Structure–Activity Study of Neuropeptide FF: Contribution of N-Terminal Regions to Affinity and Activity†

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Twenty neuropeptide FF (NPFF) analogs having various lengths were synthesized by solid-phase peptide synthesis to gain more information on the role of N-terminal residues for the NPFF receptor affinity. The affinities were evaluated in the rat spinal cord membrane preparations, and the biological activities were measured on morphine analgesia in the mouse tail-flick test. Shortening of the NPFF sequence from the N-terminus produced only a moderate decrease in affinity until NPFF(4-8) was reached. In the same way, NPFF(3-8) significantly decreased morphine analgesia, while NPFF(4-8) had no significant effect at a dose of 22 nmol. The introduction in the N-terminal part of NPFF of a D-enantiomer at positions 2 and 1 or the presence of an *N*-methyl group on position 3 did not modify affinity and activity. Substitution of proline[5] by the D-isomer decreased the affinity of NPFF analogs whatever their length, and [Tyr[1],D-Pro[5]]NPFF(1-8) was 2.5-fold less potent than [Tyr[1]]NPFF(1-8) in reversing morphine-induced analgesia. In contrast, the presence of a glycine residue in position 5 did not influence the affinity toward NPFF receptors. Data provide evidence that the N-terminal segment of neuropeptide FF is responsible for high-affinity binding.

Introduction

Neuropeptide FF (Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2, F8Famide, NPFF) was isolated from bovine brain by Yang et al.[1] via using antiserum directed against the molluscan neuropeptide FMRFamide[2] (Phe-Met-Arg-Phe-NH_2). Neuropeptide FF is present in the central nervous system of various species,[3] in particular at high concentrations in the dorsal spinal cord.[4–9] Superficial layers of the dorsal horn of the spinal cord also contained a high density of specific binding sites to NPFF.[10–12]

Neuropeptide FF has been implicated in the modulation of a variety of behavioral and physiological functions. After intracerebroventricular administration, neuropeptide FF was reported to attenuate stress and opiate-induced analgesia in rats[13,1] or mice.[14–16] Several studies using the icv route of injection have reported a potential role of neuropeptide FF in opioid tolerance and dependence.[1,13,17–19] Third ventricular injection in non-dependent rats induced a morphine-withdrawal-like behavioral syndrome.[18] In morphine-dependent rats, a significant increase of NPFF immunoreactivity was observed in cerebrospinal fluid,[17] and antiserum directed against NPFF reversed naloxone-precipitated withdrawal in morphine-dependent rats.[18]

Some studies have also shown pro-opioid effects of NPFF in addition to its antiopioid action. Thus, synthetic analogs of neuropeptide FF were reported to inhibit morphine analgesia while others produced analgesic effects following icv injections.[16] We have recently observed after intrathecal administration of NPFF a strong analgesic effect in rats,[20] although it reduced the electrophysiological activity induced by the μ-receptor.[21] Similarly, morphine and NPFF decreased colonic bead expulsion time in mice[22] and intestinal transit in mice and rats,[23,24] although NPFF blocked opiate effects on ileum contractions.[25]

These features strongly suggest that NPFF may act as an endogenous modulator of opioid functions. The characterization of physiological activities of neuropeptide FF requires probes showing both high binding affinity and resistance to enzymatic breakdown. However, detailed information on the structure–activity relationships of NPFF receptor binding is lacking at this time; only binding affinities of analogs of the molluscan peptide FMRFamide have been recently reported.[26]

In order to study further the structural determinants of the high-affinity binding of neuropeptide FF, we have synthesized an extensive series of analogs and shorter homologs of neuropeptide FF and investigated their properties. Like their parent compounds, these synthetic peptides contain Gln-Arg-Phe-NH_2 in their C-terminal region but have a different N-terminal structure. We now describe the binding and pharmacological properties of these peptides and discuss the structural features that determine the affinity toward the NPFF receptors.

