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Effect of Neuronal Nitric Oxide Synthase Inhibitors and Antioxidants on the Development of Tolerance by Different Opioid Agonists in the Rat Locus Coeruleus

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Abstract: Nitric oxide (NO) is involved in acute μ-opioid receptor (MOR) desensitization in the locus coeruleus (LC) and in the neuroadaptations following chronic morphine administration. However, the role of NO and NO-derived reactive oxygen species (ROS) in the development of cellular tolerance to different opioids remains unclear. Herein, we examined the effect of the selective neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI; 30 mg/kg/12 h, i.p.) and the antioxidants Trolox + ascorbic acid (TX+AA; 40 and 100 mg/kg/day, respectively, i.p.) and U-74389G (10 mg/kg/day, i.p.) on the development of cellular tolerance induced by morphine, methadone and fentanyl. For induction of morphine tolerance, rats were treated subcutaneously with a slow release emulsion containing free base morphine (200 mg/kg, 3 days). For methadone (60 mg/kg/day, 6 days) and fentanyl (0.2 mg/kg/day, 7 days), tolerance was induced by s.c. implantation of osmotic pumps. Concentration-effect curves for the inhibitory effect of Met5-enkephalin (ME; 0.05-12.8 μM, 2x, 1 min) on the firing rate were performed by single-unit extracellular recordings of LC neurons from rat brain slices. Morphine, methadone and fentanyl treatments shifted to the right concentration-effect curves for ME and increased the EC_{50} by 2-4 folds. Co-administration of TX+AA or U-74389G in morphine-treated animals prevented the development of tolerance in LC neurons. Conversely, co-treatments with U-74389G or 7-NI failed to affect the induction of cellular tolerance after methadone or fentanyl treatments. Our results suggest that MOR agonists with different intrinsic efficacies cause variable degrees of cellular tolerance in LC cells. Moreover, NO/ROS pathways are differentially involved in opioid tolerance after prolonged treatments with morphine, methadone and fentanyl.

Keywords: slice; μ-opioid receptor; tolerance; reactive oxygen species; locus coeruleus; nitric oxide; morphine; methadone; fentanyl; electrophysiology

1. Introduction

Morphine, methadone or fentanyl are among the most common clinically used opioid agonists. However, their long-term utility is greatly limited due to the development of tolerance and dependence (Inturrisi 2002). Numerous mechanisms have been reported to contribute to opioid tolerance. Thus, tolerance may result from adaptive changes such as enhanced MOR desensitization, receptor internalization or receptor down-regulation, among other mechanisms (Williams et al., 2013). In addition, nitric oxide (NO) has been proposed to be involved in opioid tolerance (Heinzen and Pollack, 2004). In the brain, NO is produced by the neuronal NO synthase (nNOS) and targets the heme group of guanylate cyclase, which elevates 3',5'-cyclic guanosine monophosphate (cGMP) concentrations. Moreover, high, sustained concentrations of NO promote the generation of reactive nitrogen species and reactive oxygen species (ROS), such as the extremely oxidant molecule peroxynitrite (Radi, 2013).

The locus coeruleus (LC), the major noradrenergic nucleus in the brain, has long been used as a model for examining the cellular mechanisms of opioid tolerance and dependence (Nestler et al., 1994). It contains a homogeneous population of neurons that almost exclusively express the MOR (Williams and North, 1984). There is lacking evidence regarding the implication of NO/ROS pathway in the adaptations triggered by chronic treatments with different opioid agonists in the LC. Therefore, the aim of this work was to investigate whether NO and ROS regulate the tolerance induced by opioids with different pharmacologic profiles, such as morphine, methadone and fentanyl.

