

A novel form of oxytocin in New World monkeys

Alex G. Lee^{1,*}, David R. Cool², William C. Grunwald Jr¹, Donald E. Neal¹, Christine L. Buckmaster¹, Michelle Y. Cheng³, Shellie A. Hyde¹, David M. Lyons¹ and Karen J. Parker¹

¹Department of Psychiatry and Behavioral Sciences, Stanford University, 1201 Welch Road, Palo Alto, CA 94305, USA

²Boonshoft School of Medicine, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435, USA

³Department of Biology, Stanford University, 371 Serra Mall Stanford, CA 94305, USA

*Author for correspondence (tree101@stanford.edu).

Oxytocin is widely believed to be present and structurally identical in all placental mammals. Here, we report that multiple species of New World monkeys possess a novel form of oxytocin, [P8] oxytocin. This mutation arises from a substitution of a leucine to a proline in amino acid position 8. Further analysis of this mutation in *Saimiri sciureus* (squirrel monkey) indicates that [P8] oxytocin is transcribed and translated properly. This mutation is specific to oxytocin, as the peptide sequence for arginine vasopressin, a structurally related nonapeptide, is unaltered. These findings dispel the notion that all placental mammals possess a ‘universal’ oxytocin sequence, and highlight the need for research on the functional significance of this novel nonapeptide in New World monkeys.

Keywords: oxytocin; mesotocin; arginine vasopressin; New World monkey; oxytocin receptor; squirrel monkey

1. INTRODUCTION

Oxytocin is a cyclic nonapeptide hormone with a wide spectrum of reproductive and social effects in mammals, ranging from uterine contractions and lactation to affiliative behaviour and parenting [1,2]. With the exception of cartilaginous fishes, the amino acid sequence of oxytocin in vertebrates is highly conserved, with relatively few changes over the course of 400 Myr [3–5]. The evolutionary lineage of oxytocin can be traced to isotocin ([S4,I8]-oxytocin), which is found in different classes of bony fishes (e.g. Neopterygii, Crossopterygii). Isotocin is the predecessor to mesotocin ([I8]-oxytocin), found in lungfishes, amphibians, reptiles, birds and most marsupials. Oxytocin evolved with the emergence of eutherian mammals (placental mammals) [2–6]. The amino acid sequence of oxytocin (CYIQNCPLG) is identical in all placental mammals examined to date, suggesting a strong selective pressure to withstand sequence variation [3,4]. However, we demonstrate herein that multiple New World monkey species possess a position-8 amino acid substitution in the oxytocin peptide (a leucine to a proline), resulting in a novel form of oxytocin.

2. MATERIAL AND METHODS

(a) Biological sample collection and preparation

All tissue samples were obtained opportunistically, secondary to a primary study. Blood samples were obtained from trained animals accustomed to blood sampling procedures. Biological samples were obtained from the following institutions/individuals: *Saimiri sciureus* (squirrel monkey), Stanford University; *Callithrix jacchus* (white-tufted-ear marmoset), *Macaca mulatta* (rhesus macaque) and *Cebus apella* (capuchin), Alpha Genesis Inc.; *Aotus nancymae* (owl monkey) and *Callicebus cupreus* (titi monkey) were generous gifts from Dr G. Yancey Gillespie, University of Alabama at Birmingham and Dr Karen L. Bales, University of California at Davis, respectively.

(b) Genomic sequencing and reverse transcription-PCR (RT-PCR) of primate oxytocin

