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Short Report

Large nerve cells with long axons in the granular layer and white matter of the murine cerebellum

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ABSTRACT

The murine cerebellum was investigated by light microscopy using an improved modification of Ehrlich's methylene blue supravital staining technique. The dye exhibited a special affinity for the perikarya as well as the axons of Purkinje cells. In addition, large fusiform or stellate nerve cells which were characterised by long descending axons were seen to be distributed diffusely within the granular layer and the subcortical white matter. These findings indicate the existence of a 2nd type of projection neuron besides the Purkinje cells and are therefore in full accordance with older neuroanatomical observations based on silver impregnation. When correlated with recent studies on the occurrence of different calcium-binding proteins, the results show that the large perikarya demonstrated immunohistochemically within the granular layer seem to belong to the group of methylene blue positive neurons. Nevertheless, the definitive association of a single neuron with a nerve cell class is only possible if the axon is stained and clearly identifiable. Because of its selectivity for a special type of nerve cell, including its axon, the histological method used in this study may therefore also be suitable for investigating other parts of the brain and the spinal cord.

Key words: Mouse; methylene blue; neurons; Purkinje cells.

INTRODUCTION

Golgi (1883), Retzius (1892) and Koelliker (1896) were the first to demonstrate fusiform and stellate nerve cells with long descending axons within the granular layer and white matter of the cerebellum in different species. Subsequently, a closer investigation of these neurons was carried out by Cajal (1911); he was able to trace the descending axons for a considerable distance in the white matter. All these studies were based on the Golgi silver impregnation method. Later, Estable (1923) confirmed these results by impregnation of neurofibrils. These data were verified in man by Jakob (1928; see also Jansen & Brodal 1958). These findings suggested the existence of a 2nd type of projection neuron besides the Purkinje cells in the cerebellar cortex. In later studies, however, these neurons received less attention (Eccles et al. 1967; Palay & Chan-Palay, 1974). Nevertheless, Braak (1974) showed by an improved neurolipofuscin staining technique that relatively high numbers of large perikarya are present in the granular layer of the human cerebellar cortex and the white matter. Furthermore, Braak & Braak (1983) demonstrated by silver impregnation that some of these neurons represent large fusiform or stellate cells in addition to the typical Golgi cells. Unfortunately the impregnation of the axons ceased close to the soma in that study. The aim of the present study was therefore to investigate these neurons more closely, especially the shape and course of their axons. For this purpose, Ehrlich's methylene blue (MB) supravital staining technique was chosen (Ehrlich, 1886). In the past, this method was repeatedly applied for studying the cerebellar cortex (Dogiel, 1896; Turner & Hunter, 1899; Cajal, 1896, 1911; O'Leary et al. 1968). The reason for its use here was that MB exhibits an exceptionally high affinity for the Purkinje cells and their axons, as Cajal (1896, 1911) and O'Leary et al. (1968) showed. It was thus decided to establish

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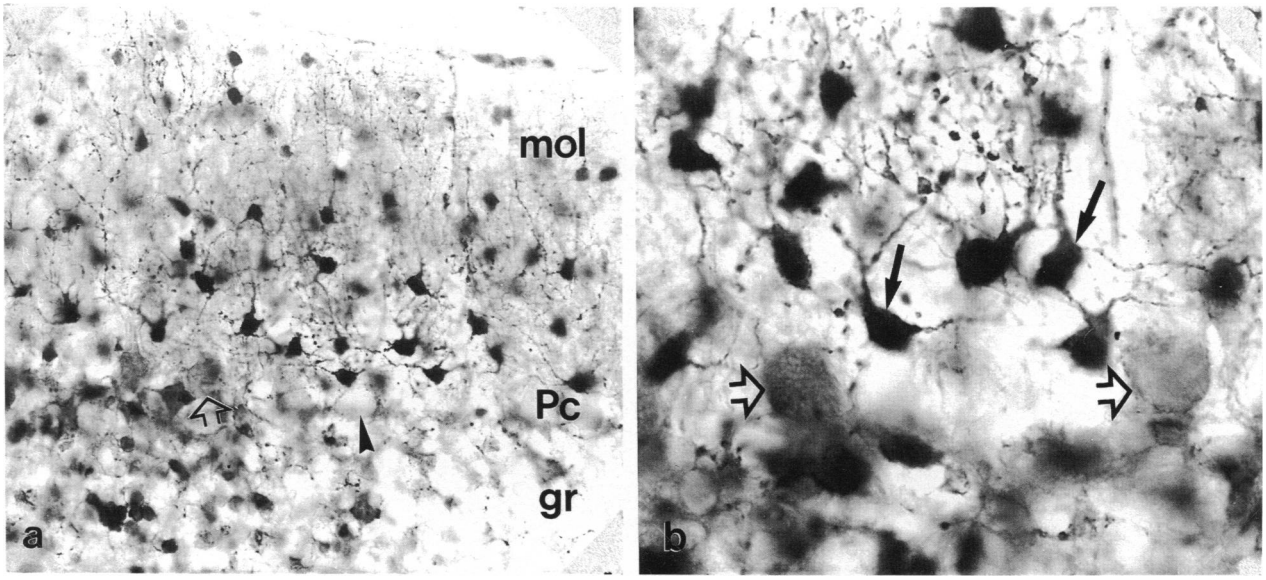

