Chapter 2: Dissecting the functions of conserved prolines within transmembrane helices of the D2 dopamine receptor*

2.1 Abstract

G protein–coupled receptors (GPCRs) contain a number of conserved proline residues in their transmembrane helices, and it is generally assumed these play important functional and/or structural roles. Here we use unnatural amino acid mutagenesis, employing α-hydroxy acids and proline analogs, to examine the functional roles of five proline residues in the transmembrane helices of the D2 dopamine receptor. The well–known tendency of proline to disrupt helical structure is important at all sites, while we find no evidence for a functional role for backbone amide *cis–trans* isomerization, another feature associated with proline. At most proline sites, the loss of the backbone NH is sufficient to explain the role of the proline. However, at one site – P210$^{5.50}$ – a substituent on the backbone N appears to be essential for proper function. Interestingly, the pattern in functional consequences that we see is mirrored in the pattern of structural distortions seen in many GPCR crystal structures.

2.2 Introduction

Proline stands apart from the other 19 canonical amino acids. Its cyclic side chain uniquely shapes protein structure and facilitates protein dynamics. As proline's side chain substantially restricts its backbone psi angle and removes a backbone hydrogen bond donor, proline disrupts the regular structures of both α-helices and β-sheets and can

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introduce flexibility into these structures.[1-4] Further, proline's backbone N–substitution biases its peptide bonds to the *cis* conformer, relative to other peptide bonds. The *cis*–*trans* isomerization of proline peptide bonds is well documented to mediate protein folding and mature protein function.[5] Here we examine which of the above properties contribute to function of the D2 dopamine G protein–coupled receptor (GPCR) at conserved proline sites within its transmembrane α-helices (TMs), using conventional and unnatural amino acid mutagenesis.

Transmembrane proline residues are a characteristic feature of GPCRs and are found in five of the D2 receptor's seven TMs. These prolines are also highly conserved; three (P201$^{5.50}$, P388$^{6.50}$, and P423$^{7.50}$) are the most conserved residue of their respective helix among Class A GPCRs and the remaining two (P89$^{2.59}$, P169$^{4.59}$) are conserved among aminergic GPCRs. (Superscripts refer to Ballesteros–Weinstein numbering, in which the most conserved residue of helix X is denoted X.50).[6] A wealth of conventional mutagenesis studies has already established that these prolines play some significant functional role in GPCRs.[7-14] Proline kinks have long been hypothesized to mediate the helical movements involved in GPCR activation, acting as pivot points, hinges, and/or swivels to expose a G protein binding site at the intracellular end of the helical bundle.[15,16] A recent crystal structure of the closely related D3 dopamine receptor confirms that the prolines investigated here are associated with helical kinks, especially prominent in TMs 2, 6, and 7 (Figure 2.1).[17] P169$^{4.59}$ sits only one helical turn from the N–terminus of TM4, although the pre–proline turn is indeed kinked relative to the rest of the helix. Interestingly in the D3 and other GPCR crystal structures, TM5 is only slightly kinked, but instead possesses a prominent bulge in the helical turn preceding P201$^{5.50}$.[17-22]

