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A radioiodinated linear vasopressin antagonist:

A ligand with high affinity and specificity for V_{1a} receptors

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A linear vasopressin antagonist, Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (Linear AVP Antag) (Phaa = Phenylacetyl), was monoiodinated at the phenyl moiety of the tyrosylamide residue at position 9. This antagonist appeared to be a highly potent anti-vasopressor peptide with a pA_2 value in vivo of 8.94. It was demonstrated to bind to rat liver membrane preparations with a very high affinity (K_i = 0.06 nM). The affinity for the rat uterus oxytocin receptor was lower (K_i = 2.1 nM), and affinities for the rat kidney- and adenohypophysis-vasopressin receptors were much lower (K_i = 47 nM and 92 nM, respectively), resulting in a highly specific vasopressin V_{1a} receptor ligand. Autoradiographical studies using rat brain slices showed that this ligand is a good tool for studies on vasopressin receptor localization and characterization.

V_{1a} vasopressin receptor; Iodinated linear antagonist; Autoradiography

1. INTRODUCTION

Receptors for vasopressin and oxytocin have now been characterized in a large number of tissues and cell types using different radiolabelled ligands (see Table I). Many of these studies, in particular the autoradiographical analysis of receptor distribution in heterogeneous tissues like brain and kidney, have been hampered by the low specific radioactivity and the lack of specificity of these ligands which do not discriminate efficiently between the vasopressin and the oxytocin receptors. Radioiodinated vasopressin and oxytocin analogues of high specific radioactivity and good selectivity are very valuable tools for studying this distribution. We have recently developed an oxytocin antagonist (ligand 8, Table I) which has a very good affinity and a very good selectivity for oxytocin receptors [5].

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Abbreviations: Phaa, Phenylacetyl (C_6H_5-CH_2CO-); D-Tyr-Et), *O*-ethyl D-tyrosine; D-Tyr(Me), *O*-methyl D-tyrosine; (Linear AVP Antag) is used to denote the AVP linear V_{1a} antagonist having the following structure:

1 2 3 4 5 6 7 8 9
Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2.

Its monoiodo and radioiodinated derivatives are designated by: ([I]-Linear AVP Antag) and by ([^{125}I]-Linear AVP Antag), respectively. AVP, arginine vasopressin; OVT, ornithine vasotocin; OT, oxytocin d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Thy^4,Tyr-NH_2^{9}]OVT, [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-*O*-methyltyrosine, 4-threonine, 8-ornithine,9-tyrosylamide]vasotocin

Highly selective ligands for the three subtypes of vasopressin receptors V_{1a}, V_{1b} and V_2 having a high specific radioactivity and a good affinity are still awaited. We have previously prepared a radioiodinated vasopressin antagonist (ligand 9, Table I). This ligand labelled the V_{1a} hepatic vasopressin receptors with a high affinity (0.28 nM). However, it does not discriminate between vasopressin and oxytocin receptors, and it shows a high nonspecific binding in autoradiographical studies [6]. A V_1-vasopressin receptor antagonist (ligand 10, table I) has been prepared and radioiodinated by Kelly et al. [4] and Gerstberger and Fahrenholz [7]. It has an affinity of 3.0 nM for the rat liver vasopressin receptor. Unfortunately, its affinity for the oxytocin receptor has not been estimated, and although this ligand may be useful for autoradiographical studies in the kidney, the very high non-specific binding observed in the brain studies [7,8] makes its use in the brain difficult.

We recently reported some pharmacological properties of a series of 8 linear V_{1a} antagonists containing Tyr-NH_2, designed as potential radioiodinated ligands [9,10]. These were designed by incorporating a C-terminal Tyr-NH_2 at position nine in analogues of the potent and selective linear octapeptide V_{1a} antagonist:

1 2 3 4 5 6 7 8
Phaa-D-Tyr(Et)-Phe-Gln-Asn-Lys-Pro-Arg-NH_2 [11]

having modifications at positions 1, 2 and 6, to give a series of eight Tyr-NH_2^{9} analogues [9,10] (Phaa = Phenylacetyl). The most potent V_{1a} antagonist of this series had a D-Tyr(Me)^2 and an Arg^6 in place of D-

