

## 5-HT<sub>1A</sub> receptor activation counteracted the effect of acute immobilization of noradrenergic neurons in the rat locus coeruleus

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Received 25 June 2006; received in revised form 11 September 2006; accepted 3 October 2006

### Abstract

The aim of our study was to evaluate the effect of acute stress and the 5-HT<sub>1A</sub> receptor involvement in both, the hippocampus noradrenaline (NA) tissue levels and the c-Fos immunoreactivity (c-Fos-IR) in the catecholaminergic neurons of the locus coeruleus (LC). Double immunocytochemical staining of tyrosine hydroxylase (TH) and c-Fos protein combined with stereological techniques were used to study the specific cell activation in the LC neurons in five experimental groups (control group, immobilization (1 h) group, 8-OH-DPAT group (8-OH-DPAT 0.3 mg/kg, s.c.), DPAT + IMMO group (8-OH-DPAT 0.3 mg/kg, s.c., 30' prior acute immobilization) and WAY + DPAT + IMMO group (WAY-100635 0.3 mg/kg, s.c. and 8-OH-DPAT 0.3 mg/kg, s.c., 45' and 30', respectively, before immobilization). The results showed that hippocampal NA tissue levels and c-Fos-IR in the TH positive neurons of the LC were significantly increased immediately and after 90', respectively, after the immobilization period. Pre-treatment with 8-OH-DPAT counteracted the effects induced by immobilization, but pre-treatment with WAY-100635 did not block the effects induced by 8-OH-DPAT. These results suggest that noradrenaline system is associated in a significant way with immobilization stress. The role of 5-HT<sub>1A</sub> receptor activation in this stress response is also discussed.

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**Keywords:** Immobilization stress; Locus coeruleus; Hippocampus; c-Fos immunohistochemistry; Noradrenaline; 5-HT<sub>1A</sub> receptor

Exposure to uncontrollable stressors may develop psychopathologic disorders such as panic, mood swings and anxiety. The hypothesis that the stress response courses with a mediated relationship between serotonergic and catecholaminergic central systems [4] focused attention on the interaction between the locus coeruleus nucleus (LC) and the dorsal raphe nucleus (DR) demonstrating a reciprocal control between both nuclei [25]. The serotonin released from the DR nucleus may control the firing activity of noradrenergic neurons of the LC via postsynaptic somatodendritic 5-HT<sub>1A</sub> receptor activation [25]. In keeping with this hypothesis, it has been demonstrated that the stimulation of 5-HT<sub>1A</sub> autoreceptors in the DR nucleus induces a decreased noradrenaline (NA) release in this nucleus from the

LC [18]. On the other hand, the LC also displays an important control on the activity of both dorsal and median raphe nuclei, with a NA release via stimulation of  $\alpha_1$ -somatodendritic adrenoceptors located in the hindbrain raphe nuclei, activating the serotonin release. In this way, electron microscopy [1] and retrograde tracing [22,23] have demonstrated a rich noradrenergic innervation of the DR nucleus arising from the LC. Thus, clonidine infusions in the brain leads to an important inhibition of the firing rate of the LC neurons with a subsequent decrease in extracellular NA levels in both LC and DR, being reduced the serotonin release by 80% in the former nucleus [18]. We have previously reported that 5-HT<sub>1A</sub> receptor activation counteracts the increased activation of serotonergic neurons in the dorsal and median raphe nuclei under acute stress of immobilization [19]. Furthermore, immobilization courses with increased serum corticosterone levels [20] that strongly increase the affinity of the 5-HT<sub>1A</sub> receptors in the DR [2] and also mediate in the induced long-term behavioural effects [8,9], including passive avoidance [20]. Dorsal ascending noradrenergic bundle [12]

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includes projections from the LC to the hippocampus. In addition, lesions with 6-OH-DA in the noradrenergic dorsal bundle in adrenalectomized rats with maintained infused corticosterone levels, induced an increase in the number of hippocampal type I corticosteroid receptors and a decreased affinity of type II corticosteroid receptors [10]. So that, the noradrenergic activation of the hindbrain raphe nuclei by the LC nucleus, which is negatively modulated by 5-HT<sub>1A</sub> receptor, is also coupled to a NA release into the hippocampus. This NA release could mediate the corticosterone induced long-term behavioural changes by modulating the expression and affinity of corticosterone receptors types under acute stress conditions stimulating also the hypothalamus-pituitary-axis (HPA) which leads to an increased serum corticosterone levels observed [20]. According to this, the aim of this study has been to evaluate the effects of acute immobilization on the activation pattern of LC noradrenergic neurons and NA levels in the hippocampus, as well as the role of the 5-HT<sub>1A</sub> receptor modulations in rats enduring acute immobilization stress.

