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Dopamine D2 receptor signaling dynamics of dopamine D2-neurotensin 1 receptor heteromers

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ABSTRACT

Biochemical, histochemical and coimmunoprecipitation experiments have indicated the existence of antagonistic dopamine D2 (D_2R) and neurotensin 1 (NTS1R) receptor–receptor interactions in the dorsal and ventral striatum indicating a potential role of these receptor–receptor interactions in Parkinson's disease and schizophrenia. By means of Bioluminiscence Resonance energy transfer ($BRET^2$) evidence has for the first time been obtained in the current study for the existence of both $D_{2L}R$/NTS1R and $D_{2S}R$/NTS1R heteromers in living HEK293T cells. Through confocal laser microscopy the $NTS1R^{GFP2}$ and $D2R^{YFP}$ were also shown to be colocated in the plasma membrane of these cells. A bioinformatic analysis suggests the existence of a basic set of three homology protriplets (TVM, DLL and/or LRA) in the two participating receptors which may contribute to the formation of the D_2R/NTS1R heteromers by participating in guide–clasp interactions in the receptor interface. The CREB reporter gene assay indicated that the neurotensin receptor agonist JMV 449 markedly reduced the potency of the D_2R like agonist quinpirole to inhibit the forskolin induced increase of the CREB signal. In contrast, the neurotensin agonist was found to markedly increase the quinpirole potency to activate the MAPK pathway as also studied with luciferase reporter gene assay measuring the degree of SRE activity as well as with ERK1/2 phosphorylation assays. These dynamic changes in D_2R signaling produced by the neurotensin receptor agonist may involve antagonistic allosteric receptor–receptor interactions in the $D_{2L}R$–NTS1R heteromers at the plasma membrane level (CREB pathway) and synergistic interactions in PKC activation at the cytoplasmatic level (MAPK pathway).

1. Introduction

Neurotensin (NT), a tridecapeptide originally identified in extracts of bovine hypothalamus by Carraway and Leeman [1], is widely distributed in the central nervous system (CNS) and in the periphery acting as a neurotransmitter [1,2]. In mammalian brain NT and its receptors are widely distributed especially in dopamine (DA)-enriched regions and several studies demonstrate the existence of a close functional relationship between NT and DA neurons [3,4]. The interaction between NT and DA has been implicated in the pathogenesis and treatment of schizophrenia [3–5]. The molecular mechanism underlying the NT-induced modulation of dopamine transmission, especially in the nigrostriatal, mesolimbic and meso-cortical dopamine pathways, has been proposed to be a direct antagonistic interaction between the NTS1R and the dopamine D2 receptor (D_2R), including the D2 autoreceptor [2,5–7]. The NT-induced reduction in D_2R agonist affinity is found both in striatal sections and in striatal membrane preparations [7–10], and may reflect direct allosteric NTS1R/D_2R interactions antagonizing the DA agonist affinity at the D_2R [5]. More recently, biochemical, neurochemical and coimmunoprecipitation experiments have supported the existence of direct NTS1R–D2R interactions [11].

Given the importance of D_2Rs in the pathophysiology of schizophrenia and Parkinson's disease and their treatments, it is of

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substantial interest to explore if these two receptors can form heteromers, which would represent a novel target for drug development in Parkinson's diseases and schizophrenia. In the current work we can, for the first time, demonstrate dynamic changes in D_2R signaling in D_2R-NTS1R heteromers upon agonist activation of NTS1R using gene reporter assays to follow the adenylyl cyclase (AC) and MAPK activities in line with the previous findings of an NTS1R mediated antagonistic regulation of D_2R recognition. The discovery of the existence of both $D_{2L}R$-NTS1R and $D_{2S}R$-NTS1R heteromers in living HEK293T cells by means of Bioluminiscence Resonance Energy Transfer ($BRET^2$) has also been made. A bioinformatic analysis suggests the existence of a basic set of three homology aminoacid protriplets (TVM, DLL and LRA) in the two participating receptors that may contribute to the formation of the D_2R-NTS1R heteromers by being part of the receptor interface.

2. Materials and methods

2.1. Plasmid constructs

The constructs presented herein were made using standard molecular biology techniques employing PCR and fragment replacement strategies. In brief, the human NTS1R coding sequences without their stop codons were amplified from 3xHA-NTS1R-pcDNA vectors using sense and antisense primers harboring unique NheI and XhoI sites. The fragments were then subcloned in-frame into humanized $pGFP^2$-N1 vectors (PerkinElmer, Waltham, MA, USA). The other constructs used are described previously [12,13].