Table I

Radiolabelled ligands for vasopressin and oxytocin receptors

Radiolabelled ligand [1]	Dissociation constants (nM) for binding to receptors from:			
	Rat kidney (V2)	Rat liver (V1a)	Rat hypophysis (V1b)	Rat uterus (OT)
1 [^{3}H]-AVP	0.4	0.6-3	1-3	1.7*
2 [^{3}H]-LVP	7	8	4	1.7*
3 [^{3}H]-OT	370*	78*	250*	1.0-2.5
4 [^{3}H]-[Thr^4-Gly7]OT	>10,000*	>8,000*	ND	1
5 [^{3}H]-des Gly^9-d(CH$_2$)$_5$-[D-Tyr(Et)^2]VAVP	0.4	0.2	ND	ND
6 [^{3}H]-d(CH$_2$)$_5$-[Tyr(Me)^2]AVP	ND	0.3[a]	ND	ND
7 [^{3}H]-dDAVP	0.8[b]	ND	ND	ND
8 [^{125}I]d(CH$_2$)$_5$-[Tyr(Me)^2-Thr^4-Tyr(NH$_2$)^9]OVT	10.2*	13.6*	ND	0.03
9 [^{125}I]d(CH$_2$)$_5$-[Tyr(Me)^2-Tyr(NH$_2$)^9]AVP	1.0*	0.28	ND	0.13*
10 [^{125}I]-d(CH$_2$)$_5$[Sar7]AVP	ND	3.0[c]	ND	ND

[1] For references, see Jard and Barberis [1]
*Values determined from competition experiments using the appropriate ligand. ND, non-determined.
[a] Cornett and Cate [2]
[b] Marchingo et al. [3]
[c] Kelly et al. [4]

Tyr(Et)^2 and Lys^6, respectively. It has the following structure:

Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$ (Linear AVP Antag) [9,10].

In preliminary iodination studies the monoiodinated derivative of this peptide, i.e. [^{125}I]-linear AVP Antag was also found to retain high affinity for V_{1a} receptors. It was thus selected for the studies reported here. We now describe the synthesis, radioiodination and estimation of the activities of this linear vasopressin antagonist ([^{125}I]-Linear AVP Antag) which contains no sulfhydryl groups. It has a very high affinity for vasopressin receptors, a good selectivity for the V_{1a} receptor and a high specific radioactivity. Preliminary autoradiographic studies show good specific labelling with low non-specific binding in areas known to contain vasopressin receptors.

2. MATERIALS AND METHODS

2.1. *Synthesis of Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$ (Linear AVP Antag)*

The protected precursor required for the synthesis of Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$ was synthesized by the manual solid-phase method [12] starting from Boc-Tyr(Bzl) resin following previously described procedures [11,13]. Successive cycles of deprotection, neutralization and coupling gave the protected acyl octapeptide resin. Ammonolysis in methanol [11,13] yielded crude Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg(Tos)-Pro-Arg(Tos)-Tyr(Bzl)-NH$_2$ (A), which was purified by precipitation from dimethylformamide/water; yield 76%, m.p. 190–194°C, $[\alpha]_D^{25}$ = 26.3° (c = 1, DMF). Thin layer chromatography in three different systems showed that this material was pure: R_F(A) = 0.54, R_F(B) = 0.55, R_F(C) = 0.34 (A = butanol/acetic acid/water, 4:1:1; B = butanol/acetic acid/water, 4:1:5 (upper phase); C = chloroform/methanol, 4:1). An aliquot of the protected precursor (A) (110 mg) was reduced with Na/liquid NH$_3$ [14] and the crude peptide was purified in a two-step procedure involving gel filtration on Sephadex G-15 in 50% acetic acid, and on Sephadex LH-20 in 2 N acetic acid as previously described [11,13,15]. The final lyophilization gave the desired product Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$ (linear AVP Antag) as a white powder, 74 mg (88% yield); $[\alpha]_D^{25}$ = −49° (c = 0.1, 50% AcOH), R_F(A) = 0.22, R_F(B) = 0.21, R_F(D) = 0.40 (D = butanol/acetic acid/water, 2:1:1). Amino acid analysis: Asp 1.05; Glu 1.03; Pro 1.02; Tyr 1.85; Phe 1.04; Arg 2.03; NH$_3$ 3.1. The homogeneity of the product was additionally confirmed by HPLC. Some pharmacological properties for this peptide (Linear AVP Antag) [9,10] and its monoiodo derivative ([I]-Linear AVP Antag) are given in Table II.

