Blockade of analgesic effects following systemic administration of *N*-methyl-kyotorphin, NMYR and arginine in mice deficient of preproenkephalin or proopiomelanocortin gene

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ABSTRACT

Kyotorphin is a unique biologically active neuropeptide (L-tyrosine-L-arginine), which is reported to have opioid-like analgesic actions through a release of Met-enkephalin from the brain slices. *N*-methyl-L-tyrosine-L-arginine (NMYR), an enzymatically stable mimetic of kyotorphin, successfully caused potent analgesic effects in thermal and mechanical nociception tests in mice when it was given through systemic routes. NMYR analgesia was abolished in μ-opioid receptor-deficient (MOP-KO) mice, and by intracerebroventricular (i.c.v.) injection of naloxone and of *N*-methyl L-leucine-L-arginine (NMLR), a kyotorphin receptor antagonist. In the Ca^{2+}-mobilization assay using CHO cells expressing $G\alpha_{qi5}$ and hMOPr or hDOPr, however, the addition of kyotorphin neither activated MOPr-mechanisms, nor affected the concentration-dependent activation of DAMGO- or Met-Enkephalin-induced MOPr activation, and Met-enkephalin-induced DOPr activation. NMYR-analgesia was significantly attenuated in preproenkephalin (PENK)- or proopiomelanocortin (POMC)-KO mice. The systemic administration of arginine, which is reported to elevate the level of endogenous kyotorphin selectively in midbrain and medulla oblongata, pain-related brain regions, caused significant analgesia, and the analgesia was reversed by i.c.v. injection of NMLR or naloxone. In addition, PENK- and POMC-KO mice also attenuated the arginine-induced analgesia. All these findings suggest that NMYR and arginine activate brain kyotorphin receptor in direct and indirect manner, respectively and both compounds indirectly cause the opioid-like analgesia through the action of endogenous opioid peptides.

1. Introduction

Kyotorphin is an analgesic dipeptide (L-tyrosine-L-arginine), which was isolated from bovine brain by use of in vivo analgesic assay system [1]. As kyotorphin causes an in vitro release of Met-enkephalin from the striatal slices [1,2], but shows neither binding activity to opioid receptors nor inhibiting activity of enkephalin degrading enzymes [1,3,4], this dipeptide is known as an enkephalin releaser. Although details remain elusive, there are several studies showing that opioid receptor antagonist, naloxone blocked various pharmacological or physiological actions of kyotorphin [5,6,3,7]. Kyotorphin was found to bind to putative G_i-coupled receptor in brain membranes through reconstitution experiments using purified G_{i1} and membrane putative receptor, which has high-affinity to [³H]-kyotorphin [8]. It should be noted that L-leucine-L-arginine (Leu-Arg) inhibits the [³H]-kyotorphin binding and kyotorphin-induced GTPase activation, a sign of G-protein activity, but Leu-Arg has no agonist activity on G proteins [8], suggesting that Leu-Arg could be considered as a pure kyotorphin receptor antagonist [9,10]. Regarding the biosynthesis, we have reported that kyotorphin is synthesized from L-tyrosine and L-arginine by partially

purified synthetase from rat brains, and the distribution and subcellular localization of the synthetase [11] are similar to those of kyotorphin [12]. However, the possibility cannot be excluded that this dipeptide is generated by the enzymatic processing of precursor proteins [13]. Most recently we have reported that tyrosyl-tRNA synthetase is a potential kyotorphin synthetase and has similar biochemical characteristics to partially purified rat kyotorphin synthetase [14]. Based on the finding that Km value for substrate arginine is much higher than the plasma concentration [11,14], we examined the change in brain kyotorphin levels following the systemic administration of arginine. The elevation of kyotorphin contents was uneven throughout brain regions [14], and it was higher in the midbrain and medulla oblongata, being consistent to the brain regional distribution of partially purified kyotorphin synthetase in the rat brain [11]. Kawabata et al. [15] reported that the systemic administration with L-arginine inhibited the carrageenan-induced inflammatory hyperalgesia, being consistent to the characterization of kyotorphin synthetase.

Through various attempts to design stable and potent derivatives of kyotorphin, we found an *N*-methyl-derivative NMYR has promising potencies in a unique and very sensitive peripheral nociception test [16]. In that study, the intraplantar injection of sub-femtomoles of NMYR caused nociceptive responses, which was reversed by the co-administration of similar dose of kyotorphin antagonist *N*-methyl-Leu-Arg, NMLR. Following this study, several papers have reported that kyotorphin-amide modification and its derivatives successfully show enzymatical stability and potent analgesic activity even by systemic administration [17,18], though no attempt has been done to examine whether kyotorphin-amide analgesia is blocked by kyotorphin antagonist, Leu-Arg or NMLR. Regarding the characterization as 'opioid-like' analgesia, they used intrathecal injection (i.t.) of naloxone, an opioid receptor antagonist to demonstrate the opioid-like analgesia of kyotorphin-amide [17]. However, as this report lacks the data with a high dose (50 μg) of naloxone (i.t.) alone, it remains elusive how much the possible hyperalgesic actions of naloxone affected the analgesic action of kyotorphin-amide. In addition, the attempt to see involvement of brain opioid mechanisms through endogenous opioid peptides also remains to be determined with the analgesia of kyotorphin derivatives.

