Proc. Natl. Acad. Sci. USA
Vol. 75, No. 6, pp. 2977–2980, June 1978
Neurobiology

Ultrastructural localization of γ-aminobutyric acid receptors in the mammalian central nervous system by means of [^{3}H]muscimol binding

(cerebellar cortex/nuclei/electron microscope autoradiography)

VICTORIA CHAN-PALAY* AND SANFORD L. PALAY†

Departments of * Neurobiology and † Anatomy, Harvard Medical School, Boston, Massachusetts 02115

Contributed by Sanford L. Palay, February 21, 1978

ABSTRACT **This study utilizes tritiated muscimol binding and electron microscope autoradiography (Ilford L4 emulsion and phenidone development) to localize γ-aminobutyric acid (GABA) receptor sites in the cerebellum of the rat. In the cerebellar cortex, silver grains were associated with somata and dendrites of basket and stellate cells in the molecular layer, with somata and primary and secondary dendritic shafts of Purkinje cells, axons and terminals of basket cells in the *pinceau* or basket, initial axonal segments and myelinated axons of Purkinje cells, and dendrites of granule and Golgi cells in the granular layer, and with somata and dendritic shafts of large and small cells in the cerebellar nuclei. These data correspond well to the light-microscope-autoradiographic observations in the cerebellum previously reported [Chan-Palay, V. (1978) *Proc. Natl. Acad. Sci.* 75, 1024–1028]. Label over GABA receptor sites can be localized to the plasma membranes between pre- and postsynaptic elements at synaptic junctions, of which 88.9% of the samples are axodendritic and the remaining 11.1% are axosomatic. GABA receptor sites are also found along axonal membranes of the GABA-containing basket cell within the basket surrounding Purkinje cells, where true axo-axonal synapses are rare. It is speculated that GABA receptors in the basket may suggest a possibility of the basis for synchronization, either self-inhibition or facilitation within the basket formation, or presynaptic suppression of inhibitory action of basket cell on Purkinje cell.**

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system, and deficits of GABA have been implicated in certain neurological and psychiatric disorders, such as Huntington's chorea, Parkinson's disease, and schizophrenia. Considerable interest attaches to the identification of GABA-containing neurons and receptor sites for GABA. Although cells and processes with GABA-synthesizing enzymes and with GABA uptake systems have been identified morphologically (1–3), the receptor binding sites for GABA have been visualized only recently (4, 5). These two recent reports provided detailed quantitative information on the distribution of GABA receptors in the cerebellum (4) and the hippocampus (5) as determined by light microscopic autoradiography. In these studies, muscimol (M, 3-hydroxy-5-aminomethylisoxazole), a psychotomimetic constituent of *Amanita muscaria* (6) and a potent GABA agonist (7–9), was used in its tritiated form as a histochemical marker for GABA receptor sites. Tritiated muscimol ([^{3}H]M) has a free amino group that enables the substance, when it is bound to receptor sites in tissue constituents, to be covalently bound by aldehyde fixation. Subsequent manipulations are then possible for autoradiographic visualization of the bound [^{3}H]M (5). Histological control experiments performed with [^{3}H]M on tissue slices and *in vivo* (5) have shown that labeling in autoradiograms is not affected by pretreatment with two inhibitors of GABA uptake and transport, (−)nipecotic acid and guvacine, but can be eliminated by pretreatment of the tissue with bicuculline hydrochloride, unlabeled GABA, or unlabeled GABA with amino-oxyacetic acid, or by treatment *in vivo* with M and [^{3}H]M given intravenously (5). These data support the suggestion that sites labeled with [^{3}H]M are GABA receptor sites.

It is imperative to know the subcellular locations of these GABA receptor sites, and the present communication provides their ultrastructural definition in the nervous system. The cerebellum was chosen for this study because its structure and function have been investigated in detail (10–12) and because it contains the highest amount of [^{3}H]GABA- and [^{3}H]M-binding activity in the brain. This paper describes the subcellular distribution of GABA receptors in particular cells of the cerebellar cortex and nuclei by electron microscopic autoradiography with [^{3}H]M. The major questions to be addressed include whether or not GABA receptors are associated with (*i*) membranes of neuronal or neuroglial elements, (*ii*) synaptic sites, (*iii*) specific cellular types, or (*iv*) unusual nonsynaptic relationships.

