Neuropsychopharmacology (2003) 28, S98–S107

www.neuropsychopharmacology.org

Cellular and Behavioral Effects of D_2 Dopamine Receptor Hydrophobic Eigenmode-Targeted Peptide Ligands

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Patterns in G-protein-coupled receptors' hydrophobically transformed amino-acid sequences can be computationally characterized as hierarchies of autocorrelation waves, 'hydrophobic eigenmodes,' using autocovariance matrix decomposition and all poles power spectral and wavelet transformations. L- or D-amino acid (retro–inverso) 12–18 residue peptides targeting these modes can be designed using eigenvector templates derived from these computations. In all, 12 human long-form D_2 dopamine receptor eigenmode-targeted 15 mer peptides were designed, synthesized, and shown to modulate and/or indirectly activate the extracellular acidification response, EAR, in stably receptor-transfected CHO and LtK cells, with an 83% hit rate. Representative L- and D-amino-acid retro–inverso peptides injected bilaterally in the nucleus accumbens demonstrated changes in rat exploratory behavior and prepulse inhibition similar to those observed following parenteral amphetamine. In contrast with geometric models used for ligand design, such as pharmacophores, the hydrophobic eigenmode approach to lead modulatory peptide design targets hydrophobic eigenmode-bearing subsequences, including those not visible from X-ray and NMR studies such as extracellular segments and loops.
Neuropsychopharmacology (2003) **28,** S98–S107 doi:10.1038/sj.npp.1300134

Keywords: D_2 dopamine receptor; hydrophobicity; wavelets; retro–inverso; peptide design; amphetamine

INTRODUCTION

Computational analyses can elucidate hierarchical autocorrelational wave patterns in amino-acid sequences (Mandell *et al*, 1997c) that have been transformed into their relative hydrophobicities, $aa_i \rightarrow hb_i$, in *kcal/mol* (Manavalen and Ponnuswamy, 1978; Nozaki and Tanford, 1971). Of these patterns, the most frequently discussed in the literature are helical turns and beta strands and turns, with wavelengths of $\omega^{-1} \approx 3.6aa$, $\omega^{-1} \approx 2.2aa$, and $\omega^{-1} \approx 2.0aa$ respectively, consistent with X-ray and NMR evidence of the protein's secondary structure (Eisenberg *et al*, 1984; Irback *et al*, 1996; Irback and Sandelin, 2000; Lazovic, 1996; Mandell, 1984; Mandell *et al*, 1987; Milner-White and Poet, 1987; Penel *et al*, 1999; Rackovsky, 1998; Rose, 1978; Rose and Wetlaufer, 1977; Schiffer and Edmundson, 1967; Wilmot and Thornton, 1988).

The availability of X-ray crystallographic and NMR three-dimensional structures of proteins appears to make the use of one-dimensional sequential pattern analyses less essential for characterizing target proteins. For example, rational peptide ligand designs, in contrast with high throughput screening of randomly varied ligand libraries (Zysk and Baumbach, 1998), are dominated by pharmacophores, three-dimensional models of their putative active or regulatory binding sites (Guner, 1999; Hruby and Agnes, 1999; Takeuchi *et al*, 1998). Similarly, polypeptide–protein and protein–protein interactions of physically characterized structures are explored using docking algorithms (Janin, 1995; Makino and Kuntz, 1997; Sandak *et al*, 1998; Stahl and Bohm, 1998).

However, an increasing number of studies (Romero *et al*, 1998) have demonstrated protein sequences and/or subsequences that are without stable tertiary structure. These conformationally disordered polypeptides (Dyson and Wright, 2002; Wright and Dyson, 1999) are defined by their failure to fold into stable secondary or tertiary structures. This is evidenced by X-ray crystallographic missing electron densities, nuclear magnetic resonance sharp peaks, and the absence of secondary structural NOEs and/or circular dichroism on spectroscopic examination with low-intensity signals from 210 to 240 nm (Romero *et al*, 2001). These subsequences characteristically become ordered upon ligand binding, going through a

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Received 01 September 2002; revised 03 November 2002; accepted 03 December 2002

disorder–order transition and achieving X-ray and NMR demonstrable, stable tertiary structure (Dyson and Wright, 2002; Kriwacki *et al*, 1996; Wright and Dyson, 1999).

Disordered protein sequences have been shown to play significant roles in polypeptide-polypeptide and protein–protein interactions, making them logical targets in rational peptide ligand design (Dunker *et al*, 1998, 2001; Dunker and Obradovic, 2001). The disordered loop sequences of globular proteins and membrane receptors participate in intramacromolecular signaling as active, allosteric, and antibody binding sites (Rondard and Bedouelle, 2000). They also act as signal-invoked 'switches', modulating access to active sites (Branden and Tooze, 1999; Ulloa-Aguirre and Conn, 2000).

