Author Accepted Manuscript (AAM)

Evidence for the Existence of Dopamine D2–Oxytocin Receptor Heteromers in the Ventral and Dorsal Striatum with Facilitatory Receptor–Receptor Interactions

W. Romero-Fernández; D.O. Borroto-Escuela; L.F. Agnati; K. Fuxe

Published article: Romero-Fernández W, Borroto-Escuela DO, Agnati LF, Fuxe K. Evidence for the Existence of Dopamine D2–Oxytocin Receptor Heteromers in the Ventral and Dorsal Striatum with Facilitatory Receptor–Receptor Interactions. Molecular Psychiatry. 2013;18(8):849–850. doi:10.1038/mp.2012.103.

This document is the peer-reviewed author accepted manuscript (AAM). The final Version of Record is available at the publisher via the DOI link above.

Repository deposit note: please cite the published version when referencing this work.

Evidence for the existence of dopamine d2-oxytocin receptor heteromers in the ventral and dorsal striatum with facilitatory receptor-receptor interactions

Wilber Romero-Fernandez[a], Dasiel O. Borroto-Escuela[a],

Luigi F. Agnati[b] and Kjell Fuxe[a]

[a]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden. Email: (WRF) Wilber.Romero-Fernandez@ki.se, (DOBE) Dasiel.Borroto-Escuela@ki.se and (KF) Kjell.Fuxe@ki.se

[b]IRCCS Lido Venice, Italy. E-mail: luigiagnati@tin.it

Corresponding author: Kjell Fuxe, Professor, Retzius väg 8, 17177 Stockholm, Sweden. Tel: +46 8 52487077; Fax: +46 8 315721; E-mail: Kjell.Fuxe@ki.se.

The pioneering work of the Insel group has demonstrated a key role of oxytocin neurons and their striatal oxytocin receptors (OTR) in producing pair bonding in the monogamous prairie vole female,[1,2] with the oxytocin neurons likely communicating mainly via volume transmission.[3,4] The prairie vole, but not the polygamous montane vole, displays a high density of OTR in the nucleus accumbens (Acb) and the dorsal striatum. The oxytocin neurons appear to be also the key regulators of social and emotional behaviours in mice, rats, sheep and in humans, including love, bonding and trust.[5--7] Research has indicated a therapeutic role of oxytocin in patients with autism spectrum disorders.[8]

Ascending dopamine neurons are also needed for social attachment in female prairie voles and partner preference is blocked by a dopamine D2 receptor (D_2R) but not a D_1R antagonist.[2] Microinjection of a D_2R agonist into the Acb can cause partner preference in the absence of mating, a finding blocked by either a D_2R or an OTR antagonist. These results indicate that coactivation of D_2R and OTR in the Acb is of importance for pair bond formation and maintenance.[1,2,9,10] In the current study, we have tested the hypothesis that the molecular mechanism for these interactions is to a great extent due to dopamine D2 receptor--oxytocin receptors (D_2R--OTR) heteromers in the striatum possessing facilitatory receptor--receptor interactions.

The *in situ* proximity ligation assay (PLA) reveals the existence of red clusters (blobs) of

D_2R and OTR representing D_2R--OTR heteromers in the neuropil of Acb and in the dorsal striatum, but not in the anterior commissure (Figure 1, Supplementary Figures 1A and B), nor in the external capsule (Supplementary Figure 1B). These results are validated in HEK293T cells transiently cotransfected with human D_2R and OTR as to colocalisation (Supplementary Figure 2) and development of PLA-positive signals (Supplementary Figure 3). Bioluminescence resonance energy transfer (BRET) analysis of the HEK293T cells after cotrans-fection of the D_2R^{Rluc} and OTR^{GFP2} further validated the striatal PLA findings by demonstrating a strong BRET signal as seen from the $BRET_{max}$ values (Table 1, Supplementary Figure 4).

Our results indicate the existence of a facilitatory allosteric receptor--receptor interaction in the demonstrated D_2R--OTR heteromer. Oxytocin at 3 nM but not at 1 or 100 nM produced a significant increase by 52±9% in the B_{max} value without a change in the K_D value of the D2-likeR antagonist [^{3}H]--raclopride-binding sites in membrane preparations from the Acb (Table 1, Supple-mentary Figure 5). Thus, the allosteric receptor--receptor inter-actions in the heteromer induced by oxytocin may make more D_2R available for binding the D2 antagonist to its orthosteric site, which likely leads to increased D_2R recognition. Furthermore, in membrane preparations from the Acb oxytocin at 3 nM, but not at 1 and 100 nM, highly significantly increased the affinity of the high-(Ki_H) but not the low-affinity (Ki_L) agonist state of the D_2R, as seen from a marked reduction of the Ki_H value from 16.58±0.74 to 1.89±0.70 nM (Table 1, Supplementary Figure 6). The major elevation of the OTR-induced increase in D_2R recognition may therefore result from an increase in the affinity of the high-affinity state of the D_2R. The oxytocin actions at 3 nM on D_2R-like recognition are all blocked by the OTR antagonist L-368,899 (30 nM, Supplementary Figure 6).