In the present study we aimed to first examine whether potent analgesic is obtained by the systemic administration of NMYR that we have previously developed [16]. Secondly, we attempted to pharmacologically characterize the analgesic effects NMYR and arginine in terms of the involvement of brain opioid peptides using mice deficient of preproenkephalin (PENK) or proopioimelanocortin (POMC) gene, as well as in vivo NMLR antagonism and in vivo and/or in vitro opioid receptor-involvements.

2. Materials and methods

2.1. Materials

Synthetic peptides, kyotorphin, NMYR and NMLR were purchased from PH Japan Co., Ltd. (Hiroshima, Japan). [D-Ala^2, *N*-Methyl-Phe^4, Gly^5-ol] enkephalin (DAMGO) and naloxone hydrochloride was purchased from Sigma Aldrich (St. Louis, MO), Met-enkephalin and L-arginine were from WAKO (Osaka, Japan). In in vivo experiments, NMYR was administered through subcutaneous (s.c.), per os (p.o.), intracerebroventricular (i.c.v.) or intrathecal (i.t.) routes, while NMLR was given by i.c.v. or i.t. injection. For the culture experiments to see opioid receptor signaling, DMEM/HAM-F12 medium, geneticine, hygromycin B were purchased from Wako, Hank's Balanced Salt Solution (HBSS) and pluronic acid were from Life Technologies (Grand Island, NY), Fluo-8 was from AAT Bioquest (Sunnyvale, CA), amaranth, probenecid, and DAMGO were from Sigma Aldrich.

2.2. Animals

Male C57BL/6 J mice (15–30 g) were purchased from TEXAM (Nagasaki, Japan) and used for most of experiments. Animals were housed in a room maintained at 22 ± 3 °C and 55 ± 5% relative humidity with a 12 h light/dark cycle (light on 8:00 A.M. to 8:00 P.M.). Food and water were available ad libitum. In some experiments, male μ opioid receptor (MOPr) gene-deficient (MOPr-KO) mice, which had been kindly supplied by Brigitte Kieffer (McGill Univ. Douglas Institute, Montreal, Canada) and backcrossed to the inbred C57BL/6J mice for at least 10 generations were used, as reported previously [19]. In some other experiments, we used male preproenkephalin-deficient (PENK-KO) mice and proopiomelanocortin-deficient (POMC-KO) mice from The Jackson Laboratory (Bar Harbor, ME) possessing C57BL/6J and 129S2/SvPas mixed genetic background, as reported previously [20]. These mice were backcrossed to the inbred C57BL/6J mice for at least 10 generations before using for behavioral experiments. All procedures were approved by the Nagasaki University Animal Care Committee (Nagasaki, Japan) and complied with the recommendations of the International Association for the Study of Pain [21]. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [22–24].

2.3. Nociception tests

Thermal paw withdrawal, paw pressure and tail-flick tests were performed as, previously reported [25–27]. In some experiments, the analgesic activities were evaluated by use of area under the curve (AUC) as described in figure legends (Figs. 3 and 4).

2.4. Cells

The CHO cells stably co-expressing human μ opioid receptor (hMOPr) and C-terminal modified $G\alpha_q$ chimeric G protein, in which last 5 amino acids of C-terminal $G\alpha_q$ were replaced by corresponding $G\alpha_i$ amino acids to make $G\alpha_{qi5}$, and the CHO cells stably co-expressing human δ opioid receptor (hDOPr) and C-terminal modified $G\alpha_q$ chimeric G protein, in which last 5 amino acids of C-terminal $G\alpha_q$ with G66D mutation were replaced by corresponding $G\alpha_i$ amino acids to make $G\alpha_{qG66Di5}$. Both CHO cells expressing hMOPr and $G\alpha_{qi5}$ or hDOPr and $G\alpha_{qG66Di5}$ were prepared, as reported [28], and generously given by Dr. Girolamo Calo at University of Ferrara, Italy. These cells were maintained with DMEM/HAM-F12 supplemented with 10% FBS, 200 μg/mL of geneticine, 100 μg/mL of hygromycin B, 100 IU/mL penicillin and 100 IU/mL streptomycin, and incubated at 37 °C in a 5% CO_2 atmosphere.

