UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

A EVOLUÇÃO MOLECULAR DO SISTEMA DA OXITOCINA EM PRIMATAS

PEDRO VARGAS PINILLA

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Maria Cátira Bortolini

Orientadora

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A los que con el anhelo de su presencia, hacen que miles de kilómetros se vuelvan segundos a la velocidad de un abrazo.

A los que te dan dos regalos todos los días y aunque siempre son los mismos, cada vez son diferentes.

A los que esperan que vuelvas corriendo en la noche, pero te animan a seguir volando durante años.

A los que te educan con su ejemplo, te moldean con amor y te iluminan con su espíritu.

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Sumário

LISTA DE ABREVIATURAS E SIGLAS

AVP – Vasopressina

AVPR1a – Receptor da vasopressina 1a

AVPR1b – Receptor da vasopressina 1b

AVPR2 – Receptor da vasopressina 2

GP – Glicopetídeo

GPCRs – Receptores acoplados as proteínas G

NP – Neurofisina

OXT – Oxitocina

OXTR – Receptor da oxitocina

PVN – Núcleo paraventricular

SP – Peptídeo sinalizador

RESUMO

A oxitocina (OXT) é um nonapeptídio envolvido em um amplo espectro de funções fisiológicas e comportamentais. Até recentemente acreditava-se em uma conservação de milhões de anos da sequência de aminoácidos, levando a pensar numa única proteína para todos os mamíferos placentários. Neste estudo foi analisada a oxitocina de 29 espécies de primatas, e desse grupo, foram estudados também os receptores de oxitocina (OXTR) de 21 espécies. Registramos aqui uma quebra na linha de conservação descrevendo 3 novas formas de OXT em macacos do Novo Mundo e reportamos um aminoácido (OXT-8Pro) com seleção positiva na família Cebidae; essa mesma posição registrou uma significância estatística (p= 0.003), numa análise de correlação com o tamanho da ninhada. Reforçando essa correlação, descrevemos uma nova forma de OXT (OXT-3Val-8Pro) nos *Saguinus* (Cebidae), um gênero com um pronunciado cuidado parental dos machos aparentados ou não. Em OXTR também foram detectados aminoácidos sob seleção positiva, assim como processos de coevolução intramolecular e intermolecular com seu ligante, OXT. Com os resultados aqui obtidos, propomos possíveis cenários da interação dessas novas formas de OXT com seus receptores e propomos perspectivas para o estudo do sistema OXT-OXTR e sua relação com outros sistemas.

ABSTRACT

Oxytocin (OXT) is a nonapeptide involved with a wide range of physiological and behavior functions. Until recently it was believed that an unmodified oxytocin sequence was present in all placental mammals. This study analyzed the oxytocin in 29 primate species, and the oxytocin receptor (OXTR) was also investigated in 21 of these species. We reported here an unprecedented lack of conservation, describing three novel OXT forms in the New World Monkeys. A signal of positive selection was detected in OXT- 8Pro in the Cebidae family and the same position showed a statistically significance (p= 0.003) correlation with litter size. Reinforcing this correlation, we describe here a novel OXT form (OXT-3Val-8Pro) in *Saguinus* (Cebidae), a genus with a pronounced male parental care. In OXTR amino acids under positive selection as well as intramolecular and intermolecular

coevolutionary process with his ligand, OXT, were detected. We suggest some interaction scenarios of the novel OXT forms with their receptors and we propose perspectives for the study of the OXT-OXTR system as well as its relationship with other systems.

1. INTRODUÇÃO

Um sistema que facilita a reprodução em todos os mamíferos: assim podem ser definidos o nonapeptídeo cíclico oxitocina (OXT) e as demais proteínas estruturalmente ligadas a ele. A oxitocina tem sido relacionada com um amplo espectro de efeitos centrais e periféricos, entre eles a modulação de reflexos neuroendócrinos e o estabelecimento de complexos comportamentos sociais (Bielsky & Young, 2004; Donaldson & Young, 2008; Gimpl & Fahrenholz, 2001; Soloff et al., 1979).

Dentro desse amplo espectro, pode-se falar dos sistemas reprodutivos. Os comportamentos monogâmicos e poligâmicos têm sido largamente estudados e são um exemplo da importância de se analisar a ação da oxitocina e das proteínas a ela relacionadas em animais (Young et al., 1998).

De 3-5% das espécies de mamíferos têm comportamentos monogâmicos (Kleiman, 1977). Uma delas é o arganaz do campo (*Microtus ochogaster*), que vive nas pradarias da América do Norte e apresenta relações monogâmicas estáveis. Após um intenso período de união inicial, o macho e a fêmea formam um casal e vivem juntos em um único ninho. O macho defende furiosamente sua parceira, e ambos os progenitores cooperam no cuidado a longo prazo de seus filhotes. Ao contrário, o arganaz montanhês (*Microtus montanus*), que vive em terras altas, é pouco sociável e bastante promíscuo. Cada indivíduo vive em um ninho isolado, os machos não tomam parte nos cuidados com os filhotes, e as fêmeas cuidam da ninhada por pouco tempo (Wang & Young, 1997; Young, et al., 1996).

Qual seria a razão dessas diferenças no comportamento, se ambas as espécies pertencem ao mesmo gênero e partilham 99% da informação genética? (Magon & Kalra, 2011). Ao se observarem os processos endócrinos, pôde-se achar uma possível resposta. Quando os arganazes de campo copulam, dois hormônios são liberados da pituitária anterior: oxitocina (OXT) e vasopressina (AVP). Se a liberação desses hormônios é bloqueada, o comportamento do arganaz do campo fica muito semelhante ao de seus primos da montanha (Neumann, 2008). Por outro lado, se forem aplicadas injeções de OXT

e de AVP nos arganazes de campo, ao mesmo tempo em que a cópula é evitada, eles ainda têm preferência pelo seu parceiro (Ross et al., 2009; Ross & Young, 2009), evidenciando a importância desses dois hormônios neste comportamento para a espécie do campo.

No entanto, quando OXT e AVP são aplicadas no arganaz montanhês, não é determinada nenhuma mudança no seu comportamento. É possível especular que a ausência de resposta, nesse caso, se deva ao fato de esses animais não terem receptores em determinadas regiões do cérebro para interagir com os hormônios nas condições fornecidas pelo experimento.

Esse exemplo ilustra muito bem o fato de que, assim como os hormônios, seus receptores — OXTR para OXT e AVPR1a, AVPR1b e AVPR2 para AVP — são igualmente importantes para determinar um fenótipo comportamental como os descritos anteriormente.

De fato, se os receptores são inativados por antagonistas, a formação do casal arganaz do campo é evitada pela fêmea, por meio da inativação de OXTR, e pelo macho, por meio da inativação de AVPR1A, AVPR1B e AVPR2 (Winslow et al., 1993).

Esses achados sugerem hipóteses muito interessantes acerca da evolução de comportamentos sociais complexos. Dentre essas poderíamos nos perguntar: em que medida as mudanças no repertório de comportamentos reprodutivos como os acima referidos seriam explicadas por mutações nos genes dos hormônios e/ou quantas seriam decorrentes de mutações em seus receptores?

Complexo OXT-OXTR e outros sistemas relacionados

Desde 1906, quando H.H. Dale, farmacologista britânico, descreveu o que ele denominou como *ergot*, têm-se estudado as propriedades da oxitocina (Dale, 1906). O primeiro processo com o qual relacionou-se esse hormônio foi o parto, determinando o seu nome – oxitocina –, um neologismo grego que significa "parto rápido". Anos depois, Ott e Scott (1910) propuseram sua relação com a lactação. Já a sequência de nove aminoácidos que compõem a oxitocina foi revelada em 1953 (Du Vigneaud, Ressler, & Trippett), e sintetizada um ano depois (Du Vigneaud, 1954). Posteriormente, foi descoberta a presença de um pré-hormônio, constituído pela oxitocina e pela neurofisina, a qual participa

do transporte da OXT do hipotálamo à pituitária posterior (Brownstein et al., 1980). O gene do receptor da oxitocina (*OXTR*), por sua vez, foi clonado em 1992 (Kimura, et al., 1992).

Esses e muitos outros estudos têm definido as funções da oxitocina bem como de seu receptor. É importante salientar o duplo papel que ela exerce, já que atua tanto como neurotransmissor quanto como hormônio, seja em humanos ou em outros mamíferos. As ações periféricas do complexo ativado incluem a estimulação para a dilatação cervical, a contração uterina durante o parto, a liberação do leite em mães lactantes e o orgasmo durante a atividade sexual. Uma recente revisão (Lucion & Bortolini, 2014) traz detalhes sobre abordagens adotadas para o entendimento dos mecanismos envolvidos no cuidado parental, dentre estas, aspectos fisiológicos e genéticos, dos quais os sistemas OXT-AVP têm papel de destaque. No cérebro, a OXT influencia nas funções cognitivas, emocionais e sociais e, também, em comportamentos maternos e sexuais (Ebstein, et al., 2012).

A OXT é um nonapeptídeo composto por Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly, sequência na qual as duas cisteínas (Cys) são conectadas por uma ponte disulfeto (Figura 1). Sua estrutura é similar à da AVP, diferenciando-se em dois aminoácidos. Seus respectivos genes (*OXT* e *AVP*), são conservados através de inúmeras espécies de diferentes *taxa* (Hoyle, 1998; Tessmar-Raible et al., 2007).

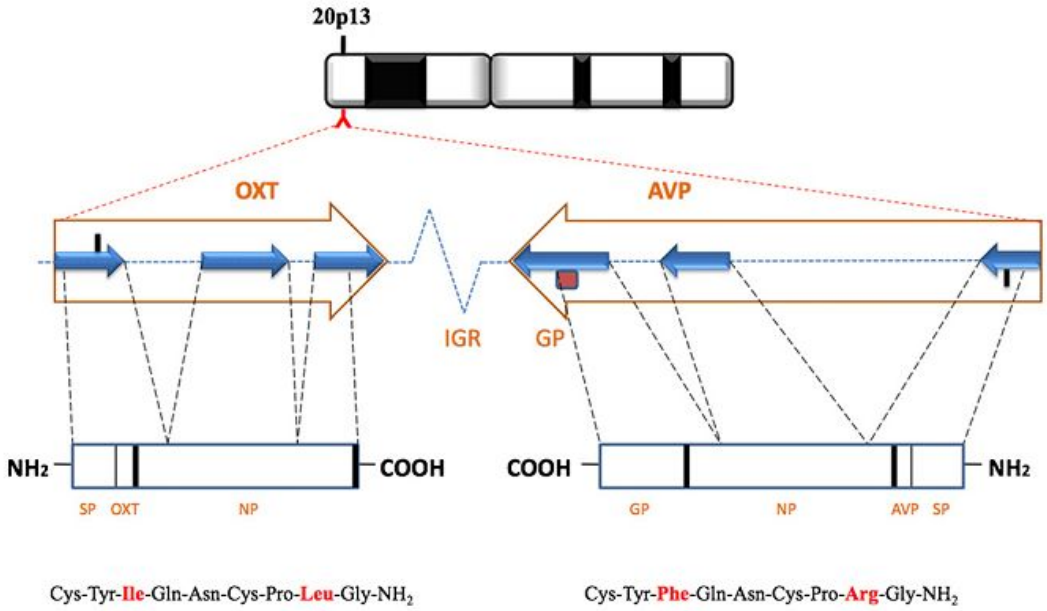


Figura 1. Diagrama dos genes de oxitocina e vasopressina (respectivamente, OXT e AVP, representados pelas setas grandes), enquanto os pré-pró-hormônios são ilustrados pelas caixas. A sequência de aminoácidos de cada um dos nonapeptídeos aparece abaixo e a localização destes no

cromossomo 20 humano é ilustrada acima. Os éxons são mostrados como setas azuis pequenas, e os íntrons como linhas pontilhadas. IGR representa a região intergênica; SP, peptídeo sinalizador; NP, neurofisina; GP, glicopeptídeo. Os resíduos de aminoácidos que diferem entre as moléculas estão representados em vermelho (Extraído de Lee et al., 2009).

O gene *OXT* está localizado no mesmo cromossomo onde também se localiza o gene *AVP*, mas ambos estão orientados numa direção transcricional contrária (cromossomo 2 em camundongos, e 20 em humanos; Figura 1). Esse fenômeno é visto ainda em outros mamíferos. Ambos os genes possuem três éxons e dois íntrons com uma alta identidade e estão separados por uma região intergênica que varia em todas as espécies (1,1 kb em ratos e humanos, e 3,6 kb em camundongos). Essa porção, normalmente conservada, possui sequências reguladoras (Gainer, et al., 2001).

A Figura 1 também mostra que o chamado pré-hormônio é formado pela sequência da *OXT*, da neurofisina (*NP*) e do peptídeo sinal (*SP*), enquanto o outro pré-hormônio relacionado envolve a sequência da *AVP*, da neurofisina (*NP*) e do peptídeo sinal (*SP*), acrescida da sequência de um glicopeptídio (*GP*).

Evolução do sistema OXT/OXTR e proteínas relacionadas

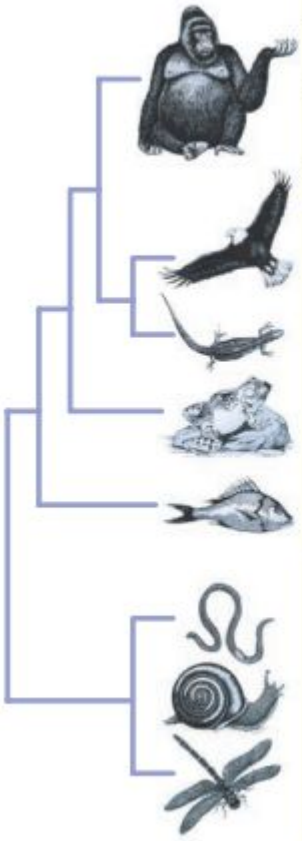
Virtualmente, todas as espécies de vertebrados possuem sistemas como o da OXT e da AVP ou proteínas similares a essas, contabilizando um total de 13 nonapeptídeos (Tabela 1). A vasotocina, por exemplo, similar à vasopressina e à OXT, foi encontrada nos Ciclostomados (lampreias) e em peixes ósseos. A diferença entre AVP e vasotocina é a presença de uma Ile (vasotocina) ou Phe (AVP) na posição 3; por outro lado, a diferença entre OXT e vasopressina é uma Arg (vasotocina) ou uma Leu (OXT) na posição 8 (Figura 2). A mesotocina, por sua vez, difere da OXT pela presença de uma Ile na posição 8 (Leu em OXT) e pode ser observada em peixes pulmonados africanos e australianos, anfíbios, répteis e aves, encontrando-se também em marsupiais, particularmente em duas espécies que apresentam, além da mesotocina, a OXT (Tabela 1).

A pouca variação do hormônio pode ser observada em todos os vertebrados ósseos já investigados, com exceção dos peixes cartilaginosos, que apresentam variabilidade e dualidade no sistema, como mostra a Tabela 1. Por exemplo, além da vasotocina, as raias da subclasse Selachii possuem o hormônio glumitocina (presença de uma glicina na posição

8, quando comparada à oxitocina), enquanto os tubarões possuem dois peptídeos: aspargtocina, (presença de uma asparagina na posição 4) e valitocina (presença de uma valina na posição 8) quando comparadas à oxitocina. A mesma comparação pode ser feita com o cação, no qual se encontra a asvatocina (uma asparagina na posição 4, e uma valina na posição 8) e a phasvatocina (fenilanina, asparagina e valina nas posições 3, 4 e 8 respectivamente).

Esses peixes cartilaginosos utilizam a ureia como elemento de osmorregulação, e uma gama maior de hormônios similares à OXT pode ter ajudado nessa função, de acordo com alguns autores (Acher, et al., 1995).

Figura 2. Homólogos de oxitocina e vasopressina. Extraído de Donaldson e Young, (2008).



Vasopressin (mammals)
Cys-Tyr-Phe-Gln-**Asn-Cys-Pro**-Arg-**Gly**-NH_2

Lysipressin (pigs, marsupials)
Cys-Tyr-Phe-Gln-**Asn-Cys-Pro**-Lys-**Gly**-NH_2

Phenypressin (marsupials)
Cys-Phe-Phe-Gln-**Asn-Cys-Pro**-Arg-**Gly**-NH_2

Oxytocin
Cys-Tyr-Ile-Gln-**Asn-Cys-Pro**-Leu-**Gly**-NH_2

Vasotocin
Cys-Tyr-Ile-Gln-**Asn-Cys-Pro**-Arg-**Gly**-NH_2

Mesotocin
Cys-Tyr-Ile-Gln-**Asn-Cys-Pro**-Ile-**Gly**-NH_2

Isotocin
Cys-Tyr-Ile-Ser-**Asn-Cys-Pro**-Leu-**Gly**-NH_2

Annepressin (annelid worms)
Cys-Phe-Val-Arg-**Asn-Cys-Pro**-Thr-**Gly**-NH_2

Conopressin (snails, cones, sea hare, leeches)
Cys-Phe/Ile-Ile-Arg-**Asn-Cys-Pro**-Lys/Arg-**Gly**-NH_2

Inotocin (some insects)
Cys-Leu-Ile-Thr-**Asn-Cys-Pro**-Arg-**Gly**-NH_2

Tabela 1. Oxitocina e peptídeos relacionados.

Hormônio	Aminoácidos[a]				Animais onde se faz(em) presente(s)
	2	**3**	**4**	**8**	
Oxitocina	Tyr	Ile	Gln	Leu	Mamíferos placentários, alguns marsupiais
Mesotocina	*	*	*	Ile	Marsupiais, tretrápodos não mamíferos[b]
Isotocina	*	*	Ser	Ile	Osteíctios (peixes ósseos)
Aspargtocina/ Valitocina	*	*	Asp/*	*/Val	Condrictios (peixes cartilaginosos)
Asvatocina/ Phasvatocina	*	*/Phe	Asp	Val	Condrictios (peixes cartilaginosos)
Cephalotonina	*	Phe	Arg	Ile	Moluscos (*Octopus vulgaris*)
Annetocina	Phe	Val	Arg	Thr	Anelídeos
Vasotocina	*	*	*	Arg	Vertebrados não mamíferos, ciclóstomos
Vasopressina	*	Phe	*	Arg	Mamíferos
Lysipressina	*	Phe	*	Lys	Porco, alguns marsupiais
Fenipressina	Phe	Phe	*	Arg	Macropódidos (Marsupiais)
Locupressina	Leu	*	Thr	Arg	Insetos (*Locusta migratoria*)
Conopressina	Ile/Phe	*	Arg	Arg/Lys	Moluscos

[a]Os aminoácidos 1Cys, 5Asn, 6Cys,7Pro e 9Gly (NH2) são conservados.