2.2. *Chloramine-T iodination*

The AVP V_{1a} antagonist (Linear AVP Antag) was iodinated on the 9-tyrosylamide by means of the oxidant chloramine-T (Merck, Germany) as previously described [6]. The unlabelled monoiodinated antagonist ([I]-Linear AVP Antag) was prepared by ICl iodination [5]. It was used to identify the [^{125}I]-Linear AVP Antag on HPLC and to establish inhibition constants for AVP and OT receptors (Tables I and II).

Table II

Biological activities of Linear AVP Antagonist and [I]-Linear AVP Antagonist

Peptide	Anti-vasopressor pA$_2$[c]	Antidiuretic U/mg
Linear AVP Antagonist[a,b]	8.94 ± 0.03	0.042 ± 0.008
[I]-Linear AVP Antagonist	8.70 ± 0.03	0.034 ± 0.004

[a] This has the following structure: Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH$_2$
[b] Data from references [9,10]
[c] Antagonistic properties represent pA$_2$ values ± SE. pA$_2$ is the negative log. of A$_2$ which is the estimated concentration of antagonist reducing the response to 2 × units of agonist to equal the response to 1 × unit administered before the antagonist

2.3. *Bioassays*

Vasopressor antagonistic potencies and antidiuretic agonistic activities were estimated from assays on rats as described by Manning et al. [11].

2.4. *Peptides*

[^{3}H]AVP was obtained from New England Nuclear (Boston, MA). It was purified by HPLC and affinity chromatography on a neurophysin-Sepharose column [16]. [^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2, Thr^4,Tyr-NH$_2^9$]OVT was prepared as described by Elands et al. [5].

2.5. *Membrane preparation*

Female Wistar rats were purchased from Iffa Credo (Lyon, France). Rat liver membranes were obtained according to the method described by Neville up to step 11 [7] and stored in liquid nitrogen until use. Rat kidney membranes were prepared as described by Butlen et al. [18]. Estrogenized rat uterus membranes were prepared as described by Elands et al. [19]. Rat anterior pituitary membranes were prepared as described by Gaillard et al. [20].

2.6. *Binding assays*

Membranes (2 μg for liver, 80 μg for kidney, 2 μg for uterus and 56 μg for adenohypophysis) were incubated in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mg/ml BSA and varying concentrations of labelled and unlabelled peptides. Incubation at 30°C lasted for 20 min ([^{3}H]-AVP, adenohypophysis), 30 min ([^{3}H]-AVP, kidney), 40 min ([^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT, uterus) or 45 min ([^{125}I]-Linear AVP Antag, liver). Non-specific binding was determined in the presence of a 250-fold excess of unlabelled peptide. Bound and free radioactivity were separated by filtration either over Millipore (RAWP, 1.2 μm) filters for the tritiated peptide or over Whatman GF/C filters, presoaked in 10 mg/ml BSA (for at least 2 h) for the radioiodinated peptides, as described [21]. Inhibition constants for the non-labelled analogues were determined in competition experiments in the presence of 0.06 nM [^{125}I]-Linear AVP Antag (liver); 1–1.3 nM [^{3}H]-AVP (kidney); 5.9 nM [^{3}H]-AVP (adenohypophysis), 0.06 nM [^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT (uterus).

