Expression of tachykinin receptors (*tacr1a* and *tacr1b*) in zebrafish: influence of cocaine and opioid receptors

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Abstract

Opioid and tachykinin receptors (TACRs) are closely related in addiction and pain processes. In zebrafish, opioid receptors have been cloned and characterized both biochemically and pharmacologically. However, the *tacr1* gene has not yet been described in zebrafish. The aim of this research was to identify the *tacr1* gene, study the effects of cocaine on *tacr1*, and analyze the interaction between *tacr1* and opioid receptors. We have identified a duplicate of *tacr1* gene in zebrafish, designated as *tacr1a* and *tacr1b*. Phylogenetic analyses revealed an alignment of these receptors in the Tacr1 fish cluster, with a clear distinction from other TACR1s of amphibians, birds, and mammals. Our qPCR results showed that *tacr1a* and *tacr1b* mRNAs are expressed during embryonic development. Whole-mount *in situ* hybridization showed *tacr1* expression in the CNS and in the peripheral tissues. Cocaine (1.5 μ M) induced an upregulation of *tacr1a* and *tacr1b* at 24 and 48 h post-fertilization (hpf; except for *tacr1a* at 48 hpf, which was downregulated). By contrast, HEK-293 cells transfected with *tacr1a* and *tacr1b* and exposed to cocaine showed a downregulation of *tacr1s*. The knockdown of ZfDOR2 and ZfMOR, opioid receptors, induced a down- and upregulation of *tacr1a* and *tacr1b* respectively. In conclusion, *tacr1a* and *tacr1b* in zebrafish are widely expressed throughout the CNS and peripherally, suggesting a critical role of these *tacr1s* during embryogenesis. *tacr1a* and *tacr1b* mRNA expression is altered by cocaine exposure and by the knockdown of opioid receptors. Thus, zebrafish can provide clues for a better understanding of the relationship between tachykinin and opioid receptors in pain and addiction during embryonic development.

Key Words

- *TACR1* gene
- opioid receptors
- cocaine
- zebrafish

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Introduction

The biological actions of the tachykinin family – substance P (SP), neurokinin A (NKA) and NKB, hemokinin-1, and endokinins – are mediated by transmembrane G-protein-coupled receptors (GPCRs) and they have been classified within the NK₁, NK₂, and NK₃ receptor types (Regoli *et al.* 1994, Maggi 2000, Harrison & Geppetti 2001, Pennefather

et al. 2004), which are encoded by tachykinin receptor 1 (*TACR1*), *TACR2*, and *TACR3* genes respectively (Zhou *et al.* 2012). SP, NKA, and NKB bind to NK₁, NK₂, and NK₃ receptors (also named *TACR1*, *TACR2*, and *TACR3*) respectively (Regoli *et al.* 1987, Regoli *et al.* 1994, DeVane 2001, Harrison & Geppetti 2001). NK₁ receptor (NK₁R) is

considered the SP receptor (Maggi 1995). Several studies have shown that NK₁ is widely distributed throughout the CNS (basal ganglia, dorsal tegmental areas, inferior colliculus, olfactory bulb, hypothalamus, hippocampus, substantia nigra, cerebral cortex, septum, striatum, mesencephalon, medulla oblongata, and the dorsal horn of the spinal cord; Shults *et al.* 1984, Maeno *et al.* 1993) and its activation has been implicated in different processes such as pain, synaptic transmission, neurogenic inflammation, neurotoxicity, mood disorder, stress, anxiety, and addiction (Regoli *et al.* 1994, Harrison & Geppetti 2001, Yu *et al.* 2002, Gadd *et al.* 2003, Gamboa-Esteves *et al.* 2004, Commons 2010).

The analgesic effect of opioids is mediated mainly by MOR (Matthes *et al.* 1996) but, as several studies have shown that MOR and NK₁R are co-localized (Aicher *et al.* 2000a,b) and present interaction between them (Pfeiffer *et al.* 2003), it is possible that analgesic effects of MOR can be modified by NK₁R or vice versa. Moreover, *in vitro* experiments reported that MOR and NK₁R form heterodimers in HEK-293 (NK₁–MOR) where the activation of either type, by SP and DAMGO respectively, induces endocytosis (Pfeiffer *et al.* 2003). Besides, it has also been described that NK₁R activation by SP inhibits the endocytosis of MOR (Yu *et al.* 2009), indicating that SP and its receptor are involved in the modulation of MOR. Several studies have also shown that tachykinin and opioid systems are related in the addiction process, as NK₁R and MOR are localized in different reward-related regions (Nakaya *et al.* 1994, Pickel *et al.* 2000, Garzón & Pickel 2001, Gadd *et al.* 2003, Laurent *et al.* 2012). Likewise, it has been suggested that SP acts in a similar manner to cocaine, producing an increase in dopamine neurotransmitter levels in the synaptic cleft (Kombian *et al.* 2009).

The tachykinin system has been cloned and characterized both biochemically and pharmacologically in different species (for reviews, see Maggi (1995) and Pennefather *et al.* (2004)), and numerous investigations using the zebrafish have proven that this organism is a valuable vertebrate animal model for study of human diseases, pharmacology and drug discovery, and development studies in vertebrates (Chakraborty *et al.* 2009, Lohi *et al.* 2012, Santoriello & Zon 2012). The study of the tachykinin system using the zebrafish could improve our understanding of different clinical situations where the tachykinin system is involved. This will also help to better understand of the interaction of the tachykinin system with other systems, like the opioids system that are involved in pain and addiction.

To date, the tachykinin system has not been described completely in zebrafish. Recently, the *tac1*, *tac2*, and *tac3* precursors and the Tacr3s (Tacr3a1, Tacr3a2, and Tacr3b; Ogawa *et al.* 2012, Zhou *et al.* 2012) have been cloned. Taking the above into consideration, we aimed to i) clone the *tacr1* gene and study its expression during embryogenesis, ii) determine the evolutionary history of the *tacr1s* using phylogenetic analyses, iii) investigate the effects of cocaine on the *tacr1s*, and iv) study the interrelationship between the tachykinin system and opioid receptors during embryonic development.

