Adult Mouse Dorsal Root Ganglia Neurons in Cell Culture

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SUMMARY

A method has been developed for the long-term culture of dissociated adult mouse dorsal root ganglia (DRG). Of critical importance to the success of this technique was a three-hour incubation in collagenase which softened the DRG and permitted gentle dissociation. The morphological and electrophysiological features of the dissociated adult DRG were similar to those observed in previous studies of immature (i.e., embryonic and newborn) DRG in culture and also to those of adult DRG in situ.

With regard to electrophysiological work, the adult DRG neurons are superior to embryonic and newborn neurons because of their larger size and greatly increased survival in culture (no degeneration for first six days, and thereafter a relatively slow decrease). The adult neurons regenerated nerve fibers to an extent comparable to that of immature neurons. Therefore, the adult DRG cultures might be useful to study factors influencing regeneration in the adult mammalian nervous system. The adult cultures might also be useful to investigate factors influencing the aging process.

INTRODUCTION

Since the inception of tissue culture by Harrison in 1907, tissue from various parts of the nervous system and from various species has been successfully cultured and used in a great many neurobiological investigations (see reviews by Murray, 1965; Nelson, 1975; Crain, 1976). However, the vast majority of these studies, including that of Harrison, have utilized embryonic or early postnatal nervous tissue and the relatively few studies which have attempted to use adult tissue have met with varying degrees of success (Murray, 1965, p. 399; Crain, 1976, p. 15). Adult peripheral nervous system (PNS) tissue has been successfully cultured and shown to have regenerative capacity by several workers (adult human sympathetic ganglia, Murray and Stout, 1974; adult rat sympathetic ganglia, Silberstein, Johnson, Hanbuaser, Bloom, and Kopin, 1971; adult frog sympathetic ganglia, Hill and Burnstock, 1975; adult frog dorsal root ganglia, J. C. Padjen, Forman, and Siggins, 1975). Most attempts to culture adult mammalian central nervous system (CNS) tissue met with less success (human cortex, Costero 1976).
and Pomerat, 1951; Hogue, 1953; Geiger, 1958; individual vestibular neurons of
the rabbit, Hillman and Sheikh, 1968; rat spinal cord and cerebellum, Kiernan
and Pettit, 1971; Drayton and Kiernan, 1973; \textit{dissociated} rat hypothalamus,
Wilkinson, Gibson, Bressler, and Inman, 1974). Greater success was achieved
recently by De Boni, Seger, Scott, and Crapper, (1976) who described regener-
ation of nerve fibers and neurotypic electrical and synaptic activity in cultures
of adult teleost CNS maintained for periods of up to 19 weeks. Also, Crain and
Peterson (1973), using “subcultures” of spinal cord explants prepared from
15-day fetal rodents, but maintained in culture for prolonged periods (four
months), have demonstrated the ability of these \textit{in vitro}—“matured” neurons
to regenerate nerve fibers for a second time in culture, to exhibit electrical ac-
tivity, and to reform synaptic relationships with other comparably “matured”
nurons or with adult muscle cells (see also Crain, 1976).

The present communication describes a method whereby adult mouse DRG
have been dissociated and maintained in culture for periods up to five months
during which time the neurons exhibited neurotypic cytological and electrophysiological features.

\section*{MATERIALS AND METHODS}

The method for preparing cell cultures of adult mouse DRG is a modification of that used pre-
viously for embryonic chick DRG (Scott, Engelbert, and Fisher, 1969) and for fetal human DRG
(Scott, 1971). In each experiment, two adult mice (three to six months of age) were ether anesthe-
tized, killed by cervical dislocation, and sterilized by dipping briefly in 65\% alcohol. The entire spinal
column was dissected from each mouse, placed in a Petri dish of phosphate-buffered saline (PBS),
cleaned as much as possible of attached tissue, e.g., skin, muscle, fascia and viscera, and finally
transferred to a fresh dish of PBS. Two longitudinal lateral cuts were made through the ventral
surface, making it possible to remove a central ventral strip from the spinal column thus revealing
the underlying spinal cord with attached dorsal roots. By gently pulling on the latter, the ganglia
were made clearly visible and were removed with microforceps. In this way 30 to 40 DRG were re-
moved from each spinal cord.