MATERIALS AND METHODS

The cerebellar cortex and nuclei in anesthetized adult Sprague–Dawley rats were injected with 20 μl of 2 nM [^{3}H]M, as described (4). [^{3}H]M obtained from New England Nuclear, specific activity 10.3 Ci/mmol, was checked for purity by silica-gel thin-layer cochromatography with unlabeled purified muscimol in butanol/glacial acetic acid/water (4:1:1) and detected with 5% (wt/vol) ferric chloride (5). The animals were perfused with 1% (vol/vol) glutaraldehyde and 1% (vol/vol) formaldehyde in 0.12 M phosphate buffer, pH 7.4. The tissue slices containing the injected sites were postfixed in osmium tetroxide, stained *en bloc*, and embedded in epoxy resin (10). Autoradiograms at the light microscopic level provided the means to study large areas of entire folia of cerebellar cortex and nuclei (4). For ultrastructural study, we selected certain areas that were well oriented in the sagittal plane and contained several Purkinje cell somata and dendritic fields, molecular layer, granular layer, and regions of the dentate nucleus. Electron microscopic autoradiograms were prepared from 150 serial thin sections by using the parlodion-coated slide/film-stripping technique (11, 13). The slides were coated with Ilford L4 emulsion (1:4) and exposures of up to 5 weeks were used. Phenidone was selected as the best developer (14) after com-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: M, muscimol; [^{3}H]M, tritiated muscimol; GABA, γ-aminobutyric acid.

