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# NEUROANATOMICAL DISTRIBUTION OF OXYTOCIN AND VASOPRESSIN 1a RECEPTORS IN THE SOCIALLY MONOGAMOUS COPPERY TITI MONKEY (*CALLICEBUS CUPREUS*)

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**Abstract—The coppery titi monkey (*Callicebus cupreus*) is a socially monogamous New World primate that has been studied in the field and the laboratory to investigate the behavioral neuroendocrinology of primate pair bonding and parental care. Arginine vasopressin has been shown to influence male titi monkey pair-bonding behavior, and studies are currently underway to examine the effects of oxytocin on titi monkey behavior and physiology. Here, we use receptor autoradiography to identify the distribution of arginine vasopressin 1a receptor (AVPR1a) and oxytocin receptors (OXTR) in hemispheres of titi monkey brain ($n = 5$). AVPR1a are diffuse and widespread throughout the brain, but the OXTR distribution is much more limited, with the densest binding being in the hippocampal formation (dentate gyrus, CA1 field) and the presubiculum (layers I and III). Moderate OXTR binding was detected in the nucleus basalis of Meynert, pulvinar, superior colliculus, layer 4C of primary visual cortex, periaqueductal gray (PAG), pontine gray, nucleus prepositus, and spinal trigeminal nucleus. *OXTR* mRNA overlapped with OXTR radioligand binding, confirming that the radioligand was detecting OXTR protein. AVPR1a binding is present throughout the cortex, especially in cingulate, insular, and occipital cortices, as well as in the caudate, putamen, nucleus accumbens, central amygdala, endopiriform nucleus, hippocampus (CA4 field), globus pallidus, lateral geniculate nucleus, infundibulum, habenula, PAG, substantia nigra, olivary nucleus, hypoglossal nucleus, and cerebellum. Furthermore, we show that, in the titi monkey brain, the OXTR antagonist ALS-II-69 is highly selective for OXTR and that the AVPR1a antagonist SR49059 is highly selective for AVPR1a. Based on these results and the fact that both ALS-II-69 and SR49059 are non-peptide, small-molecule antagonists that should be capable of crossing the blood–brain barrier, these two compounds emerge as excellent candidates for the pharmacological manipulation of OXTR and AVPR1a in future behavioral experiments in titi monkeys and other primate species.** 

**Key words: neuropeptides, receptor binding, nonhuman primate, neuroanatomy, monogamy, pair bonding.**

## INTRODUCTION

In the last several decades, the neuropeptides oxytocin (OT) and arginine vasopressin (AVP) have emerged as important modulators of social behavior in mammalian species ranging from rodents to sheep to humans (Donaldson and Young, 2008; Freeman and Young, 2013). These molecules are capable of influencing a range of social behaviors including, but not limited to, parental care (Pedersen and Prange, 1979; Pedersen et al., 1982, 2006; Wang et al., 1994), territoriality and aggression (Ferris et al., 1984, 1985; Albers et al., 1986; Albers, 2012; Bosch, 2013), affiliation and social attachment (Winslow et al., 1993; Williams et al., 1994; Young et al., 1999a; Lim and Young, 2006), and social recognition memory (Ferguson et al., 2000, 2001; Bielsky et al., 2005; Skuse et al., 2013). These neuropeptides have been extensively studied in the socially monogamous rodent, the prairie vole (*Microtus ochrogaster*), and both peptides play a critical role in pair bond formation between opposite sex adult mates (Cho et al., 1999; Young and Wang, 2004).

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*Abbreviations:* $^{125}$I-LVA, $^{125}$I-linear vasopressin-1a antagonist; $^{125}$I-OVTA, $^{125}$I-ornithine vasotocin analog; AChE, acetylcholinesterase; AVP, arginine vasopressin; AVPR1a, vasopressin 1a receptor; CA1, CA1 field of the hippocampus; CA4, CA4 field of the hippocampus; Cd, caudate nucleus; CeA, central amygdala; DG, dentate gyrus of the hippocampus; GP, globus pallidus; Hipp, hippocampal formation; LG, lateral geniculate nucleus of the thalamus; LS, lateral septum; NAcc, nucleus accumbens; NBM, nucleus basalis of Meynert; NP, nucleus prepositus; OBD, optical binding density; OT, oxytocin; OXTR, oxytocin receptor; PAG, periaqueductal gray; PG, pontine gray; PSB, presubiculum; Pu, putamen; Pv, pulvinar; SC, superior colliculus; Sp5, spinal trigeminal nucleus; SN, substantia nigra; SuG, superficial gray layer of the superior colliculus; V1, 4C, layer 4C of the primary visual cortex; V1, 5-6, layers 5 and 6 of the primary visual cortex; V2, secondary visual cortex.

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OT and AVP may play similar roles in modulating pair bonding and social attachment in socially monogamous primate species as they do in monogamous rodents. The coppery titi monkey (*Callicebus cupreus*) is a monogamous New World primate known for the selective attachment that develops after mating between male and female partners, who spend extended time in side by side contact, often with their tails twined (Mendoza and Mason, 1986; Mason and Mendoza, 1998). Male titi monkeys treated intranasally with AVP increased contact time with their pair-mate compared to a stranger female (Jarcho et al., 2011). In another socially monogamous primate, the black-penciled marmoset (*Callithrix pencillata*), treatment with OT increased pair-mate huddling, and treatment with an OT receptor (OXTR) antagonist decreased proximity and food sharing between pair-mates (Smith et al., 2010). These studies indicate that OT and AVP can influence species-specific, pair-bond-related behaviors in socially monogamous primates.

While behavioral pharmacology has been useful in demonstrating that OT and AVP can modulate pair bonding and related behaviors in primates, the brain mechanisms by which these peptides modulate pair bonding behaviors are unclear. Identifying the locations of the receptors for OT and AVP in the brain can provide insights into the neural circuits modulated by these peptides to effect species-specific social behaviors. The most commonly used technique for localizing OXTR and the vasopressin 1a receptor (AVPR1a) is receptor autoradiography. This technique has been successfully used to determine the neuroanatomical distribution of OXTR and AVPR1a in several species of rodent, ultimately leading to the identification of brain regions involved in modulating social behavior (Ferguson et al., 2001; Lim and Young, 2004; Lim et al., 2004a,b; Bielsky et al., 2005; Gobrogge et al., 2009). One of the most interesting characteristics of the OXTR and AVPR1a system is the diversity of expression patterns, even among closely related species. For example, the highly social monogamous prairie vole and the relatively asocial, promiscuously breeding meadow vole have dramatically different distributions of each receptor in the brain, and these species differences in receptor distribution are associated with species differences in mating strategies (Insel and Shapiro, 1992; Lim et al., 2004a).