The GTPgS accumulation assay results give evidence that oxytocin 3 nM also increases the D_2R/Gi/o coupling as seen from a significant increase in the B_{max} values (33±1%) and a significant reduction of the EC50 values (Table 1, Supplementary Figure 7). The oxytocin enhancement of DA-induced GTPgS accumulation, known to be mediated by D_2R was counteracted by L-368,899. These results are in line with our demonstration of an oxytocin-induced increase in D_2R-like recognition (increased agonist affinity and density) at this concentration, which may to a high degree mediate the increase in D_2R/Gi/o coupling produced by oxytocin in the membrane preparations from the Acb.

The results give experimental support to our hypothesis that facilitatory allosteric D_2R--OTR interactions in the Acb receptor heteromers may represent at least a significant part of the molecular mechanism for oxytocin-induced changes in social and emotional behaviour.[1,2,7] However, the existence of D_3R--OTR heteromers in the

Acb with similar receptor--receptor interaction cannot be excluded. D_2R--OTR heteromers may become a new target for drug development and treatment of dysfunctions in the emotional networks of the brain.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Research Council (04X-715), Hja¨rnfonden, Telethon TV3's La Marato´ Foundation 2009 and M.M. Wallenberg Foundation to KF, Karolinska Institutets Forskningsstiftelser 2010 and 2011 to DOB-E. and KF.