Materials and methods

Animals

Adult zebrafish (AB strain) were maintained on a 14 h light:10 h darkness cycle at 26 °C at our Fish Facilities. Embryos were raised at 28.5 °C and maintained in dishes in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄ in distilled water; Sigma). Embryo ages were expressed as hours post-fertilization (hpf). All procedures and experimental protocols were carried out in accordance with the guidelines of the European Communities Council directive of 24 November 1986 (86/609/EEC), and to the current Spanish legislation for the use and care of animals (BOE 252/34367-91, 2005).

Drug treatment

Zebrafish embryos at 5 hpf were exposed to 1.5 µM cocaine hydrochloride (HCl) and were then collected at 24 and 48 hpf; both stages are important during zebrafish embryonic development: at 24 hpf, the CNS is being formed and differentiated and primary organogenesis is finished at 24 hpf. HEK-293 cells (expressing zebrafish TACRs) were treated with 1.5 µM cocaine HCl over 24 hpf. The doses of 1.5 µM cocaine HCl was chosen as this dose is comparable to the concentration present in human neonates (Dempsey *et al.* 1999).

Cloning of *tacr1a* and *tacr1b* receptors in zebrafish

The PCR program used for *tacr1a* and *tacr1b* amplification was 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 59 °C (for *tacr1a*) and 62 °C (for *tacr1b*), and 3 min at 72 °C and a final extension temperature of 72 °C for 10 min. PCR primers (Table 1) were based on the predicted sequences of the *tacr1a* and *tacr1b* genes from PubMed (XM_687377.2, XM_001343037.3). The desired *tacr1a* and

Table 1 Oligonucleotides used in this study

Genes	Forward	Reverse	Amplicon	T (°C)
<i>ef1a</i>	GTACTTCTCAGGCTGACTGTG	ACGATCAGCTGTTTCACTCC	136	55
<i>tacr1a</i> (qPCR)	CGCTATTGCGCTCGACAGA	GCTACATTGACTGGCCCGA	186	55
<i>tacr1b</i> (qPCR)	CCTGCTGGCTCCCTATCA	GGAATCCCGCTCGAAACCT	173	55
<i>tacr1a</i> (riboprobe)	CCTCCAGTCAAGAAACAATCGT	GGTTGGCATTCTGTTTCCATGAC	1364	59
<i>tacr1b</i> (riboprobe)	TCCCCTTGTTAAATGCCTGTTCT	ACCGTTTTGGAGCTCACTAGC	1506	62
<i>tacr1a</i> -pEGFP-C3	^a ctgtaCTCGAGGATTCTGTCATCACTTCC ^b	^a tcgtaGGTACCTCATTCTGTAGGTTATTAC ^b	1278	64
<i>tacr1b</i> -pDsRed1-N1	^a tgataAAGCTTATGGATCCGCTGTACATCAC ^c	^a agataCCGCGGTGCTACGTTGTTACTGGAAT ^c	1236	64
<i>GAPDH</i>	ATGAGAAGTATGACAACAGCCT	CAGTGATGGCATGGACTGTG	138	57

^aLower-case letters represent adaptador sequences.

^bThe nucleotides underlined are recognition sites of the XhoI and KpnI restriction enzymes respectively.

^cNucleotide sequences underlined are specific sites of recognition for HindIII and SacII restriction enzymes respectively.

tacr1b amplicons were subcloned using the pCRII vector (Invitrogen). TOP 10/F cells (Invitrogen) were transformed with the constructs, and miniprep (ZYMO Research Corporation, CA, USA) and midiprep (Sigma) were performed. These constructs of both *tacr1a* and *tacr1b* with pCRII were digested with KpnI and EcoRV for 1 h at 37 °C. Then, the purified products were sequenced using the T7 and SP6 sequencing primers.

Phylogenetic analyses

The prediction of the open reading frames (ORFs) and translations of *tacr1a* and *tacr1b* into amino acid sequences were performed using the ORF Finder program from SMS (<http://www.bioinformatics.org/sms2/>). The *Tacr1a* and *Tacr1b* amino acid sequences from zebrafish and other orthologs of several vertebrates were aligned in NBRF format using the ClustalW Software. Molecular Evolutionary Genetic Analysis (MEGA 5) was used to construct neighbor-joining (NJ) and maximum likelihood (ML) trees.

Expression of *tacr1a* and *tacr1b* from zebrafish in mammalian HEK-293 cells

In order to insert the DNA sequences of both *tacr1a* and *tacr1b* in-frame with the vector sequences, two restriction endonuclease (RE) sites from the multiple cloning sites of the plasmid vector were chosen: XhoI and KpnI for pEGFP-C3 (Clontech) and HindIII and SacII for pDsRed1-N1 (Clontech). The RE recognition sequences were added to the ends of the primers (extreme 5'-terminal), which were used to amplify the *tacr1a* and *tacr1b* (Table 1). The vector and inserted genes were digested with both REs in a double digest for 1 h at 37 °C, and after their respective purification, the inserts were ligated using the TA cloning Kit (Invitrogen) in the plasmid vectors pEGFP-C3 (GFP)

and pDs1Red-N1 (DsRed). The PCR program used for *tacr1a* and *tacr1b* amplification was 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 64 °C, and 3 min at 72 °C and a final extension temperature of 72 °C for 10 min. Plasmid transfection was accomplished using the Effectene Transfection Reagent Kit (Qiagen). The expression vectors encoded neomycin/kanamycin resistance, which permitted us to select and obtain stable clones of *tacr1a* and *tacr1b* with 0.5 mg/ml geneticin (Gibco BRL). After an ~2-month selection period, stably transfected HEK-293 cells expressing *tacr1a*-GFP and *tacr1b*-DsRed were grown in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml geneticin at 37 °C under a 5% CO₂ atmosphere. HEK-293 cells expressing *tacr1a*-GFP and *tacr1b*-DsRed were exposed to 1.5 μM cocaine HCl for 24 hpf.