Isolation of genomic DNA or RNA was performed using the DNeasy Blood and Tissue Kit (Qiagen) and RNeasy Plus Kit (Qiagen), respectively. All molecular biology experiments were conducted using standard procedures and kits from either Invitrogen or Qiagen. Melting temperatures were calculated with OLIGO CALC. We used the same sets of primers for capuchin, marmoset, squirrel monkey and owl monkey (FW: CCAGCGCACCCGCACCAT, REV: AAGCGAGTGGCGCGCTTCGA), (FW: TGTGCCCA-GACCATTAGCCA, REV: TTCTGGATGTAGCAGGCGGA). These two primer sets cover both the 5' UTR region and span across all three exons of the oxytocin gene. For titi monkeys, the primers used were (FW: TGCTGTCTGCTCGGCCTCCT, REV: GGTCCGAAGCAGCGGCCTTT). DNA samples from PCR reactions were run on 1 per cent agarose gel, purified with a QIAquick Gel Extraction Kit (Qiagen) and sent for sequencing (Quintara Biosciences). RT-PCR primers for squirrel monkeys were: oxytocin (FW: CCAGCGCACCCGCACCAT, REV: AAGCGAGTGGCGCGCTTCGA), arginine vasopressin (FW: TTCCTCGGCCTAC TGGCCTTC, REV: CAGCTCTCGTTCGTTGCAGCA), oxytocin receptor (FW: ATGGAGGGCGCGTTCGCAG, REV: TCACGC CATGGATGGCTGGG).

(c) Matrix assisted laser/desorption ionization-time of flight (MALDI-TOF) mass spectrometry of squirrel monkey pituitary tissue

For direct analysis of squirrel monkey neurointermediate lobe pituitary peptides, pituitaries were processed according to standard protocols and analysed on a Bruker Daltonics Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). For sequence analysis, the 991 and 1013 peaks were fragmented using TOF/TOF Lift technology, and identified using BioTools software. The fragments were analysed using MASCOT.

(d) Bioinformatics

Oxytocin sequences for multiple species were acquired from public databases, Ensembl, NCBI and UCSC genome browser. These sequences were either previously deposited by researchers or predicted from genomic scaffolds using either the default algorithm from the respective database or GENESCAN. To ensure valid transcripts, sequences were further validated by alignment with the existing human oxytocin sequence (accession no. NM_000915) and excluded if they did not cover at least 40 per cent of the complete coding sequences. Oxytocin sequences previously published in primary journal articles or sequenced directly by our group were also included in this analysis. Sequences obtained were deposited into GenBank with the following accession numbers for oxytocin: owl monkey (JF315861), titi monkey (JF315862), marmoset (JF315863), capuchin (JF315864), squirrel monkey (JF315865 and JF315866) and the oxytocin receptor: squirrel monkey (JF330026).

3. RESULTS

Genomic region coding for oxytocin in squirrel monkeys revealed a surprising single in-frame mutation from a thymine to a cytosine (figure 1a). This mutation results in a single amino acid substitution, from a leucine to a proline, in position 8 of the oxytocin nonapeptide, [P8] oxytocin (figure 1b). In addition, we sequenced the oxytocin gene in a diverse population of wild-caught and captive-bred squirrel monkeys ($n = 15$). All monkeys possessed this same base substitution, suggesting that this mutation is common among squirrel monkeys and not restricted to a few genetically similar individuals.