2.2. Cell culture, transfection and confocal microscopy

HEK293T cells (American Type Culture Collection, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 10% (v/v) fetal bovine serum (FBS) at 37 °C and in an atmosphere of 5% CO_2. For transfection, cells were plated in 6-well dishes at a concentration of 1×10^6 cells/well or in 75 cm^2 flasks and cultured overnight before transfection. Cells were transiently transfected using TransFectin (Bio-Rad, Sweden). Transiently transfected HEK293T cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS containing 20 mM glycine and mounted with Vectashield immunofluorescence medium (Vector Laboratories, UK). Microscope observations were performed with a Leica TCS-SL confocal microscope (Leica, USA).

2.3. $BRET^2$ saturation assay

Forty-eight hours after transfection, HEK293T cells were transiently transfected with constant (1 μg) or increasing amounts (0.12–7 μg) of plasmids encoding for $D_{2L}R^{Rluc}$ or $D_{2S}R^{Rluc}$ and $NTS1R^{GFP2}$ respectively. The $BRET^2$ was preformed as previously described [14,15], see also Supplementary Material.

2.4. $BRET^2$ competition assay

Forty-eight hours after transfection, HEK293T cells were transiently transfected with constant amounts (1 μg) of plasmids encoding for $D_{2L}R^{Rluc}$ and $NTS1R^{GFP2}$ and increasing amounts (0.1–8 μg) of plasmids encoding for wild-type $D_{2L}R$ or NTS1R and the mock pcDNA3.1+, respectively.

2.5. Luciferase reporter gene assay

Dual luciferase reporter assay was performed as previously described [12], see also Supplementary Material.

2.6. ERK1/2 phosphorylation assay

ERK1/2 phosphorylation assay was performed as previously described [15], see also Supplementary Material.

2.7. Bioinformatic analysis of the receptor-receptor interface

Based on a bioinformatic approach, a set of amino acid triplet homologies have been deduced that may be responsible for receptor–receptor interactions among receptor heteromers [16]. In this study the $D_{2L}R$-NTS1R heteromer has been analyzed for the existence of a basic set of common triplets in the two participating receptors that may be responsible for the receptor–receptor interactions.

2.8. Statistical analysis

The number of samples (n) in each experimental condition is indicated in Figure legends. For statistical evaluation of the biochemical data, unless otherwise specified, one-way analysis of variance (ANOVA) was performed followed by Tukey's Multiple Comparison post-test. The P value 0.05 and lower was considered significant.

3. Results

3.1. Dopamine $D_{2L}R/D_{2S}R$ and neurotensin receptor 1 heteromerization

By confocal microscopy analysis $D_{2L}R^{YFP}$ (pseudocolor in green) was found to be colocated with $NTS1R^{GFP2}$ (pseudocolor in red) in the plasma membrane of HEK293T cells as shown from the development of a yellowish fluorescence which appeared to be true also in the cytoplasmatic compartment (Supplementary Fig. 1).

The $BRET^2$ assay was performed on HEK293T cells co-transfected with a constant amount of $D_{2L}R^{Rluc}$ construct while increasing the concentrations of a $NTS1R^{GFP2}$ plasmid. As a positive control, cells expressing a GFP^2-*R*luc tandem fusion protein were used (data not shown). As shown in Fig. 1A, significant and strong $BRET^2$ signals were found for the $D_{2L}R$-NTS1R pair, giving the highest maximum $BRET^2$ values ($BRET^2_{max}$: 85.7 ± 3.7 mBU; Fig. 1A). A slightly lower, $BRET^2_{max}$ signal (70.6 ± 3.5 mBU) was obtained with the $D_{2S}R$-NTS1R pair which was shown to possess a similar $BRET_{50}$ value to that found for the $D_{2L}R$-NTS1R pair (Fig. 1A). No specific $BRET^2$ signal was obtained from a mixture of singly expressing $D_{2L}R^{Rluc}$ cells and $NTS1R^{GFP2}$ cells (Fig. 1A, control (−)). In all cases $BRET^2$ signaling in cotransfected cells increased as a hyperbolic function to the increasing concentration of the GFP^2 fusion construct, reaching an asymptote at the highest concentrations used. Thus, as the negative control led to a quasi-linear curve, the specificity of the saturation (hyperbolic) assay for the $D_{2L}R^{Rluc}$ and $NTS1R^{GFP2}$ pair could be established. The specificity of this interaction is also indicated in Fig. 1B where increasing concentrations of expressed WT receptor ($D_{2L}R$, $D_{2S}R$ and NTS1R) in combination with the protomers of the BRET pair (constant ratio 1:1) leads to a concentration dependent disappearance of the $BRET^2$ signal. Instead co-transfection of pcDNA3.1+ in the same concentration range failed to diminish the signal. Taken together, these results give evidence that under basal conditions the NTS1R directly and specifically interacts with both $D_{2L}R$ and $D_{2S}R$ in living cells and forms $D_{2L}R$-NTS1R and $D_{2S}R$-NTS1R heteromers.