REFERENCES

1 Young LJ, Lim MM, Gingrich B, Insel TR. *Horm Behav* 2001; 40: 133--138.

2 Young LJ, Wang Z. *Nat Neurosci* 2004; 7: 1048--1054.

3 Fuxe K, Borroto-Escuela DO, Romero-Fernandez W, Ciruela F, Manger P, Leo G *et al. Brain Res* 2012 (in press).

4 Ross HE, Young LJ. *Front Neuroendocrinol* 2009; 30: 534--547.

5 Lee HJ, Macbeth AH, Pagani JH, Young III WS. *Prog Neurobiol* 2009; 88: 127--151.

6 Petersson M, Diaz-Cabiale Z, Angel Narvaez J, Fuxe K, Uvnas-Moberg K. *Brain Res* 2005; 1049: 234--239.

7 Neumann ID. *J Neuroendocrinol* 2008; 20: 858--865.

8 Green JJ, Hollander E. *Neurotherapeutics* 2010; 7: 250--257.

9 Aragona BJ, Liu Y, Curtis JT, Stephan FK, Wang Z. *J Neurosci* 2003; 23: 3483--3490.

10 Gingrich B, Liu Y, Cascio C, Wang Z, Insel TR. *Behav Neurosci* 2000; 114: 173--183.

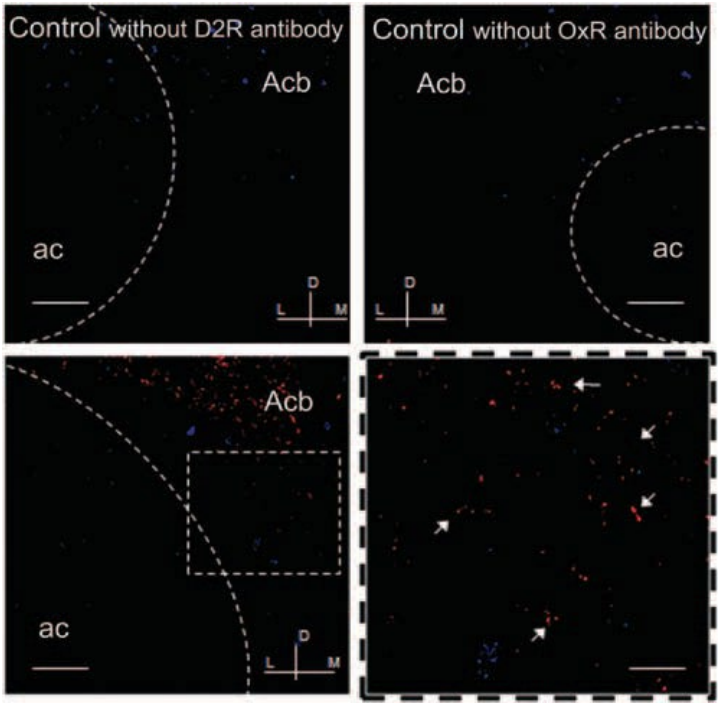


Figure 1. *In situ* proximity ligation assay (PLA)-detected dopamine D2 receptor--oxytocin receptor (D_2R--OTR) heteromers in striatal sections of rat. *In situ* PLA was performed using primary antibodies of different species directed to D_2R and OTR followed by PLA reagents. The detected receptor heteromers are represented by the fluorescent PLA rollingcircle products (seen as red clusters indicated by arrows). Each punctum represents a high concentration of fluorescence from the single-molecule amplification resulting from a several hundred-fold replication of the DNA circle formed as a result of the probe proximity. Specific D_2R--OTR heteromers were visua-lised in coronal sections (Bregma 1.0 mm) within Acb, and almost absent within the anterior commisure (ac). Control experiments employed only one primary antibody. Nuclei are shown in blue (DAPI). Scale bars, 50 mm (left) and 20 mm (right). M (medial), L (lateral) and D (dorsal) direction.

Table 1. Evidence for facilitatory allosteric receptor-receptor interaction in the D_2R-OTR heteromer

Receptors D_2R-OTR	*BRET² $D_{2L}R^{RLuc}/OxR^{GFP2}$*		*[³H]-raclopride binding saturation*		*[³H]-raclopride competition with dopamine*		*Dopamine-induced Gi/o protein stimulation*	
	$BRET_{max}$	*$BRET_{50}$*	*B_{max} (fmol mg^{ÿ1} protein)*	*K_D (nM)*	*Ki_H (nM)*	*Ki_L (mM)*	*EC_{50} (mM)*	*E_{max} (% over basal)*
Without oxytocin	62.4 ± 2.8	0.21 ± 0.03	154 ± 20	1.21 ± 0.34	16.58 ± 0.74	1.23 ± 0.82	1.33 ± 0.19	24 ± 2
With oxytocin (3 nM)	ND	ND	235 ± 23*	1.40 ± 0.27	1.89 ± 0.70***	1.51 ± 0.88	0.21 ± 0.14*	33 ± 1**

Abbreviations: BRET, bioluminescence resonance energy transfer; D2R--OTR, dopamine D2 receptor--oxytocin receptors; ND, not determined. Modulations by oxytocin (3 nM) are reported on D_2R recognition and D_2R-Gi/o coupling in Acb membranes of the rat. The $BRET_{max}$ and $BRET_{50}$ values are also shown. Data are presented as the mean ± s.e.m. from three--five independent experiments performed in triplicate. The D_{2L} represent the long isoform of the D_2R. *P<0.05; **P<0.01; ***P<0.001 by Student *t*-test. ND, not determined.

DOPAMINE D2-OXYTOCIN RECEPTOR HETEROMERS AND THEIR RECEPTOR-RECEPTOR INTERACTION IN THE VENTRAL AND DORSAL STRIATUM. RELEVANCE FOR SOCIAL BEHAVIOUR

Supplementary Material

Wilber Romero-Fernandez[a], Dasiel O. Borroto-Escuela[a],

Luigi F. Agnati[b] and Kjell Fuxe[a]

[a]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden. Email: (WRF) Wilber.Romero-Fernandez@ki.se, (DOBE) Dasiel.Borroto-Escuela@ki.se and (KF) Kjell.Fuxe@ki.se

[b]IRCCS Lido Venice, Italy. E-mail: luigiagnati@tin.it

Corresponding author: Kjell Fuxe, Professor, Retzius väg 8, 17177 Stockholm, Sweden. Tel: +46 8 52487077; Fax: +46 8 315721; E-mail: Kjell.Fuxe@ki.se.

Online Supplementary Information contents :

Materials and Methods

Animals

All studies involving animal were performed in accordance with guidelines from the Swedish National Board for Laboratory Animal and EU guidelines for accommodation and care of Laboratory Animals (appendix A). Male Sprague-Dawley rats, 10 weeks old, weighing 310–350 g were obtained from Charles River Laboratories (Germany). The animals were housed one week before experiments under 12-h light/dark cycle, with ambient temperature of 21 ± 2 °C, relative humidity of 50-5%. Food and water available *ad libitum*. Shelter and nesting material were used as environment enrichments.

Membrane Preparation

Nucleus accumbens rat membrane preparations were prepared for their use in radioligand binding experiments. Rats were sacrificed by decapitation, and the brains were removed and cooled for 2 min in ice-cold saline. The Nucleus accumbens from each animal was dissected, removed, and immediately processed. Nucleus accumbens tissue was placed in 10 ml of 50 mM Tris-HCl, pH 7.4, containing 100 nM NaCl, 7 mM MgCl2, 1mM EDTA and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). The tissue was homogenized using a mechanical tissue homogenizer and samples were subjected to centrifugation at 4°C for 30 min at 40,000 x g. The pelleted membranes were resuspended and homogenized in the same buffer and centrifuged an additional three times. The protein concentration was determined for the membrane homogenates by means of BCA Protein Assay (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standard. Pelleted membranes were resuspended to a concentration of 2 mg/ml, immediately used or stored at -80°C until required.