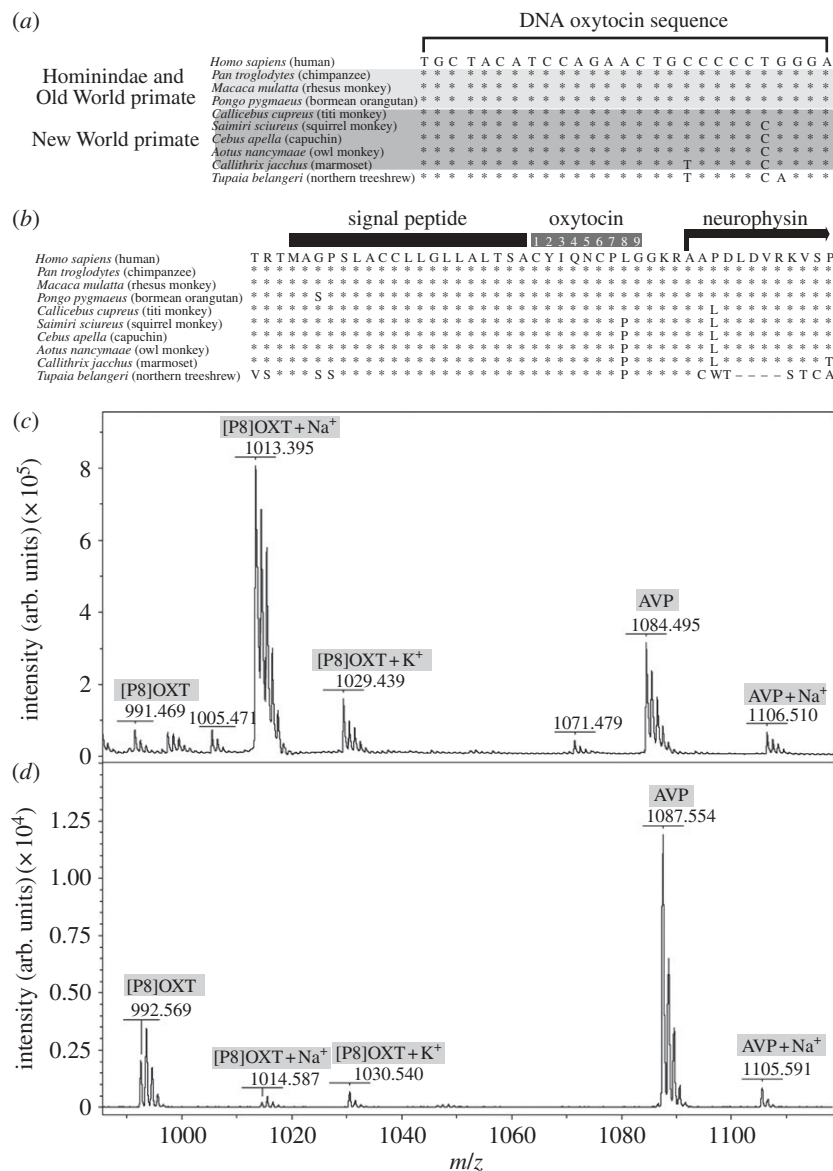


Figure 1. Genomic DNA, amino acid alignment for multiple species with and without the [P8] oxytocin mutation and representative mass spectrometry analysis of [P8] oxytocin (OXT) and arginine vasopressin (AVP) peptide from squirrel monkey pituitary tissue. Asterisks indicate nucleotides or amino acids that are identical to the corresponding residue in the human oxytocin sequence. All squirrel monkey [P8] OXT and AVP were compared with synthetic controls based on the cDNA sequence. Ion peaks represent positive detection of the corresponding peptide. (a) An alignment of genomic DNA for multiple species. A total of 27 nucleotides are depicted in the diagram. This region represents the DNA portion necessary to encode the final oxytocin nonapeptide. (b) Domain organization and amino acid alignment of the pre-oxytocin hormone for multiple species. Numbers below the region marked 'oxytocin' represent the amino acid residues corresponding to each of the nine amino acids that comprise the oxytocin peptide. (c) Ion peaks generated from squirrel monkey pituitary tissue. (d) Ion peaks generated from synthetic peptide based on the mutated squirrel monkey cDNA sequence, [P8] OXT (CYIQNCPPG) and AVP (CYFQNCPRG).

Genomic DNA analysis of the closely related white-tufted-ear marmoset revealed that there are two in-frame mutations in this primate species. The first mutation does not change the amino acid composition; however, the second mutation translates into the same proline-8 substitution as documented in squirrel monkeys (figure 1*a,b*). The marmoset sequence was obtained from DNA extracted from whole blood, the Ensembl database and UCSC genome browser. Additional genomic sequencing determined that owl monkeys and capuchin monkeys have the same mutated base pair substitution as squirrel monkeys, whereas titi monkeys have the conserved oxytocin

sequence (figure 1*a*). Finally, sequence analyses obtained from Ensembl revealed that the *Tupaia belangeri* (northern treeshrew) has multiple point mutations in the region coding for oxytocin, but results in the same amino acid substitution to that of the squirrel monkey. The proline-8 amino acid substitution therefore was the result of a single in-frame mutation of a cytosine to a thymine in all species with this mutation.

