

# Intravenously administered hypocretin-1 alters brain amino acid release: an *in vivo* microdialysis study in rats

Joshi John, Ming-Fung Wu, Tohru Kodama and Jerome M. Siegel

Neurobiology Research (151A3), Veterans Affairs Greater Los Angeles Health Care System, North Hills, CA 91343 and Department of Psychiatry and Brain Research Institute, University of California at Los Angeles, Los Angeles, CA 91020, USA

We have reported that intravenous administration of hypocretin (Hcrt or orexin) reverses the symptoms of narcolepsy in genetically narcoleptic dogs. We have also reported that the onset of symptoms in canine genetic narcolepsy is accompanied by degenerative changes in forebrain regions, particularly the septal nucleus and amygdala. In the present *in vivo* microdialysis study we have investigated the effect of intravenous administration of Hcrt-1 (orexin-A) to anaesthetized rats on glutamate and GABA release in the amygdala, a region with moderate Hcrt innervation, and in the cerebellar cortex, a region with sparse or no Hcrt innervation. We found that intravenous Hcrt administration caused a marked (> 60%) and sustained (> 50 min) increase in glutamate release within the amygdala, but no change in release in the cerebellar cortex. We did not detect a significant change in GABA release. When calcium-free artificial cerebrospinal fluid was used as the microdialysis perfusate, Hcrt-1 no longer produced an increase in glutamate release. Hcrt may act via the calcium-dependent regulation of glutamate release in certain nuclei of the central nervous system.

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**Corresponding author** J. M. Siegel: UCLA/Neurobiology Research (151A3), Sepulveda VAMC, 16111 Plummer Street, North Hills, CA 91343, USA. Email: jsiegel@ucla.edu

The somata of hypocretin (Hcrt, also called orexin) neurons are localized to the perifornical and lateral hypothalamic regions. These neurons project widely but unevenly throughout the brain (Peyron *et al.* 1998; Nambu *et al.* 1999; van den Pol, 1999; Fung *et al.* 2001). Hcrt neurons have also been described in the rat enteric nervous system (Kirchgeßner & Liu, 1999). Certain brain regions such as the cerebellar cortex are virtually devoid of Hcrt projections and receptors, whereas forebrain limbic regions including the amygdala and some brainstem nuclei are heavily innervated and have high levels of Hcrt receptor mRNA (Peyron *et al.* 1998; Trivedi *et al.* 1998; Nambu *et al.* 1999; Hervieu *et al.* 2001; Marcus *et al.* 2001). Human narcolepsy has been shown to be linked to a loss of 85–95% of Hcrt cells (Thannickal *et al.* 2000) and to undetectable Hcrt mRNA (Peyron *et al.* 2000). Narcoleptic dogs show degenerative changes in the hypothalamus, amygdala and other forebrain regions, with peak levels of axonal loss at the time of symptom onset (Siegel *et al.* 1999). We have reported recently that the amygdala contains a population of cataplexy-related neurons (Gulyani *et al.* 2002).

We have also reported that motor activation produces a marked increase in Hcrt release (Kiyashchenko *et al.* 2002; Wu *et al.* 2002). Therefore, in the current study we monitored the effects of Hcrt administration in anaesthetized rats to eliminate the possibility of motor mediation, and to prevent other effects that are inhibited by barbiturate anaesthesia (Matsukawa *et al.* 1993; Lim *et al.* 1997). Hcrt-1 crosses the blood–brain barrier by diffusion (Kastin & Akerstrom, 1999). We found that systemic administration of Hcrt-1 reverses sleepiness and disrupted night-time sleep, and reduces cataplexy in canine narcolepsy (John *et al.* 2000). It has been shown in *in vitro* studies that Hcrt application to hypothalamic slices causes changes in glutamate and GABA release (van den Pol *et al.* 1998). In the study reported here, we have administered Hcrt-1 intravenously while monitoring glutamate and GABA release with *in vivo* microdialysis and HPLC in the amygdala and cerebellum, regions of high and low Hcrt innervation and receptor concentration, respectively, to determine whether systemic administration of Hcrt produces changes in glutamate and GABA release in Hcrt-innervated regions. These data have been reported in abstract form (John *et al.* 2001).