Results and Discussion

Various strategies could be used in the design of specific ligands with high affinity for NPFF receptors. We have chosen the classical approach of amino-acid

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† Abbreviations: Symbols and standard abbreviations for amino acids and peptides are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) (*Biochem. J.* **1984**, *219*, 345. *Eur. J. Biochem.* **1984**, *138*, 9). Additional abbreviations are as follows: NPFF, neuropeptide FF, Phe-Leu-Phe-Gln-Pro-Gln-Arg-PheNH_2; HPLC, high-performance liquid chromatography; DCC, *N,N'*-dicyclohexylcarbodiimide.

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Table 1. Analytical Characterization of the Peptides Synthesized in This Work

compounds	HPLC t_R[a]	FAB-MS[b] [M + H] obs	FAB-MS[b] [M + H] calcd
NPFF	28.2	1081.5	1081.5
[Tyr^1]NPFF	25.9	1097.5	1097.5
[Tyr^3]NPFF	25.5	1097.5	1097.5
NPFF(2-8)	22.5	934.3	934.5
NPFF(3-8)	17.4	821.4	821.4
NPFF(4-8)	11.4	674.1	674.3
NPFF(5-8)	10.2	545.7	546.2
NPFF(6-8)	5.0	448.6	449.2
[Tyr^1,D-Pro^5]NPFF	25.2	1097.5	1097.5
[D-Pro^5]NPFF(2-8)	21.2	934.3	934.4
[D-Pro^5]NPFF(3-8)	19.2	821.5	821.4
[D-Pro^5]NPFF(4-8)	11.1	674.0	674.3
[D-Pro^5]NPFF(5-8)	10.2	545.6	546.2
[D-Tyr^1,(NMe)Phe^3]NPFF	29.5	1111.5	1111.5
[D-Tyr^1,D-Leu^2,(NMe)Phe^3]NPFF	29.3	1111.5	1111.5
[D-Tyr^1,D-Leu^2,D-Phe^3]NPFF	25.4	1097.5	1097.5
[D-Tyr^1,D-Leu^2]NPFF	27.7	1097.6	1097.5
[Gly^4]NPFF	29.1	1010.4	1010.5
[Gly^5]NPFF	26.4	1041.4	1041.5
[Gly^6]NPFF	28.1	1010.3	1010.5

[a] Retention time (t_R, min) for the following conditions: a reverse-phase C8, 7 μm column eluted in a linear gradient from 100% mobile phase A (0.1% TFA) to 60% mobile phase B (75% CH_3CN in 0.09% TFA) in 50 min at a flow rate of 1.0 mL/min. All peptides were monitored at $\lambda = 214$ nm. [b] FAB-MS, fast atom bombardment mass spectrometry. Primary structure of neuropeptide FF: Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2.

substitution and deletion.[27,28] The fragments and analogs were synthesized by solid-phase peptide synthesis using DCC or DCC and 1-hydroxybenzotriazole (HOBT) as a coupling reagent. Cleavage of peptide–resin and removal of the protecting groups from the amino-acid side chains were achieved in the presence of liquid hydrogen fluoride. As shown by analytical HPLC (Table 1), highly purified (>98%) peptide was obtained.

NPFF receptor affinities were determined by displacement of [^{125}I][Tyr^1]NPFF binding from rat spinal cord membranes.[10] The effects of NPFF analogs have been examined on opiate-induced analgesia in a thermal pain test, the tail-flick test in mouse as previously described.[16] In this test, NPFF and opioid antagonists similarly reversed morphine analgesia but NPFF acts indirectly since it exhibits a very low affinity toward opioid receptor.[10] [Tyr^1]NPFF produced an inhibition of morphine analgesia that resembled an all-or-none response (Figure 1) similar to that observed in others tests,[23,24] and the dose–response curve presented a sharp transition. For this reason, the effects of NPFF analogs were compared using the minimum effective dose of analogs significantly inhibiting morphine analgesia.