Next, we extracted total RNA from squirrel monkeys and demonstrated with RT-PCR that [P8] oxytocin is transcribed in this species. Oxytocin is a product of a pro-hormone consisting of four components encoded by a single gene (figure 1*b*). In the

		oxytocin								
		1	2	3	4	5	6	7	8	9
		C	Y	I	Q	N	C	P	L	G (NH ₂)
placental mammals	<i>Homo sapiens</i> (human)									
	<i>Pan troglodytes</i> (chimpanzee)	*	*	*	*	*	*	*	*	*
	<i>Macaca mulatta</i> (rhesus monkey)	*	*	*	*	*	*	*	*	*
	<i>Pongo pygmaeus</i> (bornean orangutan)	*	*	*	*	*	*	*	*	*
	<i>Microcebus murinus</i> (grey mouse-lemur)	*	*	*	*	*	*	*	*	*
	<i>Callicebus cupreus</i> (titi monkey)	*	*	*	*	*	*	*	*	*
	<i>Rattus norvegicus</i> (brown rat)	*	*	*	*	*	*	*	*	*
	<i>Cavia porcellus</i> (guinea pig)	*	*	*	*	*	*	*	*	*
	<i>Microtus ochrogaster</i> (prairie vole)	*	*	*	*	*	*	*	*	*
	<i>Mesocricetus auratus</i> (golden hamster)	*	*	*	*	*	*	*	*	*
	<i>Dipodomys ordii</i> (Ord's kangaroo rat)	*	*	*	*	*	*	*	*	*
	<i>Spermophilus tridecemlineatus</i> (ground squirrel)	*	*	*	*	*	*	*	*	*
	<i>Mus musculus</i> (house mouse)	*	*	*	*	*	*	*	*	*
	<i>Sorex araneus</i> (Eurasian shrew)	*	*	*	*	*	*	*	*	*
	<i>Oryctolagus cuniculus</i> (European rabbit)	*	*	*	*	*	*	*	*	*
	<i>Procavia capensis</i> (rock hyrax)	*	*	*	*	*	*	*	*	*
	<i>Felis catus</i> (cat)	*	*	*	*	*	*	*	*	*
	<i>Canis familiaris</i> (domestic dog)	*	*	*	*	*	*	*	*	*
	<i>Sus scrofa</i> (wild boar)	*	*	*	*	*	*	*	*	*
	<i>Bos taurus</i> (domestic cattle)	*	*	*	*	*	*	*	*	*
<i>Bubalus bubalis</i> (water buffalo)	*	*	*	*	*	*	*	*	*	
<i>Ovis aries</i> (sheep)	*	*	*	*	*	*	*	*	*	
<i>Equus caballus</i> (horse)	*	*	*	*	*	*	*	*	*	
<i>Pteropus vampyrus</i> (large flying-fox)	*	*	*	*	*	*	*	*	*	
lungfishes, non-mammalian tetrapods, most marsupials	<i>Didelphys virginiana</i> (opossum)	*	*	*	*	*	*	*	L/I	*
	<i>Macropus eugenii</i> (tammar wallaby)	*	*	*	*	*	*	*	I	*
	<i>Macropus rufus</i> (red kangaroo)	*	*	*	*	*	*	*	I	*
	<i>Taeniopygia guttata</i> (zebra finch)	*	*	*	*	*	*	*	I	*
	<i>Protopterus annectens</i> (lungfish)	*	*	*	*	*	*	*	I	*
bony fishes	<i>Halichoeres trimaculatus</i> (three-spot wrasse)	*	*	*	S	*	*	*	I	*
	<i>Takifugu niphobles</i> (grass puffer)	*	*	*	S	*	*	*	I	*

Figure 2. Oxytocin and orthologous peptides. Asterisks represent amino acid residues that are identical to the corresponding residues in the human oxytocin sequence.

human oxytocin gene, exon-1 encodes a translocator signal, the entire nine amino acids of oxytocin, a glycine residue coupled to a paired basic residue processing signal (GKR), and the first nine amino acids of neurophysin I. Exons 2 and 3 encode the rest of the neurophysin I. Comparison of squirrel monkey cDNA to squirrel monkey genomic DNA suggests that [P8] oxytocin is spliced similarly to that of humans.

