Histol Histopathol (1998) 13: 1019-1026
DOI: 10.14670/HH-13.1019
http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Methylene blue supravital staining: an evaluation of its applicability to the mammalian brain and pineal gland

T. Müller
Institute for Anatomy, University of Mainz, Germany

Summary. Methylene blue supravital staining of mammalian brain reveals typical staining patterns in different brain regions. Within the cerebellum of the mouse, the dye showed a peculiar affinity for the somata and the axons of Purkinje cells. Additionally, large polymorphic neurons characterized by long descending axons were detected within the granular layer and the white matter. These cells might represent another type of projection neuron. In the stratum pyramidale and stratum oriens of the murine hippocampus, a subpopulation of non-pyramidal cells, i.e. intrinsic interneurons, were selectively stained. Additionally, a labelling of perineuronal nets of extracellular matrix covering single cells could be achieved; this phenomenon might be due to the occurrence of strong anionic residues which attract the cationic dye. Therefore, perineuronal nets might also trap other cations and play an important role in the control of cell excitability. The electron microscopic investigation revealed drop-like dye accumulations within the cytoplasm and a staining of material at the site of the plasma membrane. Throughout the pineal gland, a network of a subpopulation of polymorphic cells with manifold long processes could be visualized. Syncytial connections of cells seemed to occur. The oxygen-dependent selective staining is probably functionally connected with the generation of oxygen radicals and subsequent oxidative stress for the cells. This reaction indicates a certain vulnerability to hypoxia; therefore, the intracellular dye-uptake might be interpreted as an early sign of metabolic disturbance.

Key words: Phenothiazine, Antipsychotic, Nitric oxide, Calmodulin, Reactive oxygen

Introduction

Ehrlich (1886) was the first to report that the phenothiazine dye methylene blue (MB) stains supravitally neurons after intravascular injection, when the skulls are subsequently removed and the tissues are exposed to the air. Since MB is soluble in water and alcohols, the production of microtome sections was not possible until ammonium heptamolybdate was found to be the appropriate dye-precipitating reagent, i.e. fixative (Bethe, 1895). This technical advance enabled the first investigators to apply this staining procedure to the mammalian brain (Cajal, 1896a,b, 1897; Dogiel, 1896). Here, it has to be emphasized that Cajal's neuron-theory is also based on his early MB-studies. Due to the fact that the practical details of the method were only insufficiently handed down to the posteriority, later studies did not completely reach the high quality of the publications of Cajal and Dogiel (Feindel et al., 1949; O'Leary et al., 1968; Fukuda, 1971). Unfortunately, Cajal's and Dogiel's publications were merely illustrated with drawings. Thus, it was a scientific challenge to attack this problem again and to work out a simple and reliable staining and fixation technique for the production of microtome sections (Müller, 1990, 1996b).

Moreover, a method was developed by the author to visualize the accumulation sites of the dye in neurons also at the electron microscopic level (Müller, 1995; Müller and Reutter, 1995). The results should be compared with studies on the pharmacological effects of MB to the nervous tissue published in the last decade in large numbers. Meanwhile, MB finds common application as an inhibitor of cytosolic guanylyl cyclase, the physiological receptor of nitric oxide (Gruetter et al., 1981). Furthermore, it was established recently that MB can also be regarded as an direct inhibitor of nitric oxide-synthase (Mayer et al., 1993). The antipsychotic effect of the dye is also a topic of intensive research (Naylor et al., 1986); this phenomenon is explained by possible interactions of MB with the calcium binding protein calmodulin (Müller, 1992); the sequestering of calmodulin by the dye might also be followed by an indirect inhibition of nitric oxide-synthase since the enzyme's activity is calmodulin-dependent (Deutsch et al., 1997). But the intraneuronal distribution pattern of the dye appears to be more complicated than hitherto assumed (Müller, 1995; see also Results and Discussion). With special regard to these functional aspects, supravital staining with MB was applied to the

Offprint requests to: Dr. Thomas Müller, Institute for Anatomy, University of Mainz, Saarstr. 19-21, D-55099 Mainz, Federal Republic of Germany. FAX: (49) (61 31) 39 54 01

mammalian brain (Müller, 1992, 1994, 1995); the results of these publications are also discussed in the present review of the author's work done during the last years. In addition, interesting new findings are presented and discussed.

Materials and methods

Human principles of animal care were applied throughout these experiments and all procedures complied with the German law on the protection of animals. Adult mice (*Mus Musculus*) and golden hamsters (*Mesocricetus auratus*) were used for the experiments. The animals were killed by an overdose of inhaled ether. Immediately thereafter, about 2 ml of an aqueous, 37 °C warm dye solution (MB med. puriss., C.I. 52015; Chroma, Köngen, Germany) were injected into the left cardiac ventricle until the skin became blue. For the investigation of the hippocampus and the cerebellum, the dye was administered at a concentration of 20%. For the selective staining of the pineal gland, a MB-concentration of 3.5% was found to be best suited.

After 1 hour at room temperature (20 °C), the brains were removed, cut into approximately 1 mm-thick slices with a razor blade, and exposed to air in a moist chamber for 1 hour at room temperature (20 °C). This led to a blueing of the specimens, i.e. oxidation of leuco-MB, since the dye had been primarily reduced to its colourless form in situ.

