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Morphological characterization of six subpopulations of adult human DRG neurons at the light microscopic level

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Dorsal root ganglion (DRG) neurons are composed of physiologically distinct subpopulations, each responding to a different sensory stimulus. One can morphologically discriminate between two broad populations of adult rat and frog DRG neurons by their appearance under the light microscope. These groups are called large clear and small dark. However, additional subpopulations have not been identified by visual observation. Such identification requires application of immunochemistry or biophysical techniques. Although these are useful techniques, they do not allow the rapid discrimination of different neuron subpopulations, which would be useful for pharmacological studies on unique neuron subpopulations. Such experiments would be greatly facilitated if viable DRG neuron subpopulations could be identified based on their morphology at the light microscopic level. Just as for adult frog and rat DRG neurons, when adult human DRG neurons are observed under phase optics, two subpopulations can be seen, small dark and large light. However, under bright-field illumination, six distinct subpopulations can be distinguished based solely on morphological features. Five subpopulations contain rusty-colored cytoplasmic inclusions with different sized granules and differences in the size and density of the granule clusters, while one is granule-free. Analysis of the soma diameter distribution shows each of the six granule-containing and the non-granule-containing (clear) neuron subpopulations has a statistically significant difference in size distribution. We propose that neurons with different morphologies correspond to unique physiological subpopulations of DRG neurons. Experiments are underway using immunochemical techniques to determine whether neurons with the unique morphologies correspond with unique physiological functions.

Key words: Sensory neurons, DRG subpopulations, Human neurons, Cytoplasmic granules

Dorsal root ganglion (DRG) neurons are a heterogeneous population of sensory neurons composed of subpopulations responding to different modalities of stimuli, having different receptors, containing various neuropeptides, and responding to different pharmacological agents. Studies of the different subpopulations of DRG neurons are essential for understanding their receptor properties, and the pharmacological agents that enhance or block their receptors.

Generally, studies on dissociated adult rat and adult frog DRG neurons in vitro are performed by randomly selecting a neuron to study (5,7,8,12,20,21). Then one analyzes the results only from neurons that respond to the specific stimuli of interest, such as heat, pH, transmitters, and various drugs and their antagonists, and characterize the properties of their specific ion channels (2,5,11-14,17,19,21). Although this approach is fruitful, the yield of useful data from primary sensory neurons could be significantly increased if there were an easy and reliable means for identifying the different DRG neuron subpopulations under light microscope based solely on the morphology of the neuron subpopulations.

Under phase illumination, adult frog and rat DRG neurons can be divided into 2 broad-based populations with different biophysical properties based upon their appearance: "small dark" and "large light" neurons (6,14,15, 18,22). Combined fluorescence and bright-field illumination show the "small dark" neurons are neurofilament-negative, while the "large light" neurons are neurofilament-positive (22).

Thus, one can reduce the randomness in selecting the neurons for study by choosing between these 2 subpopulations.

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Another approach for increasing the success of recording from a particular neuron subpopulation of DRG neurons is to select neurons based on the soma diameter (15), membrane capacitance (7), or combination of cell diameter and membrane capacitance (4). Although the dark and light classifications of DRG neurons have been used for many years, there has been no systematic analysis to determine whether further discrimination can be made within these subsets.

Alternative approaches for identifying specific DRG neuron subpopulations involve the use of histochemical stains, such as cobalt uptake, which is specific for capsaicin-sensitive neurons (22,23), or back-labeling neurons with dye via their axons (3). One can also apply a specific antibody, or compounds, such as the isolectin IB4, that bind selectively to specific DRG neuron subpopulations (1,8,9). Other combinations of techniques have been used to discriminate between DRG neuron subpopulations (10,24). However, most of these techniques require fixing the neurons, which precludes subsequent physiological studies.

In one series of experiments, we studied dissociated adult frog DRG neurons using the whole cell patch-clamp technique and the recordings were annotated as from large clear vs. dark neurons (14). The membrane properties of the large clear neurons were relatively consistent from neuron to neuron. However, the membrane properties of the dark neurons showed significant variability (14). These results suggested that the clear neurons were composed of possibly only one neuron subpopulation, whereas the dark neurons were composed of more than one neuron subpopulation. This raised the question of whether there might be a simple, but more comprehensive, method for discriminating between the different DRG neuron subpopulations.

