Culture of major pelvic ganglion neurons from adult rat

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Abstract Successful culturing of neurons from adult animals has been historically difficult for a relatively long time. In this study, we reported the development of a novel method for the isolation and the culture of major pelvic ganglion (MPG) neurons from adult rat. The cultured cells were identified by neuron morphology and staining with neuronal marker (neurofilament-200, NF-200). The results demonstrate that the new protocol we used was reliable in obtaining a relatively high yield of MPG neurons. Furthermore, it improves the speed and simplicity in neuronal isolation. The viability of neurons can be maintained for about 2 weeks, which should be sufficient for investigating physiological and pathological processes occurring in mature major pelvic ganglia. And this may provide a useful assessment to currently available techniques for the culture of adult neurons.

Keywords Adult · Major pelvic ganglion · Culture

Introduction

Pelvic ganglia are comprised of a mixed population of cholinergic parasympathetic neurons as well as noradrenergic sympathetic ganglion cells (Keast 1995). Various neurons of this mixed population innervate several pelvic structures, including the lower urinary, digestive tracts and internal reproductive organs (Keast and de Groat 1995; Keast et al. 1995). A lot of researches have described the structural and histochemical features of the major pelvic ganglion in several kinds of species (Keast 1999). In most species, these numerous small ganglia locate close to the pelvic organs. As to the rat, functionally identical groups of neurons are clustered in two large major pelvic ganglia and a small adjacent clump of accessory ganglia (Dail et al. 1975).

Until recently, few reliable protocols have been developed for the primary culture of major pelvic ganglion neurons from adult rat, despite an early attempt has been made under serum conditions (Tuttle and Steers 1992). Embryonic neurons represent an easy and effective source of primary neurons, however, the anatomical region of MPG in adult rat has precisely been identified, which in turn allowed the isolation of MPG. In addition, their developmental stage is not always appropriate for the topics of interest. Whether the behavior of these embryonic neurons in culture is like their developed adult counterparts is still an unsolved question. The developmental immaturity of embryonic cells may be

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primarily observed in mature and aged nervous tissue. During the course of development and differentiation, many structural and functional changes of neurons can be observed. Typical examples are Alzheimers disease, Huntingtons disease and Parkinsons disease. Adult peripheral nervous system retains the capability of substantial remodeling in response to functional demands. Previous studies have reported that abnormal growth of bladder causes corresponding changes in the growth of their innervating neurons, which maintains the functionally active innervations (Steers et al. 1990). Changes in size of the bladder are often accompanied by corresponding growth, or shrinkage of axons, dendrites and cell soma of the innervating neurons (Keast 1990). Studies concerning the plasticity in the adult and ageing nervous systems have confirmed the need of a technique to culture primary neurons.

The Neurobasal medium and the B27 supplement were developed for the serum-free isolation and culture of neurons, by optimising the osmolarity and by including antioxidants (Brewer 1997). The pioneering study of Tuttle and co-worker demonstrated that adult MPG neurons could survive and regenerate under serum conditions in vitro (Tuttle and Steers 1992). The presence of serum may confound the effects of specific growth factors added as experimental treatments with the effects of growth factors present in serum. Based on Tuttle and Steers (1992), Keast (1990) described culturing MPG neurons from adult rat in Neurobasal medium with B27 supplement, some work has been done to examine the effects and mechanisms of neurturin signaling in adult sacral parasympathetic neurons (Wanigasekara and Keast 2005; Nangle and Keast 2011), but they described the procedure briefly, signaling mechanisms underlying this neurotrophic support rather than dissociation and culture technique were the focus of their work.

The primary purpose of our study is to introduce a method for the isolation and culture of major pelvic ganglion neurons from adult rat in a simple and fast way without special isolation procedures, or preparation of a glial feeding layer. Furthermore, the existence of a defined, serum-free growth and maintenance medium would be of particular value for the straightforward identification of factors released by the MPG neurons in vitro. It would also provide a simplified, reproducible environment for studying the maturation and physiology of the MPG neurons and its response to

pharmacologic treatments. Here, we give a detailed delineation of our currently used culturing techniques. This procedure has the advantages in that it improves speed and simplicity of the preparation while with low cost.