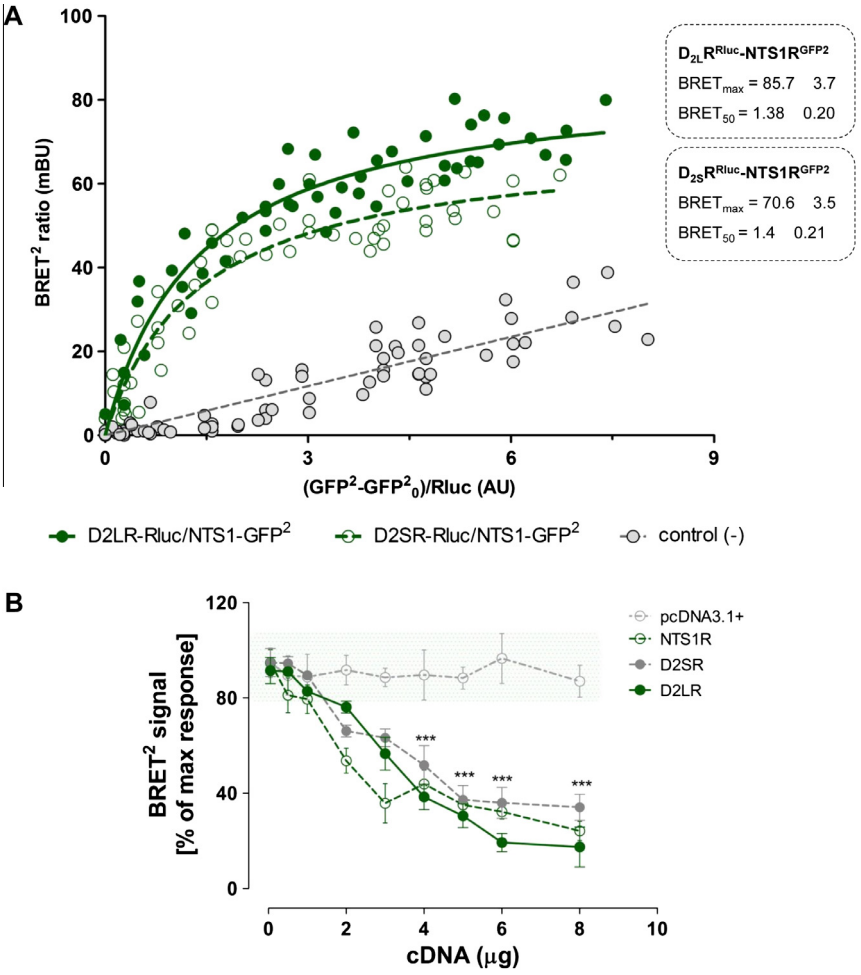

Fig. 1. BRET^2 studies on D_2R and NTS1R heteromerization in HEK293T cells. (A) BRET^2 saturation curves for both the D_{2L}R-NTS1R and D_{2S}R-NTS1R heteromers with increasing expression levels of the GFP^2 tagged NTS1 receptor. Cells singly expressing $D_{2L}R^{Rluc}$ were mixed prior to exposition to h-coelenterazine with cells singly expressing $NTS1R^{GFP2}$ as a negative control. Plotted on the *X*-axis is the fluorescence value obtained from the GFP^2, normalized with the luminescence value of $D_{2L}R^{Rluc}$ expression 10 min after h-coelenterazine incubation. Mean ± S.E.M.; $n = 10$, in triplicate. The D_{2L}R-NTS1R and D_{2S}R-NTS1R curve fitted better to a saturation curve than to a linear regression as found with a mixed pool of cells from cells singly expressing $D_{2L}R^{Rluc}$ + $NTS1R^{GFP2}$ (*F* test ($P < 0.001$). Data are means ± S.E.M. ($n = 5$). (B) BRET^2 competition experiment for the D_{2L}R-NTS1R heteromers. A fixed ratio (1:1) of expression levels of the $D_{2L}R^{Rluc}$/$NTS1R^{GFP2}$ tagged receptors was used in presence of increasing concentrations of wild-type receptors. Plotted on the *X*-axis is the concentration of cDNA transfected per competitor. Mean ± S.E.M.; $n = 6$ in triplicate. ***Significantly different compared to pcDNA3+ in the range from 4–8 µg cDNA) ($P < 0.001$) by two-way analysis of variance (ANOVA).