Despite the extensive work done in rodents to map these receptors, the distributions of OXTR and AVPR1a in the brains of primate species are still being discovered. This limitation is due, in part, to the pharmacological profiles of the two commercially available radioligands used for receptor autoradiography: the OXTR radioligand $^{125}$I-ornithine vasotocin analog ($^{125}$I-OVTA) and the AVPR1a radioligand $^{125}$I-linear vasopressin-1a antagonist ($^{125}$I-LVA). While these two radioligands are highly selective for the respective receptors in rodent tissue, they are less selective for primate receptors and exhibit a high, subnanomolar affinity for both OXTR and AVPR1a in human and rhesus macaque tissue (Freeman et al., 2014; Manning et al., 2012). Consequently each radioligand binds to both OXTR and AVPR1a, making it difficult to discriminate with confidence the distribution of the two receptors. This lack of selectivity of the radioligands for the primate receptors brings into question the specificity of the receptor binding results in earlier OXTR and AVPR1a mapping studies in human (Loup et al., 1989, 1991) and rhesus macaque (*Macaca mulatta*) brain tissue (Toloczko et al., 1997).

These issues also highlight the importance of overcoming the promiscuous binding profile of the radioligands in primate tissue by using a competitive binding design. This approach involves co-incubating the tissue with the radioligand and a selective, unlabeled competitor to displace the radioligand from one receptor and reveal the localization of only the receptor of interest. Thus, the goals of the current study were to (i) map the distributions of OXTR and AVPR1a in the brain of the socially monogamous coppery titi monkey using a pharmacologically optimized competitive binding receptor autoradiography protocol previously validated in the rhesus macaque (Freeman et al., 2014), and (ii) to determine the selectivity profile for two antagonists that may be useful in future behavioral pharmacology studies. Due to the particular lack of specificity for the OXTR radioligand when used in the macaque brain (Toloczko et al., 1997; Freeman et al., 2014), we also used *in situ* hybridization to confirm *OXTR* mRNA expression patterns in adjacent tissue sections.

## EXPERIMENTAL PROCEDURES

### Animals

Animals were housed at the California National Primate Research Center in cages (1.2 m × 1.2 m × 2.1 m) and were on a 12:12 light:dark cycle with lights on at 0600 h and lights off at 1800 h. Temperature was maintained at 21 °C. Housing conditions are identical to what has been previously described (Valeggia and Mendoza, 1999). Animals were fed a diet of monkey chow, banana, marmoset jelly, cottage cheese, apple, and carrot at 0800 h and 1300 h. Animals were euthanized on veterinary advice due to health reasons, none of which included a neurological component, and brains were harvested opportunistically. Two males (aged 6.97 and 5.21 years) and three females (ages: 4.28, 4.33, and 18.81 years) were used for the study. All animals were in stable, long-term pair bonds, and the females had all previously had infants; the males had not previously reproduced. All animal procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee and adhered to the legal requirements for non-human primate research in the United States.

### Tissue preparation

Titi monkey brains were removed promptly after death, rinsed with phosphate-buffered saline (PBS), and cut into two hemispheres. The hemispheres were blocked coronally, allowed to freeze completely on dry ice, and placed at −80 °C until sectioning. Hemisphere blocks were removed from −80 °C and brought up to −20 °C

for sectioning. The hemispheres were sectioned at 20 μm on a cryostat and mounted on Fisher Frost-plus slides. Slides were stored in a sealed slide box with desiccant and kept at −80 °C until use.

### Receptor autoradiography

Sections of titi monkey brain hemispheres were allowed to thaw in sealed slide boxes containing a desiccant packet for 1 h at 4 °C followed by 1 h at room temperature in a vacuum desiccator. The slides were processed for OXTR and AVPR1a receptor autoradiography as described previously, with slight modifications (Lim et al., 2004a). Specifically, sections were incubated for 1 h with one of two different radioligands: 50 pM $^{125}$I-LVA to target AVPR1a or 50 pM $^{125}$I-OVTA to target OXTR. Sets of three adjacent sections were co-incubated in three different competitive binding treatments for each radioligand: (i) 50 pM radioligand alone, (ii) 50 pM radioligand plus 10 nM SR49059, which is a human-selective AVPR1a ligand (Tocris, Minneapolis, MN; (Gal et al., 1993), or (iii) 50 pM radioligand plus 20 nM ALS-II-69, which is a human-selective OXTR ligand synthesized by our own lab (Smith et al., 2013). These unlabeled competitors were incubated at concentrations that were determined by previous competitive binding pharmacology experiments to be ideal for displacing the OXTR ligand from the human AVPR1a or displacing the AVPR1a ligand from the human OXTR, and thus increasing the specificity of the radioligand signal (Freeman et al., 2014).

We felt it was appropriate to use these data from human receptor pharmacology to inform our experimental approach to target the titi monkey receptors, due to high levels of homology across the two species. The titi monkey OXTR differs from the human OXTR in only 17 positions out of 389 (95.6% homology), with only three of these substitutions belonging to the N-terminus of the receptor, which forms the putative ligand-binding pocket (D.R. Ren and J.A. French, Department of Psychology, University of Nebraska-Omaha, personal communication). For AVPR1a, there are 25 amino acid substitutions between the titi monkey and human receptor out of a total of 419 (94% homology), and 11 of those are in the extracellular N-terminus domain (D.R. Ren and J.A. French, Department of Psychology, University of Nebraska-Omaha, personal communication). The 43 amino acids comprising the N-terminus putative ligand-binding pocket of the titi monkey OXTR have 93.0% homology with humans and 76.8% homology with mice. Likewise, the 54 amino acids comprising the N-terminus putative ligand-binding pocket of the titi monkey AVPR1a have 79.6% homology with humans and 64.8% homology with mice. These results support our approach that competitor ligands shown to be selective in humans are more appropriate for use in the titi monkey than ligands that are selective in rodents.

The slides were exposed to BioMax MR film (Kodak, Rochester, NY, USA) for 3 days with a set of $^{125}$I autoradiographic standards (American Radiolabeled Chemicals, St. Louis, MO, USA), then developed and quantified directly from the film as described below without image enhancement. Digital images for the figures were obtained from the films using a light box and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI, USA) connected to a computer. Brightness and contrast of the images were equally adjusted for all the sections using Adobe Photoshop (San Jose, CA, USA).

### Quantification and statistical analysis

Quantification of the optical binding density (OBD) was conducted on the resulting autoradiogram images on film on a light box in the following manner using AIS software (Imaging Research, Inc., St. Catharines, ON, Canada). After determining a flat field correction for luminosity levels, optical binding values from the set of standards were loaded into the software and used to generate a standard curve, from which binding density values from brain regions of interest could be extrapolated. In each animal, two separate measurements were made per brain region per treatment and averaged. OBD averages were calculated for six regions of interest (ROI): three for $^{125}$I-LVA binding and three for $^{125}$I-OVTA binding. Two-tailed, paired *t*-tests (alpha = 0.05) were performed to compare results from each of the competitor conditions to the radioligand alone condition. Finally, to generate a measurement of the overall efficacy of the competitors in reducing radioligand binding in this species, the OBD values were transformed to reflect the percentage of the radioligand alone condition and then averaged across the three ROIs for the two competitor conditions.

Due to the variation in the location of the midline bisection in each animal and to some slight issues with tissue integrity at the edges, some brain regions, especially hypothalamic areas and regions near the sagittal midline, were either not present or non-quantifiable and were therefore excluded from analyses. For one ROI (dentate gyrus), tissue from only four of the five animals was available for quantification.