2.5. Ca^{2+} mobilization assay

Ca^{2+} mobilization assay using the CHO cells (CHO_{hMOP}-$G\alpha_{qi5}$ and CHO_{hDOP}-$G\alpha_{qG66Di5}$) was previously described [29]. Briefly, CHO_{hMOP}-$G\alpha_{qi5}$ and CHO_{hDOP}-$G\alpha_{qG66Di5}$ cells were harvested using 0.5 mM EDTA, centrifuged, and re-suspended in culture medium described above. The cells were plated on a 384-well plate at the density of 1.0×10^4 cells/well/30 μL. Following overnight incubation, the medium was removed and the cells were loaded with 3 μM Fluo-8 dissolved in 20 mM HEPES/HBSS solution (pH7.4) containing 1 mg/mL amaranth, 2.5 mM probenecid, and 0.01% pluronic acid. After 1 h incubation, the cells were stimulated with either 10 μM kyotorphin or vehicle for 5 min. Then, Met-enkephalin or DAMGO at defined concentrations was added to the cells in the presence or absence of 10 μM kyotorphin. The fluorescence was recorded by Functional Drug Screening System/μCell (Hamamatsu Photonics K.K., Hamamatsu, Japan) and the fluorescence intensity was described as signal ratio (tested value/basal value) or fold induction. Dose-response curves were plotted as mean ± S.E.M using GraphPad prism (Graphpad Software. San Diego, CA).
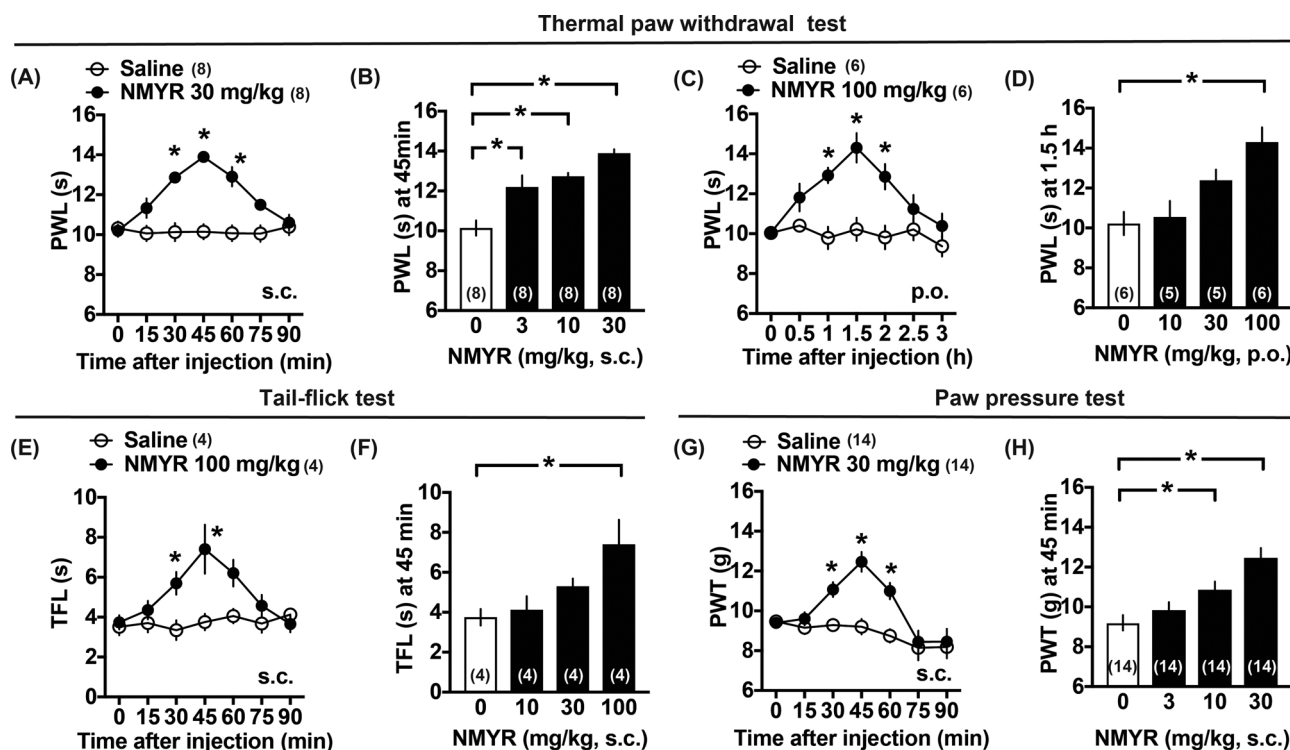
Fig. 1. Analgesic effects of NMYR given through systemic administration.
(A–D): Time course (A, C) and dose-dependency (B, D) of analgesia in the thermal paw withdrawal test after the s.c. (A, B) or p.o. (C, D) injection of NMYR or saline. Results represent the paw withdrawal latency (PWL) in seconds (s). The number of parenthesis indicates the number of mice used. In the dose-dependency test, the data show the mean ± S.E.M. E–H: Time course (E, G) and dose-dependency (F, H) of NMYR (s.c.)-induced analgesia in the tail-flick test (E, F) or paw pressure test (G, H). A, C, E, G: *$P < 0.05$, vs. saline at each time point, two-way ANOVA followed by Bonferroni's multiple comparisons test. B, D, F, H: *$P < 0.05$, vs. dose 0 (means saline injection), one-way ANOVA followed by Dunnett's multiple comparisons test.

