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Dual effects of [Tyr^6]-γ2-MSH(6–12) on pain perception and *in vivo* hyperalgesic activity of its analogues

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[Tyr^6]-γ2-MSH(6–12) with a short effecting time of about 20 min is one of the most potent rMrgC receptor agonists. To possibly increase its potency and metabolic stability, a series of analogues were prepared by replacing the Tyr^6 residue with the non-canonical amino acids 3-(1-naphtyl)-L-alanine, 4-fluoro-L-phenylalanine, 4-methoxy-L-phenylalanine and 3-nitro-L-tyrosine. Dose-dependent nociceptive assays performed in conscious rats by intrathecal injection of the MSH peptides showed [Tyr^6]-γ2-MSH(6–12) hyperalgesic effects at low doses (5–20 nmol) and analgesia at high doses (100–200 nmol). This analgesic activity is fully reversed by the kyotorphin receptor-specific antagonist Leu–Arg. For the two analogues containing in position 6, 4-fluoro-L-phenylalanine and 3-nitro-L-tyrosine, a hyperalgesic activity was not observed, while the 3-(1-naphtyl)-L-alanine analogue at 10 nmol dose was found to induce hyperalgesia at a potency very similar to γ2-MSH(6–12), but with longer duration of the effect. Finally, the 4-methoxy-L-phenylalanine analogue (0.5 nmol) showed greatly improved hyperalgesic activity and prolonged effects compared to the parent [Tyr^6]-γ2-MSH(6–12) compound. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: rMrgC; [Tyr^6]-γ2-MSH(6–12); hyperalgesia; non-canonical amino acids

Introduction

The recently identified Mas-related gene receptors (Mrgs) belong to the family of G-protein-coupled receptor and share high sequence homology (35%) to the MAS1 oncogene [1]. These receptors are localized in small diameter sensory neurons of the dorsal root and trigeminal ganglion [1–3]; correspondingly they are also named SNSRs [3]. There are three receptor subfamilies in mice and rat, MrgA, MrgB and MrgC [1], and six in humans, MrgX1–MrgX6 [3]. So far, only the MrgA and MrgC gene are found to be expressed in the rat [4].

Several ligands for Mrgs have been identified with their specificities determined: FLRF-amide for *m*MrgA, neuropeptide FF (NPFF) and neuropeptide AF (NPAF) for *m*MrgA1 and *m*MrgA4 [1], adenine for *r*MrgA, BAM22 and BAM8-22 for *h*MrgX1 [3], cortistatin-14 for *h*MrgX2 [5] and γ2- MSH, γ2-MSH(6–12) and its analogue [Tyr^6]-γ2-MSH(6–12) for *r*MrgC [6]. Among these ligands, BAM(8–22) and [Tyr^6]-γ2-MSH(6–12) are the most potent agonists for the rat MrgC (SNSR1) [6], but [Tyr^6]-γ2-MSH(6–12) was less efficient than BAM(8–22) 20 min after injection.

As replacement of Phe^6 in γ2-MSH(6–12) by a tyrosine residue with its enhanced hydrophilicity was found to produce a threefold increase in *r*MrgC-binding affinity [6], in this study four new related analogues with altered size, hydrophilicity and electronic characteristics, but with retained aromaticity were synthesized and compared to [Tyr^6]-γ2-MSH(6–12) for the *in vivo* hyperalgesic activity in rats by tail-withdrawal assay upon intrathecal administration.

Materials and Methods

Animals

All experiments were carried out according to the protocols approved by Ethics Committee of Animal Experiments at Lanzhou University, and the guidelines from China Council on Animal Care and the International Association for the Study of Pain Committee for Research and Ethical Issues. Every effort was made to minimize the numbers and suffering of the animals used in the following experiments. Adult male Wistar rats (Animal Center of Lanzhou University, Lanzhou, China), weighing 180–230 g were housed with a standard light/dark cycle (12 h light/12 h dark) at temperature (21 ± 2 °C). Food and water were available *ad libitum*.