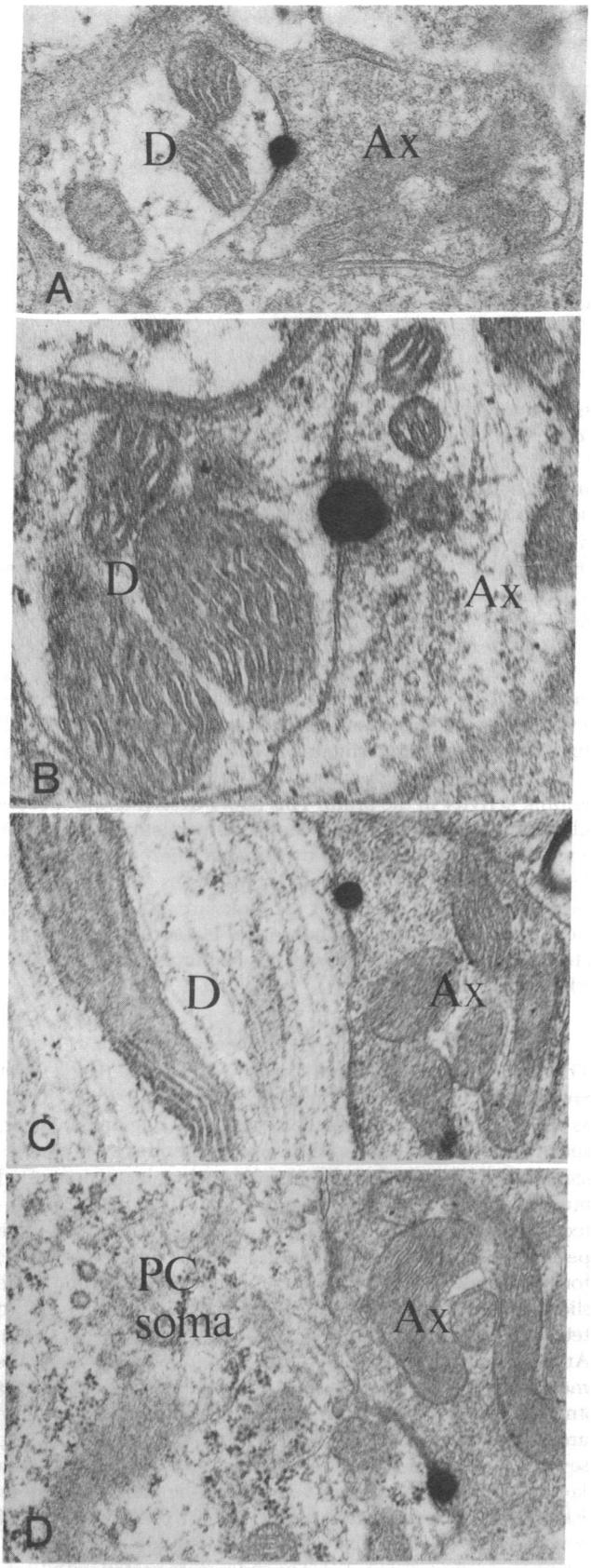

FIG. 1. (*A–C*) GABA receptor sites labeled on axodendritic synapses in the cerebellum. The silver grains produced by bound [^{3}H]M and developed by phenidone are single and round, and they are selectively deposited on the pre- and postsynaptic membranes at or near the synaptic junction. The dendrite, D, and axonal terminals, Ax, are labeled. (*A*, ×55,000; *B*, ×41,000; *C*, ×31,000.) (*D*) GABA receptor sites labeled on an axosomatic synapse of an axon terminal, Ax, on the perikaryal surface of a Purkinje cell, PC soma. (×30,000.)

parative trials with Microdol-X and physical developers (11). The phenidone grains were small, round, and well suited to the precise localization of [^{3}H]M radiation sources. The other major technical concern was to keep the background to a minimum by careful selection of exposure and development times in these procedures. All available sections were examined in a Philips EM-300. Approximately 350 electron micrographs containing silver grains were obtained. The position of every grain in every micrograph was quantitatively analyzed with respect to its relationship to the nearest adjacent membrane. Because of the harsh procedures used in the preparation of these autoradiograms, it was difficult to study the cytoplasmic morphology of axons. The identification of other cellular elements was made on the basis of their location and structure and the known connections of the various cell types. The selectivity of the method and the paucity of silver grains developed (in order to reduce background) made it impossible to estimate the true density of GABA receptor sites by means of the number of silver grains. The ratio of grains to receptor site density is not 1:1; nevertheless, the presence of a silver grain indicates the presence of labeled receptors.

RESULTS AND COMMENTS

Cellular localization

The deposition of phenidone-developed silver grains was selectively localized to the plasma membranes of several neuronal types and their processes. Background label was low. Serial-section autoradiograms exposed for 3 weeks exhibited half the number of silver grains encountered in those exposed for 5 weeks; however, the grains were on comparable structures in adjacent serial sections exposed for the two durations. Silver grains in the cerebellar cortex were associated with plasma membranes of the following structures: (*i*) somata and primary dendrites of basket and stellate cells in the molecular layer; (*ii*) somata and primary and secondary dendritic shafts of Purkinje cells; (*iii*) axons and axon terminals of basket cells in the *pinceau* region; (*iv*) initial axonal segments and myelinated axons of Purkinje cells; (*v*) the dendrites of granule cells and Golgi cells in the granular layer; (*vi*) somata and dendritic shafts of large and small neurons in the cerebellar nuclei.

These data correspond well to the distribution and density of GABA receptors on cells in the cerebellar cortex and nuclei seen in light microscopic autoradiograms (4). In almost all cases the label was associated with neuronal plasma membranes and not with the neuroglial investment. However, one example was found of label on the membranes of an opposing axon and neuroglial cell in the dentate nucleus. Furthermore, the label occurred in relation to cellular surface membranes and not to internal cytoplasmic membranes.

Synaptic terminal localization

One hundred and thirty-five electron microscopic autoradiograms were obtained in which silver grains were deposited on membranes belonging to axonal varicosities and to postsynaptic elements bearing visible synaptic junctions (see Fig. 1). In 88.9% of these examples, the label was associated with axodendritic synapses on Purkinje cells and in 11.1% the label was associated with axosomatic synapses on Purkinje, stellate, and basket cells.