### Acetylcholinesterase (AChE) staining

Following receptor autoradiography and film development, slides were counterstained for AChE to delineate the brain regions for image analysis as previously described using a modified protocol for the traditional AChE protocol that has been shown to amplify AChE signal in tissue previously used for receptor autoradiography (Lim et al., 2004a). Images of the resulting counterstained sections were compared with images from a red-bellied titi monkey brain atlas (www.brainmuseum.org), a rhesus macaque brain atlas (Paxinos et al., 1999), a common marmoset brain atlas (Newman et al., 2009), and a tufted capuchin brain atlas (Manocha et al., 1968) to determine neuroanatomical landmarks and identify regions.

### *In situ* hybridization

An antisense *OXTR* riboprobe derived from cDNA encoding the human *OXTR* was used for *in situ* hybridization for the titi monkey brains. A sense *OXTR* probe was used as a negative control. Preliminary data indicates that the nucleotide sequences for the titi

*OXTR* gene and the human *OXTR* gene are 95.6% identical (D.R. Ren and J.A. French, Department of Psychology, University of Nebraska-Omaha, personal communication), and we have previously shown that the signal generated from this probe is similar in distribution to OXTR binding sites in the rhesus macaque (Freeman et al., 2014). The human *OXTR* fragment, corresponding to nucleotide 631–1751 of human *OXTR* mRNA (NM_000916.3), was amplified from a human *OXTR* plasmid (gift of Dr. Bice Chini) using polymerase chain reaction (PCR) and a forward primer (5′-CGCGCTCGCAGCCAACTGGA-3′) and reverse primer (5′-TGCGATGGCTCAGGACAAAGGA-3′). This specific portion of the human *OXTR* gene is 96% identical to the same portion of the titi monkey *OXTR*. PCR products were inserted to pCRII vector (Invitrogen, Grand Island, NY, USA). $^{35}$S-UTP-labeled sense and antisense probes were generated from linearized plasmids using T7 or SP6 RNA polymerases.

Sections were allowed to thaw as described above, then hybridized with the probes and washed as described previously with minor modifications (Inoue et al., 2004, 2013). Specifically, the length of the proteinase K treatment was increased from 2 to 6 min, and the parameters for the high stringency wash step were changed as follows: 5% beta-mercaptoethanal was added to the 50% formamide/2× standard sodium citrate solution, and the temperature for this wash step was decreased from 65 to 62.5 °C. The sections were then exposed to BAS-IP TR 2025 E phosphorimaging screens (FujiFilm, Tokyo, Japan) for 28 days and then to BioMax MR film (Kodak, Rochester, NY) for 100 days. Phosphorimaging screens were analyzed using a BAS5000 phosphorimager (FujiFilm, Tokyo). After film development, digital images were obtained from the films using a light box and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) connected to a computer. Brightness and contrast of the images are equally adjusted for all the sections using Adobe Photoshop (San Jose, CA).

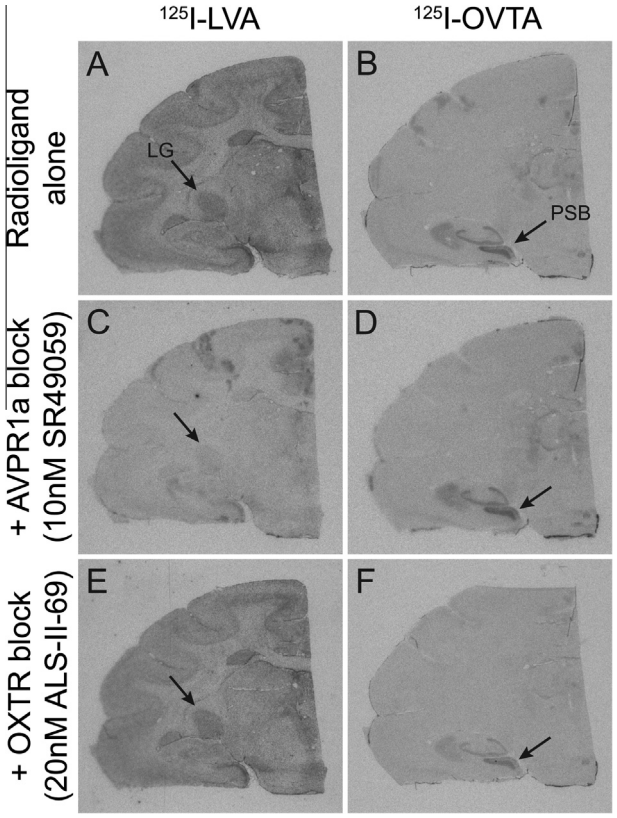


**Fig. 1.** Selectivity of radioligand binding in titi monkey brain tissue. Binding of the vasopressin 1a receptor (AVPR1a) radioligand $^{125}$I-LVA (A, C, E) and the oxytocin receptor (OXTR) radioligand $^{125}$I-OVTA (B, D, F) in adjacent titi monkey brain sections. Arrows highlight two representative regions with specific AVPR1a binding (lateral geniculate, LG) and OXTR binding (presubiculum, PSB). (A, B) Radioligand binding alone. (C, D) Radioligand binding in the presence of the AVPR1a antagonist SR49059. (E, F) Radioligand binding in the presence of the OXTR antagonist ALS-II-69. Note the diffuse $^{125}$I-LVA binding throughout the brain, including the LG, which is reduced by SR49059 but not ALS-II-69. Likewise, $^{125}$I-OVTA is reduced by ALS-II-69 but not by SR49059. These results suggest that both radioligands are highly selective for the respective titi monkey receptors.

## RESULTS

### Selectivity of radioligands

The radioligands $^{125}$I-LVA and $^{125}$I-OVTA produce distinct and mostly non-overlapping patterns of binding in the titi monkey brain (Fig. 1A, B). This result is strikingly different from what is seen when these compounds are used in rhesus macaque brain tissue, where they produce overlapping patterns of binding due to a lack of selectivity of the OXTR ligand (Toloczko et al., 1997; Young et al., 1999b; Freeman et al., 2014). SR49059 significantly reduces binding of $^{125}$I-LVA in the lateral geniculate nucleus (LG) ($t_4$ = 8.750, $p$ = 0.0009; Fig. 1A, C), the primary visual cortex (V1) and central amygdala (CeA) (Table 1). ALS-II-69, in contrast did not reduce $^{125}$I-LVA binding in these regions (Table 1), demonstrating that the radioligand binds selectively to AVPR1a and not OXTR.

In competition with $^{125}$I-OVTA, ALS-II-69 significantly reduces $^{125}$I-OVTA binding in the presubiculum (PSB) ($t_4$ = 5.439, $p$ = 0.0055; Fig. 1B, F) and dentate gyrus, and the reduction in the CA1 field of the hippocampus approached significance (Table 2). In contrast SR49059 does not reduce binding in any of these regions (Fig. 1B, D; Table 2). These data suggest that $^{125}$I-OVTA labels OXTR and not AVPR1a at the concentration used in the assay.