The DRG were collected in 1 ml of 0.25\% collagenase in PBS. The collagenase (Type CLS,
Worthington Biochemical, New Jersey, U.S.A.) was preselected for its ability to dissociate the adult
DRG from several batches of the “normal blend” of crude collagenase (CLS). The collagenase so-
lution was divided into 2-ml quantities and stored frozen at ~80^\circ C and thawed overnight at 4^\circ C
just before use. There was some slight variability in the aliquots of a single batch of collagenase
perhaps due to the freezing and thawing procedure.

After being dissected and collected in collagenase, a procedure which took about one hour (two
people dissecting), the ganglia were somewhat softened and it was easy to remove the attached nerve
and connective tissue. Each ganglia was gently split open with the microforceps so as to expose the
internal contents to the collagenase. The DRG were then incubated at 36^\circ C for three hours, followed
by two rinses with 10 ml of PBS. Dissociation was carried out in two stages. First, the DRG were
dissociated very gently using a 500 $\mu$m heat-polished tip micropipette in two ml of culture medium
(CM, described below), allowed to stand until the undissociated fragments settled to the bottom
and the supernatant removed and set aside. Two more milliliters of CM were added and the second
stage of dissociation carried out by more vigorous trituration, first with the 500-$\mu$m pipette and then
with a 400-$\mu$m pipette until all the DRG fragments were completely dissociated. The cell suspensions
from each of the two triturations were then mixed together. Four drops (0.04 ml/drop) of this sus-
pension containing approximately 2.5 \times 10^4 neurons/ml was added to each culture. The neuron
density which varied by \pm 25\% in different experiments was not critical. Each culture consisted
ADULT NEURONS IN CULTURE

of a collagen-coated coverslip lying on the bottom of a plastic Petri dish (Falcon 1006). Twenty-five cultures were set up in each experiment.

The culture medium (CM) was identical to that used previously to culture embryonic and newborn mouse DRG (Scott, 1977) and consisted of 10% fetal calf serum (Gibco, Grand Island, New York) in CMRL-1415 (Connaught Medical Research Laboratory, Toronto, Ontario, Canada) with the glucose raised to 5.0 g/l, choline chloride raised to 5.0 mg/l, and penicillin and streptomycin present at 50 units/ml and 50 μg/ml, respectively.

Cultures were incubated at 36° C with the carbon dioxide adjusted to give a pH of 7.4 to 7.5. Cultures were fed daily with 0.3 ml of medium for the first five days. On day six the cultures were flooded with 2.0 ml of medium and thereafter fed twice weekly.

Ten experiments (25 cultures per experiment) were carried out successfully using this procedure. Mice of two different types have been used without observable difference in the results (outbred Swiss albino, Connaught Medical Research Laboratories, Toronto, and inbred BALB/c Jackson Laboratory, Bar Harbour, Maine, supplied by Dr. D. St. John, Department of Zoology, University of Toronto).

The procedure and equipment for carrying out the electrophysiological investigation was identical to that described previously in a study of chick embryo DRG (Scott, Englebert, and Fisher, 1969).

RESULTS

During the period in culture which ranged up to five months, the dissociated adult DRG exhibited a pattern of progressive morphological development which, except for a few differences described below, was similar to that observed in previous studies of DRG of chick embryo (Scott, Engelbert, and Fisher, 1969), fetal human (Scott, 1971), embryonic and newborn mouse (Scott, 1977) and embryonic rat (Scott, unpublished). The latter studies of “immature” (i.e., embryonic and newborn) neurons serve as a basis of comparison in the following descriptions of both the morphological and the electrophysiological features of the adult neurons.

Immediately after dissociation, the cultures consisted of single cells and degenerating fragments of myelinated nerve fibers [Fig. 1(a)]. The neurons were easily identified by their large size (up to 50 μm), smooth round outline, the occasional presence of a short nerve stump, and their bright yellow appearance when viewed by negative phase-contrast microscopy. The smaller cells with irregular outlines and less refractivity were non-neural cells, e.g., capsule, fibroblasts, and Schwann cells. The dissociation procedure freed most neurons of their usual intimate capsule cell covering although many neurons had non-neural cells loosely associated with them.