Double-Immunolabeling

HEK293T cells expressing D_2R-OxR were washes twice with Tris buffer saline (TBS), pH 7.4 containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 1% Triton X-100 for 5 min, slices were treated with 10% FBS and 0,5% Triton X-100 in TBS (buffer B). After 1 h at room temperature, receptors were double immunostained with the indicated primary antibody at 4°C overnight, extensively washed, and stained with the indicated fluorescence labelled secondary antibody conjugated with Alexa dyes for 1 h at room temperature. The coverslips were rinsed in TBS (0.1 % Tween-20), mounted in a Vectashield immunofluorescence medium (Vector Laboratories, UK) and visualized with a 40x oil immersion objective in a Leica SP2 confocal microscope (Leica, USA). The primary antibodies used were as follows: rabbit anti-D_2R antibody (1:800; Bjelke et al. 1996 (Bjelke et al., 1996); a kind gift from Dr. Stanley Watson, University of Michigan), goat polyclonal anti-oxytocin receptor (5µg/ml; ab87312 from abcam, Sweden). The secondary antibodies used were as follows: Alexa Fluor 546-conjugated goat anti-rabbit IgG (1:2000; Invitrogen, Stockholm, Sweden), Alexa Fluor 488-conjugated donkey anti-goat IgG (1:2000; Invitrogen, Stockholm, Sweden). The amounts of D_2R/OxR complex colocalization are shown as a single z-scan image.

Proximity ligation *in situ* assay (Duolink)

To investigate whether dopamine D_2R has the capability to interact with OxR in HEK293T cells transiently expressing D_2R-OxR or rat striatum slide sections we used *in situ* proximity ligation assay (PLA) (Borroto-Escuela et al., 2011c;Trifilieff et al., 2011;Borroto-Escuela et al., 2012). *In situ* PLA was performed according to manufacturer's instructions (Duolink *in situ* PLA detection kit (Olink, Sweden)). The primary antibodies of different species directed to D_2R (rabbit anti-D_2R antibody

(1:800; (Bjelke et al., 1996); a kind gift from Dr. Stanley Watson, University of Michigan) and to OxR (5µg/ml; goat polyclonal anti-oxytocin receptor; ab87312 from abcam) was used. Control experiments employed only one primary antibody or HEK293T cells transfected with cDNAs encoding only one type of receptor. The products were visualized using a Leica SP2 confocal microscope (Leica, USA).

D_2R-OxR interaction using BRET^{2}assay

A BRET assay was performed as previously described (Borroto-Escuela et al., 2011a;Borroto-Escuela et al., 2011b;Romero-Fernandez et al., 2011;Borroto-Escuela et al., 2012) . Briefly, for BRET^2 saturation assay, forty-eight hours after transfection, HEK293T cells transiently transfected with constant (1 µg) or increasing amounts (0.25–9 µg) of plasmids encoding for $D_{2L}R^{Rluc}$ and OxR^{GFP2}, respectively, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20 µg protein) were distributed in duplicate into the 96-well microplate black plates with a transparent bottom (Corning 3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination.

For BRET^2 measurement, coelenterazine-400a, also known as DeepBlue™C substrate (VWR, Sweden), was added at a final concentration of 5 µM, and readings were performed 1 min after using the POLARstar Optima plate reader (BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings [410 nm (with 80 nm bandwidth) and 515 nm (with 30 nm bandwidth)]. The BRET^2 ratio is defined as previously described (Borroto-Escuela et al 2010).

[^{3}H]-raclopride binding Experiments

Saturation binding experiments with the D2-likeR antagonist [^{3}H]-raclopride (specific activity 82.8 Ci/mmol, PerkinElmer Life Sciences, USA) was performed as previously described (Dasgupta et al., 1996;Torvinen et al., 2004). Briefly, nucleus accumbens rat membrane preparations (100 µg protein/ml) were incubated with increasing concentrations of [^{3}H]-raclopride (ranging from 0.1 nM - 10 nM) in 250 µl of incubation buffer (IB; 50 mM Tris-HCl pH 7.4, containing 100 mM NaCl, 7 mM MgCl2, 1 mM EDTA and 1 mM dithiothreitol) for 60 min at 30 °C, in the presence or absence of 3 nM ocytocin. Nonspecific binding was defined as the binding in the presence of 100 µM dopamine hydrochloride (Sigma-Aldrich, Aldrich, St. Louis, MO, USA). The incubation was terminated by rapid filtration through Whatman GF/B filters (Maidstone, Kent, UK) using a MultiScreen™ Vacuum Manifold 96-well (Millipore Corp, Bedford, MA), followed by three washes (~250µl per wash) with ice-cold washing buffer (50 mM Tris-HCl pH 7.4). The filters were dried, 5 ml of scintillation cocktail was added and the bound ligand was determined after 12 h for 4 min each sample by liquid scintillation spectrometry.