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Abbreviations used: *MSH, melanocyte-stimulating hormone; rMrgC, rat Mas-related gene C; Nal, 3-(1-naphtyl)-L-alanine; 4FPhe, 4-fluoro-L-phenylalanine; 4MPhe, 4-methoxy-L-phenylalanine; 3NTyr, 3-nitro-L-tyrosine; RP-HPLC, reverse phase high performance liquid chromatography; SNSR, sensory neuron-specific G-protein-coupled receptor; TFA, trifluoroacetic acid.*

Table 1. Sequences of γ2-MSH(6–12) and analogues

Peptide	Sequence	R_t (min)	Mass	
			m/z (found)	M_r (Cacl.)
γ2-MSH(6–12)	Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	11.0	983.3 $[M]^+$; 492.7 $[M+2H]^{2+}$	982.5
[Tyr^6]-γ2-MSH(6–12)	Tyr-Arg-Trp-Asp-Arg-Phe-Gly-OH	10.6	999.7 $[M]^+$; 500.7 $[M+2H]^{2+}$	998.5
[4FPhe^6]-γ2-MSH(6–12)	4FPhe-Arg-Trp-Asp-Arg-Phe-Gly-OH	11.1	1000.2 $[M]^+$; 500.6 $[M+2H]^{2+}$	999.5
[3NTyr^6]-γ2-MSH(6–12)	3NTyr-Arg-Trp-Asp-Arg-Phe-Gly-OH	11.0	1044.7 $[M]^+$; 522.6$[M+2H]^{2+}$	1043.5
[Nal^6]-γ2-MSH(6–12)	Nal-Arg-Trp-Asp-Arg-Phe-Gly-OH	11.0	1033.4 $[M]^+$; 517.7 $[M+2H]^{2+}$	1032.5
[4MPhe^6]-γ2-MSH(6–12)	4MPhe-Arg-Trp-Asp-Arg-Phe-Gly-OH	11.6	1013.3 $[M]^+$; 507.6 $[M+2H]^{2+}$	1012.5

Peptides

For peptide synthesis commercially available intermediates, reagents and solvents were used. The amino acids Nal, 4MPhe, 4FPhe, 3NTyr and dipeptide Leu–Arg were purchased from GL biochem (Shanghai, China). The peptides γ2-MSH(6–12), [Tyr^6]-γ2-MSH(6–12), [Nal^6]-γ2-MSH(6–12), [4MPhe^6]-γ2-MSH(6–12), [4FPhe^6]-γ2-MSH(6–12) and [3NTyr^6]-γ2-MSH(6–12) were prepared by manual solid-phase synthesis with standard Fmoc strategy, and purified by preparative RP-HPLC. Preparative HPLC was performed on Gilson equipped with a Hypersil 10 µ C18 20 × 250 mm column by elution with: γ2-MSH(6–12), linear gradient from 30 to 70% solvent B [0.08% TFA in CH_3CN] in solvent A [0.1% TFA in H_2O] in 30 min at a flow rate of 10 ml/min; [Tyr^6]-γ2-MSH(6–12), 30 to 60% B in 25 min; [Nal^6]-γ2-MSH(6–12), 35 to 65% B in 25 min; [4MPhe^6]-γ2-MSH(6–12), 30 to 70% B in 25 min; [4FPhe^6]-γ2-MSH(6–12) and [3NTyr^6]-γ2-MSH(6–12), 35 to 75% B in 30 min. The purity (~98%) of the synthetic peptides was assessed by analytical HPLC, which is Waters 600–2996 equipped with a Waters Sunfire™ 5 µ C18 4.6 × 150 mm column by elution with linear gradient 5–95% solvent B (0.08% TFA in CH_3CN) in solvent A (0.1% TFA in H_2O) at a flow rate of 1 ml/min in 18 min, and characterized by electrospray ionisation-mass spectrometry (ESI-MS) (Table 1). Naloxone hydrochloride dihydrate was purchased from Fluka (Beijing, China). The peptides were dissolved in saline (0.9% NaCl), and injected in volumes of 10 µl followed by an additional 10 µl saline to flush the catheter. The stock solutions were stored at −20 °C, and diluted to the desired concentration with saline immediately before each experiment.