After one or two days the myelin fragments disintegrated and the resulting fine debris was gradually removed during the subsequent feedings. Some neurons regenerated a halo of very fine nerve fibers which radiated from the cell body. Other neurons regenerated nerve fibers which appeared flattened against the collagen substrate and were up to 10-μm wide. After two or three days most nerve fibers were of the usual round refractile type, a micrometer or two in diameter and several hundred micrometers in length [Fig. 1(b)] and were quite comparable in morphology and length to those regenerated by immature neurons in culture. Most neurons exhibited eccentric nuclei indicating chromatolysis during this initial period although the reaction did not appear as intense as seen
Fig. 1. Phase contrast micrograph of living cultures of dissociated adult DRG. (a) Immediately after dissociation. The large, round, phase-bright neurons (large arrows) and the smaller, irregular shaped, non-neural cells (small arrows) are easily distinguished. Occasionally a short nerve stump (curved arrows) or even longer myelinated segments of nerve (hollow arrows) have survived dissociation. (b) Three-day culture. Neurons (large arrows) have regenerated long nerve fibers (arrowheads), and some of the non-neural cells (small arrows) have flattened out. Bar = 50 μm.
Fig. 2. Phase-contrast micrograph of living cultures of dissociated adult DRG. (a) Six-day culture. Non-neural cells (small arrows) have proliferated and formed a nearly confluent monolayer on which are neurons (large arrows). Eccentric nucleus (hollow arrows) indicates chromatolysis. (b) Forty-two-day culture. Non-neural cells (small arrows) have become more tightly packed together in some areas. Neurons (large arrows) are still plentiful. Many have central nuclei (hollow arrows) indicating recovery from chromatolysis. The non-neural cell network obscures the regenerated nerve fibers in (a) and (b). Bar = 50 μm.
Fig. 3. Intracellular recordings from adult mouse DRG neurons in culture. (a) Action potentials accompanying penetration of microelectrode into neuron. Note overshoot of 35 mV and the slightly biphasic falling phase. Fifty-two days in culture. (b)–(d) Action potentials elicited by intracellular microelectrode stimulation. (b) Stimulus duration ($t$) = 10 msec, depolarizing stimulus current ($i$) = 0.4 nA. Note again large overshoot of 35 mV and the negative afterpotential of 15 mV. Thirty-nine days in culture. (c) $t$ = 10 msec, $i$ = 0.9 nA. (d) $t$ = 0.1 msec, $i$ = 22.3 nA. Note slightly shorter duration of action potential and almost linear falling phase. (c) and (d) are both neurons maintained in culture for three months. Top horizontal white line represents zero potential. Arrow indicates resting potential level. Vertical bar represents 20 mV in all photos, horizontal bar equals 5.0 msec in (a) and equals 2 msec in (b)–(d).

In immature neurons. Even after two or three days some of the neurons still had not attached to the collagen substrate whereas immature neurons attached within two or three hours.

During the initial two or three days the non-neural cells were attaching and proliferating at a slow rate so that most of the regenerated nerve fibers were completely free of Schwann or other non-neural cells. This is in great contrast to the cultures of embryonic DRG where a confluent monolayer of non-neural cells was formed during the first 24 hours. After three days the adult non-neural cells began to proliferate more rapidly and by six days had formed a nearly confluent monolayer which obscured the nerve fibers [Fig. 2(a)]. Further proliferation resulted in a close-packed confluent monolayer with a definite linearity produced by the parallel alignment of the cells. In most of the older cultures (one to five months) the continued proliferation of the non-neural cells had formed a multilayered connective tissue sheet. Neurons remained on the surface
of this sheet [Fig. 2(b)], but the regenerated nerve fibers travelled both over the surface and also within the connective tissue sheet, as revealed by silver staining [described previously in chick embryo DRG cultures (Scott, Engelbert, and Fisher, 1969)].

