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EVALUATION OF A C-TERMINALLY TRUNCATED CHIMERIC OPIOID PEPTIDE [DES-PHE^{12}]YFA ON ANTINOCICEPTIVE ACTIVITY, RECEPTOR SPECIFICITY, AND RECEPTOR REGULATION ON CHRONIC ANALGESIC EFFICACY

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ABSTRACT
The physiologic function of the NPFF/FMRFa peptide group is still complex, and their exact mode of action is unclear. Structure on the current line of inquiry, our previous chore describes a couple of chimeric concepts derived from Metenkephalin (Tyr-Gly-Gly-Phe-Met) and FMRFa (Phe-Met-Arg-Phe-NH_2) YGGFMKKKKFMRFamide (YFa) and Y(D-Ala)GFMKKKKFMRFamide ([D-Ala^2]YFa). The aforementioned hybrid peptide has been shown to induce naloxone-reversible antinociceptive effects and to reduce the onset of tolerance associated with morphine-induced analgesia. Moreover, in the presence of FMRFa and NPFF peptides, the antinociception of [D-Ala^2]YFa was markedly reduced. In addition to investigating the contribution of the C-terminal FMRFamide motif to trouble transition and receptor selectivity, we rationally developed and synthesized a recent analogy where the essential Phe residue from twelfth position was eliminated, YGGFMKKKFMRamide ([Des-Phe^{12}]YFa). The present work investigates the antinociceptive potential of [Des-Phe^{12}]YFa following intraperitoneal administration to rats, measuring using the tail-flick assay, and analysing their receptor specificity using the antagonistic target, and opioid receptor. Moreover, the efficacy of the treatment was measured throughout the duration of treatment, together with changes in opioid receptor expression evaluated using western blotting with real-time reverse transcriptase polymerase chain reaction (RTPCR).
The results show that YFa has a receptor intercede, dose-dependent antinociception, although its magnitude was lower than that detected in association with the caregiver peptide YFa. Its analgesic effects remained stable, and its receptor expression was selectively increased in parallel to the same transcript and protein stages. Together, these pharmacological and molecular discoveries show that although the engineered YFa functions essentially as an opioid antagonist, the abbreviated –RFa part contributes to the overall antinociceptive profile of the amphiactive chimeric peptide.

KEYWORDS: Chimeric peptides; Met-enkephelin; MERF, Opioid receptor regulation; FMRFamide; Real-time RTPCR; NPFF.

INTRODUCTION

Met-enkephalin-Arg^6-Phe^7 (YGGFMRF; MERF) is a peptide extensively dispersed in the focal nervous system of many mammalian species [1, 2] and grouped into the opioid group [3]. The unique feature of MERF lies in its overlap sequence motifs, Met-enkephalin (YGGFM) and FMRF (Phe-Met-Arg-Phe), which suggests that MERF and its associated peptide may not bind merely via the classical opioid nerve pathway but may also interact with an extra binding site [4, 5, 6]. The relatively low affinity of NPFF/FMRFa peptide to the opioid receptor [7] furthermore state that the aforementioned neuropeptides interact with different receptor systems and interfere with their effects via a concentrated binding site [8]. The evidence suggests that NPFF can antagonize the antinociception induced by morphine along with the activities of certain endogenous opioids [9, 10] supporting the idea that NPFF/FMRFa peptide might act as endogenous anti-opioid modulators [11, 12], thereby promoting opioid tolerance and dependence [13, 14]. Interestingly, NPFF has also been reported to produce opioid pleasure responses, such as decreased colonic bead ejection in mice [15] and arousal of analgesia following intrathecal administration in rats [16]. Taken together, these findings underscore the multifaceted nature of NPFF/FMRFa peptides. Their physiological role appears highly complex, and it is yet unclear exactly what processes underlie their activities [17]. Continuing our current research line, our previous studies have shown that YGGFMKKKFMRFamide (YFa), a chimeric peptide based on MERF, designed by combining the pharmacophoric elements of Met-enkephalin and FMRFamide, exhibits selective antinociceptive activity and significantly delays the beginning of resistance to the analgesic impacts of morphine [18]. In particular, prolonged intraperitoneal use of YFa makes it difficult to achieve tolerance [19]. An enzymatically stable analogue, Y-(D-Ala)-GFMKKKFMRFamide [(D-

Ala²)YFa], together with its parent peptide, was found to produce antinociceptive effects that were reversed by naloxone. Notably, these analgesic responses were significantly attenuated following treatment with the NPFF and FMRFamide peptides YAGFMKKKFMRFamide. These results imply that identical chimeric peptides could possibly work through NPFF/FMRFa, an alternatively non opioid adhesion site, beyond the classical opioid receptors [20].

In addition to determining the contribution of the –RFa motif to antinociception and receptor selectivity, a rationally planned analogue was synthesized wherein the C terminal, 12th Phenylalanine residue of YFa was eliminated, resulting in YGGFMKKKFMRamide ([Des-Phe^{12}]YFa). The –RFamide sequence has been identified as essential for the binding and function of the NPFF/FMRFa peptide [21]. Therefore, the present study measures the antinociceptive profile of [Des-Phe^{12}]YFa after intraperitoneal administration to rats using the tail-flick assay and investigates its receptor specificity together with and antagonist. Moreover, its efficacy during chronic treatment has been measured in parallel to the effect of differential opioid receptor expression to allow an analogous effect with the caregiver peptide YFa.