## METHODS

This study was conducted on 15 adult male Sprague-Dawley rats (275–350 g) in accordance with National Research Council's 'Guide for the Care and Use of Laboratory Animals'. All animal use protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California, Los Angeles and the V.A. Greater Los Angeles Health Care System.

### Surgical procedures

Rats were anaesthetized with 50 mg kg<sup>-1</sup> (i.p.) pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, USA) and placed in a stereotaxic frame. Under aseptic conditions, 23 G stainless-steel guide cannulae were implanted above the amygdala (central nucleus; A 6.5, L 4.2, H 2) and cerebellum (preculminate fissure 4; A -1.3, L 1.5, H 6.7; Paxinos & Watson, 1986), 2 mm dorsal to their targets. A supplemental dose of Nembutal (30 mg kg<sup>-1</sup>) was given 3 h after the first dose. Body temperature was maintained using a water-circulating heating pad (Gaymar Industries, NY, USA). Microdialysis probes (Type A-I-01; Eicom, Kyoto, Japan) with 1 mm long membranes (cut-off MW 50 000) were inserted through the guide cannulae to the targets. The recovery rate of the microdialysis probes for amino acids was between 10 and 14%. Artificial cerebrospinal fluid (ACSF; Harvard Apparatus, MA, USA) was continuously perfused at 4  $\mu$ l min<sup>-1</sup> through the amygdalar and cerebellar probes starting 2 h prior to the experiment, using an infusion pump (EPS-64, Eicom). Hcrt-1 (orexin-A, Phoenix Pharmaceuticals, Mountain View, CA, USA) dissolved in normal saline was administered (3  $\mu$ g kg<sup>-1</sup> in 200  $\mu$ l) through the tail vein, using a glass syringe. As a control, saline was administered in the same manner. Hcrt-1 or control injections in randomized order were administered between 13.30 h and 14.30 h. Three rats were used to study the Ca<sup>2+</sup> dependency of the transmitter release by omitting Ca<sup>2+</sup> from the ACSF. In these studies, the amygdalar probe on one side was perfused with ACSF of the following composition (mM): 147 NaCl, 2.5 KCl, 0.9 MgCl<sub>2</sub> and 2.2 CaCl<sub>2</sub>. The probe on the other side was perfused with Ca<sup>2+</sup>-free ACSF (Reid *et al.* 2000). Dialysate was collected for 30 min before and until 90 min after the Hcrt-1/saline injection, at 10 min intervals using an automated and refrigerated fraction collector (Eicom). The collecting polyethylene vials were maintained at 5°C during collection and then stored at -80°C until analysis.

### Amino acid assay

The concentration of amino acid in the dialysate was detected by HPLC (EP-300, Eicom) with fluorescence detection (Soma S-3350: excitation/emission = 340/440 nm), as described in our prior studies (Nitz & Siegel, 1997; Kodama *et al.* 1998). The sample was injected using an autoinjector (ESA 540). Pre-column derivatization was performed with *o*-phthaldialdehyde and 2-mercaptoethanol at 5°C for 3 min. The derivatives were then separated in a liquid chromatography column (MA-5ODS, 2.1  $\times$  150 mm, Eicom) at 30°C with 30% methanol in 0.1 M phosphate buffer (pH 6.0), being degassed by an online degasser (DG-100, Eicom). Quantification was achieved with a PowerChrom analysis system (ADI Instruments, Australia) using external amino acid standards (Sigma, USA). Neurotransmitter concentrations were calculated by comparing the HPLC peak of glutamate and GABA in microdialysis samples with the peak area of known concentrations of the same compounds analysed on the same day. The detection limit for glutamate and GABA was 20 fmol. Release of glutamate and GABA was stable 2 h after the implantation of

the dialysis probe, as reported earlier (Herbison *et al.* 1990). Thus, the mean value of glutamate and GABA from three consecutive samples during the next 30 min was taken as the basal output. The values obtained subsequently are expressed as a percentage of this basal value. In some cases ( $n = 3$ ), Hcrt-1 injection was performed within 60 min of the start of Ca<sup>2+</sup>-free ACSF perfusion into the amygdala. Three samples were taken immediately prior to the Hcrt-1 injection and used as the baseline for further comparison with postinjection levels.

