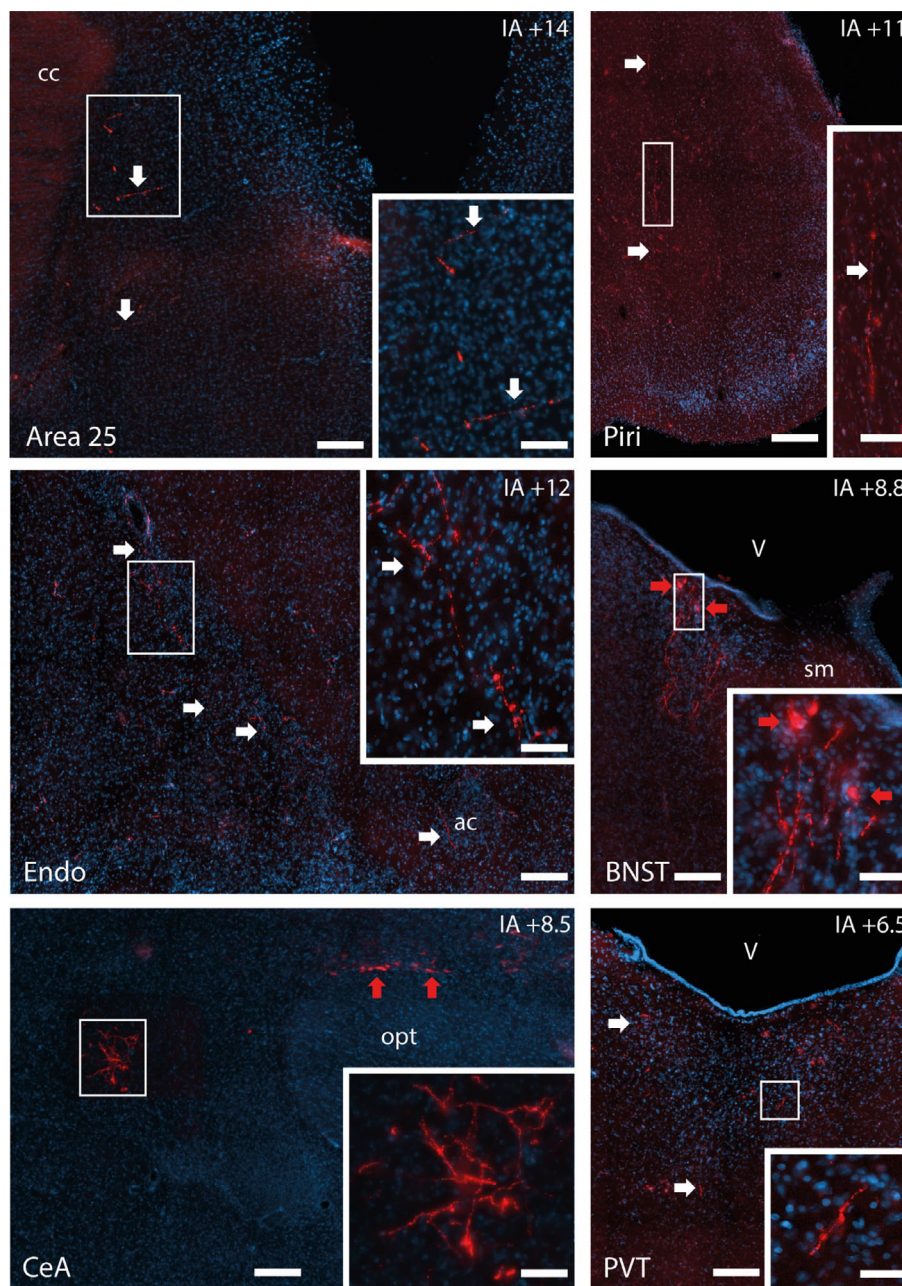
FIGURE 2 Fluorescent photomicrographs of coronal hypothalamic slices containing oxytocin neurons (in red). AC, anterior commissure; IA, interaural level; Opt, optic tract.