As indicated, under phase optics one sees only two subpopulations of dissociated adult frog and adult rat DRG neurons, large clear and small dark. Under bright-field illumination these differences are seen to result from the presence of cytoplasmic granules in the dark neurons, and the absence of granules in the clear neurons. For the adult rat DRG neurons the pale granules do not allow discrimination of more than two subpopulations of DRG neurons. However, for adult frog DRG neurons, bright-filed illumination under, even under low power of a dissecting microscope, six neuron subpopulations can be recognized based upon their morphology (Castro & Kuffler, in press). Four subpopulations contain bright rusty-colored cytoplasmic granules, which appear in clusters of different densities, with each neuron type having a unique distribution of soma diameter. Two subpopulations are granule-free, or clear, but have unique distributions of soma diameter (Castro & Kuffler, in press). These observations led us to examine dissociated adult human DRG neurons to determine whether it was also possible to discriminate morphologically between different subpopulations of the neurons. Experiments similar to those performed on adult frog DRG neurons were carried out on dissociated adult human DRG neurons. The present results show that 6 subpopulations of adult human DRG neurons can be identified based on neuron morphology and size.

Experimental Procedures. DRG were isolated from adult organ donors, following appropriate written consent from the families. The DRG were removed within 1 hour of cross clamping the aorta. The time between aortic cross clamp and DRG were removal was dictated by the time required for the transplant surgeons to remove the required organs. As soon as they were finished, an en bloc vertebralectomy was performed and the first 3 pairs of thoracic DRG were removed together with their surrounding tissue and with the DRG connective capsule tissue in tact.

As each DRG was removed it was placed on a sterile cloth at room temperature (20°C). Once the six DRG had been removed they each was placed a 50 ml sterile plastic tube containing tissue culture medium (DMEM + garamycin) (Sigma Chemical) at pH 7.6 and at 4°C and the tubes were placed on ice. The DRG were transported to the laboratory. Working at 20°C, the tissue and connective capsule surrounding the DRG were cut away using irredectomy scissors.

About 2 hours following the DRG removal the DRG were dissociated as described previously (16). Briefly, the DRG were then cut into small pieces using irredectomy scissors and placed in a siliconized glass dish (Sigmacoat, Sigma Chemical) containing collagenase P (3 mg/ml, Boehringer-Mannheim), neutral dispase II (8 mg/ml, Boehringer-Mannheim), and DNase (0.3 mg/ml, Boehringer-Manheim), in DMEM (Sigma chemical) tissue culture medium at pH 7.2 containing garamycin (10 mg/l). The dish was placed in a CO_2 incubator at 37°C in water saturated environment, and triturated every 15 minutes until completely dissociated (ca. 1 hour).

The dissociated neurons were picked up in a siliconized micropipette, with a fire-polished tip with an opening of about 100 im, and plated onto a glass cover slip that had been treated with laminin followed by poly-l-lysine and then laminin (20 mg/ml) (1-hour incubation each). Healthy neurons adhere to the cover slip immediately upon contact, while the damaged neurons do not adhere. After 15 minutes to allow good neuron adhesion, fetal bovine serum (FBS) (Sigma Chemical) (final concentration 10%) was added to the neurons. Addition of FBS prior to plating the neurons inhibits their adhesion. The cultures were placed in a 5% CO_2

incubator at 37°C and with a water saturated environment.

After 2 days the neurons were fixed in 4% glutaraldehyde, the cover slip with the neurons mounted on a slide, the neurons examined under bright field illumination on a Zeiss Axiovert 100 microscope and photographed with a Hamamatsu 3 color camera and using Universal Imaging Corp. Metamorph software.

Results

Subpopulations of adult human DRG neurons. Under phase illumination two subpopulations of adult human DRG neurons are observed, clear and dark. However, under bright field illumination seven subpopulations of neurons can be identified based solely on the distinct morphologies of the subpopulations. The rusty-colored cytoplasmic granules are clearly visible and allow easy discrimination between adjacent neurons (Figure1).

Five of the 6 subpopulations contain rusty colored cytoplasmic granules, while one subpopulation is granule-free, or clear. The granules in the 5 granule-containing subpopulations differ in the number and distribution of the granules. Based on the granule morphology the dark neurons were divided into subpopulations called: (1) sparse, (2) small loose cluster, (3) large loose cluster, (4) chunky, (5) dense cluster, and (6) clear.