SPF male Sprague–Dawley rats of 250–300 g (Charles River, Barcelona, Spain) were used. Rats were housed six per cage in a temperature-controlled room (22–24 °C) and maintained on a 12:12 h light:dark cycle (light at 7:00 am) in the central vivarium of the University of Malaga. Food and water were available ad libitum. The experiments were performed following the European Communities Council directive (86/609/EEC) for animal care and experimental procedure and the experiments were approved by the Ethical Committee for Animal Research of the University of Malaga. Animals were divided into five experimental groups ( $n=4$  for immunohistochemistry and  $n=6$  for HPLC): (1) SALINE, control group (without acute immobilization but with saline injection); (2) IMMO, group submitted to acute immobilization (1 h of immobilization in Plexiglas tubes (5.5 cm × 21 cm) 30 min after saline injection); (3) D + I, group receiving immobilization plus 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) pre-treatment 30 min before acute immobilization; (4) 8-OH-DPAT, group pre-treated with 8-OH-DPAT without acute immobilization; (5) W + D + I, group enduring acute immobilization with *N*-tetra-butyl-3-(4-(2-methoxyphenyl)piperazine-1-yl)-2-phenyl-propionamide (WAY-100635) 15 min prior 8-OH-DPAT plus immobilization treatment. The drugs were provided by Sigma (Sigma, Spain) and were made up in 0.9% saline and administered subcutaneously with a final concentration of 0.3 mg/kg because it induces neuroendocrine and behavioural effects [13]. After the drug administration and waiting periods, the rats were immediately anaesthetised with sodium pentobarbital (Mebumal; 100 mg/kg body weight, i.p.) before being intracardially perfused with 200 ml isotonic ice-cold saline followed by 200 ml of fixation fluid (4 °C) during 8 min for immunocytochemical staining. The fixative consisted of 4% paraformaldehyde (w/v) in saline 0.1 M sodium PB (PBS) (pH 7.4). The brains were removed, postfixed for 2 h in the same fixative and cryoprotected in sucrose (10% for 24 h followed by 30% for 1 week, at 4 °C). The brainstem was cut on a cryostat (Micron, HM 500M, Walldorf, Germany) in 30 μm coronal sections sampled every eight sections with a random start. A double immunocytochemistry for c-Fos plus tyrosine

