Visualization of six unique morphological subpopulations of adult frog dorsal root ganglion neurons at the light microscopic level

IDANIS BERRIOS, BS; CRISTINA CASTRO, BS; DAMIEN P. KUFFLER, Ph D

Subpopulations of adult frog dorsal root ganglion (DRG) neurons respond to different physiological stimuli, and have unique biophysical and pharmacological properties. Two broad-based subpopulations of DRG neurons appear under phase optics, "large clear" and "small dark" neurons, while immunochemical and electrophysiological techniques allow identification of additional subpopulations. Nevertheless, most studies of DRG neurons involve randomly selected neurons. Under bright field illumination, we found dark and clear DRG neurons are distinctly different, with dark neurons composed of four subpopulations, each with unique numbers and distribution of bright rusty-colored cytoplasmic granules, and statistically significant difference in the soma diameter distribution. The clear neurons are granule-free, but the two subpopulations have statistically significant differences in soma size distributions. Thus, morphological criteria alone allow identification of six distinct subpopulations of DRG neurons in the light microscope, although further studies are required to determine whether they correspond to physiologically different subpopulations of sensory neurons.

Key words: Sensory neurons, DRG subpopulations, Frog neurons, Cytoplasmic granules.

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

Generally, studies on dissociated adult rat and adult frog DRG neurons in vitro are performed by randomly selecting a neuron to study (4, 6-7, 11, 19-20). 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 channel (1, 4, 9, 11-12, 14-15, 18, 20). Although this approach is extremely fruitful, the yield of useful data from primary sensory neurons could be significantly increased if there were easy and reliable means for identifying the different neuron subpopulations under bright-field illumination based solely on the neuron's morphology.

Institute of Neurobiology, Medical Sciences Campus, University of Puerto Rico, San Juan PR 00901.

Address correspondence to: Damien Kuffler, Ph D, Institute of Neurobiology, 201 Blvd. del Valle, San Juan, PR 00901, Tel: (787) 721-1235, FAX: (787) 725-1289, E-mail: dkuffler@hotmail.com

One approach for reducing the randomness of selecting adult rat and frog neuron for study is to take advantage of the broad-based morphological differences that can be seen under the light microscope. Two subpopulations can be identified, which are referred to as "small dark" and "large light" neurons (5, 15-17, 21). Combined fluorescence and bright-field illumination show the "small dark" neurons to be neurofilament-negative and the "large light" neurons to be neurofilament-positive (21).

An increased success in recording from a particular population of neurons is to select neurons based on soma diameter (16), membrane capacitance (6), or combination of cell diameter and membrane capacitance (3). Although the dark and light classifications of DRG neurons have been used for many years, there has been no systematic analysis of the differences between these neuron subsets.

Alternative approaches for identifying specific subsets of DRG neurons for study involve the use of histochemical stains, such as cobalt uptake that is specific for capsaicin-sensitive neurons (21-22), or back-labeling neurons with dye via their axons (2). One can also apply a specific antibody, or compounds, such as the isolectin IB4, that bind selectively to specific DRG neuron subpopulations (7, 10, 13). Other combinations of techniques have been used to discriminate between DRG neuron subpopulations (8, 23). However, most of these techniques require fixing the neurons, which precludes subsequent physiological studies.

In one series of experiments, dissociated adult frog DRG neurons were selected based upon their being large clear vs. dark neurons, and the recording were annotated according to neuron type (15). 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 (15). These results suggested that the clear neurons were composed of possibly only a single population of neurons, whereas the dark neurons were composed of a larger number of subpopulations. This raised the question of whether there was a simple, but more comprehensive, method that could be developed for discriminating between the different subpopulations. The present results show that six subpopulations of adult frog DRG neurons can be identified based upon neuron morphology and size.