Size distribution of adult human DRG neurons. We analyzed the neurons to determine whether the neurons with different morphologies belong to distinct subpopulations or unique diameter distributions, or whether they are in neurons with a continuity of diameter sizes. The diameter of each neuron of each morphological subpopulation was measured. These results are plotted as the number of neurons with each type of morphology against the neuron diameter (Figure 2).

Adult human DRG neurons have a size distribution of from 11 to 118 ìm. The mean diameters of each subpopulation and its percentage of the total neuron population are: sparse, 33.7 ìm / 12.1%, small loose cluster, 43.3. ìm / 11.3%; chunky, 53.7 ìm / 6.9%, large loose cluster, 57.1 ìm / 33.5%, dense cluster 72.6 ìm / 33.5%, and clear 73.6 ìm / 7.4%.

Analysis of the size distribution of the 6 populations by the Kolmogorov-Smirnov Normality Test shows they do not have a normal size distribution ($P < 0.0001$). Comparison of neurons with different types of cytoplasmic morphologies shows 6 distinct groups, with only minimal overlap of the largest and smallest neurons in these groups. Two populations, the clear and the dense cluster, have no statistical difference in their size distribution ($P = 0.866$). However, because one has granules and the other does not, these are clearly different neuron subpopulations. The Mann-Whitney Rank Sum Test showed that the

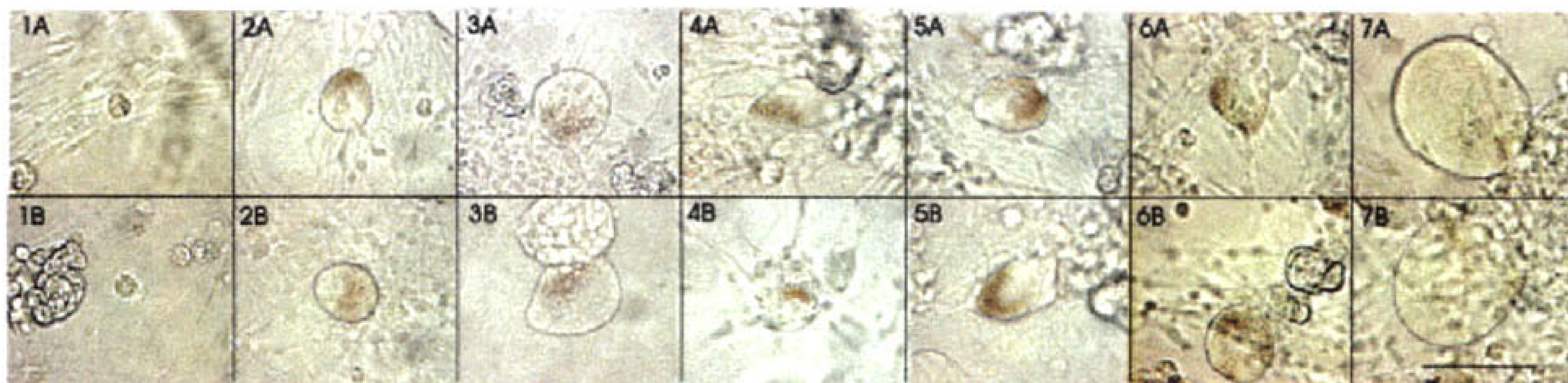

Figure 1. Bright field images of dissociated adult human DRG neurons (2 examples of each of the 6 subpopulations of neurons) identified by the morphology of their cytoplasmic granules, or their absence of granules. The neurons are composed of six populations of dark (granule-containing) neurons, called sparse, small loose cluster, large loose cluster, chunky, dense cluster, dense plus loose cluster and 1 population of clear (non-granule-containing) neurons, called clear neurons. Calibration bar: 70 µm.