2.6. Statistical analysis

All Data were presented as means ± S.E.M. and analyzed using the GraphPad prism 7.0. Data were analyzed using the unpaired t-test, one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons test and two-way ANOVA followed by Tukey's or Bonferroni's multiple comparisons test. The criterion of significance was significance was set up at $P < 0.05$.

3. Results

3.1. Potent analgesic effects of NMYR by systemic injection

An enzymatically stable kyotorphin derivative NMYR [16] at a dose of 30 mg/kg (s.c.) showed potent analgesic effects with peak effects at 45 min in the thermal paw withdrawal test, and the analgesic action was completely disappeared at 90 min, the significant analgesic effects of NMYR were observed at as low as 3 mg/kg (s.c.), and there was a dose-dependency in the range of 3–30 mg/kg (s.c.), as shown in Fig. 1 A, B . Similar potent dose-dependent analgesic effects of NMYR were also observed by the administration through a per os (p.o.) route (Fig. 1C, D). The peak effects at 100 mg/kg (p.o.) were 1.5 h and the analgesic effects lasted for 3 h. In the tail-flick test, another thermal nociception test, systemic NMYR showed potent analgesia, which lasted for 90 min with a dose-dependency in the range of 10–100 mg/kg, s.c. (Fig. 1E, F). Similar potent analgesia was also observed in the paw pressure test (Fig. 1G, H).

3.2. Antagonism of NMYR-induced analgesia by a putative antagonist NMLR and naloxone

When 3 nmol of NMYR was given i.c.v., significant analgesic effect

at 15 and 30 min, and dose-dependent effects were observed in the range of 0.3–3 nmol in the thermal paw withdrawal test (Fig. 2 A, B). However, the analgesia by NMYR given i.t. was less potent, and short-acting even with 10 nmol (Fig. 2C, D). A kyotorphin receptor antagonist, NMLR [16] at doses of 0.3–3 fmol (i.c.v.) blocked the NMYR (30 mg/kg, s.c.)-induced analgesia in a dose-dependent manner (Fig. 2E). Significant, but partial antagonism of NMYR (s.c.)-analgesia was also observed by i.t. injection of NMLR at 30 fmol (Fig. 2F). The NMYR (s.c.)-analgesia was also abolished by naloxone at as low as 0.1 nmol (approximately 40 ng, i.c.v.), which alone has no effect on the basal threshold.

3.3. Involvement of brain opioid peptides in NMYR-induced analgesia

The analgesia by 30 mg/kg (s.c.) of NMYR in the thermal paw withdrawal test was abolished in MOPr-KO mice (Fig. 3A, B). The analgesic effects of NMYR (30 mg/kg, s.c.) were also inhibited in PENK-KO mice (Fig. 3C). Quantitative analysis using AUC at periods of 15–75 min showed that NMYR analgesic activity was significant attenuated by approximately 20% in PENK-KO mice (Fig. 3D). Similar attenuation of NMYR analgesia by approximately 25% of AUC was observed in POMC-KO mice (Fig. 3E, F).