MATERIALS AND METHODS

Peptide synthesis

Standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry was used in the solid phase technique of peptide synthesis on an ACT-90 peptide synthesizer manufactured by unconventional ChemTech, USA. After synthesis, a linear gradient of 10–50% acetonitrile in water containing 0.05% trifluoroacetic acid was used in reverse phase HPLC (Waters, India) to achieve purification. The Automated Peptide Sequencer (Applied Biosystems 490, CA, USA) is used to verify peptide sequences, while the MALDI TOF mass spectrometer (Kratos, Manchester, UK) is used to verify molecular masses.

Chemicals

The reagents Binaltorphimine, naltrindole, naloxonazine hydrochloride, and bestatin, along with primary antibodies focused against μ- and δ-opioid receptors and the corresponding secondary antibodies, obtained from Sigma (St. Louis, MO, USA). Santa Cruz Biotechnology (USA) sold the κ-opioid receptor primary antibody individually. Each peptide was injected intraperitoneally after being newly distributed in a physiological saline solution. Bestatin (1mM) was added to the saline solution to reduce the enzymatic impairment of the administered neuropeptides. Preliminary tests confirm that intraperitoneal administration of bestatin (0.2 mL, 1 mM) does not produce measurable analgesic response.

Animals

The 200–250 gramme male Wistar rats were acquired from the Vallabhbhai Patel Chest Institute, Delhi. Animals were kept in collectives of 3 per cage within a regulated 12-hour light and dark cycle, with unrestricted availability of regular lab meals and water. They were acclimated for one week at a temperature of 22–25 °C in an animal holding facility. The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, approved the guidelines for all animal trials, which were approved and reviewed by the Institutional Animal Ethics Committee. This ensured that the faunas were treated ethically and that their welfare was maintained throughout the study.

Tail-flick test

To measure antinociceptive activity, a radiant heat tail-flick device from Inco Pvt. Ltd. (Ambala, India) was utilized, following the procedure referred by Gupta et. al. and group [18]. At the beginning, the heat stimulation's strength was adjusted to create a response in the untreated rat (control) inside the range of 3–5 seconds. In order to avoid tail tissue damage, a 20 second cut-off time was set. A five-day acclimation period was provided to allow the animals to adjust to the testing environment. Throughout this period, the rats were also conditioned to stand on the apparatus to reduce handling-induced stress and ensure reliable behavioural measurements. Three baseline trials were then recorded, spaced 10 minutes apart. To get the percentage of maximum probable outcome (%MPE) The following formula was used, which represents the degree of reserve of the tail-flick response: $\%MPE = [(T_1 - T_0)/(T_2 - T_0)] \times 100$, where T_0 denotes the baseline response time, T_1 represents the response time recorded after peptide or saline administration, and T_2 indicates the predetermined cut-off time.

Pharmacological studies

To evaluate the antinociceptive properties of [Des-Phe^{12}]YFa, a dose response study was performed in a group of rats (n = 6–7 per group) obtaining either saline solution (100 μl/rat) or a mesured dose of the peptide (14, 28.7, 43.1, and 57.5 μmol/kg). At five, fifteen, thirty, forty-five, and sixty minutes, the tail-flick latencies were restrained after administration, compared to the response of the parent peptide YFa.

The contribution of individual opioid receptor subtypes was investigated using selective pharmacological antagonists. Nor-binaltorphimine, naloxonazine, and naltrindole were administered to specifically inhibit μ-, δ-, and κ-opioid receptors, respectively, thereby enabling calculation of receptor-specific activity. The animals were pretreated by a standardised dose of the above antagonists [19], 5 minutes prior to intraperitoneal administration of [Des-Phe^{12}]YFa (43.1 μmol/kg) and tail-flick reactions were quantified simultaneously at the same time interval.

For chronic examination, a pair of rat cohorts (n = 18 respective) were administered either saline solution (200 μl/rat/day) or [Des-Phe^{12}]YFa (43.1 μmol/kg/day) twice habitual for 6 consecutive days. Fifteen minutes following the injection, the tail-flick responses were computed. Rats were killed following the second dose of the day, and brain and spinal cord samples were taken soon after each day's conclusion. From these samples, complete RNA and protein were extracted, with particular emphasis on the lumbar spinal cord region, owing to its high density of opioid receptor mRNA.

Real-time RTPCR

Complementary DNA (cDNA) was created using the First Strand cDNA Synthesis Kit (Amersham Biosciences, UK) from whole RNA and gene-specific antisense primers procured from TCGA, Delhi, India. Primer sequences for the real-time RT-PCR analysis of MOR1, KOR1, DOR1, and cyclophilin A were designed using Primer Express™ software (Perkin Elmer Applied Biosystems, USA) and are presented in Table 1. Using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, USA) and SYBR Green PCR Master Mix (Applied Biosystems, USA), quantitative real-time PCR was done, following the methodology described previously [22–24]. Gene expression levels were normalized against the relative expression and endogenous control were estimated by the comparative $2^{-\Delta\Delta Ct}$ method, with saline-treated animals serving as the reference group.

