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PNA-Based Multivalent Scaffolds Activate the Dopamine D_2 Receptor

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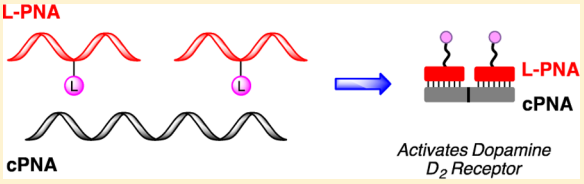
Supporting Information

ABSTRACT: Peptide nucleic acid scaffolds represent a promising tool to interrogate the multivalent effects of ligand binding to a membrane receptor. Dopamine D_2 receptors (D_2R) are a class of G-protein coupled receptors (GPCRs), and the formation of higher-ordered structures of these receptors has been associated with the progression of several neurological diseases. In this Letter, we describe the synthesis of a library of ligand-modified PNAs bearing a known D_2R agonist, (±)-PPHT. The D_2R activity for each construct was assessed, and the multivalent effects were evaluated.



KEYWORDS: *Peptide nucleic acid, multivalent display, dopamine D_2 receptor, self-assembly*

Dopamine receptors are a class of G protein-coupled receptors (GPCRs) known to modulate cognitive function associated with several disease states and have been identified as drug targets for Parkinson's disease,[1] schizophrenia,[2,3] drug abuse,[4] and obesity.[5−7] Recent studies have shown that dopamine D_2 receptors (D_2R) are expressed as dimers in cell lines and brain tissue;[8] and higher order oligomeric structures have been observed in mammalian cells.[9,10] Moreover, evidence suggests that the dimerization and/or oligomerization of D_2Rs may play an important role in the pathophysiology of neurological diseases.[3,4,11−15] Thus, the development of new pharmacological probes is crucial to understanding the mechanism and regulation of D_2R dimerization/oligomerization and its role in disease pathophysiology.

Bivalent ligands have emerged as a valuable tool to interrogate proposed higher-ordered GPCRs.[16−19] This strategy, pioneered by Portoghese,[20,21] typically consists of two discrete pharmacophores covalently linked by a spacer of an appropriate length and can lead to higher affinity, potency, and/or selectivity. While many bivalent ligands have been developed to investigate D_2R dimerization/oligomerization, as well as to improve the affinity and potency of known D_2R pharmacophores,[22−26] multivalent (>2 ligands) species targeting dopamine receptors remain unexplored. Multivalent ligand systems allow for multiple simultaneous interactions with binding sites or receptors and can therefore dramatically enhance binding affinity, avidity, and/or specificity.[27,28] Until recently, multivalent ligand systems for the interrogation of biological systems were constructed on flexible scaffolds, polymers, dendrimers, and nanoparticles.[27,29,30] A major drawback of these constructs has been the poorly defined spatial arrangement and/or ligand density, which can complicate data interpretation. To overcome this limitation, self-organized scaffolds, using DNA or PNA templates, have been developed by us[31−33] and others[27,34−39] to assemble highly defined supramolecular structures in a rapid and reproducible manner. This method offers significant advantages over previous constructs, including the ability to synthesize monodisperse materials, control the ligand valency, and precisely position functional groups.[27]

In our quest to interrogate the multivalent effects of peptide nucleic acid (PNA) scaffolds on GPCRs, we have developed a multivalent scaffold system based on peptide nucleic acid duplexes that display ligands in a well-defined, versatile manner.[31,33] In general, these duplexes contain a sequence of peptide nucleic acid residues where select residues are replaced with a synthetically modified monomer derived from lysine, $^{L}K\gamma$-PNA (Figure 1a).[32,40] A ligand can be conjugated to the side-chain of the $^{L}K\gamma$-PNA residue, and the resulting ligand-modified PNA (L-PNA) can be organized onto complementary nucleic acid oligomers to generate nanostructures that display ligands in a highly controlled, spatially defined manner.[41,42] The programmable nature of the L-PNA:PNA scaffold allows for precise changes in ligand spacing and orientation without altering the scaffold backbone, the number of rotatable bonds, or the number of atoms. This key feature of PNA scaffolds is unique among bi- and multivalent ligand systems and can provide a more detailed depiction of the effect of multivalent displays on GPCR activity. Recently, we utilized this multivalent approach to interrogate the A_{2A} adenosine receptor ($A_{2A}AR$), a GPCR that is a drug target for neurodegenerative diseases, and observed a multivalent effect indicating the assembly of high-ordered receptor oligomers.[31] In this Letter, we expand the utility of our multivalent scaffold system by generating a library of L-PNA:PNA complexes that display a