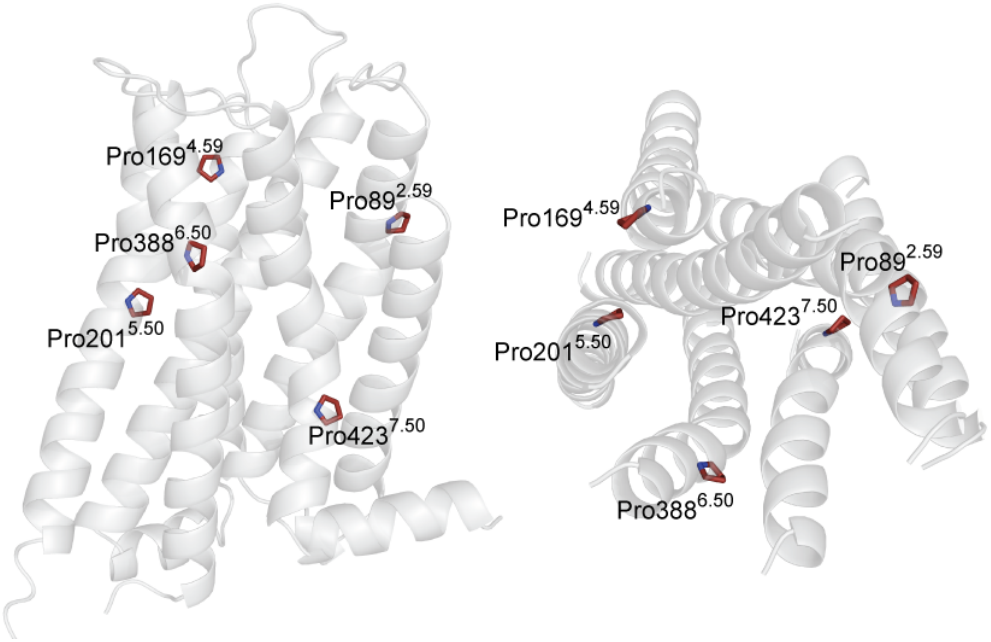


Figure 2.1. Two views of the crystal structure of the D3 dopamine receptor (PDB: 3PBL), highlighting the prolines considered here.[17] Numbering is for the corresponding residues in the D2 receptor.

Proline's ability to distort helices most obviously comes from its lack of backbone hydrogen bond donor ability, which frees its would–be hydrogen bond acceptor, the backbone carbonyl of the residue *i–4* from proline (Figure 2.2). Additionally, the proline side chain introduces a steric clash with the *i–4* carbonyl. The net effect is usually manifested as a kink, which often frees the carbonyl *i–3* to proline as well.[2,5,23,24]

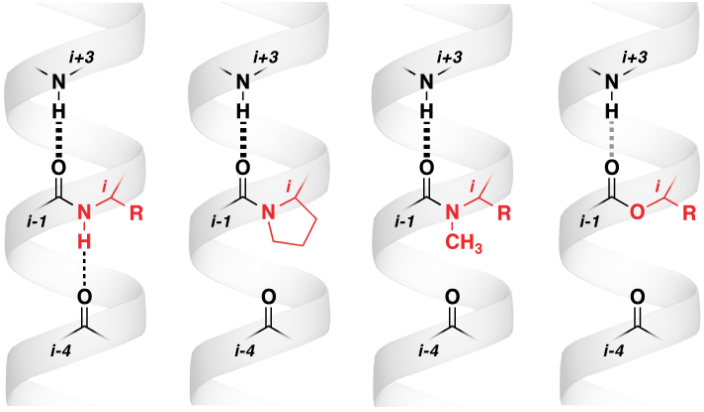
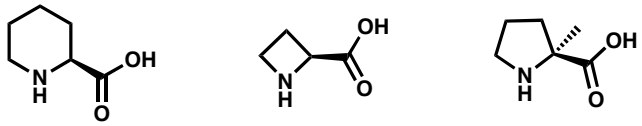


Figure 2.2. Hydrogen bonding patterns in an α–helix for (left to right) a typical amino acid; proline; an N–methyl amino acid; and an α–hydroxy acid.

Since the 19 other canonical amino acids are ill suited to probe the unique properties of proline, unnatural amino acid mutagenesis is an especially valuable tool to dissect the basis for a given proline's functional importance. As structural information on GPCRs continues to accrue, the subtle and high precision probes provided by unnatural residues will become increasingly valuable. Tests of detailed structural environments and specific functional roles are significantly facilitated by the unnatural amino acid methodology.

Here we compare the effect of replacing each of 5 TM prolines of the D2 receptor with unnatural α–hydroxy acids, and an N–methyl amino acid (Figure 2.2). We also consider cyclic proline analogs and conventional amino acids that correspond to the α–hydroxy acids. Unnatural α–hydroxy acids introduce a backbone ester that, like the proline peptide bond it replaces, is not a hydrogen bond donor (Figure 2.2). N–Me–Ala, effectively proline lacking its side chain C_γ, shares N–substitution (and thus lack of hydrogen bond donor ability) with proline, but has greater conformational freedom. Finally, proline analogs (Figure 2.3) that vary the size of the ring or introduce substituents can probe tolerance for subtle changes to the proline side chain as well as *cis–trans* isomerization. Pipecolic acid (Pip) and azetidine–2–carboxylic acid (Aze), six– and four–membered ring analogs of proline, respectively, have a greater *cis* bias relative to proline, while 2–methylproline (2–Me–Pro) is substantially more *trans*–biased.[5,6]