Fixation and embedding in paraffin were realized in a modified manner according to the author's descriptions (Müller, 1994): the first fixation was performed at 4 °C (refrigerator) for 5 hours (stock solution: 100 ml of a 9% aqueous ammonium heptamolybdate solution with the addition of 9 drops of 25% hydrochloric acid and 0.9 ml 30% hydrogen peroxide). After a short rinse in distilled water, a second fixation took place for 2 hours 30 minutes at 4 °C [stock solution: 100 ml of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 1.8% phosphomolybdic acid and 0.1% hydrogen peroxide (final pH 5.0)]. Subsequently, the specimens were washed overnight in distilled water.

The tissues were dehydrated in 100% tertiary butanol (melting point: 25 °C) for 48 hours. The first alcohol change was performed after 15 minutes, the second after 1 hour and the third after 7 hours. For these three preliminary dehydration steps, phosphomolybdic acid was added to the alcohol in a concentration of 0.05%. The tissues were then transferred into pure tertiary butanol. After dehydration, they were stored for 1 hour in a mixture of 8 parts decahydronaphthalene (Dekalin®; Chroma, Köngen, Germany) and 2 parts methyl benzoate. Before being embedded in paraffin, they were immersed for another hour in 100% decahydronaphthalene.

20 μm-thick microtome sections were mounted on glass slides. After drying, they were deparaffinized in xylene and coverslipped with DePeX® (Serva, Heidelberg, Germany). Due to the thickness of the paraffin sections combined with the 3-dimensional structure of the neurons, it was focused and photographed in different planes. Photomontages were therefore required to visualize the shape and routes of the nerve cells including their processes.

Part of the stained hippocampi were embedded in Araldite® (Fluka, Buchs, Switzerlands) according to the procedure introduced by the author (Müller, 1995; Müller and Reutter, 1995): the first fixation was done at 4 °C for 2 hours 30 minutes [stock solution: 100 ml of 2% paraformaldehyde and 1.75% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 1.8% phosphomolybdic acid and 0.1% hydrogen peroxide (final pH 5.8)]. After a short rinse in distilled water overnight, a second fixation was performed in aqueous 5% aqueous ammonium heptamolybdate for 5 hours at 4 °C. After subsequent washing in distilled water overnight, a third immersion was performed in an aqueous mixture of 0.5% osmium tetroxide and 0.5% phosphomolybdic acid for 1 hour at 4 °C. The specimens were then washed overnight in distilled water. After dehydration in tertiary butanol, they were transferred to pure ethanol for 1 hour; the alcohol was changed several times. Then, the tissues were embedded in Araldite®. The ultrathin sections were viewed under the electron microscope without any counterstaining.

Results

Cerebellum

At a depth of approximately 200 μm from the surfaces, primarily the intrinsic interneurons were intensely stained. In deeper regions of the slices, the staining results were completely the reverse. These differences in the staining pattern were due to the dependence of the dye's accumulation on the oxygen which diffused into the tissue. In addition to the Purkinje cells, a few large neurons located within the granular layer and the white matter, including their long descending axons, showed a selective dye uptake (Figs. 1, 2). In addition, many myelinated nerve fibers of the white matter also became visible. The extracellular matrix of the molecular layer was stained, but not as intensively as the Purkinje cells. The interneurons of the molecular layer except for their nuclei remained unstained.

Hippocampus

In the superficial regions of slices which could be reached by the penetrating oxygen, a selective staining pattern was apparent under the light microscope. Strongly stained polymorphic nerve cells were seen in the stratum pyramidale and stratum oriens of the cornu Ammonis (Figs. 3, 4). In general, their dendrites were spineless. Their delicate varicose axons appeared to form networks primarily within the stratum pyramidale (Fig.

4). No selective staining of cells was obtained in the fascia dentata, whereas some labelled neurons were found to be loosely distributed within the subiculum.

Moreover, some non-pyramidal cells exhibited only a staining of perineuronal nets of extracellular matrix on the outer surfaces of their somata and proximal dendrites (Figs. 5-8).

The electron microscopic investigation of stained neurons revealed that MB had been transformed to an electron-dense precipitate. In addition to a homogeneous staining of the cytoplasm and chromatin, sites of special dye concentrations could be detected: MB had accumulated in the shape large of drop-like structures in the cytoplasm and was observed to be bound to material at the plasma membrane, which possibly belongs to the perineuronal nets (Fig. 6).

Pineal gland

Within the whole gland, a subpopulation of cells were stained (Figs. 9-14); many of them showed processes of different length and thickness. Due to the vascularization, the cells in the outer periphery of the organ appeared to be earlier stained than those in the centre. The cells showed a complicated arborization pattern of their processes. Partially, syncytial connections between cells seemed to occur (Figs. 11, 12).

Discussion

Staining pattern

From their fusiform and stellate features, the large neurons stained in the granular layer and white matter of the cerebellum should better be considered as another type of projection neuron than merely ectopic Purkinje cells (Müller, 1994). In general, there was some similarity apparent compared to the images obtained by the immunohistochemical demonstration of the calcium-binding proteins parvalbumin and calbindin D-28k (Celio, 1990; Müller, 1994).