As shown in Fig. 3G, kyotorphin in the range of 0.1 nM to 10 μM has no agonist activity in MOPr-expressing cells. The addition of 10 μM kyotorphin did not affect the concentration-dependent Ca^{2+}-mobilization responses by DAMGO, a selective MOPr agonist (Fig. 3H). The EC50 of DAMGO in the absence or presence of kyotorphin was 1.8 ± 0.07 nM (n = 4), and 1.9 ± 0.1 nM (n = 4), respectively. Similarly, the EC50 of Met-enkephalin in the absence or presence of 10 μM kyotorphin was 1.3 ± 0.08 nM (n = 4), and 2.1 ± 0.02 nM (n = 4), respectively (Fig. 3I). Furthermore, 10 μM kyotorphin also has no allosteric effects on DOPr-expressing cells (Fig. 3J). EC50 of Met-
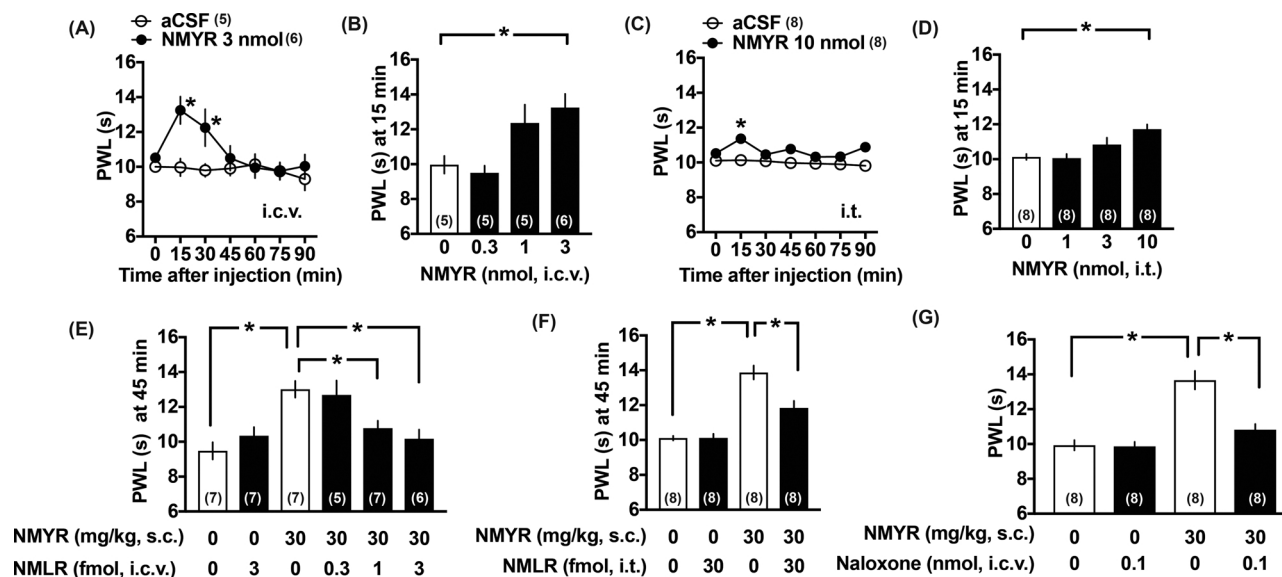
Fig. 2. Brain mechanisms of NMYR-induced analgesia.

All experiments in this figure were performed in the thermal paw withdrawal test. (A–D): Time course (A, C) and dose-dependency (B, D) of analgesia in the thermal paw withdrawal test after i.c.v. (A, B) or i.t. (C, D) injection of NMYR or aCSF. E, F: Potent or weak antagonism by i.c.v. (E) or i.t. (F) injection of NMLR against the systemic NMYR-induced analgesia. (G) Antagonism of NMYR (s.c.)-induced analgesia in the thermal paw withdrawal test by naloxone (i.c.v.). Other details are shown in the legends of Fig. 1A. (C): *$P < 0.05$, vs. saline at each time point, two-way ANOVA followed Bonferroni's multiple comparisons test. (B, D): *$P < 0.05$, one-way ANOVA followed by Dunnett's multiple comparisons test. (E, F, G): *$P < 0.05$, one-way ANOVA followed by Tukey's multiple comparisons test.

enkephalin in the absence or presence of kyotorphin was 0.53 ± 0.05 nM (n = 4), and 0.47 ± 0.04 nM (n = 4), respectively.

3.4. Involvements of kyotorphin receptor and endogenous opioid peptides in arginine-induced analgesia

As we have recently observed that the ʟ-arginine-treatment increases brain levels of kyotorphin [14], we tested whether this treatment shows the analgesia and its mechanism is related to brain opioid system. As shown in Fig. 4A, the systemic ʟ-arginine treatment at a dose of 1 g/kg (p.o.) showed potent analgesia with a peak effect at 1.5 h in the thermal paw withdrawal test. The analgesia was dose-dependent in the range of 0.1–1 g/kg (p.o.) of ʟ-arginine (Fig. 4B). The analgesia by 1 g/kg (p.o.) of ʟ-arginine was completely abolished by i.c.v. injection of NMLR (Fig. 4C) and naloxone (Fig. 4D). Furthermore, the analgesia was significantly attenuated in PENK-KO mice by 46% (Fig. 4E, F), and in POMC-KO mice by 36% (Fig. 4G, H) by use of quantitative analysis using AUC (1–2 h).

4. Discussion

The first issue to note is the experimental evidence that *N*-methyl derivatives of neuro-dipeptide kyotorphin (NMYR) and its antagonist NMLR pharmacologically behave as enzymatically stable and potent kyotorphin receptor agonist and antagonist, respectively, being consistent to our previous report using peripheral nociception test [16]. The present study revealed that systemic administration of NMYR, through s.c. or p.o. routes has potent analgesic actions in various thermal and mechanical nociception tests, though systemic kyotorphin administration has no significant analgesic action (data not shown). The enhancement of analgesia by *N*-methylation was consistent to the previous report using kyotorphin-amide [30]. The analgesic action of NMYR was reproduced largely by brain (i.c.v.) injection, but weakly by spinal (i.t.) injection. As the brain administration of antagonist NMLR in the dose range of as low as 0.3–3 fmol, blocks the systemic NMYR (s.c.)-induced analgesia, while the spinal (i.t.) administration at 30 fmol showed a blockade at the level less than the case with 1 fmol (i.c.v.), the major mechanisms appear to be attributed to the brain actions. The

second issue to note is the experimental evidence that NMYR exerts analgesic effects through brain endogenous opioids. The analgesia by NMYR was abolished in MOPr-KO mice and by naloxone (i.c.v.), being consistent to the previous observation that naloxone blocks the analgesia induced by kyotorphin given through an i.c.v. or intracisternal route [1].