[b]Inclui peixes pulmonados.

*Aminoácidos iguais aos presentes na oxitocina (Acher et al., 1995).

Receptores, interação com os hormônios e expressão diferencial.

O único receptor conhecido para a oxitocina, o OXTR, é um polipeptídio de 389 aminoácidos, com sete domínios transmembrana, que faz parte dos receptores acoplados à proteína G de classe I denominados GPCRs (G-protein coupled receptors). No genoma humano, o gene *OXTR* está localizado na região 3p25–3p26.2 (Kimura et al., 1992) e apresenta 17 kb, tendo 4 éxons e 3 íntrons. No entanto, somente os éxons 3 e 4, os quais estão separados por um íntron de 12 kb, são traduzidos. O quarto éxon codifica o último domínio transmembrana, como ilustra a Figura 3.

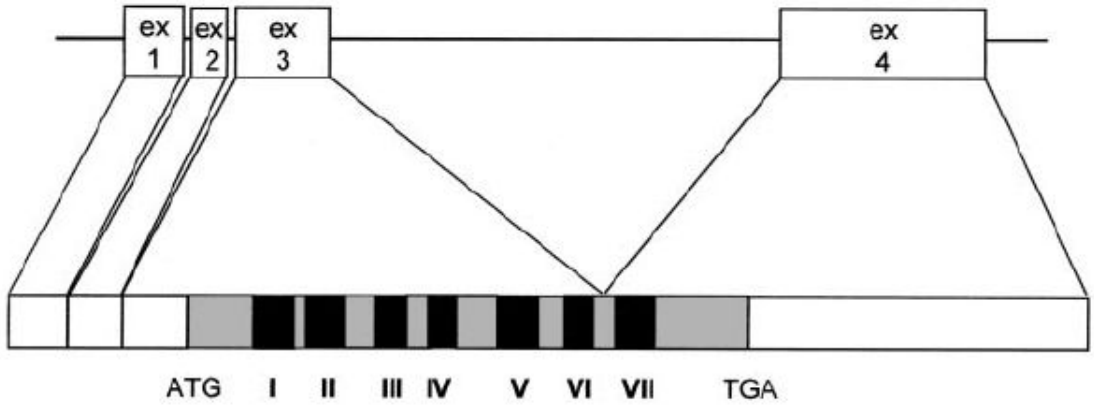


Figura 3. Esquema gráfico do gene OXTR e sua correspondência com a estrutura do receptor da oxitocina. As áreas escuras, numeradas por algarismos romanos, correspondem aos domínios transmembrana de OXTR (extraído de Gimpl & Fahrenholz, 2001).

O RNAm do receptor foi reportado como tendo dois tamanhos: 3,6 kb no seio de mães lactantes e 4,4 kb no ovário, endométrio uterino e miométrio. Isso pode ter influência sobre o incremento do número de receptores no trabalho de parto, pois um RNAm maior (cauda poli A) aumenta a estabilidade da molécula e facilita a tradução (Kimura et al., 1992).

A classe I da família de receptores GPCR, à qual pertence o receptor OXTR, apresenta um mecanismo comum de ativação e tradução de sinal à proteína G. Essa ativação se caracteriza por uma mudança na orientação do domínio transmembrana 3 e 6 (Figura 5), liberando os sítios de união à proteína G (Gimpl & Fahrenholz, 2001).

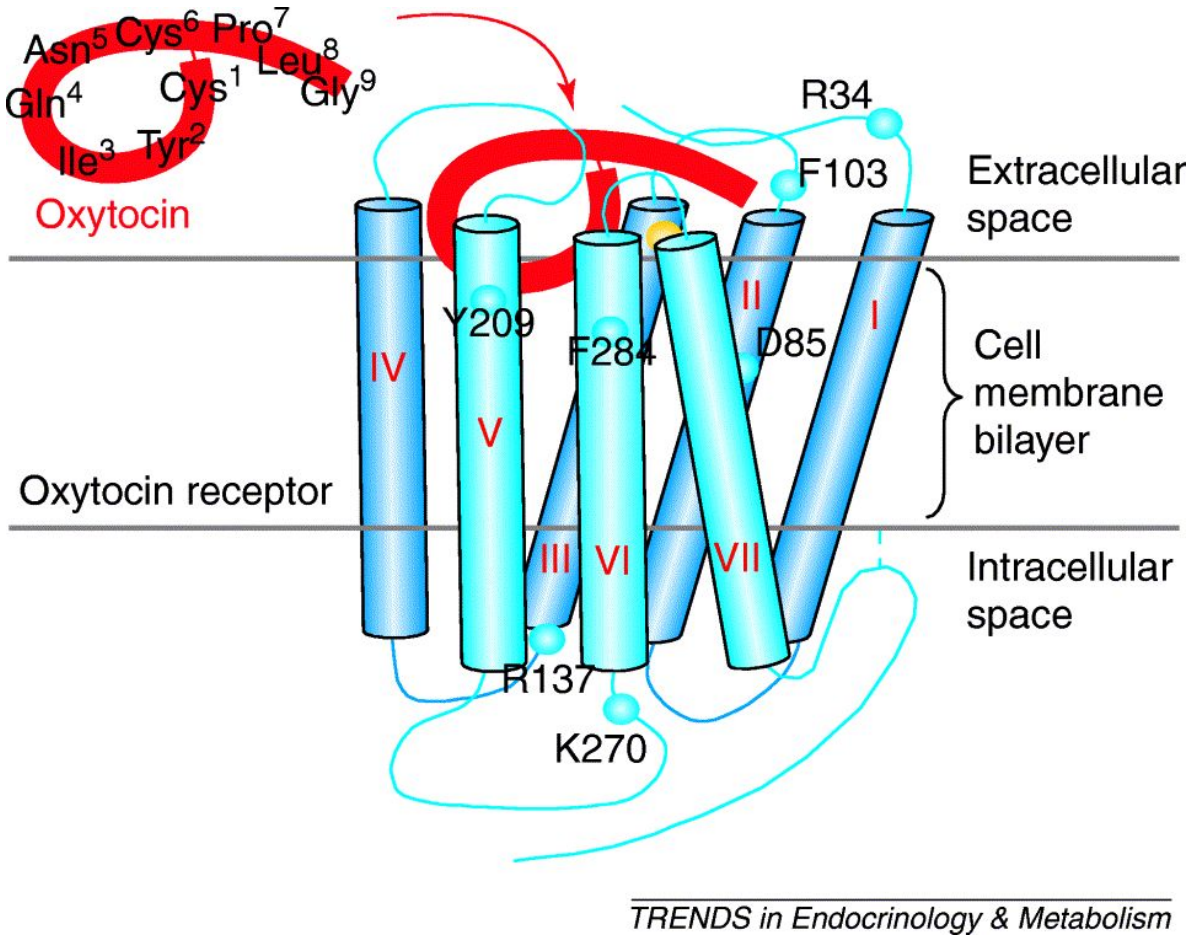


Figura 4. Modelo esquemático da estrutura da OXT e de OXTR. A oxitocina é mostrada acima, à esquerda, em vermelho, com seus resíduos de aminoácidos numerados de 1 a 9. O receptor, em azul, mostra a interação do hormônio quando o complexo está ativado. Os sete domínios intermembrana de OXTR estão indicados por números romanos. O círculo amarelo denota os resíduos L114, V115 e K116, pontos de contato específicos dos antagonistas da OXT. Compilado de (Zingg & Laporte, 2003).

Vários estudos mostraram também que a parte cíclica da OXT interage com o terço superior do receptor, especificamente com os domínios transmembrana 3, 4 e 6 (Figura 5). Por outro lado, a parte carboxiterminal da OXT permanece mais em contato com os domínios 2 e 3 na superfície e com o primeiro *loop* do receptor OXTR (Zingg & Laporte, 2003).

Através de construtos quiméricos e truncados, tem se demonstrado que a região N terminal do receptor é fundamental para a união à OXT. Dentro dessa região, a arginina, na posição 34 da cadeia de aminoácidos do OXTR (Arg-34-OXTR), teria um papel

fundamental, já que é conservada nas posições correspondentes nos receptores de hormônios de diferentes *taxa*, como é o caso da mesotocina, isotocina, vasotocina, bem como nos três tipos de receptores da vasopressina (Wesley et al., 2002).

Chini e colaboradores (1996), por sua parte, demonstraram que a fenilalanina na posição 103 da cadeia de aminoácidos do OXTR (Phe-103-OXTR), localizada na região da primeira alça extracelular, é muito importante para a efetivação da ligação com OXT, especificamente com o oitavo aminoácido, a leucina (Tabela 2). Vale lembrar que é nesse sítio da cadeia de aminoácidos da oxitocina que está localizada uma das diferenças entre esse hormônio e a vasopressina; na posição 8, há uma leucina na oxitocina e uma arginina na vasopressina (Arg-8-AVP; Figuras 1 e 2).

Um ano antes, o mesmo autor (Chini et al., 1995), descreveu a possível interação entre Arg-8-AVP e a tirosina 115 do receptor AVPR1A (Tyr-115- AVPR1A). Curiosamente, Tyr-115- AVPR1A está localizado na primeira alça extracelular do receptor, sendo homóloga à posição Phe-103-OXTR, mostrando uma interação significativa ao substituir esse aminoácido (Tyr) pelo equivalente no OXTR (Phe), evidenciando, deste modo, o papel importante desse sítio na afinidade e na seletividade do receptor (Tabela 2).

Estudos adicionais mostraram que os aminoácidos Tyr 209 e Phe 284 do OXTR apresentam papel importante na interação com o hormônio, interagindo possivelmente com o segundo resíduo aromático (Tyr) e o terceiro hidrofóbico (Ile) da OXT (Figura 3). É interessante notar que a Ile na posição 3 (Ile-3-OXT) é o resíduo que difere entre a oxitocina e a vasopressina (Phe-3-AVP) na parte circular da molécula (Figura 2). Essas diferenças poderiam estar contribuindo com o reconhecimento específico da molécula com seu receptor, (Chini et al., 1996; Tabela 2). Vale lembrar ainda que a AVP pode agir como um agonista completo ou parcial do receptor OXTR humano (Chini et al., 1996).

Deve-se considerar, também, que os aminoácidos Arg-137-OXTR e Asp-85-OXTR, entre outros, foram objeto de estudos através de mutações induzidas em organismos modelos e simuladas computacionalmente (Fanelli et al.,1999). Mutações Arg-137-Ala e Asp-85-Ala levaram a um receptor constitutivamente ativo e inativo, respectivamente. Com isso, podem-se inferir seus papéis na ativação do complexo, no reconhecimento do agonista, bem como na interação com a proteína G (Fanelli et al.,1999).

Tabela 2. Alguns aminoácidos importantes do OXTR que interagem direita ou indiretamente na ativação do complexo.

Aminoácido	Posição (aa)	Provável função	Referência
Arg	34	União ao hormônio	(Wesley et al., 2002)
Asp	85	Ativação, reconhecimento do agonista, interação com a proteína G	(Fanelli, et al., 1999)
Phe	103	União ao hormônio; OXT-8-Leu	(Chini, 1996)
Arg	137	Ativação, reconhecimento do agonista, interação com a proteína G	(Fanelli et al., 1999)
Tyr	209	União ao hormônio; OXT-3-Iso	(Chini, et al., 1996)
Phe	284	União ao hormônio; OXT-3-Iso	(Chini et al., 1996)
Trp	288	Ativação	(Fanelli et al., 1999)
Glu	307	Ativação	(Fanelli et al., 1999)

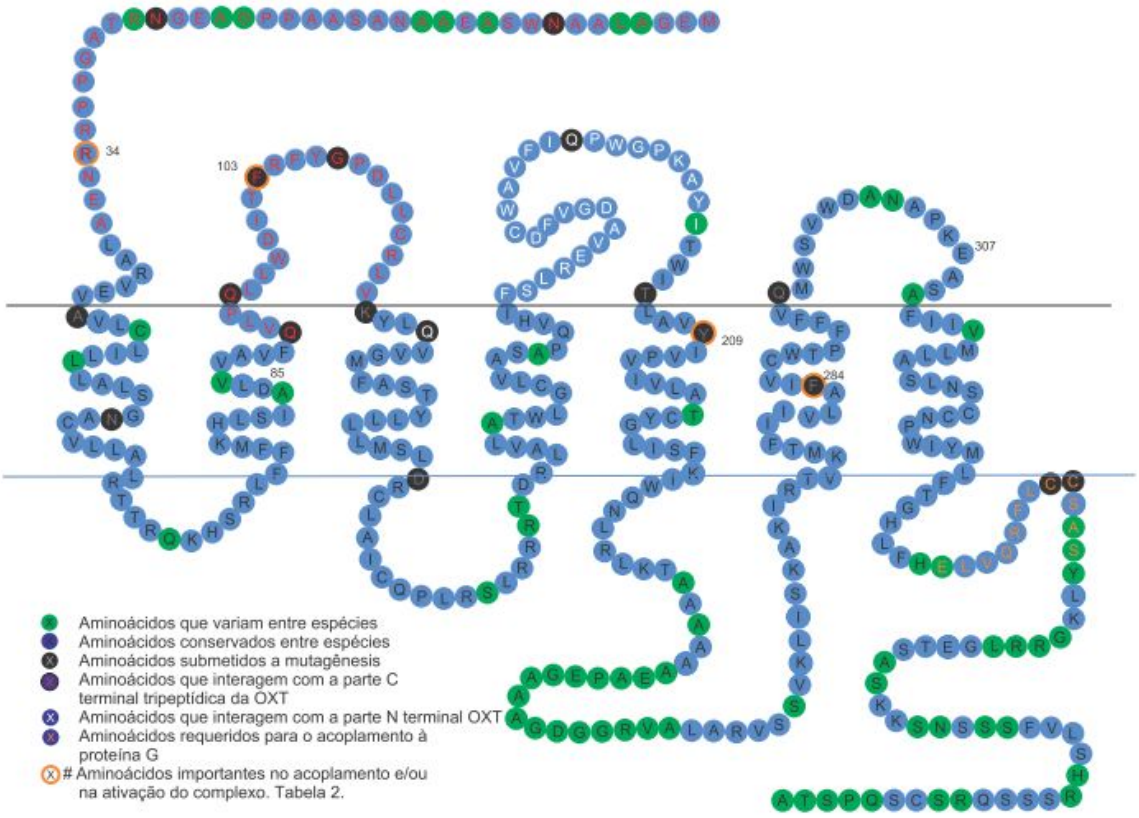


Figura 5. Modelo esquemático do OXTR indicando quais são aminoácidos conservados (azuis) entre os receptores de diferentes espécies (*Homo sapiens, Macaca mulatta, Sus scrofa, Bos taurus, Ovis aries, Ratus ratus* e *Mus musculus*; Gimpl & Fahrenholz, 2001). Os aminoácidos verdes apresentam variações entre as espécies. Em preto, aminoácidos que foram objeto de estudos através de mutações induzidas em organismos modelos e simuladas computacionalmente, ver texto. Aminoácidos com letra laranja são importantes no acoplamento e/ou ativação da proteína G. Aminoácidos delimitados por círculo laranja participam na ligação ao hormônio e/ou na ativação do complexo (Tabela 2). Tomado e modificado de (Gimpl & Fahrenholz, 2001).

Diferentemente de muitos outros membros da família de receptores acoplados à proteína G, OXTR apresenta mudanças marcantes nos níveis de expressão em células específicas. No útero, a expressão do OXTR aumenta duas vezes durante a gestação. Depois do parto, há queda dessa expressão, enquanto que, nas glândulas mamárias, essa é mantida durante o período de lactação. Essa distribuição tecido-específica permite que a interação OXT-OXTR seja mais ou menos ativa em períodos específicos (Zingg & Laporte, 2003).

Camundongos nocaute são ferramentas importantes para estudos que buscam informações sobre a funcionalidade de um determinado gene. Por exemplo, os camundongos sem nenhum RNA mensageiro que codifique a OXT, mas sem alterações na expressão de AVP, possuem um comportamento sexual e fertilidade aparentemente normais. Assim, pode-se interpretar que a AVP pode compensar a ausência de OXT nos camundongos nocaute para este gene (Bales & Perkeybile, 2012). Takayanagi (2005), por sua vez, gerou camundongos nocaute para OXTR, as fêmeas não apresentaram déficits evidentes de fertilidade ou comportamento reprodutivo. No entanto, após um parto normal, foi observado que elas apresentaram problemas na lactação. Filhotes sem o receptor emitiram menor quantidade de vocalizações ultrassônicas em resposta ao isolamento social. Na idade adulta, os machos mostraram déficit na discriminação social e elevados índices de comportamentos violentos.

Experiências como estimulação precoce de recém-nascidos, alterações da estrutura familiar, até agentes mais extremos, como estresse neonatal e manipulações farmacológicas, podem potencialmente produzir mudanças na expressão dos receptores do sistema da oxitocina.

Ruthschilling et al. (2012) avaliaram as respostas em mães camundongo que lambiam sua prole de forma frequente ou infrequente, e perceberam que as mães que lambiam infrequentemente tinham níveis mais elevados do RNAm do OXTR no bulbo olfatório e no hipocampus, quando comparadas com aquelas que lambiam suas crias frequentemente.

Esse e outros trabalhos vêm demonstrando que a expressão dos receptores pode ser induzida por estímulos sociais positivos, tais como calor, contato corporal, presença parental, como também por estímulos sociais negativos, por exemplo, separação da figura de apego ou outros relacionamentos em dificuldades (Bales & Perkeybile, 2012). A Tabela 3 mostra experimentos onde a expressão desses hormônios, bem como de seus receptores, foi avaliada sob diferentes condições experimentais.

Tabela 3. Experimentos farmacológicos e sociais relacionados com OXT e seus registros em longo prazo dos níveis OXTR e AVPR em diferentes zonas do cérebro.

