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.; Astus nancymaeae (owl monkey) and Callitrichus 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: CCAGCGCAGCCGGACCAT, REV: AAGCGAGTGGCGCGTCGCA, FW: TTGGCCCAAAGCATAGGCA, REV: TTTCGGATGTAAGACGCGG). 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: TGGTCTGCTGGGCTGCCCTTCTT, REV: GGTCCGAAAGCAGGCCGGTTT). 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: CAGCGCCACCGCCGACCAT, REV: AAGCGAGTGGCGCGTCGCA), arginine vasopressin (FW: TTGGCCCAAAGCATAGGCA, REV: TTTCGGATGTAAGACGCGG).

(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 sequences 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 monkey 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 sequence. All squirrel monkey [P8] OXT and AVP were compared with synthetic controls based on the cDNA sequence. Numbers below the region marked 'oxytocin' represent the amino acid residues corresponding to each of the nine amino acids that comprise the oxytocin peptide. (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 1a,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 1a). 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 1b). In the
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.

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