### Selectivity profile of the small molecule antagonists

Our results indicate that the competitors used in the current study are effective at selectively displacing radioligand binding in the titi monkey brain. Across brain regions, 10 nM SR49059 reduced binding of $^{125}$I-LVA by an average of 74.0 ± 1.7%, without affecting the binding of $^{125}$I-OVTA (Fig. 2, dark gray bars). Similarly, 20 nM ALS-II-69 is capable of reducing the binding of $^{125}$I-OVTA by an average of 43.0 ± 5.5%, without affecting the binding of $^{125}$I-LVA (Fig. 2, light gray bars). These results provide strong evidence that these two

**Table 1.** Quantification of competitive displacement of $^{125}$I-LVA by SR49059 and ALS-II-69. Optical binding densities (mean ± SEM) in three representative brain regions for each binding condition. Paired *t*-tests (alpha = 0.05) reveal a significant reduction in binding by the vasopressin 1a receptor (AVPR1a) block (+SR49059) compared to radioligand alone. The oxytocin receptor (OXTR) block (+ALS-II-69) did not significantly change binding compared to radioligand alone. *Abbreviations:* CeA, central amygdala; LG, lateral geniculate; V1, primary visual cortex

| | $^{125}$I-LVA Alone (dpm/mg) | $^{125}$I-LVA + SR49059 (dpm/mg) | $^{125}$I-LVA + ALS-II-69 (dpm/mg) | Alone vs. + SR49059 | Alone vs. + ALS-II-69 |
|---|---|---|---|---|---|
| CeA | 6712 ± 357.1 | 1530 ± 141.1 | 6169 ± 417.0 | $t_3 = 23.39$; $p = 0.0002$* | $t_3 = 2.292$; $p = 0.1057$ |
| LG | 5310 ± 600.0 | 1344 ± 150.1 | 5029 ± 550.1 | $t_4 = 8.750$; $p = 0.0009$* | $t_4 = 0.6409$; $p = 0.5564$ |
| V1 | 5244 ± 758.9 | 1464 ± 323.3 | 5291 ± 609.3 | $t_4 = 8.049$; $p = 0.0013$* | $t_4 = 0.1420$; $p = 0.8939$ |

* $p < 0.05$

**Table 2.** Quantification of competitive displacement of $^{125}$I-OVTA by SR49059 and ALS-II-69. Optical binding densities (mean ± SEM) in three representative brain regions for each binding condition. Paired *t*-tests (alpha = 0.05) reveal a significant reduction in binding by the oxytocin receptor (OXTR) block (+ALS-II-69) compared to radioligand alone in two out of the three regions. The vasopressin 1a receptor (AVPR1a) block (+SR49059) did not significantly change binding compared to radioligand alone. *Abbreviations:* DG, dentate gyrus; PSB, presubiculum; CA1, CA1 field of the hippocampus

| | $^{125}$I-OVTA Alone (dpm/mg) | $^{125}$I-OVTA + SR49059 (dpm/mg) | $^{125}$I-OVTA + ALS-II-69 (dpm/mg) | Alone vs. + SR49059 | Alone vs. + ALS-II-69 |
|---|---|---|---|---|---|
| DG | 3267 ± 675.0 | 3413 ± 301.3 | 1793 ± 230.5 | $t_3 = 0.1693$; $p = 0.8764$ | $t_3 = 3.284$; $p = 0.0463$* |
| PSB | 5079 ± 585.6 | 5644 ± 1246 | 2665 ± 244.7 | $t_4 = 0.4498$; $p = 0.6761$ | $t_4 = 5.439$; $p = 0.0055$* |
| CA1 | 2808 ± 487.8 | 2418 ± 165.8 | 1719 ± 188.5 | $t_4 = 0.8937$; $p = 0.4220$ | $t_4 = 2.701$; $p = 0.0541$ |

* $p < 0.05$

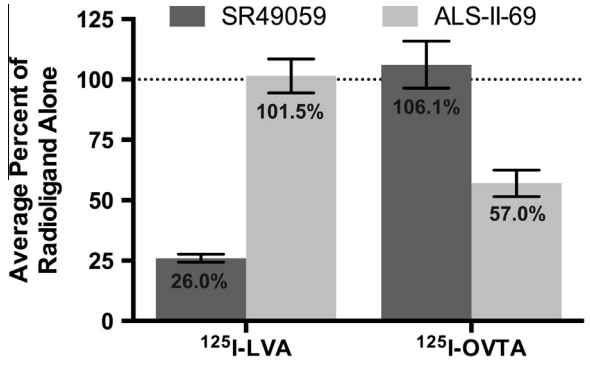


**Fig. 2.** Overall efficacy of the small molecule antagonists for displacing radioligand binding. Percent reduction in radioligand binding by SR49059 and ALS-II-69, averaged across three brain regions (LG, CeA, V1 for $^{125}$I-LVA and DG, PSB, CA1 for $^{125}$I-OVTA across animals for each radioligand. 10 nM SR49059 displaces off 74.0 ± 1.7% (average ± SEM) of binding of $^{125}$I-LVA without significantly reducing binding of $^{125}$I-OVTA (106.1 ± 9.7%). 20 nM ALS-II-69 displaces off 43.0 ± 5.5% of binding of $^{125}$I-OVTA without significantly reducing binding of $^{125}$I-LVA (101.5 ± 7.1%).

small-molecule, nonpeptide antagonists are effective and selective ligands for use in the titi monkey.

## AVPR1a distribution

The distribution of AVPR1a in the titi monkey brain is generally diffuse and widespread (Fig. 3A). AVPR1a binding exists throughout the cortex, especially in the insular (Ins), cingulate (Cg), and occipital cortices. In the occipital cortex, it is most dense in primary visual cortex (V1) in layers 5–6 and is also present in secondary visual cortex (V2). AVPR1a was also observed in the claustrum (Cl).

AVPR1a binding is also prominent in several regions of the basal ganglia (Fig. 3A, Panel 1). In the dorsal striatum, it is expressed throughout the caudate nucleus (Cd), and putamen (Pu). In the ventral striatum, it is expressed in the rostral nucleus accumbens (NAcc). AVPR1a binding also appears in and the globus pallidus (GP), in both the internal (GPi) and external (GPe) segments, although to a lesser degree than in the caudate and Pu. The substantia nigra (SN) is a major input nucleus for the basal ganglia, and we also detected AVPR1a binding in this region (Fig. 3A, Panel 2).

In the temporal lobe, AVPR1a can be detected in several areas (Fig. 3A, Panel 2). Rostrally, very dense binding is seen in what we believe to be the endopiriform nucleus (EN). We also observed dense binding in CeA. In the hippocampus, AVPR1a is expressed in the CA4 pathway of the hippocampus (CA4), also commonly referred to as the hilus of the dentate gyrus. There is also modest binding for AVPR1a in layer II of the presubiculum (PSB, II).