The present study kyotorphin did not activate MOPr-mechanisms in the Ca^{2+}-mobilization assay using CHO cells expressing hMOPr and $G\alpha_{qi5}$. Furthermore, as kyotorphin did not affect the concentration-dependent activation of DAMGO- or Met-enkephalin-induced MOPr activation, and also did not affect Met-enkephalin-induced DOPr activation, it appears that this dipeptide also has neither direct nor indirect action through positive allosteric modulation to opioid receptors, unlike the cases with opioid mimetics [31,32]. In addition, there are reports that kyotorphin failed to inhibit enkephalin-degrading enzymes [4,33]. Thus, promising mechanisms would be the release of endogenous opioids, such as Met-enkephalin or β-endorphin, since NMYR-induced analgesia was attenuated in PENK- or POMC-KO mice, though each blockade seems to be insufficient for complete blockade in the quantitative analysis using AUC by 21 or 25%, respectively. Thus, it is interesting to examine whether NMYR analgesia is fully reversed in PENK- and POMC-double KO mice. There are reports that POMC contains adrenocorticotropic hormone and α-melanocyte stimulating hormone have anti-opioid activities [34,35]. Accordingly, the loss of anti-opioid activities may attenuate the blockade of NMYR analgesia due to the loss of β-endorphin in POMC-KO mice. Furthermore, as often discussed in many other cases with KO mice, it is also possible that embryonic deficiency of POMC or PENK gene may cause compensational machineries to make up the lost functions during development.

We have previously described a partially purified kyotorphin synthetase, an enzyme catalyzing the chemical reaction: ʟ-tyrosine + ʟ-arginine + ATP → kyotorphin + AMP + PPi in the presence of Mg^{2+}. The *K*m values for ʟ-tyrosine and ʟ-arginine are 25.6 μM and 926 μM, respectively [11]. As plasma levels of ʟ-tyrosine and ʟ-arginine are both 50–100 μM [36], the levels of ʟ-arginine may be a rate-limiting substrate. Based on this fact, Kawabata et al. [15] reported that the systemic administration with ʟ-arginine inhibited the carrageenan-induced inflammatory hyperalgesia in a Leu-Arg (i.c.v.)-reversible manner. The
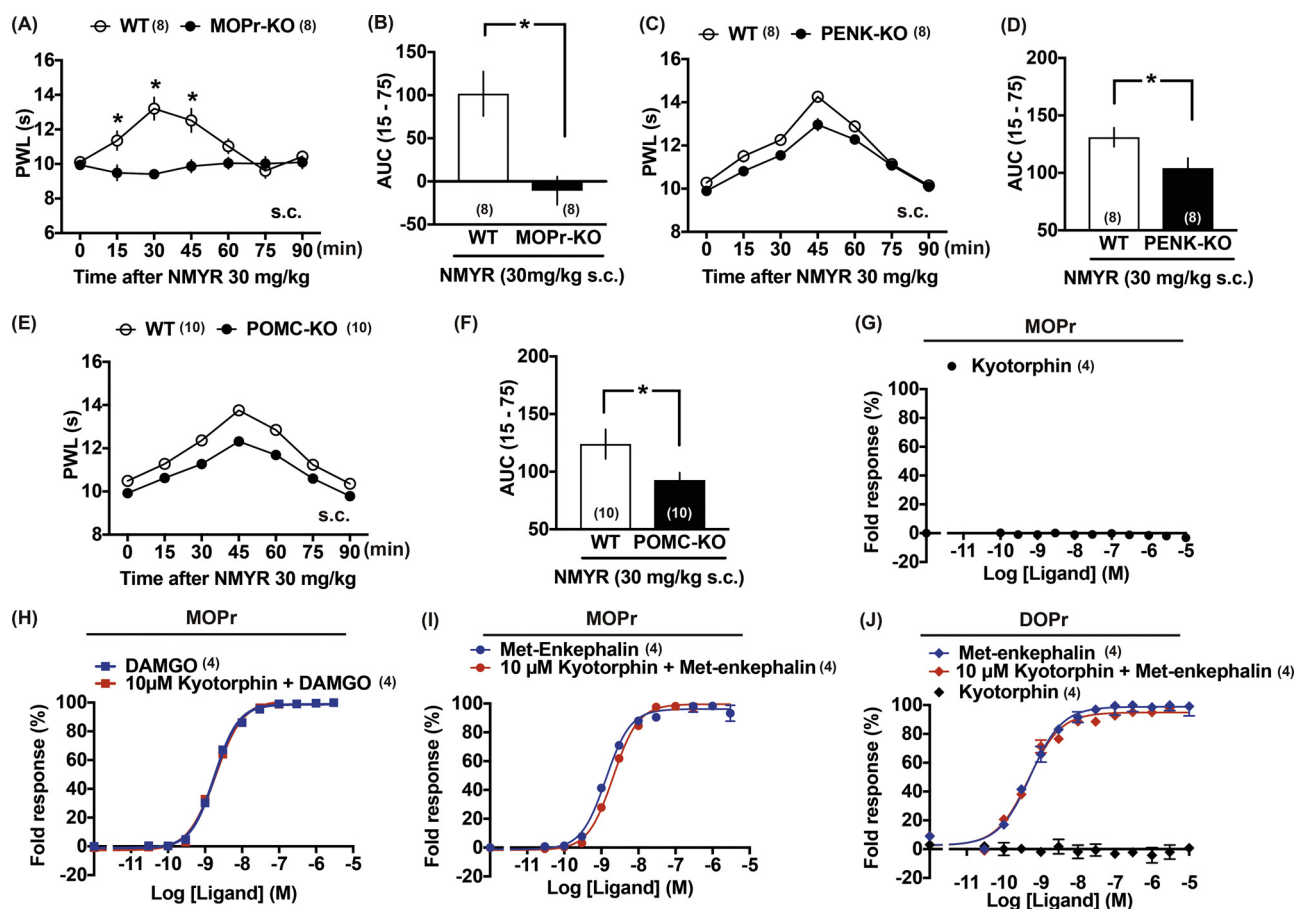
Fig. 3. Involvements of brain opioid system in the systemic NMYR-induced analgesia.

