Published in final edited form as:

Chem Biol Drug Des. 2016 October ; 88(4): 615–619. doi:10.1111/cbdd.12789.

Various modifications of the amphipathic dynorphin A pharmacophore for rat brain bradykinin receptors

Yeon Sun Lee^{1,*}, Robert Kupp¹, Michael Remesic¹, Cyf Ramos-Colon¹, Sara M. Hall¹, Christopher Chan¹, David Rankin², Josephine Lai², Frank Porreca², and Victor J. Hruby¹

¹Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ 85721, USA

²Department of Pharmacology, University of Arizona, Tucson, AZ 85721, USA

Abstract

As a unique endogenous opioid ligand, dynorphin A shows paradoxical neuroexcitatory effects at bradykinin receptors, and the effects are known to be amplified by the upregulation of dynorphin A under chronic pain and inflammatory conditions. In our earlier structure-activity relationship studies, the amphipathic dynorphin A fragment, [Des-Arg⁷]-Dyn A-(4–11), was identified as a pharmacophore for the bradykinin receptors along with key structural features. Here, further modifications of the pharmacophore showed that the position of a Pro residue is also an important feature because of its role in making (or disrupting) a β -turn or 3_{10} helix structure which is crucial for receptor recognition.

Graphical abstract

AA = amino acid;

AA¹ = a hydrophobic amino acid (or a hydrophobic peptide fragment);

AA² = a basic amino acid (or a basic amino acid fragment or Phe);

m $\geq$ 1

Various modifications of amphipathic dynorphin A pharmacophore for central bradykinin receptors showed that the position of a Pro residue in analogues is an important feature for the receptor due to the role in making (or disrupting) β -turn or 3_{10} helix.

*Corresponding authors: Yeon Sun Lee; Tel.: +1 520 626 2820; Fax: +1 520 621 8407; yeon@email.arizona.edu.

Conflict of Interest

Authors declare that there is none.

Keywords

Non-opioid dynorphin A; Structure-Activity relationship; Amphipathicity; Proline; Bradykinin receptors; Chronic pain

Identifying a pharmacophoric structure of an endogenous ligand for a receptor is crucial for the design of novel ligands, the ultimate goal of which is to develop therapeutic agents for the receptor related disease. In our previous studies, we identified the amphipathic pharmacophore of dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH) for central bradykinin receptors (BRs) which can attenuate neuroexcitatory effects caused by non-opioid Dyn A ([Des-Tyr¹]-Dyn A analogues) (1–4). The neuroexcitatory effects cannot be attenuated by opioid antagonists, because opioid receptors are not involved in the process. As a lead ligand, [Des-Arg⁷]-Dyn A-(4–11) **1** (Table 1) blocked Dyn A-(2–13)-induced hyperalgesia and motor impairment in in vivo tests using naïve animals and reversed thermal hyperalgesia and tactile hypersensitivity in nerve injured animals in a dose-dependent manner (1). Since the 1980s, many studies have repeatedly demonstrated the neuroexcitatory (pronociceptive) effects of Dyn A in the central nervous systems (CNS) and the involvement of non-opioid receptors such as BRs and N-Methyl D-aspartate receptors in the CNS (5–11). Interestingly, there is no evidence to show the existence of endogenous ligands for the BRs such as bradykinin and kallidin or their precursors in the CNS even with the receptor's involvement in the area. Therefore, Dyn A's interaction with the central BRs suggests that Dyn A may be an endogenous ligand for the receptor in the CNS. It has also been shown that under pathological conditions of chronic pain, nerve injury, and inflammation, Dyn A is up-regulated and interacts with the BRs to cause hyperalgesia in the CNS (9–12). Taken together, the central BRs are a critical mediator of the pathological pain states in response to endogenous Dyn A or its analogues.