The morphological characteristics of the stained hippocampal neurons indicate that these nerve cells have to be regarded as non-pyramidal cells. This sub-population of neurons is well-known to express different

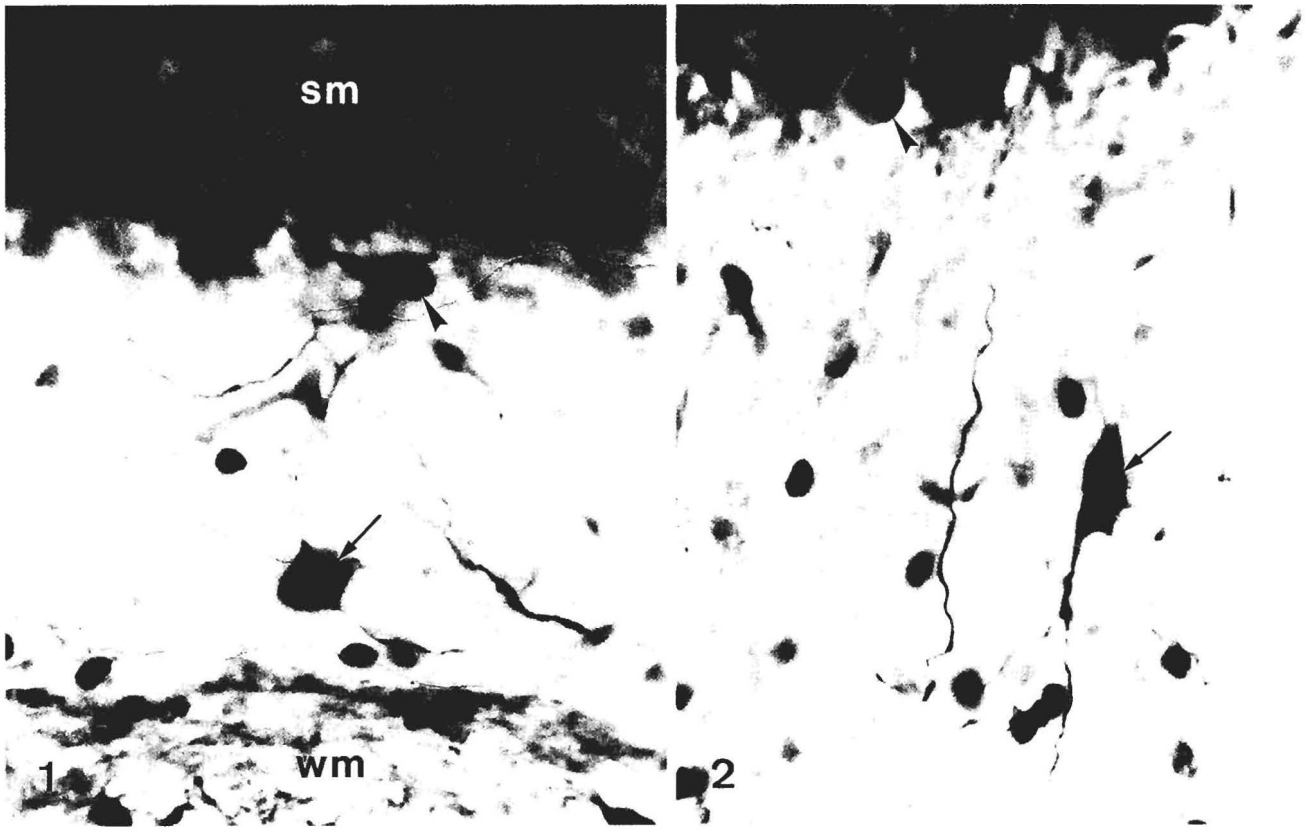


Fig. 1. Cerebellum. Mouse. A large neuron (arrow) including its arising axon is located in the basal portion of the granular layer above the white matter (wm). A Purkinje cell is also visible (arrowhead). The extracellular matrix of the stratum moleculare (sm) is intensively stained. x 400

Fig. 2. Cerebellum. Mouse. A large perikaryon (arrow) with a descending axon is situated in the granular layer. The Purkinje cell layer (arrowhead) is stained, too. x 400

neuropeptides as well as the calcium binding protein parvalbumin; most of these nerve cells are inhibitory interneurons and contain GABA (Danos et al., 1991).

Regarding the demonstrated staining of perineuronal nets around a subpopulation of hippocampal neurons, it is known from the literature that Cajal (1897) was the only person who succeeded in staining such structures with MB. But his results was merely documented by drawings. Up to now, nobody has been able to reproduce his study by supravital MB staining (Celio and Blümcke, 1994). In the hippocampus, such perineuronal nets are preferentially associated with parvalbumin containing neurons; thus, the composition of the extracellular matrix might cooperate with parvalbumin in the control of excitability and the protection of neurons (Celio, 1993). This explanation of the function of perineuronal nets is in accordance with the interpretation of the oxygen-dependent MB-staining of these neurons; this phenomenon is possible due to a strong affinity of a subpopulation of non-pyramidal cells to oxygen and