Experimental Procedures

Adult male frogs (Rana pipiens) were killed by decapitation. DRGs were dissociated and the neurons plated as described previously (Philippi et al., 1995). Briefly, DRGs were isolated, cleaned of their connective tissue capsule, and placed in a siliconized glass dish (Sigmacoat, Sigma Chemical). The DRGs were cut into small pieces, treated with collagenase P (3 mg/ml, Boehringer-Mannheim), neutral dispase II (8 mg/ml, Boehringer-Mannheim), and DNase (0.5 mg/ml, Boehringer-Manheim), in Liebowitz-15 (L-15, Gibco) tissue culture medium (diluted 10 parts L-15 + 3 parts water) containing garamycin (10 mg/l) for 1 hour, and triturated to complete dissociation. The neurons were picked up in a siliconized micropipette, with a fire-polished tip with an opening of about 100 μm, and plated onto a glass cover slip that had been treated with laminin followed by poly-l-lysine (1-hour incubation each) in L-15 medium. No neurotrophic or other factors were added to the medium. The neurons adhered to the cover slip immediately upon contact. The neurons in culture medium were left in ambient air at 23°C. The neurons were examined immediately after plating up to 1 week later. The neurons were 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

When dissociated adult frog and adult rat DRGs are viewed under phase optics, two populations of neurons are seen, referred to as “dark” and “clear.” The basis for these differences is granules within the cytoplasm that give rise to the dark appearance, and absence of granules giving rise to the clear neurons. When dissociated adult rat DRG neurons are viewed under a compound microscope under bright field illumination, the granules can be seen more clearly, but still, only the single subpopulation of neurons can be identified.

In contrast to the adult rat DRG neurons, even when intact adult frog DRG are viewed under the dissecting microscope one can easily recognize different subpopulations of DRG neurons. Some appear clear and translucent, while others have a golden color with dark spots within their cytoplasm. When the DRGs are dissociated and the neurons are examined under bright field illumination under a compound microscope, the difference between the dark and clear neurons becomes strikingly apparent. The dark neurons contain bright rusty-colored granules within their cytoplasm, while the clear neurons are granule-free.

Granules

The cytoplasmic inclusions are clearly visible and allow easy discrimination between adjacent neurons (Figure 1).

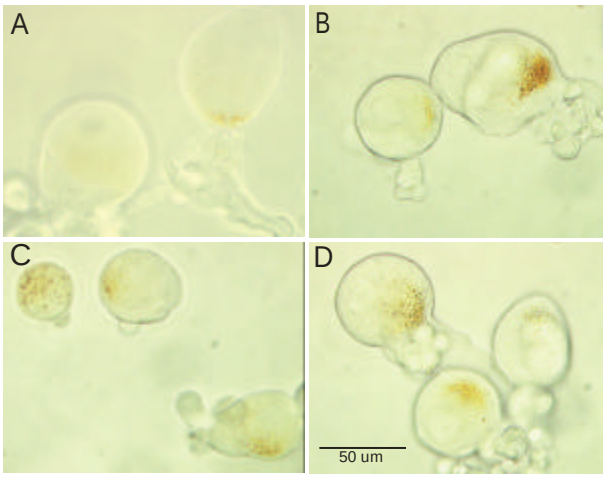

Figure 1. Bright-field illumination of neurons showing several neurons per visual field. A: Two large clear neurons, one of which has faint granules, occasionally seen in large clear neurons, but which do not appear to be a unique subpopulation of neurons. B: One small loose cluster neuron and one dense cluster neuron. C: One sparse and two small loose cluster neurons. D Two dense cluster and one large clear neuron.

The cytoplasmic granules of the subpopulations of dark DRG neurons are qualitatively different in the number of granules per neuron and the density of granule distributions. Based purely on the granule morphology, the dark neurons can be divided into 4 distinct subpopulations of granule-containing neurons, which we have defined as (1) sparse, (2) small loose cluster, (3), large loose cluster, and (4) dense cluster (Figure 2).