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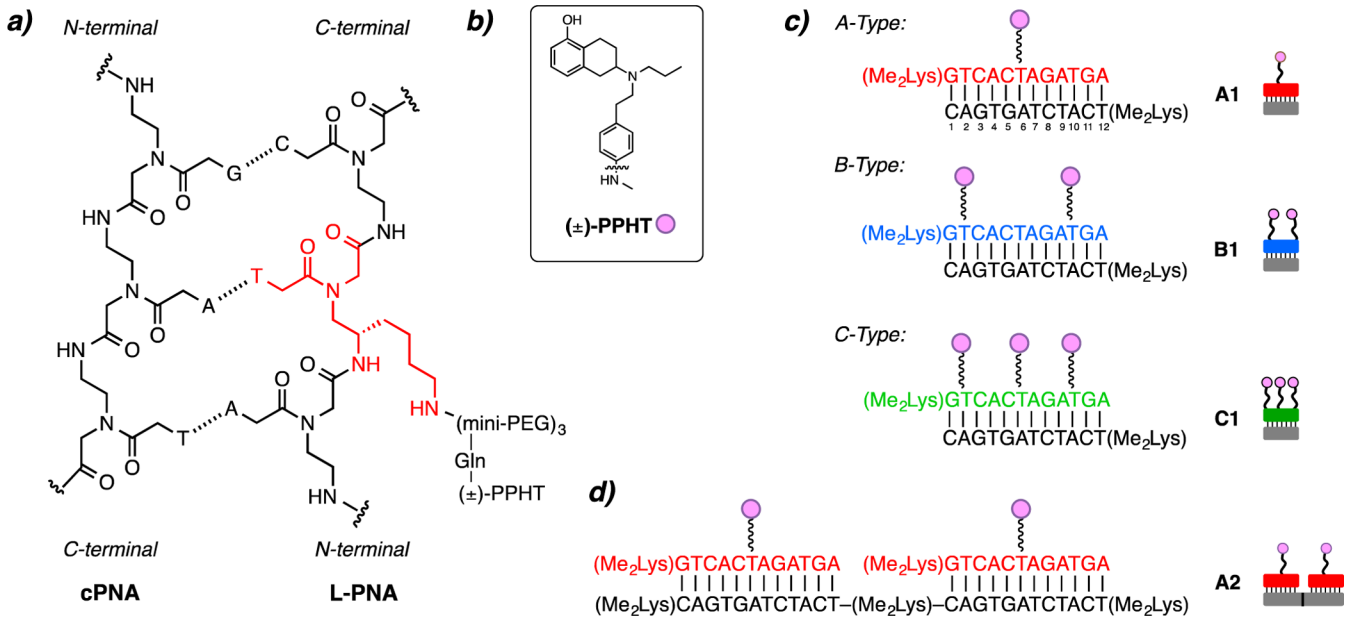

Figure 1. Ligand modified PNAs. (a) Chemical structure of L-PNA:PNA duplex containing the $^{L}K\gamma$-PNA side chain highlighted in red. (b) Chemical structure of D_2R agonist (±)-PPHT (represented as pink circles). (c) L-PNA oligomer bound to complementary PNA with one (±)-PPHT ligand (A-type, red), two (±)-PPHT ligands (B-type, blue), and three (±)-PPHT ligands (C-type, green) per PNA. (d) Each L-PNA sequence is identified by its constituent parts; for example, an A2 complex contains 2 A-type L-PNA units annealed along a 24-residue cPNA.

known D_2R agonist to probe the effects of multivalent ligand display on D_2R activity.