Of the 135 examples of labeled synaptic terminals, 24% had the silver grain directly upon the opposing pre- and postsynaptic

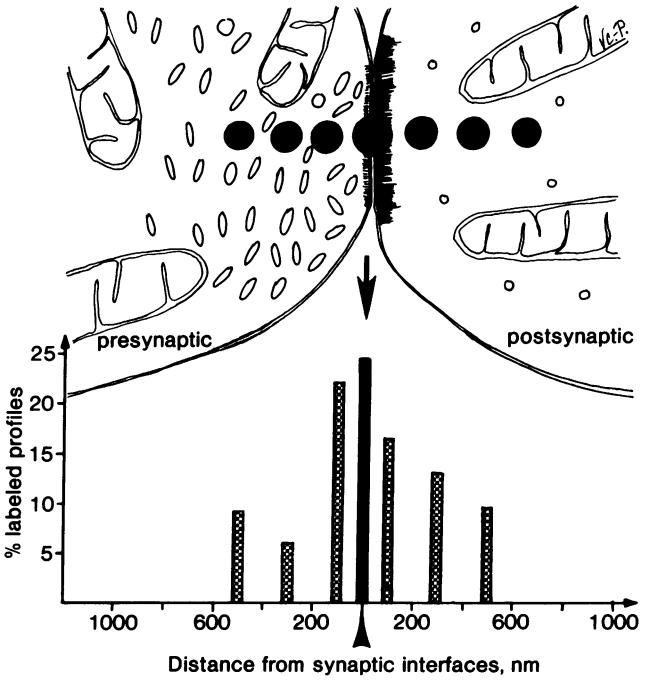

FIG. 2. Diagram summarizing the distribution of phenidone-developed autoradiographic silver grains produced by bound [^{3}H]M on the membranes of profiles bearing synaptic junctions. The sample includes 135 profiles, 24% of which have grains on the synaptic interfaces; the remaining 37.5% and 38.5% were disposed on pre- and postsynaptic sides, respectively, within 500 nm of the synaptic interfaces. This scatter of grains is within the limits of resolution of the autoradiographic method for a tritium source and the Ilford L4 emulsion.

membranes at or near the synaptic junction. The remaining examples had silver grains scattered on either the pre- or postsynaptic side, close to the synaptic membranes. Of the original population, 37.5% had label on the presynaptic side, 22.2% of which contained silver grains within the first 100 nm of the synaptic junction; the remaining 15.3% had silver grains within 450 nm of the synaptic junction. Of the original population, 38.5% had silver grains on the postsynaptic side, all of which lay within the first 500 nm of the synaptic membranes. These results are summarized diagrammatically in Fig. 2. The data show that the silver grains were clearly localized upon and near synaptic junctions. The spread of grains reported here reflects the scatter of β particles from the bound [^{3}H]M. The 500-nm maximal spread is well within the limits of resolution of this technique utilizing tritium and Ilford L4 emulsion (15).

The diameters of silver grains ranged from 110 to 150 nm. The neuronal plasma membrane was approximately 8 nm wide and the extracellular space between neuronal appositions was 20–25 nm wide. At symmetric synapses the synaptic cleft was 20–25 nm wide, whereas at asymmetric synapses the cleft was usually widened to 30 nm (16). Therefore, the diameter of one silver grain is 3–4 times the combined widths of pre- and postsynaptic membranes and the synaptic cleft. Thus, although the electron microscope autoradiographic technique indicates that labeling occurs directly in conjunction with membranes at or near synapses, it is not possible to decide whether or not the source for the label—i.e., [^{3}H]M bound to GABA receptors—is associated with either the presynaptic or postsynaptic membrane.