Injection Direct to Dorsal Root Ganglia

Peptides were administrated by direct transcutaneous intraganglionar injection according to literature protocols [7–9]. Rats were held securely with mouth and paws protected by gauze. The vertebral column was flexed around L3-L5 level, widening these intervertebral spaces. Using the anterior part of the iliac crest as a tactile landmark for the L5-L6 intervertebral level, the vertebral lumbar puncture was performed using a 50-µl microinjection syringe and a 27-gatige needle was inserted between the L5 and L6 vertebrae. A sudden lateral movement of the tail was observed when the puncture was successful. The delivery volume is 20 µl (i.t., 20 µl/60 s). Control animals were given an equivalent volume of saline. The proper injection site was verified in pilot experiments by administration and localization of methylene blue dye. To study the relationship between [Tyr^6]-γ2-MSH(6–12) and its analogues to opioidergic system, the non-selective antagonist naloxone was used by i.p. administration, which was injected at a dose of 1 mg/kg, 2 min prior to peptides administration. The specific antagonist of kytophin receptor Leu–Arg was administrated by i.t.

Tail-Withdrawal Test

Thermal sensitivity was studied using hot water tail immersion test [6]. Every rat was used only once. Tails were immersed in water set at 48.5 °C. The time length between immersing the tails in water and the rats' removing of its tail was registered as tail-withdrawal latency (TWL). Only those rats with baseline latency within the range of 5–7 s were selected for further studies, and a cutoff latency was set at 10 s to avoid damage to the tail. Before each drug trial, a series of six sequential pre-drug administration latency measurements were made to establish a stable baseline, each with a 10-min interval. The latencies of the last four tests were averaged as a control value. Typically, these control values varied by <10%. Post-drug latency measurements were performed at 5, 10, 20, 30, 40, 50 and 60 min.

Data Analysis

The hyperalgesia effects in the above tests were calculated as the percentage change of TWL from the baseline level according to the formula: percentage change of TWL = (test latency/baseline latency) × 100. The data were expressed as means ± SEM of *n* experiments, where *n* refers to the number of rats used in the test. Each group consisted of 7–8 rats per drug and each animal was used only once The statistical comparison for pain threshold as indicated by tail-flick latencies was made using a one-way analysis of variance (ANOVA) and that for antinociceptive or nociceptive effect was made by two-way ANOVA. A value of $P < 0.05$ was selected as the indicative of significant difference.

Results

Thermal Hyperalgesic and Analgesic Effects Induced by [Tyr^6]-γ2-MSH(6–12)

As shown in Figure 1, doses of 0.5 nmol [Tyr^6]-γ2-MSH(6–12) produced nearly no effect in the tail-withdrawal test; however, significant hyperalgesia was observed after injection of 5–20 nmol peptide. Consistent with previous reports, the hyperalgesia lasted for 20 min with a maximum around 10 min [6,10]. Because of the weak analgesic effect found between 20 and 30 min at doses of 20 nmol, larger doses of [Tyr^6]-γ2-MSH(6–12) were examined. With 100 nmol injections, an increased latency of 124% ($P < 0.001$) was obtained which reached a value of 153% ($P < 0.001$) at 200 nmol [Tyr^6]-γ2-MSH(6–12), clearly indicating analgesic effects at