Extramembranous segments and loop sequences in seven transmembrane G-protein-coupled and Type I tyrosine kinase-coupled receptors are, in the sense defined above, disordered, and participate in polypeptide signaling and regulation, in contrast to structural and/or transmembrane scaffolding involved segments (Finkelstein and Ptitsyn, 1987; Branden and Tooze, 1999; Bruccoleri *et al*, 1988; Howl and Wheatley, 1996; Lin *et al*, 1998; Milner-White and Poet, 1987; Qu *et al*, 1999). For these reasons, computational analysis of sequential polypeptide patterns as $aa_i \rightarrow hb_i$ in *kcal/mol* continues to have value in characterizing disordered sequences, particularly with respect to targeting them for rational peptide ligand design. This has proven particularly true for designing peptides with away-from-the-active-site, indirect agonist or antagonist, un- or noncompetitive modulatory influences (Mandell *et al*, 1998a, 2001, 1997c, 2000a).

For over 20 years, our laboratory group has exploited the methods of statistical physics and measure theoretic approaches to nonlinear dynamical systems to uncover and characterize patterns in biologically relevant, apparently disordered, real numbered sequences (Mandell, 1983, 1984; Mandell and Russo, 1981; Mandell and Selz, 1997; Selz and Mandell, 1991; Selz *et al*, 1995). This naturally led to our applications of some of these and related techniques (Broomhead *et al*, 1987; Broomhead and King, 1986; Ott *et al*, 1994) to hydrophobically transformed amino-acid sequences and/or subsequences (Manavalen and Ponnuswamy, 1978; Nozaki and Tanford, 1971; Reynolds *et al*, 1974; Zimmerman *et al*, 1968), including those that are conformationally disordered in globular proteins (Mandell *et al*, 1997a, b, 1998b), polyproteins (Mandell *et al*, 1998c), membrane channels and transporters, (Mandell *et al*, 1998a; Selz *et al*, 1998) and Type I (Mandell *et al*, 2001) and G-protein-coupled (Mandell *et al*, 1997c, 2000b) membrane receptors. Recently, other groups have begun applying some of these techniques of one-dimensional signal analysis to protein structural characterization and prediction (Hirakawa *et al*, 1999; Murray *et al*, 2002; Lio and Vannucci, 2000; Giuliani *et al*, 2002; Wouters *et al*, 2000).

For the physicochemical transformation $aa_i \rightarrow hb_i$, we have, from the beginning (Mandell, 1984; Mandell *et al*, 1987), used the oldest complete equilibrium, binary solvent partition-derived, amino-acid hydrophobic scale, normalized and parameterized in *kcal/mol* (Manavalen and Ponnuswamy, 1978; Nozaki and Tanford, 1971) (see http:\\pref.etfox.ht/split/scales.html for alternative scales).

Initially, the relatively short hb_i data series lengths of protein sequences and the characteristic multimodality of their wavelengths upon simple Fourier transformation led to difficulties in the isolation of potential hydrophobic binding modes (Mandell, 1984; Mandell *et al*, 1987). Their signals were often buried under strongly hydrophobic structural domains and/or highly hydrophobic segments, such as those of N-terminal signal sequence or seven transmembrane sequences. We were therefore led to approach this problem as an autocovariance matrix eigenvalue/eigenvector decomposition and eigenfunction construction problem. This group of techniques allows the computational removal of the masking hydrophobic modes of structural segments, permitting the identification and characterization of the underlying potential binding modes.

Hierarchical sets of orthogonal wave patterns, we called them hydrophobic eigenmodes, can be computationally extracted using linear transformations of the hydrophobically transformed sequences of any sufficiently long amino-acid sequence, even those of disordered proteins and/or segments (Mandell *et al*, 1998a, 1997c, 2000a). The hb_i series is used to create a sequentially lagged data matrix, with lags of $0, 1, 2, \ldots, M$, $M = 12$–$18aa$. The data matrix can be linearly decomposed through its $M \times M$ autocovariance matrix, C_M. The eigenvectors, χ_i of the autocovariance matrix can be ordered by the value of their associated eigenvalues. Each of the leading eigenvectors are then composed with the original hydrophobic series, hb_i, to form the set of orthogonal autocovariance eigenfunctions, ψ_i (Broomhead *et al*, 1987; Broomhead and King, 1986). The M value (for the D_2 human long-form dopamine receptor, $M = 15$), which determines the length of the eigenvector template, and therefore the length of the peptides to be designed, was chosen such that the correlation between the leading eigenfunction, ψ_1, dominated by the transmembrane segments, and the nearest neighbor averaged hydropathy plot (Kyte and Doolittle, 1982), also dominated by the transmembrane segments, was maximal (Mandell *et al*, 1997c).