retrochiasmatic part of the SON was evident between interaural +9.0 and +7.8 mm (Figure 2). As expected,¹⁶ we observed high densities of OT neurons in both the PVN and SON (Figure 2), while a small number of OT positive cells were also evident in the Stria Terminalis close to the ventricle junction (lateral and third) around interaural +8.6 mm. Finally, few OT cells were seen in between PVN and SON, between interaural +9.5 and +8.0 mm, but as opposed to rodents^{21,44,45} no accessory nucleus was observed in marmosets (Figure 2).

Because of the paucity of data on the distribution of OT fibers in the primate brain, we next systematically quantified OT fibers through the brains of all four marmoset subjects. This analysis revealed OT fibers are evident in considerably more areas of the primate brain than had previously been reported.^{14,16} First, we observed high innervation (not quantified) of all hypothalamic areas, including the preoptic area (median, medial, and lateral), the anterior, lateral, ventro- and dorso-medial, and posterior hypothalamus as well as the mammillary body and zona incerta. At the sub-cortical level, we found a dense plexus in

the endopiriform nucleus that extended to the claustrum (Figure 3), as well as medium innervation to the nucleus accumbens (Figure 3). In addition, we found several regions close to the hypothalamus with an elevated OT fibers count such as the amygdala (in both central and basolateral parts), the bed nucleus of stria terminalis, the horizontal diagonal band of Broca and Meynert nucleus (Figure 3). Interestingly, we also found several cortical areas that contained OT fibers. These included mostly median areas like the anterior cingulate cortex (such as area 24, 25, and 32) and ventrolateral areas in the orbital and temporal cortices (such as area 13, 14, temporal areas, entorhinal and insula). Finally, in the posterior half of the brain, light but consistent OT innervation was evident in the brain stem, mostly directed to the periaqueductal gray with sparser fibers to other nuclei such as the inferior and superior colliculus, the nucleus of tractus solitarius and the locus coeruleus. We did not observe differences between the male and female brains nor between left and right hemispheres (see Figure 4A). A comprehensive list of brain regions found innervated by

FIGURE 3 Photomicrographs of coronal slices containing oxytocin fibers (in red). Fibers are indicated by white arrows, cell bodies by red arrows (found in BNST and SON). Scale bar = 300 μ m (100 μ m for insets). ac, anterior commissure; BNST, bed nucleus of the stria terminalis; cc, corpus callosum; CeA, central amygdala; Endo, endopiriform nucleus; IA, interaural level in mm; Opt, optic tract; Piri, piriform cortex; PVT, paraventricular nucleus of the thalamus; sm, stria medullaris; V, ventricle.



OT fibers can be found in Figure 4A and a representative schema in Figure 4B. We also provide an estimation of OT fiber density per region (fiber segments/ mm^3) to account for the different volumes of each region (Table 1).

4 | DISCUSSION

OT is synthesized mostly by neurons in two nuclei of the hypothalamus, the PVN located close to the third ventricle and the SON located above the optic tract,^{48,49} an organization that is highly conserved across vertebrate taxa,⁴⁰ including humans.¹⁵ In the present study, we investigated and mapped OT fibers throughout the male and female marmoset brain and observed OT fibers in numerous brain regions,

including multiple cortical areas. We observed numerous extra hypothalamic OT fibers, especially in the anterior half of the brain, most of which were found close to the midline (ACC, BNST, PVT, PAG, and brainstem), ventral (OFC, Olf, HDB, basal ganglia, Meynert nucleus, amygdala) or ventro temporal (claustrum, hippocampal formation, temporal cortex). In the posterior half of the brain, we only found OT fibers in the PAG and the brainstem, with a very similar distribution to what was previously found in macaques¹⁴ and rodents,³⁰ albeit the lower quality of data from the brainstem prevented us to quantify the fibers. Although no significant differences were observed between males and females, subtle differences may not have been evident because of the small number of marmoset brains analyzed, and the nature of the analysis performed here. It should however be noted that sex and hemispheric differences in rodents are only evident when