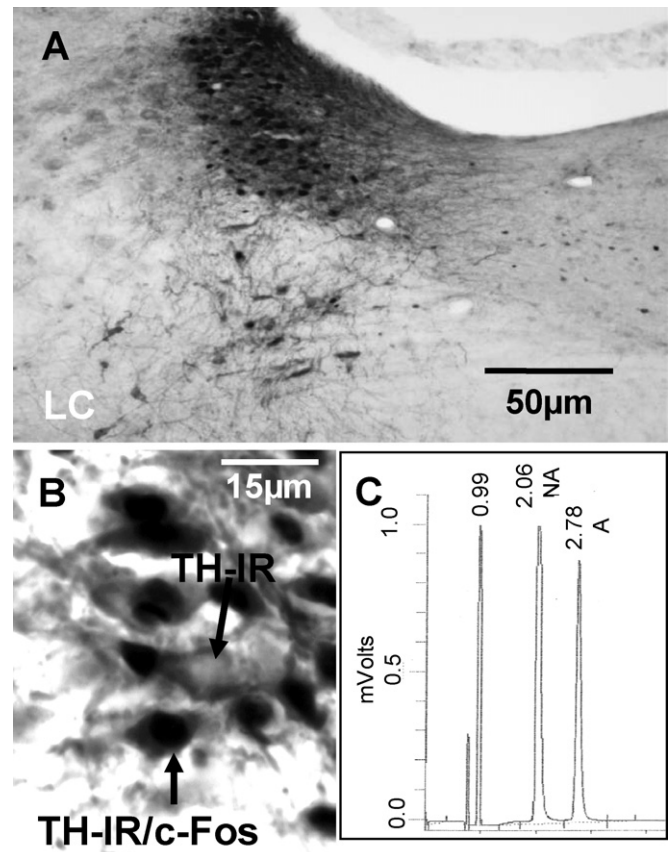


Fig. 1. (A) Unilateral micrograph of the LC; (B) high resolution micrograph of immunostained neurons in the LC. Arrows correspond to c-Fos-IR noradrenergic cell bodies (TH-IR/c-Fos) or tyrosine hydroxylase immunoreactive cell bodies (TH-IR); (C) chromatogram of standards for HPLC-ED analysis of monoamines.

hydroxylase (TH) was performed to quantify the number of activated catecholaminergic neurons per volume unit. A rabbit polyclonal antibody raised against the c-Fos protein (Sc52, Santacruz, USA) at a final concentration of 1:5000 was used following a methodology described in a previous paper [19]. 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Spain) (0.03%, w/v) was used as chromogen intensified with nickel chloride hexahydrate (Sigma, Spain) (0.04%, w/v) giving a black nuclei staining (see Fig. 1B). The TH immunocytochemistry was performed following the same protocol but using a monoclonal antibody raised against TH (Sigma, Spain) at a final concentration of 1:10000. Nickel chloride was not added to the chromogen solution in the second incubation for immunostaining in order to get a brownish reaction. Controls for the specificity of the used primary antibodies were tested following the methodology performed in our previous studies [14]. Sections from every animal from each experimental group were processed simultaneously. After mounting the sections on gelatin-chromalum coated slides, the sections were dehydrated and coverslipped with DPX (Panreac, Barcelona, Spain). Thus, every section was numbered according to the rostrocaudal level determined with the Patxinos and Watson [15] rat brain atlas. c-Fos immunoreactivity (c-Fos-IR) in noradrenergic cell bodies (dark nucleus and brownish cytoplasm double immunostaining) and total c-

Fos-IR cell nuclei were counted bilaterally in the LC using the CAST-Grid software package (Olympus, Glostrup, Denmark). The quantification was performed by systematically sampling in the region (see Fig. 1A) using the stereological methodology [19,27]. The resulting densities were averaged in order to obtain the mean of c-Fos-IR noradrenergic cell bodies per volume (Nv). Differences between cell densities were evaluated using the SPSS software (SPSS Inc., 11.5.1 version) package by the Kruskal–Wallis test for the whole groups and Mann–Whitney test for differences between group pairs. NA levels of fresh samples from hippocampus for equal experimental groups as in the immunocytochemical study were also quantified ( $n=6$ , per group). The animals were immediately decapitated after the immobilization period or after 1 h of waiting period in the control group for ice-cold extraction of fresh hippocampus. Trunk blood was collected in 5 ml plastic tubes. NA levels were determined by reversed phase isocratic high performance liquid chromatography with electrochemical detection (HPLC-ED) using a mobile phase, flow and detection conditions as previously described in literature for monoamines analysis [21]. For this purpose, hippocampus were dissected out on ice-cooled iron racks, then weighed and diluted in cold 0.05 N perchloric acid before being homogenized by sonication, and finally centrifuged, filtered and stored at  $-80^{\circ}\text{C}$  until analysis. A volume of  $20\ \mu\text{l}$  of the extract was injected, being the retention time for NA 2.06 min (see Fig. 1C). One-way ANOVA test for mean NA levels comparison followed Fisher's least significant difference was performed using also the SPSS software package.

