Monolayer cultures of perikarya isolated from postnatal rat cerebellum

D.N. Currie[1], G.R. Dutton[2] and J. Cohen[3,4]

Brain Research Group, Department of Biology, The Open University, Milton Keynes MK7 6AA (England), 16 May 1978

Summary. Viable cerebellar perikarya of mixed cell type obtained from 7-9-day-old rats were maintained in monolayer culture for up to 12 days. During this time extensive neurite formation and outgrowth occurred. The large majority of the cells developing in culture were tentatively identified as granule neurons. This identification was based on the large number isolated from the starting tissue, and the cells' general morphological features in culture such as perikaryal and nuclear size, the bipolar nature of neurite extension, and their migratory behaviour.

We demonstrated in previous work that it is possible to obtain high yields of perikarya[5] representing mixed cell types from postnatally developing rat cerebella[6]. These preparations, from rats up to 14 days of age, were largely debris free, and the cells retained a functional and ultrastructural integrity comparable to that of the intact tissue[7]. Here we report on the survival of this preparation in monolayer culture, and give evidence for a tentative identification of the major cell types obtained.

Materials and methods. Perikaryal suspensions were prepared from rat cerebella (Wistar CFHB) as previously described[7]. Briefly, this involved the sequential treatment of chopped tissue with trypsin (0.025%, 15 min, 37 °C), trypsin inhibitor, and EDTA in Krebs Ringer buffer solutions containing BSA and glucose, followed by trituration of the tissue blocks in the presence of DNAse and centrifugation through a layer of 4% BSA at low speed ($100\times$ g). Cell numbers were determined using a model ZB Coulter Counter (Coulter Limited, U.K.).

Monolayer cultures were prepared from cerebellar perikarya resuspended in Eagle's minimal essential medium (MEM, Gibco-Biocult) containing 2.5% chick embryo extract (Flow Labs. Ltd, U.K.), 10% fetal calf serum (Gibco-Biocult, U.K.), and 33 mM glucose. Cells were counted, transferred to 35 mm Falcon plastic petri dishes (2×10^5 cells/cm^2) and incubated at 37 °C in an H_2O-saturated atmosphere of 95% air/5% CO_2. Gentamycin (100 µg/ml) (Flow Labs. Ltd) was used to prevent bacterial growth[8,9]. Fluorodeoxyuridine (FUdR, 8×10^{-5} M, Sigma) was added to cultures after 24 h to suppress the proliferation of the dividing non-neuronal cells[10]. In some experiments polylysine (Type I-B, mol.wt 230,000, Sigma) coated plastic petri dishes were used[11]. Medium changes were made every 3-4 days. Optimal growth conditions were assessed by maximizing length of survival time, extent of neurite formation and 'plating efficiency'. Plating efficiency was defined as the amount of DNA in cells adhering to the substrate after 24 hrs divided by the amount of DNA in cells present at zero-time. DNA analyses were performed by the method of Zamenhof[12] on cultures grown in 6-cm plastic petri dishes (Falcon).

Results and discussion. Immediately after isolation cerebellar perikarya from 7-9-day-old rats were spherical and varied in diameter from about 7 to greater than 20 µm (figure a). 80% were in the size range of 7-10 µm. As previously reported, over the age range studied here (7-9 days) total yields and the relative proportions of the perikaryal sizes were very similar[6]. Thus, the starting material was considered to be equivalent in all these experiments.