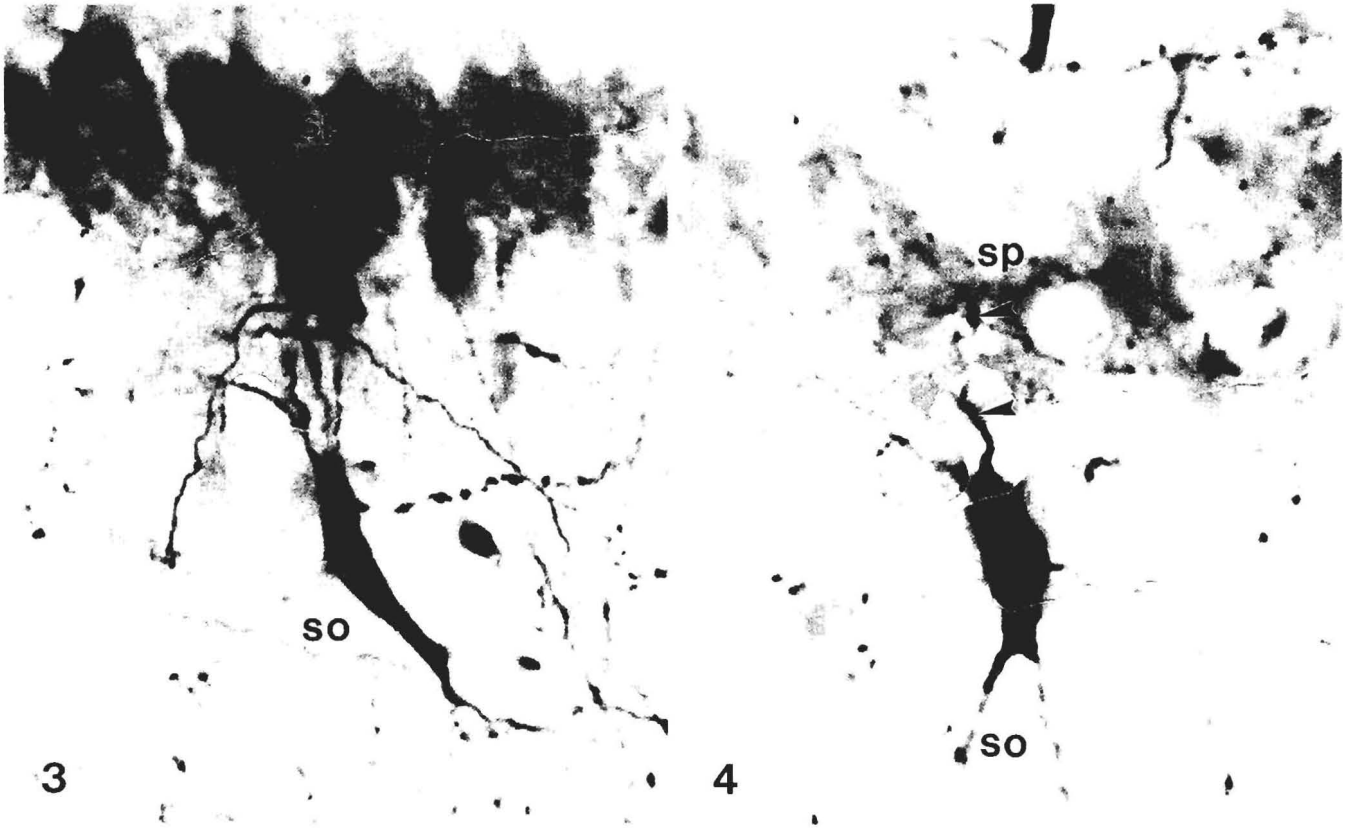


Fig. 3. Hippocampus. Mouse. In the stratum pyramidale (sp) and stratum oriens (so) of the cornu Ammonis, polymorphic neurons are selectively stained. x 750

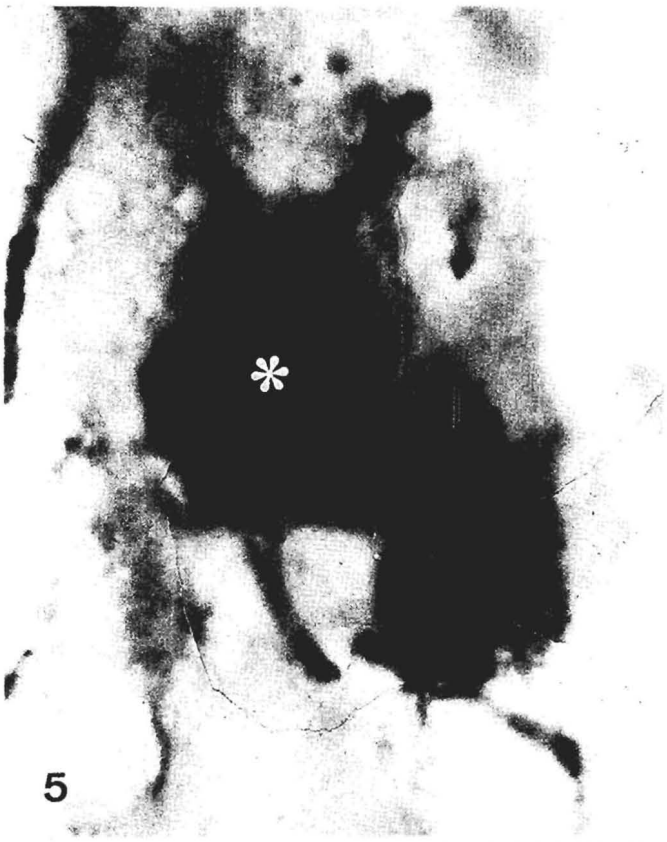
Fig. 4. Hippocampus. Mouse. A large multipolar neuron is located in the stratum oriens (so) and sends off a delicate axon (arrowhead) which penetrates the only slightly stained stratum pyramidale (sp). x 750