Kruskal–Wallis test for the comparison of means for density values of c-Fos-IR noradrenergic cell bodies in the locus coeruleus showed significant differences between groups ( $P=0.05$ ). As seen in Fig. 2, acute immobilization induced a significant five-fold increase in the density of c-Fos-IR noradren-

ergic cell bodies [ $133 \pm 54$  (Mean  $\pm$  S.D.) double positives per  $10^3\ \mu\text{m}^3$ ] in comparison with the density of c-Fos-IR noradrenergic neurons in the SALINE group ( $23 \pm 4$  double positives per  $10^3\ \mu\text{m}^3$ ;  $P<0.05$ ). Although, the group treated with the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT alone showed a clear tendency to increase the density of c-Fos-IR noradrenergic cell bodies, it was not a statistically significant effect ( $69 \pm 29$  double positives per  $10^3\ \mu\text{m}^3$ ;  $P>0.05$ ), but pre-treatment with this 5-HT<sub>1A</sub> agonist before immobilization (D + I group) clearly counteracted the increased density of c-Fos-IR noradrenergic neurons observed in IMMO group ( $P<0.05$ ), yielding a mean density value ( $43 \pm 11$  double positives per  $10^3\ \mu\text{m}^3$ ) not statistically different to the mean obtained in the saline group ( $P>0.05$ ). In addition, pre-treatment with the specific 5-HT<sub>1A</sub> antagonist WAY-100635 (W + D + I group) did not counteract the effect of the pre-treatment with 8-OH-DPAT in immobilized animals ( $48 \pm 19$  double positive scores per  $10^3\ \mu\text{m}^3$ ;  $P>0.05$ ). In the case of NA tissue levels in the hippocampus, one-way ANOVA test followed by Fisher's least significant difference post hoc-test also showed statistically significant differences ( $P<0.05$ ). As seen in Fig. 3, bar-graph for NA tissue levels in hippocampus showed a similar pattern than the density of c-Fos immunoreactivity in TH positive neurons of the LC. IMMO group showed a statistically significant two-fold increase [ $223.7 \pm 26.6$  pg/mg of fresh tissue (f.t.)] compared to SALINE group ( $114.3 \pm 12.0$  pg/mg f.t.;  $P<0.05$ ), while treatment with 8-OH-DPAT alone did not show significant differences ( $118.8 \pm 18.8$  pg/mg f.t.;  $P>0.05$ ) compared to the SALINE group. The group of animals pre-treated with 8-OH-DPAT before acute immobilization (D + I) also showed similar tissue levels of NA to the control group ( $148.8 \pm 28.6$  pg/mg f.t.) and were significantly different from mean NA tissue levels in IMMO group ( $P<0.05$ ), so the pre-treatment with 8-OH-DPAT before immobilization counteracted the increased of NA tissue levels induced by immobilization

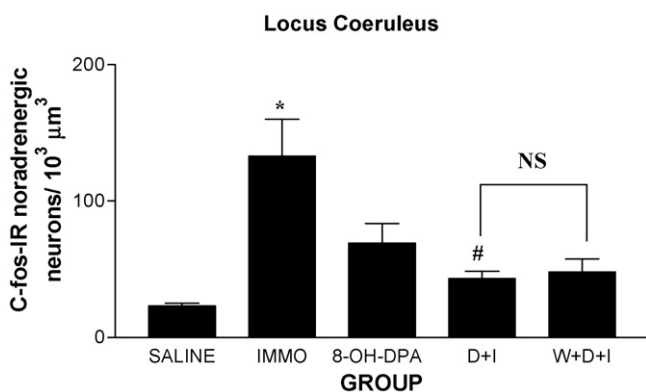


Fig. 2. Density of c-Fos-IR noradrenergic neurons in the locus coeruleus expressed as neurons per  $10^3\ \mu\text{m}^3$ . Data are shown as mean  $\pm$  S.D.; \* $P<0.05$  compared to SALINE group; # $P<0.05$  compared to IMMO group; NS, differences statistically not different (one-way ANOVA test for mean NA levels comparison followed Fisher's least significant difference); SALINE, control group (without acute IMMO but with saline injection); IMMO, group submitted to acute IMMO (1 h of immobilization after saline injection); D + I, group receiving IMMO plus 8-OH-DPAT pre-treatment 30 min before acute IMMO; 8-OH-DPAT, group pre-treated with 8-OH-DPAT without acute IMMO; W + D + I, group enduring acute IMMO with WAY-100635 15 min prior 8-OH-DPAT plus IMMO treatment.