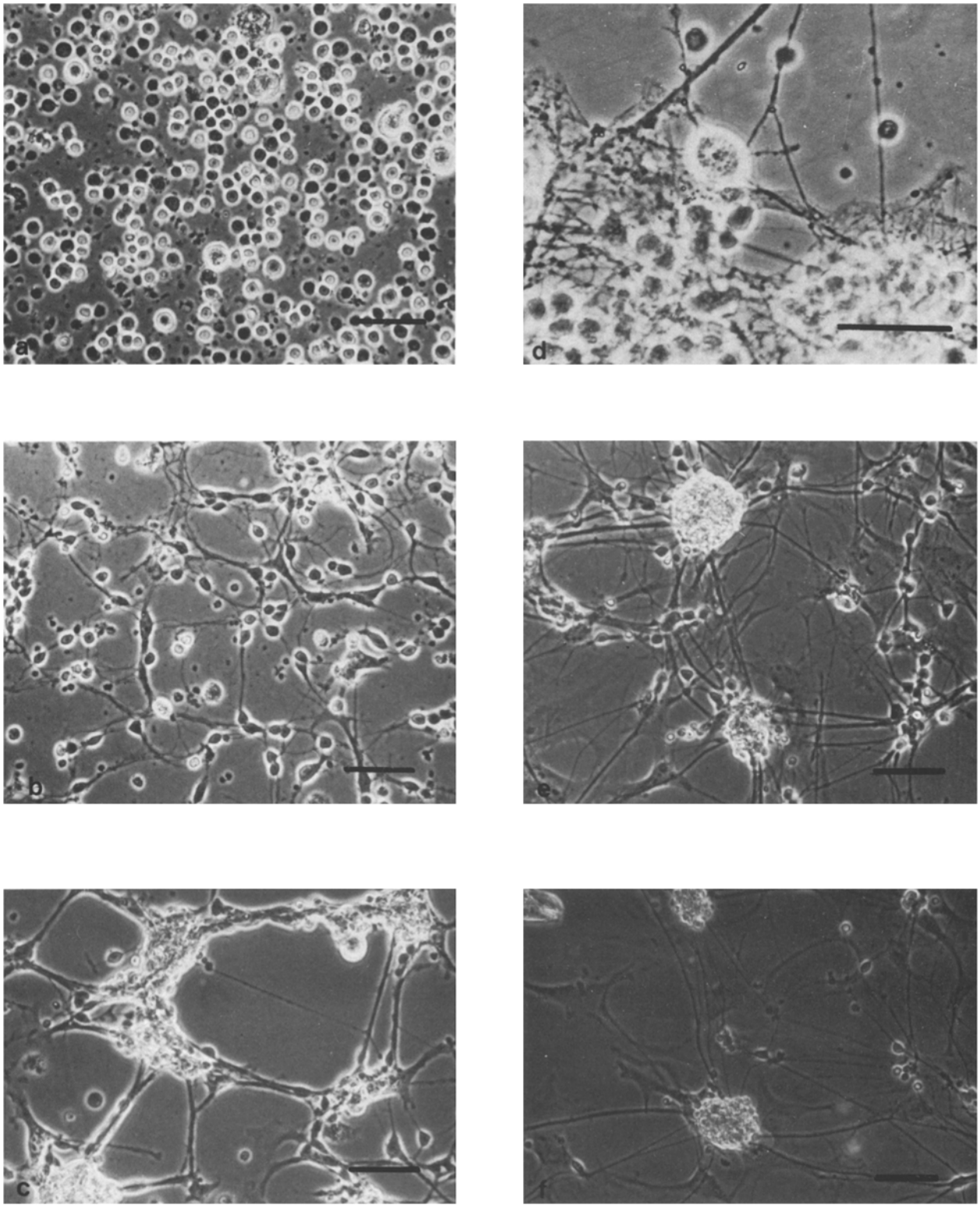
Cells adhered to and extended neurites on plastic or poly-L-lysine[12] substrates giving plating efficiencies of 40.7% (mean of 2 experiments) and 88.2 ± 2.5 (4 experiments, $\pm$SEM), respectively. By 48 h in vitro an extensive array of neurites had formed between cell bodies on both plastic (figure c) and poly-L-lysine (figure b) substrates. However, the extent of initial cell aggregation during this period was considerably greater in cultures growing on plastic (figure c), and largely involved perikarya of 7-10 µm in diameter. Poly-L-lysine initially prevented this phenomenon (compare figures b and c), but the difference persisted for only a few days. The extent of aggregation was the same in 12-day cultures grown on poly-L-lysine (figure f) and in 8-day cultures on plastic (figure e). Thus, the extent of cell aggregation increased with increasing time in culture, and beyond 1 week became independent of the substrate used.

In other studies this cellular migratory process has been clearly observed using time-lapse cinematography[13,14]. Movement of the cell body appeared as an active translocation along its bipolar processes. These movements may be analogous to the in situ migration of the granule cell body down a leading cell process from the external to the internal layer of the developing cerebellar cortex[15], and thus may reflect an inherent property of this cell type. After about 1 week in culture (and thereafter), the neurites associated into large bundles to form thicker, cable-like structures (compare figures b and e). Also during this period, the soma of these neurite-forming cells remained rounded. Other cells, representing a small proportion of the total population, spread and flattened to form a layer upon which the neurite-forming cells appeared to rest (figures e and f). When cells were cultured in the absence of FUdR, there was a rapid proliferation of these flat[9] non-neuronal cells which dominated the culture within 7-10 days (not shown). The overgrowth by these cells was accompanied by the loss of most of the neurite-forming cells. However, with the addition of FUdR at 24 h, as used in all experiments shown here, the flat cells did not proliferate.