It was shown that an amphipathic property of Dyn A to distribute positive charges of basic amino acid residues is the key structural feature for interaction with central BRs (1,3–4). This was the identification of the pharmacophoric structure of Dyn A for the receptor which has a potential to treat pathological pain states. However, the lead ligand **1** even with the truncation of a Arg residue which can avoid enzymatic degradation followed by the recognition of paired Arg residues exhibited very low enzymatic stability (half-life < 1 h in rat plasma) (13,14). Thus, there remained a need to improve the enzymatic stability and so various modifications were performed on the ligand. As per the results, it was found that i) C-terminal amidation, which is one of the simplest ways for improving stability, is not tolerated, ii) the replacements of basic amino acid residues and/or hydrophobic amino acid residues with other non-natural basic amino acids and/or non-natural hydrophobic amino acids are endured in most cases as long as the amphipathicity is retained, iii) modifications in the N-terminal region do not affect the affinities but modifications in the C-terminal region are critical.

Reversal of the direction of peptide bonds in the backbone (retro-), chirality of the α -carbons (inverso-), and their combination (retroinverso-), in general, create a plethora of structural changes in relatively simple and easy ways (15,16). Retroinverso- and inverso-

modified peptides can enhance their stability because of the inverted chirality which is not easily recognized by enzymes. Retro-peptides are being used to investigate secondary structure stabilization factors dependent on backbone orientation (17). Helix formation and stabilization appeared to be very similar in both normal and retro peptides, despite the unfavorable charge-macrodipole interactions and bad N-capping in the retro peptide. Therefore, the comparative SAR analysis of parent, retroinverso-, inverso-, and retro-peptides along with other modified peptides were performed to investigate factors that conserve the topographical structure without changes in amino acid compositions and to obtain an additional structural insight for the BRs.

All analogues were synthesized by standard solid phase peptide synthesis using N^α-9-fluorenylcarboxy (Fmoc)-chemistry on amino acid preloaded Wang resin in high yields (overall yield > 30% for ligands **1–11** and > 20% for ligands **13–14**) (1). Chain elongation was carried out by repetitive coupling (3 equiv 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate/3 equiv N-hydroxybenzotriazole/6 equiv diisopropylethylamine in *N,N*-dimethylformamide (DMF), 1 h) and deprotection (20% piperidine in DMF, 20 min). Crude peptide was detached from the resin using a cocktail solution (95% trifluoroacetic acid: 2.5% triisopropylsilane: 2.5% water, 3 h) and could be isolated by preparative reverse phase-high performance liquid chromatography (RP-HPLC) using gradient (10–40% acetonitrile in water containing 0.1% trifluoroacetic acid in 15 min) in a short time (t_R < 15 min; < 40% acetonitrile) owing to their hydrophilic characters (aLOGPs < -1) (Table 2). The purified analogues were validated by analytical RP-HPLC (purity > 97%) and high resolution mass spectroscopy and tested for their binding affinities by radioligand competition analysis using [³H][des-Arg¹⁰, Leu⁹]-kallidin (DALKD) in rat brain membranes where non-specific binding is defined by 10 μM kallidin. Data were analyzed by non-linear least squares analysis using GraphPad Prism 4. Logarithmic values were determined from nonlinear regression analysis of data collected from at least two independent experiments in duplicates.

In our earlier studies, it was shown that simple amphipathic ligand **9**, which is composed of 3 Arg, 2 Nle, a Pro, and a Phe, retained the same good binding affinity (IC₅₀ = 87 nM) as lead ligand **1** at BRs (2,3). This is the case for ii as discussed above. The simple amphipathic ligand **9** was obtained by replacing hydrophobic amino acids (Leu and Ile) and basic amino acid (Lys) in ligand **1** with Nle residues and an Arg residue, respectively. The high affinity of ligand **9** supported our suggestion of the amphipathic pharmacophore as another good fit for the BRs. In an effort to improve enzymatic stability, as described above, retro-inverso and inverso modifications were performed on ligand **9** but the resulting ligands **10** and **11** did not show any interaction with the BRs (no competition). The loss of binding in ligand **10** was simply attributed to the lack of a C-terminal basic amino acid which is necessary for receptor recognition along with amphipathicity. The loss of affinity in ligand **11**, which satisfies the two structural requirements, a C-terminal basic amino acid and amphipathicity, indicated that D-chirality in the amphipathic structure, especially at the C-terminal part (case for iii), was not tolerated for the BRs. It is clear that both retro-inverso and inverso modifications, which cause the reversal of the chirality, cannot be applied for the interactions of the amphipathic pharmacophoric structure and the receptor even with many advantages.