In mammals, arginine vasopressin is often present in addition to oxytocin, encoded by a separate gene. The sequence of arginine vasopressin (CYFQNCPRG) differs from oxytocin in positions 2 and 8. In order to test whether the oxytocin mutation in squirrel monkeys is related to concomitant changes in arginine vasopressin, we also sequenced the squirrel monkey arginine vasopressin gene. Arginine vasopressin is expressed in squirrel monkeys and the amino acid sequence is identical to humans. A search using Ensembl also indicates that marmoset monkeys possess the same conserved arginine vasopressin sequence.

We next investigated whether [P8] oxytocin and arginine vasopressin are processed into mature nonapeptides in squirrel monkeys. Homogenates of the posterior pituitaries from nine squirrel monkeys were analysed by mass spectrometry. [P8] oxytocin (991 Da) and [P8] oxytocin ionized with sodium

(1013 Da) or potassium (1029 Da) peak adducts all suggest the presence of [P8] oxytocin (figure 1c). In addition, we compared the homogenate spectra with an oxytocin synthetic standard and found similar peaks (figure 1d). A difference of approximately 1 Da was observed owing to C-terminal amidation of squirrel monkey [P8] oxytocin compared with carboxylation of the C-terminal in the synthetic standard. The sequence of [P8] oxytocin was confirmed by MALDI-TOF/TOF mass spectrometry LIFT fragmentation with the b- and y-ion fragments identified using the MASCOT database. Fully processed (1084.5 Da) and amidated arginine vasopressin (1105.92 Da) was also present and detected in the same spectra (figure 1c). Overall, these results indicate that both [P8] oxytocin and arginine vasopressin are transcribed and translated in squirrel monkeys, confirming that the processing of [P8] oxytocin is not impaired by the P8 substitution. Finally, we performed a comprehensive bioinformatics search, and found no other placental mammals with a position-8 amino acid substitution (figure 2).

4. DISCUSSION

Here, we report that multiple New World monkey species express a novel form of oxytocin, consisting of a proline substitution in amino acid position 8.

Not all New World monkey species possess the novel form of oxytocin, as titi monkeys have the conserved oxytocin sequence. These findings suggest that the mutation arose in a group of highly related New World monkey species. Indeed cladistic analysis using combined molecular datasets (β -2M, EPSILON, G6PD and IRBP) suggests that marmosets, owl monkeys, squirrel monkeys and capuchins belong to a single clade in the Cebidae family, whereas titi monkeys belong to the Pitheciidae family [7]. Although New World monkey taxonomies are debated, our oxytocin dataset is consistent with this phylogeny [7–9]. Future research should examine additional members of the Pitheciidae family to confirm the absence of the mutation as well as the inclusion of monkeys from the Atelidae family, the third New World monkey lineage. If the oxytocin mutation differs systematically between these three lineages, it may prove useful in further elucidating New World monkey cladistic organization.

In addition to these four New World monkey species, we found that northern treeshrews also possess the P8 mutation, which probably represents a parallel substitution. Our bioinformatic analysis revealed that of the other 24 placental mammals in which the oxytocin sequence is known, no additional P8 mutations were observed. At present, 10 distinct orders have been sampled, while an additional 11 mammalian orders (albeit some with very few members) remain unsampled. These findings raise, but do not answer, an important question: has the oxytocin sequence been reported to be invariant in placental mammals because of under-sampling across mammalian orders, or does the [P8] mutation in treeshrews and a subset of New World monkeys form a biologically informative exception to this rule?

Oxytocin is a small nonapeptide, and a single amino acid change might, *a priori*, be hypothesized to have a functional effect. At present, it is unclear what the biological consequences of this mutation are. However, proline adds an additional rigid-ring structure that makes it more rotationally constraining and alters protein architecture to a much larger extent than either isoleucine or leucine in amino acid position 8 [10], as found in mesotocin and oxytocin, respectively. This substitution could change the overall binding property of the mutated [P8] oxytocin peptide, therefore rendering it more or less able to regulate species-typical reproductive and social behaviours. To date, all pharmacological studies of New World monkey species have involved administration of the conserved oxytocin peptide to species with the P8 mutation [11,12]. Detailed *in vitro* assays of oxytocin, [P8] oxytocin and arginine vasopressin, as well as their respective receptors, could provide insight on the role of this mutation on binding and activation in these peptide signalling systems, and guide pharmacological studies of the *in vivo* effects of oxytocin in primate species with and without this mutation.