2.7. *Autoradiography*

Brain slices from adult female Wistar rats were prepared as described by Elands et al. [5]. They were pre-incubated at room temperature for 15 min in 50 mM Tris-HCl, pH 7.4, containing 0.1% BSA. Incubation was carried out for 1 h at room temperature in a humid chamber by covering each section with 400 μl incubation medium (50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM bacitracin and 0.1% BSA) containing 0.06 nM [^{125}I]-Linear AVP Antag alone or in the presence of 0.1 μM AVP, or 0.05 nM [^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT alone or in the presence of 0.1 μM OT. The slides were dried with cold air and then placed in an X-ray cassette in contact with hyperfilm Betamax (Amersham) for 4 days (antagonist AVP) or 7 days (antagonist OT).

3. RESULTS AND DISCUSSION

3.1. *Biological activities and affinity determinations*

The vasopressin antagonist Linear AVP Antag appeared to be a highly potent antivasopressor peptide with a pA_2 value in vivo of 8.94 (Table II). It has a weak antidiuretic activity. Iodination of the 9-tyrosylamide residue reduced the antivasopressor potency about 45% and did not change antidiuretic activity significantly. The iodinated vasopressin antagonist inhibited [^{125}I]-Linear AVP Antag binding to V_{1a} (liver), [^{3}H]-AVP binding to V_{1b} (adenohypophysis) and V_2 (kidney) vasopressin receptors and [^{125}I]-d(CH$_2$)$_5$-[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT binding to oxytocin receptors from the uterus of an estrogenized rat. In line with in vivo data, it has a very high affinity for V_{1a} receptor (Table III). It has also a fairly high but significantly lower affinity for OT receptors and a very low affinity for V_{1b} and V_2 receptors. Hill plot slopes did not differ significantly from unity, indicating an interaction with single populations of receptor sites in all cases.

3.2. *[^{125}I]-Linear AVP Antag binding*

Specific [^{125}I]-Linear AVP Antag binding to rat liver membranes was a fairly slow process. At 30°C and for 0.1 nM ligand, a concentration close to the equilibrium dissociation constant, a maximum binding was reached within 30 min. The estimated half-time was about 4 min 30 s. All further experiments were performed at 30°C and the duration of membrane incubation in the presence of [^{125}I]-Linear AVP Antag was 45 min. Specifically bound [^{125}I]-Linear AVP Antag could be partially released in a time-dependent manner by adding an excess of unlabelled [I]-Linear AVP Antag. 50% of the specifically bound radioactivity was released after 60 min incubation in the presence of unlabelled [I]-Linear AVP Antag.

Fig. 1 shows a typical Scatchard analysis of [^{125}I]-Linear AVP Antag binding to liver membranes. The high specific radioactivity of [^{125}I]-Linear AVP Antag allowed a precise saturation binding analysis to be done with only 2 μg of membrane protein per assay.

Table III

Affinities of [I]-Linear AVP Antagonist

Inhibition constants of the [I]-Linear AVP Antag were determined in competition experiments. Liver, adenohypophysis, kidney medulla and uterus membranes were incubated with 0.06 nM [^{125}I]-Linear AVP Antag for liver vasopressin receptor, 1.2–5.9 nM [^{3}H]AVP for adenohypophysis and kidney vasopressin receptors, 0.05–0.06 nM [^{125}I]-d(CH$_2$)$_5$ [Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT for uterus oxytocin receptors and varying concentrations of [I]-Linear AVP Antag. Values were obtained in independent experiments, each performed in triplicate

Peptide	Inhibition constants (nM)			
	Liver[1]	Adenohypophysis[2]	Kidney[2]	Uterus[3]
[I]-Linear AVP Antag	0.18	92	62	1.4
	0.17		33	2.8

[1]Competition experiments with [^{125}I]-Linear AVP Antag
[2]Competition experiments with [^{3}H]AVP
[3]Competition experiments with [^{125}I]-d(CH$_2$)^5[Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]OVT