(A, B): Lack of NMYR (s.c.)-induced analgesia in MOPr-KO mice in the thermal paw withdrawal test. AUC (15–75) indicates [area under the curve of PWL (s) from 15 to 75 min after NMYR injection] – [60 × PWL at time 0]. (C–F): Attenuation of NMYR analgesia in PENK- or POMC-KO mice. Time course of NMYR (30 mg/kg, s.c.)-induced analgesia in the thermal paw withdrawal test using WT or PENK-KO mice (C) or WT or POMC-KO mice (E). AUC (15–75) in the quantitation (D, F) indicates [area under the curve of PWL (s) from 15 to 75 min after NMYR injection] – [60 × PWL at time 0]. (G–I): Lack of allosteric effects by 10 μM kyotorphin on DAMGO (H)- or Met-enkephalin (I) -induced Ca^{2+} mobilization in MOPr-expressing CHO, hMOP-Gαqi5 cells. (J): Lack of allosteric effects by 10 μM kyotorphin on Met-enkephalin-induced Ca^{2+} mobilization using CHO, hDOP-GαqG66Di5 cells. Other details are shown in the legends of Fig. 1. (A): *$P < 0.05$, vs. WT mice at each time point, two-way ANOVA followed Bonferroni's multiple comparisons test. (B, D, F): *$P < 0.05$, unpaired t-test.

present study using naïve mice supported these findings, and NMLR (i.c.v.) as well as naloxone (i.c.v.) completely reversed ʟ-arginine (p.o.)-induced analgesia in naive mice, suggesting the involvement of endogenous kyotorphin and opioids in the brain.

Thus, necessary future approach to clarify the involvement of β-endorphin or Met-enkephalin in the NMYR-analgesia would be the identification of brain loci responsible NMYR-induced endogenous opioid release for the study of the direct measurement of opioid peptide release through microdialysis probe or push-pull cannula. To be successful in this strategy, we need to first identify the brain loci responsible for NMYR-actions, e.g. through an experiment to measure the NMYR-activated ^{35}S-GTPγS binding and its reversibility by NMLR, using brain slices.

As NMYR is a smallest peptide, it could be a promising prototype to be developed as pharmaceutical compound in terms of synthetic costs and drug-delivery system. NMYR shows analgesic activity through endogenous opioids, which have no ceiling effects, unlike non-steroidal anti-inflammatory drugs or acetaminophen. Therefore, better-modified kyotorphin mimetics would be added to a compound group useful at the first stage of three-step analgesic ladder for cancer pain relief proposed by world health organization.

In conclusion, we successfully confirmed the unique analgesic mechanisms of kyotorphin by use of more stable and potent derivative, NMYR. Additional findings were observed in the experimental evidence

that NMLR behaves as a kyotorphin receptor antagonist, and that opioid-like NMYR-analgesia was inhibited by naloxone (i.c.v.), and in MOPr-KO and opioid peptides-KO mice. Better kyotorphin mimetics in terms of potency and pharmacokinetics/pharmacodynamics would be promising as supportive drugs for cancer pain relief.