Materials and methods

Isolation of major pelvic ganglion neurons

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

MPGs, closely attached to the ventral part of the prostate, were dissected from adult male Sprague–Dawley rats (3–6 months). Animals were anesthetized with chloralhydrate (Sigma, St. Louis, MO, USA) as previously described (Steers et al. 1991). To obtain relatively high-purity neuron cultures, it was necessary to identify the exact location of MPG tissues. Ganglia were kept in D-Hanks solution (Gibco - Life Technologies, Carlsbad, CA, USA) on ice and desheathed carefully under a dissecting microscope, and all surrounding connective tissues were removed. Ganglia were chopped into a number of slices and treated enzymatically in the following two steps. Tissue slices were first digested with collagenase (1 mg/ml, Sigma) for 30 min at 37 °C in neurobasal medium (Gibco, Neurobasal® Medium) with gentle agitation for 10 min. After washing twice with D-Hanks solution, ganglia were digested with trypsin (1 mg/ml, Sigma) as described before. Finally, the proteases were neutralized by adding plating medium (DMEM/F12; Gibco), supplemented with 2 mM glutamine (Gibco), 10 % FBS (Sigma), 1 % penicillin/streptomycin (Gibco), 20 ng/ml nerve growth factor (NGF, Roche Applied Science, Colorado, IN, USA). Slices were triturated about 10 times using a fire-polished glass pipette. The solution was allowed to settle for 2 min and the supernatant was transferred to another tube. Fresh medium was then added, and further trituration was performed, then the cells were again removed. The sequential trituration was repeated several times until all slices were nearly or totally dissociated. The collected cell suspension was

pelleted by centrifugation at $200\times g$ for 5 min and resuspended at the concentration of 0.6×10^4 neurons/ml in plating medium. Neurons were plated onto glass coverslips that had been precoated overnight with 50 $\mu\text{g/ml}$ poly-L-lysine (Sigma). Coverslips were rinsed twice with PBS and allowed to air dry about 10 min prior to seeding the cells. Usually, 2 ml of this cell solution was added to each 35-mm plate (Corning, NY, USA).

The entire process from killing the rats to plating took approximately 3 h. Cells were allowed to be settled for 2 h, washed once with PBS to remove debris, and prewarmed plating medium was added (1.5 ml/35 mm dish). The cultures were incubated at 37 °C in 5 % CO_2 for 2 days without medium replacement. No attempt was made to control glial cell proliferation, because glial cells are a rich source for trophic factors which can promote neuronal survival and synaptic development (Ullian et al. 2001). On the 3rd day, the plating medium was replaced with the same volume of feeding medium. After that, half of the medium was replaced with fresh medium every 3–4 days. Feeding medium was composed of Neurobasal medium (Invitrogen - Life Technologies) supplemented with 2 % B27 (Invitrogen), 1 % penicillin/streptomycin, 20 ng/ml NGF. L-Glutamine was added to the media on the day of use to a final concentration of 2 mmol/l.

Evaluating cultures of major pelvic ganglion neurons

Cultured major pelvic ganglion neurons were identified by immunocytochemical staining with a monoclonal mouse antibody NF-200 (Sigma). Coverslips were rinsed three times in 0.1 M phosphate-buffered saline (PBS pH 7.4) and fixed for 20 min at room temperature with 4 % paraformaldehyde in PBS. After rinsing in PBS three times, cells were permeabilized for 5 min with 0.5 % Triton X-100 in PBS. After rinsing in PBS, non-specific sites were blocked and cells permeabilized with 1 % BSA, 0.5 % TritonX-100 for 20 min in PBS. Cells were incubated with mouse anti-NF-200 primary antibody overnight at 4 °C, at a dilution of 1:200, in a humidified chamber. After washing three times in PBS, Cells were incubated for 30 min at 37 °C in a Goat Anti-Mouse secondary antibody (Fluorescein-Conjugated ImmunoPure IgG, Life Technologies), diluted 1:100. For the

color reaction with diaminobenzidine, the ABC (avidin-biotin-horseradish peroxidase complex) method was used. Cells were incubated for 20 min at 37 °C in a Goat-Anti-Mouse secondary antibody (Biotin-Goat anti-Mouse IgG, Life Technologies), followed by incubation with ABC complex. Photographs were captured by an Olympus TH4-200 Fluorescence microscope and imaged with a CCD camera and IMAGE-PRO Discovery software.

Comparisons of different methods of primary pelvic ganglion neuron culture

To compare the advantages and disadvantages of our method with those published by Tuttle and Steers (1992) and by Keast (1992), duration of digestion, estimated yield, coating substance, culture media, plating density, maximum length of cell survival were assessed. The prevalence of duration of digestion, estimated yield plating density and maximum length of cell survival were expressed as numeric variance, while coating substance and culture media were represented as described variance.