The distribution of AVPR1a also includes several subcortical nuclei involved in the processing of visual information. The lateral geniculate nucleus of the thalamus (LG), which receives input from the retina (Hubel, 1995), has moderate AVPR1a binding (Fig. 3A, Panel 2). The superficial gray layer of the superior colliculus (SuG), which also receives visual information from the retina, has dense AVPR1a binding, and there also is binding in the deeper layers of the superior colliculus (SC). The primary visual cortex, as previously mentioned above, has dense AVPR1a binding in layers 5 and 6 (Fig. 3A, Panel 3), which project back to the LG and SC, respectively (Hubel, 1995).

We also detected AVPR1a binding in several other structures. These areas include the habenula (Hb), periaqueductal gray (PAG), the infundibulum (Inf), the olivary nuclei (ON), and the hypoglossal nucleus (HN). AVPR1a binding was also seen throughout the cerebellum (Cb).

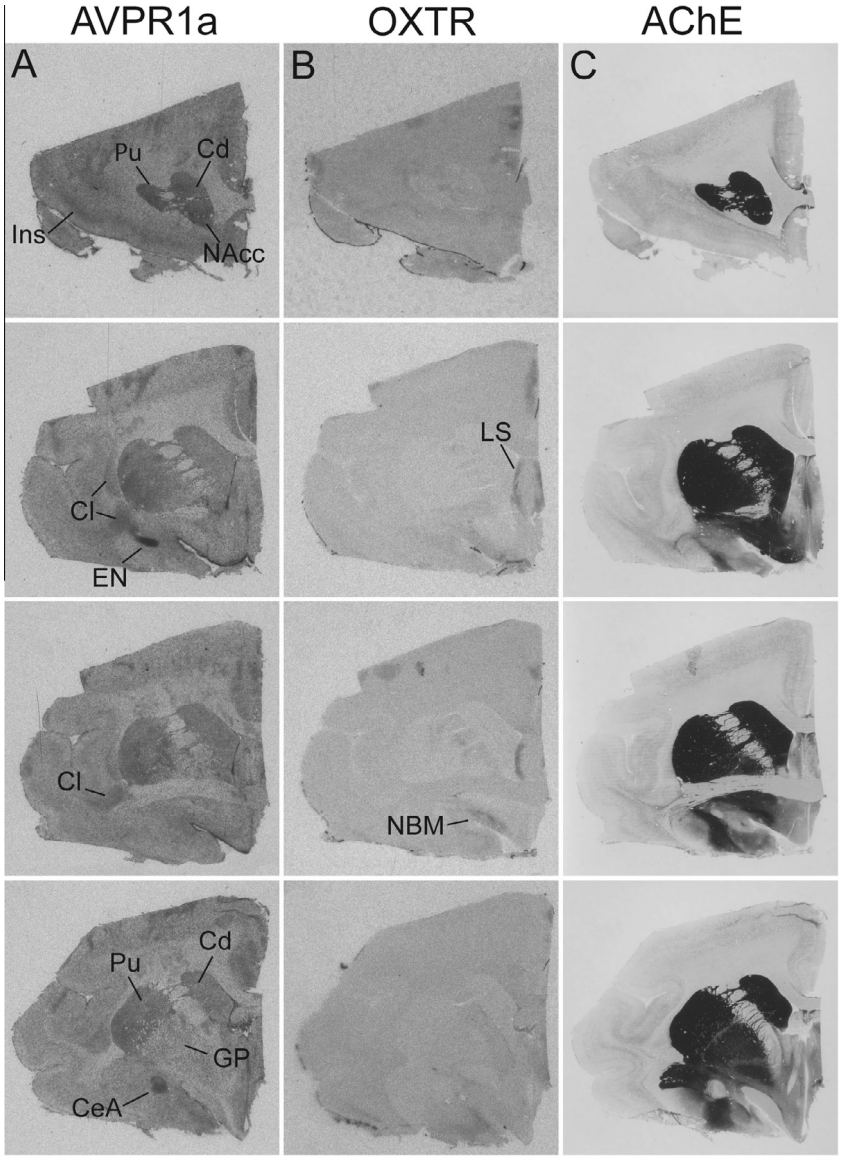


**Fig. 3.** Distribution of AVPR1a (A) and OXTR (B) in adjacent sections from one representative titi monkey brain, aligned with acetylcholinesterase (AChE) counterstain (C). Panels 1–3. Note the diffuse binding pattern of the AVPR1a radioligand throughout the cortex and striatum, while the OXTR binding pattern is much more restricted.

### OXTR distribution

The distribution of OXTR in the brain of the titi monkey is generally quite sparse and much more restricted than AVPR1a (Fig. 3B). The areas where the binding was densest were in the hippocampal formation (Hipp), particularly the CA1 field (CA1) and the dentate gyrus of the hippocampus (DG), and in layers I and III of the presubiculum (PSB,I and PSB,III, respectively) (Fig. 3B, Panel 2).

There were several other structures with modest levels of OXTR binding. These areas are the nucleus basalis of Meynert (NBM) (Fig. 3B, Panel 1), PAG, pulvinar (Pv), layer 4C of the primary visual cortex (V1,4C) in the occipital lobe, the deeper layers of the SC, and some hindbrain regions, including the nucleus prepositus (NP), pontine gray (PG), and spinal trigeminal nucleus (Sp5) (Fig. 3B, Panel 3).

Because hemispheres of brain tissue were used for this study, areas along the midline were either absent, bisected, or damaged in a few subjects, but in at least one animal we were able to detect specific OXTR binding in the lateral septum (LS) (Fig. 3B, Panel 1).

### OXTR *in situ* hybridization

*OXTR* mRNA was detected in areas where we also detected strong radioligand binding to OXTR (Fig. 4), confirming that the binding produced by the OXTR radioligand was due to binding at OXTR. These areas include LS, DG, PSB, CA1, PAG, PG, NP, and Sp5. Two areas with moderate OXTR binding, the Pv and layer 4C of V1 did not show a strong signal for *in situ* hybridization, possibly indicating that the mRNA levels in these areas are below the detectable limit or that the