3.2. The signaling of D_{2L}R-NTS1R heteromers

To explore the role of the dopamine D_{2L}R and NTS1R interactions in cotransfected cells on second messenger signaling cascades, we examined how the potent NT receptor agonist JMV 449 could modulate the D2 like agonist quinpirole induced signaling changes in different luciferase reporter assays in heterologous HEK293T cells (coexpressing the human D_{2L}R and human NTS1R receptors).

3.3. Determination of NTS1R modulation of D_2R agonist induced inhibition of the AC-CREB pathway in a CRE-luciferase reporter gene assay

It has been well documented that dopamine D2 like receptor activation via the $G_{i/o}$-AC-PKA cascade, can decrease the phosphorylation of the CREB transcription factor leading to a reduction of the activity of cAMP response element (or CRE) (in our experimental approach represented as a CRE-luciferase reporter gene). In D_{2L}R-NTS1R cotransfected cells (Fig. 2A) the concentration response curve obtained with the D_{2L}R like agonist quinpirole to inhibit forskolin-mediated CRE-Luc induction was shifted to the right by the NT receptor agonist JMV 449 (10 nM and 30 nM), showing a reduced potency of the D2 like agonist to inhibit the CRE signal.

We also observed that the forskolin-induced increase of luciferase activity through the direct activation of AC was significantly reduced by 100 nM of quinpirole, a concentration around the EC_{50} value, which is not only counteracted by 50 nM of the potent NT receptor agonist JMV 449 but even resulted in a marked and highly significant increase of CRE activity vs. control (Fig. 2B). Treatment of the cells with of the dopamine receptor antagonist raclopride (10 µM) significantly counteracted the reduction observed in the forskolin-induced increase of luciferase activity by quinpirole. The marked enhancement in forskolin-induced increase of luciferase activity in cells co-stimulated with quinpirole and JMV 449 was counteracted by the selective NT receptor antagonist SR142948 (10 µM). The NT receptor agonist modulation of the D_2R like agonist inhibition of the CRE activity was not observed in cells transfected with the D_{2L}R alone (Supplementary Fig. 2).

3.4. Determination of NTS1R modulation of D_2R agonist induced activation of the MAPK pathway by a SRE-luciferase reporter gene and ERK1/2 phsphorylation assays

The extent of phosphorylation of extracellular signal-regulated kinases (ERK1/2) was studied in response to quinpirole and upon coactivation together with JMV 449 using the principle of the