Result

To isolate and culture major pelvic ganglion neurons from adult rat, we adapted a protocol that had been developed to isolate hippocampal neurons from adult rat (Brewer 1997). In early experiments, neuron yields varied from experiment to experiment depending upon the dissociation technique. The application of enzymatic treatment to break down the association between neurons and surrounding cells was essential. Adult neurons required different enzyme treatment compared to embryonic or perinatal neurons. The use of collagenase and extended incubation times are required for the successful dissociation of adult MPG neurons. Further improvement was achieved by using sequential trituration to remove the majority of viable neurons. More than 15 triturations reduced markedly the survival rate of viable neurons. To quantify the reproducibility of our protocol, we measured the number of dissociated neurons before plating. As displayed in Table 1, the duration of digestion took only 1 h with our method. While by using Tuttle and Keast's methods, this process was beyond 2 h. In addition, the yield of our method was

Table 1 Comparisons of different methods of primary pelvic ganglion neuron culture

Protocol	Our method	Tuttle and Steers	Keast
Duration of digestion	1 h	2 h	2 h
Estimated yield	30–40 %	70 %	Unknown
Coating substance	Poly-L-lysine	Rat-tail collagen, laminin	Poly-ornithine, laminin
Culture media	Neurobasal, B27	DMEM, FBS, HS, chick embryo extract	Neurobasal, B27
Plating density	$\approx 250/\text{cm}^2$	$500/\text{cm}^2$	$\approx 1,500$ per coverslip
Maximum length of cell survival	2 weeks	More than 1 week	More than 1 week

about 2,000 neurons per well ($250/\text{cm}^2$), and the average yields of adult MPG neurons represented approximately 30–40 % of the total pool of MPG neurons in the rat. Tuttle reported that the yield after dissociation was 70 % for adult female rat. However, their protocol required fetal bovine serum, horse serum and chick embryo extract in the culture medium. By comparison, our yield after dissociation, as was expected for adult neurons, was highly reproducible under serum-free conditions. While cell attachment might be improved by poly-ornithine or

laminin, the simple culture conditions described in our method allowed the viability of neurons to be maintained for about 2 weeks. The achieved maximum length of cell survival was more than 1 week using the methods used by Tuttle and Keast

We found that neurons dissected from adult rat MPG readily adapted to cell culture. Figure 1 shows several examples of MPG neurons in culture under phase contrast microscope. We observed that MPG neurons have a rather wide size distribution in vitro. The somas were normally ovoid or fusiform but not

Fig. 1 Characteristics of major pelvic ganglion neurons from adult rats after dissociation in culture when cells were isolated using the described methodology. Phase contrast images are shown in (a–d). **a, b** Note Neurons had bright, rounded-up cell bodies with few but long processes, which extended much

longer than the length of the cell soma. After 5 days in vitro with exposure to B27/Neurobasal medium + 20 ng/ml NGF. **c, d** Note growing neurons exhibiting elaborate neuritic trees. After 8 days in vitro with exposure B27/Neurobasal medium + 20 ng/ml NGF. Scale bar 100 μm

Fig. 2 Major pelvic ganglion neurons stained with the NF-200 antibody. These neurons **a–c** were cultured for 7 days. **a** Cultured neuron, with the neuronal marker NF-200 stained

dark brown with a color reaction of DAB. **b, c** Cultured neurons identified by fluorescent labeling of NF-200, which defined the cell soma and extensive neuritic processes. Scale bar 100 μ m

exclusively so. MPG neurons usually exhibit one or no dendrite and one axon in vivo (Wanigasekara et al. 2003), but we observed that the cultured neurons did not retain the same appearance as that of neurons in vivo. Some of the neurons in culture were associated with several primary neuritic processes, secondary and tertiary neuritic processes branched or were observed as well. The branching processes exhibit typical morphology of rat peripheral neurons, representing numerous varicosities along the process length.

We also observed that adult MPG neurons tended to aggregate together rather than to grow as single cells. This tendency implied the possibility that the growth of adult neurons might be dependent on secreted factors from neighboring cells. In addition, the cells might also express adhesion factors at the surface (eventually in dependence of the culture medium) leading to aggregates. Thus, attachment and survival of adult neurons might be improved by reducing the volume of medium during culturing or by increasing the cell density during plating. In our studies, the MPG neurons were initially placed in the minimum amount of medium so that they could attach as soon as possible.

In our study we added 20 ng/ml NGF to the feeding medium, which is known to protect cultured neurons and stimulate neurite outgrowth (Wanigasekara et al. 2003). With supplementation of NGF, we observed that a larger fraction of neurons survived, and that a greater extent of processes grew than those without NGF (not shown).

Cytoskeletal components were used as specific markers to distinguish neurons from glia. NF-200 is a common intermediate filament protein restricted to

neurons. In our studies, the MPG neurons were identified by immunostaining with antibody directed against NF-200 and by their appearance. Staining with this antibody revealed the entire extent of the neuron and its processes (Fig. 2). The presence of such neurons provided an on-going confirmation of cell viability and morphology before the cells were fixed and immunostained for the expression of NF-200. In addition to these MPG neuron-like cells, we also observed the presence of astrocytes. The MPG neurons grew on the layer of astrocytes. Furthermore, the MPG neurons could be readily distinguished from the bed-layer of astrocytes by their morphological characteristics.