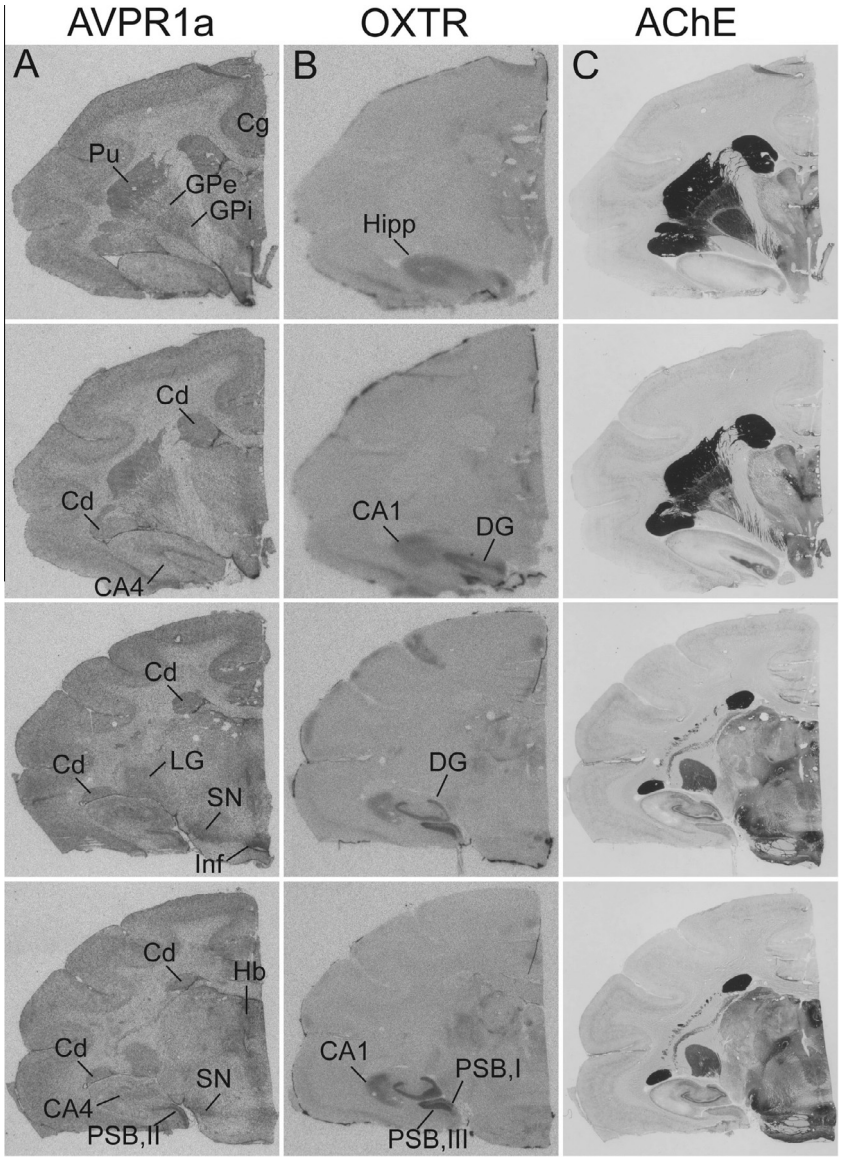


**Fig. 3** (*continued*)

OXTR protein present here is synthesized elsewhere and that the radioligand is detecting OXTR expressed on terminals. The sense strand probe, which does not target any mRNA, yielded uniform low background across all regions, whereas the antisense probe produced signal above background, although higher background was detected in some area containing white matter tracks (Fig. 4C).

## DISCUSSION

### General discussion

This is the first study to characterize the OXTR and AVPR1a distribution in the socially monogamous coppery titi monkey. The distribution of AVPR1 and OXTR are very distinct compared to that of the rhesus macaque and marmoset (Young et al., 1999b; Schorscher-Petcu et al., 2009; Freeman et al., 2014). This result is consistent with comparative studies across rodents, where there is remarkable phylogenetic plasticity among species. The present result confirms that this principle of interspecies variation also applies to primates.

The results of the current study are consistent with previous reports in the common marmoset and rhesus macaque in the sense that OXTR binding is significantly more limited in distribution than AVPR1a (Schorscher-Petcu et al., 2009; Freeman et al., 2014). In the rhesus macaque, OXTR distribution is even further restricted, with low levels of expression detectable in only five areas of the brain: NBM, ventromedial hypothalamus (VMH), SuG, trapezoid body (TB), and the pedunculopontine tegmental nucleus (PPT) (Freeman et al., 2014). In titi monkeys, OXTR binding is also present in the NBM, but mostly in areas that are not seen in the rhesus macaque: Hipp, DG, PSB, PAG, and a few hindbrain regions not identified in the rhesus tissue. In the common marmoset, there is OXTR in Sp5, as there is in titi monkeys and in humans (Loup et al., 1989, 1991).