Pip Aze 2-Me-Pro

Figure 2.3. Unnatural proline analogs employed in this study

2.3 Results

2.3.1 Experimental approach

We utilized a recently optimized electrophysiological readout of the D2 receptor that yields quantitative dose–response relationships from receptor activation of coexpressed GIRK1/4 channels in *Xenopus laevis* oocytes and is amenable to unnatural amino acid mutagenesis by nonsense suppression.[7-14,25] All unnatural amino acid and hydroxy acid mutations, as well as the valine mutants noted, were generated by nonsense suppression, while all other mutants were expressed conventionally. A wild–type rescue experiment (incorporating Pro by nonsense suppression) at each proline site yields an EC_{50} within two–fold of the wild–type value, validating the ability to perform nonsense suppression at all residues considered here.

Because of the indirect nature of the measurement, interpretation of EC_{50} requires some caution. A detailed discussion of this issue has been presented elsewhere.[15,16,26] For present purposes, we consider EC_{50} values that differ by a factor of greater than 2 to be distinguishable in this assay.

2.3.2 Strategy and general observations

Previously, we have characterized three distinct phenotypes for functionally important proline residues in ligand–gated ion channels. In the M1 transmembrane helix of both the α subunit of the muscle–type nicotinic acetylcholine receptor and the 5–HT_{3A} receptor, a conserved proline in the middle of the helix can be replaced by a range of α–hydroxy acids, giving essentially wild–type behavior.[17,27,28] In contrast, incorporating any canonical amino acid other than proline gave a nonfunctional receptor. A second phenotype was seen in the M2–M3 loop of the 5–HT_{3A} receptor.[17-22,29] In this system,

cis–trans isomerization of a proline peptide bond is critical for receptor gating, as revealed by a series of cyclic proline analogs with varying *cis* preferences. Finally, we have characterized an important proline–aromatic interaction in the Cys loop of the muscle–type nicotinic acetylcholine receptor, in which the hydrophobicity of a Phe side chain preceding proline is important for receptor function.[30] We seek to determine which of these phenotypes is most applicable to the transmembrane prolines of the D2 receptor, and we began by incorporating α–hydroxy acids at each site.

For the most part, incorporation of an α–hydroxy residue is not strongly perturbing; all but one of the hydroxy acid mutants we could characterize gave EC_{50}s within 5-fold of wild type (Figure 2.4, Table 2.1). In addition, each conventional amino acid residue is typically much more perturbing than its unnatural α–hydroxy acid analog. This trend suggests the importance of lacking a hydrogen bond donor at these sites. N–Me–Ala provides an alternative way to remove the backbone NH group, and it is also well–tolerated at all sites. Given the results for α-hydroxy residues and N–Me–Ala, it is not surprising that various cyclic proline analogs, which are much more similar to proline, are well–tolerated at all transmembrane proline sites, yielding EC_{50}s within 5-fold of wild type.

Mutations of proline to the conventional amino acids Ala, Val, and Leu range from modestly perturbing to dramatically perturbing (EC_{50} shifts of 3.5–fold to >2000–fold from wild type), with the exception of $P89^{2.59}$, discussed below. Generally, Gly is less perturbing than the other natural amino acids, with the glaring exception of $P201^{5.50}$G. These conventional mutants provide a useful reference to which we can compare the α–hydroxy mutants.