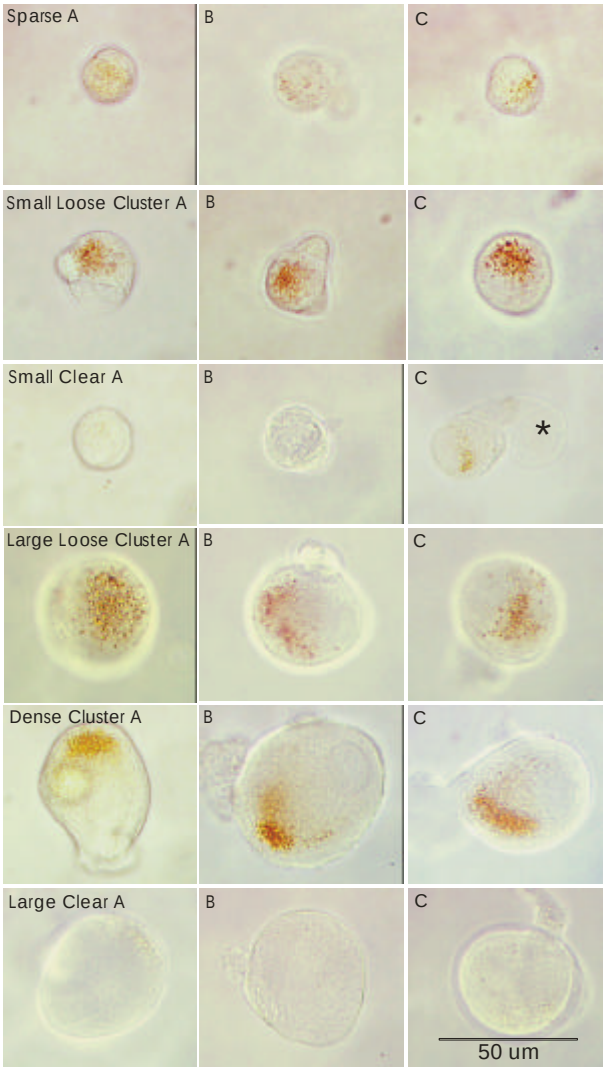

Figure 2. Bright field images of dissociated adult frog DRG neurons. Three 3 examples of each of the subpopulations of neurons identified by the morphology of their cytoplasmic granules, or their absence of granules. The neurons are composed of four populations of dark (granule-containing) neurons, called sparse, small loose cluster, large loose cluster, and dense cluster, and 2 populations of clear (non-granule-containing) neurons, called small clear and large clear neurons. The small clear neuron in C (*) is adjacent to a sparse neuron.

Clear neurons lack rusty-colored cytoplasmic granules and are thus called clear. However, the clear neurons can be subdivided under visual inspection by significant differences in their diameters, which we defined as (1) small clear and (2) large clear neurons.

Size distribution of neurons

The dark adult frog DRG neurons were analyzed to determine whether neurons with different morphologies of cytoplasmic granules correspond to subpopulations with distinctly different diameters. Culture dishes were systematically scanned at 32x and the diameter of each dark neuron measured, along an annotation of its morphology.

The size distribution of neurons, with each type of cytoplasmic granules, was plotted as a function of the number of neurons of each morphology (Figure 3). The