2. Results

Effect of chronic treatments with morphine, methadone and fentanyl on concentration-effect curves for ME in rat LC neurons

To evaluate the development of tolerance, concentration-effect curves for the inhibitory effect of ME were performed. Subchronic treatment with morphine (200 mg/kg, s.c., 3 days) induced a strong degree of tolerance in the LC, which was revealed by a rightward shift in the concentration-effect curves for ME and an increase in the EC_{50} of about 4 fold, as compared to the corresponding sham group ($p < 0.005$). Similarly, chronic treatment with methadone (60 mg/kg/day, s.c., 6 days) induced a rightward shift in the concentration-effect curves for ME and increased by about 2 fold the EC_{50} value when compared to the corresponding sham group ($p < 0.005$). Finally, chronic treatment with fentanyl (0.2 mg/kg/day, s.c., 7 days) also caused a rightward shift in the concentration-effect curves for ME with an increase of the EC_{50} of about 3 fold, as compared to the corresponding sham group ($p < 0.005$). These results indicate that morphine, methadone and fentanyl induce different degrees of tolerance to the inhibitory effect of ME in LC neurons. In all groups, the maximal effect of ME was 100% of baseline, which corresponded with an absolute inhibition from the basal firing rate.

It can be hypothesized that differences between morphine, methadone and fentanyl in their ability to induce receptor internalization and recycling (Alvarez et al., 2002; Virk and Williams, 2008) may contribute, at least in part, to the different degrees of cellular tolerance observed in this study.

Effect of the nNOS inhibitor 7-NI on opioid tolerance in rat LC neurons

Co-administration of the neuronal NOS inhibitor 7-NI in methadone- and fentanyl-treated rats (methadone/7-NI group and fentanyl/7-NI group) failed to prevent the development of tolerance. 7-NI administration in sham-treated animals (sham-7-NI group) did not modify the concentration-effect curves for ME in the LC as compared to the sham-vehicle group. No differences were found in the basal firing rate among groups.

Effect of antioxidants on opioid tolerance in rat LC neurons

Co-administration of the vitamin E analogue Trolox, together with ascorbic acid (AA), or the structurally unrelated antioxidant U-74389G in morphine-treated rats unclear. Further studies are needed to unmask the underlying mechanisms.

(morphine/TX+AA group and morphine/U-74389G group, respectively) attenuated the development of cellular tolerance, which was shown by a blockade of the rightward shift of the concentration-effect curve in this group ($p < 0.05$ and $p < 0.01$, respectively, when compared to the corresponding sham group) (Fig. 1). Administration of TX+AA or U-74389G in sham animals failed to modify the concentration-effect curves for ME when compared to the sham/vehicle group.

On the contrary, co-treatment with U-74389G in rats treated chronically with methadone or fentanyl did not prevent the development of cellular tolerance induced by these opioids (Fig. 2). In all cases, no differences were found in the basal firing rate among groups. The mechanisms by which NO modulates morphine-. but not methadone- or fentanyl-induced tolerance via ROS generation remain