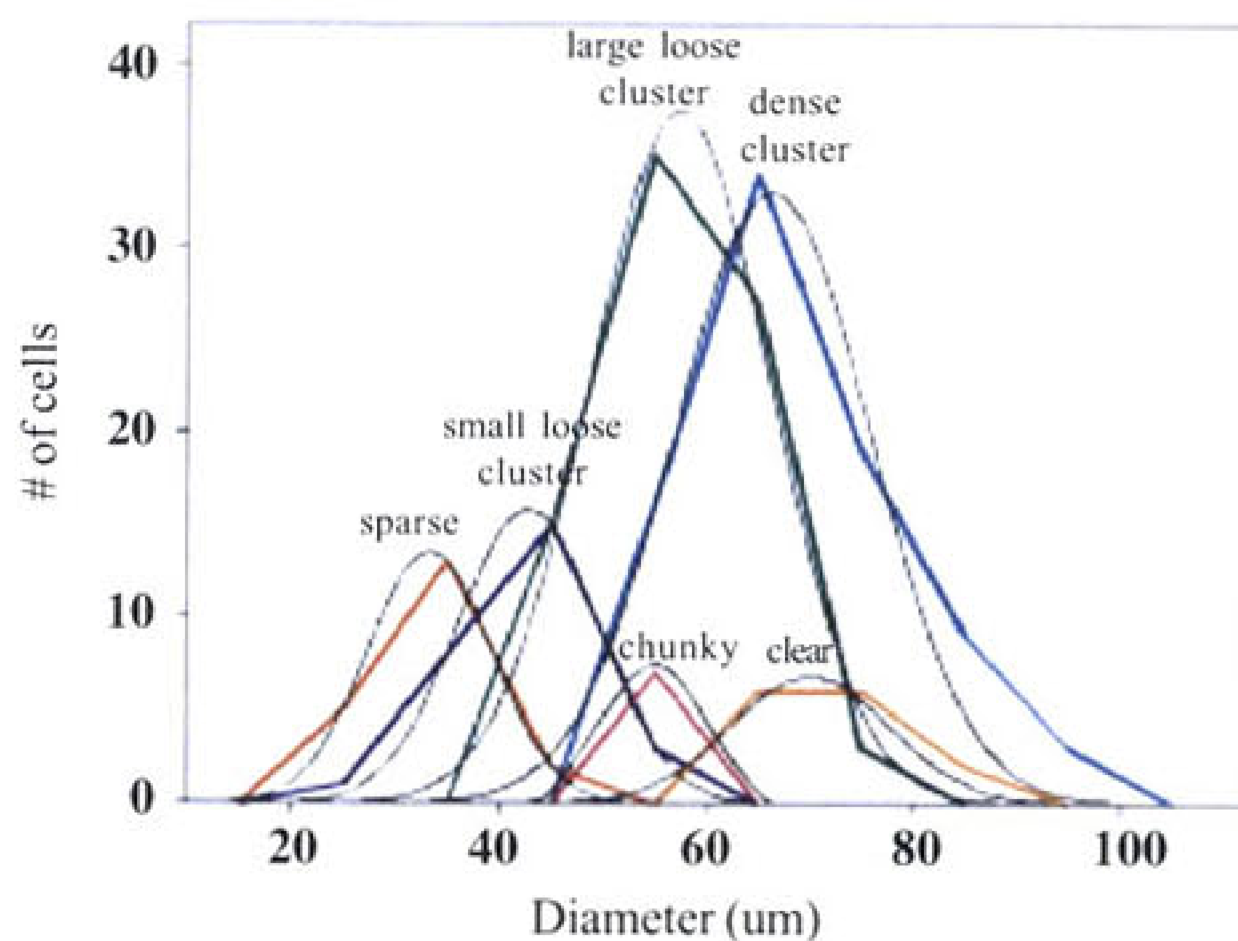

Figure 2. Plot of the distribution of subpopulations of adult human DRG neurons based on their different types of cytoplasmic inclusions vs. neuron diameters (plotted in 10 ìm bins). The curved lines show the data for each subpopulation plotted with a Gaussian curve.

neurons with large loose and with dense cluster cytoplasmic inclusions and apparently overlapping subpopulations are in fact statistically different sized populations (P = <0.001); the small loose and chunky cluster neuron populations are statistically different populations (P = 0.004); and the sparse and small loose cluster granule-containing neurons are separate subpopulation (P = 0.001). However, because the neuron populations do not have a normal size distribution it is not possible to determine whether these larger neurons constitute a separate population, or are part of the non-normal size distribution.

Cytoplasmic inclusions are not artifacts of DRG treatment. It was important to determine whether the unique morphologies of the cytoplasmic inclusion in each neuron subpopulation are physiological or an artifact of DRG isolation, DRG dissociation, or time in vitro. If the different morphologies are physiological then each should be present when the DRG are removed from the organ donor. One DRG was placed in fixative (4% glutaraldehyde) immediately after being removed from the donor. The DRG was then cut into small pieces and examined under bright field illumination. All 6 distinct of cytoplasmic granule morphologies were present, as was the single population of clear neurons.