Tipo de experiência	Sexo	Espécie	Expressão OXTR em adulto	Expressão AVPR1A em adulto	Referência
Fornecimento de OXT em recém-nascidos	Macho	*Microtus ochrogaster*	Nenhuma mudança	Elevada em PCC	(Bales et al., 2007)
Fornecimento de antagonista de OXT em recém-nascidos	Machos	*Microtus ochrogaster*	Nenhuma mudança	Inferior em MPOA, BNST e LS	(Bales et al., 2007)
Fornecimento de OXT em recém-nascidos	Fêmeas	*Microtus ochrogaster*	Nenhuma mudança	Inferior em MPOA, BNST, LS, PCC e MdThal	(Bales et al., 2007)
Fornecimento de antagonista de OXT em recém-nascidos	Fêmeas	*Microtus ochrogaster*	Nenhuma mudança	Inferior em BNST e PCC	(Bales et al., 2007)
Exposição pré-natal a estrógenos	Ambos	*Microtus bavaricus*	Diminui em CC	Não avaliado	(Engell et al., 2006)
Crescimento do recém-nascido: cuidado biparental *X* só da mãe	Ambos	*Microtus ochrogaster*	Marginalmente elevado em BNST em biparental	Nenhuma mudança	(Ahern & Young, 2009)
Crescimento do recém-nascido: comunitário *X* só mãe	Ambos	*Mus musculus*	Elevado em LS dorsal e ventral, BNST	Diminui em LS dorsal em cresc. comunitário	(Curley et al., 2009)
Mãe que lambe com frequência	Fêmeas	*Rattus norvergicus*	Elevado em CeA e BNTS	Nenhuma mudança	(Champagne, et al., 2001)
Mãe que lambe com frequência	Machos	*Rattus norvergicus*	Nenhuma mudança	Elevado em CeA	(Champagne & Meaney, 2006)
Separação materna	Macho	*Rattus norvergicus*	Inferior em InsCtx agranular, LS e CP	Inferior em Arc	(Lukas, et al., 2010)
Sem estimulação	Fêmeas	*Microtus ochrogaster*	Elevado Nacc, LS e BNTS	Nenhuma mudança	(Bales, et al., 2011)
Sem estimulação	Machos	*Microtus ochrogaster*	Elevado em BNST	Nenhuma mudança	(Bales et al., 2011)
Mãe que lambe com pouca frequência	Ambos	*Rattus norvergicus*	Elevado em OB e HP	Não avaliado	(Ruthschilling et al., 2012)
Mãe que lambe com frequência	Ambos	*Rattus norvergicus*	Diminui em OB e HP	Não avaliado	(Ruthschilling et al., 2012)
Nocaute para *OT*	Ambos	*Mus musculus*	Nenhuma mudança	Nenhuma mudança	(Nishimori et al., 1996)

Arc: Núcleo arqueado; CeA: Amígdala central; BNST: Estria terminalis; CC: Cortex cingulate; CP: Putâmen; HP: Hipocampus; InsCtx agranular: Córtex insular agranular; LS: Septum lateral; MPOA: Área pré-óptica medial; MdThal: Tálamo médio dorsal; OB: Bulbo Olfatório; PCC: Cingulate posterior. Compilado e adaptado de Bales & Perkeybile (2012).

Primatas

Os primatas são reconhecidos por um conjunto de particularidades, entre elas rosto curto, órbitas oculares frontais associadas à visão estereoscópica, capacidade craniana relativamente grande e polegares oponíveis. Também apresentam características cerebrais como perda na área olfativa e maior espaço do cérebro – especificamente do córtex – correlacionado com o incremento da dependência da visão e mudanças nos comportamentos sociais (Myers, 2000).

O registro fóssil mais antigo dos primatas corresponde ao Eoceno (54-55 milhões de anos). Tavaré (2002) sugere, a partir de dados moleculares, a presença de um ancestral comum para esse grupo, cuja existência poderia ter ocorrido há 81,5 milhões de anos.

Dentro dos primatas, dois grandes grupos são encontrados: Strepsirrhini e Haplorrhini; os primeiros apresentam nariz longo e desprotegido pela ausência de uma estrutura cartilaginosa, os dentes incisivos inferiores em forma de pente e não dispõem de uma placa óssea que separe a órbita da fossa temporal. Esse clado encontra-se dividido em Lemuriformes e Lorisiformes. A maioria das espécies são arbóreas, mas, ao mesmo tempo, alguns possuem uma especiação extrema a hábitos particulares de vida (Goodman et al., 1998).

Os *Haplorrhini*, por sua vez, apresentam uma placa que divide a órbita da fossa temporal. Dois grandes grupos aparecem no clado: *Platyrrhini* (primatas do novo mundo) e *Catarrhini* (primatas do velho mundo), e a diferença externa mais evidente entre estes encontra-se na estrutura do nariz; nos primeiros ele é achatado, com narinas dirigidas à frente, enquanto nos últimos são direcionadas para baixo.

Platyrrhini é considerado um grupo monofilético com 120 espécies reconhecidas, com pelo menos 16 gêneros. As espécies do grupo são classificadas em três famílias: Cebidae, Athelidae e Pitheciidae. Embora Goodman (1998) o classifique numa quarta família, inclui-se o macaco da noite (gênero *Aotus nancymaae*) na família Cebidae, na qual também são encontrados, entre outros, o macaco prego (*Cebus apella*), o macaco do cheiro (*Saimiri sciureus)* e o sagui (*Callithrix jacchus*). A família Atelidae inclui o macaco aranha (*Ateles*), o macaco barrigudo (*Lagothrix*), o bugio (*Allouata*) e o muriqui (*Brachyteles*). A família Pithecidae é composta pelo

sakí (*Pithecia*), o uacari (*Cacajao*), o cuxiú (*Chiropotes*) e o titi (*Callicebus*) (de Oliveira et al., 2012).

Porém, a maioria dos estudos concordam quanto à classificação dos *Platyrrhini* em 11 gêneros, em 3 clados monofiléticos: a) macacos grandes com rabo preênsil, da família Atelidae, com os gêneros *Alouatta, Ateles, Lagotrhix* e *Brachiteheles*; b) consumidores de sementes, da família Pithecidae, com os gêneros *Pithecia*, *Chiropotes* e *Cacajao* e c) pequenos macacos com garras, da subfamília Calithrichinae (Família Cebidae, gêneros *Saguinus, Leontopithecus, Callithrix* e *Cebuella*) (de Oliveira et al., 2012; Goodman et al., 1998).

A maioria das espécies de primatas vivem nos trópicos, embora alguns vivam em regiões de maior latitude e em climas extremos, como é o caso dos humanos. Exceto por alguns terrestres, os primatas são arbóreos. A ampla diversidade de tamanhos, dietas, sistemas sociais, sistemas de acasalamento, número de filhos, entre outros fatores que os caracterizam é resumidamente apresentada em *Table S2* do manuscrito no capitulo 2.

O sistema OXT e OXTR em primatas

Até o ano 2011, pensava-se que a sequência do gene *OXT* era conservado entre todos mamíferos placentários. Porém, naquele ano, Lee e colaboradores reportaram uma modificação na sequência do gene *OXT* em macacos do novo mundo (Lee et al., 2011). A mutação C ⇄ T, que leva a uma substituição de uma leucina por uma prolina na posição 8 da cadeia de aminoácidos, foi detectada em Sai*miri sciureus* (macaco de cheiro), *Cebus apella* (macaco prego), *Aotus nancymaae* (macaco da noite), *Callithrix jacchus* (sagui), e em *Tupaia belangeri* (musaranho).

Já o *Callicebus cupreus,* pertencente à família Pitheciidae, não apresentava a mutação. Esse trabalho corrobora a ideia de que, embora conservado ao longo da evolução, um gene pode mudar em alguns ramos de uma dada filogenia, como atesta um estudo de nosso grupo de pesquisa com o gene *PAX9* (Pereira et al., 2006).

Em outro estudo de nosso grupo de pesquisa, avaliou-se a diversidade de *OXTR* (Vieira, et al., 2012) em nove espécies de macacos do novo mundo, junto com sequências de outros primatas disponíveis em bancos de dados públicos. Nesse trabalho, foram detectadas 30 variações

não-sinônimas em 22 sítios diferentes. O domínio intracelular 4 mostrou ser uma região pouco conservada.

Vale ressaltar que nenhum estudo avaliou até o momento se as mudanças encontradas por Lee e seus colegas na sequência de *OXT,* estariam presentes em outras espécies de macacos do novo mundo, nem tampouco se estas mudanças estariam relacionadas com alguma alteração específica no OXTR encontradas por nosso grupo de pesquisa (Vieira et al., 2012). A conexão do sistema *OXT/OXTR* com outros correlacionados, tais como *AVP/AVPR1/AVPR2/AVPR3*, também nunca foi avaliada.

2. OBJETIVOS

Objetivo geral

Contribuir para o delineamento do cenário evolutivo em nível molecular do sistema da *OXT-OXTR*.

Objetivos específicos

a) Avaliar a variabilidade na região codificadora do gene da oxitocina (*OXT*) nos primatas;
b) Estimar índices que inferem padrões evolutivos para os genes *OXT* e *OXTR* em primatas;
c) Comparar as variantes do gene *OXTR* com aquelas encontradas no gene *OXT* e avaliar se as mesmas estariam co-evoluindo;
d) Fazer inferências sobre a variabilidade encontrada no sistema *OXT-OXTR* e se esta poderia estar relacionada com algum dos traços reprodutivos e comportamentais espécie ou grupo-específicos presentes nos primatas estudados.

3. RESULTADOS

Os resultados do presente trabalho são apresentados sob forma de um manuscrito em preparação. Ali também poderão ser encontrados detalhes sobre as amostras e métodos empregados. Na introdução do manuscrito haverá uma inevitável repetição de temas já apresentados na introdução da presente Dissertação.

4. MANUSCRITO ACEITO PARA PUBLICAÇÃO

Classification: Biological sciences (Anthropology)

Evolutionary pattern in the OXT-OXTR system in primates: Coevolution and positive selection footprints

Pedro Vargas-Pinilla[a,1], Vanessa Rodrigues Paixão-Côrtes [a,1], Pamela Paré[a], Luciana Tovo-Rodrigues[a], Carlos Meton de Alencar Gadelha Vieira[a], Agatha Xavier [a], David Comas[b], Alcides Pissinatti[c], Marialva Sinigaglia[a], Maurício Menegatti Rigo[a], Gustavo Fioravanti Vieira[a], Aldo B. Lucion[d], Francisco M. Salzano [a,2], Maria Cátira Bortolini [a,2]

[a]Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul Caixa Postal 15053, 91501-970 Porto Alegre, RS, Brazil; [b]Institut de Biologia Evolutiva (CSIC-UPF), Departament de Ciències Experimentals i de la Salut Universitat Pompeu Fabra, Spain; [c]Centro de Primatologia do Rio de Janeiro, Rio de Janeiro, RJ, Brazil, [d]Departamento de Fisiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, 90050-170 Porto Alegre, RS, Brazil

[1]These authors contributed equally to this research.

[2]To whom correspondence may be addressed. E-mails: maria.bortolini@ufrgs.br or francisco.salzano@ufrgs.br.

Author contributions: PV-P, VRP-C, CMAGV, and MCB initially conceived the project; VRP-C, FMS and MCB designed research; PV-P, CMAGV, and AX obtained the original genetic data; AP provided information about the animals investigated; PV-P, VRP-C, PP, LT-R, MS, GFV, and MMR analyzed data, supervised by MCB; PV-P, PP, VRP-C, and MCB wrote the majority of the manuscript with critical inputs by FMS, DC, ABL, and the remaining authors.

The authors declare no conflict of interest.

Abstract

Oxytocin is a nonapeptide involved in a wide range of physiological and behavioral functions. Until recently, it was believed that an unmodified oxytocin sequence was present in all placental mammals. This study analyzed oxytocin (*OXT*) in 29 primate species, and the oxytocin receptor (*OXTR*) in 21 of these species. We reported here three novel OXT forms in the New World monkeys (NWm) and a more extensive distribution of a previously described variant (Leu8Pro). In structural terms, these OXTs share the same three low energy conformations in solution during molecular dynamic simulations, with subtle differences in their side chains. A consistent signal of positive selection was detected in the Cebidae family, and OXT position 8 showed a statistically significant ($p = 0.013$) correlation with litter size. Several OXTR changes were identified, some of them promoting gain or loss of putative phosphorylation sites, with possible consequences for receptor internalization and desensitization. OXTR amino acid sites are under positive selection and intramolecular and intermolecular coevolutionary processes with OXT were also detected. We suggest that some NWm OXT-OXTR forms can be correlated to male parental care through the increase of cross-reactivity with its correlated vasopressin system.

Keywords

OXT | OXTR | primates | co-evolution | behavior | parental care

Significance Statement

It was previously believed that placental mammals present no variability in oxytocin (OXT). The present study reports novel data on *OXT* and its receptor (*OXTR*) diversity in primate species, including New World monkeys. Contrary to prior expectations, we found three novel

OXT forms and several OXTR non-synonymous changes not previously described. In the Cebidae family, signals of positive selection were found for an OXT variant at position 8, associated with larger litter sizes. We detected positive selection for OXTR forms, and report a coevolutionary process between changes in OXT and OXTR.

\body

Oxytocin has crucial functions related to physiological processes and social behaviors in primates and other placental mammals. A nonapeptide (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly;1) oxytocin (called here OXT-8Leu) is both a neurotransmitter released by neuronal cells in synapses, and a hormone, activating receptors distant from the site of its synthesis through the circulatory system (2). In mammals, OXT acts as a hormone in uterine contraction during parturition and milk ejection while lactating. It is also a key central nervous system neurotransmitter, regulating/modulating complex social and reproductive behaviors (*i.e.* pair bonding and parental care; 3-7).

Until recently, it was believed that the OXT amino acid chain was the same in all placental mammals. However, Lee et al. (8) reported a T>C change in four New World monkeys (NWm), *Saimiri sciureus, Cebus apella, Callithrix jacchus* and *Aotus nancimaae*, substituting leucine to proline at position 8 (OXT-8Pro). This form was also found in *Tupaia belangeri,* a treeshrew species of Southeast Asia (8). OXT differs from its paralog vasopressin (AVP, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly) at positions 3 and 8. Variation at position 8 also identifies non-placental OXT/AVP-like nonapeptides, such as mesotocin, present in some marsupials (7,9). These findings dispel the notion of a universal OXT amino acid sequence for placental mammals. They also suggest that residue variability at position 8, in some cases associated with variations at positions 2-5, may be connected with recognition, binding, and activation of receptors, potentially leading to species specific functional changes (7,10).

OXT activity depends on adequate interaction with its unique receptor, OXTR, although it can also bind to the vasopressin receptors (AVPR1a, AVPR1b, and AVPR2) with lower affinity (11-13). Similar to other receptors that use G-proteins as transducer signals across the cell membranes, OXTR is composed of seven transmembrane (TM1-TM7), four extracellular (N-terminal tail-ECL3) and four intracellular (ICL1-C-terminal tail) domains. ECL and ICL are important for the interaction with OXT and G-proteins respectively, while TMs are connected with both functions (7,11).

In contrast to what is observed for placental mammal *OXT*, *OXTR* presents hundreds of variants in regulatory and coding regions, including at the intraspecific level. In humans, *OXTR* single nucleotide polymorphisms have been associated with several social behavioral phenotypes (14).

The presence of *OXT-OXTR*-related systems throughout the animal kingdom indicate that their typical roles in placental mammals are likely exaptations of ancient functions, such as

regulation of fluid balance and egg-laying (15,16). Studies have attempted to investigate both the interaction of *OXT-OXTR*-like systems and their coevolution (11,17). However, our knowledge about this nonapeptide-receptor system, including the extent of its variability in the primate order, remains limited.

New World monkeys (NWm) emerged ≅ 30 millions of years ago. They are classified into 16 genera and approximately 75 species and present a wide range of reproductive and social behaviors (18,19), but little is known about their genetic variability and concurrent phenotypic variation (20).

The present study reports results about *OXT* and *OXTR* diversity in 29 primate species, including 20 New World monkey species. These analyses include original *OXT* and *OXTR* sequences for 16 and 12 NWM species, respectively. We discuss details about the coevolution of these systems, as well as possible connections between reported genetic variability, positive selection and some key species-specific biological traits.

Results

Oxytocin (OXT) Variants in Primates

Figure 1 shows that OXT-8Pro is present in all species from the Cebidae family, furthermore A>G (isoleucine > valine) at position 3 (OXT-3Val-8Pro) in *Saguinus*. The Pithecidae family presented 2 novel OXT forms, both with changes at codon 8: C>G (leucine > alanine) in *Cacajao melanocephalus* and *Chiropotes utahickae* (OXT-8Ala), and C>A (leucine > threonine) in one specimen of *Chiropotes albinasus* (OXT-8Thr).

All amino acid changes were found to be homozygous. In species tested with multiple individuals, no intraspecific variation was found, suggesting that these OXT forms are probably species or group specific.

Grantham Scores (GS) were calculated to investigate the putative functional effects of detected changes in OXT. Cebidae OXT8-Pro, Pithecidae OXT8-Ala, as well as *Chiropotes albinasus* OXT-8Thr are moderately conserved (GS = 98, 96 and 92, respectively), while a Val at position 3 in *Saguinus* is a conservative change (GS = 29). No statistically significant differences in relation to biochemical properties were found using PRIME and SIFT tools.

The available three-dimensional structure for OXT (PDB ID: 1NPO;21) was used to perform a structural evaluation of the implications of these changes. OXT-8Leu was *in silico* with

water and ions at specific physiologic concentrations in a virtual cube. Based on this molecular dynamics simulation, a free energy surface analysis revealed three main low energy conformations: Type I, Type II and Type III (Figs. S1A, B and S2; Table S1). Type I has a more open conformation than the others, while Type II and Type III are more compact. Type II also shows hydrogen bonds between the Tyr2/Cys6 and Tyr2/Asn5 residues, which lead to the typical ring structure that resembles oxytocin complexed with neurophysin, its molecular carrier (21, 22). All OXT forms showed the presence of the same three conformational types, with some small differences in the side chains (Fig. S1A).

The same experiment was conducted with AVP. Fig. S1B shows that these three conformational types are also observed there, with small changes in the side chains. This suggests preservation of the three-dimensional conformations that allow nonapeptide transport (through connection with neurophysin) and an adequate coupling with receptors.

It is difficult to imagine that these OXT changes have no evolutionary implications. Thus, we tested whether they might have been under selection through 2 different approaches. The NsSites model failed to detect codons under positive selection. However, when considered site by site, a non-synonymous/synonymous ratio (ω) of ~1 was found for OXT at position 8. This site is also recovered by Branch-site model, indicating relaxation (Fig. S3). This last detected evidence for positive selection in the Cebidae family. Clade Model D had a better performance when compared to the neutral model using a likelihood ratio test (p = 0.0368 ω = 125.99, Table S2). This result indicates that the primate ω values can vary between branches, suggesting a distinctive evolutionary pattern for the Cebidae family.