A partial description of the relevant eigenfunctions was achieved using the 'all poles' power spectral transformation, which picks out only one or sometimes two leading frequencies, their inverse being their wavelengths, ω^{-1} (or $\bar{\omega}$) in (statistical, often fractional) units of *aa*. The techniques for autocovariance matrix decomposition and power spectral transformation of the relevant eigenfunctions (Mandell *et al*, 1998a, 1997c, 2000a, b; Selz *et al*, 1998) are derived from those developed for hierarchical pattern finding in real numbered sequences, such as those generated by nonlinear dynamical systems (Broomhead *et al*, 1987; Broomhead and King, 1986; Golub and Van Loan, 1993; Madan, 1993; Press *et al*, 1988). Owing to space limitations, the reader is directed to our earlier references for detailed descriptions of the computational techniques.

When the eigenfunctions, ψ_i, are transformed using all poles power spectral transformations (Madan, 1993; Golub and Van Loan, 1993), they each manifest a single or sometimes two characteristic peaks indicating the statistical wavelength of that eigenfunction. The result is a family of hydrophobic wavelengths, of which helical turns ($\omega^{-1} \approx 3.6aa$), beta strands ($\omega^{-1} \approx 2.2aa$), and beta turn

($\omega^{-1} \approx 2.0aa$) patterns are members. By this method, we uncover a variety of characteristic wavelengths ranging from $\omega^{-1} = 2.0aa$ of porins, connexins, and some channel proteins, to $\omega^{-1} = 13.6aa$ or longer in Type I tyrosine kinase receptors such as the nerve growth factor receptor, TrkA (Mandell, 1984; Mandell *et al*, 1987, 1997a, 1998b).

Supporting the premise of polypeptide hydrophobic wave interactions is the large literature describing hydrophobic attraction and aggregation among biopolymers, particularly in charge-shielded (high dielectric constant) aqueous environments (Ben-Naim, 1980; Chothia, 1974; Eisenberg and McLachlan, 1986; Israelachvili and Pashley, 1982; Israelachvilli, 1992; Kauzmann, 1959; Leckband *et al*, 1994; Pashley *et al*, 1985; Sagvolden, 1999). The demonstrated hydrophobic mode matching found in peptide ligand-receptor pairs and self-aggregation in mode-matched polypeptides and proteins is also relevant (Bromberg and Dill, 1994; Godzik and Skolnick, 1992; Lesk and Rose, 1981; Mandell *et al*, 1998a, 2001, 1997c, 2000a ; Panchenko *et al*, 1997; Sandak *et al*, 1998). In this study, our goal was to design peptides that targeted the hydrophobic mode matchable sequential amino-acid patterns found in the vicinity of the extramembranous loops of the human G-protein-coupled, long-form, D_2 dopamine receptor (Araki *et al*, 1992; Selbie *et al*, 1989).

The first orthogonal eigenfunction, ψ_1, is associated with the greatest proportion of the hydrophobic variation of the sequence. In G-protein-coupled, transmembrane proteins, this is usually attributable to the high amplitude, long wavelength variation of the transmembrane segments. As noted above, the graphs of these ψ_1 closely resemble the proteins' hydropathy plots (Mandell *et al*, 1997c), and the choice of autocovariance lag, M, and therefore the lengths of the peptides to be designed is chosen to maximize the correlation between ψ_1 and the protein's hydropathy plot. Here it is the ψ_2 that contains the receptor's hydrophobic binding modes. Wavelet transformation of the hb_i and/or ψ_i yield patterns that confirm the wavelength and, in addition, the sequence locations of these hydrophobic segments as well the eigenmodes as described below (Mandell *et al*, 1997a, b, c; Hirakawa *et al*, 1999; Lio and Vannucci, 2000; Wouters *et al*, 2000; Murray *et al*, 2002; Giuliani *et al*, 2002).

Using the above decomposition we can look past the transmembrane source of hydrophobic variation to other, buried hydrophobic patterns latent in the sequence. This is the case for the ψ_1 of the human long-form D_2 dopamine receptor sequence. ψ_1 has a wavelength, $\omega^{-1} \approx 50aa$, corresponding to the sequential pattern of the highly hydrophobic seven transmembrane segments of the sequence.

The second most prominent eigenvector of D_2's $hb_iM = 15$ lagged autocovariance matrix is denoted χ_2. Composed with D_2's hb_i, it is expressed as the D_2 receptor's secondary eigenfunction, ψ_2. As ψ_2 exemplified both hydrophobic modes of interest for D_2-targeted peptide design, a normalized form of its associated χ_2 served as the template for our L-amino-acid proprietary peptide design process (see Figure 1 and Table 1). Intuitively, the orthogonal eigenvector template serves as a sequence of relative hydrophobic weights which when four-partitioned can be brought into correspondence with the natural four-partition of the Nozaki–Tanford–Zimmerman amino-acid hydrophobicity scale values listed below (Manavalen and Ponnuswamy, 1978). L-amino acids are randomly assigned to each sequence position in the peptide with probability weights for each group obtained from human spinal fluid (Perry *et al*, 1975) and as constrained by hydrophobic group membership and as determined by its level in the vertical four-partition of the sequence of eigenvector template weightings (Figure 1, bottom row, left).