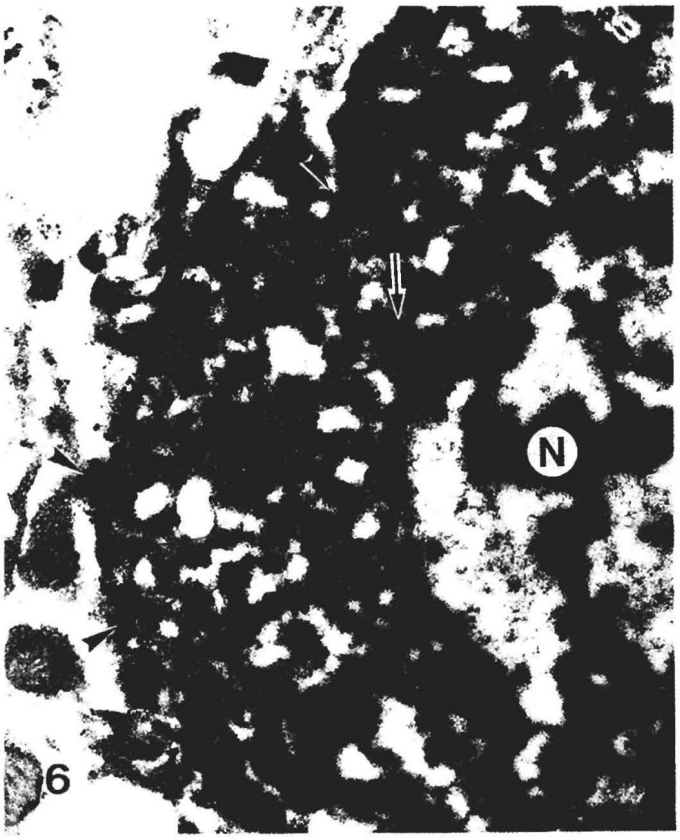
Fig. 5. Hippocampus. Mouse. One perikaryon is homogeneously stained (asterisk), whereas an adjacent neuron shows only an inhomogeneous labelling of a perineuronal net (arrow). x 2,400

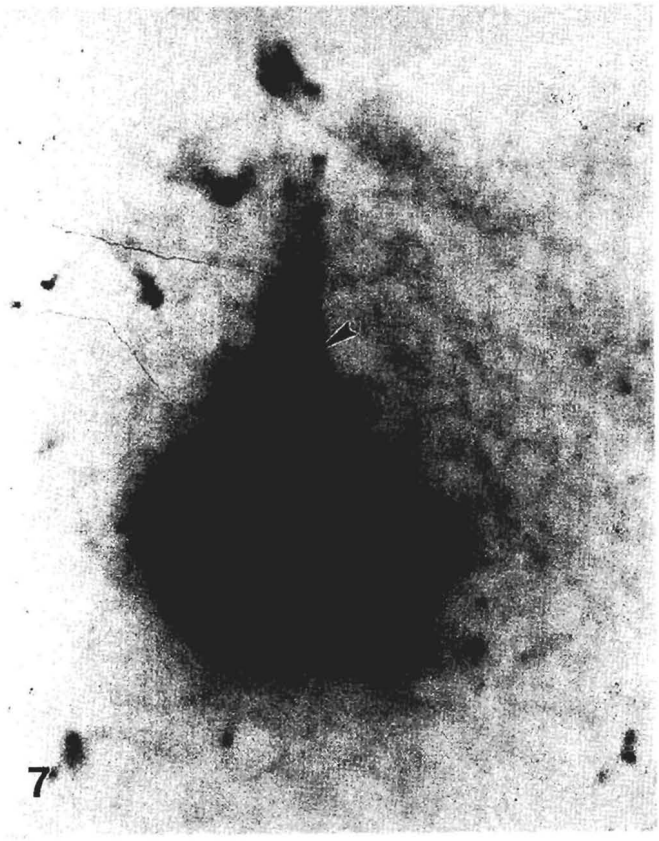
Fig. 6. Hippocampus. Mouse. Ultrastructurally, large electron-dense drop-like dye accumulations (arrow) are visible within the cytoplasm. A staining of material in the region of the plasma membrane can also be detected (arrowheads); probably, these structures belongs to the specialized extracellular matrix, i.e. perineuronal nets. Apparently due to a homogeneous staining, the cytoplasm as well as the clumped chromatin in the nucleus (N) exhibits a slight increase in electron density. x 30,000