Additionally, when the species phylogenetic tree (Fig. 1) is visually compared with the *OXT* maximum likelihood tree, different topologies can be clearly observed (Fig S7). When an incongruence of this nature is detected, a simple neutral model of mutation and drift is insufficient to to explain the pattern found.

We assessed significance associations between molecular OXT forms and specific NWm social behavior and ecological traits using Mann-Whitney or Fisher exact tests (23). Proline at position 8 was significantly associated with larger litters (p = 0.013) but not with other ecological/social primate traits (Tables S3, S4).

Oxytocin receptor (OXTR)

Our analyses reveal 73 non-synonymous (ns) OXTR changes, 17 of them not previously described (Table S5), while others are located in known human polymorphic sites. Of the total, 18 (5 novel) are located in the N-terminal tail, one of the most relevant domains for the interaction with OXT. Thirty-eight changes (6 novel) are present in the ICL3 and C-terminal tail, while 10 (6 novel) are found along TM1, TM3-TM5 (Fig. S4). The highest number of ns changes was found in *Cebus xanthosternos, Saguinus niger* and *Chiropotes utahickae* (27 each), while *Saimiri sciureus* and *Chiropotes utahickae* have the higher number of singletons (4 each).
According to the Grantham Scores, 4% of the *OXTR* changes are considered radical changes: Gly22Trp (located at the N-terminal tail, GS = 184), Trp161Gly (TM4, GS = 184) and Cys383Gly (C-terminal tail, GS = 159). Twenty percent of the changes lead to moderately radical changes and are present in the N-terminal tail, TM1, ICL2, and C-terminal domains. The amino acid change at position Trp161Gly *(Cebus xanthosternos)* was also predicted as damaging using SIFT (Table S5).

Although homology modeling and docking experiments are important to understand the OXTR-OXT system interaction, the GPCR signaling regarding intracellular domains are also fundamental to elucidate receptor response. Based on this, we analyzed the level of OXTR protein disorder, secondary structure, and solvent accessibility, as well as the putative phosphorylation sites in the intracellular domains. Our analyses indicated that OXTR has long disordered regions on N, C-terminal tails and portion of ICL3. The analyses showed that the changes do not introduce any important difference in flexibility or accessibility. Taking into account the available information for two GPCRs (protein kinase C and the G-protein coupled receptor kinase), 13 putative phosphorylation sites were identified (Table S6). Arg149Ser is sufficient to create a new phosphorylation site in *Callithrix jacchus* and *C. geoffroyi, Cebuella pygmea, Cebus xanthosternos*, and *Chiropotes albinasus*, probably compensating the loss of the phosphorylation site at position 152. *Papio anubis* and *Macaca mulatta* also gained a phosphorylation site (Phe362Thr). On the other hand, the Ser368Leu form present in three *Saguinus* species disrupts an important phosphorylation site from one of the two known OXTR serine clusters (Ser368-Ser370 and Ser377-Ser379, Fig. S4). These clusters are relevant for β arrestin binding, a protein involved in the desensitization of GPCRs (11,24). These findings suggest that some of the OXTR changes may be functional.

The NsSites maximum likelihood model (Table S7) indicated that the best-fit model (M2a) allows for sites under positive selection. In OXTR 91% (P0) of the sites are under selective constraint (ω0 = 0.036), 8% (P1) with indication of selective relaxation (ω1 = 1), and 1% (P2) of positive selection (ω2 = 5.157). Results in the same direction were obtained with another model

(M8) that admits positive selection (Table S7). The Bayes Empirical Bayes test indicated that two sites, 6Ala (N-terminal) and 255Val (ICL3), present signals of positive selection, with a probability of 98% (Fig. S5).

Additionally, the Selecton software detected three positively selected OXTR sites: 6Ala, 255Val, and 251Gly (ICL3), two of which NsSites also detected. All are located in important regions that interact with the nonapeptides or G-proteins (Fig. S4).

These results reveal that the degree of conservation and evolutionary rates were unequally distributed across the OXTR domains. The N-terminal and ICL3 regions were most variable, while TMs were most conserved. This may be due to the fact that TMs have the double function of interaction with nonapeptides and G-proteins (7,11).

OXT-OXTR **Coevolution**

Intramolecular coevolution analyses (Table S8) showed that *Callithrix geoffroyi, Callithrix jacchus, Cebuella pygmaea, Cebus xanthosternos*, and *Chiropotes albinasus* are coevolving at OXTR positions 23 (N-terminal) and 149 (ICL2). When a serine is found at position 23, a serine is also found at position 149 (posterior probability, 81%), creating a new putative phosphorylation site recognized by protein kinase C (Table S6).

Other important results emerged regarding OXTR binding sites (Arg-34, Phe-103, Tyr-209 and Phe-284) (25-28). These sites are conserved in the species sampled, except *Saimiri sciureus* which showed the Phe103Tyr change (7). OXTR-103 is a binding site for OXT at position 8 (Fig. S4), and a homolog of the binding site for AVPR1a at position 115. Remarkably, when the AVPR1a-115 site is artificially mutated in rat cells (Tyr115Phe), the affinity of AVPR1a for OXT increases (29). Keeping in mind that Tyr is the most common amino acid present in AVPR1a, the presence of a Tyr at OXTR position 103 could increase its affinity for AVP at least in *Saimiri sciureus.* These findings suggest that coevolutionary processes might involve not simply OXT and OXTR, or AVP and its three receptors, but rather a cross-reaction between these systems.

The Spidermonkey software showed two coevolutionary processes in intermolecular analyses: OXT at position 3 coevolves with OXTR (position 368, C-terminal, posterior probability of 56%) while OXT at position 8 coevolves with OXTR at position 345 (C-terminal, posterior probability of 80%; Fig S6; Table S8). More specifically, the three species of *Saguinus* have a Val at position 3 concomitantly with a Leu at OXTR 368. As mentioned, the latter change removes an

important phosphorylation site from one known OXTR serine cluster, which could modify the desensitization of G protein-coupled receptors (11, 24).

Additionally, a correlation analysis revealed that species that have the highest OXT genetic distances tend to show a similar pattern with OXTR (τ = 0.52; $p < 0.001$; Table S9), independently of their position on the phylogenetic tree. This result suggests similar evolutionary tendencies, which can be due to natural selection.

Discussion

The nonapeptide oxytocin and its paralog vassopresin perform crucial functions in physiological processes and social and reproductive behaviors in placental mammals. During the roughly ~70 million years of evolution that separate the human and mouse lineages, no amino acid replacement occurred in OXT. In contrast, NWm present five forms of OXT, with changes found exclusively at positions 3 and 8. These positions are the most important for highly selective binding to different receptor subtypes (29). Interestingly, AVP is conserved in the NWm branch (Fig. S6), at least based on 15 species studied recently(30).

Prominent examples of positive selection in the NWm lineage are rare (20). Here we obtained consistent evidence for positive selection in *OXT* for the Cebidae branch (OXT-8Pro and OXT-3Val-8Pro). For OXTR, we identified sites that seem to be relevant for the function of the OXT-OXTR system, beyond those previously identified (25-28). For three of them (sites 6, 251, and 255) signals of positive selection were detected. Intermolecular coevolutionary analysis also revealed that *Saguinus* present a Val at position 3 of OXT that seems to have coevolved with a Leu at 368 of OXTR. It is noteworthy that Smith and Ginsburg (31) demonstrated through *in vivo* and *in vitro* experiments that a Val at position 3 of OXT increases uterus contraction, indicating its functionality at least for this trait.

Since natural selection acts on phenotypes, we verified whether the amino acid changes described above would have phenotypic implications. Although the GS predictions for the new NWm-OXTs showed moderate/conservative values and no significant alteration in some physico-chemical properties, all changes deserve to be investigated. For instance, comparing isotocin (present in fishes; 2), with mesotocin (found in birds, lungfish, reptiles, amphibians, and some marsupials; 2), identified two amino acid changes, Ser>Gln (GS = 68) and Leu>Ile (GS = 5), at positions 4 and 8 respectively. Additionally, the difference between mesotocin and OXT

consists of only one alteration at position 8: Ile>Leu (GS = 5). These changes have lower GS values than those we report here in the NWm group.

Furthermore, our analysis reveals that the five OXT forms present three preferential conformations in solution, suggesting the preservation of basal characteristics of these molecules and consequently of their primordial functions in placental mammals.

As for OXT-OXTR affinity, an instigating example of coevolutionary analysis can be cited: *Saimiri sciureus* presents both OXT-8Pro and OXTR-103Tyr forms. The presence of the Tyr residue at position 103 could increase affinity with AVP at position 8, similarly to what occurs with OXT and AVPR1a when changes are introduced in homolog sites (29). Considering receptor activation and signaling, a leucine at OXTR-368 in *Saguinus* (coevolving with a Val at OXT position 3) destroys an important phosphorylation site. This could lead to instability of the OXTR-β-arrestin interaction, and consequent changes in OXTR internalization and desensitization. Also, a serine at OXTR position 149 (coevolving with a Ser at position 23 in several species) creates a new putative phosphorylation site recognized by protein kinase C.

The processes described above can be connected to ecological and social behavior traits, particularly in the Callitrichinae subfamily, where we found an association between larger litter size and a proline at position 8.

Both OXT and AVP are related to pair bonding and parental care, but apparently the OXT system plays a more important role in females, while the AVP system primarily influences males. This suggests an interesting sexually dimorphic pattern (32), which has been described in *Callithrix jacchus* (33). Another study showed that AVPR1a increases in dentritic spines of the prefontral cortex during *Callithrix jacchus* fatherhood (34). Furthermore, experimentation with prairie voles showed the importance of AVP and AVPR1a in complex male behaviors, including monogamy and paternal care (2,35). Thus, our finding of the OXT-8Pro and OXT-3Val-8Pro in the Cebidae could increase OXT affinities to AVP receptors (especially AVPR1a). Meanwhile, changes in OXTR (*i.e.* OXTR-103Tyr) could increase its affinity to AVP.

In Callitrichinae, the subfamily of the Cebidae to which *Saguinus* and *Callithrix* belong, it is well documented that males (fathers, siblings and unrelated) provide important support for infant survival (36). Studies also demonstrate that male exposure to newborns increases responsiveness for care in inexperienced alloparental Callitrichinae (37). There are well-known reasons that could justify this pattern of multiple helpers: first, high energetic costs of reproduction (birth weight of twin infants is ~30 % of Callitrichinae females' body weight), and second, females have a post-partum estrus, and may become pregnant while still nursing. The result is that infant survival rates of experienced mothers with inexperienced fathers and no

siblings are lower than those observed when both parents are experienced and siblings are present (36). Also, even in some Cebidae species where twin births are rare, paternal care is present, which could be connected to a strategy that promotes the evolution of genetic monogamy, such as observed in *Aotus azarae* (38) and *Callimico goeldii* (20).

Paternal care seems to have evolved independently at least four times in the radiation of the primate order, one of them in the Cebidae branch (39). Thus, OXT-8Pro and OXT-3Val-8Pro forms could influence behaviors through increase of affinity at least with AVPR1a. These behaviors include parental male care in Cebidae species with twin pregnancies or in other reproductive and social circumstances where male care (both parental and alloparental) is fundamental for adaptive success.

Several studies reveal intense male and female parental, as well as alloparental care in the Callitrichinae subfamily (20). Thus, Cebidae OXT-8Pro and OXT-3Val-8Pro forms can be also connected with female behaviors. Recently, Cavanaugh et al. (40) showed that treatment with OXT-8Pro in *Callithrix jacchus* facilitated fidelity in females but not in males, but they unfortunately did not test other behaviours, including parental care.

Curiously, the shrew *Tupaia belangeri* presents both OXT-8Pro and OXTR-103Tyr, as observed in *Saimiri sciureus*. The simultaneous presence of these two forms in different taxa suggests parallel evolution, providing a potential example of positive selection and adaptive evolution (41).*Tupaia belangeri* males do not take care of their offspring. A similar behavior is observed in *Saimiri*, one of only two genera of the Cebidae family without paternal care (39), indicating a possible reversion from the ancestral state (Fig. 1). Another parallelism involves the OXTR Ala218Thr (652C>T) change found in *Saimiri sciureus*. It is also reported as a human polymorphism (rs4686302), with implications in ligand-receptor affinity and preterm births (42). The implication of this change to *Saimiri sciureus* is unknown.

Finally, although some species studied here share similar reproductive and parental care behaviors, they have also intra and inter-species differences (43,44). Thus, the general genotype-phenotype connections suggested in the present study should be considered with caution, and can only be confirmed with additional population and functional studies, as well as investigations with related genes.

Conclusion

We reported novel OXT forms in New World monkeys, and a previously unrecognized lack of sequence conservation in placental mammals. Signals of positive selection and of coevolution at inter and intramolecular levels were detected. Some changes in the OXT-OXTR

system seems to be connected to specific ecological/social behavior traits, such as intense parental care.

Materials and Methods

DNA samples, sequencing and sequence alignment

Blood samples from 41 individuals belonging to 16 NWm species (Table S10) were provided by Rio de Janeiro's Primatology Center (http://mapadecultura.rj.gov.br/guapimirim/centro-de-primatologia-do-rio-de-janeiro/).

Genomic DNA was extracted and PCR products were purified and sequenced (27 and 1167 nucleotides coding for the *OXT* nonapeptide and *OXTR* systems, respectively) using Applied Biosystem Genetic Analyzer sequencers (GenBank accessions: KM186262 to KM186289).

DNA sequences from genomic databases were also included in the analyses (Table S10): a total of 29 primate species for OXT, and 21 for OXT and OXTR.

Multiple alignments were performed in the Mega software (5.1 version) using amino acid sequences with the MUSCLE algorithm (45). The alignments were also visually checked.

Evolutionary analyses

We used the phylogeny-based maximum likelihood analysis as implemented in the CODEML program of the PAML 4.7 package to test for positive selection and/or relaxation of functional constraints (46). Two approaches using the non-synonymous/synonymous ratio (dN/dS = ω where ω < 1 indicates negative or constraint selection, ω ≅1 indicates neutral or relaxing selection, and ω > 1 indicates positive selection) were applied: [1] the NsSites codon substitution model, which allows ω values to vary among sites; and [2] The Branch-site Models, which enable ω variation in different branches of the phylogeny. Unrooted trees, necessary for the construction of the input files, were created based on the Primate phylogenetic tree provided by Perelman et al. (19). Coevolution was tested at the intramolecular (within single molecules or genes) or intermolecular (between different molecules or genes) levels (47). First we considered recognized specific binding sites, OXT amino acid chain positions 3 and 8, and OXTR amino acid chain positions 34, 103, 209 and 284 (25-28). Second, we examined coevolutionary processes through the Bayesian Spidermonkey tool (48), available at the Datamonkey server

(http://www.datamonkey.org/). This test furnishes the posterior probability of a change in a site as dependent of change(s) in other(s) site(s). Intramolecular (inside same gene) and intermolecular (between different genes) analyses were performed. Third, we calculated pairwise Nei-Gojobori (49) *OXT*/*OXTR* distances for the 21 primate species, with MEGA 5.2. Correlation between genetic distance matrices was calculated using the Mantel test (GenAlEx 6.5; 50).

Molecular characteristics and predicted functionality

Changes found in NWm species were compared with the amino acid residue present in *Otolemur garnettii* and classified according to Grantham scores (51) as conservative (0-50), moderately conservative (51-100), moderately radical (101-150) or radical (>151) (52). Changes in physico-chemical properties were also evaluated using PRIME (53) and SIFT (54).

The structures of OXT and AVP variants were modeled starting from the OXT-8Leu crystal 3D structure (PDB ID: 1NPO http://www.rcsb.org/pdb). A molecular dynamics simulation was applied to the six structures (five OXT and one AVP) along 400 nanoseconds to evaluate the impact of amino acid changes in solution (two independent simulations for each system, using the GROMACS package v. 4.5.1; 55). To retrieve low energy conformers, which can reflect stable conformations in solution, a Free Energy Surface was calculated for the OXT and AVP molecules. Additionally, a clustering approach was applied to determine the most frequent conformational states over the simulation of each peptide hormone.

Although 3D crystal structures of GPCRs are known, they present < 29% similarity with OXTR. This level of similarity can be used to model G protein coupled A class receptors (13, 27). However, we opted for a more conservative approach to evaluate the OXTR changes. We took into account functional information for OXTR intracellular domains (loops and C-terminal portion) to search for phosphorylation post-translational modifications, since they are very important for GPCR regulation, as well as dimerization (24, 56, 57). Three available software packages were used to obtain a consensus result: [a] PPSP 1.06 Prediction of PK-specific Phosphorylation site (58); [b] NetPhosK 1.0 (59); and [c] GPS 2.1 Group-based Prediction System (58). For predictions, only consensus sites for kinases experimentally known to interact with OXTR, namely Protein Kinase C (PKC;60) and G protein-coupled receptor kinase (GRK;61), were considered. Additionally we analyzed the pattern of protein disorder, secondary structure, and solvent accessibility in the OXTR intracellular domains to assess the impact of changes on loop flexibility and to offer further support for phosphorylation analysis using PONDR-FIT (intrinsic

protein disorder; 62), PSIPRED and NetSurfP v.1.1 (secondary structure; 63, 64), and NetSurfP v.1.1 (solvent accessibility; 64).

OXT/OXTR forms and ecological/social behavioral traits analysis

We assessed significance associations between molecular OXT and OXTR changes and seven NWm social behavior and ecological traits (Table S3) using Mann-Whitney or Fisher exact tests (23). Differences were considered significant with a p value < 0.05 after Bonferroni correction.

A complete description of all procedures listed above is found in the Supplementary Information (SI).

Ethical approval

This project was registered in the official Brazilian system, which permits the collection of biological material for research in conservation units (SISBIO number 27951-2).