Table 1 Human Dopamine (D_2DA) Targeted Peptides

	Direct effect		Modulatory effect	
Sequence	**CHO**	**LtK**	**CHO**	**LtK**
H-SHQRWEYKGVNCIVY-OH	***	***	***	***
H-ERNRKPYRPKNKYLL-OH	NS	***	NS	***
H-ERNKLNYKNKNKYIL-OH	NS	NS	NS	***
H-SHTAYHWMSCGKIVI-OH	NS	NS	***	*
H-SHQAWRYKNVNCYVI-OH	***	NS	NS	***
H-GETAFRYVNCNVYVY-OH	**	***	NS	NS
H-SRQAFHYKNVQVLVL-OH	NS	NS	NS	NS
H-THQAFHYCNKQCLVI-OH	NS	NS	NS	NS
H-GHSAWRWKSKNVYMI-OH	***	NS	NS	NS
H-NASALHLVGVQCWVY-OH	**	NS	NS	NS
H-SEQAIRICQKGVLMY-OH	*	NS	NS	NS
H-SHSRWRIVSVNVLCY-OH	*	NS	NS	NS

*$0.05 \leqslant p < 0.01$, **$0.01 \leqslant p < 0.001$, ***$p \leqslant 0.0001$, NS, $p > 0.05$.

The χ_2 template in inverted sequence was also assigned amino acids as their D isomer. As shown in Figures 2 and 3, these *retro–inverso* hydrophobic mode conserving, peptidase-resistant peptides were also found to be modulators of the D_2 dopamine receptor-mediated function (Chaturvedi *et al*, 1981; Chorev and Goodman, 1995; Chorev *et al*, 1979; Hearn *et al*, 2000; Mandell *et al*, 2001). The retro–inverso transformation conserves the side-chain-dependent hydrophobic eigenvector mode structure, but not the back bone conformation of their L-amino acid, noninverted peptide congeners, while maintaining and prolonging the physiological actions of peptide ligands such as the enkephalins (Berman *et al*, 1983), vasopressin/oxytocin (Howl *et al*, 1999), and nerve growth factor (Beglova *et al*, 2000). An intuitive way to think about the hydrophobic mode invariance of the retro–inverso transformation involves using the chiral conformation of the threads of a helical screw as an initial reference. Viewing its image in a mirror as analogous to the L→D aa_i transformation, then turning it upside down represents the inversion of the sequence of aa_i. Taken together the two transformations recover the original chiral thread conformation of the hydrophobic mode (Guptasarma, 1992; Ribeiro *et al*, 1983; Yamazaki and Goodman, 1991).

The direct and modulatory actions of 12 computationally designed L-amino-acid D_2 receptor-targeted 15 mer peptides were tested *in vitro* using the microphysiometric assay of the extracellular acidification rate response, EAR, to dopamine in stably human long-form D_2 receptor-transfected Chinese hamster ovary, CHO, and mouse fibroblast, LtK, cells, see Table 1 (McConnell *et al*, 1992; Neve *et al*, 1992). Two of the microphysiometrically active peptides then underwent retro–inverso transformation (synthesized using D amino acids in reverse order, to $\geqslant$95% HPLC-MS purity by Multiple Peptide Systems, La Jolla, CA). These two