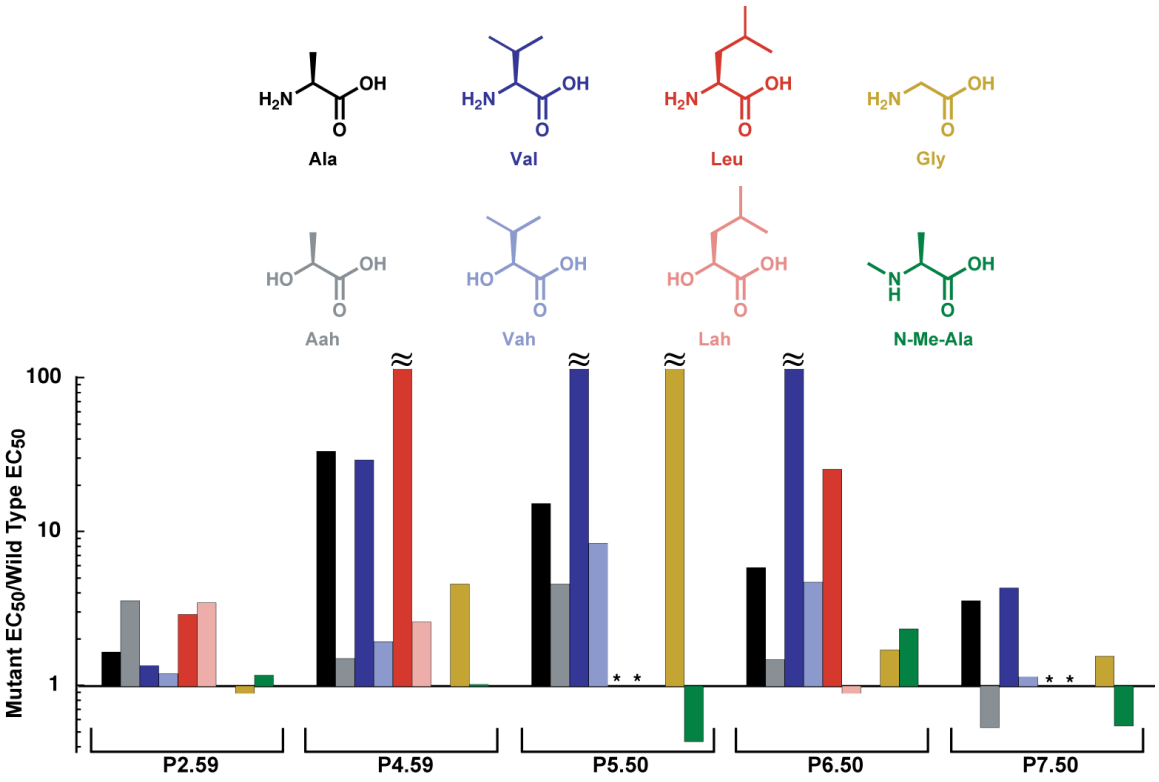


Figure 2.4. Results for incorporating α–hydroxy acids; their amino acid analogs; Gly; and N–Me–Ala. At four sites, indicated by a ≈, EC_{50} is too large to determine accurately. * = no current detected.

The $P89^{2.59}$ site, despite >70% conservation among aminergic receptors, accepts diverse mutations with only modest functional consequences. All mutations made, including conventional mutations, give wild–type EC_{50}s or relatively small shifts from wild type. Evidently, this site does not require the unique side chain and hydrogen bonding properties conferred by proline, and we will not consider it further here.

Table 2.1. Conventional amino acid, hydroxy acid, and N-Me-Ala mutations. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

Site	Mutation	EC_{50} (nM)	n_H	n
wild type		39 ± 1	1.12 ± 0.03	71
$Pro89^{2.59}$	Pro[a]	24 ± 1	1.14 ± 0.04	30
	Gly	35 ± 3	1.3 ± 0.1	12
	Ala	65 ± 4	1.32 ± 0.08	10
	Val[b]	53 ± 7	1.2 ± 0.2	11
	Leu	114 ± 5	1.33 ± 0.07	10
	Aah	140 ± 10	0.94 ± 0.05	12
	Vah	47 ± 1	1.20 ± 0.02	13
	Lah	136 ± 4	1.19 ± 0.03	13
	N-Me-Ala	46 ± 2	1.11 ± 0.04	19
$Pro169^{4.59}$	Pro[a]	41 ± 2	1.17 ± 0.05	49
	Gly	180 ± 10	1.00 ± 0.06	14
	Ala	1310 ± 80	1.12 ± 0.06	12
	Val[b]	1150 ± 70	1.03 ± 0.05	12
	Leu	>2000[c]		14
	Aah	59 ± 3	1.20 ± 0.07	15
	Vah	76 ± 4	1.42 ± 0.09	14
	Lah	102 ± 2	1.27 ± 0.03	27
	N-Me-Ala	40 ± 2	1.22 ± 0.06	11
$Pro201^{5.50}$	Pro[a]	24 ± 1	1.07 ± 0.04	37
	Gly	>2000[c]		17
	Ala	600 ± 50	1.09 ± 0.09	17
	Val	>2000[c]		20
	Leu	ND[d]		
	Aah	180 ± 10	1.3 ± 0.1	9
	Vah	330 ± 10	1.01 ± 0.04	14
	Lah	ND[d]		
	N-Me-Ala	16 ± 1	1.15 ± 0.08	14
$Pro388^{6.50}$	Pro[a]	46 ± 2	1.18 ± 0.04	34
	Gly	67 ± 3	1.24 ± 0.06	9
	Ala	230 ± 10	1.25 ± 0.06	14
	Val[b]	>2000[c]		15
	Leu	1000 ± 100	0.87 ± 0.07	10
	Aah	58 ± 2	1.43 ± 0.07	15
	Vah	185 ± 6	1.31 ± 0.05	9
	Lah	35 ± 2	1.12 ± 0.07	25
	N-Me-Ala	92 ± 4	1.26 ± 0.06	12
$Pro423^{7.50}$	Pro[a]	35 ± 2	1.13 ± 0.06	43
	Gly	61 ± 5	1.19 ± 0.09	16
	Ala	140 ± 10	1.4 ± 0.1	10
	Val[b]	170 ± 8	1.01 ± 0.04	16
	Leu	ND[d]		
	Aah	21 ± 2	1.4 ± 0.1	8
	Vah	45 ± 3	1.10 ± 0.05	16
	Lah	ND[d]		
	N-Me-Ala	21.5 ± 1	1.25 ± 0.06	10