In contrast, retro modification of lead ligand **1** retained the same good affinity ($IC_{50} = 50$ nM) even with a non-basic C-terminal amino acid, Phe, that does not meet the key structural feature (3). The Phe residue at the C-terminus was truncated resulting in ligand **3** with 19-fold reduced affinity ($IC_{50} = 930$ nM), which indicated a potential role of the electron rich aromatic moiety of the Phe residue for receptor recognition. The aromatic moiety may act like an Arg or a Lys residue for electrostatic interactions that are suggested to be crucial for interaction with the BRs. Studies showed that the reversal of backbone and chirality of a peptide sometimes results in different retention time on a RP-HPLC-column even with the same hydrophobicity because of conformational change (18). This is because the retention time is directly correlated with a peptide's ability to adopt a specific conformation such as a α -helix. An amphipathic helical structure can induce a preferred hydrophobic binding domain and result in stronger retention on RP-column (19,20). This trend was observed in our studies. Our previous NMR studies showed that ligand **1** has a β -turn or a 3_{10} helix like conformation. Unlike ligand **1**, ligand **2** showed very different retention time on HPLC even with the same amino acid composition and similar lipophilicity (ALOGPs: -1.63) (Table 2). Compared to the other ligands, **2** possesses a relatively stronger retention time ($t_R = 14.5$ min). This may hint at how the ligand forms a specific conformation binding to the BRs with its C-terminal Phe residue.

The reduced affinity of ligand **3** was not recovered by the replacement of the C-terminal Leu residue to the N-terminal Leu in ligand **6**. The resulting ligand **6** even showed 5-fold decreased affinity ($IC_{50} = 4,300$ nM). It should be noted that ligand **6** clearly fulfills the key structural requirement in terms of having a C-terminal basic amino acid and amphipathicity, but did not exhibit good affinity. Further modifications of the structure by the truncation of the C-terminal Arg residue and the attachment of an N-terminal Lys residue also did not change the binding affinity in ligands **7** ($IC_{50} = 6,000$ nM) and **8** ($IC_{50} = 6,300$ nM). Ligand **3** which did not have a C-terminal basic amino acid showed better binding affinity than ligand **6**, and the insertion of a Leu residue between Lys and the Pro in ligand **5** slightly increased binding affinity ($IC_{50} = 320$ nM). These results led us to consider a role for the Pro residue in the ligand's topographical structure. Pro is known to act as a structural disruptor in the middle of secondary structures such as α -helices and β -sheets, but nevertheless is still commonly found in the end part of a helix and assists to make β -turns (21, 22). Statistics indicates that a total of 89% of the Pro residues in α -helices occur in the first turn, with the most favored location being the second position, and there are significantly no Pro residues observed at position 5 where a hydrogen bond with position 1 is necessary to stabilize the first turn (22). In longer peptide chains, the Pro residue up to and including position 4 does not disrupt structure and is rarely found after position 4 in helices. In our case, the Pro residue was shown to cause a break of strong consecutive turns (type III β -turn or 3_{10} helix) (1). Based on the Pro position-structure relationship, the losses of binding affinity in ligands **6–8** are considered to be caused by the Pro residue being placed in the middle of the sequence to disrupt the β -turn or 3_{10} helix structure for BRs. Overall, ligands (**1–5, 9**) having a Pro residue in the end parts showed better binding affinity compared to the ligands (**6–8**) that have a Pro in the middle of the sequence regardless of the existence of a C-terminal basic amino acid. Longer chain ligands (**12–14**) which are retro-modified originally from Dyn A-(2–13) showed the same range of high binding affinity as

the parent ligand (3). It should be noted that a Pro residue is at position 4 in the longer chain ligands. In an effort to promote a helical structure that appears to be necessary for the BRs recognition and to investigate the turn structure stabilizing effect, α -aminoisobutyric acid (Aib) residues were simply introduced into the hydrophobic residue positions, because the introduction of multiple Aibs is well known to stabilize a 3_{10} helix in short peptides by restricting conformational space (23–25). The resulting simple amphipathic ligands **15–17** with multiple Aibs (2 or 3) and C-terminal Arg residue did not exhibit an interaction with the receptor and so indicated that it may be crucial to keep flexible alkyl group for hydrophobic interaction along with basic amino acids for electrostatic interaction and a high degree of constraint caused by multiple Aibs may alter the conformation extremely.