All squirrel monkey biological sample collection procedures were approved by Stanford University's Administrative Panel on Laboratory Animal Care. Collection procedures for the other species were approved by Institutional Animal Care and Use Committees at each of the organizations that provided samples.

This research was supported by the National Alliance for Research on Schizophrenia and Depression (NARSAD), Stanford University, and Public Health Service grants MH47573 and MH77884.

- 1 Young, L. J. & Wang, Z. 2004 The neurobiology of pair bonding. *Nat. Neurosci.* **7**, 1048–1054. (doi:10.1038/nn1327)
- 2 Gimpl, G. & Fahrenholz, F. 2001 The oxytocin receptor system: structure, function, and regulation. *Physiol. Rev.* **81**, 629–683.
- 3 Acher, R. 1996 Molecular evolution of fish neurohypophysial hormones: neutral and selective evolutionary mechanisms. *Gen. Comp. Endocrinol.* **102**, 157–172. (doi:10.1006/gcen.1996.0057)
- 4 McEwen, B. B. 2004 General introduction to vasopressin and oxytocin: structure/metabolism, evolutionary aspects, neural pathway/receptor distribution, and functional aspects relevant to memory processing. *Adv. Pharmacol.* **50**, 1–50. (doi:10.1016/S1054-3589(04)50001-7)
- 5 Gwee, P. C., Tay, B. H., Brenner, S. & Venkatesh, B. 2009 Characterization of the neurohypophysial hormone gene loci in elephant shark and the Japanese lamprey: origin of the vertebrate neurohypophysial hormone genes. *BMC Evol. Biol.* **9**, 47. (doi:10.1186/1471-2148-9-47)
- 6 Chauvet, J., Hurpet, D., Michel, G., Chauvet, M. T. & Acher, R. 1984 Two multigene families for marsupial neurohypophysial hormones? Identification of oxytocin, mesotocin, lysipressin and arginine vasopressin in the North American opossum (*Didelphis virginiana*). *Biochem. Biophys. Res. Commun.* **123**, 306–311. (doi:10.1016/0006-291X(84)90413-3)
- 7 Schneider, H. 2000 The current status of the New World monkey phylogeny. *An. Acad. Bras. Cienc.* **72**, 165–172. (doi:10.1590/S0001-37652000000200005)
- 8 Singer, S. S., Schmitz, J., Schwiegk, C. & Zischler, H. 2003 Molecular cladistic markers in New World monkey phylogeny (Platyrrhini, Primates). *Mol. Phylogenet. Evol.* **26**, 490–501. (doi:10.1016/S1055-7903(02)00312-3)
- 9 Steiper, M. E. & Ruvolo, M. 2003 New World monkey phylogeny based on X-linked G6PD DNA sequences. *Mol. Phylogenet. Evol.* **27**, 121–130. (doi:10.1016/S1055-7903(02)00375-5)
- 10 Tymoczko, J. L., Berg, J. M. & Stryer, L. 2010 *Biochemistry: a short course*. New York, NY: W. H. Freeman.
- 11 Parker, K. J., Buckmaster, C. L., Schatzberg, A. F. & Lyons, D. M. 2005 Intranasal oxytocin administration attenuates the ACTH stress response in monkeys. *Psychoneuroendocrinology* **30**, 924–929. (doi:10.1016/j.psyneuen.2005.04.002)
- 12 Smith, A. S., Agmo, A., Birnie, A. K. & French, J. A. 2010 Manipulation of the oxytocin system alters social behavior and attraction in pair-bonding primates, *Callithrix penicillata*. *Horm. Behav.* **57**, 255–262. (doi:10.1016/j.yhbeh.2009.12.004)