To determine whether the morphologies changed over time, dissociated neurons were left for 5 days in vitro and then examined. All 6 subpopulations of DRG neurons were still present. These results indicate that the different types of cytoplasmic granule inclusions are normal characteristics of the different subpopulations of adult human DRG neurons and are not artifacts.

Discussion

The present study was designed to determine whether, under bright field illumination, distinct subpopulations of dissociated adult human DRG neurons could be identified based on neuron morphology and diameter. When viewed under dark field optics dissociated adult frog, rat and human DRG are seen to be composed two subpopulations, small dark and large clear. Under bright field illumination, the difference between dark and clear neurons can be seen to result from cytoplasmic granules in the dark neurons, which are absent from the clear neurons.

The granules in adult the rat DRG neurons are faint, difficult to see, and do not allow the DRG neurons to be classified into different subpopulations. However, as for adult frog DRG neurons, adult human DRG neurons contain large, rusty-colored granules that are clearly visible under the dissecting microscope. The morphological differences in the cytoplasmic granules in the human and frog DRG neurons allow for the discrimination of 6 and distinct subpopulations respectively. In addition human and frog DRG neurons contain 1 and 2 subpopulations of granule-free (clear) neurons respectively.

In earlier work using the whole-cell patch-clamp technique on isolated adult frog DRG neurons, we studied the membrane properties of the dark and clear neurons subpopulations. Clear differences and similarities were recorded from the two neuron populations (14). Recordings from the dark neurons yielded widely different properties, suggesting that the dark neurons are composed of a number of different neuron subpopulations. However, time did not allow us to discriminate between the neurons with different membrane properties and the respective morphologies of their cytoplasmic granules. Recordings from the clear neurons showed fewer differences in their membrane properties, suggesting that there were composed of only one or two subpopulations.

Analysis of the size distribution of the granule-free adult human DRG neurons indicated they were composed of one distinct size subpopulation. Analysis of the diameters of the granule-containing neurons with different morphologies indicated that they had statistically different size distributions. It has not yet been possible to determine whether the different subpopulations have different biophysical or pharmacological properties.

In conclusion, these data show that neurons with different morphologies belong to distinct DRG subpopulations. We propose that these morphologically distinct subpopulations correspond to physiologically distinct subpopulations that respond to different stimuli. This will be determined by testing whether the morphologically distinct DRG neuron subpopulations possess unique receptors and neuropeptides combinations that correspond to physiologically characterized adult human DRG neurons.

Resumen

Neuronas del ganglio dorsal (DRG) están compuestas por subpoblaciones fisiologicamente distintivas, respondiendo cada cual a diferentes estimulos sensoriales. Se puede discriminar morfologicamente entre dos amplias poblaciones de neuronas del DRG en ratas y ranas adultas por su apariencia bajo un microscopio de luz. Estos grupos son llamados grande claro y pequeño oscuro. Sin embargo, subpoblaciones adicionales no han sido identificadas por observación visual. Tales identificaciones requieren aplicaciones de inmunoquímica o técnicas biofisicas. Aunque estas son técnicas útiles, no permiten la

discriminación rápida de diferentes subpoblaciones de neuronas, las que serian útiles para estudios farmacológicos en subpoblaciones de neuronas únicas. Estos experimentos serían facilitados si subpoblaciones de neuronas del DRG viables pudieran ser identificadas basado en su morfología a el nivel de microscopio de luz. Asi como las neuronas del DRG de ratas y ranas adultas, cuando las neuronas del DRG de humanos adultos son observadas bajo fases ópticas, se pueden observar dos subpoblaciones, pequeñas oscuras y grandes claro. Sin embargo, bajo iluminación "bright-field", 6 subpoblaciones distintas se pueden distinguir basado solamente en características morfológicas. Cinco subpoblaciones contienen inclusiones citoplásmicas de color "rusty" (mohoso) con granulos de diferente tamaño y diferencias en el tamaño y densidad de los agregados (clusters) de granulos, mientras que una es libre de granulos. Análisis de la distribución del diametro del soma demuestra que cada una de las seis subpoblaciones de neuronas que contienen granulos y la que no contiene granulos tiene una diferencia estadísticamente significativa en la distribución de tamaño. Proponemos que neuronas con diferentes morfologías corresponden a subpoblaciones fisiologicamente únicas de neuronas del DRG. Se estan llevando a cabo experimentos utilizando técnicas inmunoquímicas para determinar si la morfología única corresponde con funciones fisiologicas únicas.

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