[a] *Expression of the wild type receptor with the natural amino acid incorporated by nonsense suppression*
[b] *Conventional mutant generated by nonsense suppression*
[c] *Response of naïve oocytes to dopamine doses ≥ 100 μM obscures complete dose-response data*
[d] *No dopamine-induced current detected for dopamine concentrations up to 1 mM*

2.3.3 $169^{4.59}$, $388^{6.50}$, $423^{7.50}$: Importance of lacking a backbone hydrogen bond donor

For the TM4, 6, and 7 proline sites, we observe hydroxy acid mutations (to Aah, Vah, or Lah; "ah" signifying α–hydroxy) in all cases to be less perturbing than the corresponding conventional amino acid mutations (to Ala, Val, or Leu) (Figure 2.4, Table 2.1). In fact, most of the hydroxy acid mutants for these sites ($P169^{4.59}$Aah, $P169^{4.59}$Vah, $P388^{6.50}$Aah, $P388^{6.50}$Lah, $P423^{7.50}$Aah, and $P423^{7.50}$Vah) have EC_{50}s that are not meaningfully different from wild type (i.e., within the margin of error we can expect from this assay). As hydroxy acids and proline both lack backbone hydrogen bond donors, this property alone may account for proline's functional role at these sites. However, hydroxy acids do introduce additional perturbations, including weakening the hydrogen bond acceptor strength of the preceding residue's backbone carbonyl, and an electrostatic repulsion by the introduced main chain oxygen with the *i–4* carbonyl.

An alternative way to remove the backbone NH of a peptide bond is with N–Me–Ala, which contains a fragment of the proline ring and does not significantly perturb the backbone carbonyl. At the helix 4, 6, and 7 prolines, N–Me–Ala is not largely perturbing, being essentially equivalent to Aah and producing EC_{50}s within 2.5-fold of wild type. This suggests that the carbonyl mutation associated with α-hydroxy residues is not a large factor in these helices.

Given the tolerance for α-hydroxy and N–methyl residues, it is not surprising that the proline analogs Aze and Pip are also well tolerated in helices 4, 6, and 7 (Table 2.2). As described in previous work, Aze and Pip show different intrinsic *cis–trans* preferences than Pro.[5] The minimal impact of these mutations at these sites indicates that *cis–trans* isomerization is not an essential component of receptor function. While varying ring size

does not substantially alter function, introducing a methyl substituent at proline's α carbon (2–Me–Pro) does produce measurable EC_{50} shifts at the $388^{6.50}$ and $423^{7.50}$ sites, but not at $169^{4.59}$.

Table 2.2. Proline analog mutations. EC_{50} and Hill coefficient (n_H) are ± SEM for goodness of fit to the Hill equation.