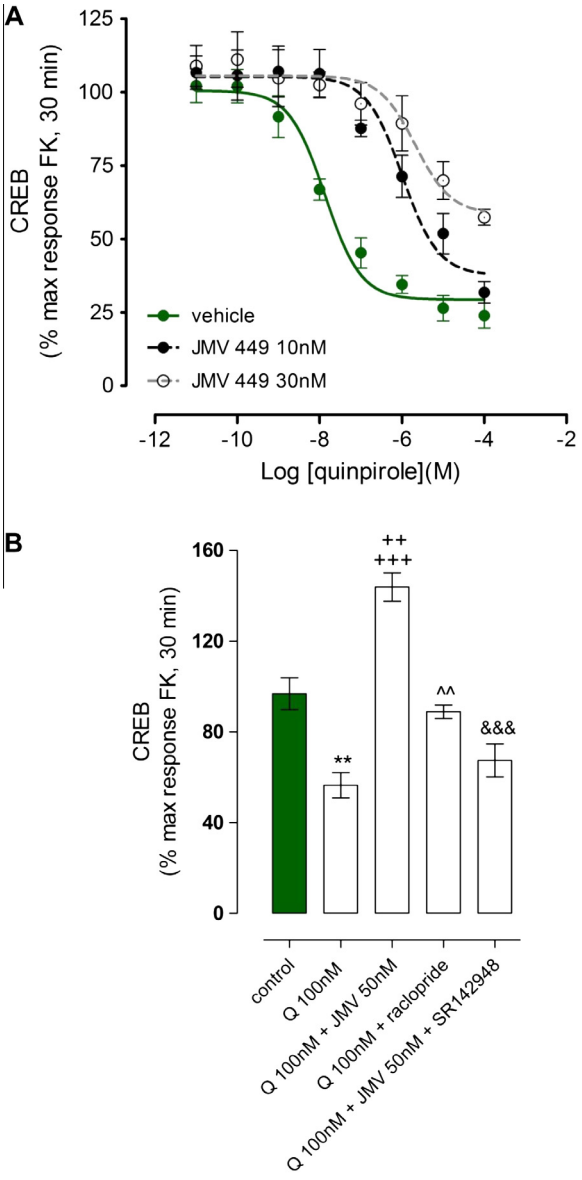

Fig. 2. Agonist-induced $D_{2L}R$ and NTS1R receptor activation in a forskolin-induced CRE-luciferase reporter gene assay. (A) Dose–response curves with quinpirole in $D_{2L}R$-NTS1R co-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 2 μg of both ($D_{2L}R$ and NTS1R) expression vectors and 1 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 h with 2 μM forskolin (sub-maximal concentration), and D2 like receptor agonist quinpirole in presence or absence of the NTS1R agonist JMV 449 (10 nM and 30 nM) and the luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of three independent experiments performed in quadruplicate. (EC_{50} ~ 13.6 nM in control group and EC_{50} ~ 1.02 μM; EC_{50} ~ 2.2 μM in the NT agonist JMV 449 groups at 10 and 30 nM, respectively). (B) HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-CRE-luc2p), 2 μg of both ($D_{2L}R$ and NTS1R) expression vectors and 1 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 h with 2 μM forskolin (sub-maximal concentration value), and agonist or antagonist (in presence of agonist) and the luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of three independent experiments performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. **Significantly different compared to control ($P < 0.01$); $^{++}$ and $^{+++}$Significantly different compared to control ($P < 0.01$) and to quinpirole 100 nM ($P < 0.001$), respectively; $^{\wedge\wedge}$Significantly different compared to quinpirole 100 nM ($P < 0.01$); $^{\&\&\&}$Significantly different compared to quinpirole 100 nM + JMV 50 nM ($P < 0.001$). Forskolin (2 μM); Q, quinpirole (100 nM); JMV, JMV 449 (50 nM); raclopride (10 μM) and SR142948 (10 μM).