Table 1 Details of primers used for real-time RTPCR

S. No.	mRNA	Primers	Product size
1.	Cyclophilin A	5'-CAAACACAAATGGTTCCCAG-3' 5'-AATGCTCATGCCTTCTTTCAC-3'	100 bp
2.	MOR1	5'-ACTTCCTGGTCATGTATGTG-3' 5'-GGTAGTTGACACTCTGAAAGG-3'	129 bp
3.	KOR1	5'-GCCATCCCTGTTATCATCAC-3' 5'-GGTCTTCATCTTTGTGTATCGG-3'	107 bp
4.	DOR1	5'-GGTACACTAAGCTGAAGACG-3 5'-GTTTCCATCAGGTACTTGGC-3'	111 bp

Table 1: Information on the primers used in real-time RTPCR. The Centre for Genomic Application in New Delhi, India, synthesized primers using Primer ExpressTM software.

Western blot analysis

After transferring 25 µl of membrane protein extract (2 µg/µl; measured by Bradford test) onto PVDF membranes, 5% skimmed milk prepared in TTBS buffer (0.1 M TrisHCl, pH 7.4, including 0.9% NaCl and 0.1% Tween 20) was blocked for two hours at room temperature (RT). After that, rabbit polyclonal antibodies against MOR1, KOR1, and DOR1 (diluted 1:500 in 5% milk/TTBS) were applied to the membranes and gently shaken for one hour at RT. Membranes were treated to goat anti-rabbit biotinylated IgG (1:1000) for one hour at RT following three 15-minute washes in TTBS. After more washing, the blots were developed using 0.05 M Tris-HCl buffer (pH 7.5) comprising 0.06% diaminobenzidine and 0.009% H_2O_2 and for kept in dark for 30 minutes after treating with avidin–biotin complex reagent. Lastly, to get rid of any remaining substrate, membranes were carefully rinsed with water that has been distilled.

Statistical Analysis

Analysis of variance (ANOVA) was used to examine the information, and the findings were reported as mean ± S.E.M. One-way or two-way ANOVA was hired as suitable for the experimental design, considering time as the repeated-measures (within-subject) factor and treatment (saline versus peptide) as the between-subject factor. Significant effects were further examined using Tukey's multiple comparison post hoc test where suitable. A prospect value of $P < 0.05$ was found to be the criteria for statistical significance.

RESULTS

Antinociception of [Des-Phe^{12}]YFa chimeric peptide

Figure 1A shows the antinociceptive effects of intraperitoneally delivered chimeric peptide at various dosages. When compared to saline controls, [Des-Phe^{12}]YFa did not significantly reduce pain at the lowest dose tested (14.4 µmol/kg). On the other hand, compared to rats given with saline, larger doses (28.7, 43.1, and 57.5 µmol/kg) significantly increased tail-flick delay ($P < 0.05$). Five minutes after treatment, analgesia became apparent, and at about fifteen minutes, maximal activity was noted (Figure 1A). Figure 1B further illustrates the comparative dose-dependent effects of the parent peptide YFa and its analogue [Des-Phe^{12}]YFa. Across all tested concentrations, the antinociceptive response of [Des-Phe^{12}]YFa was consistently and significantly ($P < 0.05$) lower than that produced by YFa, highlighting the reduced efficacy of the modified peptide.

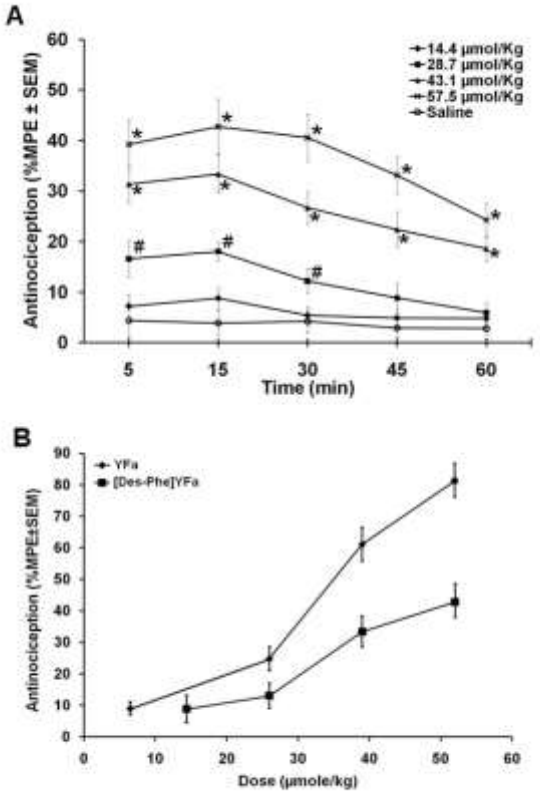

Figure 1

Figure 1: A: Antinociceptive effect (dose dependent) of chimera peptide [Des-Phe^{12}]YFa by intraperitoneal injection at doses of 14.4, 28.7, 43.1, and 57.5 µmol/kg using the rat tail flick latency test. Values of antinociception (% MPE) for saline (0.1 ml; i.p.) are also included as control. Information expressed as mean ± SEM (n=6). * Significant difference from saline ($P < 0.005$, two-way ANOVA, Tukey's test); # Significant difference from saline ($P < 0.05$, two-way ANOVA, Tukey's test). B: Antinociceptive activity contrast of YFa and [Des-Phe^{12}]YFa peptides upon their injection intraperitoneally at various doses. Each point indicates the mean value ± S.E.M. for 7 animals. All tail flick values for [Des-Phe^{12}]YFa peptide differ significantly from YFa ($P < 0.05$, two-way ANOVA,)