Nonsynaptic locations

Sixty-four examples of neuronal profiles were obtained with

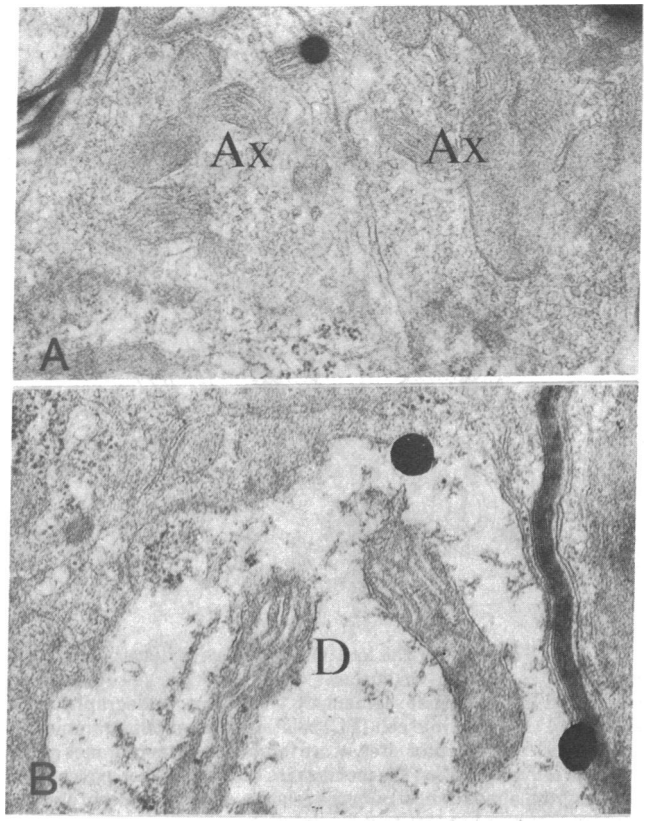

FIG. 3. (*A*) GABA receptor sites labeled by a phenidone-developed silver grain on the plasma membrane surfaces between two basket cell axons, Ax, in the basket area around the Purkinje cell where no synaptic specializations are evident. (×25,000.) (*B*) GABA receptor sites labeled on the plasma membrane of a large dendrite, D, belonging to a cerebellar dentate nucleus neuron. (×30,000.)

silver grains located on plasma membranes of structures without visible synaptic contacts. Of these, 66% were on plasma membranes between axons (Fig. 3*A*) and 34% were on dendrites (Fig. 3*B*). The basket region around the Purkinje cell body and initial axon segment presented the most examples of silver grains between axonal plasma membranes. Here, the axons belonging to the GABA-containing basket cells of the molecular layer are complexly intertwined with one another, with some neuroglial processes between them (10). Few, if any, true axo-axonal synapses exist, although specialized, septate-like junctions have been described (17–19, 10). The label present in the *pinceau* was found on the membranes between apposed basket axons that were bare of neuroglia and not at any specialized junction, as summarized in Fig. 4. These observations confirm the earlier report from light microscopic autoradiograms that the basket region around Purkinje cells was most densely labeled with [^{3}H]M-binding GABA receptor sites (4).

The presence of GABA receptors on the membranes of GABA-containing axons raises the obvious question of their function. Do the basket axon terminals within this region release GABA at nonsynaptic locations? If so, does binding of GABA to the receptors in the basket produce self-inhibition of the basket and consequently a self-regulated inhibition of discharges from the Purkinje cell axon that it surrounds or does it produce a presynaptic facilitation? Because the basket formation around each Purkinje cell consists of axon collaterals from a number of different basket cells (12, 10), the effect of activation of any one basket cell axon is difficult to predict. It could

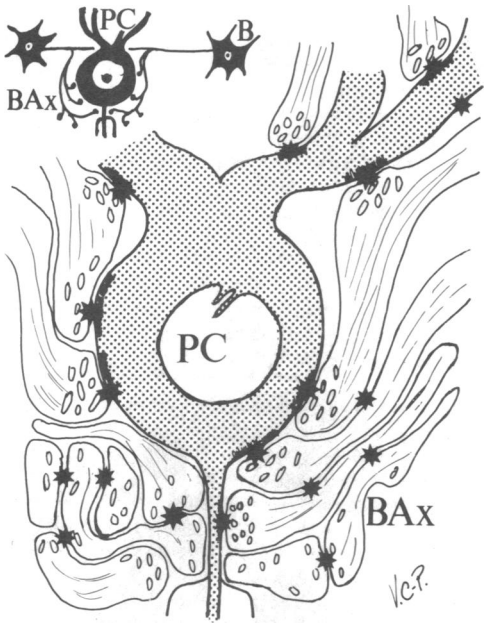

FIG. 4. Summary of the location and relative density of GABA receptor sites detected on surfaces of the Purkinje cell soma, dendrites, and initial axonal segment by [^{3}H]M autoradiography. The basket cell (B)–Purkinje cell (PC) relationships are diagrammed in the *Inset*. GABA receptor sites, *, are found on the membranes of the Purkinje cell as well as on the membranes between basket axons, BAx. The diagram aims to show only the relative density of receptors, not the absolute number (see text for details).