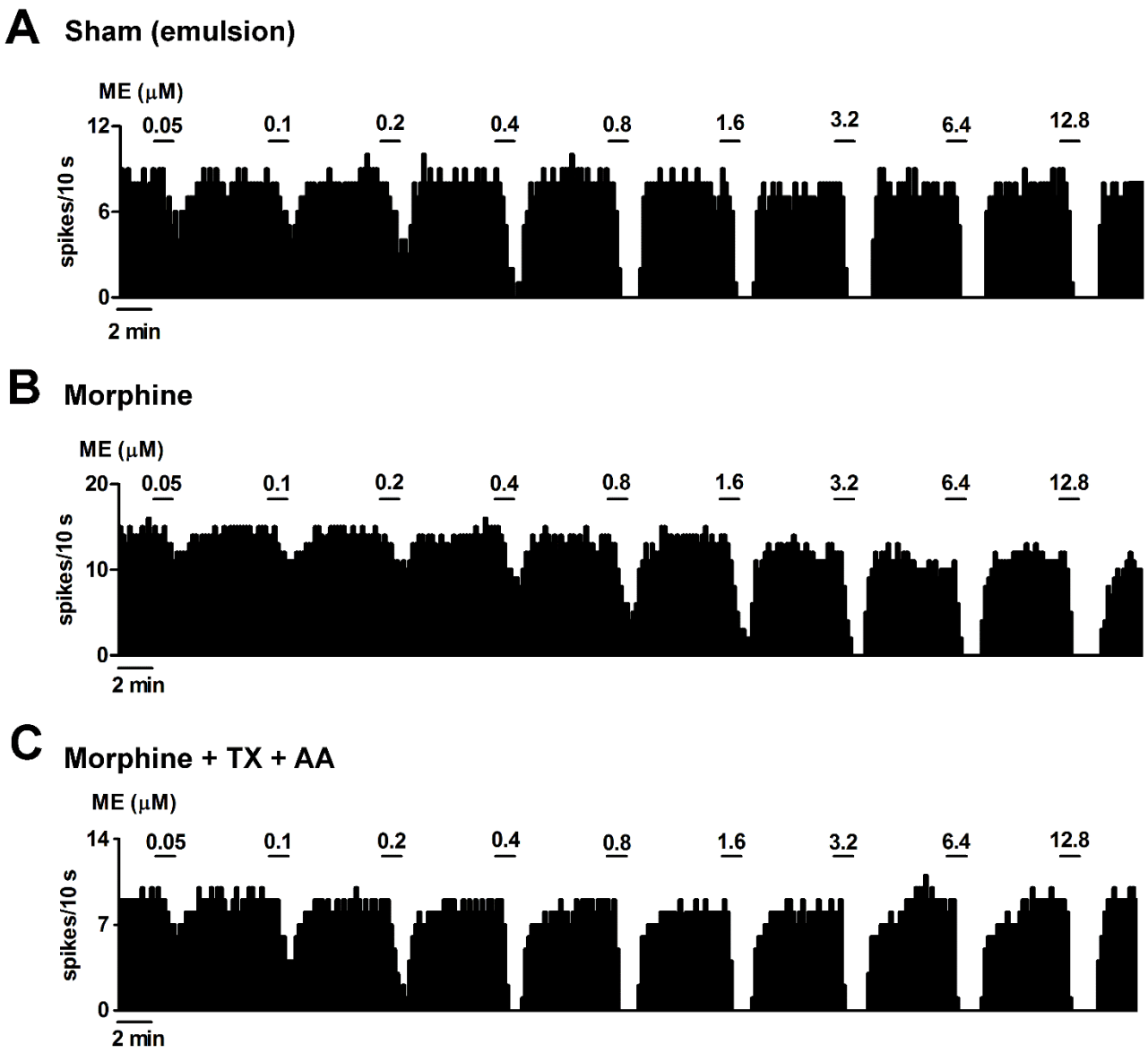

Figure 1. Effect of antioxidants Trolox + ascorbic acid (TX+AA) on the inhibition induced by ME in the LC of morphine-treated rats. **A, B, C**. Representative examples of firing-rate recording of LC cells from rats receiving the following treatments: sham (emulsion) (**A**), morphine (**B**) morphine + TX+AA (**C**). Each horizontal bar represents the period of application of each ME concentration (0.05 - 12.8 μM, 2x) and the vertical lines show the number of spikes recorded every 10 s. The inhibitory effect induced by each application was calculated as a percentage from the basal firing rate. Note that greater concentrations of ME are needed to inhibit the neuron activity in rats treated with morphine, compared with sham animals, which indicates the development of tolerance. Co-administration of TX+AA in morphine-treated rats increases the inhibitory effect of ME when compared to the morphine/vehicle group indicating an attenuation of celullar tolerance.