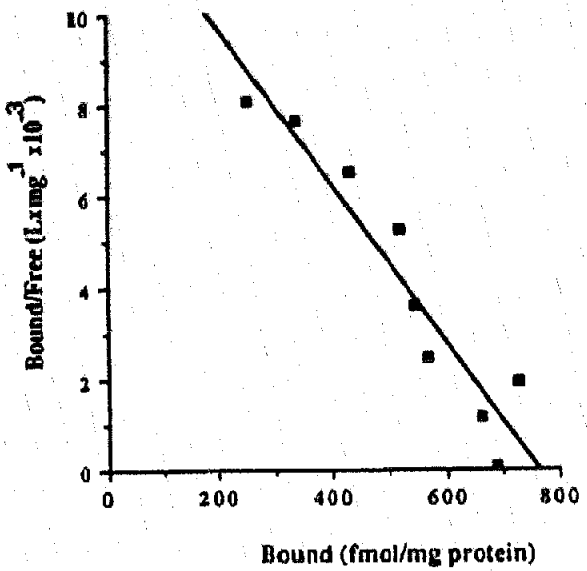

Fig. 1. Concentration-dependence of [^{125}I]-Linear AVP Antag binding (0.03–0.90 nM) to a liver membrane preparation. The values represented in the graph are from one experiment. The protein content per tube was 2 μg, the correlation coefficient of linear regression was 0.96. The dissociation constant was 0.060 nM and the maximal binding capacity 750 fmol/mg protein.

The dose-dependent specific [^{125}I]-Linear AVP Antag binding at equilibrium to rat liver membrane preparations revealed binding to one class of receptor sites with a very high affinity (K_d = 0.06 nM, results obtained from 2 separate experiments in triplicate) and a high capacity (B_{max} = 750 fmol/mg protein). There was a good correspondence in affinity constants calculated for [I]-Linear AVP Antag from competition experiments on [^{125}I]-Linear AVP Antag binding to liver vasopressin receptors (Table III) and the affinity constants calculated from the concentration-dependence analyses (Fig. 1).

At a [^{125}I]-Linear AVP Antag concentration close to the K_d value (0.06 nM), the non-specific binding was 18.7%.

[^{3}H]-AVP binding to V_{1b}- and V_2-receptor sites was inhibited with a very low potency (respectively K_i = 92 nM and K_i = 47 nM) (Table III). The V_{1a}-receptor/V_{1b}-receptor and the V_{1a}-receptor/V_2-receptor selectivity index (the calculated ratio of K_is of one ligand for two different receptor types) were respectively, 0.0018 and 0.0036. [^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT binding to OT-receptor sites was also inhibited with a low potency (K_i = 2.1 nM) (Table III). The V_{1a}-