The very fine nerve fibers which were visible on the surface of the connective tissue sheet did not stain with silver, were generally free of Schwann cells and were not myelinated. The fibers within the sheet did stain with silver, did have some Schwann-like cells in intimate association, but were not myelinated. In some cases these nerve fibers could be traced for over 20 mm. The neurons showed a progressive recovery from chromatolysis throughout the culture period and by 40 days many neurons had central nuclei [Fig. 2(b)]. Most neurons appeared to lack satellite cells and only in a few of the older cultures was any capsule cell investment of neurons seen. Very little neuron degeneration occurred even in the oldest cultures (5 months).

Resting potentials were recorded from 36 adult neurons maintained in culture from 39 days to five months and were found to range from 30 to 68 mV with a mean of 46.9 (± standard deviation of 9.6). This is in fair agreement with the range of 20 to 80 mV reported for adult cat DRG in situ (Sato and Austin, 1961) and is in even better agreement with the mean 43.2 mV (±9.5, N = 20) obtained for similar cultures of embryonic and newborn mouse DRG (Scott, unpublished). It also is in fair agreement with values obtained for DRG cultures of other species, e.g., 53.6 mV (±8.4, N = 40) for embryonic and newborn rat (Scott, unpublished), and 52.8 mV (±9.9, N = 58) for embryonic chick (Scott, Engelbert, and Fisher, 1969). It is important to point out that all of these results were obtained under identical recording conditions except that the chick neurons were maintained in 5% instead of 10% fetal calf serum.

Penetration of a microelectrode into an adult neuron was nearly always followed by one or more action potentials [Fig. 3(a)]. Direct stimulation of the neurons through the recording intracellular microelectrode also resulted in the production of action potentials [Fig. 3(b)–(d)] with durations of 2 to 3 msec, overshoots of 10 to 35 mV, and were occasionally followed by pronounced negative and/or positive afterpotentials. The falling phase of the action potentials was sometimes biphasic (Fig. 3), but this feature seemed less frequent than observed previously for immature mouse neurons (Scott, unpublished) and certainly was much less pronounced than in the case of embryonic chick neurons in culture (Scott, Engelbert, and Fisher, 1969). Measurements on nine adult neurons gave a rheobase of 0.9 nA and a chronaxie of 1.2 msec which agrees with the values obtained for cultures of immature rodent neurons of 0.8 nA and 1.0 msec, respectively (Scott, unpublished). Spontaneous action potentials were rarely observed in the adult neurons as was the case for the immature neurons. However, in two cases spontaneous repetitive spikes were observed which disappeared upon the application of 10 mV hyperpolarizing current.

DISCUSSION

In the present study a method has been developed for the long-term culture of dissociated adult mouse DRG which permitted morphological development
(including extensive nerve fiber regeneration) and the retention of typical electrophysiological function by the dissociated adult neurons. Relatively little neuron degeneration occurred even after five months in culture.

Probably the most important factor contributing to the success of the present study was the use of a three-hour incubation in collagenase to soften the DRG and permit gentle dissociation. Also important was the selection of particular batches of the collagenase which were nontoxic and yet adequately softened the DRG. Preliminary experiments showed that collagenase was much superior to trypsin but that pronase did yield viable cultures (unpublished).

Another factor which may have been important was the choice of PNS tissue since it lacks the complex synaptic structures of CNS tissue which would have been completely obliterated during the dissociation and might have caused neuron degeneration. In this regard it is relevant that Wilkinson, Gibson, Bressler, and Inman, (1974) reported a method utilizing trypsin for dissociating hypothalamus from newborn and "mature" rats. (The latter were only four or five weeks old whereas the mice of the present study were three to six months.) They reported the formation of a confluent monolayer of non-neuronal cells on top of which were clusters of neurons some of which had regenerated nerve fibers. Further cytological and functional development was interrupted by extensive neuron degeneration at four to five weeks for the "mature" rat cultures and at two weeks for the newborn rat cultures. The limited success obtained by Wilkinson, Gibson, Bressler, and Inman (1974), along with the recent report of the dissociation and maintenance for 24 hours of CNS from 10 to 20 days postnatal mice (Poduslo and McKhann, 1976), suggest that it is possible to culture dissociated adult CNS tissue. Experiments along this line are in progress.