Shorter Homologs. The first step was to establish the minimal peptide length for complete and partial activities. The ability of the synthesized NPFF analogs to inhibit the specific binding of [^{125}I][Tyr^1]NPFF to rat spinal cord membrane preparations is summarized in Table 2. As clearly illustrated in Figure 2, shortening of the neuropeptide FF sequence from the N-terminus by one amino acid at a time produced a moderate decrease in affinity until the C-terminal pentapeptide NPFF(4-8) was reached (Table 2). Deletion of the two N-terminal residues did not significantly modify peptide affinity. Thus, NPFF(3-8) displayed excellent affinity, only 2-fold lower than that of NPFF(2-8). In contrast,

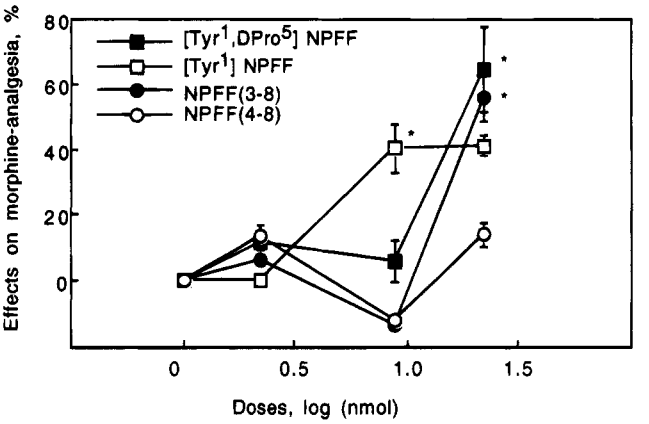

Figure 1. Inhibition of morphine analgesia by NPFF analogs in the mouse tail-flick test. Data are expressed as differences in percentage (±SEM) of analgesia measured 15 min post-intracerebroventricular injection between animals injected with morphine alone (5.5 nmol) and morphine plus NPFF analog at the dose indicated. *$P < 0.05$, significantly different from group receiving morphine alone.

Table 2. Binding Assays of NPFF Analogs[a]

compounds	K_i, nM
NPFF	0.21 ± 0.03
[Tyr^1]NPFF	0.20 ± 0.04
[Tyr^3]NPFF	0.22 ± 0.03
NPFF(2-8)	0.20 ± 0.02
NPFF(3-8)	0.43 ± 0.06
NPFF(4-8)	20.9 ± 3.1
NPFF(5-8)	15.5 ± 2.3
NPFF(6-8)	300 ± 45
[Tyr^1,D-Pro^5]NPFF	30 ± 4
[D-Pro^5]NPFF(2-8)	33 ± 5
[D-Pro^5]NPFF(3-8)	45 ± 6
[D-Pro^5]NPFF(4-8)	64 ± 9
[D-Pro^5]NPFF(5-8)	94 ± 12
[D-Tyr^1,(NMe)Phe^3]NPFF	0.16 ± 0.02
[D-Tyr^1,D-Leu^2,(NMe)Phe^3]NPFF	0.19 ± 0.03
[D-Tyr^1,D-Leu^2,D-Phe^3]NPFF	1.0 ± 0.2
[D-Tyr^1,D-Leu^2]NPFF	0.50 ± 0.07
[Gly^4]NPFF	10 ± 1
[Gly^5]NPFF	1.2 ± 0.2
[Gly^6]NPFF	0.60 ± 0.10

[a] The data are means of three determinations ± SEM. Affinities of peptides for rat spinal cord membranes are given by K_i values determined according to the formulation of Cheng and Prusoff.[32]

NPFF(4-8) and NPFF(5-8) exhibited a 100-fold lower affinity than NPFF(1-8) (Table 2). The carboxy-terminal tripeptide NPFF(6-8) (Gln-Arg-Phe-NH_2) had only a weak affinity for [^{125}I][Tyr^1]NPFF-binding sites.

The biological efficiency of NPFF analogs also decreased as the NPFF sequence was shortened (Table 3). NPFF(4-8) was ineffective in significantly reversing morphine analgesia (Figure 1), in accordance with its low affinity. In contrast, NPFF(3-8) was as potent as NPFF (Table 3). The effects of doses higher than 22 nmol were not tested due to the limits of solubility.