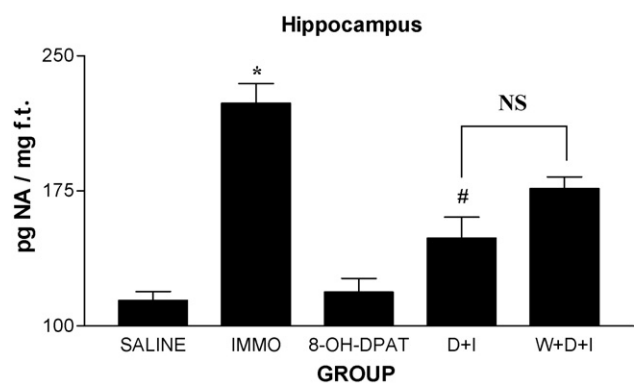


Fig. 3. Noradrenaline concentration in the hippocampus nucleus expressed as pg of noradrenaline per mg of fresh tissue (f.t.). Data are mean  $\pm$  S.D.; \* $P<0.05$  compared to SALINE group; # $P<0.05$  compared to IMMO group; NS, differences statistically not different (one-way ANOVA test for mean NA levels comparison followed Fisher's least significant difference); SALINE, control group (without acute IMMO but with saline injection); IMMO, group submitted to acute IMMO (1 h of immobilization after saline injection); D + I, group receiving IMMO plus 8-OH-DPAT pre-treatment 30 min before acute IMMO; 8-OH-DPAT, group pre-treated with 8-OH-DPAT without acute IMMO; W + D + I, group enduring acute IMMO with WAY-100635 15 min prior 8-OH-DPAT plus IMMO treatment.

stress. Finally, the W + D + I group showed NA tissue levels ( $176.3 \pm 16.0$  pg/mg f.t.) statistically not different from de mean NA tissue levels of D + I group ( $P > 0.05$ ).

Since cytoarchitectonic and immunocytochemical evidence has demonstrated that although NA is not the only neurotransmitter synthesized in the neurons of the LC nucleus, it is the only chatecolamine [7]. According to this, the TH-IR neurons, in our study, are noradrenergic ones. The increased c-Fos-IR in IMMO group (see Fig. 2), compared to control animals (SALINE group), in the noradrenergic neurons demonstrate that 1 h of acute immobilization stress activates noradrenergic neurons in the LC. This result is in close agreement with further results previously described in literature that point to the LC as a critical location related to the stress response. In this way, it has been reported that single and repeated immobilization [6] and single and repeated social stress [11] enhances the c-Fos-IR in the rat LC. HPLC-ED quantification of hippocampal NA tissue levels in this IMMO group shows that this induced activation by acute immobilization is followed by a NA release into the hippocampus (see Fig. 3). We have previously reported [20] that acute immobilization also activates the HPA axis and thus, increasing serum corticosterone levels, so the parallel NA release into the hippocampus, that induces a change in the density and affinity of corticosteroid receptors, may function as a compensatory mechanism in the stress response. Because immobilization stress has been previously reported as an emotional stressor involving limbic areas in the forebrain [28] it is not surprising to find that the hippocampus is a target region in the NA release. The administration of the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT alone did not show a significant increase in the density of c-Fos-IR noradrenergic neurons but there was a tendency to increase. In keeping with our results, it has been previously described in electrophysiological studies [16,24] that this 5-HT<sub>1A</sub> agonist has no effect on the activity of the neurons of the LC. However, immunocytochemical studies [5] have demonstrated that the administration of 8-OH-DPAT increases c-Fos-IR in this nucleus as a dose dependent effect that was also mimicked with buspirone, a partial 5-HT<sub>1A</sub> receptor agonist. So, we see that the effect of 8-OH-DPAT administration on the activation of noradrenergic neurons of the LC remains unclear, but due to the fact that the 5-HT<sub>1A</sub> receptor is coupled to the inhibitory G protein, and the fact that 8-OH-DPAT is also a partial agonist of the inhibitory  $\alpha_2$ -adrenoceptors, it is more consistent to think that the administration of 8-OH-DPAT has no effect, at least, on noradrenergic neurons of the rat LC. The differences between our results and previous immunocytochemical studies [5] may be due to the fact that those authors have included in the counting of c-Fos-IR all types of neurons of the LC where GABAergic and peptidergic neurons also exist, while we have studied the activation pattern exclusively in noradrenergic neurons. According to NA contents in the 8-OH-DPAT group, no changes in NA tissue levels in hippocampus were detected when compared to SALINE group. The administration of 8-OH-DPAT alone did not show a significant effect on the activation pattern in the LC, but pre-treatment with the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT before acute immobilization counteracted the increased activation mediated by this source of stress. We have previously reported that 5-HT<sub>1A</sub> recep-