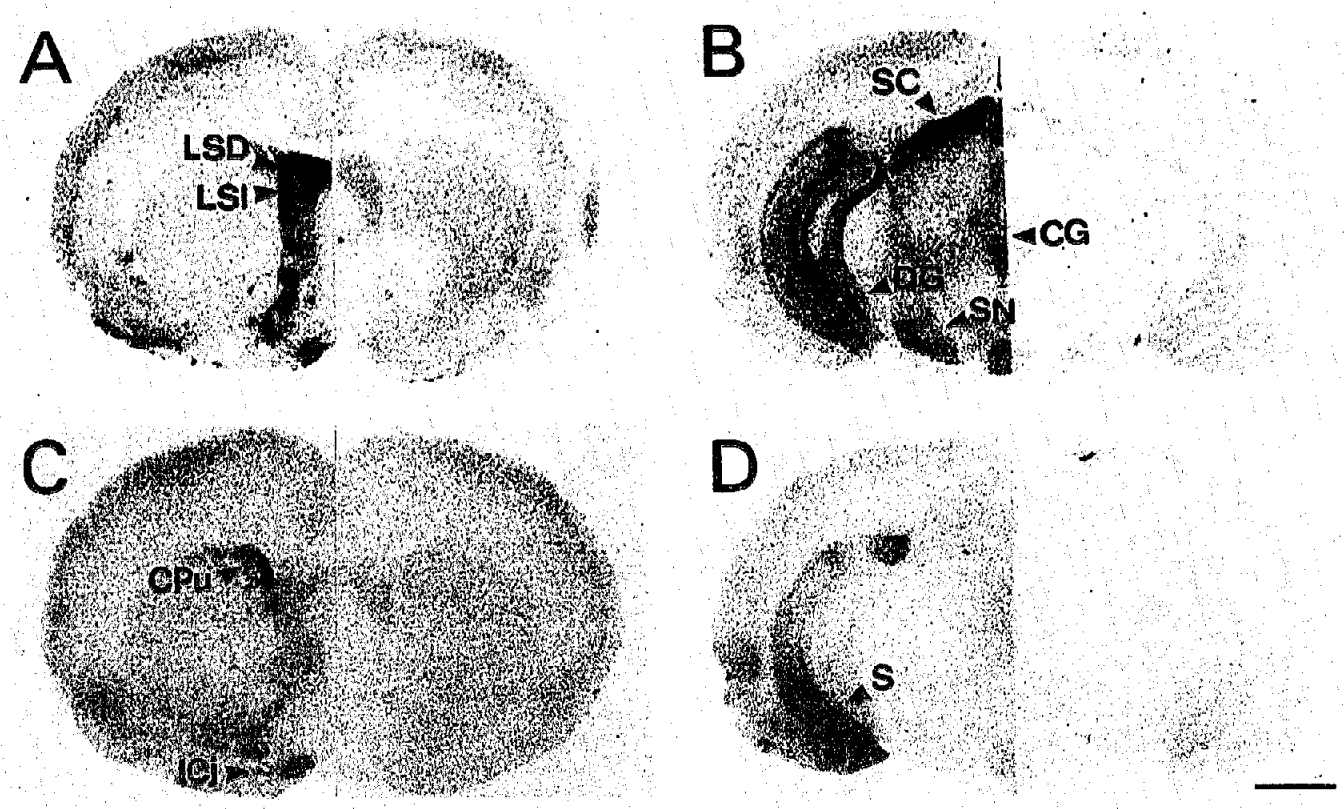

Fig. 2. Autoradiographs of female rat brains. Autoradiographs were obtained from coronal sections incubated with 0.06 nM [^{125}I]-Linear AVP Antag (A,B) exposed for 4 days or with 0.05 nM [^{125}I]-d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH$_2$^{9}]OVT (C,D) exposed for 7 days. The non-specific binding (left part of the sections) was obtained by incubating the slices in the presence of 0.1 μM AVP for vasopressin antagonist or 0.1 μM OT for oxytocin antagonist. Calibration: 2 mM.

Abbreviations: CG, central grey; CPu, caudate-putamen; DG, dentate gyrus; ICj, islands of Calleja, LSD, Laterial septum nucleus (dorsal); LSI, lateral septum nucleus (intermediate); S, subiculum; SC, superior colliculus; SN, substantia nigra.

receptor/OT-receptor selectivity index was 0.0809. Thus the [^{125}I]-Linear AVP Antag appeared to be a selective ligand for the vasopressin V_{1a} receptor.

3.3. *Autoradiography*

Specific labelling was found after a 4-day exposure in regions known to contain AVP binding sites such as the lateral septal nucleus (dorsal and intermediate) (Fig. 2A) and the hippocampus (particularly the dentate gyrus), the superior colliculus, the substantia nigra and the central grey (Fig. 2B). This labelling was completely different from that obtained in adjacent slices with the oxytocin angatonist [^{125}I]-d(CH_2)$_5$ [Tyr(Me)^2,Thr^4,Tyr-NH_2^9]OVT: moderate labelling was seen in the dorsal part of the caudate-putamen and the islands of Calleja (Fig. 2C), while intense labelling was detected in the subiculum (Fig. 2D).

Thus, the radioiodinated ligand ([^{125}I]-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 ([^{125}I]-Linear AVP Antag) appears a good ligand for V_{1a} vasopressin receptors. It is worth noting that its affinity for V_{1a} receptors (K_d = 0.06 nM) is much higher than any radiolabelled ligand developed so far for vasopressin receptors. Moreover, its high selectivity and high specific radioactivity make this ligand a very good tool for future studies on vasopressin receptor localization and characterization.

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