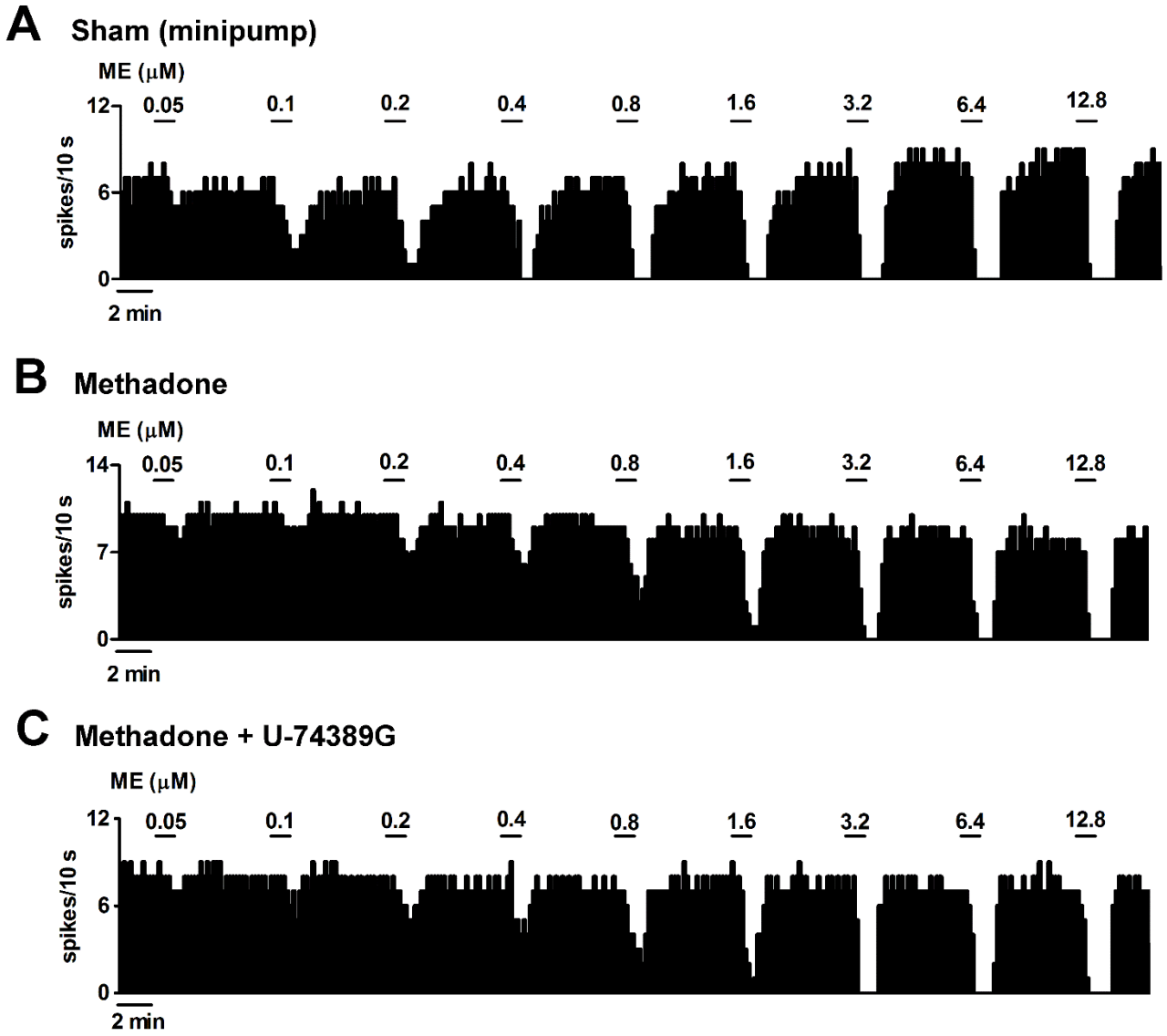

Figure 2. Effect of antioxidant U-74389G on the inhibition induced by ME in the LC of methadone-treated rats. **A, B, C**. Representative examples of firing-rate recording of LC cells from rats receiving the following treatments: sham (minipump) (**A**), methadone (**B**), methadone + U-74389G (**C**). Each horizontal bar represents the period of application of each ME concentration (0.05 - 12.8 μM, 2x) and the vertical lines show the number of spikes recorded every 10 s. The inhibitory effect induced by each application was calculated as a percentage from the basal firing rate. Note that greater concentrations of ME are needed to inhibit the neuron firing in rats treated with methadone, when compared to sham animals, indicative of tolerance However, co-administration of U-74389G in methadone-treated rats failed to prevent the development of cellular tolerance, so that concentration-effect curves for ME were not modified when compared to the methadone/vehicle group.

3. Materials and Methods

Animals and treatments Male adult Sprague-Dawley rats (200–300 g) were housed under standard laboratory

conditions (22 ºC and 12-h light/dark cycles) with free access to food and water. Principles of laboratory animal care were followed in all experimental procedures reported in this manuscript. Experimental procedures were carried out in accordance with the European Community Council Directive on "Protection of Animals Used in Experimental and Other Scientific Purposes" (86/609/EEC). The use of animals for this study was also approved by the *Animal Care and Use Committee* of the University of the Basque Country. All the efforts were made to minimize animal suffering and to reduce the number of animals used.

For induction of morphine tolerance, an oily emulsion of morphine base (200 mg/kg) was subcutaneously (s.c.) injected in the back of the rat, under slight anesthesia with chloral hydrate (200 mg/kg, i.p.). Control animals were implanted with a sham emulsion, which contained the same vehicle for morphine (mannide monooleate, liquid paraffin, and NaCl). Then, morphine or sham animals were daily injected with the antioxidants Trolox (40 mg/kg) and ascorbic acid (100 mg/kg) (TX+AA), U-74389G (10 mg/kg) or 0.9% NaCl (saline) intraperitoneally (i.p.). Electrophysiological experiments were performed 72 h after emulsion implantation.