tor activation counteracts the increased activation induced by acute immobilization in serotonergic neurons of the hindbrain raphe nucleus [19], suggesting that this serotonergic receptor is involved in the feed-back mechanisms following a serotonin synthesis and/or release induced by acute stress. In the present study, pre-treatment with 8-OH-DPAT before acute immobilization also showed a reduction of the activity in noradrenergic neurons of the LC. This reduction of the excitatory noradrenaline outputs arising from the hindbrain raphe nuclei could function as a first step on modulating the response of the serotonergic system to acute stress. This is consistent with the NA contents in the hippocampus in the D + I group were significantly lower when compared to IMMO group. Pre-treatment with the specific 5-HT<sub>1A</sub> receptor antagonist WAY-100635 was carried out to evaluate if the counteracted c-Fos immunoreactivity, in immobilized animals pre-treated with 8-OH-DPAT, was specific for 5-HT<sub>1A</sub> receptor activation. However, the pre-treatment with WAY-100635 did not counteract the effect of the administration of 8-OH-DPAT before acute immobilization. Because WAY-100635 has more affinity in binding 5-HT<sub>1A</sub> receptors, most of them may be locked by this antagonist. 8-OH-DPAT, which also functions as partial agonist of  $\alpha_2$ -adrenoceptors [3], could bind these second inhibitory adrenoceptors, yielding a similar effect as the stimulation of the inhibitory 5-HT<sub>1A</sub> receptors and subsequently, similar NA tissue levels than D + I group found in the hippocampus. Since 8-OH-DPAT also shows moderate affinity for the 5-HT<sub>7</sub> serotonergic receptor, it could mediate in the activation of the noradrenergic neurons of the LC, but no evidences have been found for the presence of this receptor in the rat LC [26]. In case of 8-OH-DPAT could bind the 5-HT<sub>7</sub> receptor in the LC, because it is coupled to an activator G protein, we should see an increased activation of noradrenergic neurons in the LC compared to D + I group. But the observed effect is similar to inhibitory receptor activation. In this sense, the mediation of  $\alpha_2$ -adrenoceptors in this effect is more consistent. Another explanation which could explain why the W + D + I group shows a similar activation pattern in the LC and similar NA contents in the hippocampus when compared to the D + I group, there could be the existence of a high reservoir of 5-HT<sub>1A</sub> somatodendritic receptors in the LC, but previous results [17] reported a moderate density of 5-HT<sub>1A</sub> receptor in this nucleus. It is not new the clinical use of therapeutics based on 5-HT<sub>1A</sub> binding for the treatment of stress related psychopathologies. In this sense our data are in tight agreement with this clinical use of 5-HT<sub>1A</sub> agonists. Furthermore, NA released in hippocampus is related to the limbic effect of the neuropathological disorders induced by stress [10].

In conclusion, our data provide new immunocytochemical and neurochemical insights suggesting that the central noradrenergic system modulates several pathways related to acute immobilization stress. The serotonergic system is activated by the LC, and the 5-HT<sub>1A</sub> somatodendritic control of the noradrenergic neurons in the LC support the idea that the serotonin released by the raphe nuclei also modulates the activity of the noradrenergic central system via 5-HT<sub>1A</sub> receptor modulation. This increased activity induced by acute immobilization leads to a raised NA content in the hippocampus that may induce

behavioural consequences modulating the corticosterone effects in this limbic area.

## Acknowledgements

This work has been supported by the Spanish DGICYT (PM99-0159). We thank Mr. Paul Godden for English revision and Mr. Manuel F. Lopez-Aranda, PhD student from the University of Malaga for technical contribution.

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