In conclusions, we have synthesized a series of amphipathic analogues modified from lead ligand **1**, tested for their binding affinities at rat brain BRs, and analyzed them to get further insights into SAR for the receptors. The SAR results suggest that the position of a Pro residue in ligands may be another important structural feature to make a β -turn or possibly a 3_{10} helix for the BRs. Now we conclude that amphipathic ligands with a C-terminal basic amino acid or an electron-rich aromatic ring moiety, which can build an active conformation through a structure forming Pro residue, can be a ligand for the central BRs like Dyn A (Figure 1).

Acknowledgments

This work has been supported by U.S. Public Health Services, NIH, and NIDA P01DA006284 and R01DA013449.

Abbreviations

Aib	α -aminoisobutyric acid
BR	bradykinin receptor
CNS	central nervous system
DALKD	[des-Arg ¹⁰ , Leu ⁹]-kallidin
DMF	<i>N,N</i> -dimethylformamide
Dyn A	dynorphin A
Fmoc	9-fluorenylmethoxycarbonyl
Nle	norleucine
RP-HPLC	reversed-phase high performance liquid chromatography
SAR	structure-activity relationship

References

1. Lee YS, Muthu D, Hall SM, Ramos-Colon C, Rankin D, Hu J, Sandweiss A, Felice MD, Xie JY, Vanderah TW, Porreca F, Lai J, Hruby VJ. Discovery of amphipathic dynorphin A analogues to