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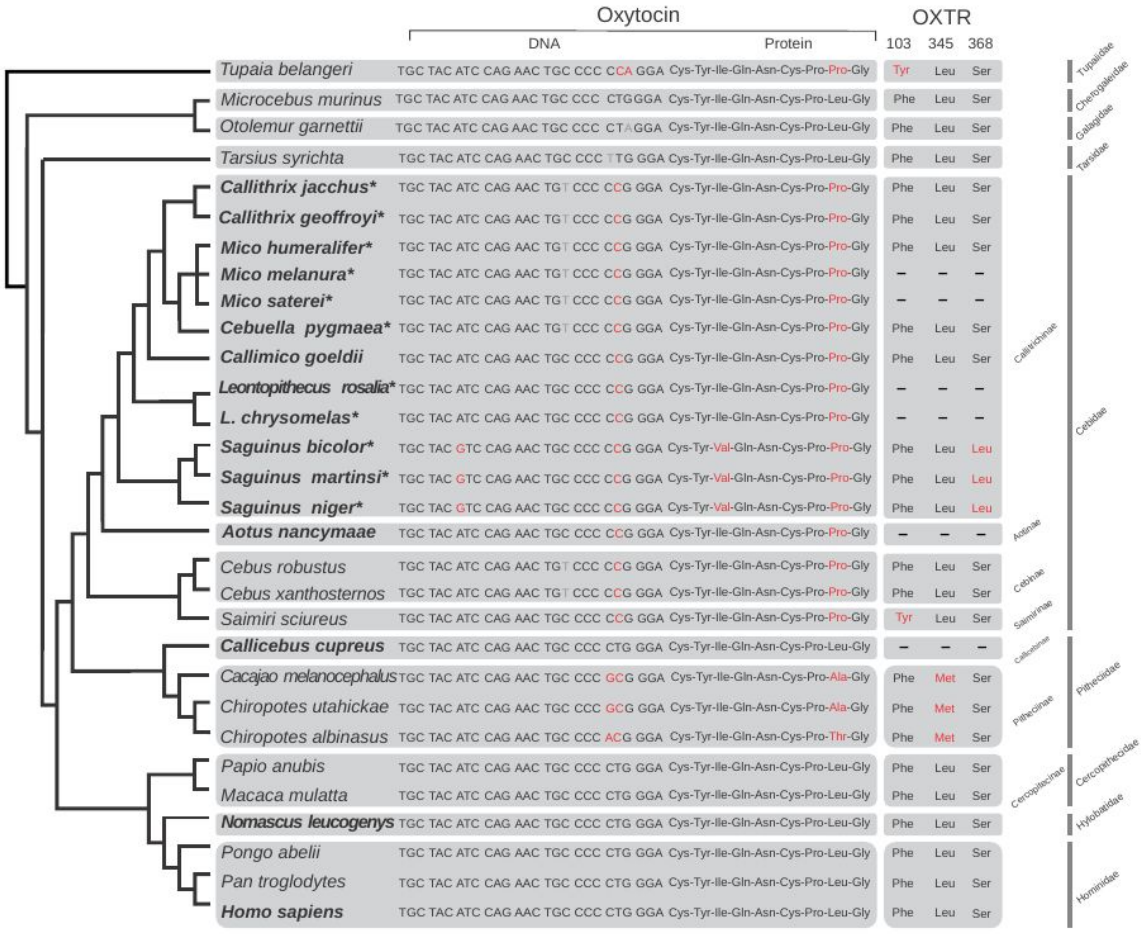

Figure 1. Oxytocin nucleotide and amino acid sequences observed in primates, with the OXTR positions coevolving with OXT. The maximum likelihood phylogenetic tree was based on an ≅ 8 Mb genomic sequence, reported by Perelman et al. (19). Non-synonymous nucleotide and amino acid changes are shown in red. Taxonomic families and subfamilies are indicated, as well as species with common male care (in bold) and with twin pregnancies (asterisks). The trace (-) indicates that no information is available.

Supplementary Information

Material and Methods

DNA samples, sequencing and data mining

Blood samples from 41 individuals belonging to 16 NWm species *(Cebus xanthosternos, Cebus robustus, Cacajao melanocephalus, Chiropotes albinasus, Chiropotes utahickae, Saguinus niger, Saguinus martinsi, Saguinus bicolor, Mico humeralifer, Mico melanura, Mico saterei, Leontopithecus chrysomelas, Leontopithecus rosalia, Calimico goeldii, Cebuella pygmaea, Callithrix geofroyi)* were provided by Rio de Janeiro's Primatology Center (CPRJ). This Center is geographically located between 22°27'S-22°32'S and 42°50'W-42°56'W, in an area of 239.54 hectares with 95% of forest cover, where the animals are kept in captivity, without public access. (http://mapadecultura.rj.gov.br/guapimirim/centro-de-primatologia-do-rio-de-janeiro/). This project was registered in the official Brazilian system, which permits the collection of biological material from conservation units for research (SISBIO number 27951-2).

For some species, the DNA of more than one individual was sequenced (total number, 41, see Table S10). Both *OXT* and *OXTR* DNA strands were sequenced. When changes were found, the samples were resequenced and the reading was made by a different researcher.

Due to technical difficulties in obtaining data, eventually some individuals investigated for *OXT* and *OXTR* were not the same (Table S10). Genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the protocol recommended by the manufacturer.

Primers and conditions described by Lee et al. (1) were used. *OXTR* exons were amplified using three primer sets constructed with the FastPCR software (2).

The products were purified and sequenced using Applied Biosystem 3130 or 3730 Genetic Analyzer sequencers. Sequences were aligned and their quality and accuracy

evaluated using the Codon Code Aligner software (4.0 version; http://www.codoncode.com/).

GenBank accession numbers for the sequences reported here are from KM186262 to KM186289.

DNA sequences of additional species obtained from genomic databases were included in the analyses for additional species (Table S10).

Evolutionary analyses

We used the phylogeny-based maximum likelihood analysis as implemented in the CODEML program of the PAML 4.7 package to test for positive selection and/or relaxation of functional constraints (3). Two approaches which use the non-synonymous/synonymous rate ratio (dN/dS = ω), where ω < 1 indicates negative selection, ω ≅1 indicates neutral or relaxing selection, and ω > 1 indicates positive selection were applied: [a] the NsSites codon substitution model, which allows ω values to vary among sites, and [b] The Branch-site Models, which enables ω variation in different branches of the phylogeny.

For the NsSites codon substitution model, likelihood ratio tests were performed between neutral models (named M0, Neutral, M1a, Nearly Neutral, and M7, Beta) and models that identify positive selection and/or relaxation of functional constraints, named M2a (Selection, which admits three ω classes, one of which may be a value > 1); M3 (Discrete, which admits three ω classes, but never values > 1); and M8 (Beta + Selection, which admits eleven ω classes, of which one or more may be values > 1). Note that the M2a Selection model is more conservative, being less likely to lead to false positive results than M8 Beta + Selection, since it admits a lower number of ω classes (4).

The Branch-site Models approach detects positive selection acting on specific phylogenetic lineages. Maximum likelihood tests were performed between the neutral one-ratio (M1a), which assumes only one ω value for all branches, and a free-ratio model (Clade Model D), which assumes different ω parameters for each branch of the tree. We also employed the Bayes Empirical Bayes test (BEB from NsSites) or Naïve Empirical Bayes (NEB from Clade Model D) approaches, implemented in CODEML, to verify which

sites could be evolving under positive selection (3-5). Additionally we used the Selecton server to visualize the sites under positive selection (6,7).

Unrooted trees, necessary for the construction of the input files, were prepared based on the Primate phylogenetic tree provided by Perelman et al. (8), edited with PhyloWidget (9).

Coevolution, reciprocal evolutionary changes between interacting species (10), was tested using an analog definition at the molecular level (11). This interaction can be intramolecular (within single molecules or genes) or intermolecular (between different molecules or genes) (12). Multiple sequence alignments (MSA) were used to detect correlated changes (13) in the OXT/OXTR system, considering functional and structural information. Three approaches were used. First we considered recognized specific binding sites (OXT amino acid chain positions 3 and 8; OXTR amino acid chain positions 34, 103, 209 and 284) (14-17). Second, we examined coevolutionary processes through the Bayesian Spidermonkey tool (18), available at the Datamonkey server (http://www.datamonkey.org/). Intramolecular and intermolecular OXT/OXTR analyses were performed. For the nonapeptide-receptor analysis, the OXT and OXTR sequences were concatenated. Third, we examined the pairwise comparison of *OXT*/*OXTR* divergence rates in 21 primate species to identify molecular evolutionary patterns, performed with the MEGA 5.2 Nei-Gojobori model (19).

Molecular characteristics and predicted functionality

The crystal 3D structure of the wild type oxytocin form (OXTLeu8, PDB ID: 1NPO) available in the RCSB Protein Data Bank (http://www.rcsb.org/pdb) was used to evaluate the putative impact of changes through molecular dynamics simulation. We changed the residues from those found in OXTwt using the PyMOL program (20) to obtain the oxytocin structural variants (OXT-8Pro, OXT-3Val-8Pro, OXT-8Ala, and OXT-8Thr) and AVP.

All OXT structures were independently subjected to an *in silico* molecular dynamic study for 400 nanoseconds, through the GROMACS package (v. 4.5.1 GROMOS96 53a6 force field; 21). Two simulations were performed for each system. The molecular

dynamics system was created based on experimental parameters from the literature. Briefly, each oxytocin form was solvated with water (Single Point Charge model - SPC) and ions (Na^+ and Cl^-) at a physiologic concentration of 0.15M in a cubic box (≈55.60 nm^3) with at least a 9 Å solvation layer around the protein. Short-range and long-range electrostatic interactions were calculated using the cut-off and particle-mesh Ewald method (distance <1.2 nm), respectively. The system was first energy-minimized with steepest descent and conjugated gradient algorithms using an integration step size of 2 fs. Then, the position of all heavy atoms of the protein was restricted with a force constant of 5000 kJ mol-1nm-1 during 500 ps at the temperature of 300K, to allow the molecule solvation. In the next 150 ps, the system temperature was decreased to 20K and the atom restraints were gradually removed. After that, the system was gradually heated to 300K for 1850 ps. The system was simulated with a md integrator until it reached 400 ns of simulation, keeping it coupled to an external thermal bath (v-rescale algorithm) with a τT of 0.1 ps and a pressure coupling (Parrinello-Rahman algorithm) with a τP of 2 ps. Vasopressin was also simulated using the same parameters. At the end of the simulation, all analyses were taken from the complete molecule trajectory of each peptide. Plots of the simulation were generated with the respective software from the GROMACS v4.5.1 package and visualized with xmgrace, the full-featured GUI-based Grace version (http://plasma-gate.weizmann.ac.il/Grace/).

To calculate the Free Energy Surface (FES), the values of Root Mean Square Deviation and the Radius of Gyration of each molecule along 400ns of simulation were used to retrieve low energy states. This analysis was performed using the fes.py script, developed by Birgit Strodell from the Multiscale Modelling Group (http://www.strodel.info/) and optimized by Cristóvão Freitas Iglesias Junior (http://lmdm.biof.ufrj.br/).

The clustering study, applied to obtain the most frequent conformational states of the different molecules along the simulation, was performed using the g_cluster analysis tool, from the GROMACS v4.5.1 package. The "gromos" method was employed (22), and a 0.25 nm cutoff was set.

Although 3D crystal structures of GPCRs are known, they present < 29% similarity with OXTR. This level of similarity can be used to model G protein coupled A class receptors (23, 17). However, we opted for a more conservative approach to evaluate the

OXTR changes. We took into account functional information for OXTR intracellular domains (loops and C-terminal portion) to search for phosphorylation post-translational modifications, since they are very important for GPCR regulation, as well as dimerization (24-26). To complement this information and to evaluate the impact of changes in loop flexibility, we also analyzed the level of OXTR protein disorder, secondary structure, and solvent accessibility since it is well known that many GPCRs have regions of protein disorder mainly in the N-terminal region, third cytoplasmic loop and C-terminal region (27, 28). Regions of long disorder content are rich in sites for post-translational modification, protein-protein interaction and degradation sites, and, for GPCR, these sites are relevant for fine-tune signaling as well as for heteromerization (26, 29). Here we evaluated, physicochemical properties of the OXTR intracellular region, pooling analyses of protein disorder through PONDR-FIT, secondary structure (PSIPred and NetSurfP) and solvent accessibility (NetSurfP). Prediction of the OXTR specific-kinase phosphorylation sites was performed using the consensus results from three available software packages: [a] PPSP 1.06 (Prediction of PK-specific Phosphorylation site (30) uses an Bayesian decision theory approach; [b] NetPhosK 1.0 (31), is based on neural network predictions of kinase specific eukaryotic protein phosphorylation sites; and [c] GPS 2.1 Group-based Prediction System (30) which is based on a group-based phosphorylation scoring algorithm. For predictions, only consensus sites for kinases experimentally known to interact with OXTR, namely Protein Kinase C (PKC; 32) and G protein-coupled receptor kinase (GRK; 33), were considered.

OXT/OXTR forms and ecological/social behavioral trait analyses

Several physical (body size in kg), social behavioral and ecological characteristics (activity cycle, habitat, locomotion, diet, social structure, mating system, gestation period, number of offspring, group size, reproductive maturity for females and males, and average life span) were compiled for the New World monkey species considered in the present study (Table S3). Seven of them [social structure, mating system, gestation period, number of offspring, group size, and reproductive maturity (female and male)] could directly or indirectly be connected with OXT and/or OXTR variants, based on the

well-known role of the OXT-OXTR system in social and reproductive behaviors (*i.e.* pair bonding and parental care). Significance associations were determined by Mann-Whitney or Fisher exact test IBM; 34). Differences were considered significant with a p value < 0.05 after Bonferroni correction.

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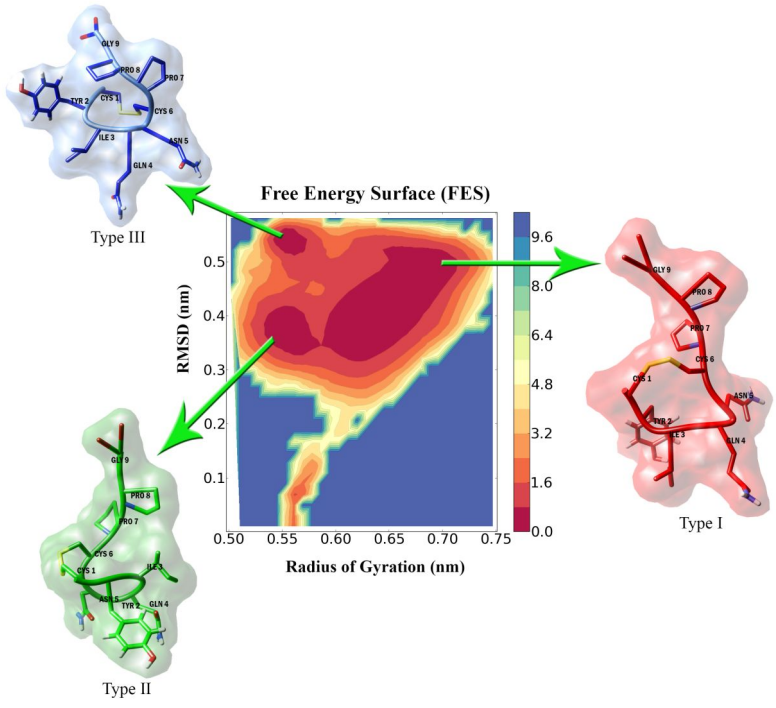
A



B

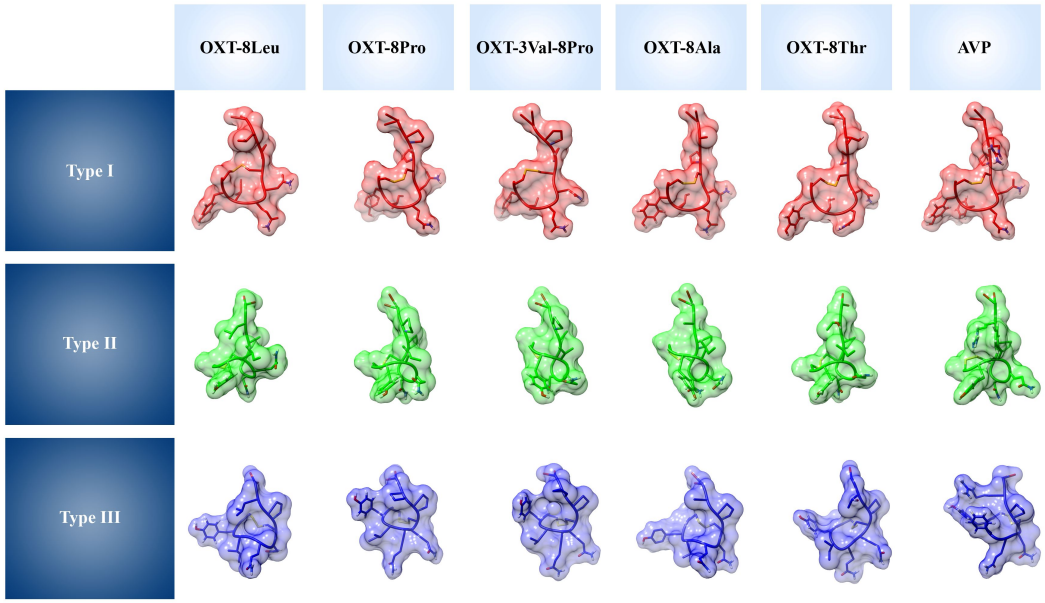


Figure S1. A) Three low energy islands oxytocin Free Energy Surface (FES) graphic, indicating its interchanging conformations observed in the simulations, for all OXT forms and AVP. The corresponding Radius of Gyration (on the x-axis) and Root Mean Square Deviation (RMSD) values (on the y-axis) for these preferential conformers can be also observed. The free energy is color-represented with the lowest to highest values represented by warm to cool colors, respectively. The Type I (red), Type II (green) and Type III (blue) conformers are representative from each low energy island. B) Average structures of representative clusters from the three main low energy conformations (Type I, Type II and Type III) for each OXT and AVP form found in primates. The specific protein backbone pattern is shown for each conformation. Subtle differences among the molecules occur due to side chain variations, as a result of amino acid changes.