[^{3}H]-raclopride (2.0-3.0 nM) binding was displaced by dopamine to determine agonist affinities from the competition curves obtained. Briefly, nucleus accumbens rat membrane preparations (100 µg protein/ml) were incubated with increasing concentrations of dopamine (ranging from 0.1 nM - 1 mM) in 250 µl of IB for 60 min at 30 °C, in the presence or absence of 3 nM ocytocin. The incubation was terminated as described above and the radioactivity content of the filters was detected by liquid scintillation spectrometry. Nonspecific binding was defined by radioligand binding in the presence of 10 µM (+)-butaclamol (Sigma Aldrich, St. Louis, MO).

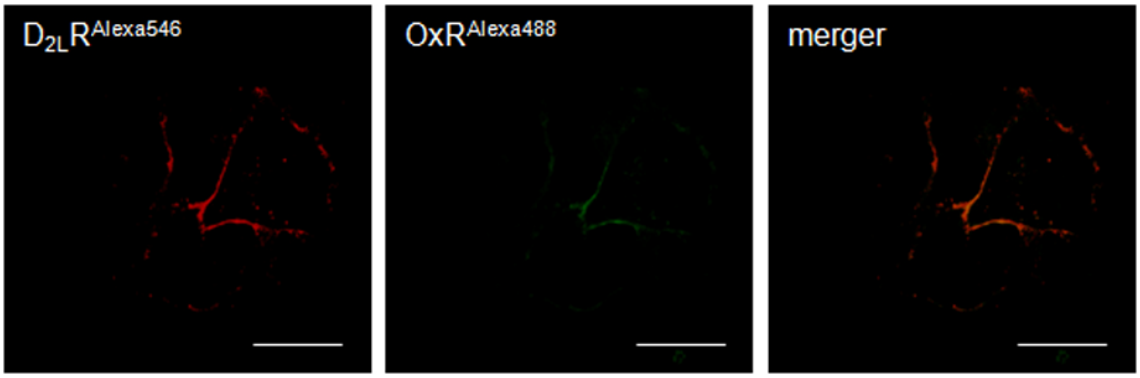
[^{35}S]-GTPγS binding assays

The [^{35}S]-labeled guanosine 5′-*O*-(γ-thio)triphosphate ([^{35}S]-GTPγS; specific activity 1250 Ci/mmol, PerkinElmer Life Sciences, USA) binding experiments were performed essentially as described by Riken et al. (1999). Briefly, aliquots of the diluted nucleus accumbens rat membrane preparations (equivalent to 25 µg protein) were incubated with 0.08-0.10 nM [^{35}S]-GTPγS, 20 µM guanosine-5'-diphosphate sodium salt (GDP; Sigma Aldrich, USA) and increasing concentrations of dopamine (1 nM - 1 mM) in 250 µl of 20 mM K-Hepes, pH 7.6 containing 7 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, in the absence or in the presence of 3 nM of oxytocin. Reactions were incubated at 30°C for 60 min. Binding reactions were terminated by rapid filtration through Whatman GF/B filters (Maidstone, Kent, UK) using a MultiScreen™ Vacuum Manifold 96-well (Millipore Corp, Bedford, MA), followed by three washes (~250µl per wash) with ice-cold washing buffer (20 mM K-Hepes, pH 7.6). The washed filters were dry and resuspended in 5 ml scintillation cocktail, vortex mixed and radioactivity was detected by a liquid scintillation counter. The non-specific binding was measured in the presence of 10 µM (+)-butaclamol. Basal [^{35}S]-GTPγS (agonist-independent binding) was defined as the specific binding when drug solvent without agonist or antagonist was added to assay well.

Statistical analysis

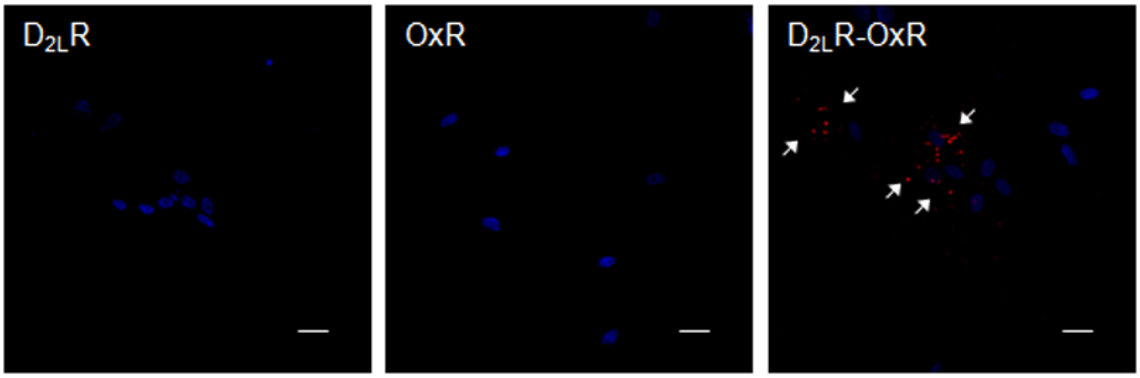
The number of samples (*n*) in each experimental condition is indicated in figure legends. Data from saturation and competition experiments were analyzed by nonlinear regression analysis using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA), and the density of the binding sites (B_{max}) and the dissociation constant (K_D) values from several independent replications were averaged to permit statistical comparisons (Student's *t*-test) between experiments carried out in the presence or absence of the oxytocin.