RNA extraction, retrotranscription, and real-time PCR (qPCR)

Total RNA from the embryos and HEK-293 cells was extracted using TRIzol reagent (Invitrogen), following the protocol recommended by the manufacturers. In all cases, RNA samples were treated with DNase I (Roche) at 37 °C for 15 min. The quantity and quality of the RNA were determined by measuring absorbance at 260 nm and 260 nm/280 nm, using NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was carried out by retrotranscription (RT) of total RNA to cDNA using the Promega RT Kit, following the protocol recommended by the manufacturers. The cDNA samples were then treated with RNase A (20 ng/μl for 7–8 μg cDNA) for 20 min at 37 °C. Following this, these products were purified using the QIAquick PCR Purification Kit (Qiagen) and eluted in DNase-free water. The oligonucleotides used for qPCR experiments are listed in Table 1. qPCR products

were measured using the curve of the SYBR-Green method. SYBR-Green was included in a 2× Master Mix from Applied Biosystems (SYBR Green dye, dNTPs, Passive Reference (ROX), Amplitaq1 Gold DNA polymerase). The ABI Prism 7300 detection system (Applied Biosystems) was used to amplify the different genes. The final volume of each reaction was 20 µl: 10 µl Master Mix, 1 µl of each oligonucleotide, 7 µl distilled water, and 1 µl cDNA at a concentration of 5 ng/µl.

Standard curves for absolute quantification were performed for the different genes studied in zebrafish embryos. A basic PCR method was implemented to amplify the fragments of the different genes to be studied. PCR products were visualized on agarose gel and the fragment corresponding to each gene was cut from the agarose gel and purified. Serial 1:10 dilutions were made from the purified PCR product, ranging from 10^{-2} to 10^{-5} ng/µl and chosen according to their cycle quantification (Cq) values and considering that perfect amplification had been obtained when the difference in the Cq values from one dilution to the next was close to 3.3, which afforded a four-point straight line with a slope of -3.3 .

Spatial expression of *tacr1a* and *tacr1b* mRNA

The constructs of both *tacr1a* and *tacr1b* with pCRII were linearized by EcoRV and KpnI digestion respectively over 1 h at 37 °C. From the linearized *tacr1a* and *tacr1b* DNAs, antisense RNAs were transcribed with T7 and SP6 promoters respectively and the RNAs were also labeled with digoxigenin. These latter products were treated with DNase I (Roche) and purified. The riboprobe (600 ng/ml) was diluted in the prehybridization solution for the *in situ* hybridization (ISH) experiments. Embryos at 24 and 48 hpf were dechorionated, fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, washed twice in PBS, 5 min each, at room temperature, and finally maintained in absolute methanol at -20 °C until use. The following day, the embryos were rehydrated in consecutive dilutions of methanol/PBS (75, 50, and 25%), 5 min for each dilution. These were washed four times for 5 min each in 100% PBS–Tween 20 (0.2%; PBT). Proteinase K (10 µg/ml) was used to permeabilize the embryos at room temperature over 20 min for 24 hpf embryos and 40 min for 48 hpf embryos. Proteinase K digestion was stopped by incubating the embryos for 20 min in 4% PFA in 1×PBS. Four washes, 5 min/wash, in 1×PBT were performed to remove residual PFA. After 2 h of prehybridization with *tacr1a* and *tacr1b* riboprobe, the embryos were left overnight at 64 °C to hybridize. Washes were performed every 20 min in each

wash solution with prehybridization/Tris-buffered saline (TBS; 50%/50%) and TBS–Tween 20 (0.2%; TBST) over 2 h. Then, the embryos were blocked with blocking buffer (goat serum + TBST) for 2 h and were incubated overnight with antidigoxigenin antibody conjugated with alkaline phosphatase (1:3000, Roche) at 4 °C. The following day, the embryos were washed with Xpho solution (1 M Tris–HCl, pH 9.5, 1 M MgCl₂, 4 M NaCl, and 20% Tween 20) for 10 min over 1 h, and finally, the hybridization was developed with fresh NBT/BCIP mix (Roche).

Knockdown of opioid receptors

Antisense morpholino oligonucleotides (MOs) of opioid receptors were used to knock down opioid receptors (Gene Tools, Philomath, OR, USA) in order to determine the relationship between opioid and TACRs (*tacr1a* and *tacr1b* expression). Approximately 3 nl of MOs were microinjected into the yolks of zebrafish embryos at the one- to four-cell stage with a micromanipulator–microinjector system from Eppendorf AG (Hamburg, Germany). The MO experimental groups (ZfMOR, ZfDOR1, ZfDOR2, and ZfDOR1–ZfDOR2) were compared with the MO control groups (microinjected with a standard MO control solution and embryos without microinjections). The following MO sequences were used: ZfMOR (AATGTTGC-CAGTGTTCATCATG), ZfDOR1 (GAATGACGGACGG-CTCCATCGCTTC), and ZfDOR2 (GGAGGCTCCATTAT-GCTCGTCCCCT). The MO concentrations used for the different opioid receptors were 0.2 µM (ZfMOR), 1 µM (ZfDOR1), 1 µM (ZfDOR2), and 1 µM (ZfDOR1–ZfDOR2).

Statistical analyses

The results are presented as means of transcripts $\pm$ S.E.M. of the values. Pairs of the data were analyzed by Student's *t*-test while multiple comparisons were evaluated by ANOVA. Follow-up analysis was performed using the Dunnett test for multiple comparisons. Differences were considered significant at $P < 0.05$. All statistical analyses were performed with Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Full-length *tacr1a* and *tacr1b* cloning in zebrafish

After RNA isolation from 48-hpf zebrafish embryos, PCR amplification, cloning, and sequencing, we obtained the full-length cDNA of two genes, which were designated