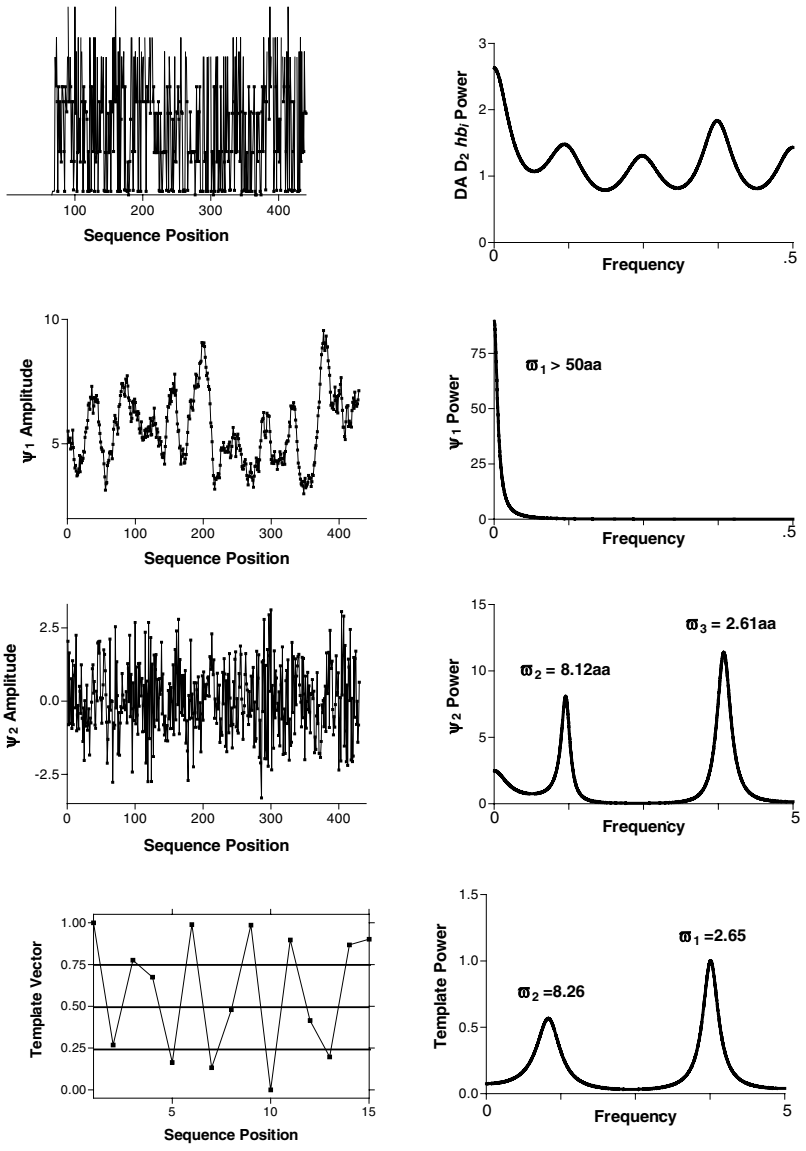

Figure 1 Graphs representing steps in the process of receptor sequence hydrophobic transformation, analyses, and the resulting hydrophobic eigenvector template extraction used in D_2 dopamine receptor-targeting peptide design (see text for details).

retro–inverso peptides were tested and kinetically characterized *in vitro* with respect to their ability to act upon the D_2 dopamine receptor-mediated EAR in receptor stably transfected LtK cells (McConnell *et al*, 1992; Neve *et al*, 1992). They both demonstrated highly significant direct and modulatory effects in this system.

One of the retro–inverso peptides was then tested *in vivo* by bilateral infusion into the rat *nucleus accumbens*, and examined for its capacity to act directly and/or modulate the effect of parenterally administered amphetamine on quantitative aspects of bounded rat exploratory behavior (Feifel *et al*, 1997; Geyer *et al*, 1987a, 1972; Kinkead *et al*, 2000; Ott and Mandel, 1995; Segal and Mandell, 1974). It is generally accepted that the *nucleus accumbens* is prominently dopaminergic. In pilot experiments (results not shown), the retro–inverso peptide was examined for its influence on the prepulse inhibition paradigm in rats in comparison with the effects of parenteral amphetamine (Caine *et al*, 1995; Davis *et al*, 1990; Ralph *et al*, 1999; Swerdlow *et al*, 1986, 1992).

METHODS AND RESULTS

Hydrophobic Transformation: $aa_i \rightarrow hb_i$

The hydrophobic free-energy values, in kcal/mol, assigned to amino acids in D_2DAR are from the solvent partition derived Nozaki–Tanford–Zimmerman hydrophobicity scale (Manavalen and Ponnuswamy, 1978; Nozaki and Tanford, 1971; Reynolds *et al*, 1974; Zimmerman *et al*, 1968), with the exception of the sequence 'structure breaker' proline, which was set to 0.00 instead of its solvent partition-derived 2.77 kcal/mol. Specifically, the values for hydrophobic free energy of the *aa* in kcal/mol used in the following computations are: $G = 0.0$, $P = 0.0$, $Q = 0.0$, $S = 0.07$, $T = 0.07$, $N = 0.09$, $D = 0.66$, $E = 0.67$, $R = 0.85$, $A = 0.87$,

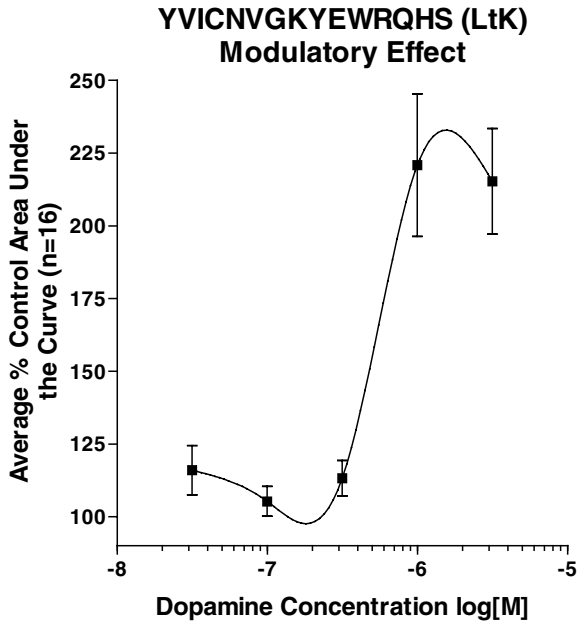

Figure 2 Kinetic interactions of dopamine and retro–inverso peptide on the extracellular acidification rate in a D2 dopamine receptor-transfected cell system (see text for details).