Effect of different opioid receptor antagonists on antinociception of [Des-Phe^{12}]YFa

Receptor antagonist experiments revealed that antinociceptive effect of [Des-Phe^{12}]YFa (43.1 µmol/kg) was highly significantly attenuated by nor binaltorphimine (1.0 µmol/kg, i.p.) pretreatment 5 min before [Des-Phe^{12}]YFa ($P < 0.001$; Figure 2). In contrast, optimised doses of naloxonazine (1.0 µmol/kg) and naltrindole (1.7 µmol/kg), administered under similar conditions, produced no measurable decrease in the analgesic response to [Des-Phe^{12}]YFa (Figure 2).

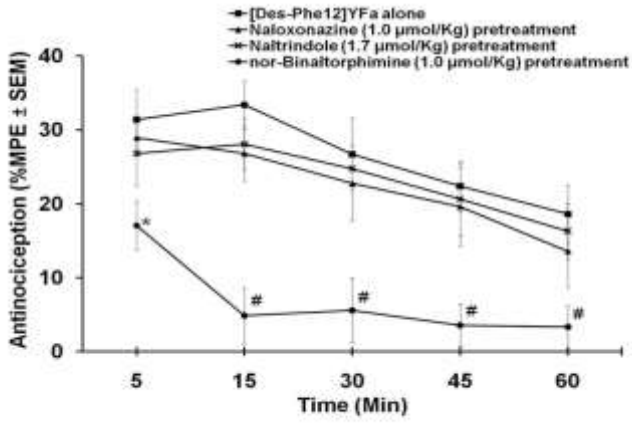

Figure 2

Figure 2: Impact of pretreatment with µ (naloxonazine; 1.0 µmol/kg; i.p.), δ (naltrindole; 1.7 µmol/kg; i.p.), and κ (nor-binaltorphimine; 1.0 µmol/kg; i.p.) opioid receptor antagonists on the duration of 43.1 µmol/kg of [Des-Phe^{12}]YFa - induced antinociception in the tail-flick test. The data is shown as mean ± SEM. Additionally shown are the antinociception (% MPE) results for the chimeric peptide [Des-Phe^{12}]YFa (43.1 µmol/kg, i.p.) alone. * denotes a

significant difference from [Des-Phe^{12}]YFa ($P < 0.05$, two-way ANOVA, Tukey's test); # denotes a significant difference from [Des-Phe^{12}]YFa ($P < 0.005$, two-way ANOVA, Tukey's test).

Importantly, administration of these same doses of antagonists 5 min prior to standard opioid agonists, dynorphin A (1-13) (61.79 µmol/kg), endomorphin-1 (65.5 µmol/kg), and (D-Pen2,5) enkephalin (24.9 µmol/kg), resulted in a complete reversal of antinociception ($P \leq 0.05$; Supplementary Figures A-C). These findings demonstrate the κ selectivity of the activity of [Des-Phe^{12}]YFa and confirm the expected receptor specificity of classical opioid agonists.

Antinociception on chronic administration of [Des-Phe^{12}]YFa

Baseline tail-flick latencies were similar in all groups and did not differ statistically prior to initial injections on day 1 of experimentation. The rats were then injected intraperitoneally with either [Des-Phe^{12}]YFa or tail-flick reflexes were assessed 15 minutes after receiving saline twice daily for six days. Treatment with [Des-Phe^{12}]YFa produced a substantial inhibition of the tail-flick response associated with saline controls ($P < 0.005$; Figure 3). Interestingly, the response values of the [Des-Phe^{12}]YFa group remained similar throughout the entire time of observation, suggesting a preserved antinociceptive efficacy upon repeated administration.

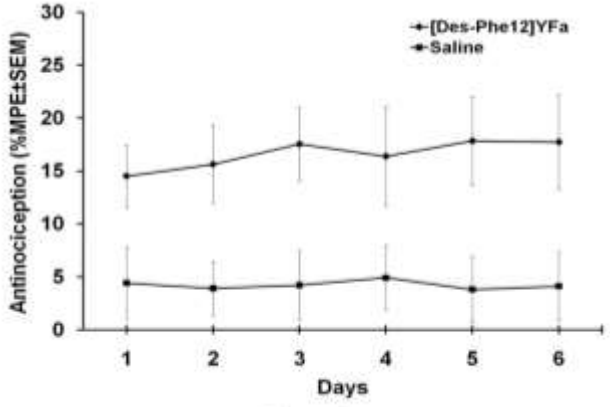

Figure 3

Figure 3: For six days, each group received 43.1 µmol/kg/day of [Des-Phe^{12}]YFa in addition to 200 µl of saline per rat per day. On the first day, baseline tail-flick latency was evaluated 15 minutes after each injection and 30 minutes before the first saline and [Des-Phe^{12}]YFa injection. The data values were displayed as mean ± SEM. Every tail-flick delay measurement for [Des-Phe^{12}]YFa differed significantly from saline ($P < 0.005$, two-way ANOVA, Tukey's test).