		N-terminal	TM1	
Human	MDNVLPVDS	DLSENIQNTSEP-----NQFVQPAWQ	IVLWAAAYTVIVVTSVVGNNVV	53
Rat	MDNVLPMD	SDLFENIQNTSES-----NQFVQPTWQ	IVLWAAAYTVIVVTSVVGNNVV	53
Mouse	MDNVLPVDS	DLFENIQNTSES-----NQFVQPTWQ	IVLWAAAYTVIVVTSVVGNNVV	53
Zf Tacrla	MDS-FIT	STDFPSNWDVNSTAQNETEIYWNQFVQPVWR	IVLWAVAYSTIVVSVVGNITV	59
Zf Tacrlb	MDPLYITS	PNSSADNGSAGSPVNDTEVFGNQFVQPVWQ	IVLWAIAYCTIVLVSVVGNITV	60
	**	..: .:	: *****.:***** *	..*:*****:*
		IL1	TM2	EL1
Human	MWII	LAHKRMRTVINYFLVNLAFAEASMAAFNTVVNFQ	YAVHNEWYYGLFYCKFHNFFPI	113
Rat	IWII	LAHKRMRTVINYFLVNLAFAEACMAAFNTVVNFQ	YAVHNVWYYGLFYCKFHNFFPI	113
Mouse	IWII	LAHKRMRTVINYFLVNLAFAEACMAAFNTVVNFQ	YAVHNVWYYGLFYCKFHNFFPI	113
Zf Tacrla	IWII	LAHKRMRTVINYFLVNLAFAEASMAAFNTVIN	FVYSVHNEWYGLYYCRFHNFFPI	119
Zf Tacrlb	IWII	LAHKRMRTVINYFLVNLAFAEASMAAFNTVIN	FVYAVHNEWYGLVYCRFHNFFPI	120
		:*****:***** *	:*****:***** *	:*****:***** *
		TM3	IL2	TM4
Human	AAVFASII	SMTAVAFDRYMAIIHPLQPRISATAQ	KVVICVIWVLALLLAFPGQYISTTET	173
Rat	AALFASII	SMTAVAFDRYMAIIHPLQPRISATAQ	KVVFVWVLALLLAFPGQYISTTET	173
Mouse	AALFASII	SMTAVAFDRYMAIIHPLQPRISATAQ	KVVIFVWVLALLLAFPGQYISTTET	173
Zf Tacrla	AAVFASII	SMTAIALDRYMAIIHPLQRM	SATQKKVIGVIWILALLLAFPGYYSDTDQ	179
Zf Tacrlb	AAVFASII	SMTAIALDRYMAIIHPLQRL	SATQKLVVCVIWAALMLAFPGYYFSTAQ	180
		:*****:***** *	:*****:***** *	:*****:***** *
		EL2	TM5	IL3
Human	MPSRVVCM	IEWPEHPIKIYEKVVYHICVTVLIYFLPLL	VIGYAYTVVGITLWASEIPGDS	233
Rat	MPSRVVCM	IEWPEHPIRTYEKAYHICVTVLIYFLPLL	VIGYAYTVVGITLWASEIPGDS	233
Mouse	MPSRVVCM	IEWPEHPIRTYEKAYHICVTVLIYFLPLL	VIGYAYTVVGITLWASEIPGDS	233
Zf Tacrla	LPGRVVCY	IDWPEYTLDFKTMVFVCVAVLIYFLPLL	VMGCAYLVGITLWASEIPGDS	239
Zf Tacrlb	LPGRVVCY	INWPEYSMWDFQKTYVVCVVLIYFLPLL	VMGCAYLVGICFLWASEIPGDS	240
		:*****:***** *	:*****:***** *	:*****:***** *
		IL3	TM6	EL3
Human	DRYHEQV	SAKRKVVKMMIVVCTFAICWLPFHIFLLPY	INPDLYLKKFIQQVYLAIMWL	293
Rat	DRYHEQV	SAKRKVVKMMIVVCTFAICWLPFHVFLLPY	INPDLYLKKFIQQVYLASMWL	293
Mouse	DRYHEQV	SAKRKVVKMMIVVCTFAICWLPFHIFLLPY	INPDLYLKKFIQQVYLASMWL	293
Zf Tacrla	DRYQEQL	SAKRKVVKMMIVVCTFAVCWLPYHVYFLIHQ	FYPHLFEHTFIQQVYLTIMWL	299
Zf Tacrlb	DRYREQL	IAKRKVVKMMIVVCTFATCWLPYHVYFIVH	QFLPHLFEERYIQQVYLGIMWL	300
		:*****:***** *	:*****:***** *	:*****:***** *
		TM7	C-terminal	
Human	AMSSTMYN	PIIYCCLNDRFRLGFKHAFRCPPFI	SAGDYEGLEMKSTRYLQTS	353
Rat	AMSSTMYN	PIIYCCLNDRFRLGFKHAFRCPPFI	SAGDYEGLEMKSTRYLQTS	353
Mouse	AMSSTMYN	PIIYCCLNDRFRLGFKHAFRCPPFI	SAGDYEGLEMKSTRYLQTS	353
Zf Tacrla	AMSSTMYN	PIIYCCLNDRFRAGFKQAFRCPCVPEGS	YEGLELKSSTRYLQTS	359
Zf Tacrlb	AMSSTMYN	PIIYCLNDRFRAGFQAFSWCPCVPGS	YEGLELKSSTRYLQTS	360
		:*****:***** *	:*****:***** *	:*****:***** *
		C-terminal		
Human	LEDTIS	TVVGAHEE-----EPEDGPKATPS	LDLQNGSS-RDQSKMTESSEFY	402
Rat	LEDTIS	TVVGAHEE-----EPEGPKATPS	LDLQNGSS-RDQSKMTESSEFY	402
Mouse	LEDTIS	TVVGAHEE-----EPEGPKATPS	LDLQNGSS-RDQSKMTESSEFY	402
Zf Tacrla	MEQVSCVM	QPSDEGHKVTFGSVRGAAVKPAARSP	REFDQNGSSBRQSKMTESSEFY	419
Zf Tacrlb	MEQVSCVM	QPSDEGHKVTFGSVRGAAVKPAARSP	REFDQNGSSBRQSKMTESSEFY	406
		:*****:***** *	:*****:***** *	:*****:***** *
Human	S-NVLS-	407		
Rat	S-NMLA-	407		
Mouse	S-NMLA-	407		
Zf Tacrla	SSNNLQE	426		
Zf Tacrlb	SSNNVA-	412		
	** *	:		

tacr1a (1281 bp; GenBank ID: 33455) and *tacr1b* (1239 bp; GenBank ID: 345672) (Fig. 1A, B and C). The ORFs of the *tacr1a* and *tacr1b* genes encoded proteins of 426 (predicted molecular weight of 48.96 kDa) and 412 amino acids (40.53 kDa) respectively (Fig. 2). Tacr1a and Tacr1b exhibited characteristic seven transmembrane domains (TMs), two consensus sites for N-glycosylation sites for Tacr1a (Asn¹³ and Asn¹⁷), and one possible site for Tacr1b (Asn¹⁴) in the N-terminal extracellular domain (N-terminal), together with two conserved Cys residues for Tacr1a (Cys¹¹¹ and Cys¹⁸⁶) and for Tacr1b (Cys¹¹² and Cys¹⁸⁷), which can form a disulfide bond between the first and second extracellular loops. The NK receptors also showed putative phosphorylation sites on serine or threonine residues: in the N-terminal, in the first intracellular loop (IL1), in IL2, in IL3; in the carboxyl terminal domain (C-terminal), and a potential palmitoylation site on Cys residues for Tacr1a (Cys³²⁹) and for Tacr1b (Cys³³⁰) in the C-terminal domain. Moreover, Tacr1a and Tacr1b had a typical DRY motif at the border between TM3 and IL2 and a K/RK/RXXK/R moiety in IL3 (Fig. 2).