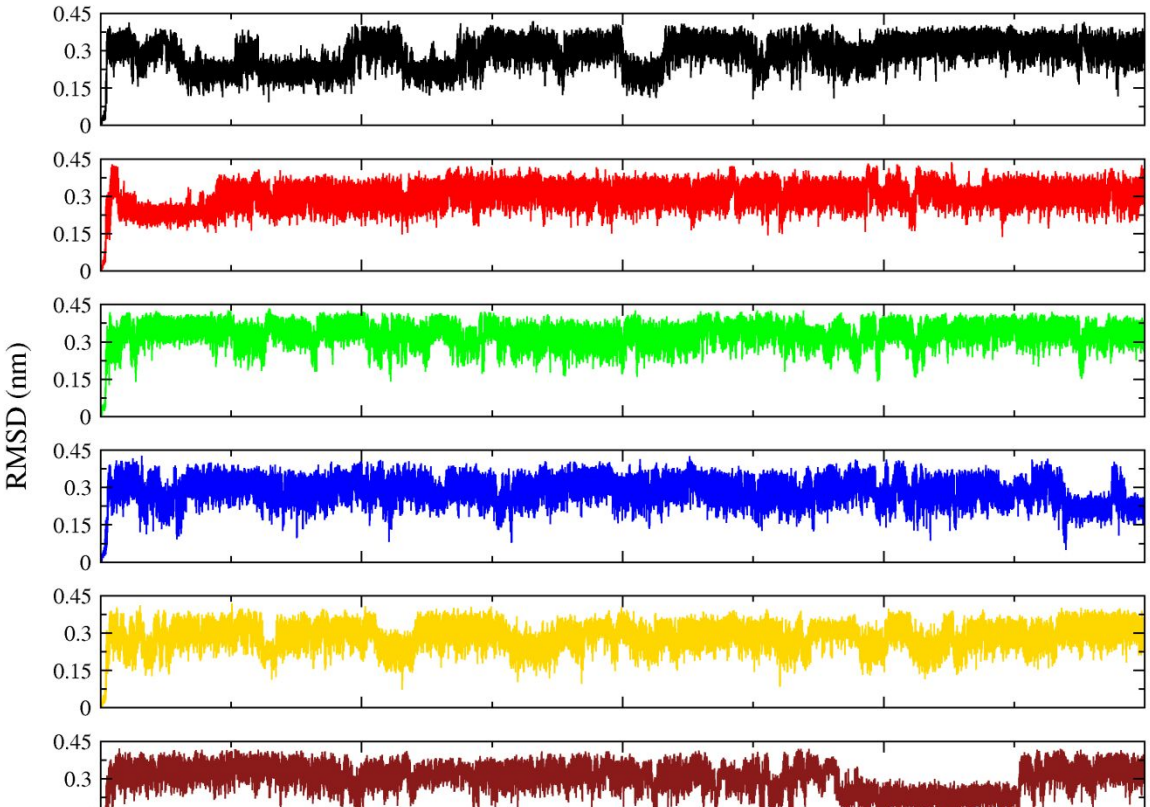

RMSD (nm)
0.45
0.3
0.15
0

Figure S2. Root mean square deviation (RMSD) of OXTs and AVP backbone atoms over a 400ns simulation. All simulations converged at similar deviation values (around 0.3 nm). Some variation can be seen in the beginning of the OXT-8Leu simulation and in the last interval of the AVP simulation, for example. This indicates the presence of alternative conformers, subsequently confirmed by FES and clustering analysis. Black - OXT-8Leu; Red - OXT-8Pro; Green - OXT-3Val-8Pro; Blue - OXT-8Ala; Yellow - OXT-8Thr; Dark red - AVP.

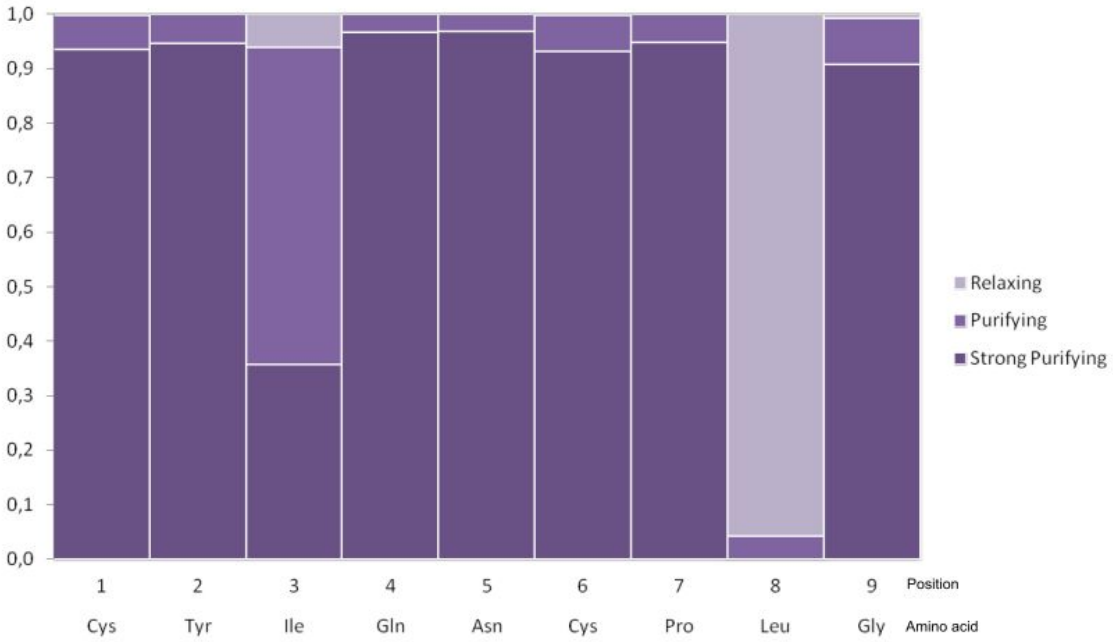


Figure S3. Posterior probabilities of the Naïve Empirical Bayes test (NEB for the BranchSites model, Clade ModelD) for each OXT amino acid position to be under strong purifying, purifying or relaxing selection. The same values were also obtained with the NsSites model.

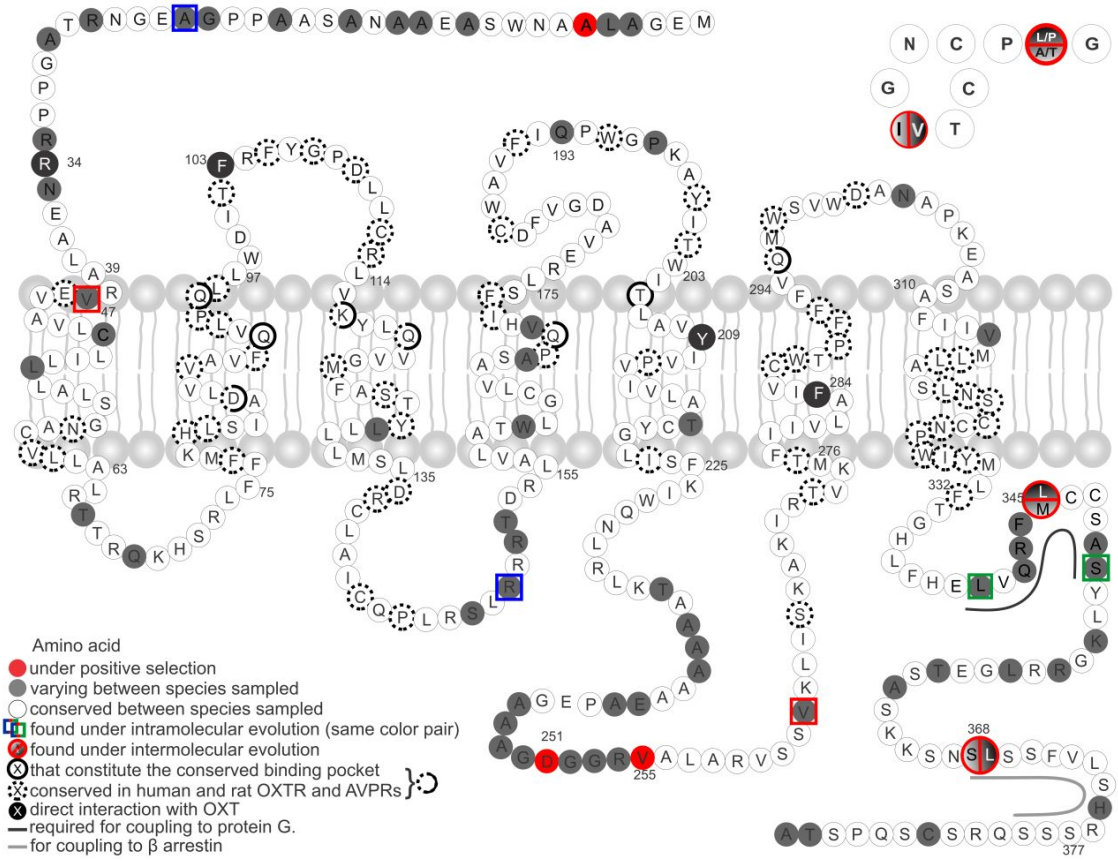


Figure S4. Snake plot of OXTR amino acid chain. Gray solid circles show interspecific variation. Amino acids in white are conserved; those in red indicate sites where positive selection was detected. White to black gradients indicate sites in which coevolution was detected. Amino acids that constitute the conserved binding pocket are solid black circles; OXTR and AVPR amino acids, which are identical in humans and rats, are shown in dotted circles. A black line indicates the amino acids required for coupling with G protein. A gray line indicates coupling-region for β arrestin. Figure includes information from the present, as well as van Kesteren and Geraertz (31), and Gimpl and Fahrenholz (32) studies.Above right, the OXT amino acid chain.

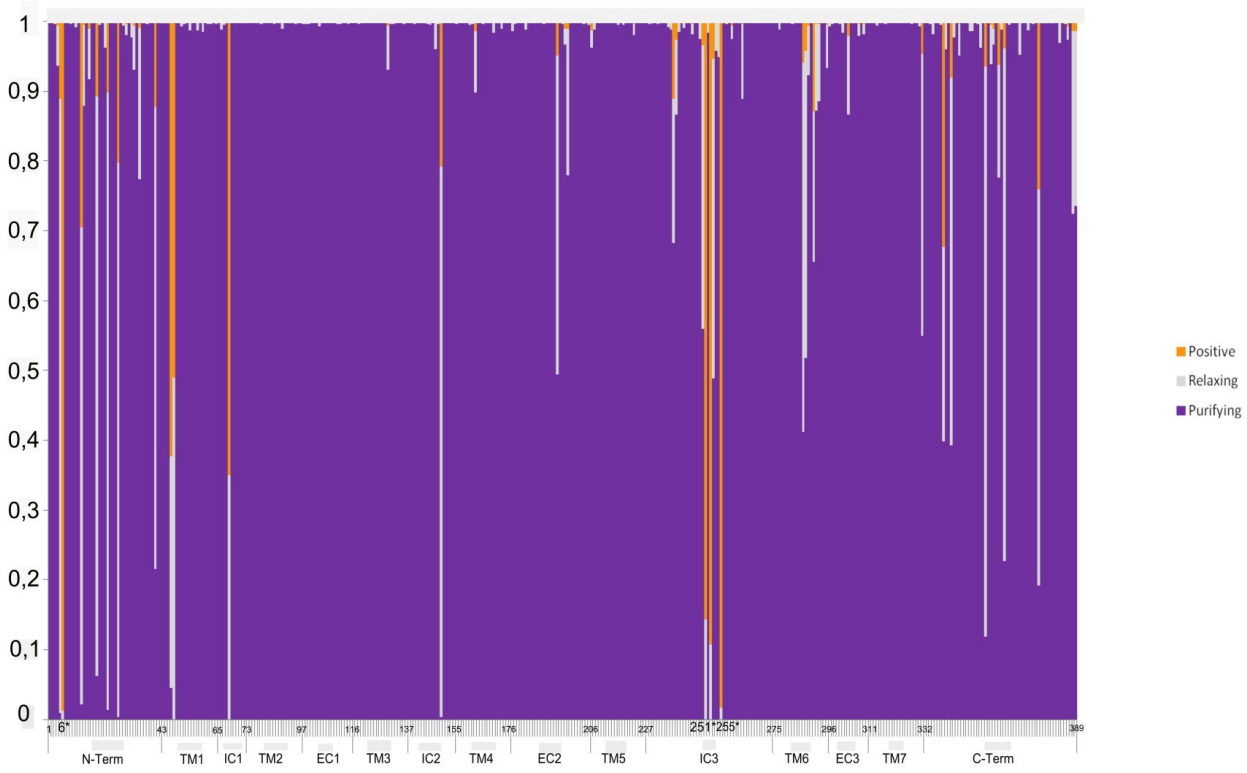


Figure S5. Posterior probabilities of Bayesian Empirical Bayes tests (BEB for NsSites; M2a-Selection model) for each OXTR amino acid position to be under purifying (purple), relaxing (grey) or positive (orange) selection.

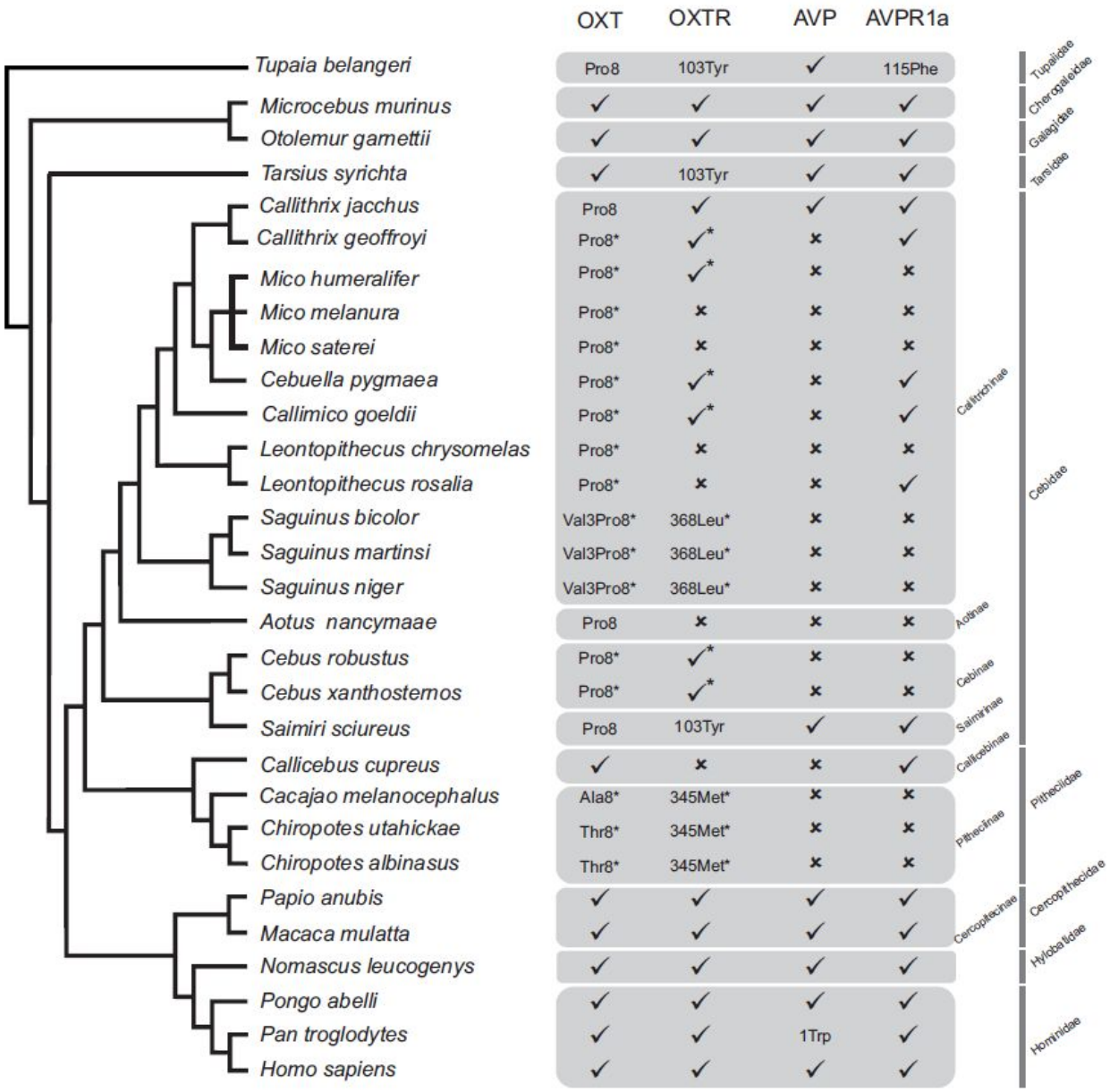


Figure S6. Nonapeptides and their receptor forms in primates. ⩵ indicate that the sequence is equal to the human OXT (1Cys-2Tyr-3Ile-4Gln-5Asn-6Cys-7Pro-8Leu-9Gly),AVP (1Cys-2Tyr-3Phe-4Gln-5Asn-6Cys-7Pro-8Arg-9Gly); and OXTR (34Arg, 103Phe, 209Tyr, 284Phe, 345Leu, 368Ser) and AVPR1a (46Arg, 115Tyr, 116Arg, 125Arg, 204Asp) at some

specific positions. Phylogenetic tree was based on genomic data (48), showing families and subfamilies. Data from the present study are indicated with an asterisk* and the others are from data bases (Table S10). ㄱ No information.

Table S1. Low energy oxytocin and vasopressin conformers obtained from molecular dynamics (400ns)

	Conformers		
	Type I	**Type II**	**Type III**
Oxytocin (OXT)			
RMSD (nm)	0.45	0.35	0.54
Radius of Gyration (nm)	0.65	0.57	0.55
Vasopressin (AVP)			
RMSD (nm)	0.50	0.42	0.53
Radius of Gyration (nm)	0.70	0.57	0.58

Table S2. Estimated parameters under different codon substitution models through the OXT Branch-site Models[1]

OXT Primates	Model Clade D (free-ratio model, admit selection)				M1a (neutral)		Probability[2]
	Proportion	Clade 1	Clade 2	LogL	Proportic	LogL	M1a vs Clade Models
Hominidae vs other Primates	P_0=0.17970 P_1=0.70750 P_2=0.11279	ω_0=0.12119 ω_1=0.00000 ω_2=2.33044	ω_0=0.12119 ω_1=0.00000 ω_2=999.00000	-66.668.484	P_0=0.87355 P_1=0.12645 ω_0=0.01008 ω_1=1.00000	-67.090.904	Models >0.999
Cebidae vs other Primates	P_0=0.17283, P_1=0.72338 P_2=0.10379	ω_0=0.11802 ω_1=0.00000 ω_2=**125.9960**	ω_0=0.11802 ω_1=0.00000 ω_2=0.00000	-62.405.368	P_0=0.87355 P_1=0.12645 ω_0=0.01008 ω_1=1.00000	-67.090.904	**0.0368**
Pithecidae vs other Primates	P_0=0.70750 P_1=0.17970 P_2=0.11280	ω_0=0.00000 ω_1=0.12119 ω_2=2.33049	ω_0=0.00000 ω_1=0.12119 ω_2=999.00000	-66.668.515	P_0=0.87355 P_1=0.12645 ω_0=0.01008 ω_1=1.00000	-67.090.904	>0.999

[1]P= Proportion of codons in each ω class, where ω = dN/dS (non-synonymous/synonymous rate ratio); Two degrees of freedom were considered.
[2]Likelihood ratio test after Bonferroni correction.