Figure 1



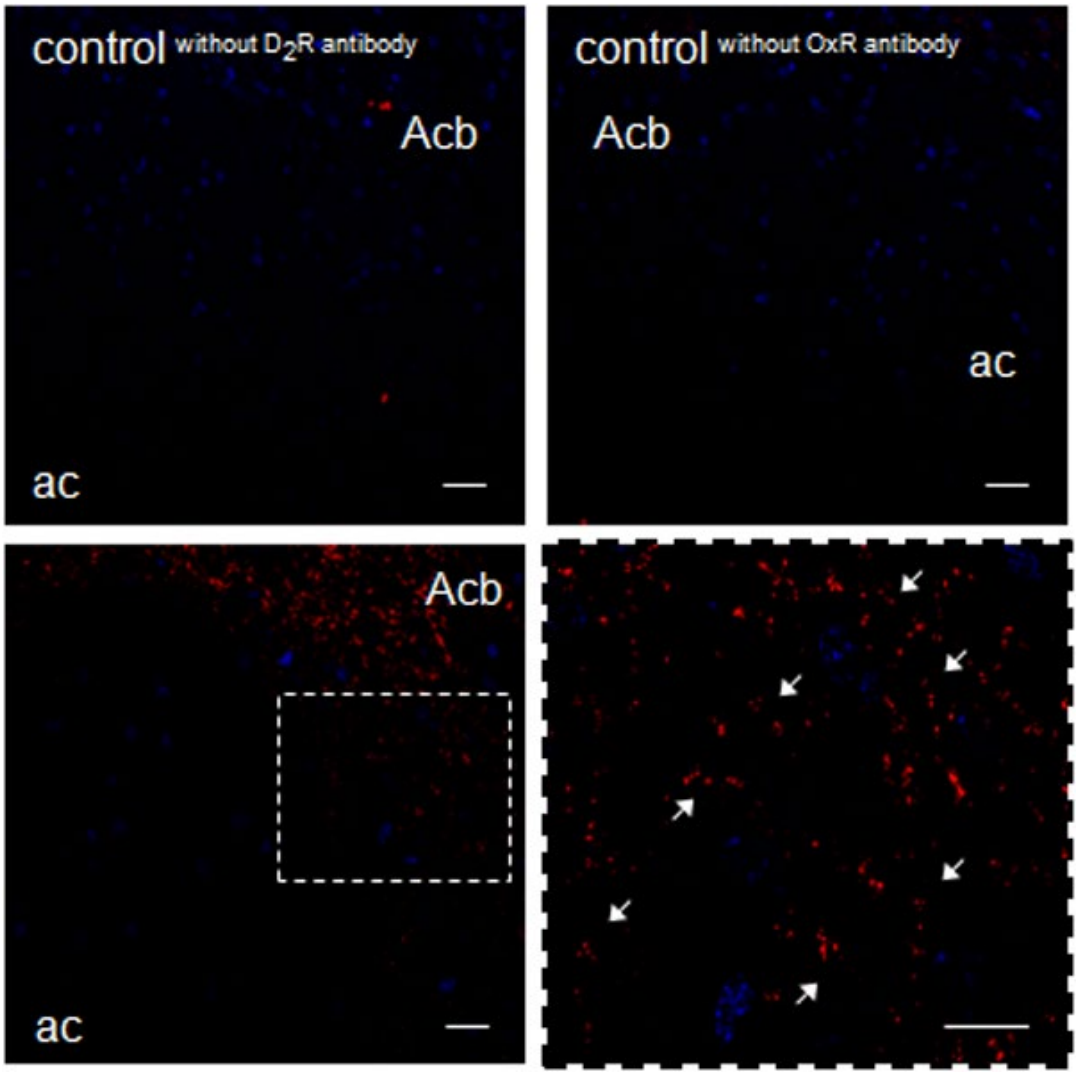
Scale bar 1:50

Figure 2



Scale bar 1:10

Figure 3



Scale bar 1:10 and 1:20

Figure 4

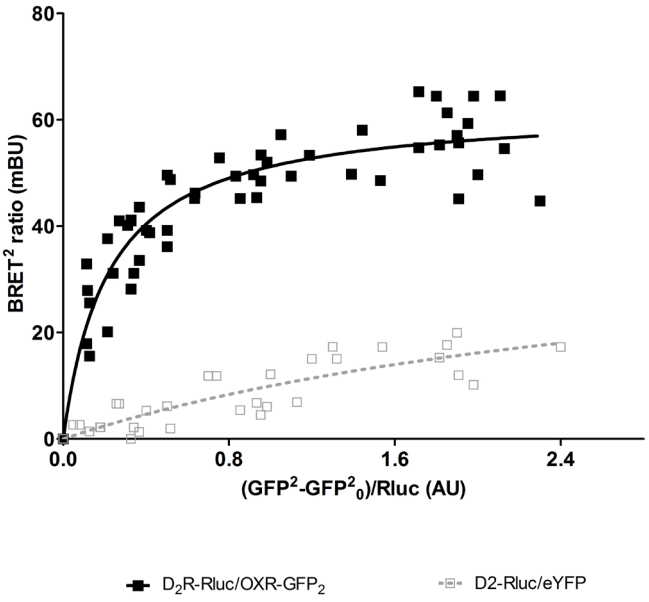
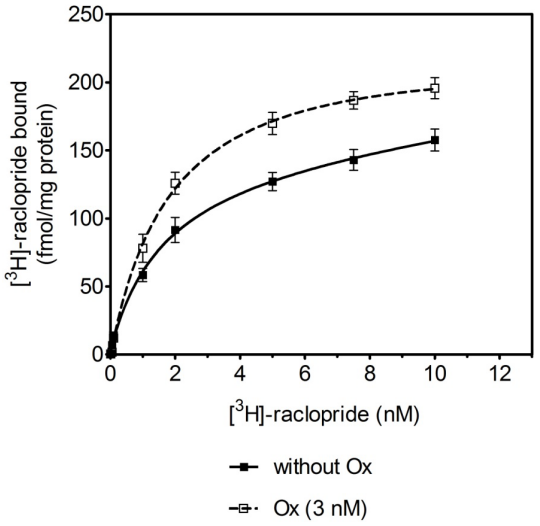