The Ensembl database (<http://www.ensembl.org/>) revealed that the *tacr1a* and *tacr1b* genes were found on chromosomes 5 and 11 respectively and formed by five exons. The zebrafish Tacr1a and Tacr1b homology with respect to human, rat, and mouse (TACR1) was 66% and when it was compared between them (Tacr1a and Tacr1b) it was 75%.

Phylogenetic analysis of zebrafish Tacr1a and Tacr1b

A clear demarcation was found between zebrafish Tacr1s (Tacr1a and Tacr1b), Tacr2, and Tacr3, and their orthologs, supported by bootstrap values of 100, 48, and 99% (NJ; Fig. 3A) and 55, 70, and 94% (ML; Fig. 3B). The zebrafish Tacr2 (predicted sequence) and Tacr3 (Zhou *et al.* 2012) amino acid sequences were used to check whether Tacr1a and Tacr1b could be aligned in Tacr2 or Tacr3 clusters and might not be duplicate genes. The phylogenetic analyses showed that Tacr1a and Tacr1b were aligned in the same

cluster of Tacr1s, suggesting that Tacr1a and Tacr1b receptors are duplicate genes and paralogs of the Tacr2 and Tacr3 from zebrafish and orthologs of the TACR1, TACR2, and TACR3 of several species. We performed a double phylogenetic analysis using two different methods in order to corroborate that the replication of the alignment of the branches was indeed due to the divergence of the amino acid sequences, rather than to the phylogenetic algorithms employed. The zebrafish opioid receptors ZfMOR (NP_571782.1), ZfDOR1 (NP_571333.1), ZfDOR2 (NP_997920.1), and ZfKOR (NP_878306.1) were included as out-groups, as these and TACR1 belong to the GPCR superfamily (Herrero-Turrión & Rodríguez 2008) and, additionally, both of them are involved in pain and analgesic processes respectively.

The following species (with their respective accession number) were employed to perform the phylogenetic analyses in the case of TACR1: human (NP_001049.1), dog (NP_001012637.1), guinea pig (NP_001166332.1), rat (NP_036799.1), mouse (NP_033339.2), chicken (NP_990199.1), frog (NP_001106489.1; *Xenopus tropicalis*), fugu (AAQ02694), zebrafish Tacr1a (GenBank ID: 33455), and zebrafish Tacr1b (GenBank ID: 345672; also named Nk1a and Nk1b respectively); for TACR2: human (NP_001048.2), rat (NP_542946.1), mouse (NP_033340.2), and zebrafish Tacr2 (XP_001341981.1) (predicted sequence); in the case of TACR3: human (NP_001050.1), rat (NP_058749.1), mouse (NP_067357.1), and zebrafish Tacr3a1, Tacr3a2, and Tacr3b (Zhou *et al.* 2012).

Temporal expression of *tacr1a* and *tacr1b* during embryogenesis

In this study, the mRNA expression of *tacr1a* was gradually increased from 8 to 72 hpf (Fig. 4A), except at 48 hpf, when *tacr1a* mRNA decreased with respect to 30 hpf. By contrast, the mRNA of *tacr1b* displayed a different expression pattern with respect to *tacr1a* (Fig. 4B). At early developmental stages, from 8 to 22 hpf, no marked changes were observed. When the CNS had been formed

Figure 2

Comparison of the deduced amino acid sequences of the TACR1s from human, rat, mouse, and zebrafish (Zf). Asterisks, colons, and dots denote the identical amino acid, conserved substitutions, and semi-conserved substitutions respectively. Putative membrane-spanning sequences are highlighted in gray and are denoted by TM. N-terminal extracellular domain (N-terminal), carboxyl terminal domain (C-terminal), and IL and extracellular loops (EL) are indicated by horizontal lines above the

sequences. The red circles indicate consensus N-linked glycosylation sites. The red line indicates the typical GPCR moieties, DRY and K/RK/RXXK/R. The black arrows denote the cysteine residues responsible for a disulfide bridge. The red arrow depicts a potential palmitoylation site. The black circles indicate potential intracellular serine and threonine phosphorylation sites. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-12-0199>.

Figure 3

Phylogenetic analyses of vertebrate neurokinin receptors. The evolutionary history was inferred using the NJ (A) and ML (B) methods. The percentage of replicate trees in which the associated taxa clustered together in the

bootstrap test (1000 replicates) is shown next to the branches. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

at 24 hpf, *tacr1b* showed a threefold increase with respect to 22 hpf. The expression of *tacr1b* decreased from 24 to 48 hpf. In addition, at 72 hpf, we observed that *tacr1b* reached the maximum peak of expression with respect to all the developmental stages studied (Fig. 4B).

The 8 hpf stage was selected because at this moment, there is a reliable mRNA expression with no maternal interference. During the early stages of 16, 19, and 22 hpf, the segmentation and differentiation of the CNS are taking place and at 24 hpf, the zebrafish already have the five lobes (telencephalon, diencephalon, epiphysis, midbrain, and cerebellum) that conform the CNS. Organogenesis takes place between 24 and 48 hpf, and at 48 hpf, the morphogenesis of the primary organ systems has been completed. The end of embryonic development occurs at 72 hpf.