Site	Mutation	EC_{50} (nM)	n_H	n
$Pro89^{2.59}$	Pip	26 ± 2	1.04 ± 0.04	11
	Aze	26 ± 3	1.3 ± 0.1	11
	2-Me-Pro	64 ± 3	1.25 ± 0.05	9
$Pro169^{4.59}$	Pip	19 ± 2	1.00 ± 0.09	12
	Aze	25 ± 3	1.1 ± 0.1	17
	2-Me-Pro	37 ± 3	1.3 ± 0.1	18
$Pro201^{5.50}$	Pip	34 ± 2	1.14 ± 0.06	21
	Aze	41 ± 2	0.90 ± 0.03	10
	2-Me-Pro	160 ± 10	0.95 ± 0.07	15
$Pro388^{6.50}$	Pip	82 ± 5	1.12 ± 0.07	17
	Aze	47 ± 3	1.26 ± 0.07	11
	2-Me-Pro	131 ± 5	0.77 ± 0.02	15
$Pro423^{7.50}$	Pip	80 ± 5	1.27 ± 0.09	10
	Aze	15 ± 2	1.1 ± 0.1	12
	2-Me-Pro	128 ± 7	0.95 ± 0.04	12

Interestingly, Gly is less perturbing than the other conventional amino acids at these sites. We do note, however, that the $P423^{7.50}$G mutant gave currents that were generally small, suggesting either poor expression or diminished receptor efficacy. While no $P423^{7.50}$ mutants produced dramatic EC_{50} shifts, cells injected with appropriate mRNA and tRNA for the Leu and Lah mutants gave no response in our assay; evidently the leucine side chain is not tolerated.

2.3.4 $201^{5.50}$: N–substitution as well as lack of a hydrogen bond donor are important

The $201^{5.50}$ site shows a qualitatively distinct pattern from the other prolines considered here. As before, α-hydroxy residues are less perturbing than their α–amino analogs. Unlike the other proline sites, however, no hydroxy acid mutation to $P201^{5.50}$

yields a wild–type EC_{50}. P201$^{5.50}$Aah and P201$^{5.50}$Vah are loss of function by 4– and 8–fold, respectively, while we were unable to characterize the P201$^{5.50}$Lah mutant (though the same was true of the Leu mutant). Interestingly, N–Me–Ala is minimally perturbing, perhaps even gain of function, and the proline analogs Pip and Aze are wild type. Taken together, these data suggest that simply deleting the hydrogen bonding ability of the backbone NH is not sufficient to produce a functional receptor. A substituent on the N is also necessary, and it can be either a ring (Pro, Pip, Aze) or a methyl (N–Me–Ala). Also in contrast to the TM4, 6, and 7 sites, Gly is highly disruptive at 201$^{5.50}$, more so than Ala. Together with the fact that no hydroxy acid tested gave a wild–type EC_{50} at this site, these findings suggests a more specific need for proline here, not merely a generic requirement for a helix–breaking residue.

2.4 Discussion

In the present work, we have used nonsense suppression techniques and the assay system we recently developed to evaluate an array of unnatural residues at five new sites of the D2 dopamine receptor. Our results establish the generality of this protocol for evaluating GPCR function with unnatural residues.

In particular, we have probed five conserved proline sites located in the transmembrane region of the D2 receptor. Transmembrane prolines are not uncommon, and are frequently considered to play important functional roles by introducing structural perturbations and/or increased conformational flexibility to the helix.[4,15] In previous work we established several types of unnatural residues to probe proline function: α-

hydroxy acids to evaluate the role of backbone hydrogen bonding,[27,28] and a series of proline analogs to evaluate any role for *cis–trans* isomerization around the proline peptide bond.[29] In many ways, the prolines considered here respond to our various mutations in a common fashion, although we find that P201$^{5.50}$ shows a functional phenotype that is distinct from the other residues.

Not surprisingly, P89$^{2.59}$, which is located in a helix that is not intimately involved in ligand binding nor conformational changes, is generally tolerant of mutations and does not show any obvious trends in the mutagenesis data.

Three prolines on more critical helices – P169$^{4.59}$, P388$^{6.50}$, and P423$^{7.50}$ – share a functional phenotype. Conventional amino acids are modestly to substantially disruptive, but their corresponding α-hydroxy acids significantly restore function. N–Me–Ala and the proline analogs Pip and Aze are also well tolerated. Taken together, these results indicate that the key function of proline at these sites is to disrupt the α helix by removing the key backbone NH. Any strategy that accomplishes this, including cyclic residues, α-hydroxy residues, or N–methyl residues, produces receptors with essentially wild type function. Interestingly, glycine is also well tolerated. Glycine is well known to have an especially low propensity for helical structures relative to other amino acids (second–lowest only to proline),[31,32] a property believed to stem both from its high conformational entropy and from its minimal burial of solvent accessible surface area in helices.[33] The good tolerance of Gly at these sites suggests a generic functional requirement for a helix breaker at these sites. Proline appears to accomplish this by lack of a backbone hydrogen bond donor, but Gly can achieve the same end by different means.