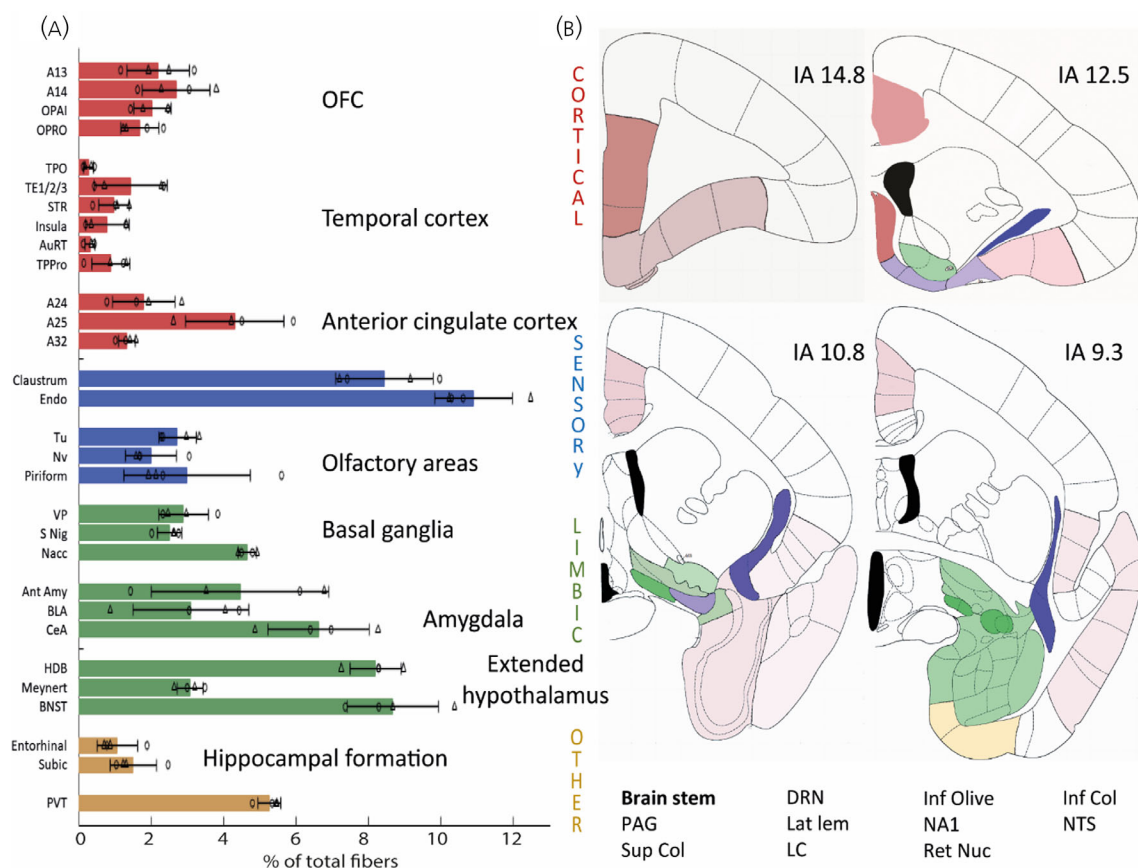


FIGURE 4 (A) Graph indicating individual (triangles and circles represent males and females, respectively) and averaged level of oxytocin (OT) innervation per region, expressed as percent of the total. Error bars represent standard deviation. Lower right: list of brainstem regions containing OT fibers (not quantified). (B) Schematics of OT fibers distribution on four representative coronal sections (IA: Inter Aural level in mm), colors indicate the type of region (same as in [A], cortical in red, sensory in blue, limbic in green and others in yellow) and color intensity indicates fiber density. Ant Amy, anterior amygdala; AuRT, auditory rostrotemporal cortex; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; DRN, dorsal raphe nucleus; Endo, endopiriform nucleus; HDB, horizontal limb of the diagonal band; Inf Oli, inferior olive; Lat lem, lateral lemniscus; LC, locus coeruleus; NA1, noradrenergic group 1; NAcc, nucleus accumbens; NTS, nucleus of the solitary tract; Nv, navicular nucleus; OPAI, orbital periallocortex; OPro, orbital proisocortex; PAG, periaqueductal gray; Piri, piriform cortex; PVT, paraventricular nucleus of the thalamus; Ret nuc, reticular nucleus; S Nig, substantia nigra; STR, superior temporal rostral area; Subic, subiculum; Sup/Inf col, superior/inferior colliculus; TE1/2/3, temporal areas 1/2/3; TPO, temporal par occ assoc; TPPro, temporopolar proisocortex; Tu, olfactory tubercle; VP, ventral pallidum. Adapted from the Paxinos atlas.⁴¹

comparing virgin and pregnant/lactating animals,^{21,22,50} and both of our female subjects were nulliparous. We also cannot rule out false negatives from our antibody which could lead to underestimation or lack of detection of some OT fibers. Other techniques, such as AAV anterograde tracing, are needed to confirm these results. The widespread prevalence of OT fibers throughout the brain notably contrasts with previous studies in marmosets¹⁶ and macaques,¹⁴ perhaps due to newer and more sensitive antibodies or more systematic investigation, and opens the door to more direct comparisons with rodents.