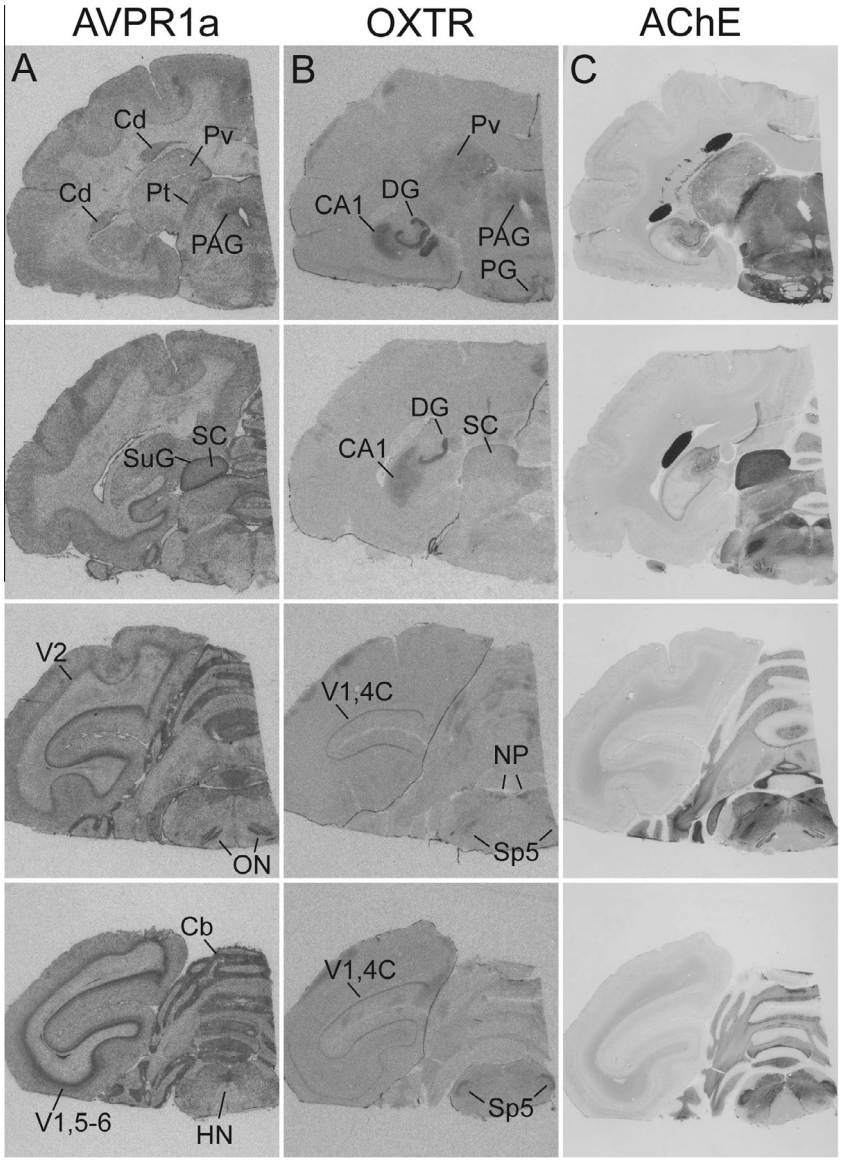


**Fig. 3** (*continued*)

Furthermore, the expression of AVPR1a in the titi brain is remarkably diffuse and widespread compared to any other species examined. Titi monkeys are the first species reported with AVPR1a expressed throughout the striatum. This suggests that the AVPR1a expression may be less tightly regulated in terms of cell-type specific expression compared to AVPR1a in other species or compared to OXTR. In contrast to the monogamous marmoset and monogamous prairie vole, which both have dense OXTR in the NAcc (Lim et al., 2004a; Schorscher-Petcu et al., 2009), OXTR in the titi monkey is largely absent in the striatum. OT acting at OXTR in the NAcc is necessary for pair-bond formation in prairie voles (Young et al., 2001). Therefore, if OT is involved in pair bonding in the titi monkey, it is likely to be by a different mechanism than in prairie voles. Due to the high levels of homology between OT and AVP as well as between their receptors, and due to the mixed affinities of OT and AVP for OXTR and AVPR1a in primates, one intriguing possibility is that OT may be acting at striatal AVPR1a to promote pair bonding in this species. This hypothesis can be tested with selective OXTR and AVPR1a antagonists in future behavioral pharmacology experiments. Alternatively, OT has been shown to promote non-reproductive social relationships in non-monogamous meadow voles by acting in brain areas other than the ones that have been shown to be important for opposite sex pair bonding in prairie voles (Beery and Zucker, 2010). Thus, it is also possible that OT could be acting at OXTR in other brain areas in titi monkeys, such as the Hipp and NBM, to promote pair bonding in this species.

Our results demonstrate that ALS-II-69 and SR49059 are highly selective ligands in the titi monkey for OXTR and AVPR1a, respectively, and can efficiently displace radioligand binding. Although these small molecule antagonists did not eliminate radioligand binding at the concentrations used in our study, it should be noted that

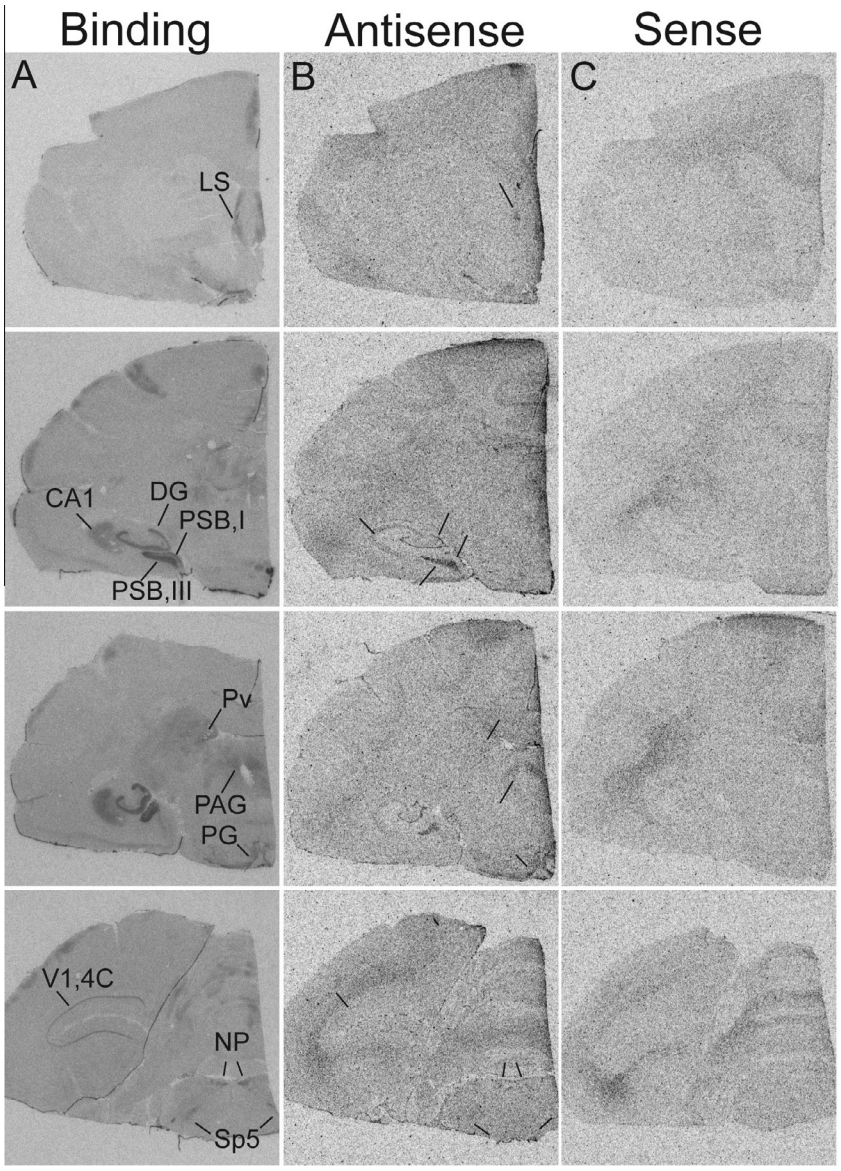


**Fig. 4.** *In situ* hybridization results for *OXTR* mRNA compared to OXTR binding. (A) Radioligand binding to OXTR. (B) Binding of the antisense probe to *OXTR* mRNA. (C) Binding of the sense probe, as a negative control to show areas of nonspecific probe binding.

the radioligands bind with much higher affinity than the endogenous ligands, OT and AVP, indicating that the small molecule antagonists should be more capable of displacing OT and AVP than displacing the radioligands used here. Furthermore, nonpeptide ligands, like the competitors used in the present study, can act as allosteric modulators to affect the affinity of the orthosteric ligand (in our case, the peptide radioligand) without directly competing it off of the putative ligand-binding pocket (Gruber et al., 2010), which can cause some of the radioligand binding to remain in the presence of the competitor. Thus, ALS-II-69 and SR49059 should be useful in behavioral pharmacology experiments to distinguish which receptor is mediating OT- or AVP-induced behavioral effects in titi monkeys.

Taken together, these comparative primate results confirm the observation from rodents that both OXTR and AVPR1a display remarkable phylogenetic plasticity in their central distributions. Thus, it is important to remember that the receptor distribution in the brain of one primate species cannot be a surrogate for the receptor distribution in another, including humans.

### Brain regions involved in visual attention and multimodal sensory processing

OXTR and AVPR1a binding are both expressed in several areas important in the processing of visual and multimodal sensory information. First, AVPR1a binding is dense in the LG, which is the primary thalamic relay nucleus for visual input from the retina to downstream regions (Hubel, 1995). The LG sends visual input from the retina to the SC, an area with both AVPR1a and OXTR in titi monkeys, and also projects to (and receives projections from) V1 (Hubel, 1995). In titi monkeys, both OXTR and AVPR1a are also observed in V1, with OXTR

in layer 4C and AVPR1a in layers 5 and 6. AVPR1a binding was also detected in the secondary visual cortex (V2) and generally throughout the occipital cortex.