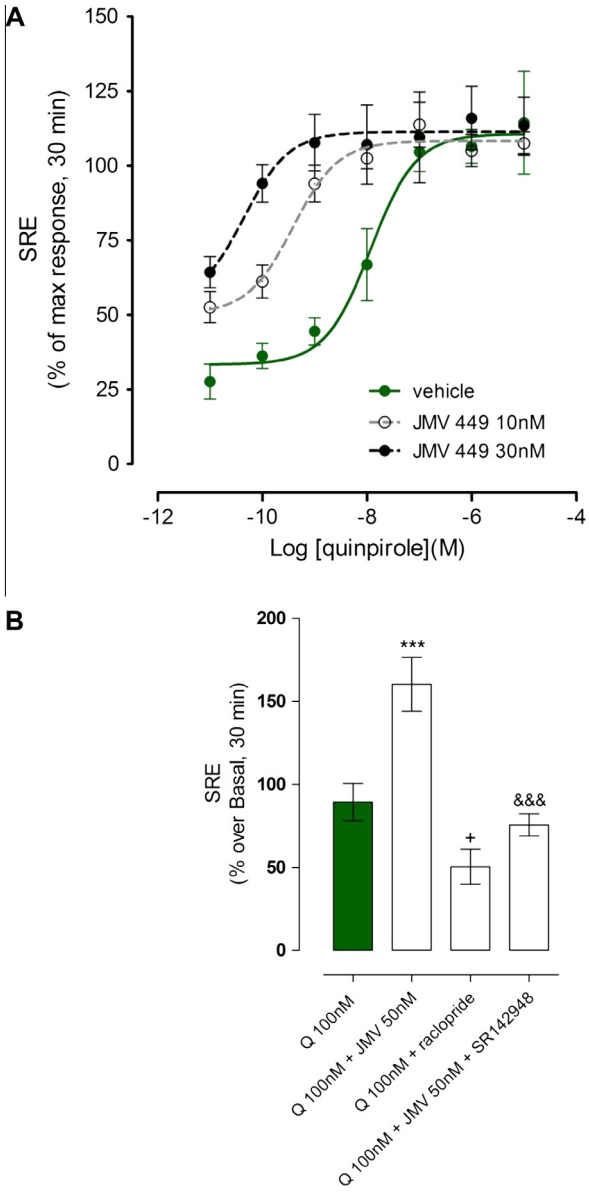

Fig. 3. SRE-luciferase reporter gene response after agonist-induced $D_{2L}R$ and NTS1R receptor activation. (A) Dose–response curves with quinpirole in $D_{2L}R$-NTS1R co-transfected HEK293T cells. HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p), 2 μg of both ($D_{2L}R$ and NTS1R) expression vectors and 1 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 h with D2 like receptor agonist quinpirole in presence or absence of the NTS1R agonist JMV 449 (10 nM and 30 nM) and the luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of three independent experiments performed in quadruplicate. (EC_{50} ~ 11.7 nM in the control group; EC_{50} ~ 383.2 pM and EC_{50} - ~ 44.2 pM with the potent NT agonist JMV 449 at 10 and 30 nM, respectively). (B) HEK293T cells were transiently co-transfected with 2 μg firefly luciferase-encoding experimental plasmid (pGL4-SRE-luc2p), 2 μg of both ($D_{2L}R$ and NTS1R) expression vectors and 1 μg *Renilla* luciferase-encoding internal control plasmid (phRG-B). Thirty-six hours after transfection, cells were treated 4 h with agonist or antagonist (in presence of agonist) and the luciferase activity was measured. Light emission is expressed as a percentage of the control forskolin-induced value. The data represent the mean ± S.E.M. of three independent experiments performed in quadruplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post-test. The *P* value 0.05 and lower was considered significant. ***Significantly different compared to quinpirole ($P < 0.001$); $^{+}$Significantly different compared to quinpirole ($P < 0.05$); $^{\&\&\&}$Significantly different compared to quinpirole 100 nM + JMV 50 nM ($P < 0.001$). Q, quinpirole (100 nM); JMV, JMV 449 (50 nM); raclopride (10 μM) and SR142948 (10 μM).