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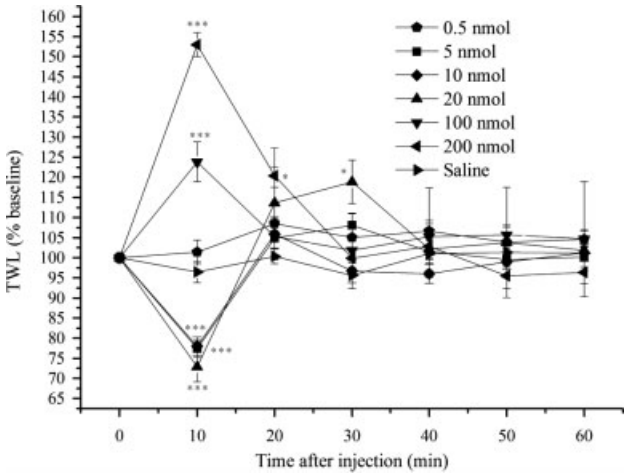

Figure 1. Dose- and time-related effects of i.t. administration of [Tyr^6]-γ2-MSH(6–12) at nanomole level in 48.5 °C warm-water tail immersion test in rats. All data at each time point are presented as % change of TWL ± SEM for $n = 7$–8 per group. At 20 nmol/rat, $n = 8$, other doses, $n = 7$. $^{*}P < 0.05$, $^{***}P < 0.001$, statistically significant differences between [Tyr^6]-γ2-MSH(6–12) *versus* saline.

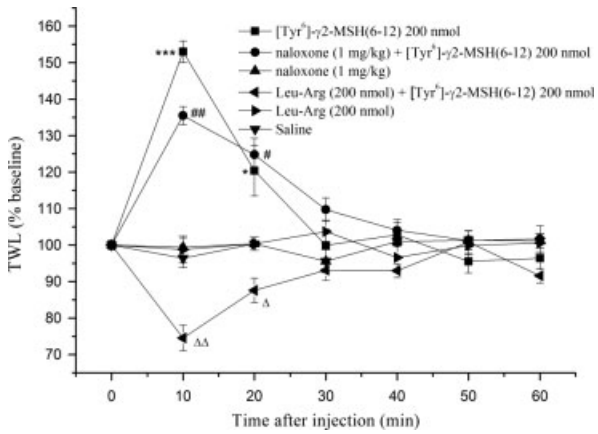

Figure 2. Dose- and time-related effects of [Tyr^6]-γ2-MSH(6–12) (200 nmol/rat), pretreatment with naloxone (1 mg/kg) or Leu–Arg (200 nmol/rat) 2 min prior to [Tyr^6]-γ2-MSH(6–12) administration in 48.5 °C warm-water tail immersion test in rats. All data at each time point are presented as % change of TWL ± SEM for $n = 7$ per group. $^{***}P < 0.001$, $^{*}P < 0.05$, statistically significant differences between [Tyr^6]-γ2-MSH(6–12) *versus* saline; $^{\#\#}P < 0.01$, statistically significant differences between ([Tyr^6]-γ2-MSH(6–12) + naloxone) *versus* [Tyr^6]-γ2-MSH(6–12) alone. $^{\#}P < 0.05$, statistically significant differences between ([Tyr^6]-γ2-MSH(6–12) + naloxone) *versus* saline; $^{\Delta\Delta}P < 0.01$, $^{\Delta}P < 0.05$, statistically significant differences between ([Tyr^6]-γ2-MSH(6–12) + Leu–Arg) *versus* saline.

these higher doses with maximum at 10 min and a duration of the overall effect of 30 min.