The morphological and electrophysiological investigation of the adult DRG cultures revealed a great degree of similarity between the adult neurons and immature (i.e., embryonic and newborn) neurons which had been investigated previously in vitro (Scott, Engelbert, and Fisher, 1969; Scott, 1971; Scott, 1976; and unpublished results) and mature neurons in situ. It is concluded that under the culture conditions of the present study, the disruption of the original tissue integrity and the isolation of the neurons from their usual in situ surroundings does not prevent expression of many of the cytological and electrophysiological features characteristic of the neurally differentiated state by the dissociated adult neurons. Also, the ability of the dissociated adult DRG to undergo a progressive morphological reorganization similar to that exhibited by dissociated immature DRG indicates that, under suitable culture conditions, dissociated adult neurons have the same independent developmental capacity as immature neurons.

No myelination of nerve fibers and little capsule cell investment of neurons was observed in the present study of dissociated adult DRG. This was also the case for cultures of embryonic and newborn DRG under similar culture conditions and is undoubtedly probably due to the use of a relatively simple culture medium consisting of just 10% fetal calf serum in synthetic medium. The use of a higher serum concentration and embryo extract may be useful in promoting myelination and capsule cell investment in adult DRG cultures.

Morphologically, the outstanding difference observed between the adult and
the immature dissociated DRG was the much slower proliferation of the non-
neural cells in the adult cultures. This difference was also observed by Wilk-
inson, Gibson, Bressler, and Inman (1974) in their cultures of newborn and
mature rat hypothalamus. However, the observed slower proliferative rate of
the adult non-neural cells does not seem to be an intrinsic limitation of these cells
since subculturing adult DRG cultures resulted in a nearly confluent monolayer
within 24 hours (Scott, unpublished).

The observation of rapid and extensive neurite regeneration in the adult cul-
tures, quite comparable to that observed in the immature DRG cultures, suggests
that factors extrinsic to the neuron might be responsible for the slow regeneration
reported for adult neurons in situ (Guth, 1975; Kerr, 1975). The two types of
nerve fibers initially regenerated by the adult DRG (the fine and thick types)
as well as differences between the regenerated fibers of fetal and adult DRG
neurons deserve further attention. Cultures of adult DRG provide a valuable
new model system for elucidating extrinsic factors controlling nerve regeneration
and for determining how regeneration may be controlled in the adult mammalian
nervous system.

Qualitatively, the electrophysiological characteristics of the adult neurons were
similar to those recorded from immature neurons, but further work would have
to be done to determine if quantitative differences do exist. A definite advantage
of the adult neurons compared to the immature neurons is their larger size which
makes them less susceptible to damage by microelectrode insertion, thus making
the electrical recordings more reliable.

A very important difference between the adult and immature DRG neurons
is the fact that, in culture, dissociated adult neurons undergo far less degeneration
that immature neurons. During the first six days in culture, neuron survival
is approximately constant at 100% in the adult cultures while in the immature
cultures, it is in the range of 5.6% to 27% depending on the precise age. The much
lower rate of degeneration in the adult DRG cultures makes them a much more
faithful representation of the neuronal population in the intact animal (Scott,
in preparation).

The increased reliability of the electrical recordings due to large neuron size
along with the increased fidelity due to the increased neuron survival makes these
adult neuron cultures superior to immature neuron cultures for quantitative
electrophysiological investigations.

Another advantage of the use of adult cultures is that one could select a donor
animal on the basis of different environmental conditions in which the animals
had been raised and thus investigate the effect of chronic exposure to a particular
factor throughout the lifetime of the animal. One could also select animals which
had developed specific pathological conditions and examine the effect of various
disease states on neural morphology and function at the cellular level. Finally,
one could select donor animals of various ages and thus test the effect of senes-
cence on the capacity of the neurons to regenerate nerve fibers and on the elec-
trophysiological functioning of the neurons. In this regard, the cultures of adult
neurons might be useful in studying the proposed immortality of cells in culture
(Loeb, 1912) and in investigating the various factors which have been implicated
in the aging process (e.g., Wright and Hayflick, 1975). With this goal in mind, experiments are in progress in which the technique of the present study has been successfully extended to culturing DRG from “old” rabbits (two years) and mice (one year).

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REFERENCES