Fig. 1. Murine cerebellar cortex after supravital injection of MB; paraffin section obtained from the superficial region of a brain slice. (*a*) Due to the influence of the ambient oxygen in the room air on the dye's binding behaviour, only the intrinsic interneurons are intensely stained within both the molecular layer (mol) and the granular layer (gr); the characteristic projection neurons of the Purkinje cell layer (Pc) are only weakly stained (open arrow) or unstained (arrowhead). × 190. (*b*) Higher magnification showing the weakly stained Purkinje cells in detail (open arrows); 2 basket cells, from which fine processes radiate, are indicated by arrows. × 400.

whether the existence of these large neurons with long axons could be verified within the granular layer and also the cerebellar white matter by MB supravital staining.

MATERIALS AND METHODS

Thirty adult mice (*Mus musculus*) were killed by diethyl ether. Immediately thereafter, about 2 ml of an aqueous warm methylene blue solution (40 °C; filtered before use) were injected into the left cardiac ventricle until the skin became blue. The dye was administered at a concentration of 20 %. After 1 h at room temperature (20 °C), the cerebelli were removed, cut into approximately 1 mm slices with a razor blade, and exposed to air in a moist chamber for 1 h at room temperature. This led to blueing of the labelled neurons, since the dye had been reduced to its colourless leuco-form before passing the blood brain barrier and therefore had to be reoxidized (Becker & Quadbeck, 1952).

Further treatment of the tissues was carried out as suggested by Müller (1992). First, fixation was performed for 5 h at 4 °C (stock solution: 100 ml of a 10 % aqueous ammonium heptamolybdate solution with 10 drops of 25 % hydrochloric acid). After a short wash in distilled water for 10 min, a 2nd fixation took place for 2 h at 4 °C in a mixture of 2 % paraformaldehyde and 1.75 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 1.75 % phosphomolybdic acid (final pH 3.0). The specimens were then washed overnight in distilled water and finally dehydrated in 100 % tertiary butanol (melting point 25 °C; changed 3 times) for 24 h. After storage for 1 h in a mixture of 8 parts decahydronaphthalene (Dekalin, Chroma, Köngen, Germany) and 2 parts methyl benzoate, the tissues were transferred through 100 % decahydronaphthalene for a further hour before embedding in paraffin and sectioning. Sections (15–20 µm) were mounted on untreated glass slides and dried. Finally, they were coverslipped with DePeX and viewed under a light microscope.

Quantitative investigation was restricted to the Purkinje cells within their characteristic layer and the large nerve cells with long axons within the granular layer. In total, about 23 700 stained cells were counted in sections which had been selected randomly from the whole available material.