- inhibit the neuroexcitatory effects of dynorphin A through bradykinin receptors in the spinal cord. *J Am Chem Soc.* 2014; 136:6608–6616. [PubMed: 24742335]
2. Bannister K, Lee YS, Goncalves L, Porreca F, Lai J, Dickenson AH. Neuropathic plasticity in the opioid and non-opioid actions of dynorphin A fragments and their interactions with bradykinin B2 receptors on neuronal activity in the rat spinal cord. *Neuropharmacol.* 2014; 85:375–383.
  3. Lee YS, Rankin D, Hall SM, Ramos-Colon C, Ortiz JJ, Kupp R, Porreca F, Lai J, Hruby VJ. Structure-activity relationships of non-opioid [des-Arg⁷]-dynorphin A analogues for bradykinin receptors. *Bioorg Med Chem Letts.* 2014; 24:4976–4979. [PubMed: 25282551]
  4. Lee YS, Hall SM, Ramos-Colon C, Remesic M, LeBaron L, Nguyen A, Rankin D, Porreca F, Lai J, Hruby VJ. Modification of amphipathic non-opioid dynorphin A analogues for rat brain bradykinin receptors. *Bioorg Med Chem Letts.* 2015; 25:30–33. [PubMed: 25434001]
  5. Walker JM, Moises HC, Coy DH, Baldrihi G, Akil H. Nonopiate effects of dynorphin and Des-Tyr-dynorphin. *Science.* 1982; 218:1136–1138. [PubMed: 6128791]
  6. Faden AI, Jacobs TP. Dynorphin-related peptides cause motor dysfunction in the rat through a non-opiate action. *Br J Pharmacol.* 1984; 81:271–276. [PubMed: 6142741]
  7. Skilling SR, Sun X, Kurtz HJ, Larson AA. Selective potentiation of NMDA-induced activity and release of excitatory amino acids by dynorphin: possible roles in paralysis and neurotoxicity. *Brain Res.* 1992; 575:272–278. [PubMed: 1349253]
  8. Vanderah TW, Laughlin T, Lashbrook JM, Nichols ML, Wilcox GL, Ossipov MH, Malan TP, Porreca F. Single intrathecal injections of dynorphin A or des-Tyr-dynorphins produce long-lasting allodynia in rats: blockade by MK-801 but not naloxone. *Pain.* 1996; 68:275–281. [PubMed: 9121815]
  9. Lai J, Luo MC, Chen Q, Ma S, Gardell LR, Ossipov MH, Porreca F. Dynorphin A activates bradykinin receptors to maintain neuropathic pain. *Nat Neurosci.* 2006; 9:1534–1540. [PubMed: 17115041]
  10. Altier C, Zamponi GW. Opioid, cheating on its receptors, exacerbates pain. *Nat Neurosci.* 2006; 9:1465–1467. [PubMed: 17128281]
  11. Podvin S, Yaksh T, Hook V. The emerging role of spinal dynorphin in chronic pain: a therapeutic perspective. *Ann Rev Pharmacol Toxicol.* 2016; 56:511–533. [PubMed: 26738478]
  12. Lai J, Ossipov M, Vanderah TW, Malan TP, Porreca F. Neuropathic pain: the paradox of dynorphin. *Mol Interventions.* 2001; 1:160–167.
  13. Wise RJ, Barr PJ, Wong PA, Kiefer MC, Brake AJ, Kaufman RJ. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc Natl Acad Sci USA.* 1990; 87:9378–9382. [PubMed: 2251280]
  14. Veenstra JA. Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. *Arch Insect Biochem Physiol.* 2000; 43:49–63. [PubMed: 10644969]
  15. Goodman M, Chorev M. On the concept of linear modified retro-peptide structures. *Acc Chem Res.* 1979; 12:1–7.
  16. Chorev M, Goodman M. Recent developments in retro peptides and proteins – an ongoing topochemical exploration. *Tiptech.* 1995; 13:438–445.
  17. Haack T, Sanchez YM, Gonzalez M-J, Giralt E. Structural comparison in solution of a native and retro peptide derived from the third helix of *Staphylococcus aureus* protein A, domain B: retro peptides, a useful tool for the discrimination of helix stabilization factors dependent on the peptide chain orientation. *J Pep Sci.* 1997; 3:299–313.
  18. Subbalakshmi C, Nagaraj R, Sitaram N. Biological activities of retro and diastereo analogs of a 13-residue peptide with antimicrobial and hemolytic activities. *J Peptide Res.* 2001; 57:59–67. [PubMed: 11168889]
  19. Zhou NE, Mant CT, Hodges RS. Effect of preferred binding domains on peptide retention behavior in reversed-phase chromatography: amphipathic alpha-helices. *Pep Res.* 1990; 3:8–20.
  20. Steiner V, Sch M, Btimsen KO, Mutter MJ. Retention behavior of a template-assembled synthetic protein and its amphiphilic building blocks on reversed-phase columns. *Chromatogr.* 1991; 586:43–50.
  21. Blaber M, Zhang XJ, Matthews BW. Structural basis of amino acid alpha helix propensity. *Science.* 1993; 260:1637–1640. [PubMed: 8503008]

22. MacArthur MW, Thornton JM. Influence of proline residues on protein conformation. *J Mol Biol.* 1991; 218:397–412. [PubMed: 2010917]
23. Schweitzer-Stenner R, Gonzales W, Bourne GT, Feng JA, Marshall GR. Conformational manifold of α -Aminoisobutyric Acid (Aib) containing alanine-based tripeptides in aqueous solution explored by vibrational spectroscopy, electronic circular dichroism spectroscopy, and molecular dynamics simulations. *J Am Chem Soc.* 2007; 129:13095–13109. [PubMed: 17918837]
24. Marshall GR, Hodgkin EE, Langs DA, Smith GD, Zabrocki J, Leplawy MT. Solid state governing helical preference of peptides containing multiple α,α -dialkyl amino acids. *Proc Natl Acad Sci USA.* 1990; 87:487–491. [PubMed: 2296604]
25. Benedetti E, Bavoso A, Blasio B, Pavone V, Pedone C, Crisma M, Bonora GM, Toniolo C. Solid-state and solution conformation of homo oligo(α -aminoisobutyric acids) from tripeptide to pentapeptide: evidence for a 3_{10} helix. *J Am Chem Soc.* 1982; 104:2437–2444.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Table 1