A library of ligand-modified peptide nucleic acids bearing a known D_2R agonist, (±)-2-(*N*-phenethyl-*N*-propyl)amino-5-hydroxytetralin, (±)-PPHT[22,43−45] (Figure 1b), was generated by systematic insertion of synthetic $^{L}K\gamma$ monomers into a 12-residue PNA oligomer (Figure 1a). To attach the ligand, the lysine moiety of the incorporated $^{L}K\gamma$ monomer was extended from the main PNA backbone using three mini-PEG (8-amino-3,6-dioxaoctanoic acid) linkers. A glutamic acid modified (±)-PPHT was then conjugated to the mini-PEG *N*-terminus to generate the desired L-PNA. The ligand valency of L-PNAs was varied from one ligand per L-PNA (A-type), to two (B-type), and three (C-type) ligands per L-PNA by incorporating one, two, or three $^{L}K\gamma$-PNA monomers, respectively (Figure 1c). In the A-type L-PNA constructs, the ligand was attached to the central residue, while in the B-type the ligands were attached at residues 2 and 10. The C-type constructs contained 3 ligands that were attached at residues 2, 6, and 10 (Figure 1c). The L-PNAs were then annealed to complementary PNA oligomers (cPNA) in accordance with traditional Watson−Crick base pairing[41,46,47] to provide a library of multivalent scaffolds with defined valency, ligand spacing, and orientation (Figure 1a). In an earlier report, we demonstrated that L-PNA:PNA duplexes are preferred to L-PNA:DNA when targeting membrane proteins such as GPCRs.[31] This preference is likely due to the minimization of the charge repulsion forces that exist between the anionic DNA backbone and the cell surface in the case of L-PNA:DNA. To identify the library constructs, we refer to each L-PNA sequence according to the constituent parts; for example, a single A-type L-PNA annealed to its 12-residue cPNA is referred to as **A1** (Figure 1c). Similarly, an **A2** complex contains two A-type L-PNA units annealed along a 24-residue cPNA (Figure 1d). In total, 15 unique L-PNA:PNA complexes were generated systematically and span a valency of 1−15 ligands (Figure 2). For longer cPNAs, an *N*,*N*-dimethyl lysine was incorporated after each 12-residue sequence to maintain aqueous solubility at longer PNA lengths.

Each member of the L-PNA:PNA library was tested for D_2R activity using a whole cell β-arrestin recruitment assay,[48,49] and the data are summarized in Figure 2a,b. Overall, attaching linkers to (±)-PPHT decreases D_2R activation by about one order of magnitude relative to the free ligand, which is consistent with previous reports.[22] Importantly, the data demonstrate that an increase in ligand valency is associated with improved EC_{50} values. Of particular interest was the dramatic change in the EC_{50} values when the valency was increased from one to two ligands, specifically in going from A1 to A2 and A1 to B1. These data were further analyzed using η values, a term that we recently introduced,[31] to evaluate the change in D_2R activity between L-PNA:PNA complexes of the same type when the change in ligand valency is normalized (i.e., comparing sequential A-type L-PNA:PNA complexes). For the purpose of this Letter, an η value of approximately one indicates that improvement in D_2R activity is proportional to the increase in ligand valency. Alternatively, η values greater than two suggest that the incorporation of additional ligands results in an increase in D_2R activation that cannot be attributed solely to increased ligand content. Using the η parameter to analyze D_2R activity, we obtained an η value of two in the transition from one to two ligands for both the A1 to A2 and A1 to B1 transitions (Figure 2b). This indicates that increasing the valency from one to two ligands significantly enhances the D_2R activity. Interestingly, the ligand spacing in both the A2 and B1 constructs did not impact D_2R activity. In contrast, the addition of a third ligand to the 12-residue L-PNA C1 had a slightly detrimental effect on D_2R activation. This is likely due to steric crowding, which does not allow for favorable ligand−receptor interactions. The η values for the remaining constructs are close to one, indicating that an increase in ligand valency beyond two ligands marginally improves D_2R activity.