RESULTS

The light microscopic investigation revealed that at a depth of about 200 µm from the surfaces only the intrinsic interneurons were intensely stained. The Purkinje cells were weakly stained (Fig. 1). In the deeper regions of the brain slices, the staining pattern was the reverse. This discrepancy in the staining pattern between superficial and deep regions was almost certainly due to the dependence of the dye's binding behaviour on the influence of the ambient

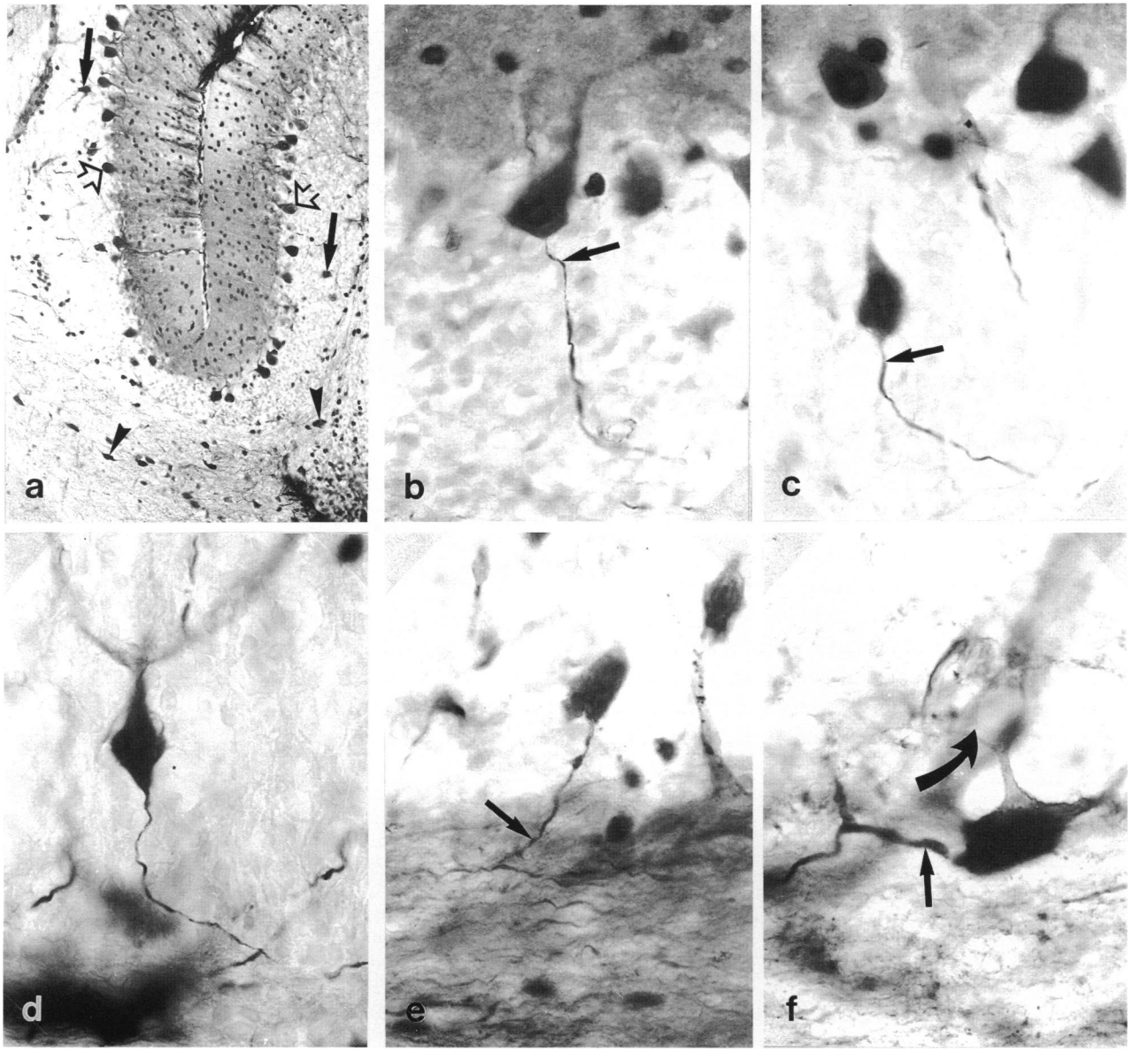

Fig. 2. Mouse cerebellum stained supravitally with MB; paraffin section obtained from the deeper regions of the tissue blocks. (*a*) Two lobules of the cerebellar cortex meet with their surfaces in apposition. The open arrows point to Purkinje cells. Additionally, large neurons are also visible within the granular layer (arrows) as well as the white matter (arrowheads). × 75. (*b*) A Purkinje cell with its long axon extending through the granular layer (arrow). × 400. (*c*) A large fusiform neuron, from which a descending axon (arrow) arises, is situated in the outer zone of the granular layer. × 400. (*d*) A fusiform neuron located in the middle of the granular layer. × 400. (*e*) A large nerve cell in the inner region of the granular layer; the axon extends into the dense fibre network of the white matter (arrow). × 400. (*f*) A large stellate perikaryon in the subcortical lamina of the white matter; the lumen of an adjacent weakly stained capillary is marked by a curved arrow. The axon is indicated by an arrow. × 550.