### Histology

Rats were deeply anaesthetized with pentobarbital sodium (60 mg kg<sup>-1</sup>) and transcardial perfusion was carried out with normal saline followed by 4% buffered paraformaldehyde. After treatment with 30% sucrose in 0.1 M phosphate-buffered saline, brains were cut at 40  $\mu$ m intervals using a cryostat. Cresyl violet staining was carried out to verify placement of dialysis probes.

### Data analysis

Changes in neurotransmitter release were analysed by one-way analysis of variance, followed by *post-hoc* comparisons using Fisher's least significant difference (LSD) test.

## RESULTS

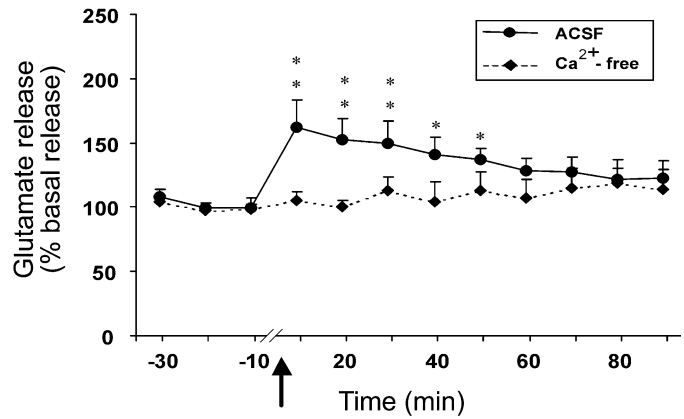
### Glutamate and GABA release in the amygdala

Intravenous administration of Hcrt-1 produced a significant change in the glutamate level in the central nucleus of the amygdala. The basal level of glutamate in the amygdala was 22.93  $\pm$  1.18 pmol per 20  $\mu$ l sample. A significant increase in glutamate level was apparent within 10 min of intravenous Hcrt-1 injection. The increase was maintained for more than 50 min ( $F_{9,99} = 2.35$ ,  $P < 0.02$ ;  $P < 0.05$ , Fisher's LSD; Fig. 1). In the amygdala perfused with Ca<sup>2+</sup>-free ACSF, a 21% decrease in basal glutamate release was seen (25.34  $\pm$  1.39 to 20.13  $\pm$  0.84 pmol). This tonic decrease in glutamate release was apparent starting 30 min after the start of Ca<sup>2+</sup>-free ACSF perfusion and was stable for 30 min before Hcrt-1 injection. The Ca<sup>2+</sup>-free ACSF perfusion abolished the Hcrt-1-induced increase in glutamate release in the amygdala. There was no increase in glutamate throughout the 90 min postinjection period ( $F_{11,22} = 1.30$ ,  $P < 0.28$ ; Fig. 1). However, the contralateral amygdala, which was perfused with normal ACSF, exhibited a significant increase in glutamate ( $F_{11,22} = 2.45$ ,  $P < 0.04$ ).

The basal level of GABA in the amygdala was 1.98  $\pm$  0.25 pmol per 20  $\mu$ l sample. The level of GABA exhibited a non-significant decrease at 40–60 min after Hcrt-1 injection in the amygdala perfused with Ca<sup>2+</sup>-containing ACSF ( $F_{9,99} = 0.68$ ,  $P < 0.71$ ; Fig. 2). Intravenous Hcrt-1 injection did not alter the level of amygdala GABA release when Ca<sup>2+</sup>-free perfusion was performed. The tonic level of GABA release decreased by 18% from the basal level (1.61  $\pm$  0.25 to 1.32  $\pm$  0.20 pmol) following Ca<sup>2+</sup>-free perfusion in this group, and remained stable for 30 min before Hcrt-1 injection. Intravenous administration of saline instead of Hcrt-1 did not change amino acid levels in the amygdala (Fig. 3).