NPFF(3-8) exhibited a K_i value 2-fold higher than that of [Tyr^1]NPFF(1-8) and similarly showed a lower potency (2.5-fold) in inhibiting morphine analgesia in the mouse tail-flick test. It could be noted however that [Tyr^1]NPFF was more effective than NPFF in inducing an in vivo effect. Quantitative differences between the binding assays and bioassays may be due to a different degradation rate and/or nonspecific adsorption of the hydrophobic molecules. The removal of the two aromatic N-terminal residues leads to an apparent decrease of lipophilicity as reflected by the decrease of HPLC

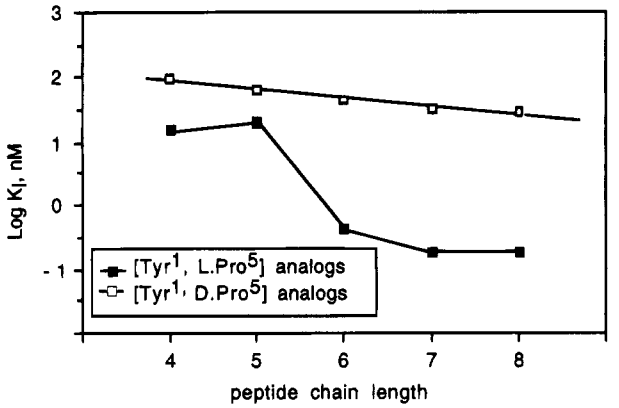

Figure 2. Differential affinities of the shorter homologs of neuropeptide FF relative to the number of amino-acid residues. log values of the affinities (K_i) are plotted against the peptide chain length. Open squares, NPFF analogs containing a D-Pro^5 residue at position 5; closed squares, NPFF analogs containing an L-Pro^5 residue.

Table 3. Relative Potency of Neuropeptide FF Analogs on Analgesia Induced by Morphine[a]

compounds	minimum effective dose, nmol
NPFF	22
[Tyr^1]NPFF(1-8)	8.8
NPFF(3-8)	22
NPFF(4-8)	>22
[D-Tyr^1,(NMe)Phe^3]NPFF(1-8)	8.8
[Tyr^1,D-Pro^5]NPFF(1-8)	22
[Gly^5]NPFF(1-8)	8.8

[a] Activity of analogs was measured in the tail-flick test (10 mice/group) 15 min after intracerebroventricular coinjection of 0, 1, 2.2, 8.8, and 22 nmol of analog with 5.5 nmol of morphine. The results are expressed as the minimum effective dose able to significantly reverse morphine analgesia.

elution times in reverse-phase chromatography (Table 1); this could modify the ability of analogs to diffuse into the tissues after intracerebroventricular injections.

Modifications of N-Terminal Residues. The implied importance played by the N-terminus of NPFF in directing the peptide toward NPFF receptors was further examined by evaluating changes induced by reversal of amino-acid chirality. The introduction of a D-enantiomer at positions 2 and 1 confers enhanced stability against peptidases.[16]

NPFF analogs containing conformational constraints such as *N*-methyl-amino acids and reversal of chirality in the N-terminal part exhibited high affinity in the same range as the parent peptide. In particular, the substitution of L- by D-Tyr^1 and L- by D-Leu^2 did not affect affinity (Table 2). Similarly, the N-methylation of the second peptidic bond did not significantly modify the K_i value. In contrast, the presence of the three D-amino acids on the N-terminus, [D-Tyr^1,D-Leu^2,D-Phe^3]-NPFF, decreased the affinity of the peptide about 5-fold. [D-Tyr^1,(NME)Phe^3]NPFF and [D-Tyr^1]NPFF displayed the same affinity (Table 2) and a similar ability to reverse morphine analgesia in mice (Table 3).

Role of Proline and Glutamine Residues. Proline, a conformationally constrained amino acid, breaks α-helices due to the constraints of the pyrrolidone ring. Furthermore, its *N*-alkyl group sterically restricts the conformation of the preceding residue in the peptide sequence.[30] Single substitution of L-Pro^5 by D-Pro in the [Tyr^1]NPFF sequence decreased the affinity by a factor 150. The shortening of the sequence of D-Pro^5 analogs from the N-terminus induced only a weak decrease in affinity (3-fold) (Figure 2). Thus, the affinity of [D-Pro^5]-NPFF(4-8) was only 3-fold lower than that of NPFF(4-8) (Table 2).