***tacr1a* and *tacr1b* mRNA spatial expression at 24 and 48 hpf**

The *tacr1a* transcript was detected at 24 hpf in different areas of the CNS (telencephalon, hypothalamus, diencephalon, midbrain, midbrain–hindbrain boundary (MHB), and hindbrain) and also peripherally in the somites (Fig. 5A and B). The expression of *tacr1b* at 24 hpf, like

that of *tacr1a*, was found in the CNS (Fig. 5E and F; telencephalon, hypothalamus, diencephalon, MHB, midbrain, hindbrain, and spinal cord) and at the periphery (somites). At 48 hpf, *tacr1a* showed a similar pattern of expression in the CNS to that at 24 hpf and it was also expressed in the medial longitudinal fasciculus (MLF). At the periphery, expression was less than at 24 hpf but was observed in somites (Fig. 5C and D). *tacr1b* transcripts at the 48 hpf stage (Fig. 5G and H) were also present in the CNS, similar to at 24 hpf, although with higher expression and with no expression in the spinal cord. At this stage, *tacr1b* was also expressed in the MLF and somites (Fig. 5G and H; but less intensely than at 24 hpf).

Effects of cocaine on the temporal expression of *tacr1a* and *tacr1b*

Embryos exposed to cocaine (at 5 hpf) and analyzed at 24 hpf showed a twofold increase in the expression of *tacr1a* with respect to the control group ($P < 0.0001$; Fig. 6A). Also, the expression of *tacr1b* was increased by the actions of cocaine: threefold higher with respect to the control group ($P = 0.0194$; Fig. 6B). At 48 hpf, cocaine decreased the expression of *tacr1a* ($P = 0.0006$; Fig. 6A). Conversely, cocaine led to a twofold increase in the

Figure 4

Expression of *tacr1a* (A) and *tacr1b* (B). qPCR analyses of transcript levels of neurokinin receptors were performed at several development stages: 8, 16, 19, 22, 24, 30, 48, and 72 hpf. Gene expression levels were measured by fluorescence intensities using qPCR. The reference gene used to normalize the expression of the different genes studied by qPCR was *eflα* (NM_131263.1). Two hundred embryos were used to extract RNA and synthesize cDNA. Error bars represent means of mRNA copies at each developmental stage $\pm$ S.E.M. Three independent experiments (each performed three times) were conducted for each stage.

expression levels of *tacr1b* ($P < 0.0359$; Fig. 6B), in both cases with respect to the control group.

Knockdown of ZfMOR, ZfDOR1, and ZfDOR2 and effects on *tacr1a* and *tacr1b* expression

Knockdown of the ZfDOR2 opioid receptor with a MO elicited nearly eight times less expression of *tacr1a* at 48 hpf with respect to the control group ($P = 0.0010$; Fig. 7A) and the MO control group. Conversely, ZfMOR MO

microinjection induced a fourfold increase in the expression of *tacr1b* ($P < 0.0001$; Fig. 7B) in comparison with the control and MO control groups. The ZfDOR1 and ZfDOR1/2 did not induce changes in the expression of any *tacr1s*.

Effects of cocaine on the level of expression of *tacr1a* and *tacr1b* transfected into HEK-293 cells

HEK-293 cells transfected with *tacr1a* and *tacr1b* coupled to pEGFP-C3 and pDsRed-N-1 respectively did not reveal morphological changes in *tacr1* (Fig. 8A and B) and *tacr1b* (Fig. 8C and D) due to exposure to cocaine. Nevertheless, PCR results showed that cocaine decreased the expression levels of *tacr1a* (Fig. 8E) and *tacr1b* (Fig. 8F). This decrease, especially that of *tacr1a*, being noteworthy (Fig. 8E). We have also corroborated by qPCR that cocaine decreased the expression level of *tacr1a* dramatically ($P < 0.0001$; Fig. 8I), and in the case of *tacr1b*, the changes produced by cocaine were more apparent than those observed before in the PCR experiment (Fig. 8F), as cocaine elicited a reduction ($P < 0.0001$; Fig. 8I). Before exposure to cocaine, we evaluated the toxic effect of cocaine on HEK-293 cells by microscopy observations and we did not find changes of cell viability in cocaine-treated and -untreated groups of cells. The doses of 0.5, 1.5, and 3 μ M were used and none produced toxicity in the cell culture. Moreover, we studied the expression of the reference gene (*GAPDH*) by PCR and qPCR (Fig. 8A and B respectively), where this reference gene does not exhibit consistent changes. This indicates that the effects of cocaine altered the expression of *tacr1* genes and is not related to the toxicity of cocaine on the HEK-293 cells.

Discussion

In this study, the *tacr1a* and *tacr1b* genes in zebrafish, which are duplicate genes of the *TAC1R* gene in mammals have been identified (Pennefather *et al.* 2004). The phylogenetic trees generated with the NJ and ML methods show that these receptors have a monophyletic origin, as the three TACRs derive from a common gene. In vertebrates, there are three TACRs (TACR1, TACR2, and TACR3; Pennefather *et al.* 2004) that were originated during evolution by duplications of a single ancestral receptor. In addition, both phylogenetic trees suggest that the TACRs (related to the pain process) were originated before the development of opioid receptors (analgesic receptors) during evolution, suggesting that during phylogenetic evolution first appeared pain and after its regulatory mechanism system.

Figure 5

Distribution of *tacr1a* and *tacr1b* at 24 and 48 hpf in embryos by whole-mount ISH. *tacr1a* is present in the telencephalon, diencephalon, midbrain–hindbrain boundary, hindbrain, and somites at 24 hpf (A and B). *tacr1b* is present in telencephalon, diencephalon, midbrain, hindbrain, spinal cord, and somites at 24 hpf (E and F). Lateral views (C and D) show that *tacr1a* is present in the telencephalon, diencephalon, midbrain, MLF, hindbrain, heart, and somites. In the case of *tacr1b* (G and H), it is present in the telencephalon, diencephalon, midbrain, MLF, hindbrain, spinal cord, and

somites. Control group (I, J, K and L). All the embryos of the control group, from both *tacr1a* and *tacr1b*, did not show staining; for this reason, we only included one control group. Scale bar, 250 μ m; 6 $\times$ magnification in A, C, E, G, I, and K and 12 $\times$ magnification in B, D, F, H, J, and L. t, telencephalon; d, diencephalon; hy, hypothalamus; m, midbrain; MLF, medial longitudinal fasciculus; h, hindbrain; ov, otic vesicle; sc, spinal cord; s, somites; H, heart. The images obtained were documented with a fluorescent stereomicroscope (Leica M165 FC).