Fig. 7. Hippocampus. Mouse. A perineuronal net is visible in the shape of an intense staining of the plasma membrane's outer surface (arrowhead). x 2,400

Fig. 8. Hippocampus. Mouse. The perineuronal net of a single neuron is inhomogeneously stained. x 2,400


5


N
6


7

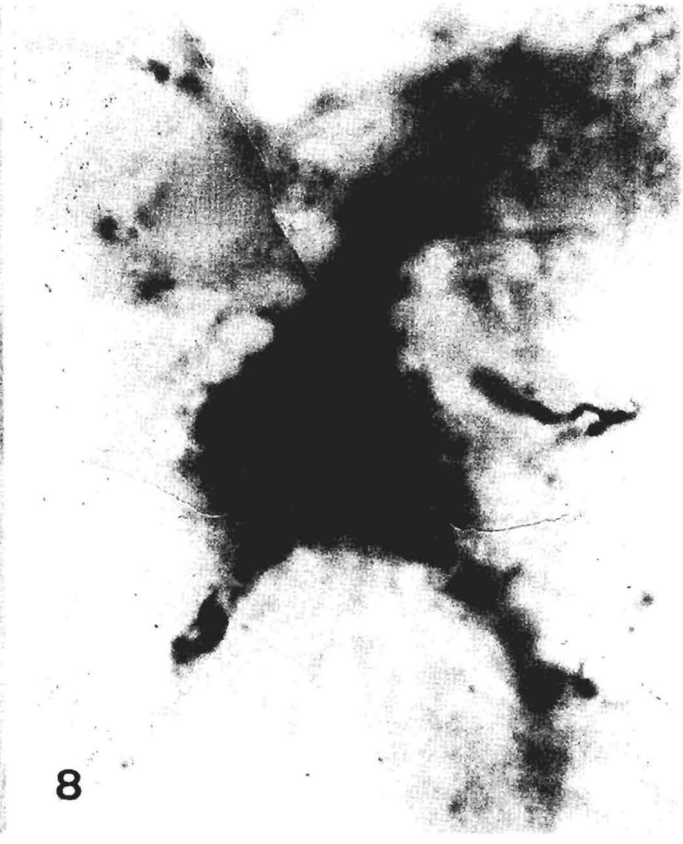

8

Fig. 9. Pineal gland. Hamster. In addition to the dense network of cell processes, several somata are intensively stained. x 520

Fig. 10. Pineal gland. Hamster. A single cell shows both a thin (arrow) and a thick process (arrowhead). x 810

Fig. 11. Pineal gland. Hamster. Groups of cells seem to form a syncytium by an anastomosis (arrow). x 1,000

Fig. 12. Pineal gland. Hamster. Polymorphic cells are apparently connected by a thick anastomosis. x 1,000

Fig. 13. Pineal gland. Hamster. A single soma (asterisk) sends off a cable-like process (arrowheads) forming a terminal plexus. x 1,000

Fig. 14. Pineal gland. Hamster. A perikaryon (asterisk) exhibits a relatively thick process (arrowhead) forming a characteristic branching point (arrow). x 1,000

suggests therefore a high vulnerability to hypoxia (Müller, 1995).

Within the pineal gland, the stained cells showed a more complicated arborization pattern of their processes than hitherto established by means of silver impregnation (Bargmann, 1943) or electron microscopy (Wartenberg, 1968). In general, the stained cells exhibited a certain similarity to opsin-like immunoreactive pinealocytes (Korf et al., 1985). In this context it has to be emphasized that no study has been published till now which was capable of staining cells in the mammalian pineal gland by means of MB-staining. In the pineal organs of lower vertebrates, MB is not stored in any preferential cell type; sensory cells, ganglionic cells as well as supporting cells could be stained (Tretjakoff, 1915; Holmgren, 1918; Paul et al., 1971). Therefore, it did not surprise that it was impossible to determine by MB-staining in the mammalian pineal gland, of which concrete phyllogenetic origin the stained cells are. Nevertheless, MB-staining shows interesting new data of the structure of the mammalian pineal gland with special regard to the delicate morphological details of the characteristic cells.

Staining mechanism and functional conclusions

Ehrlich (1886) was the first to note that MB stains oxygen-binding sites in nerve and sensory cells. Later, it was assumed that intracellular ion was involved in the simultaneous binding of oxygen and dye (Kondratjew, 1926). Furthermore, it could be demonstrated that sodium azide, an inhibitor of many metallo-enzymes, also inhibits the staining of nerve fibers (Kiernan, 1974). In the case that intracellular iron is actually responsible for selective staining, it is not associated with mitochondrial enzymes. However, many heme-proteins are situated within the cytosol. Moreover, the inhibiting influence of MB on such enzymes has functionally been associated with a direct interaction of the dye with iron (Mayer et al., 1993). Here, it is of special interest that reduced MB, i.e. leuco-MB, is able to reduce molecular oxygen to oxygen radicals intracellularly; this production of oxygen radicals is considered to be important for manifold pharmacological effects of the dye (Lee and Wurster, 1995). The generation of such reactive species might be the reason for the observed damage of the ultrastructure. In general, the oxygen-dependent selective staining of subpopulations of neurons suggests a high requirement for oxygen for these neurons and might indicate a certain vulnerability of these cells. Logically, MB-uptake might be interpreted as an early metabolic disturbance of single neurons; this phenomenon must be based on a change of the redox potential of the cells (Deane et al., 1960). In addition, since it is well-known that MB acts as a photosensitizer and causes light-induced cell damage, the presence of light does not seem to play a decisive role in the selective staining (Müller, 1995).