[Tyr^1]NPFF was more potent (2.5-fold) than [Tyr^1,D-Pro^5]NPFF in reversing morphine analgesia in the mouse tail-flick test (Table 3). The dose–response curves (Figure 1) indicated clearly that 8.8 nmol of the L-Pro analog was effective in reversing morphine analgesia while a dose 2.5-fold greater of the D-Pro analog was needed to induce the same effect. Surprisingly, a 150-fold difference in affinity produced only a 2.5-fold shift in activity.

The data in Table 2 demonstrated that the substitution of a Pro^5 residue by Gly resulted in a weak loss in receptor affinity without modifications of the activity (Table 3). Similarly, the replacement of Gln^6 by Gly did not significantly modify the K_i value. In contrast, the substitution of Gln^4 by Gly induced an important drop in affinity for NPFF receptors (50-fold). Surprisingly, although [Tyr^1,D-Pro^5]NPFF exhibited a lower affinity (Table 2) than [Tyr^1]NPFF (150-fold), the substitution of Pro^5 by Gly induced only a 6-fold change in the affinity.

Conclusions

A18Famide and NPFF share common C-terminal tetrapeptide sequences, Pro-Gln-Arg-Phe-NH_2, and exhibit the same high affinity toward specific NPFF-binding sites in rat spinal cord.[10] The critical structural differences between these two peptides lie in the N-terminal part which should be important for NPFF receptor recognition and stimulation since shortening of the neuropeptide FF sequence from the N-terminus produced both a loss of affinity and a decrease in biological activity. The C-terminal tetrapeptide sequence is not sufficient alone for high affinity. Cutting off the four hydrophobic N-terminal aminoacids produced an important loss of affinity, while NPFF(3-8) exhibited a high affinity and reversed morphine analgesia. In the same way, D-amino substitution or N-methylation of the three N-terminal residues did not affect affinity, although such a modification should enable the peptide to assume unique spatial conformations[29] and generate analogs resistant to enzymatic degradation.[16] The partial homology between FMRFamide and NPFF and the fact that FMRFamide displayed a low affinity[10] toward NPFF-binding sites in the spinal cord also indicated the essential role of Arg-Phe-NH_2 in the recognition process.

However, the results of the tail-flick test were not always found to be in quantitative agreement with the receptor-binding data. In particular, several analogs exhibiting a lower affinity than [Tyr^1]NPFF were as potent as [Tyr^1]NPFF in reversing morphine-induced analgesia in the tail-flick test.

The presence of a proline residue could suggest that NPFF is likely to adopt a β-turn in the backbone of the four C-terminal residues.[30] The structural requirements at Pro^5 are precise since the reversal of chirality leads to low-affinity compounds. The reduction in affinity of D-Pro^5 analogs may reflect a disruption of the N-terminal β-turn which leads to suboptimal spatial alignment of the octapeptide. A configurational freedom is, however, permitted because the substitution of Gln^4

or Gln[6] by glycine induced only a relatively weak change in affinity, although such substitutions could induce a profound change in a β-turn in the four C-terminal amino-acid residues.[30] Neither X-ray crystallographic nor NMR data pertaining to the ligand or ligand–receptor complex are available; thus it is difficult to ascertain that such substitutions only modify the conformation of the molecules. Results overall showed that C-terminal amino acids of the molecule would be essential for biological response while the N-terminal segments allow the formation of the appropriate conformation required for the interaction with the receptor.

Experimental Section

Reagents and Solvents. *p*-Methylbenzhydrylamine resin (0.56 mmol of amine/g) was purchased from Novabiochem (France), and N^{α}-*tert*-butyloxycarbonyl (Boc)-protected amino acids were purchased from Propeptide (France). *N,N*-Dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), *N,N*-diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were from S.d.S (France). DCC and 1-hydroxybenzotriazole (HOBT) were obtained from Aldrich Chemicals (Coger, France).

Peptide Synthesis. Neuropeptide FF and analogs were prepared by solid-phase synthesis on an automated peptide synthesizer (Applied Biosystem 430A) according to *N*-*tert*-butyloxycarbonyl (t-Boc)-amino acids and symmetric anhydride or hydroxybenzotriazole ester as activation chemistry. A *p*-methylbenzhydrylamine resin (MHBA) was used as the solid support, and DCC was used for the coupling step. Side-chain protection of α-Boc-amino acids was as follows: N^{g}-Tos(Arg), 2Br-Z(D-Tyr).