Tacr1a and Tacr1b, displayed characteristics of the seven TM receptor, the two consensus sites for N-glycosylation sites in the human TAC1R (Asn¹⁴ and Asn¹⁸; Takeda *et al.* 1991) are conserved in zebrafish Tacr1a (Asn¹³ and Asn¹⁷) but not in Tacr1b, where the asparagine residues are substituted by Asp¹⁴ and Ala¹⁸ (Fig. 2). This difference in the N-glycosylation pattern between TACR1a and TACR1b could involve changes in the bioactivity of Tacr1b and affect its physical stability as the glycosylation of TACR1 may stabilize the receptor at the plasma membrane (Tansky *et al.* 2007). Two amino acid residues -Val¹¹⁶ and Ile²⁹⁰- in the human TACR1 (Fong *et al.* 1992a) are conserved in zebrafish Tacr1a (Val¹²² and Ile²⁹⁶) and Tacr1b (Val¹²³ and Ile²⁹⁷) receptors and are not present in the rat or mouse, where they are substituted by Leu¹¹⁶ and Ser²⁹⁰. These residues appear to be determinant for the binding of the selective non-peptide antagonist CP 96 345 on the TACR1 type and they are also important in the

differentiation between species (Fong *et al.* 1992b). The replacement of Val¹¹⁶ and Ile²⁹⁰ of the human TACR1 by Leu¹¹⁶ and Ser²⁹⁰ in the rat confers sensitivity to the TACR1 antagonist RP 67 580. Likewise, a non-peptide TACR1 antagonist, WIN 51 708, binds to the rat TACR1-type receptor but not to the human TACR1. This binding is mediated by Ser²⁹⁰ in the rat TACR1 while human TACR1 has Ile²⁹⁰ (Venepalli *et al.* 1992). As zebrafish Tacr1a and Tacr1b have residues of isoleucine (Ile²⁹⁶ and Ile²⁹⁷ respectively; Fig. 2), this organism would be an alternative model in the study of TACR1 antagonist in the field of pain. Additionally, the potential putative phosphorylation sites on serine or threonine residues in zebrafish Tacr1a and Tacr1b show that these receptors may be able to shed new light on the desensitization and internalization of TACR1. It is controversial if fishes feel pain, but during the last decade, important evidence has been shown concerning the fact that fishes can perceive pain.

Figure 6

Effects of cocaine on the expression levels of *tacr1a* (A) and *tacr1b* (B) at 24 and 48 hpf. Two hundred embryos were used to extract RNA and synthesize cDNA. Error bars represent means of mRNA copies at each developmental stage $\pm$ S.E.M. Three independent experiments (each carried out three times) were performed for each stage. *P* values were calculated by a two-tailed unpaired Student's *t*-test: **P* < 0.05 and ****P* < 0.001.

In this sense, the goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) have been reported to present nociceptors that respond to mechanical, thermal, and chemical stimulations (Sneddon 2003a, Dunlop & Laming 2005, Ashley *et al.* 2007) and morphine reduced dramatically the behavioral and physiological responses after injection of a noxious stimulus (Sneddon 2003b). As a teleost fish, zebrafish, has an analgesic system, opioid system (Gonzalez-Nunez & Rodriguez 2009), and according to our findings, this organism also has the receptor of SP, Tacr1a and Tacr1b. It can be deduced that this organism through this nociceptive and tachykinin system can feel pain. Unfortunately, at present, a prototypic behavioral pain model in zebrafish has not been developed, as in mouse and rat, to corroborate the molecular and electrophysiological findings of pain in

fishes (Sneddon 2003a, Dunlop & Laming 2005, Ashley *et al.* 2007). However, all the recent studies using the zebrafish as a model point to this organism as a new potential model in the study of pain.

One interesting finding of our work is that the expression of *tacr1a* (Fig. 4A) is increasing since the early zebrafish stages of development (8 hpf, gastrulation) until the end of the CNS development (~24 hpf), primary organogenesis (48 hpf), and embryogenesis (72 hpf), suggesting that these receptors are involved in the formation of the CNS, organogenesis, and embryogenesis in general. This fact is in agreement with studies that SP, ligand of the TACR1, is increasing in number during embryonic development of the CNS (Gilbert & Emson 1979). Moreover, it has been reported that TAC1R could be important in the development of motoneurons in the rat during embryonic development (St John & Stephens 1992, St John *et al.* 1997). One characteristic shared between *tacr1a* and *tacr1b* is that both are expressed at high levels at 72 hpf, although the expression of *tacr1a* is almost threefold higher with respect to *tacr1b* (Fig. 4A and B). Also, during several embryonic stages of development, *tacr1a* mRNA expression was greater than *tacr1b*. This fact suggests that *tacr1a* could have a major role, during zebrafish embryogenesis, in comparison to *tacr1b*, as a subfunctionalization of the gene can occur after a gene duplication event (Postlethwait 2007).

In zebrafish, *tacr1a* and *tacr1b* (Fig. 5) were mainly expressed at 24 hpf in the CNS (telencephalon, midbrain, diencephalon, hypothalamus, and hindbrain) and the peripheral (nervous system somites), with the difference that *tacr1b* was also expressed in the spinal cord. The expression of *tacr1b* in the spinal cord is consistent with the findings of TACR1 (mRNA) expression in the rat spinal cord (McCarson & Krause 1994, 1996, McCarson 1999, Todd *et al.* 2002, Gamboa-Estevés *et al.* 2004), suggesting that in the spinal cord *tacr1b* might play a major role in comparison with *tacr1a*.