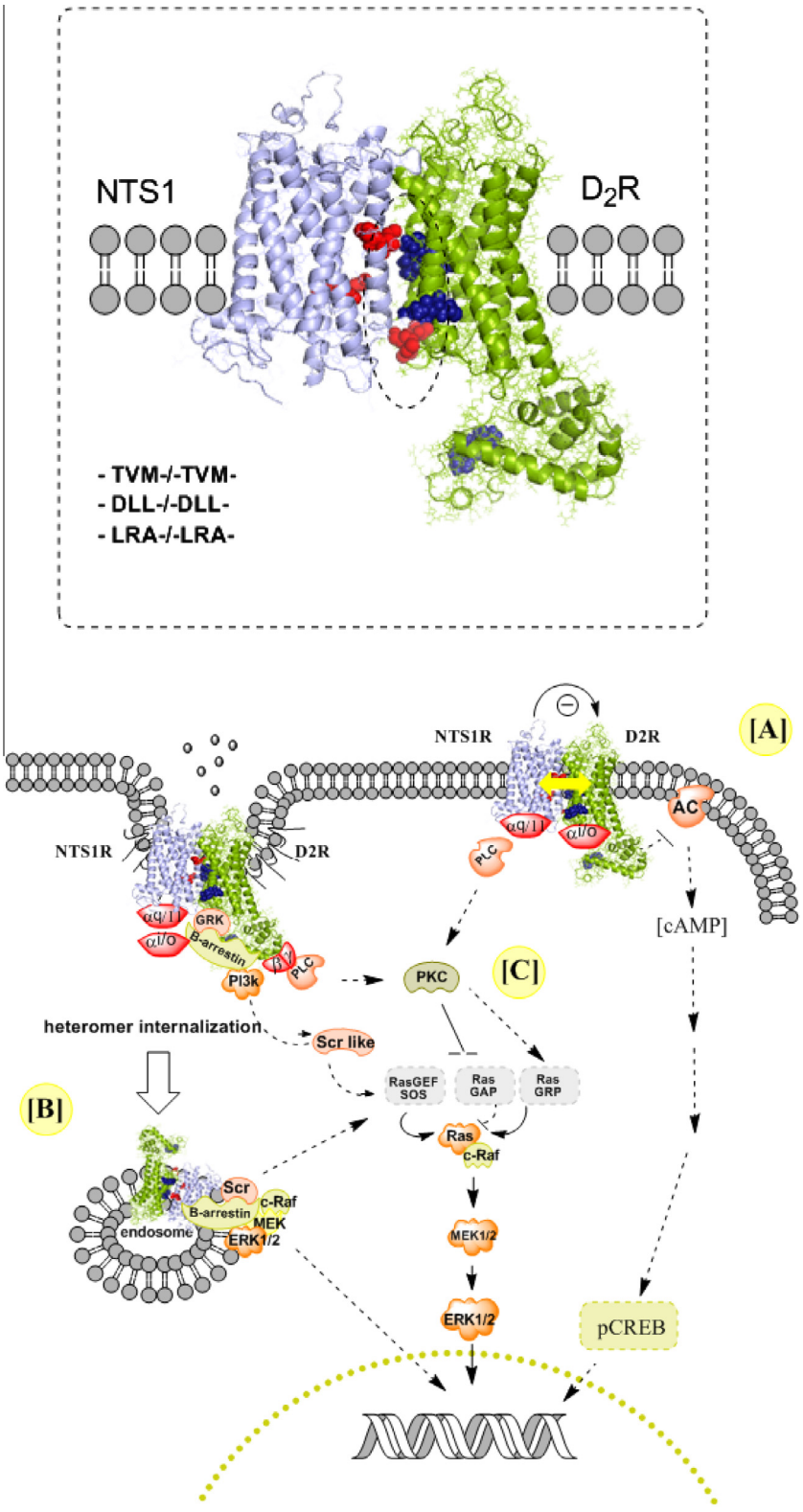

Fig. 4. (top) Three-dimensional molecular models of the $D_{2L}R$ and NTSR1 were built by means of the homology modeling program Accelrys Discovery Studio 2.5 (San Diego, CA, USA). The model with the highest PROCHECK score was selected. Amino-acid sphere-dots (in red and blue) represent the triplet of amino acid residues determined by bioinformatic analysis to be involved on the $D_{2L}R$–NTS1R heteromer interface. (bottom) Schematic cross-talk signalling pathways of D_2R and NTS1R. The D2R receptor is a GPCR which primarily produces cAMP inhibition via $G_{i/o}$ proteins (A) but also PLC/PKC activation via βγ G-protein subunits (C). In addition, G protein-coupled receptors couple to the MAPK pathway with pERK activation via both G proteins and β-arrestin recruitment (B). The latter often leads to desensitization and internalization of the GPCR. The NTS1R is a GPCR which primarily activates PLCβ via $G_{q/11}$ proteins and thus also leads to pERK activation via PKC (C). As illustrated, signaling pathways of the D2R and NTS1R thus converge, especially to PKC. In addition, we have shown that the heterodimerization of the two receptors leads to an antagonistic functional modulation by the NTS1 protomer of the D2 protomer mediated inhibition of the AC/cAMP/CREB pathway. This takes place directly at the receptor heteromer level (antagonistic allosteric receptor–receptor interaction) and the heterodimer interaction may change the preferred signaling pathways of the individual protomers and may lead to biased signaling and/or altered receptor trafficking.

SRE-luciferase reporter assay and ERK1/2 phosphorylation by means of in cell western assays.

The NTS1R agonist modulation of the D_2R agonist induced concentration–response curve in $D_{2L}R$-NTS1R cotransfected cells is displayed in Fig. 3A. We can observe that the NTS1R agonist shifts the $D_{2L}R$ agonist concentration–response of SRE activity (Fig. 3A) and ERK1/2 phosphorylation (Supplementary Fig. 3B) to the left in $D_{2L}R$-NTS1R cotransfected cells but not in cells transfected with the $D_{2L}R$ alone (Supplementary Fig. 3B). A detailed pharmacological analysis also showed that the quinpirole-induced increase of SRE-luciferase activity and ERK1/2 phosphorylation was significantly and markedly enhanced by 50 nM of JMV 449, which is fully counteracted by the potent neurotensin receptor antagonist SR142949 (Fig. 3B and Supplementary Fig. 3A). The increase in quinpirole-induced MAPK signaling was counteracted by raclopride (10 μM) in $D_{2L}R$ alone and co-expressing $D_{2L}R$-NTS1R cells, respectively. The NT receptor antagonist fully counteracted the enhancement of ERK1/2 phosphorylation by the NT agonist after quinpirole (Supplementary Fig. 3A).

3.5. Structural determinants potentially involved in D_2R-NTS1R heterodimerization based on bioinformatic analysis

Based on a bioinformatic approach, Tarakanov and Fuxe [16] have deduced a set of triplet homologies that may be responsible for receptor-receptor interactions. This set consists of two non-intersecting subsets: 'pro-triplets' and 'contra-triplets'. Any pro-triplet appears as a homology in at least one heterodimer but does not appear as a homology in any non-heterodimer. In reverse, any contra-triplet appears in at least one non-heterodimer but does not appear in any heterodimer. In the $D_{2L}R$-NTS1R heteromer three pro-triplets have been identified; DLL (Asp-Leu-Leu), in the transmembrane region II (TM-II) of both receptor protomers, the LRA (Leu-Arg-Ala) in the N-terminal part of the third intracellular loop (IL3) of D_2R and the cytosol adjacent part of TM-VI of the NTS1R and the TVM (Thr-Val-Met) in the cytosol adjacent part of TM-IV of D_2R and the N-terminal part of the third intracellular loop (IL3) of NTS1R (Fig. 4-top).