Table S3. Ecological/social primate traits for the species considered in the present study[1]

Species	Body size (kg)	Body length(mm)	Activity cycle	Habitat	Locomotion	Diet	Social structure	Mating system	Gestation period	Number of offspring	Group size (No)	Reprodutive maturity (months) Female	Male	Average life span (captivity) years
Microcebus murinus	0.055	100-140	NT	TF	AB	LV/FR	OMMF	PGA	61	3	1	21	13	15
Otolemur garnettii	0.751	230-338	NT	TF	AB	FR/IN	MMMF	PGA	130	1	4	12	20	15
Tarsius syrichta	0.120	80-160	NT	TF	AB	IN/VT	MFPB	MGY	180	1	3	24	24	14
Callithrix jacchus	0.848	120-150	DI	TF	AB	OM	MFPB	MGY	148	2	7	16	13	16
Callithrix geoffroyi	0.375	190-350	DI	TF	AB	OM	MFPB	MGY/PA	160	2	9	16.5	16.5	10
Mico melanura	0.4	180-280	DI	TF	AB	FR/IN	MFPB/OFMM	MGY-PGY-PA	145	2	9	14.5	20	16
Mico saterei	0.4	195-230	DI	TF	AB	FR/IN	MFPB/OFMM	MGY-PGY-PA	145	2	9	14.5	20	12
Mico humeralifer	0.4	200-270	DI	TF	AB	OM	MFPB/OFMM	MGY-PGY-PA	140	2	9	14.5	20	12.5
Cebuella pygmaea	0.13	130-370	DI	TF	AQ	OM	MFPB/OFMM	PA/MGY	140	2	7	15	15	16
Callimico goeldii	0.626	210-234	DI	TF	AB	IN/FR/EX	MFPB	MGY	155	1	6	14	14	10
Leontopithecus chrysomelas	0.535	200-336	DI	TF	AB	OM	MFPB/OFMM	MGY	128	2	8	18	24	21.3
Leontopithecus rosalia	0.654	100-150	DI	TF	AB	OM	MFPB/OFMM	MGY	133	2	8	18	24	22
Saguinus niger	0.45	206-300	DI	TF	AB	OM	MFPB *	PA	140	2	8	21	21	11.1
Saguinus martinsi	0.475	206-300	DI	TF	AB	OM	MFPB *	PA	140	2	8	24	24	18
Saguinus bicolor	0.43	208-283	DI	TF	AB	LV/FR	OFMM	PA	145	2	8	18	24	19
Aotus nancymaae	0.788	637	NT	TF	AB	FL/FR	MFPB	MGY	133	1	10	11	11	?
Cebus robustus	3	300-560	DI	TF	AB	OM	OMMF/SD	PGA	165	1	20	48	72	44
Cebus xanthosternos	3	350-480	DI	TF	AB	OM	MMMF	PGA	165	1	19	48	72	30
Saimiri sciureus	0.925	300	DI	TF	AB	FR/IN	MMMF	PGY	165	1	55	36	65	27
Callicebus cupreus	1.12	247	DI	TF	AB	FR/IN	MFPB	MGY	150	1	5	?	?	25
Cacajao melanocephalus	3.2	365-485	DI	TF	AQ	FR/IN/SD	MMMF	MGY	180	1	25	43	43	12
Chiropotes uthaickae	2.9	327-480	DI	TF	AQ	FR/LV/IN	MMMF	PGA-MGY	135	1	24	48	48	15
Chiropotes albinasus	3	420-380	DI	TF	AQ	FR/LV/IN	MFPB / FF	MGY	142	1	44	48	48	15
Papio anubis	20	480-760	DI	SW TF	TQ	OM	MMMF	PGA	180	1	70	7.5	8.5	25.2
Macaca mulatta	8	450-640	DI	DV	AQ	OM	MMMF	PGA	165	1	90	3.2	5.7	36
Nomascus leucogenys	5.7	450-630	DI	TF	AB/BR	FR/LV	MFPB	MGY	210	1	5	6.5	7	28
Pan trogrodytes	50	635-925	DI	TF	QM/BR	OM	FF	PGA	230	1	85	11.5	13.5	60
Pongo abelii	60	1300-1800	DI	TF	AB/BR	FR/LV/IN	SD	PGA	254	1	2	12.2	19	55
Homo sapiens	65	1600-1800	DI	DV	BP	OM	VAR	VAR	280	1	VAR	168	144	65

[1]Information collected from the Animal Diversity Web (http://animaldiversity.ummz.umich.edu/). Activity cycle: DI=Diurnal; NT=Nocturnal. Habitat: TF=Tropical Forest; DV=Diverse, SW=Savanna-Wood land. Locomotion: AB=Arboreal; TQ=TerrestrialQuadruped; AQ=ArborealQuadruped; BR=Brachiator; BP=Bipedal. Diet: OM=Omnivore; FR=Fruits; LV=Leaves; SD=Seeds; IN=Insects; EX=Exudates; VT=Small vertebrates. Social structure: SD=Solitary/scattered; FF=Fission-Fusion; MMMF=MultimaleMultifemale; OMMF=One Male Multifemales; MFPB=Multi Female Pair Bonds. Mating system: PGA=Polygynandry; PGY=Polygynous; PA=Polyandrous; MGY=Monogamy; VAR=Variable. *Only one reproductive female. ? = No information available. The offspring number was defined based on the average proportion of twin and single pregnancies;this and other traits present in this table should be taken as approximations.

Table S4. Statistical correlations between ecological/social primate traits and the OXT forms

Variable	Social structure	Mating system	Gestation period	Number of offspring	Group size	Maturity female	Maturity male
Test	Fisher	Fisher	Mann-Whitney	Fisher	Mann-Whitney	Mann-Whitney	Mann-Whitney
p	0.10622	0.24469	0.05357	0.00181	0.07281	0.31407	0.27284
*p**	0.744	1	0.375	**0.013**	0.510	1	1

p, statistical significance; *p*,* statistical significance after Bonferroni correction.

Table S5. Non-synonymous OXTR mutations in primates

			Order	Primates																					
			Suborder	Strepsirrhini	Haplorhini																				
			Family	Galaguidae	Cebidae										Pithecidae			Cercopithecidae		Hylobatidae	Hominidae			
			Subfamily		Callithrichinae								Cebinae		Saimirinae										
Protein domain	**Nucleotide**	**Amino acid**	**Grantham Score**	*Otolemurgarnettii*	*Callithrixjacchus*	*Callithrixgeoffroyi*	*Micohumeralifer*	*Cebuellapygmaea*	*Callimicogoeldii*	*Saguinus bicolor*	*Saguinusmartinsi*	*Saguinusniger*	*Cebusrobustus*	*Cebusxanthosternos*	*SaimiriscIureus*	*Cacajaomelanocephalus*	*Chiropotesalbinasus*	*Chiropotesutahickae*	*Papioanubis*	*Macacamulatta*	*Nomascusleucogenys*	*Pongoabelii*	*Pan troglodytes*	*Homo sapiens*
N-terminal tail	GCG>GAG	4 Ala>Glu	107	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
	CTC>TTC	5 Leu>Phe	22	0	1	0	1	1	0	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0
	GCA>GTA	6 Ala>Val	64	0	0	1	1	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0
	GCC>ACC	11 Ala>Thr	58	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
	GCG>GAG	13 Ala>Glu	107	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

	GTC>ATC	**14 Val>Ile**	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
	GTC>GCC	**14 Val>Ala**	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	1
	GCC>TCC	16 Ala>Ser	99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
	CGC>TGC	19 Ala>Val	64	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	GGG>TGG	22 Gli>Trp	184	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	GCC>TCC	23 Ala>Ser	99	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
	AGC>CGC	**27 Ser>Arg**	110	0	1	1	1		1	1	1	1	1	1	1	1		1			1	1	1	1
	AGC>CAC	**27 Ser>His**	89	0				1									1							
	AGC>TGC	**27 Ser>Cys**	112	0															1	1				
	GCC>TCC	29 Ala>Ser	99	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CCG>CTG	32 Pro>Leu	98	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	CGG>CAG	33 Arg>Gln	54	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
	AAC>GAC	35 Asn>Asp	23	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0
TM1	GTG>ATA	41 Val>Ile	29	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	TGT>TCT	47 Cys>Ser	112	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0
	CTC>GTC	48 Leu>Val	32	0	0	1	1	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0	0
	TTC>CTC	51 Phe>Leu	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
ICL1	ACC>ATC	66 Thr>Ile	89	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	CAC>CAG	69 His>Gln	40	0	0	0	1	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	1	1
ECL1	TTC>TAC	103 Phe>Tyr	22	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
TM3	ATG>CTG	129 Met>Leu	15	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ICL2	ACG>TCG	147 Thr>Ser	58	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

	CGC>AGC	149 Arg>Ser	110	0	1	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
TM4	TGG>GGG	161 Trp>Gli	184	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
	CTT>TTT	162 Leu>Phe	22	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	GCG>GTG	169 Ala>Val	64	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
	GTG>ATG	172 Val>Met	21	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
ECL2	CAG>GAG	193 Gln>Glu	29	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0
	CCC>TCT	197 Pro>Ser	74	0	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0
TM5	GCC>ACC	218 Ala>Thr	58	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
ICL3	ACC>AAC	235 Thr>Asn	65	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	GCT>TCT	237 Ala>Ser	110	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	CCA>GCA	238 Ala>Pro	103	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	GCG>ACG	239 Ala>Thr	71	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	GCG>TCG	247 Ala>Ser	110	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	GTG>GCG	248 Val>Ala	64	0	0	0	0	0	0	0	0	0	0	0	0	0	0		1	1	1	1	1	1
	GTG>ATG	248 Val>Met	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1						
	CTT>GTT	**249 Leu>Val**	32	0	1	1	1	1	1	1	1	1	1	1	1	1	1							
	CTT>GCT	**249 Leu>Ala**	96	0														1	1	1	1	1	1	1
	GGC>CGA	250 Gli>Arg	125	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	GGG>GCC	**251 Gli>Ala**	60	0	1	1	1		1	1	1	1	1	1	1	1								
	GGG>ACC	**251 Gli>Thr**	59	0				1									1							
	GGG>AGG	**251 Gli>Arg**	125	0														1						
	GGG>GAT	**251 Gli>Asp**	94	0															1	1	1	1	1	1

	GGG>CCG	252 Gli>Pro	42	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	GGG>GCG	253 Gli>Ala	125	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	CAG>CGC	254 Gln>Arg	54	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	GTA>ATA	**255 Val>Ile**	29	0			1			1	1	1		1		1								
	GTA>ATG	**255 Val>Met**	10	0	1	1		1	1				1		1		1	1	1	1				
	GTC>ATC	263 Val>Ile	29	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	CTG>ATG	345 Leu>Met	21	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0
C-terminal tail	TCC>GCC	349 Ser>Ala	99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
	AGC>AGG	350 Ser>Arg	110	0	0	?	?	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	AAG>AGG	353 Lys>Arg	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	AGC>AAC	**355Ser>Asn**	46	0	1	?	?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
	AGC>AAA	**355 Ser>Lys**	121	0		?	?																1	
	AGC>AGA	**355 Ser>Arg**	110	0		?	?																	1
	CCC>CTG	357 Pro>Leu	98	0	1	?	?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	ACG>ATG	360 Thr>Met	81	0	0	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	UUC>GCC	**362 Phe>Ala**	113	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1			1	1	1	1
	UUC>ACC	**362 Phe>Thr**	103	0		?	?												1	1				
	TCG>TTG	368 Ser>Leu	145	0	0	?	?	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
	CGT>CAG	**375 Arg>Gln**	43	0	1	?	?	1	1	1	1	1												
	CGT>CAC	**375 Arg>His**	29	0		?	?						1	1	1	1	1	1	1	1	1	1	1	1
	TGC>GGC	383 Cys>Gli	159	0	0	?	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

A<u>T</u>G>A<u>C</u>G	388 Met>Thr	81	0	0	?	?	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1
G<u>T</u>G>G<u>C</u>A*	**389 Val>Ala**	64	0	1	?	?	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
GT<u>G</u>>G<u>A</u>G	**389 Val>Glu**	107	0		?	?														1			
Total			0	25	21	19	26	21	25	24	27	25	27	20	25	26	27	19	18	14	15	16	17
Unique				1				1	1		3	2	1	4	1		4	1			2	1	3

Domains: EC=Extracellular; TM=Transmembrane; IC=Intracellular. 0 indicates the allele present in the reference sequence (*Otolemurgarnettii)*, whereas 1 indicates a variant allele. In **bold** different mutations in the same amino acid site: for instance, at OXTR amino acid position 27 *Otolemurgarnetti* presents a Ser, *Cebuellapygmaea* and *Chiropotesalbinasus* a His, *Papioanubis* and *Macacamulatta* a Cys, and all others a Arg ; ? = No information available.

Table S6. OTXR putative phosphorylation sites[1]

Species	PKC 147	PKC 149[2]	PKC 152[3]	PKC 262	PKC/GRK 362[4]	PKC/GRK 363	GRK 366	GRK 368[5]	PKC 374	PKC/GRK 378	PKC/GRK 379	PKC/GRK 382	PKC/GRK 384
Otolemurgarnettii	P		P	P		P	P	P	P	P	P	P	P
Callithrixjacchus	P	P		P		P	P	P	P	P	P	P	P
Callithrixgeoffroyi	P	P		P		P	?	?	?	?	?	?	?
Micohumeralifer	P		P	P		P	?	?	?	?	?	?	?
Cebuellapygmaea	P	P		P		P	P	P	P	P	P	P	P
Callimicogoeldii	P		P	P		P	P	P	P	P	P	P	P
Saguinus bicolor	P		P	P		P	P		P	P	P	P	P
Saguinusmartinsi	P		P	P		P	P		P	P	P	P	P
Saguinusniger	P		P	P		P	P		P	P	P	P	P
Cebusrobustus	P		P	P		P	P	P	P	P	P	P	P
Cebusxanthosternos	P	P		P		P	P	P	P	P	P	P	P
SaimiriscIureus	P		P	P		P	P	P	P	P	P	P	P
Cacajaomelanocephalus	P		P	P		P	P	P	P	P	P	P	P
Chiropotesalbinasus	P	P		P		P	P	P	P	P	P	P	P
Chiropotesutahickae	P		P	P		P	P	P	P	P	P	P	P

Papioanubis	P		P	P	P	P	P	P	P	P	P	P
Macacamulatta	P		P	P	P	P	P	P	P	P	P	P
Nomascusleucogenys	P		P	P		P	P	P	P	P	P	P
Pongoabelii	P		P	P		P	P	P	P	P	P	P
Pan troglodytes	P		P	P		P	P	P	P	P	P	P
Homo sapiens	P		P	P		P	P	P	P	P	P	P

[1]PKC: protein kinase C; GRK: Gprotein-coupled receptor kinase. Numbers = OXTR amino acid sites; P = presence of a phosphorylation site. ? = Non-determined. [2]Putative new phosphorylation sites in *Callithrix*,*Cebuelapygmea, Cebusxanthosternos*, and *Chiropotesalbinasus*;[3]Loss of putative phosphorylation site in *Callithrix*genus,*Cebuelapygmea, Cebusxanthosternos*, and *Chiropotesalbinasus;*[4]Putative new phosphorylation sites in *Papioanubis* and *Macacamulatta*. [5]Loss of putative phosphorylation site in *Saguinus*.

Table S7. Estimated parameters under different OXTR codon substitution models using NSsites

Model[1]	*dN/dS*[2]	Estimated parameters[3]	Likelihood ratio test	p-*value*
M0-Neutral	0.0934	ω=0.0934	-3848.845622	
M3- Discrete	0.1334	P_0=0.81694, P_1=0.16642 (P_2=0.01664) ω_0=0.01537, ω_1=0.40250, ω_2=1	-3755.871525	<<0.0001
M1a- Nearly Neutral	0.1195	P_0=0.91198, (P_1=0.08802) (ω_0=0.03448), (ω_1=1.00000)	-3767.747104	
M2a- Selection	**0.1526**	**P_0=0.91279, P_1=0.07946, (P_2=0.00775)** **(ω_0=0.03634), (ω_1=1.00000), ω_2=5.15725**	-3760.902484	**0.00106517**
M7- Beta	0.1198	[p=0.09359 q=0.67877]	-3768.205890	3.65059e-06
M8- Beta + Selection	0.1340	[p=0.14724 q=1.52768] P_0=0.98456 (P_2=0.01544) ω_2=3.37957	-3755.685268	

[1]Neutral or nearly neutral models: M0, M1a and M7; Models that identify positive selection and/or relaxation of functional constraints: M2a, M3, and M8; M0 vs M3: chi-square with four degrees of freedom (df); M1a vs M2a: chi-square with two df; M7 vs M8: chi-square with two df; [2]dN/dS = non-synonymous/synonymous rate ratio (ω); where ω < 1 indicates negative selection, ω ≅1 indicates neutral or relaxing selection, and ω > 1 indicates positive selection; [3]Within parentheses: fixed parameters; [4]Withinbrackets: Beta parameters p and q; P= proportion of codons in each ω class.