Second, both AVPR1a and OXTR binding were present in the pulvinar nucleus of the thalamus (Pv), which also receives input from the retina, as well as from the SC (Berman and Wurtz, 2011). Inhibiting neurotransmission by injection of GABA-related drugs into the Pv of behaving monkeys disrupts performance on a visual attention task (Petersen et al., 1987). Furthermore, electrophysiological evidence suggests that neuronal activity in the Pv is related to the salience of visually presented objects (Robinson and Petersen, 1992). The medial portion of the Pv has higher densities of both receptor types in titi monkey brain tissue than the rest of Pv, and the medial subdivision has been shown to be connected to limbic areas, like the prefrontal cortex and amygdala, and has been suggested to be important in directed attention and changes in visual orientation (Romanski et al., 1997). These results suggest a possible functional link between OT and AVP and visual, attentional, and emotional processing.

Third, AVPR1a and OXTR are found in two areas that are especially important for the control of eye direction and gaze: the SC and the NP. The dense AVPR1a binding in the SuG layer of SC in the titi monkey closely resembles the dense OXTR binding in the SuG of the rhesus macaque (Freeman et al., 2014) and the marmoset (Schorscher-Petcu et al., 2009). In the deeper layers of the SC in the titi monkey, there is also modest OXTR binding as well as dense AVPR1a binding. The layers of the SC play distinct and important roles in stabilizing gaze direction, issuing motor commands to produce saccadic eye movements, and incorporating cognitive information to orient to (or away from) a stimulus (Gandhi and Katnani, 2011). Furthermore, OXTR was detected in NP, a brainstem nucleus that is part of the horizontal gaze holding system and is considered an important neural integrator for the oculomotor system (McCrea and Horn, 2006). The NP also has OXTR binding in the human brain (Loup et al., 1989) and seems to have low levels of OXTR binding in the marmoset brain, which was overlooked in the report (Schorscher-Petcu et al., 2009). OT and AVP acting in these areas may be important regulators of social visual attention, gaze shifting, and gaze stabilization.

Lastly, one striking commonality between the OXTR and AVPR1a distributions of the titi monkey and the binding patterns seen in other primates is the expression of this class of receptors in areas that are important in the allocation of attention to sensory stimuli. The titi monkey, human, and rhesus macaque all have OXTR expression in the NBM (Loup et al., 1991; Freeman et al., 2014), an area that is critical for mediating attention to visual stimuli (Muir et al., 1993). The common marmoset also has OXTR (and AVPR1a) binding in the NBM (S.M. Freeman, unpublished observations), although in the published report (Schorscher-Petcu et al., 2009), OXTR binding in this area was labeled as the nearby and closely related structure, the diagonal band of Broca. Therefore, while there may be species differences between OXTR and AVPR1a distributions in primates, one main commonality across species is that these neurohypophyseal receptors are expressed in areas which process visual stimuli, allocate attention, and control gaze direction and eye movement. These functions are highly relevant to the processing of social stimuli in primates, which use vision as the primary modality for social interactions.

### AVPR1a in the CeA

We observed dense AVPR1a binding in the CeA in titi monkeys, which is an area of dense AVPR1a expression in rhesus macaques as well (Young et al., 1999b; Freeman et al., 2014). In macaques, neurons in the amygdala respond to the head direction and gaze direction (Tazumi et al., 2010) and to the identity and facial expression (Gothard et al., 2007) of images of monkey faces. In rodents, AVPR1a is expressed in the CeA of rats, mice, prairie voles, and hamsters (reviewed in (Beery et al., 2008). In rats, OXTR and AVPR1a are expressed in distinct subpopulations of the CeA (Veinante and Freund-Mercier, 1997), and OT and AVP activate these subdivisions to differentially regulate fear behavior (Huber et al., 2005; Viviani et al., 2011). Specifically, in the CeA of rats, AVP acts to increase fear responses, while OT acts to decrease it (Huber et al., 2005; Viviani and Stoop, 2008; Viviani et al., 2011; Knobloch et al., 2012). Interestingly, we did not detect OXTR in the CeA of titi monkeys, despite it being present in the CeA of the vast majority of rodents studied to date (reviewed in (Beery et al., 2008). While future studies are needed in primates to investigate the effect of AVP and OT on fear behavior, this comparative evidence from rats suggests that AVPR1a the CeA of titi monkeys is likely important for fear behavior. Evidence from macaques indicates that this brain area may also be important for face processing.

### Brain regions important for reinforcement, learning, and memory

We did not detect OXTR in the NAcc or AVPR1a in the ventral palladium, two regions of the mesolimbic dopamine reward pathway that have been shown in prairie voles to be important for pair-bond formation (Young and Wang, 2004). However, these receptors were located in several areas of the brain that mediate various aspects of learning and memory, as well as the processing of rewarding or novel stimuli. For example, the three main forebrain regions in the titi monkey brain where we detected strong OXTR binding—CA1, DG, and PSB—are highly interconnected temporal lobe structures critical for learning and memory (Demeter et al., 1985; Squire and Zola-Morgan, 1991; Squire, 1992). We also detected modest AVPR1a binding in the hippocampus, as well as in the Cd, Pu, GP, and SN. The striatum (Cd, Pu, GP) is highly important in reinforcement learning (Schultz et al., 1992, 2003), and SN is a main source of dopaminergic input into the striatum. Thus, it is possible that OT and AVP are acting in titi monkeys via different neural circuits than those in prairie voles or marmosets to contribute to the acquisition and/or recall of social memories and the processing of social reward.

## CONCLUSIONS

The OXTR and AVPR1a distributions in titi monkeys are unique among primates and also among rodents more broadly. The differences between titi monkey receptor distributions and those of prairie voles and marmosets suggest that if these peptides play a role in social bonding in titi monkeys, the mechanisms may differ from those elucidated in prairie voles. In future studies, the localization of these receptors can be used to generate hypotheses about potential mechanisms of action of these peptides, which could then be tested by using pharmacological manipulations with the small molecule antagonists evaluated in the present study.

Finally, although the distribution of receptors differs across all primate species examined to date, OXTR expression appears to be consistently concentrated in areas involved in visual processing and attention, such as the SC and NBM. This result is in contrast to rodents, where OXTR tends to be concentrated in areas involved in olfactory processing, including the olfactory bulb. This redistribution of receptors from olfactory to visual and multimodal processing regions is consistent with the switch from olfactory to vision and audition as primary social information processing pathways during the evolution of primates.

## CONTRIBUTIONS AND DISCLOSURES

SMF and LJY designed the study. KLB supplied the tissue. SMF and HW performed all experiments except the *in situ* hybridization, which was performed by KI. ALS designed and synthesized the novel OXTR antagonist used in the study under guidance and support from MMG. LJY and KLB funded the research. SMF performed all analysis and wrote the first draft of the manuscript. LJY and KLB edited the manuscript. All authors have approved the final manuscript. This work was supported by NIH grants MH090776 and 1P50MH100023 to LJY and NIH HD053555 and the Good Nature Institute to KLB. Training support for SMF was provided by T32MH073525-06. Financial support to HW was provided by The Wenner-Gren Foundation. Additional support was provided by Office of Research Infrastructure Programs/OD P51OD011132 (formerly NCRR P51RR000165) to YNPRC and P51OD011107 to CNPRC. The authors have no conflicts of interest to disclose.

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