oxygen in the room air (Ehrlich, 1886; see also O'Leary et al. 1968). Besides the Purkinje cells, some large neurons within the granular layer and the white matter, including their long axons, were characterised by intense MB uptake (Fig. 2). In addition, many myelinated nerve fibres in the white matter were stained. The neuropil of the molecular layer was stained blue, but not as intensively as the Purkinje cells and the other large neurons (Fig. 2*a–c*). In general terms, there was some similarity to the images obtained by the immunohistochemical demonstration of the calcium-binding proteins parvalbumin and calbindin D-28k (see Discussion). The large cells in the outer regions of the granular layer were mainly fusiform (Fig. 2*c–e*), whereas in the inner zones, as well as in the white matter, the stellate form predominated (Fig. 2*f*). At the level of the Purkinje cell layer, a clear differentiation from the Purkinje cells was not always possible. Like the Purkinje cells, the dendrites of these cells were only weakly stained;

however, their axons showed a high affinity for the dye. Within the cerebellar cortex, the number of the solitary neurons (1100) amounted to about 5% of the Purkinje cells (22600). The projection area of their axons remained unclear.

DISCUSSION

The most interesting result of this study was the observation that large fusiform and stellate nerve cells with long descending axons definitely exist in the granular layer as well as in the white matter of the murine cerebellum in significant numbers. This fully confirms early neuroanatomical descriptions which were based on silver impregnation. In comparison with those silver studies, however, the main advantages of the MB procedure that was applied in the present investigation were that the long axons were intensely stained and nearly all projection neurons were demonstrated in a single section. Subsequent counting of these neurons thus became possible and showed that these cells occur in the granular layer about half as frequently as the Golgi interneurons. Eccles et al. (1967) reported a ratio of about 1 Golgi cell per 10 Purkinje cells. These values indicate a greater importance of the described cells contrary to the estimations of Palay & Chan-Palay (1974) that these neurons represent very rare variations.

Additionally, the present results have to be compared with those of recent immunohistochemical studies on the distribution of different calcium-binding protein containing neurons in the cerebellum. Those investigations exhibited uncertainty as to the identity of stained solitary perikarya within the granular layer because their axons were not visible; Garcia-Segura et al. (1984) (rats), Fournet et al. (1986) and Scotti & Nitsch (1992) (human), interpreted them as Golgi neurons. Braun et al. (1986) (birds) were in doubt as to whether these cells should be considered as ectopic Purkinje cells or large Golgi cells. Furthermore, Stichel et al. (1986) (cats), Rogers (1989) (chicks and rats) and Celio (1990) (rats) denied any immunoreactivity of Golgi neurons. But it is logical to conclude that these solitary cells possess functional similarities to Purkinje cells characterised by a high content of calcium-binding proteins. They therefore seem to belong to the group of fusiform and stellate cells with long axons displayed by MB staining. However, the term 'displaced Purkinje cell' as used by Rogers (1989) and Alonso et al. (1992) may not be completely wrong, since Cajal & Illera (1907), Schaffer (1913) and Estable (1923) demonstrated pericellular axonal baskets around large cell bodies of the granular layer through silver impregnation of neurofibrils. Nevertheless, their equivalence with Purkinje cells would only be acceptable if these neurons exhibited the same shape of the perikarya and the typical dendritic tree. In most cases, however, this is not so (see also Cajal 1911; Braak & Braak 1983), although transitional forms between the 2 cell types may exist, mainly at the level of the Purkinje cell layer. From their fusiform and stellate features, they should better be regarded as a 2nd class of projection neurons in addition to the Purkinje cells; their definitive projection area has still to be elucidated.

In summary, because of its demonstrated selectivity for special types of nerve cells and the intense axonal staining, the demonstrated modification of Ehrlich's old methylene blue technique seems also suitable for other regions of the brain and the spinal cord.

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