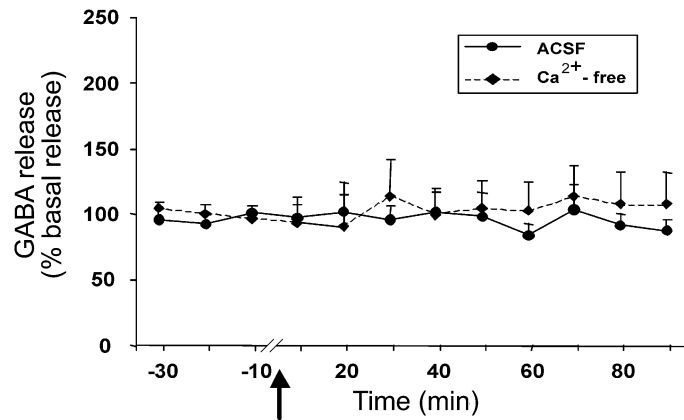
**Figure 1. Intravenous hypocretin (Hcrt)-1 administration elevates glutamate release, and this elevation is calcium dependent**

This graph shows the level of glutamate release in the amygdala from 30 min before to 90 min after the intravenous administration of Hcrt-1 ( $3 \mu\text{g kg}^{-1}$ ). The arrow indicates the point of injection. The continuous line shows data from the artificial cerebrospinal fluid (ACSF)-perfused site ( $n = 12$ ) and the broken line shows data from the  $\text{Ca}^{2+}$ -free ACSF-perfused site ( $n = 3$ ). Note that the  $\text{Ca}^{2+}$ -free ACSF perfusion completely blocked the effect of Hcrt-1. All values are means  $\pm$  S.E.M;  $**P < 0.01$ ,  $*P < 0.05$ , compared to the corresponding pretreatment level, Fisher's LSD test.



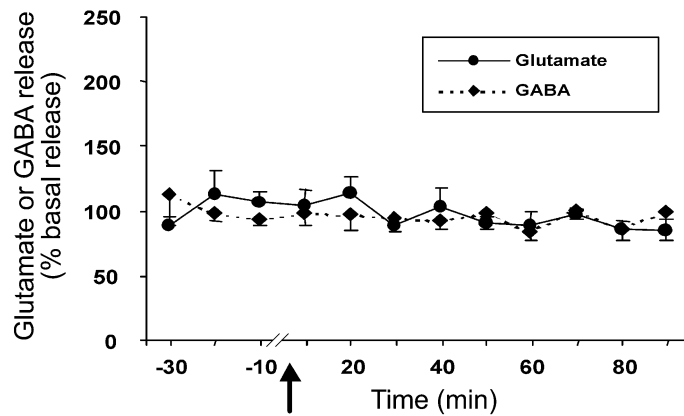
**Figure 2. Intravenous Hcrt-1 administration does not significantly alter GABA release**

GABA levels in the amygdala from 30 min before to 90 min after intravenous administration of Hcrt-1 ( $3 \mu\text{g kg}^{-1}$ ). The arrow indicates the point of injection, the continuous line shows data from the ACSF-perfused site ( $n = 12$ ) and the broken line shows data from the  $\text{Ca}^{2+}$ -free ACSF-perfused site ( $n = 3$ ). All values are means  $\pm$  S.E.M.



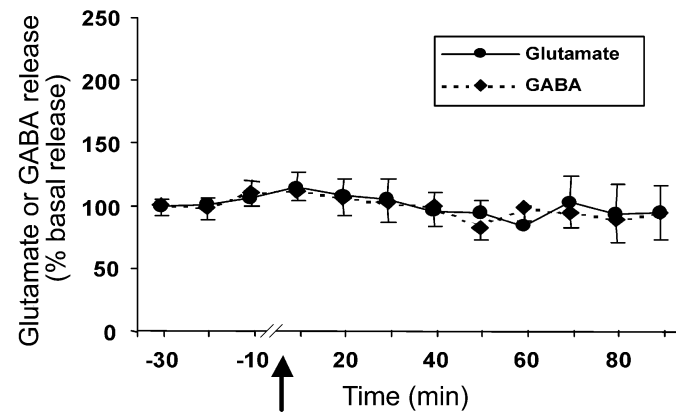
**Figure 3. Intravenous saline administration does not alter glutamate and GABA levels**

Glutamate and GABA levels in the amygdala from 30 min before to 90 min after intravenous administration of saline. The arrow indicates the point of injection, the continuous line shows glutamate levels ( $n = 9$ ) and the broken line shows GABA levels ( $n = 9$ ). All values are means  $\pm$  S.E.M.