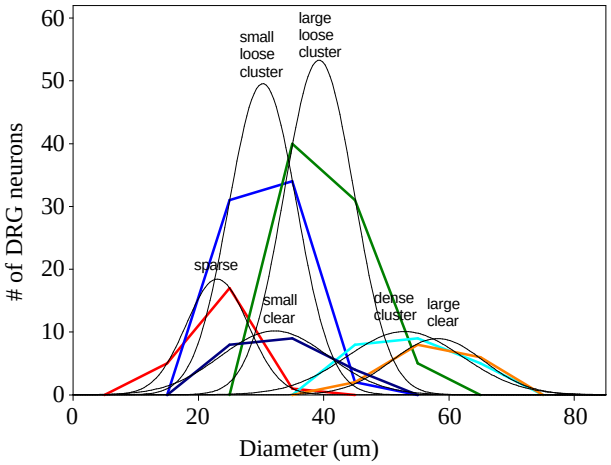

Figure 3. Size distribution of dissociated adult frog DRG neurons. The number of neurons within a 10 µm bin size, from 5 to 80 µm, was plotted against the neuron diameter. The straight lines represent the plot of the raw data. The curved lines represent the data fitted with a Gaussian curve. Six distinct subpopulations of adult frog DRG neurons can be seen, 4 dark (granule-containing) dark and 2 clear (granule-free). Although each subpopulation has overlap of the largest and smallest diameter neurons with the adjacent subpopulations, the difference in the size distribution has a statistically significant difference. This indicates that based upon the morphology of the cytoplasmic granules and neuron diameter, the DRG neurons can be sorted into 6 distinct and separate subpopulations. The clear neurons are composed of 2 distinct subpopulations with some size distribution overlap, but, they have statistically different size distributions, indicating that they are entirely separate subpopulations.

size distribution data show that the dark neurons are composed of four distinct subpopulations. The dark neurons have mean diameters of: (1) sparse 23.0 µm (±0.72, N= 23) (range 14.6 – 30.2 µm), (2) small loose cluster, 30.7 µm (±0.4, N= 67) (range 20 – 42.5 µm), (3) large loose cluster, 40.7 µm (±0.7, N= 76) (range 30,3 – 59 µm), and (4) dense cluster, 54.1 µm (±1.5, N= 22) (range 42.9 – 66.8 µm).

The clear, or non-granule-containing neurons, were analyzed for the distribution of their soma diameters. The size distribution data show these neurons are composed of two subpopulations which have statistically significant differences in their size distributions (P = < 0.001). The

mean diameters of the clear neurons are (1) small clear 34.6 μm (±1.4, N= 21) (range 26.5 – 45.2 size distribution) and (2) large clear, 57. 5 μm (±1.4, N= 16) (range 46.7 – 66 size distribution). This data is plotted in Figure 3. The diameters of the neurons were first organized into 10-micron bins (solid lines) before plotting the data. Each data set was fit with a Gaussian curve (smooth lines).

Analysis of the size distribution of the six populations by the Kolmogorov-Smirnov Normality Test shows they do not have a normal size distribution (P = < 0.0001). The analysis of the different types of dark neurons, with cytoplasmic granules, yielded four distinct groups, while the clear neurons yielded two distinct groups. The data for the dark and clear subpopulations have minimal overlap of the largest and smallest neurons in these groups.

The Mann-Whitney Rank Sum Test showed that the neurons with large loose cluster and with dense cluster cytoplasmic granules, with apparently overlapping subpopulations, are in fact statistically different sized populations (P = < 0.001). The sparse and small loose cluster granule-containing neurons are also separate subpopulation (P = < 0.001). The small clear and the large clear granule-free neurons are also separate subpopulation (P = < 0.001).

Two populations, small clear and small loose cluster, have no statistical difference in their size distribution (P = 0.866). However, because one has granules and the other does not, these are different neuron subpopulations.

Cytoplasmic inclusions are not artifacts of DRG treatment

The four forms of unique morphology of the cytoplasmic granules are clearly visible within individual neurons even under the dissecting microscope, and when the DRGs were fixed immediately after being removed from the animal. The different morphologies of cytoplasmic granules also remain constant when the dissociated are maintained in vitro for 5 days. These results indicate that the different morphologies of cytoplasmic granules are distinct characteristics of the different subpopulations of adult frog DRG neurons, and are not artifacts of dissociation or the time the neurons are in vitro.

Discussion

The present study was designed to determine whether, under bright field illumination, dissociated adult frog DRG neurons could be categorized into separate populations based upon neuron morphology and neuron diameter. When viewed under dark field optics both adult frog and adult rat dissociated DRG neurons are composed of clear and dark neurons. Under bright field illumination, the difference between dark and clear neurons is seen to result from the presence of cytoplasmic granules in the dark neurons, and the absence of granules from the clear neurons.