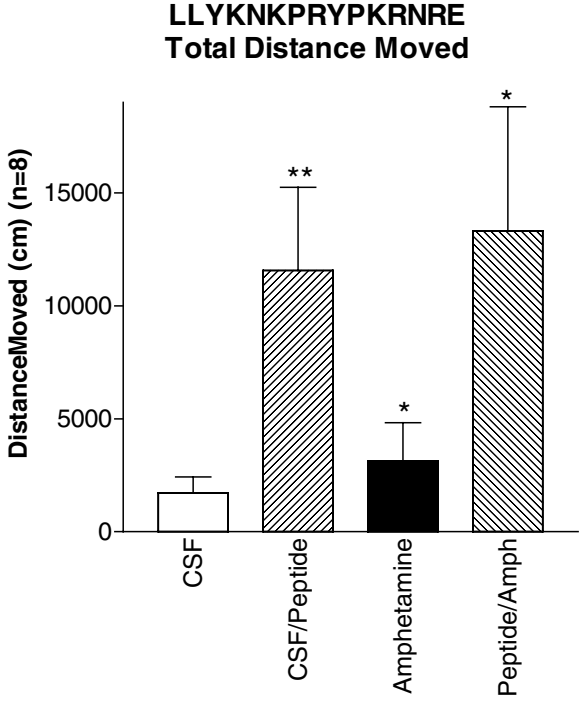

Figure 3 Changes in the properties of rat exploratory behavior following bilateral nucleus accumbens infusion of retro–inverso peptide and/or parenteral amphetamine (see text for details).

$H = 0.87$, $C = 1.52$, $K = 1.65$, $M = 1.67$, $V = 1.87$, $L = 2.17$, $Y = 2.76$, $F = 2.87$, $I = 3.15$, $W = 3.77$. Note the naturally disjunctive four-partition of these values: {0.0–0.09}, {0.66–0.87}, {1.52–1.87}, and {2.17–3.77}. These group memberships are matched to the four-partitioned eigenvector template that is used in the peptide design process.

Computational Hydrophobic Autocorrelation Eigenmode Analyses and Peptide Design Targeting the Putative Binding Modes of D_2DAR's hb_i

Figure 1 (top row, left) illustrates the results of the $aa_i \rightarrow hb_i$ transformation, in kcal/mol, of the human long-form D_2 dopamine receptor, and (top row, right) the all poles power spectral transformation of this undecomposed sequence. It manifests the expected noisy, broadband multiplicity of modes (see discussion above). The second row (left) illustrates the result of the decomposition of the $M = 15$ lagged autocovariance matrix, C_M, of this hb_i. The C_M's leading eigenvector, χ_1, when composed with the original hb_i, generated the leading eigenfunction, ψ_1, shown in the graph. Its peaks are consistent with the ≈ 7 highly hydrophobic transmembrane segments characteristic of the family of G-protein-coupled receptors (Mandell *et al*, 1997c). When ψ_1 was power spectrally transformed (second row, right) it revealed a dominant inverse frequency of $\omega^{-1}(\bar{\omega}) \geqslant 50aa$, the average intertransmembrane plus transmembrane repeat wavelength of the D_2DAR in *aa*.

Figure 1 (third row, left) portrays ψ_2, resulting from the composition of the C_M's χ_2 (fourth row, left) with hb_i (top row, left). The third row (right) portrays the all poles power spectrum of ψ_2, with wavelengths of $\omega^{-1}(\bar{\omega}) = 8.12aa$ and $2.61aa$. They represent the hydrophobic wavelengths of the putative ligand–receptor hydrophobic binding eigenmodes. The corresponding power spectral transformation of χ_2 is shown on the fourth row left, demonstrating the conservation of mode wavelengths from ψ_2. A normalized and four-partitioned version of χ_2 serves as the template for hydrophobic group-specific, human spinal fluid amino-acid distribution-constrained (Perry *et al*, 1975), random amino-acid assignment in our peptide design (Mandell *et al*, 2001) (fourth row, right).

As described above, the template (Figure 1, fourth row, left) was equipartitioned into four levels that correspond to the natural four-partition of the Nozaki–Tanford–Zimmerman hydrophobicity scale as described above. The quartile occupied by each of the 15 values of the template determined the choice among the four corresponding hydrophobic classes of amino acids within which a random assignment of specific amino-acid members of each Nozaki–Tanford–Zimmerman hydropathy group was made from probabilities weighted by their relative normalized levels within each group in human cerebrospinal fluid, a reflection of the brain's free amino-acid distribution available for peptide synthesis (Perry *et al*, 1975). In all, 12 15 mer, D_2DAR-targeted peptides were designed in this manner and then synthesized to $\geqslant 95\%$ purity using the HPLC-MS criteria by Multiple Peptide Systems (La Jolla, CA) (Table 1).