For chronic treatments with methadone or fentanyl, osmotic mini-pumps were implanted subcutaneously in the rat. Animals were treated with methadone (60 mg/kg/day) or fentanyl (0.2 mg/kg/day). Sham, methadone-, or fentanyl-treated animals were treated every 12 h with the nNOS inhibitor 7-NI (30 mg/kg) or its vehicle. In another group of experiments, sham, methadone, or fentanyl-receiving rats were daily injected with the antioxidant U-74389G (10 mg/kg) or its vehicle (saline), i.p.

In vitro electrophysiology

Brain slice preparation

Animals were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and sacrified by decapitation. The brain was rapidly removed and a block of tissue containing the brainstem was placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 130 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 21 mM $NaHCO_3$, 2 mM $CaCl_2$, and 2 mM $MgSO_4$. Coronal slices of 500–600 μm thickness containing the LC were cut using a vibratome (FHC Inc., Brunswick, USA). The tissue was allowed to recover from the slicing for 90 min in oxygenated aCSF. Next, slices were placed on a nylon mesh in a modified Haas-type interface chamber maintained at 33°C and continuously perfused with oxygenated aCSF saturated with (95% O_2/ 5% CO_2, pH = 7.34–7.38) at a flow rate of 1–1.5 ml/min.

Recording procedures

Single-unit extracellular recordings of LC cells were performed as described previously (Mendiguren and Pineda, 2007). The recording electrode, an Omegadot glass micropipette was pulled and filled with NaCl (50 mM). The tip was broken back to a diameter of 2–5 μm (3–5 MΩ). The electrode was placed in the LC, visually identified as a dark oval area on the lateral borders of the central gray and the fourth ventricle, just anterior to the genu of the facial nerve. The extracellular signal from the electrode was passed through a high-input impedance amplifier and displayed on an oscilloscope and monitored with an audio unit. Individual neuronal spikes were isolated from the background noise with a window discriminator.