The granules in adult rat DRG neurons are faint and difficult to see, and do not allow the rat neurons to be classified into different subpopulations. However, the cytoplasmic granules in the adult frog DRG neurons are large, rusty-colored, easy to see, and the four subpopulations of dark neurons have granules with distinctly different morphologies. Thus, the dark adult frog DRG neurons can be categorized into subpopulations based upon the morphology of the cytoplasmic granules.

In earlier work using the whole-cell patch-clamp technique on isolated adult frog DRG neurons, we took advantage of the clear and dark appearance of the neurons to study the membrane properties of the two subpopulations. For each neuron from which a recording was made, the data was annotated as coming from a clear or dark neuron. Clear differences and similarities were recorded from the two neuron populations (15). Although we found different responses from the dark neurons indicating that they were composed of different neuron subpopulations, time did not allow us to discriminate between the neurons based upon their properties and the different morphologies of their cytoplasmic granules.

Analysis of the size distribution of the clear neurons, granule-free, indicated they were composed of two distinct subpopulations. These populations had statistically significant differences in size distribution.

Although six different adult frog DRG neuron subpopulations were characterized, it was beyond the scope of the present experiment to characterize the composition of the cytoplasmic granules in the different neuron subpopulations. Similarly, it was not possible to determine whether the different subpopulations have different biophysical or pharmacological properties. Finally, further analysis is required to determine whether the neurons of the different subpopulations correspond to different physiological subpopulations of DRG neurons, with different receptors and membrane properties. If such a correspondence exists, this will allow the simple and rapid identification, under bright field illumination, of unique subpopulations of sensory neurons for analysis of their biophysical properties and responses to pharmacological agents.

Resumen

Las sub-poblaciones de neuronas aisladas de ganglios dorsales de rana adulta responden a diferentes estímulos fisiológicos y tienen propiedades biofísicas y

farmacológicas únicas. Dos amplias sub-poblaciones de neuronas de ganglios dorsales se pueden observar bajo óptica de fase, neuronas "grandes claras" y "pequeñas obscuras, mientras que a través de técnicas inmunoquímicas y electrofisiológicas se pueden identificar otras sub-poblaciones. Sin embargo, la mayor parte de los estudios de neuronas aisladas de ganglios dorsales se hacen seleccionando las neuronas al azar. Utilizando iluminación de campo claro, encontramos que las neuronas obscuras y las claras son distintivamente diferentes. Las neuronas obscuras se componen de 4 sub-poblaciones, cada una con un número y distribución única de gránulos citoplásmicos de color rojizo y brillante, y con diferencias estadísticamente significantes en el diámetro del soma. Las neuronas claras no contienen gránulos, pero se encontraron dos sub-poblaciones que tienen diferencias estadísticamente significativas en la distribución del tamaño del soma. De este modo, la utilización únicamente de un criterio morfológico nos permitió la identificación de 6 sub-poblaciones distintas de neuronas a través de un microscopio de luz. Aunque, se requieren otros estudios para determinar si estas sub-poblaciones corresponden fisiológicamente a diferentes sub-poblaciones de neuronas sensoriales.

Acknowledgements

We thank Dr. Viktoria Vlachova for assistance with the statistical analysis of the neuron size distributions. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee approval, and in accordance with regulations on the treatment of animals.