Direct and Modulatory D_2 Receptor-Mediated Extracellular Acidification Responses to χ_2 Template-Designed Peptides

The *in vitro* evaluation of the direct and dopamine-modulatory actions of the 12 algorithmically designed and synthesized peptides listed in Table 1 involved measuring the integrated area under the curve (AUC) of the extracellular acidification rate, EAR, in human long-form D_2 stably transfected Chinese hamster ovary, CHO, and

mouse fibroblast, LtK, cell systems using Cytosensor microphysiometry (McConnell *et al*, 1992; Neve *et al*, 1992). A mouse fibroblast, LtK, cell line expressing the D_2DAR was generously provided by Frederick Monsma (Hoffman La-Roche, Basel) (Sibley *et al*, 1993). A Chinese hamster ovary, CHO, cell line expressing D_2DAR was kindly provided by Richard Mailman (UNC, Chapel Hill) (Lawler *et al*, 1999).

Changes in cellular metabolism and/or Na^+/H^+ exchanges across cellular membranes alter extracellular protonic concentrations, $[H^+]$. Increased extracellular H^+ neutralizes the charge on the surface of the silicon, photocurrent-driven, semiconductor sensor, and reduces the current conductance at a rate linearly related to H^+ production. Integration of the time-dependent H^+ production as AUC is computed by the trapezoidal approximation. Perfusion of buffer through the Cytosensor's cell chambers generated baseline AUC values, which served as the within-chamber control. Measures of the response of the cell system to peptide perfusion and peptide treatment followed by dopamine perfusion of the cell chambers served as indicators of the direct peptide and peptide-modulated, dopamine-induced, D_2DAR-mediated cell activity (Bouvier *et al*, 1993; Neve *et al*, 1992). A direct effect is indicated by a change in the AUC of the EAR in response to the peptide alone in buffer as compared with control, and a modulatory effect is indicated by a change in response to dopamine perfusion alone compared with dopamine perfusion preceded by peptide perfusion. In our hands, this technique demonstrated sensitivity in the range of 0.001 pH unit and changes of as little as 2% of the control were replicable using the Cytosensor® Microphysiometer (Molecular Devices, Sunnyvale, CA).

Of the 12 peptides in Table 1, 10 demonstrated statistically significant direct and/or modulatory *in vitro* EAR activity in one or both D_2-transfected CHO and LtK cell systems.

It has generally been the expectation that high throughput screening of 100 000 randomly ordered sequences from general peptide libraries would yield 2–4 promising hits (Guner, 1999; Spencer, 1998). Assuming this estimate as a basis for a Bayesian prior assumption, then a relevant theorem (Cox and Hinkley, 1974) says that at a random survey success rate of 5 per 100 000, $p(B) = 0.00005$, the probability of the algorithmic design's observed rate of hits, $p(A) = 0.83$, would occur at a chance expectation of

$$\frac{p(A|B)p(B)}{p(A)} = \frac{0.0000415 \times 0.00005}{0.83} = 0.25 \times 10^{-8}.$$

Two of the 12 template-designed peptides indicated in Table 1, SHQR… and ERNR…, that were active *in vitro* underwent hydrophobic eigenmode, but not backbone orientation preserving (see above), retro–inverso transformation: (L-*aa*) SHQR… → (D-*aa*) YVIC… and (L-*aa*) ERNR… → (D-*aa*) LLYK… (Chorev and Goodman, 1995; Goodman *et al*, 1992). The nonlinear, variable slope, sigmoid concentration-activity behavior of these retro–inverso congeners (Figure 2) is consistent with a hydrophobically mediated (eigenmode-matching) mechanism (Boysen *et al*, 1999). The D-amino-acid peptide, being resistant to L-amino-acid peptidases, augers an increased time and/or potency of action over the L-amino-acid peptides.

The kinetic function portrayed in the graph in Figure 2 is the standard, iteratively computed best fit, four-parameter logistic, variable Hill coefficient (slope), sigmoidal concentration–response curve generated by (Ferscht, 1977)

$$y = \min + \frac{(\max - \min)}{1 + 10^{\log EC_{50} - [\text{ligand}] \times \text{Hill coefficient}}}.$$

Figure 2 exemplifies the characteristic away-from-the-active-site, modulatory actions observed with both our ortho (Table 1) and retro–inverso peptides. For each point, $n = 16$ and standard errors of each point is indicated. Figure 2 shows that with the preinfusion of a fixed, 1 μM concentration of the D-amino-acid, retro–inverso peptide YVIC…, the integrated (AUC) EAR dopamine concentration–response curve was nonlinearly augmented up to ≈225% compared with the ($n = 16$) dopamine alone values. The sigmoidal kinetic function is consonant with an away-from-the-active-site, modulatory, 'allosteric,' action (Hall, 2000; Hoare *et al*, 1996) by the D_2 receptor-targeted, hydrophobic mode-matched retro–inverso peptide. Similar positive sigmoidal modulatory effects following the preinfusion of fixed, 1 μM concentrations of dopamine across increasing peptide concentrations (not shown) have also been observed. This suggests mechanisms involving interactions between heterosteric and allosteric sites as receptor protein 'linked-functions' (Hall, 2000; Lumry, 1995; Proska and Tucek, 1995; Wyman and Gill, 1990). These issues will be examined parametrically in future studies.