Furthermore, there is some evidence that MB is reduced to its uncharged lipophilic leuco-form immediately before entering nerve cells; this is followed by intracellular reoxidation to the blue positively-charged form (Becker and Quadbeck, 1952; Harris and Peters, 1953). In protoplasts, a reductase associated with the plasma membrane is presumed to be responsible for the initial reduction of the dye (Swain and De, 1997). Subsequently, the regenerated cationic blue dye is able to bind to other intracellular structures, for example the chromatin within the nucleus as observed. Additionally, a binding to microtubules and neurofilaments seems to occur (Chapman, 1982; Wischik et al., 1996). In contrast to these assumptions, an additional partial oxidation of leuco-MB in the extracellular space during the air exposure appears to be likely, since also a staining of perineuronal nets could be detected; these sites of specialized extracellular matrix are known to be rich in strong anionic residues (Celio and Blümcke, 1994). The same explanation concerns the staining of the nodes of Ranvier and homologous structures (Müller, 1991, 1992). These locations are also rich in extracellular matrix with negative charges (Seeger et al., 1994). Therefore, these locations might attract the dye in its cationic blue form. A similar staining phenomenon in the peripheral nervous system seems to be the intensive dye uptake by the extracellular amorphous substance located at the apical microvilli of taste bud cells (Müller and Reutter, 1995). Therefore, perineuronal nets might be capable of attracting other cations and play an important role in the control or mediation of cell excitability.

In conclusion, due to its capacity to visualize morphological details of subpopulations of neurons in the brain and cells within the pineal gland, the described method seems to be a helpful supplement in neuroanatomy. Moreover, the phenothazine dye MB might represent a model substance for studying the distribution pattern and the effects of other phenothiazines which are well known to act as antipsychotic drugs (Müller, 1992; 1996a; Deutsch et al., 1997). In future, studies have to be performed which correlate MB supravital staining directly with biochemical and electrophysiological experiments; this would give more insight regarding the extra- as well as intracellular metabolic pathway of the dye.

Acknowledgments. The author is grateful to Mrs. B. Becker-Ruoff, Institute for Anatomy, University of Tübingen, and Mrs. A. de Cuvry, Institute for Anatomy, University of Mainz, for skillful technical assistance.

References

Bargmann W. (1943). Die Epiphysis cerebri. In: Handbuch der Mikroskopischen Anatomie des Menschen - Innersekretorische Drüsen. Vol. VI/1 part 4. von Möllendorff W. (ed.). Springer Verlag. Berlin. pp 309-502.

Becker H. and Quadbeck G. (1952). Tierexperimentelle

Untersuchungen über die Funktionsweise der Bluthirnschranke. Z. Naturforsch. 7, 493-497.

Bethe A. (1895). Studien über das Centralnervensystem von Carcinus maenas nebst Angaben über ein neues Verfahren der Methylenblaufixation. Arch. Mikrosk. Anat. Entwicklungsgesch. 44, 579-622.

Cajal S.R. (1896a). Las espinas colaterales de las células del cerebro. Rev. Trimest. Micrograf. 1, 123-136.

Cajal S.R. (1896b). El azul de metileno en los centros nerviosos. Rev. Trimest. Micrograf. 1, 151-203.

Cajal S.R. (1897). La red superficial de las células nerviosas centrales. Rev. Trimest. Micrograf. 3, 163-178.

Celio M.R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35, 375-475.

Celio M.R. (1993). Perineuronal nets of extracellular matrix around parvalbumin-containing neurons of the hippocampus. Hippocampus 3, 55-60.

Celio M.R. and Blümcke I. (1994). Perineuronal nets - a specialized form of extracellular matrix in the adult nervous system. Brain Res. Rev. 19, 128-145.

Chapman D.M. (1982). Localization of methylene blue paramolybdate in vitally stained nerves. Tissue Cell 14, 475-487.

Danos P., Frotscher M. and Freund T.F. (1991). Non-pyramidal cells in the CA3 region of the rat hippocampus: relationship of fine structure, synaptic input and chemical charcteristics. Brain Res. 546, 195-202.

Deane H.W., Barrnett R.J. and Seligman A.M. (1960). Histochemische Methoden zum Nachweis der Enzymaktivität. In: Handbuch der Histochemie. Vol. VII/1. Graumann W. and Neumann K.H. (eds.). G. Fischer Verlag. Stuttgart. pp 1-229.

Deutsch S.I., Rosse R.B., Schwartz B.L., Fay-McCarthy M., Rosenberg P.B. and Fearing K. (1997). Methylene blue adjuvant therapy of schizophrenia. Clinic. Neuropharmacol. 20, 357-363.

Dogiel A.S. (1896). Die Nervenelemente im Kleinhirne der Vögel und Säugethiere. Arch. Mikrosk. Anat. Entwicklungsgesch. 47, 707-719.