Table S8. Comparison of several non-synonymous OXT and OXTR variants in different primate species

Bayesian posterior probabilities	Intermolecular										Intramolecular **OXTR**											
	0.56				0.80						0.81				0.54				0.55			
	OXT		**OXTR**		**OXT**				**OXTR**		**23 (N-Term)**		**149 (ICL2)**		**41 (TM1)**		**263 (ICL3)**		**340 (C-Term)**		**350 (C-Term)**	
	3		**368 (C-Term)**		**8**				**345 (ICL4)**													
Species	Ile	Val	Ser	Leu	Pro	Leu	Ala	Thr	Leu	Met	Ala	Ser	Arg	Ser	Val	Ile	Val	Ile	Leu	Phe	Ser	Arg
Otolemurgarnettii	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Callithrixgeoffroyi	X	-	X	-	X	-	-	-	X	-	-	X	-	X	X	-	X	-	X	-	X	-
Callithrixjacchus	X	-	X	-	X	-	-	-	X	-	-	X	-	X	X	-	X	-	X	-	X	-
Micohumeralifer	X	-	X	-	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Cebuellapigmea	X	-	X	-	X	-	-	-	X	-	-	X	-	X	-	X	-	X	X	-	X	-
Callimicogoeldii	X	-	X	-	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Saguinus bicolor	-	X	-	X	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Saguinusmartinsi	-	X	-	X	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Saguinusniger	-	X	-	X	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Cebusrobustus	X	-	X	-	X	-	-	-	X	-	X	-	X	-	X	-	X	-	-	X	-	X
Cebusxanthosternos	X	-	X	-	X	-	-	-	X	-	-	X	-	X	X	-	X	-	-	X	-	X
Saimiriscireus	X	-	X	-	X	-	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Cacajaomelanocephalus	X	-	X	-	-	-	X	-	-	X	X	-	X	-	X	-	X	-	X	-	X	-
Chiropotesutahickae	X	-	X	-	-	-	X	-	-	X	X	-	X	-	X	-	X	-	X	-	X	-
Chiropotesalbinasus	X	-	X	-	-	-	-	X	-	X	-	X	-	X	-	X	-	X	X	-	X	-
Papioanubis	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Macacamulatta	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Nomascusleucogenys	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Pongoabelii	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Pan troglodytes	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-
Homo sapiens	X	-	X	-	-	X	-	-	X	-	X	-	X	-	X	-	X	-	X	-	X	-

Table S9. Pairwise Nei-Gojobori distances for *OXT* (upper right) and *OXTR* (lower left)

OXTR \ OXT	C	C	C	M	C	C	S	S	S	C	C	S	C	C	C	F	M	M	F	F	H
Otolemur garnettii		0,05	0,05	0,05	0,05	0,05	0,105	0,105	0,105	0,05	0,05	0,05	0,105	0,114	0,105	0	0	0	0	0	0
Callithrix jacchus	0,035		0	0	0	0	0,05	0,05	0,05	0	0	0	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Callithrix geoffroyi	0,036	0,005		0,00	0,00	0,00	0,05	0,05	0,05	0,00	0,00	0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Mico humeralifer	0,032	0,010	0,008		0,00	0,00	0,05	0,05	0,05	0,00	0,00	0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Cebuella pygmaea	0,040	0,007	0,009	0,016		0,00	0,05	0,05	0,05	0,00	0,00	0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Callimico goeldii	0,032	0,008	0,008	0,009	0,010		0,05	0,05	0,05	0,00	0,00	0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Saguinus bicolor	0,034	0,013	0,010	0,002	0,018	0,012		0,00	0,00	0,05	0,05	0,05	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,105
Saguinus martinsi	0,032	0,010	0,010	0,002	0,016	0,009	0,002		0,00	0,05	0,05	0,05	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,105
Saguinus niger	0,037	0,015	0,013	0,005	0,020	0,014	0,005	0,005		0,05	0,05	0,05	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,11	0,105
Cebus robustus	0,036	0,016	0,012	0,008	0,021	0,013	0,010	0,010	0,013		0,00	0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Cebus xanthosternos	0,036	0,012	0,009	0,006	0,017	0,015	0,008	0,008	0,010	0,007		0,00	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Saimiri sciureus	0,029	0,017	0,020	0,016	0,023	0,016	0,019	0,016	0,021	0,020	0,020		0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Cacajao melanocephalus	0,034	0,014	0,012	0,003	0,019	0,013	0,006	0,006	0,008	0,009	0,007	0,017		0,05	0,00	0,11	0,11	0,11	0,11	0,11	0,105
Chiropotes albinasus	0,040	0,008	0,010	0,017	0,003	0,014	0,019	0,017	0,021	0,020	0,016	0,021	0,016		0,05	0,11	0,11	0,11	0,11	0,11	0,105
Chiropotes utahickae	0,040	0,020	0,018	0,012	0,023	0,019	0,014	0,014	0,017	0,015	0,015	0,024	0,011	0,020		0,105	0,105	0,105	0,105	0,105	0,105
Papio anubis	0,029	0,029	0,031	0,029	0,035	0,027	0,031	0,029	0,033	0,031	0,032	0,023	0,030	0,033	0,033		0,00	0,00	0,00	0,00	0
Macaca mulatta	0,028	0,028	0,030	0,027	0,033	0,026	0,030	0,027	0,032	0,030	0,031	0,022	0,029	0,032	0,032	0,001		0,00	0,00	0,00	0
Nomascus leucogenys	0,023	0,024	0,024	0,023	0,030	0,021	0,025	0,023	0,028	0,024	0,026	0,020	0,024	0,029	0,028	0,009	0,008		0,00	0,00	0
Pongo abelii	0,025	0,025	0,026	0,023	0,030	0,023	0,026	0,023	0,028	0,026	0,027	0,020	0,024	0,029	0,028	0,012	0,011	0,005		0,00	0
Pan troglodytes	0,027	0,027	0,028	0,023	0,032	0,025	0,025	0,023	0,027	0,026	0,026	0,022	0,024	0,031	0,028	0,012	0,010	0,005	0,007		0
Homo sapiens	0,026	0,029	0,030	0,025	0,035	0,027	0,028	0,025	0,030	0,028	0,029	0,023	0,026	0,033	0,030	0,014	0,013	0,007	0,009	0,005	

Genetic distances increase from blue to red. Correlation (Mantel test) between the genetic distance matrices (¬] = 0.52; p = 0.0001).

Table S10. Reference data for the original/or database material and primers used in the present study[1]

Species	OXT	OXTR	AVP	AVPR1a
Tupaia belangeri	Lee,et al., 2011	XM_006152039.1	XM_006163955.1[c]	XM_006154152.1[c]
Microcebus murinus	ENSMICT00000013788.1 [b]	ENSMICG00000002030.1 [b]	NM_176854[c]	NM_001104990[c]
Otolemur garnettii	XM_003788219.1[a]	XM_003785470.1[a]	NM_213952[c]	NM_001199792[c]
Tarsius syrichta	Blat[b]	Blat[b]	XM_008047997.1[c]	XM_008074126.1[c]
Callithrix jacchus	XM_002747304.1 [b]	XM_002758625.1c	XM_002747402.1[c]	KJ641425.1[c]
Callithrix geoffroyi	KM186262[d]	KM186278[d]	*	KJ641427.1-KJ641449.1[c]
Mico melanura	KM186263[d]	*	*	*
Mico saterei	KM186264[d]	*	*	*
Mico humeralifer	KM186265[d]	KM186279[d]	*	*
Cebuella pygmaea	KM186266[d]	KM186280[d]	*	KJ641423.1-KJ641445.1[c]
Callimico goeldii	KM186267[d]	KM186281[d]	Ren	KJ641429.1-KJ641451.1[c]
Leontopithecus chrysomelas	KM186268[d]	*	*	
Leontopithecus rosalia	KM186269[d]	*	*	KJ641430.1-KJ641452.1[c]
Saguinus niger	KM186270[d]	KM186284[d]	*	*
Saguinus martinsi	KM186271[d]	KM186283[d]	*	*
Saguinus bicolor	KM186272[d]	KM186282[d]	*	*
Aotusnancy maae	JF315861.1[b]	*	*	*
Cebus robustus	KM186273[d]	KM186285[d]	*	*
Cebusxanthosternos	KM186274[d]	KM186286[d]	*	*
Saimiri sciureus	JF_315866.1[a]	JF_330026.1 a	XM_003941111.1[c]	KJ641433.1-KJ641455.1[c]
Callicebus cupreus	JF315862.1[b]	*	*	KJ641441.1-KJ641463.1[c]
Cacajao melanocephalus	KM186275[d]	KM186287[d]	*	*
Chiropotes utahickae	KM186276[d]	KM186288[d]	*	*
Chiropotes albinasus	KM186277[d]	KM186289[d]	*	*
Papio anubis	XM_003904999.1[b]	XM_003894143.1[b]	XM_003905000.1[c]	XM_003906711.1[c]
Macaca mulatta	XM_001115045.2[b]	NM_001044732.1[c]	XM_001115061.2[c]	XM_001116798[c]
Nomascusleucogenys	XM_003277949.2[b]	XM_003264935.2[b]	XM_003277950.1[c]	XM_003252729.2[c]
Pongo abelii	XM_001160221.3[b]	XM_001144020.3[b]	XM_002830097.2[c]	XM_002823469.1[c]
Pan trogrodytes	XM_002830099.1[a]	XM_002813482.1[b]	XM_001160259.3[c]	XM_003952135.1[c]
Homo sapiens	NM_000915.3[b]	NM_000916.3[b]	NM_000490.4[c]	NM_000706.4[c]

Information from database centers: [a]UCSC; [b]Ensembl; [c]NCBI. [d]Species whose DNA was sequenced in our laboratory, now reported in NCBI. *None available.
Primers for OXTR exon 3: A: Forward CGTAAAGGGCTCGAAGGCCG, Reverse ATGCCCACCACCTGCAAGTAC; B: Forward TGCTGTGGGACATCACCT; Reverse: TCCCAGACGCTCCACATCTG; for OXTR exon 4: Forward CTGCTGCAACCCCTGGATCTA; Reverse: AGAACACTGGACTTCCTGACCCA. The PCR cycling protocol used consisted of 30 sec for 95°C, 30 sec for 59.1º(3A); 59.6º (3B) and 61°C(4), and 40 sec at 72°C for 39 cycles. Primers and conditions for amplifications of coding OXT region were obtained from Lee et al. (1).

Reference

1. Lee AG, Cool DR, Grunwald WC, et al. A novel form of oxytocin in New World monkeys. *Biol Lett.* 2011;7(4):584-587.

5. DISCUSSÃO

O sistema OXT-OXTR fornece uma oportunidade promissora para analisar as interações receptor-ligando, bem como para avaliar a coevolução de genes/proteínas. Quando essas questões podem ser investigadas em primatas, novas e instigantes questões são apresentadas, dada a complexidade comportamental e cognitiva desses animais.

Tendo em vista que o manuscrito já discute os resultados, somente observações mais gerais serão aqui consideradas, embora seja inevitável alguma redundância do que já foi discutido.

As novas formas de OXT apresentadas neste estudo sugerem que a variabilidade do sistema pode se dar em nível da sequência de aminoácidos, diferente da variabilidade mais conhecida e reportada, que se dá através das modificações na distribuição do receptor no cérebro e outros órgãos, ou seja, apenas em nível de regulação (Insel & Young, 2000; Wang, et al., 1996).

As modificações descritas em OXT mostram sinais de seleção positiva. Além disso, foram detectados sinais de coevolução com mudanças específicas no receptor OXTR que parecem ser concomitantes com aquelas presentes no nonapeptídeo. Vale lembrar que estudos

anteriores já reportaram achados de coevolução intermolecular (nonapaptídeo-receptor), no caso entre isotocina e seu receptor, e mesotocina e seu receptor (Koehbach et al. 2013).

Ao mesmo tempo, ao se compararem as distâncias genéticas descritas dentro do neuropeptídio com as descritas dentro do receptor (*Table S7* do manuscrito), pode-se identificar que os grupos de espécies que apresentam as maiores distâncias genéticas para OXT também tendem a apresentar as maiores distâncias quando se considera o receptor OXTR. Esses eventos, segundo Park et al., (2002), descrevem um fenômeno comum no nível molecular, já que as forças evolutivas estão dirigidas para manter a afinidade ligante-receptor, garantindo assim eficiência na ativação, no processamento e na degradação das moléculas envolvidas. Salienta-se aqui que é a primeira vez que o fenômeno é descrito em macacos do Novo Mundo considerando o sistema OXT-OXTR.

Diferentes estudos através de um amplo espectro de espécies demonstram que AVP é mais abundante e possui maiores efeitos em machos do que em fêmeas, especialmente na região hipotalâmica. A mesma relação pode ser descrita nas fêmeas em comparação com os machos no que diz respeito à OXT (Grober & Sunobe, 1996; Insel & Young, 2000).

A relação de AVPR1a com comportamento tem sido estudada em diferentes organismos como macacos do Novo Mundo (Babb et al., 2010), chimpanzés (Hopkins et al., 2012) e humanos (Hammock & Young, 2006). Especial atenção têm recebido os arganazes (McCall & Singer, 2012; Young & Hammock, 2007). Neles foi descrita a presença de regiões repetitivas, determinantes nos padrões de expressão do receptor no cérebro, que, por sua vez, influenciam nas diferenças de estrutura social entre as espécies. Todos esses trabalhos descrevem variações intraespecíficas no receptor da vasopressina, sugerindo possíveis ligações deste com os comportamentos das espécies estudadas. Aqui apresentamos evidência estatística significativa de uma correlação entre caracteres ecológicos com mutações em OXT. Sugerimos que as modificações em OXT poderiam ter um efeito, tanto na interação com seu receptor nativo, OXTR, como com receptores da vasopressina (AVPR1a, AVPR1b e AVPR2). Assim, a associação significativa de OXTs e partos de gêmeos, especialmente nos Cebidae, pode estar relacionada com comportamentos colaborativos dos machos (parentes e não parentes) no cuidado com os infantes.

Por outro lado, devem-se levar em conta outras variáveis no sistema que podem estar relacionadas com comportamento — incluindo aqueles ligados ao cuidados com a prole e infantes —, tais como as diferenças dos efeitos do hormônio em machos e fêmeas, fatores epigenéticos, dentre outros (McCall & Singer, 2012). No entanto, no caso dos *Saguinus* não se acham mudanças no nível de expressão da oxitocina, quando se comparam machos e fêmeas (Snowdon et al., 2010). Também em *Callithrix jaccus* não se acha nenhuma diferença no cérebro na distribuição de neurônios imunorreativos à OXT, mas sim observa-se um padrão dismórfico sexual na distribuição de células reativas à vasopressina, sendo os machos os que mais as possuem nos núcleos supraótico e paraventricular no hipotálamo (Wang et al., 1997).

Em uma observação geral dos eventos evolutivos nas espécies estudadas, pode-se denotar uma tendência do sistema OXT-OXTR em aumentar as afinidades com a AVP e seus receptores (Figura 6); assim, a modificação encontrada no *Saimiri sciureus* poderia aumentar a afinidade de OXTR por AVP, reportada como 100 vezes menor que a afinidade de OXTR por OXT (Chini et al., 1995).

De outra parte, as modificações no gênero *Saguinus* poderiam influenciar na afinidade de OXT por AVPRs, que pode ser até 1.000 vezes menor do que a afinidade de AVP por seus receptores (Figura 6).

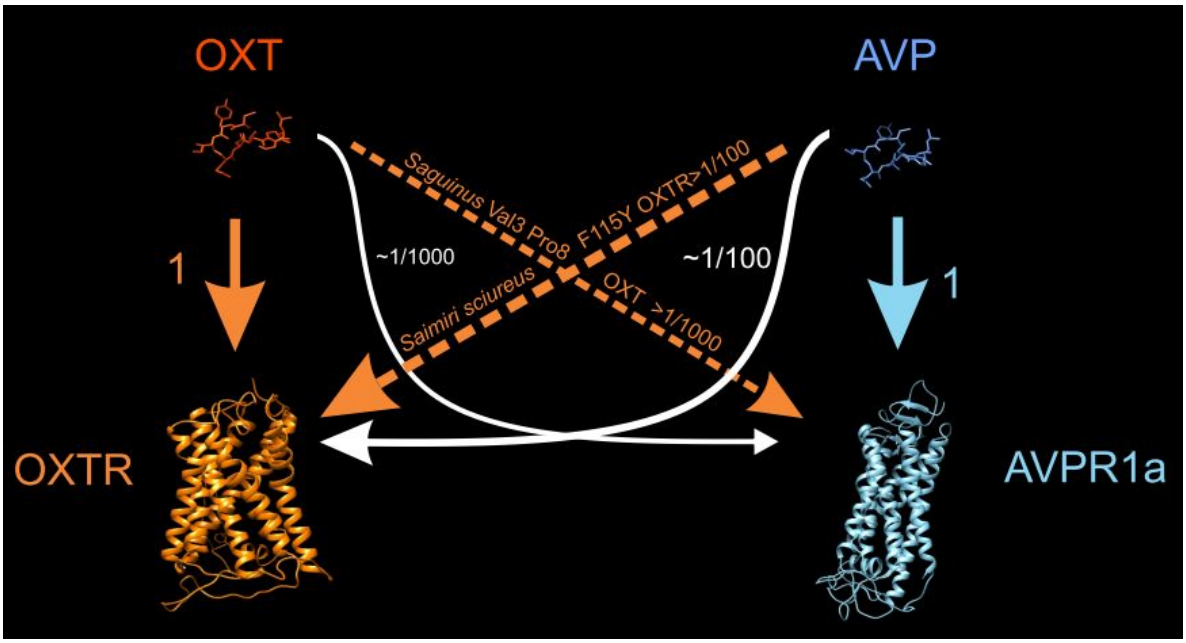


Figura 6. Sistema de ativação cruzada de OXT e AVP com seus receptores (no caso AVPR1a). Flechas diagonais pontilhadas mostram um cenário possível para explicar nossos resultados que aumentariam afinidades cruzadas.

Finalmente, esses resultados evidenciam somente modificações na cadeia de aminoácidos de OXT e OXTR. Não se pode pretender que o sistema OXT-OXTR seja uma unidade evolutiva isolada, de modo que outras moléculas que interagem com eles devem ser consideradas, em especial AVP e seus receptores.

6. CONCLUSÃO

No presente estudo, detectamos uma quebra na linha de conservação mantida na oxitocina por milhões de anos, reportando 3 novas formas de OXT em macacos do Novo Mundo. Registrou-se um aminoácido (OXT-8Pro) com seleção positiva na família Cebidae; esse mesmo resíduo teve uma significância estatística na sua correlação com cuidado parental. Reforçando essa associação, descrevemos uma nova forma de OXT (OXT-3Val-8Pro) nos *Saguinus* (Cebidae), um gênero com um pronunciado cuidado parental dos machos, aparentados ou não. No OXTR foram detectados aminoácidos sob seleção positiva, assim como processos de coevolução intramolecular e intermolecular com seu ligante OXT.

Formulamos aqui possíveis cenários na interação dessas novas formas de OXT com seus receptores e propomos perspectivas sobre o estudo do sistema OXT-OXTR e sua relação com outros sistemas.

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