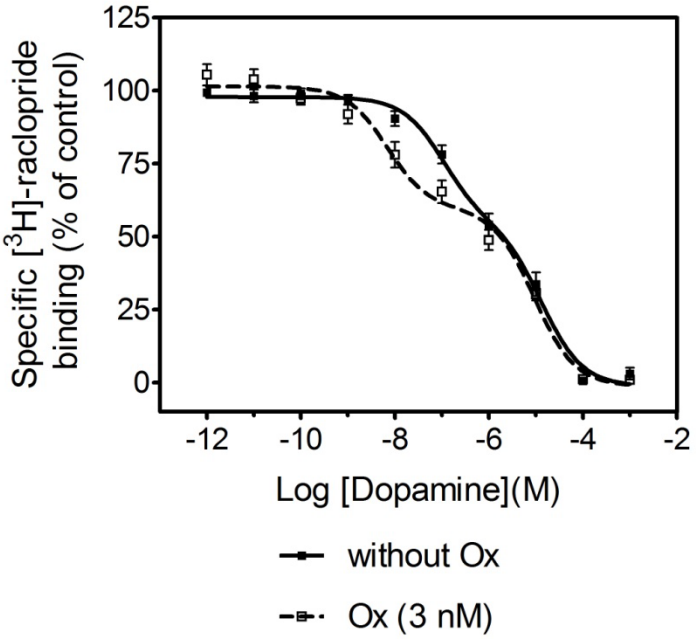


Figure 5



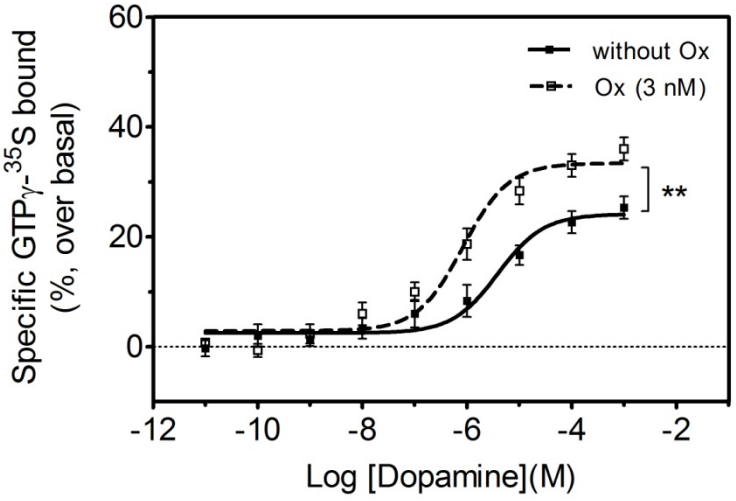
Receptors	B_{max} *(fmol/mg protein)*	K_D *(nM)*
D2R-OxR	146 ± 38	1.60 ± 0.76
D2R-OxR + Ox (3nM)	248 ± 50*	1.93 ± 0.64