There is evidence of interaction of DOR/PPTA, as it has been reported that DOR coexist with PPTA, in the rat dorsal root ganglia in the primary afferent neurons (Minami *et al.* 1995) and PPTA can modulate the sensitivity of nociceptive afferents to opioids (Guan *et al.* 2005). In the present work, we report that knockdown of ZfDOR2 downregulates the expression of *tacr1a*, which suggests that DOR/TAC1R can have an important role during embryonic development and also in pain modulation (Minami *et al.* 1995, Guan *et al.* 2005). The difference of our work in relation to Minami *et al.* (1995) and Guan *et al.* (2005) is that we studied the mRNA

Figure 7

Effects of MOs on the expression levels of *tacr1a* (A) and *tacr1b* (B) at 48 hpf. MOs of ZfMOR, ZfDOR1, ZfDOR2, and ZfDOR1–ZfDOR2 (ZfDOR1/2) were microinjected into the yolk in the one- to four-cell stage. Two hundred embryos were used for RNA extraction. Each bar represents the number of copies of cDNA $\pm$ S.E.M. For each stage, three experiments were performed, each three times. Between-group differences in *sp*, *tacr1a*, and *tacr1b* levels were analyzed using ANOVA with the *post hoc* Dunnett's test: * $P < 0.05$ and *** $P < 0.001$.

expression (of Tacr1a and Tacr1b) in whole-mount zebrafish embryos in one stage of embryonic development (48 hpf) while the other studies were done in adult rats and mice. Also, several studies have described that TACR1/MOR functionally interact and mediate the regulation of MOR–TACR1 trafficking, where the activation of TACR1 by SP can induce or inhibit the endocytosis of MOR (Pfeiffer *et al.* 2003, Yu *et al.* 2009). Our results show that downregulation of ZfMOR increases the expression of *tacr1b*, suggesting that MOR could be positively regulating TACR1. According to our results, a possible mechanism of ZfDOR2 and ZfMOR antisense knockdown leading to changes in expression of *tacr1a* and *tacr1b* could be explained as an interaction of these receptors in the cell membrane or gene expression, but these postulates have to corroborate with future studies.

The importance of our research is that the tachykinin system and the opioid receptors are closely related, where changes induced in the tachykinin system could modify the functions of opioid receptors. As DOR and MOR interact (in the cell membrane) and DOR modulates the function of MOR (Gomes *et al.* 2004), TACRs (via MOR or indirectly via DOR) would modulate the activity of the opioid receptors. In this study, the knockdown of ZfDOR2 and ZfMOR regulated the expression of *tacr1a* and *tacr1b* respectively, indicating that tachykinin and the opioid systems are closely interrelated in zebrafish, as has been described in mammals (Minami *et al.* 1995, Pfeiffer *et al.* 2003, Guan *et al.* 2005, Yu *et al.* 2009). As the set of MOs of opioid receptors was effective, as previously demonstrated by Sanchez-Simon *et al.* (2010), and replicated in our study, we are convinced that the changes on the expression of the TACRs are a consequence of the downregulation of the opioid receptors. As we are studying the changes of gene expression *in vivo* whole-mount zebrafish embryos, it is difficult to know whether the effects that we observe are specifically produced by opioid receptors as other genes or elements can also regulate both the opioid and the tachykinin system.

SP is considered a neurotransmitter of the primary sensory neurons that mediate the synaptic transmission of pain (Otsuka & Yoshioka 1993, Seybold 2009), which means that changes observed by cocaine actions in the mRNA expression of *tacr1a* and *tacr1b* (Fig. 6A and B respectively) could affect pain processes and alterations in the development of pain and hyperalgesia (Stanley & Safford 2004) such as in embryogenesis. Conversely, our results show that cocaine exerts different actions in human cells and in zebrafish embryos, probably because in whole organisms cocaine must cross different barriers before exerting its actions on its targets, and also, it should be considered that it can be metabolized. As cocaine presents a high liposolubility, this drug rapidly passes through the cell membrane by passive transport (Schenker *et al.* 1993); this could explain the alterations of the expression of the *tacr1a/b* genes transfected in the HEK-293 cells. It would be interesting to study the amount of cocaine that is passing through the HEK-293 cell membrane. Moreover, according to our previous study, ~12% 1.5 μ M cocaine is passing the chorion of zebrafish embryos (R López-Bellido, K Barreto-Valer, FM Sanchez-Simon & RE Rodriguez 2012, unpublished observations) and up to 80% the placenta (Schenker *et al.* 1993). Thus, as we found that 1.5 μ M cocaine did not induce toxicity in the HEK-293 cells and produced no evident changes in the expression of *tacr1a* and *tacr1b* and the reference gene

Figure 8

HEK-293 cells expressing *tacr1a* and *tacr1b* from zebrafish. HEK-293 cells were transfected with *tacr1a*-GFP receptor (A and B) and *tacr1b*-DsRed receptor (C and D). The pictures on the left and on the right correspond to the control (A and C) and cocaine groups (B and D) respectively. Scale bar, 100 μ M. The pictures were taken using epifluorescent microscope (Eclipse TE2000-S, Nikon). Cocaine induced a decrease in *tacr1a* (E) and *tacr1b* (F), *GAPDH* was

used as a reference gene for both *tacr1a* (G) and *tacr1b* (H) expression in the PCR results. Effects of cocaine on the expression of *tacr1a* and *tacr1b* (I and J) analyzed by qPCR. Cocaine induced a decrease in *tacr1a* and *tacr1b* expression (I), *GAPDH* was used as a reference gene (J). Three independent experiments, each performed three times, were carried out. *P* values were calculated by unpaired two-tailed Student's *t*-test: ****P* < 0.001.

GAPDH, we believe that changes observed in the expression of *tacr1s* are due to the action of cocaine. HEK-293 cells do not express the dopamine transporter (DAT; Storch *et al.* 1999), and for this reason, we believe that cocaine is not working via DAT.

In conclusion, the *tacr1s* from zebrafish, *tacr1a* and *tacr1b*, are the product of a gene that became duplicated during evolution. The expression of *tacr1a* and *tacr1b* mRNAs in the CNS and at the periphery during the embryonic stages of development shows that the tachykinin system could exert important actions in embryogenesis in zebrafish. Also, opioid receptors can regulate the expression of *tacr1a* and *tacr1b*, which indicates that the opioid and tachykinin systems are tightly linked. Finally, the influence of cocaine on the expression level of *tacr1a* and *tacr1b* could affect embryogenesis in zebrafish.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R L-B and R E R conceived and designed the experiments. R L-B performed the experiments. R L-B and R E R analyzed the data. R L-B and K B-V contributed reagents/materials/analysis tools. R L-B and R E R wrote the manuscript.

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