Structurally, all three prolines seem to play a similar role also. In various structures of GPCRs, helices 4, 6, and 7 all display a significant kink associated with the proline.[17-21] Movements of helices 6 and 7 are considered to be critical to receptor activation, with the kinks playing a prominent role.[16] Indeed, a recent crystal structure thought to represent an active conformation of the β2 adrenergic receptor shows displacement of TM6 accomplished by a slight "unwinding" of the helical turn preceding the proline at position 6.50.[34] The movement originates at residue 6.46, the would–be backbone hydrogen bond acceptor to position 6.50, potentially providing a direct link between proline 6.50's lack of hydrogen bond donor ability and functionally important helix flexibility.

Because the α-hydroxy residues do not seriously compromise receptor function at these sites (the way their α–amino analogs do), we can use the α–hydroxy data to consider the effects of side chain variation. The largest perturbations are seen for Lah at P169$^{4.59}$ and Vah at P388$^{6.50}$. This suggests that the 169$^{4.59}$ site is sensitive to size, while 388$^{6.50}$ is sensitive to β-branching. No response was detected for cells injected with appropriate mRNA and tRNA for P423$^{7.50}$L and P423$^{7.50}$Lah mutations, suggesting this site is especially sensitive to steric bulk. Supporting this notion, in the D3 receptor crystal structure, the P423$^{7.50}$ side chain points directly at TM1, while P169$^{4.59}$ and P388$^{6.50}$ are directed more toward the lipid bilayer.[17] Curiously, the P423$^{7.50}$G mutant gives small signals (though a near-wild-type EC_{50}), suggesting this site may play an important role in GPCR folding, transport, and/or function, possibilities we cannot differentiate with our assay.

P201$^{5.50}$ shows a different pattern in response to the array of mutations considered here. α–Hydroxy residues are more strongly perturbing, while N–Me–Ala and the cyclic proline analogs Pip and Aze are wild type. This suggests that simply removing the NH is not enough to achieve the function of the native proline residue. There must be a substituent on the backbone N. Consistent with this, glycine is highly perturbing at P201$^{5.50}$, in contrast to the other sites.

It is clear from Figure 2.1 that the structural perturbation associated with the proline of helix 5 is distinct from what is seen in helices 2, 4, 6, and 7. Instead of a prominent kink, helix 5 has a bulge. It has been proposed that this bulge is functionally significant, as residues in the bulge directly contact ligands in the agonist binding site. Specifically, the side chain of S197$^{5.46}$, which is *i–4* relative to P201$^{5.50}$ and located at the center of the bulge, is thought to contribute a hydrogen bond that is important to agonist binding.[35,36] Evidently, the steric effect of proline's N–substitution is key to producing this bulge.

2.5 Conclusions

In all functionally important transmembrane proline sites we have investigated to date – the TM 4, 5, 6, and 7 prolines in this study and the M1 transmembrane proline of two different ligand–gated ion channels – we find that lack of a backbone hydrogen bond donor is important for function.[27,28] A general phenotype has emerged in which loss of function mutations to conventional amino acids are mitigated by the corresponding α–hydroxy acids. In contrast, α–hydroxy residues produced nonfunctional human 5–HT_3

receptors when replacing a proline that appears to undergo *cis–trans* isomerization.[29] This proline lies not in an α–helix, but in a loop/turn region, where *cis–trans* isomerization seems much more plausible.

We do observe variations on the general transmembrane proline phenotype. Unlike the D2 receptor's TM4, 6, and 7 prolines, the requirement for lacking a backbone hydrogen bond donor is absolute at the ion channel M1 proline sites; conventional amino acids, including glycine, produced completely nonfunctional channels.[27,28] The TM5 proline of the D2 receptor additionally requires N–substitution for fully wild–type function. Taken together, these studies establish the power of unnatural amino acid methodology for dissecting out the various consequences of the proline residue's unique structural features.