The firing rate was analyzed by a PC-based custom-made software, which generated histogram bars representing the cumulative number of spikes in consecutive 10 s bins (HFCP®, Cibertec S.A., Madrid, Spain).

Pharmacological procedures

We performed concentration-effect curves for the inhibitory effect of the MOR agonist ME in sham and opioid-treated animals. Thus, we perfused increasing concentrations of ME (0.05–12.8 μM, each concentration applied for 1 min) at 5-min intervals. ME was chosen because its washout is fast even at high concentrations and its action is mediated almost exclusively by MOR in LC neurons (Williams and North, 1984). The inhibitory effect of each ME concentration was calculated as follows:

$$E(\%) = \frac{FR_{pre} - FR_{post}}{FR_{basal}} \cdot 100$$

where FR_{pre} is the average firing rate for 60 s before each ME application, FR_{post} is the average firing rate for 90 s after each ME perfusion, and FR_{basal} is the firing rate for 60 s of each cell at the beginning of the recording. Curve fitting analysis was performed by the computer program GraphPad Prism (version 5.0 for Windows, San Diego, CA, USA) to obtain the best simple nonlinear fit to the following three-parameter logistic equation:

$$E(\%) = \frac{E_{max}}{1 + \left(\frac{EC_{50}}{A}\right)^{n}} \cdot 100$$

where E is the effect induced by each concentration of ME (A), Emax is the maximal effect, EC_{50} is the concentration of ME needed to elicit a 50% of the maximal effect, and n is the slope factor of the concentration-effect curve. These parameters were determined by the nonlinear analysis. EC_{50} values were finally expressed as negative logarithm (pEC_{50}).

Drugs and reagents

The following drugs were purchased from Sigma-Aldrich Química (Madrid, Spain): Fentanyl, methadone, L-ascorbic acid and 7-nitroindazole (7-NI). Met^5-enkephalin acetate salt (ME) was obtained from Bachem (Weil am Rhein, Germany). Morphine base was purchased from Alcaliber (Madrid, Spain). Trolox and U-74389G were obtained from Enzo Life Sciences (Lausen, Switzerland). For subchronic treatments with morphine, an oily emulsion containing morphine free base (200 mg/ml) in a mixture of mannide monooleate (Sigma-Aldrich Química S.A., Madrid, Spain), liquid paraffin (Sigma-Aldrich Química S.A., Madrid, Spain), and NaCl (0.9 %) (0.08:0.42:0.5, *v/v/v*) was prepared. Methadone, fentanyl and ascorbic acid were dissolved in saline. 7-NI was dissolved in peanut oil. Trolox was dissolved in NaOH (1 M), neutralized with HCl (1 M) and diluted to the last volume with saline. U-74389G was dissolved in 0.05 M HCl. The final pH prior to i.p administration was in all cases 6-7. ME stock solutions were prepared in Milli-Q water, stored at −25 °C and, on the day of the experiment, diluted in aCSF to their final volume.

Data analysis and statistics

Data are expressed as mean ± standard error of the mean (SEM). For statistical analysis, the EC_{50} values were transformed to the corresponding logarithmic data to convert them to a Gaussian distribution. Data among groups were compared by one-way analysis of variance

(ANOVA) followed by Tukey´s *post hoc* test using the computer program GraphPad Prism (version 5.0). A probability level of 0.05 was accepted as statistically significant.

4. Conclusions

Our results suggest that MOR agonists with different intrinsic efficacies cause variable degrees of cellular tolerance in LC cells. Moreover, NO/ROS pathways are differentially involved in opioid tolerance after prolonged treatments with morphine, methadone and fentanyl.

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Author Contributions

- P. Pablos performed the experiments and analyzed the data.
- A. Mendiguren and J. Pineda designed the research study
- P. Pablos, A. Mendiguren and J. Pineda wrote the manuscript

Conflicts of Interest

The authors declare no conflict of interest.

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