**Figure 4. Intravenous administration of Hcrt-1 ( $3 \mu\text{g kg}^{-1}$ ) has no effect on glutamate and GABA levels in the cerebellar cortex, a region that lacks significant Hcrt innervation**

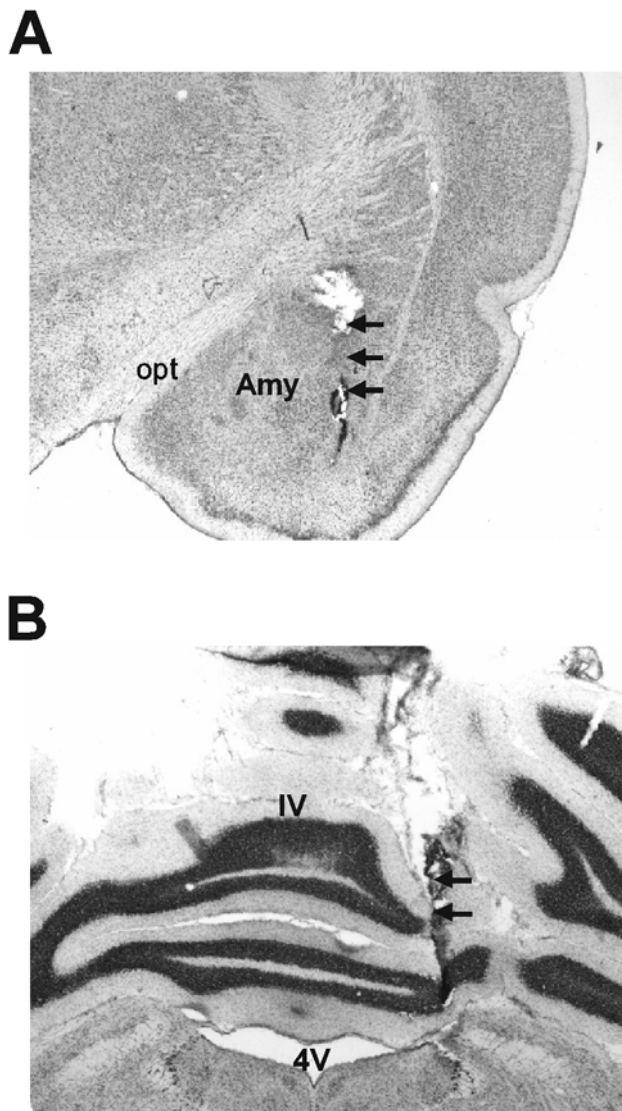
Glutamate and GABA levels in the cerebellar cortex from 30 min before to 90 min after intravenous administration of Hcrt-1 ( $3 \mu\text{g kg}^{-1}$ ). The arrow indicates the point of injection, the continuous line shows glutamate levels ( $n = 7$ ) and the broken line shows GABA levels ( $n = 5$ ). All values are means  $\pm$  S.E.M.



### Glutamate and GABA release in the cerebellum

The basal level of glutamate and GABA in the cerebellar cortex was  $5.71 \pm 0.93$  pmol per  $20 \mu\text{l}$  sample and  $1.53 \pm 0.48$  pmol per  $20 \mu\text{l}$  sample, respectively. There was no significant change in the glutamate ( $n = 7$ ) or GABA ( $n = 5$ ) level in the cerebellum after intravenous administration of Hcrt-1 (Fig. 4).

Cresyl violet staining of brain sections revealed that the microdialysis probes were located in the central nucleus of the amygdala and the cerebellar cortex (Fig. 5).



**Figure 5. Cresyl-violet-stained coronal brain section showing location of the microdialysis probes**

A, the amygdala. B, the cerebellar cortex. The locations of the microdialysis probes are indicated by arrows. Opt: optic tract; Amy: central nucleus of amygdala; 4V: 4th ventricle; IV: preculminate fissure 4.