The identity of the flat cells has not yet been determined unambiguously. However, preliminary experiments suggest that they have a high affinity uptake system for γ-aminobutyric acid (GABA) which is inhibited by β-alanine. A preliminary report of this has appeared[16]. These observations, aside from their general morphological characteristics, would suggest they are glial cells. The vast majority of the flat cells are perhaps astrocytes, and as yet we have seen no evidence of myelination occurring in the older cultures.

Because their size, general bipolar morphology, and number are comparable to those seen in the external layer of immature cerebellum in situ, the small diameter neurite-forming cells were tentatively identified as immature granule neurons differentiating in culture. It is not yet known whether the tendency of these small neurite-forming cells to form aggregates is a reflection of their natural migration from an external to an internal position in the intact developing cerebellum. The larger perikarya (~ 20 µm), seen clearly in the total cell suspension at zero-time (figure a), are only infrequently seen growing in cultures of cells isolated from animals of more than 6 days of age (figure d). These large perikarya represent 1-3% of the total number isolated from 7-9-day rats, and some may be Purkinje cells which are thought to be present in the starting cell suspension[6-7].

In the present study we have extended our earlier results by demonstrating the ability of perikarya obtained from postnatally developing rat cerebellum to survive and develop in monolayer culture for up to 12 days. We believe that this



a Freshly prepared total cell suspension (TCS) of cerebellar. Perikarya isolated from 8-day-old rats. Over the age range shown in these figures (7–9 days) the size distribution and yield of perikarya were the same. All cultures contained FUdR at 24 h, see 'materials and methods' for details. Bar represents 50 µm in all frames. *b* TCS, 2 days in culture. Cells isolated from 9-day-old animals and grown on polylysine-coated plastic. *c* TCS, from same experiment as *b*, but cells grown on plastic. Note higher degree of aggregation compared to cells in *b*. *d* Large cell growing in 3-day culture of TCS from 7-day-old rats. Plastic substrate. *e* TCS, 5 days in culture growing on plastic from 8-day-old rats. Note increased aggregation compared with younger cultures (*b* and *c*). *f* TCS grown 12 days in culture. Cells from 8-day-old rats. Polylysine substrate.

system represents a well-defined and reliable starting material for the in vitro study of the developmental neurobiology of the cerebellum. These studies are our first steps towards establishing cultures of more purified, and better identified single cell types of the developing cerebellum.

1 D.N. Currie was supported by a grant from Hoechst Pharmaceuticals, Ld, U.K.
2 To whom correspondence should be addressed.
3 J. C. was supported by an S.R.C. Grant to S.P.R. Rose, present address: Medical Research Council Neuroimmunology Project, Department of Zoology, University College London, U.K.
4 The authors would like to thank Kathleen Tear for excellent technical assistance and members of the Brain Research Group for helpful discussions.

5 The terms perikarya and cells, while not strictly equivalent, are used interchangeably here.
6 J. Cohen, R. Balazs, F. Hajos, D.N. Currie and G.R. Dutton, Brain Res. *148*, 313 (1978).
7 G.P. Wilkin, R. Balazs, J.E. Wilson, J. Cohen and G.R. Dutton, Brain Res. *115*, 181 (1976).
8 A. Rudin, A. Healey, C.A. Phillips, D.W. Gump and B.R. Forsyth, Appl. Microbiol. *20*, 989 (1970).
9 T.W. Schafer, A. Pascale, G. Shimonaski and P.E. Came, Appl. Microbiol. *23*, 565 (1972).
10 E.W. Godfrey, P.G. Nelson, B.K. Schrier, A.C. Brener and B.R. Ransom, Brain Res. *90*, 1 (1975).
11 E. Yavin and Z. Yavin, J. Cell Biol. *62*, 540 (1974).
12 S. Zamenhof, H. Bursztyn, K. Rich and P.J. Zamenhof, J. Neurochem. *11*, 505 (1964).
13 D.N. Currie and G.R. Dutton, Trans. Am. Soc. Neurochem. Abstr. *8*, 181 (1977).
14 E. Trenkner and R.L. Sidman, J. Cell Biol. *75*, 915 (1977).
15 J. Altman, J. comp. Neurol. *163*, 427 (1975).
16 D.N. Currie, K.L. Fields and G.R. Dutton, 6th int. Meet. int. Soc. Neurochem., Abstr. *6*, 365 (1977).