Effect of Naloxone and Leu–Arg on [Tyr^6]-γ2-MSH(6–12)-Induced Thermal Analgesia

As the analgesic effects induced by [Tyr^6]-γ2-MSH(6–12) at high doses could be mediated by the opioid system, naloxone (1 mg/kg, i.p.) was administered 2 min prior to 200 nmol [Tyr^6]-γ2-MSH(6–12). With the latency dropping from 153 to 135%, naloxone was found to slightly decrease the analgesic effects (Figure 2), suggesting that analgesia might not directly be mediated by the opioid system. Inspired by the similarity of the N-terminal dipeptide sequence of [Tyr^6]-γ2-MSH(6–12) and kyotorphin [11–13] (Table 1), the specific antagonist Leu–Arg of the kyotorphin receptor was used to prove if [Tyr^6]-γ2-MSH(6–12) can activate this receptor and by this way induce analgesia. Surprisingly, when rats were co-injected with Leu–Arg (200 nmol, i.t.), the analgesic effect was totally suppressed and hyperalgesia was observed (Figure 2), although at lower potency but at longer duration than that produced by [Tyr^6]-γ2-MSH(6–12) at a 5 nmol dose. These results indicate that at large doses [Tyr^6]-γ2-MSH(6–12) can bind to kyotorphin receptor and then promote enkephalin release to produce analgesia, which in turn is partially blocked by naloxone.

Comparison of γ2-MSH(6–12), [Tyr^6]-γ2-MSH(6–12) and Its Analogues

As the most potent hyperalgesic effects were observed at 10 min after injection (Figure 1), the values at 10 min and at doses of 10 nmol were chosen for comparing the hyperalgesic effects of the analogues. When Phe^6 of γ2-MSH(6–12) was replaced by 4FPhe or 3NTyr, practically no hyperalgesia was observed (data not shown); however, with [Nal^6]-γ2-MSH(6–12) the hyperalgesic activity was similar to that of γ2-MSH(6–12) (Figure 3(A)). Among all analogues, [4MPhe^6]-γ2-MSH(6–12) was the most potent in terms of hyperalgesic effects. Because the maximum values at 10 nmol doses were too close for comparison, 5 nmol was chosen as the dose for further comparative analysis (Figure 3(B)). The effective time of [4MPhe^6]-γ2-MSH(6–12) was 40 min (Figures 3(B) and 4(A)), while the effect of γ2-MSH(6–12) and [Tyr^6]-γ2-MSH(6–12) lasted only for 20 min (Figure 3). As shown in Figure 4(A) by the dose- and time-dependency of the hyperalgesic effects of [4MPhe^6]-γ2-MSH(6–12), the strongest hyperalgesia was observed at 20 nmol. Even at 0.5 nmol this analogue shows significant hyperalgesia ($P < 0.001$), while at this dose [Tyr^6]-γ2-MSH(6–12) is inactive (Figures 1 and 4(B)). At higher doses up to 200 nmol, [4MPhe^6]-γ2-MSH(6–12) still produces hyperalgesic effects, but the maximum is not higher than that at 20 nmol (Figure 4).

Discussion

The hyperalgesic/analgesic-related effects of [Tyr^6]-γ2-MSH(6–12) and Bam(8–22) have been reported by several groups [6,10,14–19]. However, to our knowledge, the possible mechanism of [Tyr^6]-γ2-MSH(6–12) activating kyotorphin receptor at high dose to induce analgesic effects has not been previously reported. Kyotorphin is an opioid-like neuropeptide which promotes the release of Met-enkephalin and then produces morphin-like analgesic effects [11–13]. After administration of 200 nmol [Tyr^6]-γ2-MSH(6–12), significant analgesia was observed, which could be totally blocked by Leu–Arg (Figure 1). This dose is similar to that of kyotorphin used to study its analgesic action; indeed 20–500 nmol doses/rat were used and at 200 nmol the analgesic effect was similar to that observed with [Tyr^6]-γ2-MSH(6–12) at this dose [20]. Based on these results, we suggest that a possible mechanistic interpretation of this analgesia is that [Tyr^6]-γ2-MSH(6–12) binds predominantly to rMrgC at low doses to induce nociceptive effects, and that binding to kyotorphin receptor occurs to produce analgesia when rMrgC is saturated. Such explanation is supported by the fact that co-administration of 200 nmol/rat