Ehrlich P. (1886). Ueber die Methylenblaureaction der lebenden Nervensubstanz. Dtsch. Med. Wochenschr. 12, 49-52.

Feindel W.H., Allison A.C. and Weddell G. (1949). Intravenous methylene blue for experimental studies on the Central Nervous System. J. Neurol. Neurosurg. Psychiatry 11, 227-242.

Fukuda T. (1971). The place of intra-arterial methylene blue vital stain and histochemistry of acetylcholinesterase in experimental neurology and psychiatry. Trab. Inst. Cajal Invest. Biol. 63, 169-187.

Gruetter C.A., Gruetter D.Y., Lyon J.E., Kadowitz P.J. and Ignarro L.J. (1981). Relationship between cyclic guanosine 3',5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl nitrate, nitroprusside, nitrite and nitric oxide: effects of methylene blue and methemoglobin. J. Pharmacol. Exp. Ther. 219, 181-186.

Harris J.E. and Peters A. (1953). Experiments on vital staining with methylene blue. Q. J. Microsc. Sci. 94, 113-124.

Holmgren N. (1918). Zum Bau der Epiphyse von Squalus acanthias. Ark. Zool. 11, 13-28.

Kiernan J.A. (1974). Effects of metabolic inhibitors on vital staining with methylene blue. Histochemistry 40, 51-57.

Kondratjew N.S. (1926). Eine neue Methode der elektiven makroskopischen Färbung des Nervensystems. Z. Anat. Entwicklungsgesch. 78, 669-681.

Korf H.W., Foster R.G., Ekström P. and Schalken J.J. (1985). Opsin-like immunoreaction in the retinae and pineal organs of four mammalian species. Cell Tissue Res. 242, 645-648.

Lee Y.S. and Wurster R.D. (1995). Methylene blue induces cytotoxicity in human brain tumor cells. Cancer Lett. 88, 141-145.

Mayer B., Brunner F. and Schmidt K. (1993). Inhibition of nitric oxide synthesis by methylene blue. Biochem. Pharmacol. 45, 367-374.

Müller T. (1990). Supravital staining of murine brain with methylene blue according to the Cajal method: a simple and reliable preparation technique for embedding in paraffin. J. Neurosci. Methods 32, 223-226.

Müller T. (1991). Methylene blue filling of the dorsal giant fibres of the earthworm by electrophoresis. Tissue Cell 23, 223-226.

Müller T. (1992). Light-microscopic demonstration of methylene blue accumulation sites in mouse brain after supravital staining. Acta Anat. 144, 39-44.

Müller T. (1994). Large nerve cells with long axons in the granular layer and white matter of the murine cerebellum. J. Anat. 184, 419-423.

Müller T. (1995). Light and electron microscopic demonstration of methylene blue accumulation sites in the murine cornu Ammonis after supravital staining. Ital. J. Anat. Embryol. 100, 179-187.

Müller T. (1996a). Electron microscopic demonstration of intracellular promethazine accumulation sites by a precipitation technique: application to the cerebellar cortex of the mouse. J. Histochem. Cytochem. 44, 531-535.

Müller T. (1996b). Morphological differences among nerve fiber endings in the rat oral mucosa as revealed by methylene blue staining. Histol. Histopathol. 11, 659-666.

Müller T. and Reutter K. (1995). Light and electron microscopical demonstration of methylene blue accumulation sites in taste buds of fish and mouse after supravital dye injection. Anat. Embryol. 192, 537-545.

Naylor G., Martin B., Hopwood S.E. and Watson Y. (1986). A two-year doubleblind cross over trial of the prophylactic effect of methylene blue in manicdepressive psychosis. Biol. Psychiatry 21, 915-920.

O´Leary J.L., Petty J., Harris A.B. and Inukai J. (1968). Supravital staining of mammalian brain with intraarterial methylene blue followed by pressurized oxygen. Stain Technol. 43, 197-201.

Paul E., Hartwig H.G. and Oksche A. (1971). Neurone und zentralnervöse Verbindungen des Pinealorgans der Anuren. Z. Zellforsch. Mikrosk. Anat. 112, 466-493.

Seeger G., Brauer K., Härtig W. and Brückner G. (1994). Mapping of perineuronal nets in the rat brain stained by collodial iron hydroxide histochemistry and lectin cytochemistry. Neuroscience 58, 371-388.

Swain D. and De D.N. (1997). Active dye uptake: A new mechanism of vital staining of some plant protoplasts. Indian J. Exp. Biol. 35, 180-187.

Tretjakoff D. (1915). Die Parietalorgane von Petromyzon fluviatilis. Z. Wiss. Zool. 113, 1-112.

Wartenberg H. (1968). The mammalian pineal organ: electron microscopic studies on the fine structure of pinealocytes, glial cells and on the perivascular compartment. Z. Zellforsch. Mikrosk. Anat. 86, 74-97.

Wischik C.M., Edwards P.C., Lai R.Y.K., Roth M. and Harrington C.R. (1996). Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines. Proc. Natl. Acad. Sci. USA 93, 11213-11218.

Accepted May 8, 1998