Unexpected features of the interaction between individual primary afferents and spinal motoneurones

A.I. Shapovalov[1] and B.I. Shiriaev

Laboratory of Physiology of the Nerve Cell, Sechenov Institute, Leningrad (USSR), 18 September 1978

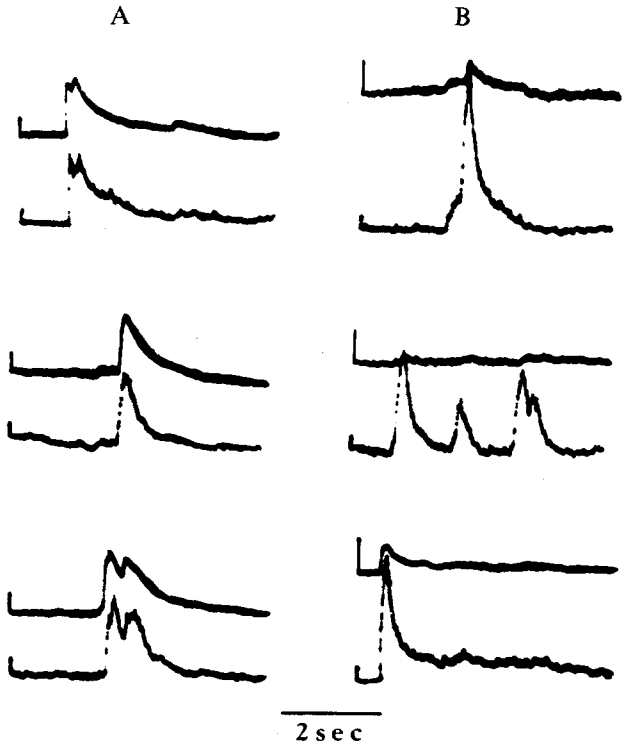
Summary. Simultaneous intracellular recording from spinal motoneurone and dorsal root fibres afferent to it in the isolated amphibian cord revealed a specific correlation between the slow spontaneous depolarizing activity in motoneurones and primary afferents.

It has recently been found in the isolated frog spinal cord preparation that in Mg^{2+}-free Ringer's solution the ventral roots exhibit high fluctuating levels of spontaneous activity and poor response stability to stimulation of the dorsal roots[2]. We developed a method which allows simultaneous microelectrode penetration of ventral horn motoneurone and dorsal root fibres afferent to it, and found that both elements reveal spontaneous graded depolarizing activity. We present here evidence that there is a specific correlation between the spontaneous activity in motoneurones and individual primary afferent fibres. This correlation may reflect an important communication mechanisms between neuronal ensembles within the spinal cord.

Isolated hemisected spinal cords from *Rana ridibunda* were prepared as described elsewhere[3] and continuously perfused with Ringer's solution of the following initial composition (standard saline) in mM: NaCl 98.0; KCl 2.0; $CaCl_2$ 1.8; $MgCl_2$ 0.01–0.05; NaH_2PO_4 1.2; Na_2HPO_4 2.0; $NaHCO_3$ 6.0; glucose 5.5; pH 7.4–7.6.

One microelectrode filled with 3 M KCl was used to impale a primary afferent fibre located in the dorsal root entry zone, and a second microelectrode filled with 3 M KCl or 2 M K-citrate was inserted into a motoneurone. A pen writer provided a continuous record of membrane potential fluctuations which were also photographed from a double-beam oscilloscope.

When the spinal cord was perfused with the standard solution, slow spontaneous potential fluctuations were waxing and waning at irregular intervals with a mean frequency of 2–4/sec. These fluctuations could be detected practically in all impaled motoneurones. The shape of the observed fluctuations was usually complex and unstable and their amplitude ranged from a few mV to over 30 mV. The maximal size of depolarizing fluctuations was observed in motoneurones with resting membrane potentials over



Examples of the spontaneous depolarizing potentials recorded from the same motoneurone (lower traces) and 2 different dorsal root fibres afferent to it (upper traces). A: records from the fibre establishing monosynaptic contact with the motoneurone. B: records from the fibre establishing polysynaptic connections with the motoneurone. Calibration pulse 1 mV precedes each record. Note different voltage gain in A and B.