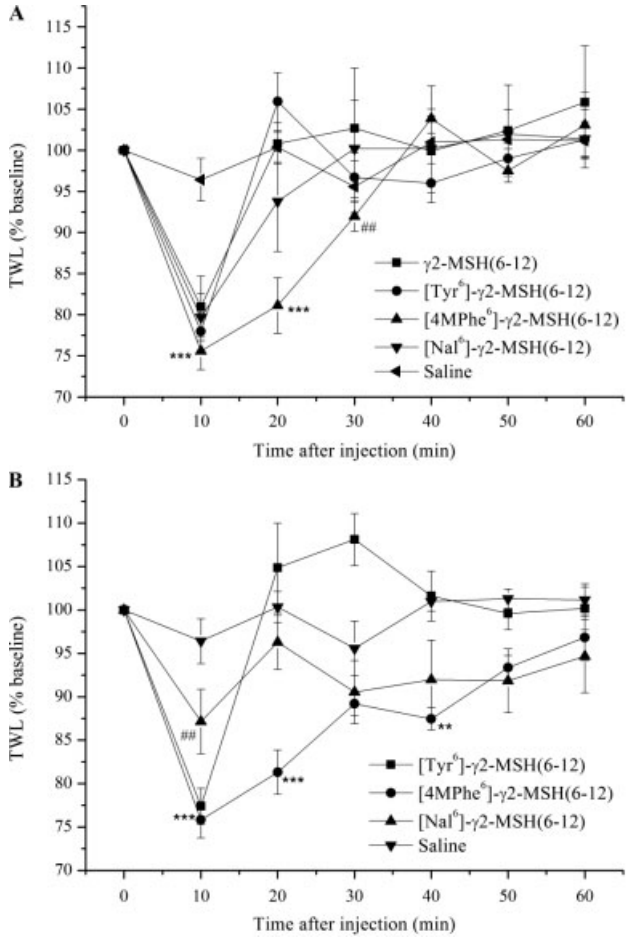

Figure 3. Comparison of γ2-MSH(6–12) and its analogues at nanomole level in 48.5 °C warm-water tail immersion test in rats. (**A**) Effect of 10 nmol γ2-MSH(6–12), [Tyr^6]-γ2-MSH(6–12), [4MPhe]-γ2-MSH(6–12) and [Nal]-γ2-MSH(6–12). (**B**) Effect of 5 nmol [Tyr^6]-γ2-MSH(6–12), [4MPhe]-γ2-MSH(6–12) and [Nal]-γ2-MSH(6–12). All data at each time point are presented as % change of TWL ± SEM for $n = 7$ per group. $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$, statistically significant differences between analogues *versus* saline; $^{\#\#}P < 0.05$, statistically significant differences between [Nal]-γ2-MSH(6–12) (**B**) or [4MPhe]-γ2-MSH(6–12) (**A**) *versus* saline.