Binding affinities of modified Dyn A analogues at BRs in rat brain

no	Dyn A (Tyr ¹ -Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro- Lys-Leu-Lys-Trp-Asp-Asn-Gln ¹⁷ -OH) Analogue	BR ^a , [³ H]DALKD	
		Log[IC ₅₀] ^b	IC ₅₀ (nM)
1	Phe-Leu-Arg-Ile-Arg-Pro-Lys	-7.16±0.09	69 ^c
2	Lys-Pro-Arg-Ile-Arg-Leu-Phe	-7.30±0.09	50 ^d
3	Lys-Pro-Arg-Ile-Arg-Leu	-6.03±0.21	930 ^d
4	Lys-Pro-Arg-Ile-Arg-Phe	-6.23±0.11	590
5	Lys-Leu-Pro-Arg-Ile-Arg-Leu	-6.50±0.06	320
6	Leu-Lys-Pro-Arg-Ile-Arg	-5.37±0.15	4300
7	Leu-Lys-Pro-Arg-Ile	-5.09±0.13	8100
8	Lys-Leu-Lys-Pro-Arg-Ile	-5.20±0.10	6300 ^d
9	Phe-Nle-Arg-Nle-Arg-Pro-Arg	-7.06±0.10	87 ^e
10	DArg-DPro-DArg-DNle-DArg-DNle-DPhe	-	n.c. ^d
11	DPhe-DNle-DArg-DNle-DArg-DPro-DArg	-	n.c. ^d
12	Lys-Leu-Lys-Pro-Arg-Ile-Arg-Arg-Leu-Phe-Gly-Gly	-7.11±0.06	78 ^d
13	Lys-Leu-Lys-Pro-Arg-Ile-Arg-Leu-Phe-Gly-Gly	-6.82±0.15	150 ^d
14	Leu-Leu-Lys-Pro-Arg-Ile-Arg-Arg-Leu-Phe-Gly-Gly	-7.03±0.10	93 ^d
15	Arg-Aib-Arg-Aib-Arg	-	n.c.
16	Aib-Arg-Aib-Arg-Aib-Arg	-	n.c.
17	Arg-Aib-Arg-Aib-Arg-Aib-Arg	-	n.c.

^aCompetition assays were carried out using rat brain membranes.^bLogarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments in duplicate using GraphPad Prism 4.^cReference 1.^dReference 4.^eReference 3. n.c.: no competition.

Table 2

Analytical Data of [des-Arg⁷]-Dyn A analogues

no	molecular formula	LR MS ^a	HR MS ^b		HPLC ^c , (t _R min)	ALOGPs ^d
			observed	calcd. error (ppm)		
4	C ₃₈ H ₆₅ N ₁₃ O ₇	816.5	816.5178	816.5202 2.9	11.8	-1.91
5	C ₄₁ H ₇₈ N ₁₄ O ₈	895.1	895.6201	895.6200 -0.2	12.8	-1.73
6	C ₃₅ H ₆₇ N ₁₃ O ₇	781.5	781.5501	781.5519 2.2	10.6	-2.02
7	C ₂₉ H ₅₅ N ₉ O ₆	625.4	625.4263	625.4269 0.9	10.3	-1.77
15	C ₂₆ H ₅₂ N ₁₄ O ₆	-	657.4257	657.4267 1.5	3.3	-3.45
16	C ₃₀ H ₅₉ N ₁₅ O ₇	-	742.4788	742.4795 0.9	3.3	-3.12
17	C ₃₆ H ₇₂ N ₁₉ O ₈	-	898.5795	898.5806 1.2	3.3	-3.15

^a (M + H)⁺, ESI method (Finnigan, Thermoelectron, LCQ classic).

^b (M + H)⁺, FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF.

^c Performed on a Hewlett Packard 1100 (C-18, Microsorb-MV^{TM}, 4.6 mm x 250 mm, 5 μm) using gradient system (10–100% of acetonitrile containing 0.1% TFA within 45 min, 1 mL/min).

^d <http://www.vcclab.org/lab/alogsps>.