Modulation of opioid receptor expression induced by chronic [Des-Phe^{12}]YFa administration

MOR1

The general profile of MOR1 expression, both in terms of mRNA and protein, was comparable to what was seen in controls treated with saline during 6 days of chronic administration. Western blot and real-time RT-PCR analysis consistently demonstrated this in individually brain and spinal cord tissues (Figure 4A–D).

KOR1

When compared to saline controls, a persistent upregulation of KOR1 transcripts was observed in the spinal cord and brain over the course of the experiment (Figure 5A, B). From day 2 to day 6, there were notable increases in the brain compared to day 1, with fold changes of 2.9 ($P < 0.05$), 3.2 ($P < 0.05$), 4.0 ($P < 0.005$), 4.0 ($P < 0.005$), and 4.4 ($P < 0.001$), respectively (Figure 5B). The spinal cord showed a similar pattern, with fold increases of 2.2 ($P < 0.05$), 3.6 ($P < 0.05$), 4.2 ($P < 0.005$), 4.3 ($P < 0.005$), and 4.5 ($P < 0.001$) observed on days 2 through 6 in comparison to day 1 (Figure 5A).

KOR1 protein expression mirrored the mRNA profile in both tissues, increasing gradually from day 1 to day 6. The single exception occurred on day 6, when protein levels were somewhat lower than corresponding mRNA countenance in the spinal cord (Figure 5C, D). KOR1 protein was quantified in the spinal cord (C) and brain (D) of rats treated with saline and [Des-Phe^{12}]YFa using immunoblotting for days 1–6. The pictures displayed are the most accurate depiction of the data from the three repetitions of the immunoblots. The comparative integrated densitometry values (IDV) measured and normalised by the GAPDH signal using AlphaEaseFC software are displayed graphically. The values show means ± S.D. and are stated as a percentage of those achieved in the group of control. Western blot study of loading control GAPDH in rats treated chronically with saline and [Des-Phe^{12}]YFa for days 1–6. The brain protein is represented by each lane, and anti-GAPDH antibody was used to create the immunoblots.

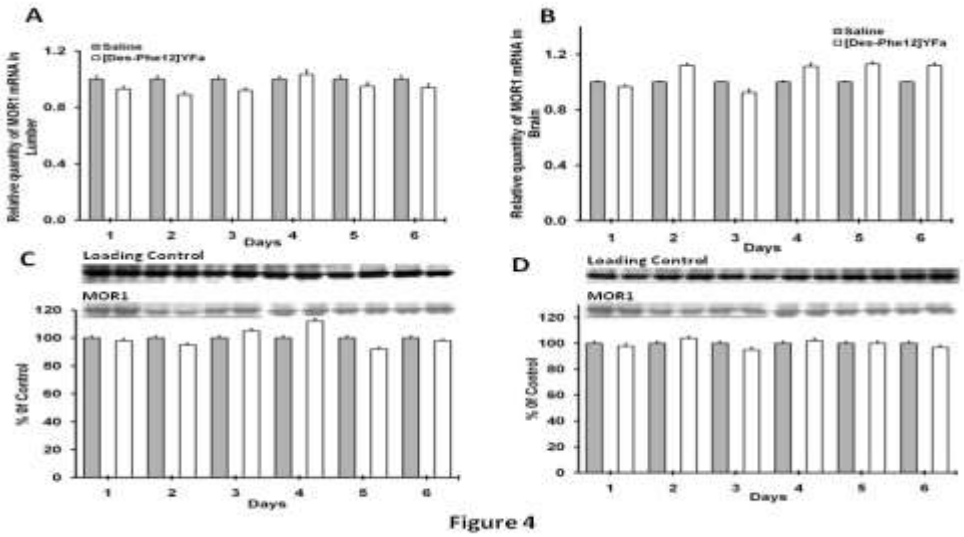

Figure 4

Figure 4: On days 1-6 of chronic treatment with saline and [Des-Phe^{12}]YFa, the relative expression of MOR1 mRNA in the spinal cord (A) and brain (B) measured using real-time RTPCR employing gene-specific primers. The relative expression of mRNA was determined using the $2^{-\Delta\Delta Ct}$ method, and the magnitude of the change is displayed. ΔCt values were determined using cyclophilin A as an internal control. The pictures displayed are the most accurate depiction of the data from the three repetitions of the immunoblots. The relative integrated densitometry values (IDV) measured and normalized by the GAPDH signal by means of AlphaEaseFC software are displayed graphically. # indicates a significant difference ($P < 0.05$) among the relative quantity of KOR1 mRNA on day 1 and saline in (A) and (B); * indicates a substantial variance ($p < 0.001$) between the relative quantity of KOR1 mRNA on days 2, 3, 4, 5, and 6 and day 1.