The products were removed from the resin and simultaneously deprotected by reaction with liquid anhydrous hydrofluoric acid (10 mL) in the presence of anisol (5%) and *m*-cresol (5%), as a cation scavenger, for 120 min at −5 °C. After evaporation, precipitation and washings with anhydrous diethyl ether, and solubilization with aqueous acetic acid (30%), the crude peptides were lyophilized to yield amorphous powder. For [D-Tyr[1],(NMe)Phe[3]]NPFF and [D-Tyr[1],D-Leu[2],-(NMe)Phe[3]]NPFF, Boc-N(Me)Phe (5 equiv), Boc-Leu (5 equiv), and Boc-2Br-Z-(D-Tyr) (4 equiv) were coupled manually with DCC/HOBT in *N*-methyl-2-pyrrolidone. Coupling was monitored by a ninhydrin colorimetric test.[31]

Peptide Purification. The products were purified by preparative HPLC on an Aquapore column (C8, 10 × 100 mm, 20 μm; Brownlee Labs) with a linear gradient from 10% to 60% of solvent B (75% acetonitrile, 0.09% trifluoroacetic acid) in solvent A (0.1% trifluoroacetic acid) over 50 min. The purity of the final products was assessed by analytical reverse-phase liquid chromatography and fast atom bombardment mass spectrometry on a ZAB-HS double focusing spectrometer (VG Analytical, U.K.).

Binding Assays. Rat spinal cord membrane preparations and binding assays were performed as previously described.[10] Briefly, membranes were incubated at 25 °C for 30 min, with 0.15 nM [^{125}I][Tyr[1]]NPFF in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 0.5% bovine serum albumin (BSA), 0.1 mM bestatin, 1 mM EDTA, and 0.5 mM diisopropylfluorophosphate. Under standard conditions, 350 μL aliquots of homogenate (about 50 mg of protein per assay) were dispersed in 1.5 mL Eppendorf vials with 100 μL of increasing concentration of unlabeled peptides (5×10^{-11}–10^{-6} M). The nonspecific binding was defined in the presence of 10^{-6} M unlabeled peptide [Tyr[1]]NPFF. The reaction was initiated by adding 50 μL of [^{125}I][Tyr[1]]NPFF at a final concentration of 0.1 nM, a value close to the K_D value.[10] The reaction was ended by addition of 1 mL of ice-cold Tris-HCl buffer containing 2% BSA and rapid centrifugation (10000*g*, 5 min, 4 °C). Vials were washed twice by cold buffer and recentrifuged to eliminate the free radioactivity in the pellet. The tissue-bound radioactivity in the pellet was quantified by γ-spectrometry. The affinity constant (K_i) was calculated from the IC_{50} value according to Cheng–Prusoff's equation.[32]

Antinociceptive Activity. Male CDF1 mice weighing 18–25 g were housed in stainless steel cages in a room maintained at 22 °C on a 12 h dark/light cycle. Food and water were provided ad libitum. Animals were normally prepared in groups of 10. Intracerebroventricular injections were held as described by Haley and McCormick.[33] These experiments were carried out in compliance with the E.E.C. guidelines on animal experiments.

Morphine hydrochloride (Francopia, France) and naloxone hydrochloride (Sigma) were dissolved in 150 mM NaCl. A dose of 5.5 nmol of morphine was chosen as it produced a submaximal analgesia (76 ± 15% analgesia). Pain thresholds were determined at 15 min intervals. The tail-flick reflex was evoked by focused heat applied to the tail.[34] The analgesic effect was determined as the increase of response time in seconds. Radiant heat was adjusted to attain a mean base line of 2–3 s in controls. To minimize damage to the skin of the tail, maximal inhibition of the tail-flick reflex was defined as a latency of 6 s (100% analgesia). The percentage of antinociception was calculated according to $(T_t - T_c/6 - T_c) \times 100$, where T_t is the latency time obtained for treated animals and T_c the control latency time (1.5–2.5 s). Comparisons were made using the one-way analysis of variance followed by the Student's *t* test for paired or unpaired values (Statview program, Macintosh).

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