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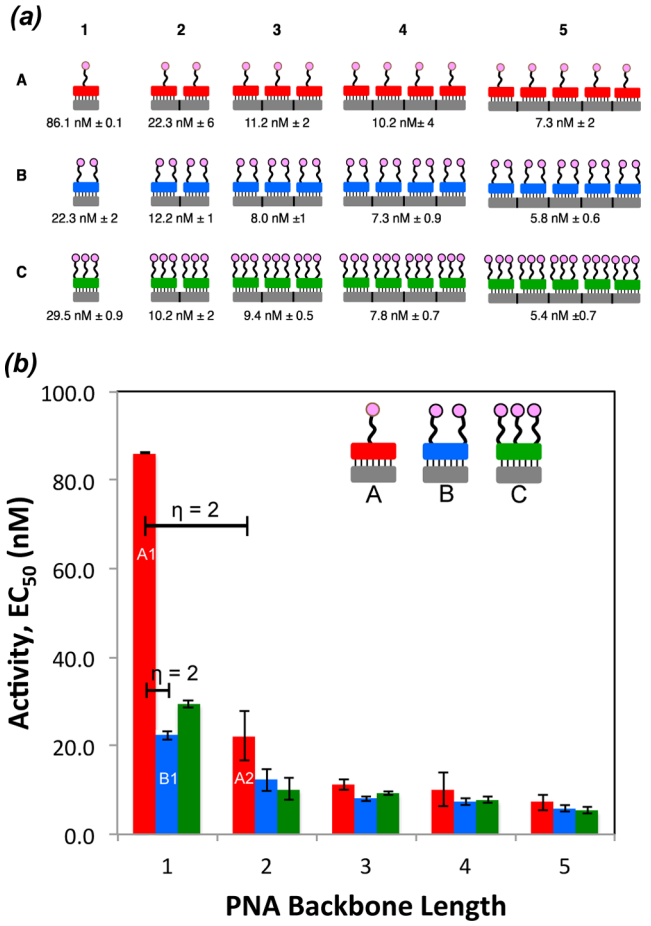

Figure 2. L-PNA:PNA multivalent library and landscape. (a) L-PNA:PNA multivalent library and the associated EC_{50} values for D_2R activity. (b) Multivalent landscape highlighting the relationships between the A (red), B (blue), and C (green) type L-PNA constructs when annealed to various lengths of DNA. Key η values are included and indicate an increase in the individual ligand binding affinity.

We also examined the nonspecific binding effects using an acetylated A type PNA that did not contain the (±)-PPHT ligand. We did not observe any nonspecific binding for this construct. Additionally, shorter linkers were considerably less active compared to the three mini-PEG linker. Taken together, these data demonstrate that the most significant activation of D_2R is observed when the ligand valency is increased from one to two and that additional ligands only slightly improve activity.

The highly programmable and versatile nature of the PNA scaffold lends itself to the rapid assembly of multivalent tools in a predictable manner. The ability to rigorously and precisely control the ligand content, density, and spatial orientation of the PNA scaffold represents a clear advantage over traditional bi- and multivalent approaches to investigate GPCRs. In this work, a multivalent scaffold system based on L-PNA:PNA duplexes was used to explore the effects of multivalency on D_2R activity. A library of 15 unique L-PNA:PNA complexes bearing a known D_2R agonist, (±)-PPHT, was prepared, and the D_2R activity was evaluated. A significant increase in D_2R activity was observed when the valency was increased from one to two ligands in both the A1 to A2 and A1 to B1 constructs. Using η values to further examine the A1 to A2 or B1 transitions, we conclude that the substantial increase in D_2R activity is due to a multivalent effect that cannot be attributed solely to the change in ligand valency. The incorporation of additional ligands in the remaining constructs improved activity proportionally to the increase in ligands. These data suggest that the formation of discrete receptor dimers may be responsible for the enhanced D_2R activity when comparing constructs with one versus two ligands, which would agree with previous observations of dimeric receptors in cell lines and brain tissue.[8,9] It is important to note that the presence of the PNA construct could drive dimer formation, and the receptor does not associate in the absence of ligand. It is also possible that ligand rebinding effects could account for the increases in activity with additional ligands, although the high affinity of the ligands for the receptor likely minimize significant contribution from rebinding.[51] With mounting evidence suggesting the importance of oligomeric GPCRs in disease pathophysiology, the L-PNA scaffold represents an important pharmacological tool to probe the effects of multivalent ligand displays on GPCR activity. In future work, we intend to probe the multivalent profiles of other ligand−receptor systems.