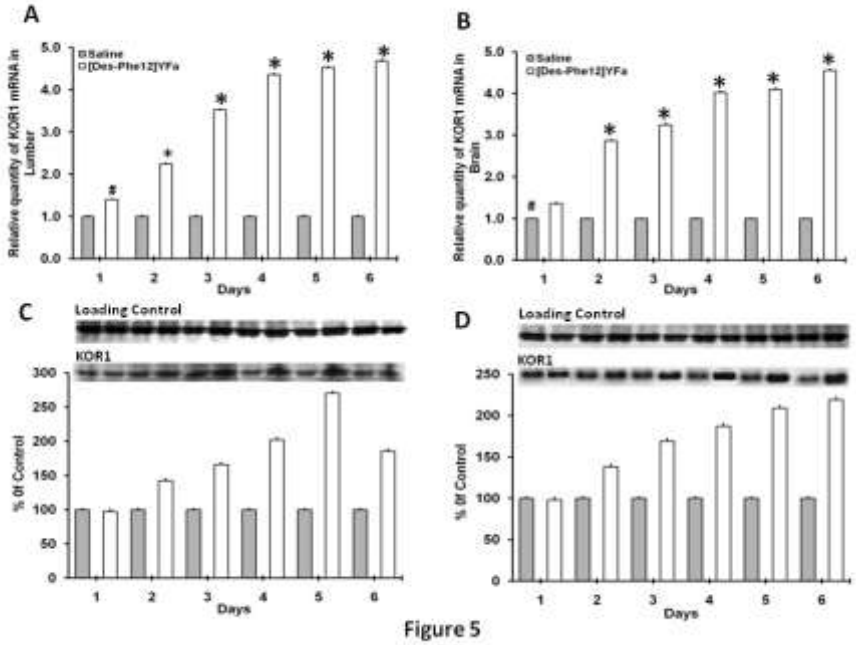

Figure 5

Figure 5: On days 1-6 of chronic treatment with saline and [Des-Phe^{12}]YFa, the relative expression of KOR1 mRNA in the spinal cord (A) and brain (B) measured using real-time RTPCR employing gene-specific primers. The relative expression of mRNA was determined using the $2^{-\Delta\Delta Ct}$ method, and the magnitude of the change is displayed. ΔCt values were determined using cyclophilin A as an internal control. The pictures displayed are the most accurate depiction of the data from the three repetitions of the immunoblots. The relative integrated densitometry values (IDV) measured and normalized by the GAPDH signal by means of AlphaEaseFC software are displayed graphically. # indicates a significant difference ($P < 0.05$) among the relative quantity of KOR1 mRNA on day 1 and saline in (A) and (B); * indicates a substantial variance ($p < 0.001$) between the relative quantity of KOR1 mRNA on days 2, 3, 4, 5, and 6 and day 1.

DOR1

During six days of chronic [Des-Phe^{12}]YFa treatment, the expression profile of DOR1 remained mostly unchanged compared to saline controls. Both mRNA and protein stages in the brain and spinal cord followed MOR1 patterns, according to real-time RT PCR and western blot examination (Figure 6A-D).

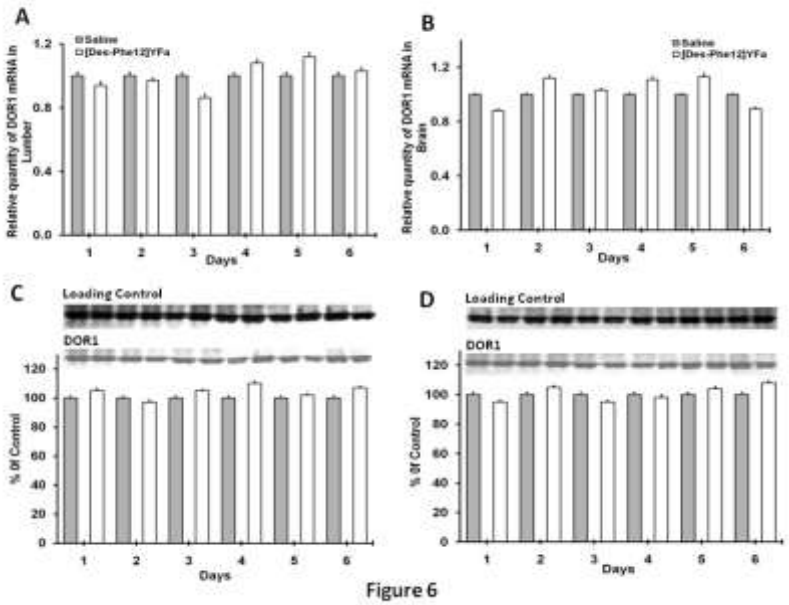

Figure 6

Figure 6: On days 1-6 of chronic treatment with saline and [Des-Phe^{12}]YFa, the relative expression of DOR1 mRNA in the spinal cord (A) and brain (B) measured using real-time RTPCR employing gene-specific primers. The relative expression of mRNA was determined using the $2^{-\Delta\Delta Ct}$ method, and the magnitude of the change is displayed. ΔCt values were determined using cyclophilin A as an internal control. The pictures displayed are the most accurate depiction of the data from the three repetitions of the immunoblots. The relative integrated densitometry values (IDV) measured and normalized by the GAPDH signal by means of AlphaEaseFC software are displayed graphically. # indicates a significant difference ($P < 0.05$) among the relative quantity of KOR1 mRNA on day 1 and saline in (A) and (B); * indicates a substantial variance ($p < 0.001$) between the relative quantity of KOR1 mRNA on days 2, 3, 4, 5, and 6 and day 1.