result in inhibition of the other members of the basket and thus a reduction of the effect. Alternatively, it is conceivable that the membrane events in the terminals could lead to synchronized activation of the whole formation and to reinforcement of the effect of exciting a single basket cell. Moreover, is it possible that presynaptic suppression of inhibitory action of basket cell on the Purkinje cell could result? The inhibitory effect of basket cell activation on Purkinje cell discharges builds up and decays relatively slowly (12, 20, 21). Some aspects of these electrical properties may be explainable by the general distribution of GABA receptors throughout the axons of the basket formation. The present findings suggest that re-examination of the physiology of the complicated synapse between basket and Purkinje cells would be useful.

We thank Dr. Povl Krogsgaard-Larsen for donation of the unlabeled muscimol, (−)nipecotic acid, and guvacine used in these studies; Ms. J. Hilsz and Mr. H. Cook for excellent technical and photographic assistance, respectively; and Ms. J. Bellizia for typing the manuscript. This work was supported in part by Research Grants NS 10536 and NS 03659 and Training Grant NS 05591 from the National Institute of Neurological and Communicative Diseases and Stroke and by a gift for research in Parkinson's disease.

1. Barber, R. P. & Saito, K. (1976) in *GABA in Nervous System Function*, eds. Roberts, E., Chase, T. N. & Tower, D. B. (Raven, New York), pp. 113–132.
2. McLaughlin, B., Wood, J. G., Saito, K., Barber, R., Vaughn, J. E., Roberts, E. & Wu, J.-Y. (1974) *Brain Res.* **76**, 377–391.
3. Hökfelt, T. & Ljungdahl, Å. (1970) *Brain Res.* **22**, 391–396.
4. Chan-Palay, V. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1024–1028.
5. Chan-Palay, V. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2516–2520.
6. Eugster, C. H. (1969) *Fortsch. Chem. Org. Naturst.* **27**, 261–321.
7. Krogsgaard-Larsen, P., Johnston, G. A. R., Curtis, D. R., Game, C. J. A., & McCulloch, R. M. (1975) *J. Neurochem.* **25**, 803–809.
8. Johnston, G. A. R., Krogsgaard-Larsen, P., Stephanson, A. L. & Twitchin, B. (1976) *J. Neurochem.* **26**, 1029–1032.
9. Enna, S. J., Collins, J. F. & Snyder, S. H. (1977) *Brain Res.* **124**, 185–190.
10. Palay, S. L. & Chan-Palay, V. (1974) *Cerebellar Cortex, Cytology and Organization* (Springer-Verlag, Berlin).
11. Chan-Palay, V. (1977) *Cerebellar Dentate Nucleus, Organization, Cytology and Transmitters* (Springer-Verlag, Berlin).
12. Eccles, J., Ito, M. & Szentágothai, J. (1967) *The Cerebellum as a Neuronal Machine* (Springer-Verlag, Berlin).
13. Chan-Palay, V. (1975) *Anat. Embryol.* **148**, 235–265.
14. Lettré, H. & Paweletz, N. (1966) *Naturwissenschaften* **53**, 268–271.
15. Fertuck, H. C. & Salpeter, M. M. (1976) *J. Cell Biol.* **69**, 144–158.
16. Peters, A., Palay, S. L. & Webster, H. de F. (1976) *The Fine Structure of the Nervous System; The Cells and Their Processes* (Saunders, Philadelphia), 2nd Ed.
17. Gobel, S. (1971) *J. Cell Biol.* **51**, 328–333.
18. Sotelo, C. & Llinás, R. (1972) *J. Cell Biol.* **53**, 271–289.
19. Mugnaini, E. (1972) in *The Comparative Anatomy and Histology of the Cerebellum: The Human Cerebellum, Cerebellar Connections and Cerebellar Cortex*, eds. Larsell, O. & Jansen, J. (University of Minnesota Press, Minneapolis, MN), pp. 201–265.
20. Eccles, J. C., Llinás, R. & Sasaki, K. (1966) *Exp. Brain Res.* **1**, 1–16.
21. Eccles, J. C., Sasaki, K. & Strata, P. (1967) *Exp. Brain Res.* **3**, 81–94.