EXPERIMENTAL PROCEDURES

PNA Oligomer Synthesis. Commercial-grade reagents and solvents were used without further purification unless indicated. The resin (MBHA, 100−200 mesh, 1% divinylbenzene, 0.3 mmol g^{-1}, Advanced Chemtech) was prepared by swelling in CH_2Cl_2 and downloading the resin with *N*,*N*-dimethyl lysine to 0.1 mmol g^{-1} capacity. Boc-protected aegPNA monomers were purchased from PolyOrg. PNA oligomer synthesis was carried out on a 5 μmol scale on an Applied BioSystems 433A Automated Peptide Synthesizer. The resin was swelled with CH_2Cl_2 for 105 min before synthesis. The $^{L}K\gamma$-PNA monomer was synthesized according to published procedures.[32,40] Activated $^{L}K\gamma$-PNA monomer was allowed 90 min to couple. A further treatment of trifluoroacetic acid deprotection solution was also used to remove the *N*-Boc protecting group from $^{L}K\gamma$-PNA residues. The lysine side chains of $^{L}K\gamma$-PNA monomers (Fmoc) were orthogonally deprotected with 20% piperidine in DMF. When multiple $^{L}K\gamma$-PNA residues were present in the PNA oligomer (PNA-B and PNA-C), the primary amines on the side chains were deprotected and coupled to mini-PEG residues in tandem, followed by coupling to (±)-PPHT. Purification of PNA oligomers was carried out using an XBridge Prep BEH 130 C18 5 μm (10 mm × 250 mm) column on an Agilent 1100 HPLC. In all cases, 0.1% aqueous trifluoroacetic acid and acetonitrile were used as solvents. Additional information can be found in the Supporting Information.

General Annealing Conditions for Formation of L-PNA:PNA Duplexes. In RNA/DNAase free microfuge tubes, L-PNA, cPNA, and PBS buffer were combined at room temperature. Equivalents of PNA were calculated based on the number or repeating 12-residue sequences in the PNA. For example, to generate L-PNA:PNA multi5, a 5:1 molar ratio of L-PNA:cPNA was used. The solution was heated to 90 °C, held for 5 min, then slowly allowed to cool down to 25 °C over a period of 3 h. Additional information can be found in the Supporting Information.

***β*-Arrestin Recruitment Assay.** Agonist-mediated recruitment of β-arrestin-2 was determined using the DiscoveRx PathHunter complementation assay (DiscoveRx Inc., Fremont, CA), as previously described.[49,50] Briefly, CHO-K1 cells stably expressing the D_2R were seeded in cell plating (CP) media (DiscoveRx) at a density of 2625 cells/well in 384-well black, clear-bottom plates. Following 24 h of incubation, the cells were treated with multiple concentrations of compound in PBS buffer containing 0.2 mM sodium metabisulfite and incubated at 37 °C for 90 min. DiscoveRx reagent was then added to cells according to the manufacturer's protocol followed by a 60 min incubation in the dark at room temperature. Luminescence was measured on a Hamamatsu FDSS μ-cell reader (Hamamatsu, Bridgewater, NJ), and data was collected using the FDSS software.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.V.D. participated in research design and execution (chemistry) and contributed to writing the manuscript; J.L.C. participated in research design and execution (biology); K.M.G.R. participated in research execution (chemistry) and contributed to writing the manuscript; D.R.S. participated in research design (biology), acquired funding, and contributed to the writing of this manuscript; D.H.A. participated in research design (chemistry), acquired funding, and contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptors; D_2R, D_2 receptor; PNA, peptide nucleic acid; L-PNA, ligand modified PNA; $A_{2A}AR$, A_{2A} adenosine receptor; PPHT, 2-(*N*-phenethyl-*N*-propyl)amino-5-hydroxytetralin; cPNA, complementary PNA

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