DISCUSSION

The current research focuses on pharmacological and molecular studies of rationally synthesized chimeric peptide [Des-Phe^{12}]YFa. It generated considerable dose-dependent antinociception when administered i.p. compared to saline (Figure 1A). The beginning of antinociception occurred within 5 minutes, with a peak impact reported 15 minutes after injection. Importantly, in comparison to parent peptide YFa, the –RFa- site modified chimeric peptide [Des-Phe^{12}]YFa, induced significantly lower antinociception (figure 1B). This clearly suggests that in chimeric peptides such as YFa, in addition to the N-terminal opioid site (YGGFM), the C-terminal -FMRFa- site also contributes to the overall observed antinociception rather than antagonizing/neutralizing it. This could only possibly when NPFF/FMRFa and opioid receptors are of physically associated with each other as suggested by [25]. Another possibility could, if opioid and FMRFa are not physically associated or not in vicinity with each other than chimeric peptide might be separately discretely interacting with both type of receptors. Therefore, when FMRFa sequence was modified in [Des-Phe^{12}]YFa, the -RFa site contribution toward antinociception was abated because of its inability to effectively bind with respective receptor. The observed antinociception of [Des-Phe^{12}]YFa was substantially lower ($P < 0.05$) than the parent peptide YFa. These data further enhance and establish that NPFF and FMRFa related peptides may cause opioid agonist and antagonist actions and mediation can be done by various anti-opioid receptor subtypes via difference G-protein Gi (G-inhibitory) and Gs (G-stimulatory) coupling [26-28]. Alternatively, the chimeric peptide might be behaving as putative opioid agonist through N-terminal met-enkephalin site and antagonist to FMRFa/NPFF receptor via C-terminal FMRFa site either conjointly or separately [29]. Therefore, the observed antinociception could be combined effect of opioid receptor(s) stimulation and/or simultaneous FMRFa/NPFF receptor(s) antagonism. However, we recognize that thoroughly examine this phenomenon, more research is necessary.

Furthermore, we found that the κ specific antagonist nor-binaltorphimine significantly reduced the antinociceptive action of [Des-Phe^{12}]YFa. Whereas μ and δ selective antagonist naloxonazine and naltrindole, respectively does not induced significant attenuation in its antinociception indicating that [Des-Phe^{12}]YFa mediate its observed antinociception predominantly through κ opioid receptors (figure 2). The same receptor specificity of [Des-Phe^{12}]YFa and parent peptide YFa indicates that change at C-terminal -RFa- site does not alter the opioid receptor specificity of these chimeric peptides rather primarily affect its antinociceptive effectiveness.

Opioid receptor agonists cause tolerance when administered continuously [30-34], and receptor down-regulation is thought to be a key mechanism underlying opioid tolerance [35, 36]. To examine the behavior of [Des-Phe^{12}]YFa throughout long-term administration, we investigated the antinociception during a period of six days of long-term action of [Des-Phe^{12}]YFa and evaluated its impact on discrepancy expression of μ, δ, and κ opioid receptors. Importantly, [Des-Phe^{12}]YFa induced tolerance devoid antinociception throughout the chronic treatment (figure 3). This tolerance free antinociception during chronic treatment might be the result subsequent up-regulation (mRNA and protein) in KOR1

expression (figure 5A-D). Similar, results were obtained for parent peptide YFa which induced tolerance free antinociception with synchronized up-regulation in KOR1 receptor expression, whereas the μ-receptor peptide agonist endomorphin-1, induced significant tolerance (by 49%) with consequent MOR1 expression down-regulation upon chronic treatment [19]. The increase in KOR1 expression was maybe due to the result of active opioid motif (YGGFM) present in [Des-Phe^{12}]YFa. Being endogenous, this motif seems to hinder the binding of endogenous ligands and thus, prevent receptor down-regulation [37-40]. Beside this, chimeric peptide also seems to increase the recycling of opioid receptors via internalization and re-sensitization [41, 42, 43] a phenomenon specific for peptide ligands [37, 44-48], thereby preserves the down-signaling of opioid receptors and counteracting the development of tolerance [49-52].Changes in the other two opioid receptors' expression (MOR1 and DOR1) were anticipated during chronic treatment of [Des-Phe^{12}]YFa because co-administration of parent peptide YFa, with morphine results in additive antinociception and attenuation in the formation of morphine analgesia tolerance [18]. However, it was observed that long-lasting treatment of [Des-Phe^{12}]YFa does not induce any substantial variation in expression of MOR1 and DOR1 receptors because of the fact that [Des-Phe^{12}]YFa mainly uses KOR1 to mediate its antinociception rather than MOR1 and DOR1. Therefore, it largely induced variations in KOR1 expression and not in other two opioid receptors. This observation further potentiates our pharmacological results that [Des-Phe^{12}]YFa mediate its antinociception through κ opioid receptor (figure 2).