2.6 Experimental

2.6.1 Molecular biology

In these experiments, the cDNA for GIRK1 and GIRK4 was in pBSMXT plasmids and for the D2 receptor (human long form) was in the pGEMhe plasmid. Site–directed mutagenesis was performed using the Stratagene QuikChange protocol to generate the appropriate codon. For unnatural amino acid mutants and conventional mutants generated by nonsense suppression, the site of interest was mutated to the TAG stop codon. Plasmids were linearized with the appropriate restriction enzymes (the GIRK plasmids with SalI and the D2 receptor with NheI or SbfI). Receptor mRNA was

prepared by *in vitro* runoff transcription using the Ambion T7 mMessage mMachine kit and GIRK1 and GIRK4 mRNA was prepared with the T3 kit.

Hydroxy or amino acids, all commercially available, were appended to the dinucleotide dCA and enzymatically ligated to truncated 74mer THG73 tRNA as previously described.[37,38] The 74mer tRNA was prepared using the Ambion T7 MEGAshortscript™ kit by transcription from a DNA oligonucleotide template with its 5' terminal two nucleotides bearing methoxy groups at the ribose C2' position to enhance RNA transcript homogeneity, as described in the literature.[39] Crude tRNA–amino acid or tRNA–hydroxy acid product was used without desalting, and the product was confirmed by matrix–assisted laser desorption ionization time–of–flight mass spectrometry on a 3–hydroxypicolinic acid matrix. Deprotection of the NVOC group on the tRNA–amino acids or NB group on tRNA–Aah was carried out by 5–min photolysis on a 1 kW xenon lamp with WG–335 and UG–11 filters immediately prior to injection. tRNA–Vah and –Lah were unprotected and were injected directly.

2.6.2 Oocyte preparation and RNA injection

Stage V–VI oocytes of *Xenopus laevis* were harvested and injected with RNAs as described previously.[37] For nonsense suppression experiments, each cell was injected with 15 ng each of GIRK1 and GIRK4 mRNA approximately 64 hours before recording, then 4 – 30 ng receptor mRNA and approximately 25 – 60 ng appropriate tRNA approximately 48 hours before recording. Mutants yielding small responses required a second injection of receptor mRNA and appropriate tRNA 24 hours before recording.

For wild type experiments, each cell received a single injection of 0.16 ng receptor mRNA and 10 ng each of GIRK1 and GIRK4 mRNA approximately 48 hrs before recording. Conventional amino acid mutants (except those generated by nonsense suppression) were prepared identically, except 1 ng receptor mRNA was required. Small responses were obtained for P423$^{7.50}$G, which required 10 ng receptor mRNA. Injection volumes for each injection session were 25 – 75 nL per cell.

As a negative control for suppression experiments at each site, unacylated full length tRNA was co–injected with mRNA in the same manner as charged tRNA. These experiments yielded negligible responses for all sites. Wild–type recovery conditions (injecting tRNA–Pro and appropriate mRNA) were injected alongside mutant nonsense suppression conditions to control for data variability.

2.6.3 Electrophysiology

Oocyte recordings were made in two–electrode voltage clamp mode using the OpusXpress^{TM} 6000A (Axon Instruments). Recording buffers were ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, pH 7.5) and high K^+ ringer (96 mM NaCl, 24 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 1.8 mM $CaCl_2$, pH 7.5). Solution flow rates were 2 mL min^{-1} and drug application flow rates were 2.5 mL min^{-1}. Initial holding potential was –60 mV. Data were sampled at 125 Hz and filtered at 50 Hz. Cells were subjected to a ND96 pre–wash for 10 s, a high K^+ application for 50 s to establish basal currents, and dopamine application in high K^+ ringer for 25 s, followed by high K^+ and ND96 washings for 45 s and 90 s in duration, respectively. Dopamine–induced currents were measured over the basal K^+ current as described previously.[25] Dopamine (Sigma–Aldrich) solutions in high K^+ ringer were prepared immediately before recording

by dilutions from a 1 M stock in ddi water. Dose–response data were obtained for a minimum of eight concentrations of dopamine, for a minimum of two cell batches, and for a minimum of 8 cells total. Dose–response relations were fitted to the Hill equation, $I_{Norm} = 1/(1+(EC_{50}/A))^{nH}$, where I_{Norm} is the normalized current peak at [dopamine] = A, EC_{50} is the concentration of agonist that elicits a half–maximum response, and n_H is the Hill coefficient. EC_{50} values were obtained by averaging the I_{Norm} values for each cell at a given dose and fitting those averaged I_{Norm} data to the Hill equation.

2.7 References

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