### DISCUSSION

We found that intravenous Hcrt administration causes a marked and sustained increase in glutamate release in the amygdala. Because these studies were conducted in anaesthetized animals, the observed changes in release could not have been mediated by behavioural changes.  $\text{Ca}^{2+}$ -free ACSF perfusion abolished the Hcrt-1-induced glutamate release, indicating that these changes were dependent on vesicular release. Hcrt-1 has been shown to cross the blood–brain barrier (Kastin & Akerstrom, 1999). Therefore, the observed effect is probably mediated by Hcrt activation of Hcrt-1 and Hcrt-2 receptors in the amygdala (Trivedi *et al.* 1998; Marcus *et al.* 2001). The lack of Hcrt innervation (Peyron *et al.* 1998; Nambu *et al.* 1999) and equivocal evidence for Hcrt receptors in the cerebellar cortex (Trivedi *et al.* 1998; Hervieu *et al.* 2001; Marcus *et al.* 2001) is consistent with the lack of change in glutamate and GABA release in this structure.

In the *in vivo* situation, Hcrt could act by receptor-mediated presynaptic regulation of glutamate release near the collection site or through action at a distant site, affecting glutamate release in the amygdala through a multisynaptic pathway. The *in vitro* release of glutamate by Hcrt has been shown to be present even when axonal transmission was blocked (van den Pol *et al.* 1998), suggesting presynaptic regulation. The changes in GABA release that we saw were not significant, although an increase was reported in an *in vitro* study of hypothalamic tissue. It remains to be seen whether this difference is a function of the regions sampled, the dose of Hcrt to which the tissue was exposed, differences between the slice and *in vivo* preparations, anaesthesia or other factors. The distribution of Hcrt-1 receptor protein-like immunoreactivity is very dense in the hypothalamus compared to the amygdala (Hervieu *et al.* 2001) and this could also affect the GABA response to Hcrt administration.

It is possible that an indirect effect through peripheral pathways might trigger glutamate release, although barbiturate anaesthesia has an inhibitory effect on many peripheral pathways (Matsukawa *et al.* 1993; Lim *et al.* 1997). Evidence for peripheral Hcrt release and receptors has been contradictory (Gautvik *et al.* 1996; Sakurai *et al.* 1998; Kirchgessner & Liu, 1999). The presence of  $\text{Ca}^{2+}$ -dependent glutamate release in the amygdala, with its high levels of Hcrt receptors, and the absence of release in the cerebellar cortex, a region relatively devoid of Hcrt receptors, can be most parsimoniously explained by the hypothesis that systemically administered Hcrt may be acting directly on Hcrt receptors in the amygdala. This is consistent with a recent study showing increased levels of glutamate in the locus coeruleus after Hcrt administration (Kodama & Kimura, 2002). Recent work by our group has demonstrated that the Hcrt effect on trigeminal motoneurons is mediated by the local release of glutamate (Peever *et al.* 2003).

It has been found that most human narcolepsy is due to a loss of Hcrt neurons (Thannickal *et al.* 2000) or of the mRNA of the Hcrt peptide (Peyron *et al.* 2000). Narcolepsy is characterized by overwhelming sleepiness and sudden losses of muscle tone, known as cataplexy. We have reported previously that systemic administration of Hcrt produces motor activation and reverses the symptoms of narcolepsy in genetically narcoleptic dogs (John *et al.* 2000). We have hypothesized that these effects are mediated by Hcrt-1 receptors, which are functional in these Hcrt-2-receptor-mutant animals. In a recent relevant abstract (Fujiki *et al.* 2001), there was no report of any effect of systemic Hcrt administration on cataplexy, although the other indicators of narcoleptic symptomatology monitored by John *et al.* (2000), including waking and sleep amounts and continuity, were not measured. Other studies of the effects of systemically administered Hcrt on centrally mediated responses have reported both positive (Dyer *et al.* 1999; Mitsuma *et al.* 1999; Kodama & Kimura, 2002) and negative results (Takahashi *et al.* 1999; Chen *et al.* 2000). The causes of these differences remain to be identified, but it is likely that the dose, time course and method of assessment of effects are critical. The present results suggest that amino acid release is an important mediator of the effects of systemically administered Hcrt.

Prior work has shown that application of Hcrt to Hcrt-1-receptor-rich regions in the brainstem and intracerebroventricularly increases motor activity and alters muscle tone (Hagan *et al.* 1999; Kiyashchenko *et al.* 2001; Peever *et al.* 2003). Hcrt release is maximal during periods of motor activation (Kiyashchenko *et al.* 2002; Wu *et al.* 2002). Regulation of amino acid release may play a major part in these and other actions of Hcrt in the central nervous system.

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