The triplet homologies DLL and LRA can also be found in the integrins of marine sponges and the integrin receptors of marine sponges are αβ-heterodimers [17]. The DLL homology is present in ten protomers of receptor heteromers (Supplementary Table 1). The topological analysis of the DLL homology demonstrates it to be present in TM-II of these heteromers [18]. Therefore, this triplet may participate in the receptor interface of these D_2R heteromers including the D_2R-NTS1R heteromer. These triplets may 'guide-and-clasp' direct protein–protein interactions by electrostatic interactions of aspartate in the triplet DLL (Asp-Leu-Leu) and arginin in the triplet LRA (Leu-Arg-Ala). A flexibility of the protein skeleton may also be provided by the hydrophobic properties of leucine-rich motifs in the above triplets DLL.

4. Discussion

The BRET^2 experiments in HEK cells give evidence for the existence of $D_{2L}R$-NTS1R and $D_{2S}R$-NTS1R heteromers indicating that the previously reported antagonistic D_2R-NTS1R receptor–receptor interactions on D_2R recognition in striatum [2,5,8] and co-immunoprecipitation of D_2R and NTS1R in cell preparations [11] may reflect the existence of D_2R-NTS1R heteromers. The YFP tagged-$D_{2L}R$ and GFP^2 tagged NTS1R also showed a high degree of colocation (yellow) associated with the plasma membrane. It is of substantial interest that both the long and short forms of the D_2R formed heteromers with NTS1R to a similar extent. Thus, e.g. the D2 autoreceptor represents the short form and most postjunctional D_2Rs the long form; and the microdialysis work on D_2R–NTS1R interactions gives evidence that these receptor–receptor interactions take place both pre and postjunctionally in striatal DA neurotransmission [2,4,6].

The bioinformatic analysis demonstrated three pro-triplet amino acid homologies (TVM–TVM, DLL–DLL and LRA–LRA) that according to the molecular modeling may be located in the heteromer interface. These results therefore support the theory of the triplet puzzle introduced by Tarakanov and Fuxe [16] stating that triplet homologies may guide the protomers in the formation of the receptor heteromer.

The CREB reporter gene assay indicated that the NT receptor agonist JMV 449 markedly reduced the potency of the D_2R like agonist quinpirole to inhibit the forskolin induced increase of the CREB signal. Thus, it seems that the antagonistic allosteric D_2R-NTS1R receptor–receptor interaction previously observed on striatal D_2R recognition [5,9,10] also exists in the regulation of the D_2R/Gi/o coupling inhibiting the AC activity and leading to a reduction of the CREB mediated signaling (Fig. 4-bottom). These results explain the antagonistic functional interactions seen with NT and DA ligands in microdialysis and behavioural experiments [2,4]. It should be noticed that at the highest concentration of the NT agonist used quinpirole could no longer fully counteract the NT agonist action even in the high concentrations. It is of substantial interest that at 50 nM the NT agonist not only counteracted the D_2R agonist induced inhibition but even significantly increased the CREB signal vs. control. It may be speculated that this event can reflect a conformational change in the D_2R protomer induced by the NT agonist which via allosteric receptor–receptor interaction also leads to a Gs coupling of the D_2R protomer.

In contrast, the NT agonist was found to markedly increase the quinpirole potency to activate the MAPK pathway as also studied with luciferase reporter gene assay measuring the degree of SRE activity as well as with ERK1/2 phosphorylation by means of in cell western assays. The NT receptor agonist induced enhancement of the D_2R agonist produced increases of MAPK signaling was blocked by the NT receptor antagonist SR142948. One mechanism for this enhancement of the MAPK signaling of the D_2R receptor may be the NT receptor agonist induced activation of PKC via the Gq-PLCβ-PKC pathway [19] (Fig. 4-bottom). Agonist activation of D_2Rs is also known to activate PKC involving Gi/o-βγ release from and βarrestin binding to the D_2R followed by activation of PLCβ [20]. Fig. 4-bottom. Thus, a synergistic activation of PKC by D_2R and NTS1R protomers may be the mechanism for the ability of agonist coactivation of the D_2R and NTS1R protomers to markedly enhance D_2R protomer signaling via the MAPK pathway. In this case there is likely no allosteric receptor–receptor interaction involved as proposed for the NTS1R mediated antagonism of the Gi/o signaling to AC. Taken together, the integrative activity of the D_2R-NTS1R heteromer leading to dynamic changes in D_2R signaling with reduction of the CREB pathway and increased activity of the MAPK pathway may involve antagonistic allosteric receptor–receptor interactions at the plasma membrane level and synergistic interactions with PKC at the cytoplasmatic level, respectively.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/J.BBRC.2013.04.058.

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