Funding

Conflicts of interest

The authors declare no conflict of interest.

Contributions

HN contributed to the study design, behavioral experiments and their data analysis except for the study using PENK- and POMC-KO mice. YH and MN contributed to behavioral experiments and their data analysis in the study using PENK- and POMC-KO mice. RT contributed to the study of opioid receptor signaling. KT contributed to the study
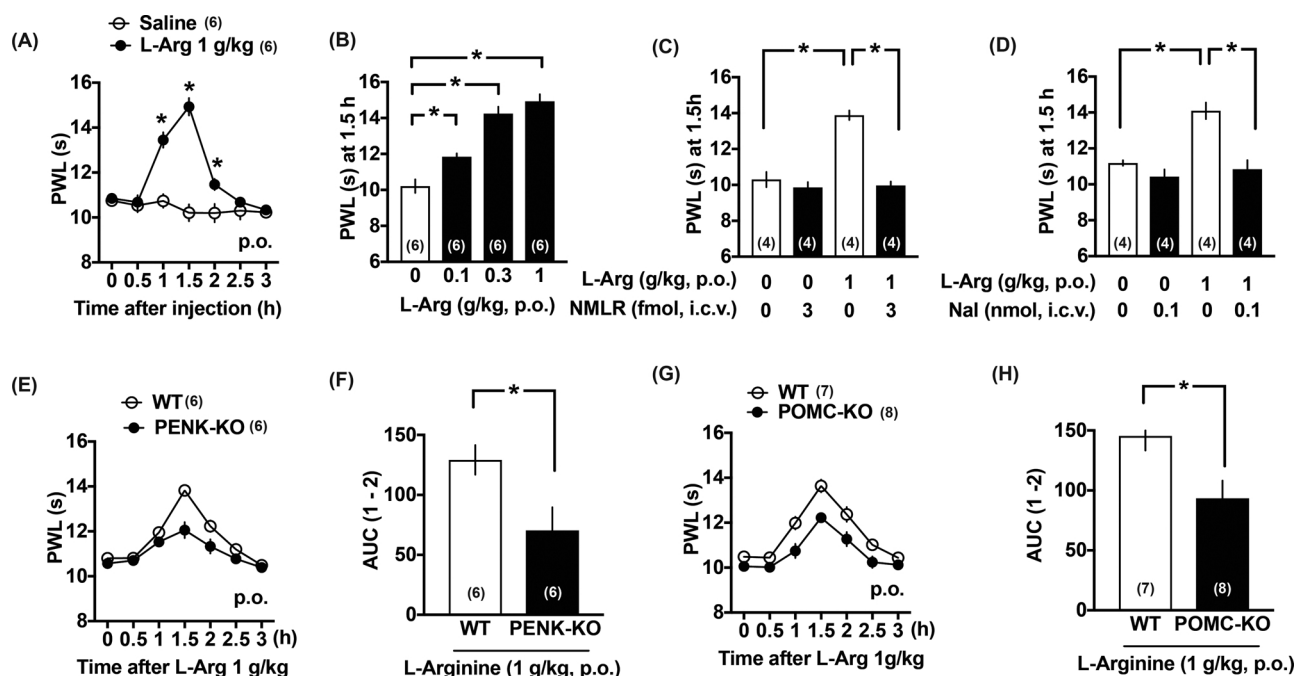
Fig. 4. Orally administered L-arginine mimics analgesic mechanisms by systemic NMYR.
All results in this figure represent the nociceptive threshold in the thermal paw withdrawal test. (A, B): Time course (A) and dose-dependency (B) of L-arginine (p.o.)-induced analgesia. L-arginine was consecutively injected at 0, 0.1, 0.3 and 1 mg/kg (p.o.) at day 0, 1, 2, 3. A: *$p < 0.05$, vs. saline at each time point, two-way ANOVA followed by Bonferroni's multiple comparisons test. B: *$P < 0.05$, one-way ANOVA followed by Dunnett's multiple comparisons test. (C, D): Blockade of L-arginine-induced analgesia by i.c.v. injection of NMLR (C) or naloxone (D). *$P < 0.05$, one-way ANOVA followed by Tukey's multiple comparisons test. (E–H): Time course (E, G) of L-arginine (p.o.)-induced analgesia using WT or PENK-KO mice (E) or WT or POMC-KO mice (G). AUC (1–2) in the quantitation (F, H) indicates [area under the curve of PWL (s) from 1 to 2 h after L-arginine injection] – [60 × PWL at time 0]. *$P < 0.05$, unpaired t-test.

design and manuscript preparation. HU contributed to the study design, data analysis of behavioral and opioid receptor signaling experiments and writing of manuscript. All authors approved the final version of the manuscript for publication and agree to be accountable for all aspects of the study.

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