Two Behavioral Characterizations of the Actions of a D_2 Receptor Eigenmode-Matched Retro–Inverso Peptide, LLYK…, 1 μg Administered Bilaterally into the Nucleus Accumbens of Rats and Interactions with Amphetamine

The bar graph in Figure 3 indicates the means and standard errors of a well-established quantitative aspect of a rat exploratory behavioral paradigm (Geyer *et al*, 1987a), total distance traveled in the arbitrary time (2 h) of observation. Compared is this quantity following bilateral nucleus accumbens infusions of artificial CSF, parenteral administration of amphetamine, 1 mg/kg, bilateral infusion of artificial CSF followed by 1 μg peptide into the nucleus accumbens, and bilateral infusion of artificial CSF followed by parenteral administration of amphetamine, 1 mg/kg.

The animals were housed in groups of three under reversed dark : light conditions, with food and water *ad libitum*. All animal studies were conducted under NIH guidelines, publication 85-23. The behavior was determined over 2 h in 225–250 g Harlan Sprague–Dawley rats, $n = 8$, for each point. Each was placed in a 75 cm × 75 cm white Plexiglas arena and videotaped continuously under red light conditions. The animals locations were automatically determined using Ethovision 2.0 (Noldus Information Technology, The Netherlands). The threshold for movement was defined as a greater than 3 cm change in position of the center of mass of the rat within 2 s. Postprocessed videotapes of the behavior were digitized at six frames per second, and integrated over time with respect to the total distance moved in centimeters. The anatomical locations of the intracerebral cannulae were confirmed histologically.

From the data indicating a peptide-induced increase in receptor-mediated activity shown in Figure 2, an augmentation hypothesis led to the use of a one-tailed *t*-test to assess statistical significances of the average total distance traveled in 2 h as graphed in Figure 3. For CSF/peptide *vs* CSF, $t_{(7)} = 3.588$, $\rho = 0.0089$; CSF/peptide *vs* amphetamine, $t_{(7)} = 3.275$, $\rho = 0.0136$; peptide/amphetamine *vs* CSF, $t_{(7)} = 3.510$, $\rho = 0.0099$; peptide/amphetamine *vs* amphetamine, $t_{(7)} = 3.259$, $\rho = 0.0139$; CSF *vs* amphetamine approached significance, $t_{(7)} = 2.276$, $\rho = 0.057$; and CSF/peptide *vs* peptide/amphetamine was not significant.

Another experimental model for evaluating the putative D_2 (but not D_3 or D_4) dopamine receptor-mediated amphetamine-like effects on rat behavior is its disruption of prepulse inhibition in a rat startle paradigm (Geyer *et al*, 1999; Ralph *et al*, 2001). We used this well-established experimental model and instrumentation (San Diego Instruments) to look for convergent evidence for the amphetamine-like behavioral effect of the bilateral nucleus accumbens administration of the D_2 hydrophobic mode-matched retro–inverso peptide, LLYK… (Bakshi *et al*, 1995; Mansbach *et al*, 1988; Binder *et al*, 2001a, b). Pilot studies using intraventricular administration of the peptide by Mark Geyer's group (UCSD) and bilateral nucleus accumbens administration of the peptide by Becky Kinkead's group (Emory University) demonstrated the expected, statistically significant amphetamine-like reversal of prepulse inhibition following peptide alone, and a peptide-induced augmentation of the amphetamine reversal of prepulse inhibition. Systematic dose–response and time of action studies will be required for definitive results with respect to the prepulse inhibition paradigm.

SUMMARY

De novo L- and D-amino-acid 15 mer peptides were designed targeting the human long-form D_2 dopamine receptor using the secondary eigenvector of its hydrophobically transformed autocovariance matrix as the template. These peptides augmented the dopamine-induced increase in integrated extracellular acidification rate, AUC of EAR, in receptor stably transfected cell systems with an 83% hit rate. A D-amino-acid, receptor eigenmode-matched, retro–inverso exemplar, LLYKNKPRYPKRNRE, demonstrated a positive cooperative influence on dopamine-induced augmentation of AUC of EAR *in vitro*. When LLYK… was infused bilaterally into the *nucleus accumbens* of rats, it increased the amount of exploratory behavior and heightened the amphetamine-induced increase in motoric activity induced by the parenteral administration of amphetamine. Pilot work demonstrated that following intraventricular or *nucleus accumbens* administration, LLYK… also disrupted prepulse inhibition in rats. Receptor hydrophobic eigenmode-matched targeting may be a useful new approach to modulatory neuropeptide design.

ACKNOWLEDGEMENTS

This work is supported by MH 58026. Some of the methods and all of the amino-acid sequences designed by these methods and contained in this work are proprietary (Patents 09/490,792; 09/490,701; 09/767,460 (January 24, 2000)).

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