References

1. Houweling DA, Brook GA, Gieling RG, et al. Differential distribution of immunoreactivity in the developing rat spinal cord revealed by the monoclonal antibody Py. Brain Res Dev Brain Res 1999;116:87-96.
2. Greffrath W, Nemenov MI, Schwarz S, et al. Inward currents in primary nociceptive neurons of the rat and pain sensations in humans elicited by infrared diode laser pulses. Pain 2002;99:145-55.
3. Vyklicky L, Philippi M, Kuffler DP, et al. GABAA membrane currents are insensitive to extracellular acidification in cultured sensory neurons of the frog. Physiol Res 1993;42:313-7.
4. Kocsis JD, Rand MN, Lankford KL, et al. Intracellular calcium mobilization and neurite outgrowth in mammalian neurons. J Neurobiol 1994;25:252-64.
5. Waxman SG, Cummins TR, Dib-Hajj S, et al. Sodium channels, excitability of primary sensory neurons, and the molecular basis of pain. Muscle Nerve 1999;22:1177-87.
6. Holohean AM, Rodriguez CA, Hackman JC, et al. Voltage-gated calcium currents in whole-cell patch-clamped bullfrog dorsal root ganglion cells: effects of cell size and intracellular solutions. Brain Res 1996;711:138-45.
7. Vyklicky L, Lyfenko A, Kuffler DP, et al. Vanilloid receptor TRPV1 is not activated by vanilloids applied intracellularly. Neuroreport 2003;14:1061-5.
8. Kuffler DP, Lyfenko A, Vyklicky L, et al. Cellular mechanisms of nociception in the frog. J Neurophysiol 2002;88:1843-50.
9. Chung MK, Guler ADCaterina MJ. Biphasic currents evoked by chemical or thermal activation of the heat-gated ion channel, TRPV3. J Biol Chem 2005;
10. Kissin I, Davison NBradley EL, Jr. Perineural resiniferatoxin prevents hyperalgesia in a rat model of postoperative pain. Anesth Analg 2005;100:774-80, table of contents.
11. Oshita K, Inoue A, Tang HB, et al. CB(1) Cannabinoid Receptor Stimulation Modulates Transient Receptor Potential Vanilloid Receptor 1 Activities in Calcium Influx and Substance P Release in Cultured Rat Dorsal Root Ganglion Cells. J Pharmacol Sci 2005;97:377-85.
12. Philippi M, Vyklicky L, Kuffler DP, et al. Serotonin- and proton-induced and modified ionic currents in frog sensory neurons. J Neurosci Res 1995;40:387-95.
13. Scroggs RSFox AP. Distribution of dihydropyridine and omega-conotoxin-sensitive calcium currents in acutely isolated rat and frog sensory neuron somata: diameter-dependent L channel expression in frog. J Neurosci 1991;11:1334-46.
14. Winter J. Characterization of capsaicin-sensitive neurones in adult rat dorsal root ganglion cultures. Neurosci Lett 1987;80:134-40.
15. Hayes NV, Holmes FE, Grantham J, et al. A60, an axonal membrane-skeletal spectrin-binding protein. Biochem Soc Trans 1995;23:54-8.
16. Tandrup T, Woolf CJCoggeshall RE. Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. J Comp Neurol 2000;422:172-80.
17. Dube GR, Kohlhaas KL, Rueter LE, et al. Loss of functional neuronal nicotinic receptors in dorsal root ganglion neurons in a rat model of neuropathic pain. Neurosci Lett 2005;376:29-34.
18. Wood JN, Winter J, James IF, et al. Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. J Neurosci 1988;8:3208-20.
19. Cristino L, Florenzano F, Bentivoglio M, et al. Nitric oxide synthase expression and cell changes in dorsal root ganglia and spinal dorsal horn of developing and adult Rana esculenta indicate a role of nitric oxide in limb metamorphosis. J Comp Neurol 2004;472:423-36.
20. Kobayashi S, Sasaki S, Shimada S, et al. Changes of calcitonin gene-related peptide in primary sensory neurons and their central branch after nerve root compression of the dog. Arch Phys Med Rehabil 2005;86:527-33.
21. Murinson BB, Hoffman PN, Banihashemi MR, et al. C-fiber (Remak) bundles contain both isolectin B4-binding and calcitonin gene-related peptide-positive axons. J Comp Neurol 2005;484:392-402.
22. Ivanavicius SP, Blake DR, Chessell IP, et al. Isolectin B4 binding neurons are not present in the rat knee joint. Neuroscience 2004;128:555-60.
23. Yoshimura N, White G, Weight FF, et al. Patch-clamp recordings from subpopulations of autonomic and afferent neurons identified by axonal tracing techniques. J Auton Nerv Syst 1994;49:85-92.