The main limitation of the study is the manual approach to quantify OT fibers, that is susceptible to experimenter bias and redundant counting of the same fiber due to slicing. To reduce experimenter bias, data were analyzed independently by two experimenters (A.L. and J.M.) and showed a good correlation ($r > 0.9$ for all brains). Furthermore, because the number of fibers counted could vary with different preprocessing and analysis, we presented the data as percentage of the total, a normalized measure less susceptible to variation between

laboratories. Ultimately, our findings should be confirmed by automatic labeling techniques. Finally, because we only stained for OT, we cannot infer whether the fibers reported here are forming synapses or are passing by. However, due to the nature of OT neurotransmission being not strictly synaptic,⁴⁷ we considered that any immunoreactive fiber could release OT vesicles and influence surrounding neurons.

The pattern of OT projections observed in marmosets here is consistent with analogous studies in rodents,^{21,25,29,30,44} with the notable exception of lateral septum. While this structure is well innervated with OT fibers in rats,⁵¹ none were detected in marmoset lateral septum. We also failed to detect OT positive fibers in the hippocampus fields (some were detected in subiculum). While a few studies indicated that this region is innervated by OT neurons using viral techniques,^{21,52,53} others did not observed it.^{29,30} To our knowledge, no study has reported OT immunoreactive fibers in the hippocampus, thus it could be that this structure receives only glutamatergic innervation from OT neurons. Because hippocampus is surrounded by CSF,

TABLE 1 Fibers per cubic millimeter for each area in which we found OT immunoreactive fibers.

Region	Fibers/mm ³	Region	Fibers/mm ³	Region	Fibers/mm ³
A13	6.62	A24	2.46	NaccSh	25.75
A14	25.61	A25	30.92	HDB	306.21
OPAI	36.69	A32	5.51	Ant Amy	76.91
OPRO	20.18	Clastrum	43.4	BLA	11.42
TPO	0.7	Endopiriform	95.57	CeA	102.72
TE1/2/3	1.11	Tu	16.65	Meynert nucleus	122.63
STR	2.58	Nv	11.92	BNST	75.63
Insula	2.13	Piriform	9.52	Entorhinal cortex	1.79
AuRT	1.32	VP	116.33	Subiculum	3.55
TPPro	4.27	Snc	80.06		