Discussion

Adult neurons have been grown earlier in culture either as explants (Cowen et al. 1997) or as dissociated cells in the presence of serum and non-neuronal cells (Uchida and Tomonaga 1985). In this study, we developed a new protocol for the dissociation and culture of major pelvic ganglion neurons from adult rats under serum-free conditions. As summarized in Table 1, in comparison to the treatment with collagenase and trypsin at the same time (Tuttle and Steers 1992; Wanigasekara and Keast 2005; Nangle and Keast 2011), our digestion procedure improved the speed greatly. Tuttle reported that the yield after dissociation was 70 % for adult female rat. However, their protocol required fetal bovine serum, horse serum and chick embryo extract in the culture medium (Tuttle and Steers 1992). By comparison, our yield after dissociation was approximately 30–40 % under serum-free conditions. As was expected for adult

neurons, our yield was highly reproducible. While cell attachment might be improved by poly-ornithine or laminin, the simple culture conditions described in our method allowed the viability of neurons to be maintained for about 2 weeks, which should be sufficient for most cytochemical, electrophysiological and neurotoxicological studies.

The most important steps that appear to determine the extent of viability are the enzyme digestion regime. The use of collagenase and extended incubation times are vital for the successful dissociation of adult MPG neurons. We found that the purity of the cultured neuronal cells was depended on the effective treatment of protease prior to plating, and the careful washing of the attached cells. Shorter enzyme incubation period and lower enzyme concentration resulted in dirty cultures containing glia cells, but no neurons survived after plating. The requirement for different digestion and dissociation regimes for adult neurons is likely to reflect changes in the composition of the ganglionic extracellular matrix with increasing age. Adult dissociated neurons were more tightly bound to the ganglion matrix than younger neurons. As a result, these adult dissociated neurons not only changed the matrix, their length and complexities of their dendritic arborisations were also increased (Andrews et al. 1996).

The trituration procedure might be another critical factor to the loss of viability. The proportion of viable neurons obtained was a crucial issue in the *in vitro* studies. When cells were triturated with more than 15 strokes, the survival rate of adult MPG neurons was reduced markedly. So we removed the MPG neurons at each stage by decanting the supernatant medium and replacing it with fresh medium before the next sequence of triturations. It is important to avoid foaming during trituration, as cells at an air–liquid interface can be lysed (Ren and Miller 2003). The procedure described of sequential trituration increased the yield significantly compared to the conventional trituration methods.

During isolation of adult MPG neurons, some cells were lysed and inevitably produced debris. Survival and regeneration of axons and dendrites were inhibited by large amounts of cellular debris produced during the dissociation procedure (Brewer et al. 2001). Debris could further interfere with cell attachment. We found that cells were able to attach to the coverslips within

the first 2 h. Therefore, medium should be aspirated from the plate 2 h later after plating in order to optimize cell survival and to improve yield. For adult MPG cells, the amount of neurons decreased dramatically in dishes that were either left unwashed or washed 24 h later after plating, demonstrating the importance of removing debris.

The reasons of the improved yield and survival of the MPG neurons need further study. The Neurobasal/B27 medium may be part of the explanation. The antioxidant components of B27-catalase, glutathione, superoxide dismutase, and vitamin E have been proven to be essential for the long-term survival of cultured cortical neurons (Perry et al. 2004). The presence of these antioxidants might thus account for the improved yield over physiological saline as isolation medium. Furthermore, specialized media with altered osmolarity can reduce neuronal death of adult neurons. Our work with Neurobasal/B27 culture medium suggests that this medium is favorably beneficial not only to the maintenance of cell viability, but also to the growth-stimulating effect of the neurons.

For their survival, developing neurons depend on a wide variety of neurotrophic factors, produced either by their target tissue or by other neighbouring cell type (Henderson 1996). Using proper neurotrophic substances may be beneficial for the survival and neurite outgrowth in cultures derived from adult animals. Tuttle and Steers have shown that nerve growth factor (NGF) can enhance the survival and neurite outgrowth of MPG neurons derived from adult rats (Tuttle and Steers 1992). So in our study, we used NGF to enhance the survival and neurite outgrowth of different cell populations. Our results demonstrated that some of adult MPG neurons remained responsive to exogenous growth factors.

In conclusion, our culturing conditions described above allowed good yields of healthy adult MPG neurons with fewer steps, less labor, and at lower costs. It is desirable to develop a reliable protocol for the culture of adult MPG neurons. Our study may facilitate new studies on the development and extent of plasticity of the adult MPG under well-controlled conditions.

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