Figure 6



Receptors	Ki_H *(nM)*	Ki_L *(µM)*
D2R-OxR	16.58 ± 0.18	1.23 ± 0.15
D2R-OxR + Ox (3nM)	1.89 ± 0.19***	1.51 ± 0.13

Figure 7



Receptors	EC_{50} *(μM)*	EC_{max} *(% over basal)*
D2R-OxR	3.87 ± 0.19	24 ± 2
D2R-OxR + Ox (3nM)	0.83 ± 0.14***	33 ± 1**

Bjelke, B., Goldstein, M., Tinner, B., Andersson, C., Sesack, S.R., Steinbusch, H.W., Lew, J.Y., He, X., Watson, S., Tengroth, B., and Fuxe, K. (1996). Dopaminergic transmission in the rat retina: evidence for volume transmission. *J Chem Neuroanat* 12**,** 37-50.

Borroto-Escuela, D.O., Romero-Fernandez, W., Mudo, G., Perez-Alea, M., Ciruela, F., Tarakanov, A.O., Narvaez, M., Di Liberto, V., Agnati, L.F., Belluardo, N., and Fuxe, K. (2012). Fibroblast growth factor receptor 1- 5-hydroxytryptamine 1A heteroreceptor complexes and their enhancement of hippocampal plasticity. *Biol Psychiatry* 71**,** 84-91.

Borroto-Escuela, D.O., Romero-Fernandez, W., Tarakanov, A.O., Ciruela, F., Agnati, L.F., and Fuxe, K. (2011a). On the existence of a possible A2A-D2-beta-Arrestin2 complex: A2A agonist modulation of D2 agonist-induced beta-arrestin2 recruitment. *J Mol Biol* 406**,** 687-699.

Borroto-Escuela, D.O., Romero-Fernandez, W., Tarakanov, A.O., Marcellino, D., Ciruela, F., Agnati, L.F., and Fuxe, K. (2011b). Dopamine D2 and 5-hydroxytryptamine 5-HT(A) receptors assemble into functionally interacting heteromers. *Biochem Biophys Res Commun* 401**,** 605-610.

Borroto-Escuela, D.O., Van Craenenbroeck, K., Romero-Fernandez, W., Guidolin, D., Woods, A.S., Rivera, A., Haegeman, G., Agnati, L.F., Tarakanov, A.O., and Fuxe, K. (2011c). Dopamine D2 and D4 receptor heteromerization and its allosteric receptor-receptor interactions. *Biochem Biophys Res Commun* 404**,** 928-934.

Cabello, N., Gandia, J., Bertarelli, D.C., Watanabe, M., Lluis, C., Franco, R., Ferre, S., Lujan, R., and Ciruela, F. (2009). Metabotropic glutamate type 5, dopamine D2 and adenosine A2a receptors form higher-order oligomers in living cells. *J Neurochem* 109**,** 1497-1507.

Dasgupta, S., Ferre, S., Kull, B., Hedlund, P.B., Finnman, U.B., Ahlberg, S., Arenas, E., Fredholm, B.B., and Fuxe, K. (1996). Adenosine A2A receptors modulate the binding characteristics of dopamine D2 receptors in stably cotransfected fibroblast cells. *Eur J Pharmacol* 316**,** 325-331.

Romero-Fernandez, W., Borroto-Escuela, D.O., Tarakanov, A.O., Mudo, G., Narvaez, M., Perez-Alea, M., Agnati, L.F., Ciruela, F., Belluardo, N., and Fuxe, K. (2011). Agonist-induced formation of FGFR1 homodimers and signaling differ among members of the FGF family. *Biochem Biophys Res Commun* 409**,** 764-768.

Torvinen, M., Kozell, L.B., Neve, K.A., Agnati, L.F., and Fuxe, K. (2004). Biochemical identification of the dopamine D2 receptor domains interacting with the adenosine A2A receptor. *J Mol Neurosci* 24**,** 173-180.

Trifilieff, P., Rives, M.L., Urizar, E., Piskorowski, R.A., Vishwasrao, H.D., Castrillon, J., Schmauss, C., Slattman, M., Gullberg, M., and Javitch, J.A. (2011). Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D2-adenosine A2A receptor complexes in the striatum. *Biotechniques* 51**,** 111-118.