Note: For perspective, large dense core vesicles (LDCV, containing OT) are thought to be at a concentration of 0.7 LDCV/ μm of axon.⁴⁶ Each LDCV has been calculated to activate OTR in a 55 μm sphere around release site,⁴⁷ thus, 1429 LDCV would be required to cover an entire millimeter. This equates to 2041 μm of OT axon. Given that we only counted fibers if they were extending over 100 μm , this means that regions with >20 fibers/mm³ can be considered as highly innervated by OT (with enough OT to cover the entire area, theoretically) while those with <2 fibers/mm³ can be considered as weakly innervated (not enough OT to cover 10% of the volume, theoretically). Note that these calculations rely on several estimations and are here to illustrate the numbers in this table.

OT might reach it through volume transmission.^{49,54–56} More research is needed, both in rodents and primates, regarding OT in the hippocampus. The consistent similarities in OT fibers across taxa are of critical importance because we can now hypothesize that these shared pathways have similar functions in rodents and primates. For instance, OT release in the amygdala is known to have anxiolytic effects²¹ but also to be important for social recognition.^{52,57} In the nucleus accumbens, OT is involved in social reward⁵⁸ and regulates pair bonding in voles.⁴ It would thus be very interesting to manipulate OT in these areas in marmosets, as these highly social monkeys also display pair bonding. Furthermore, recent studies have demonstrated that in rodents, OT in medial prefrontal cortical areas is critical for social motivation and sociability, notably in the medial prefrontal cortex.^{59,60} This finding is potentially significant because the human mPFC (area 24, 25, 32) has been found to be a hub for neuropeptidergic transmission.⁶¹ Finally, it should be noted that some of the most innervated areas in both rodents and marmosets such as the PVT and the endopiriform nucleus have been scantily studied. The largely conserved neurobiology of OT between rodents and marmosets provides a roadmap in which to more precisely interrogate the functional role of these pathways in a primate to improve therapeutic treatments for the myriad disorders affected by OT given that intranasal OT administration has yielded such poor results.

Many of the brain areas innervated by OT in marmosets are involved in vocal communication, such as auditory and temporal cortices, ACC areas 32 and 24 and amygdala. As OT is important for social behaviors¹ and marmosets are known to be highly social and voluble,³⁴ the occurrence of these fibers in these neural substrates is perhaps not surprising but demonstrates an important link between this neurohormone and multiple social brain structures in primates. This pattern is also consistent with the theory that OT and oxytocin receptors (OTR)

are particularly present in the sensory brain regions mostly used by a species to interact (olfactory in mice, auditory in songbirds, visual in macaques).^{48,62} One noteworthy exception to this pattern was the absence of OT fibers in vIPFC (area 44 and 45), neocortical substrates known to be involved in the production and perception of primate vocalizations, including marmosets.^{36,63,64} A possible explanation is that OT reaches this cortical region by cerebrospinal fluid diffusion,^{49,54–56} though further research looking at the presence of OTR in these areas is needed. This mechanism may also explain whether the mismatch between OT fibers and OTR observed in rodents,⁴⁸ and the sexually dimorphic OTR distribution,^{65,66} also exist in primates. Future studies are needed to address these questions, as well as the potential overlap between OT and AVP fibers, and their receptors, in the marmoset. Overall, our results suggest that OT could act at different levels of the vocal communication system, ranging from perception (auditory areas), decision making and selection of vocalizations (amygdala and area 25/32) as well as motor control (area 24), and highlight that marmosets are a keystone model in which to study the effects of OT on acoustic communication in the primate brain.

AUTHOR CONTRIBUTIONS

Arthur Lefevre: Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing – original draft; writing – review and editing. **Jazlynn Meza:** Data curation; investigation; methodology; writing – review and editing. **Cory T. Miller:** Conceptualization; funding acquisition; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors of the manuscript have no conflicts of interest to declare.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/jne.13397>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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