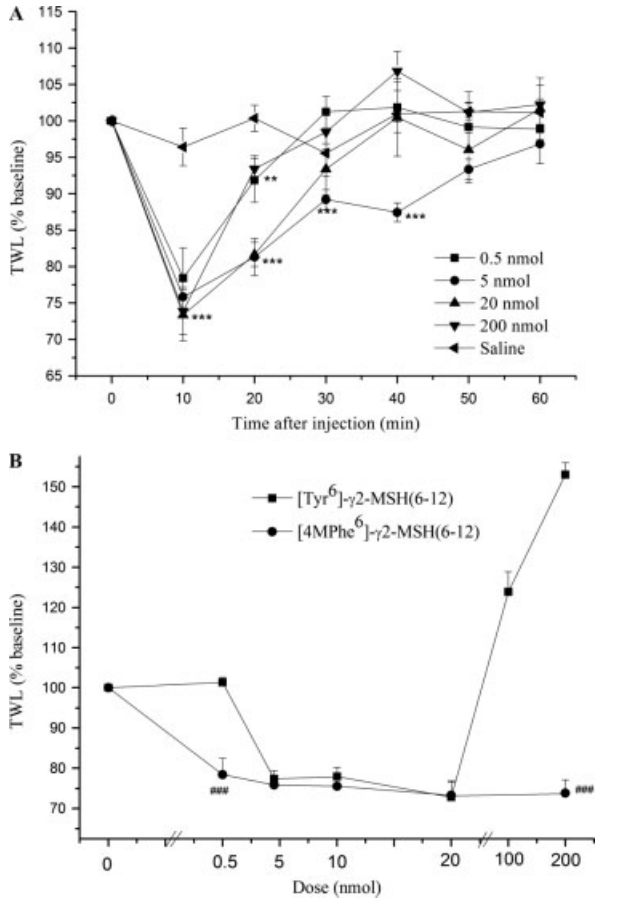

Figure 4. The dose- and time-dependent hyperalgesic effect of [4MPhe^6]-γ2-MSH(6–12) (**A**) and the comparison of dose-dependent nociceptive effects of [Tyr^6]-γ2-MSH(6–12) and [4MPhe^6]-γ2-MSH(6–12) (**B**). All data at each time point are presented as % change of TWL ± SEM for $n = 7$–8 per group, at 20 nmol/rat [4MPhe^6]-γ2-MSH(6–12), $n = 8$, other groups, $n = 7$. $^{***}P < 0.001$, $^{**}P < 0.01$, statistically significant differences between [4MPhe^6]-γ2-MSH(6–12) *versus* saline; $^{\#\#\#}P < 0.001$, statistically significant differences between [4MPhe^6]-γ2-MSH(6–12) *versus* [Tyr^6]-γ2-MSH(6–12).

[Tyr^6]-γ2-MSH(6–12) and Leu–Arg leads to reversion of analgesia to hyperalgesia, whose effective time was also prolonged to 40 min compared to the 10–20 min duration caused by 5 nmol doses. Such time-extended activity could derive from the large amount of [Tyr^6]-γ2-MSH(6–12) available when the kyotorphin receptor is blocked.

Previous structure-activity studies of [Tyr^6]-γ2-MSH(6–12) suggested that the phenolic group of Tyr^6 is crucial to its biological activity. In our study, this was confirmed by the weaker hyperalgesic activity of γ2-MSH(6–12), which contains no such hydroxyl group at this sequence position. As fluorine is generally considered as isosteric to oxygen, 4FPhe was introduced into γ2-MSH(6–12). Probably because of the higher chemical inertness and stronger hydrophobicity of 4FPhe [21], a greatly decreased hyperalgesic effect was caused by [4FPhe^6]-γ2-MSH(6–12) compared to [Tyr^6]-γ2-MSH(6–12). Practically no hyperalgesic effect was observed when Tyr^6 was substituted by 3NTyr; this is probably caused by electron withdrawing property of the nitro group [22]. Although Nal in position 6 exhibits a larger steric bulk and stronger hydrophobicity, the hyperalgesic activity of the related analogue was similar to that of γ2-MSH(6–12). Among all analogues, [4MPhe^6]-γ2-MSH(6–12) showed the highest hyperalgesic activity, which has to be attributed to the methoxy group in [4MPhe^6]-γ2-MSH(6–12) with its enhanced hydrogen bonding capacity compared to the hydroxy group of Tyr. These interesting findings are compelling for additional studies which are currently in progress.

In summary, intrathecal injection of [Tyr^6]-γ2-MSH(6–12) leads to dual effects in rats by tail-withdrawal test. It not only binds to rMrgC at low dose to produce hyperalgesic effects, but also to kyotorphin receptor at high dose to induce analgesia. Substitution of Tyr^6 with 4FPhe or 3NTyr resulted in complete lost of hyperalgesic potency, while the analogue with Nal^6 produced similar effects as γ2–MSH(6–12). Substitution with 4MPhe led to a significantly increased and prolonged hyperalgesic potency compared to [Tyr^6]-γ2-MSH(6–12).

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