CONCLUSION

The present work indicates that [Des-Phe^{12}]YFa on i.p. injection induces κ specific dose dependent antinociception in rats, which was much lower than the parent peptide YFa. Chronic treatment results in significant antinociception in rats and selective up-regulation of κ opioid receptors (KOR1). Further, molecular and pharmacological activity of [Des-Phe^{12}]YFa demonstrate that, while amphiactive chimeric peptides like YFa primarily operate as opioid agonists but the FMRFa site also contributes to total observed antinociception.

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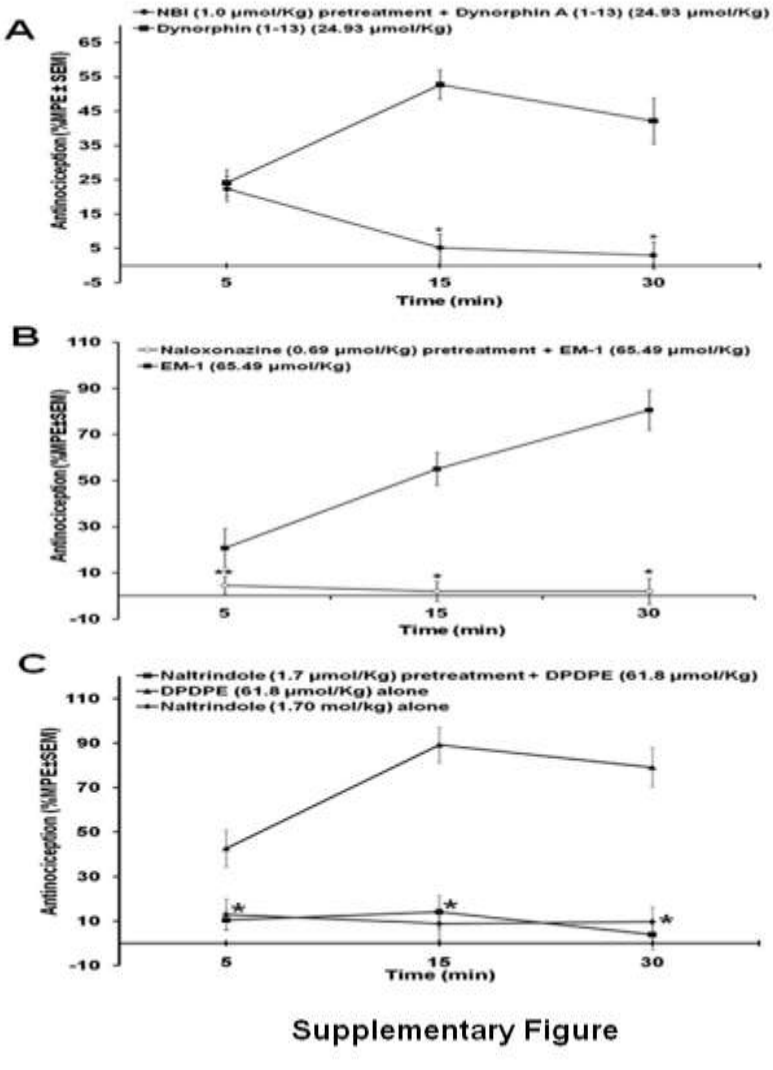

Supplementary Figure

Supplementary Figure:

A: Effect of pretreatment of κ opioid receptor antagonist nor-Binaltorphimine on the time course of Dynorphin A (1-13) (24.93 µmol/kg, i.p.) induced antinociception in the tail-flick test. Data represented as mean ± SEM. Antinociception (%MPE) values for Dynorphin A (1-13) (24.93 µmol/Kg, i.p.) alone and NBI (1.03 µmol/Kg) alone were also represented. * indicate significantly different from Dynorphin A (1-13) alone ($p<0.001$, two way ANOVA, Tukey's test).

B: Effect of pretreatment of µ opioid receptor antagonist naloxonazine on the time course of endomorphine-1 (EM-1) (65.49 µmol/Kg, i.p.)-induced antinociception in the tail-flick test. Data represented as mean ± SEM. Antinociception (%MPE) values for EM-1 (65.49 µmol/kg, i.p.) alone were also represented. * indicate significantly different from EM-1 alone ($p<0.001$, two-way ANOVA, Tukey's test); ** indicate significantly different from EM-1 alone ($p<0.05$, two way ANOVA, Tukey's test).

C: Effect of pretreatment of the δ opioid receptor antagonist naltrindol (1.7 µmol/kg; i.p.) on the time course of DPDPE (61.8 µmol/kg, i.p.) induced antinociception in the tail-flick test. Data values are represented as mean ± SEM. Antinociception (% MPE) values for DPDPE (61.8 µmol/kg; i.p.) alone and naltrindol (1.7 µmol/kg; i.p.) alone are also represented. *Significantly different from DPDPE alone ($P < 0.001$, two-way ANOVA, Tukey's test).