Spinal Cord Tissue Cultures – a Model for Autoradiographic Studies on Uptake of Putative Neurotransmitters such as Glycine and GABA

The cellular and fine structural localization of the putative neurotransmitters glycine and γ-aminobutyric acid (GABA) have recently been studied with autoradiographic techniques[1-10]. Since these substances do not pass the blood brain barrier, the labelled amino acids were administered either in vitro to brain slices[1-3,5-7,9] or by intraventricular[8] or direct intracerebral[4] injections. All these techniques are associated with certain disadvantages. Thus, incubated brain slices show an inferior morphology, especially when examined in the electron microscope (for ref. see[11]). Furthermore, the uptake patterns reveal remarkable diffusion gradients of the isotopes within the slices[1-3,5].

Tissues culture techniques[10,12] offer unique possibilities to avoid these problems. In the present paper we should like to present some preliminary results on the localization of (^{3}H)-glycine and (^{3}H)-GABA in rat spinal cord cultures as revealed with autoradiography at the light and electron microscopic level.

Material and methods. The cultures were prepared from the spinal cord of new-born Wistar rats and grown on collodion-collagen-coated[13] or collagen-coated coverslips for 15–26 days in vitro in the Maximov assembly[12,14]. After a brief rinse in Tyrode solution, the cultures were incubated at 37 °C in Hank's solution containing (^{3}H)-glycine or (^{3}H)-GABA (5×10^{-7} to $10^{-5} M$; specific activity 4.7 C/mM and 5.6 C/mM, respectively; New England Nuclear Corp.) for 5 to 15 min. The cultures were then rinsed 3 times in Tyrode solution, fixed in 1.5 to 3% glutaraldehyde in 0.05–0.1 M phosphate buffer. At this stage the cultures were sent by air from Basle, Switzerland to Stockholm, Sweden. After remaining approximately 24–48 h in the aldehyde, the cultures were rinsed in 0.1 M phosphate buffer, removed from the coverslips, postfixed in 1% osmium tetroxide, rinsed in Ringer solution, dehydrated in ethanol and embedded in Epon[15]. Semithin sections – cut on an LKB Pyramitome or LKB Ultrotome and mounted on objective glass slides – and ultrathin sections – cut on a Reichert Om U3 ultramicrotome, contrasted with uranyl acetate and lead citrate[16] and coated with carbon – were covered with Ilford L4 emulsion (Ilford Ltd, Essex, England) according to the loop techniques[17-19] and exposed at +4 °C for 2–12 weeks. Development with Kodak D19b (light microscopy) and an amidol developer (electron microscopy) was followed by fixation with 25% sodium thiosulphate. The autoradiographs were examined in a Leitz Orthoplan light microscope and a Philips 300 electron microscope.

Results and discussion. The outgrowth of the spinal cord cultures as seen in the light and electron microscope was similar to that described in an earlier paper[20]. After incubation with either (^{3}H)-glycine or (^{3}H)-GABA specific uptake patterns were observed. The two amino acids were taken up both in neurones and in glial cells (Figures 1–3). The labelled cells were mostly found throughout the entire section (Figure 1), indicating a good penetration of the amino acids. A remarkably high activity was also found over the neuritic-meningeal outgrowth zone (Figure 1). After incubation with (^{3}H)-glycine a relatively large number of neurones of varying size showed an autoradiographic reaction, whereas after incubation with (^{3}H)-GABA mainly small neurones were covered by grains. However, there was a rather high proportion of neurones which were covered only by a few or no grains after incubation with (^{3}H)-glycine or (^{3}H)-GABA (Figures 2 and 3). As was observed in cultures of rat medulla oblongata[10], the intensity of labelling was dependent on the concentration and time of incubation.

The present results demonstrate that tissue cultures provide a suitable model for autoradiographic studies on the cellular and subcellular localization of the uptake of putative neurotransmitters such as glycine[21,22] and GABA[23-27]. In contrast, for instance to incubated brain slices, the cellular components of the explant show a good preservation of general morphology. Furthermore, the occurrence of labelled cells throughout the explant indicate a good penetration of the isotope.

In the present study no attempts were made to determine to what extent the isotopes responsible for the autoradiographic reaction reflect the unchanged amino acid or a metabolite. However, from studies on the uptake of (^{3}H)-GABA[28] or (^{3}H)-glycine[29,30] in brain slices, as well as of the uptake of (^{3}H)-GABA in intact brain[8,31], it was shown that the metabolites formed are rapidly removed from the tissues. Therefore, most of the autoradiographic reaction is due to the localization of the isotopes used.

Little is known about the compartmentalization of the exogenously administered amino acids. It has been shown that both glycine[30] and GABA[28] are incorporated into proteins only to a very limited extent under in vitro

[1] T. HÖKFELT and Å. LJUNGDAHL, Brain Res. *22*, 391 (1970).
[2] T. HÖKFELT and Å. LJUNGDAHL, Brain Res. *32*, 189 (1971).
[3] T. HÖKFELT and Å. LJUNGDAHL, in *Histochemistry of Nervous Transmission* (Ed. O. ERÄNKÖ; Elsevier Publ. Comp., Amsterdam, London, New York); Progr. Brain Res. *34*, 87 (1971).
[4] T. HÖKFELT and Å. LJUNGDAHL, Exp. Brain Res. *14*, 354 (1972).
[5] T. HÖKFELT and Å. LJUNGDAHL, in *Studies on Neurotransmitters at the Synaptic Level* (Eds. L. L. IVERSEN and E. COSTA, Raven Press, New York); Adv. Biochem. Psychopharmac., in press.
[6] F. E. BLOOM and L. L. IVERSEN, Nature, Lond. *229*, 628 (1971).
[7] L. L. IVERSEN and F. E. BLOOM, Brain Res. *41*, 131 (1972).
[8] F. SCHON and L. L. IVERSEN, Brain Res. *42*, 503 (1972).
[9] A. J. MATUS and M. E. DENNISON, Brain Res. *32*, 195 (1971).
[10] L. HÖSLI and E. HÖSLI, Brain Res. *45*, 612 (1972).
[11] T. HÖKFELT, Z. Zellforsch. *91*, 1 (1968).
[12] M. M. MURRAY, in *Handbook of Neurochemistry* (Ed. A. LAJTHA; Plenum Press, New York, London 1971), p. 373.
[13] L. DESCARRIES, J. Microsc. *6*, 313 (1967).
[14] E. HÖSLI and L. HÖSLI, Brain Res. *30*, 193 (1971).
[15] J. H. LUFT, J. biophys. biochem. Cytol. *9*, 409 (1961).
[16] E. S. REYNOLDS, J. Cell Biol. *17*, 208 (1963).
[17] L. G. CARO and R. P. VAN TUBERGEN, J. Cell Biol. *15*, 173 (1962).
[18] O. L. MILLER JR., G. E. STONE and D. M. PRESCOTT, J. Cell Biol. *23*, 654 (1964).
[19] T. NAGATA and T. NAWA, Histochemie *7*, 370 (1966).
[20] J. R. WOLFF, E. HÖSLI and L. HÖSLI, Brain Res. *32*, 198 (1971).
[21] D. R. CURTIS, L. HÖSLI, G. A. R. JOHNSTON and I. H. JOHNSTON, Expl. Brain Res. *5*, 235 (1968).
[22] R. WERMAN, R. A. DAVIDOFF and M. H. APRISON, J. Neurophysiol. *31*, 81 (1968).
[23] K. KRNJEVIĆ and S. SCHWARTZ, Expl. Brain Res. *3*, 320 (1967).
[24] K. OBATA, M. ITO, R. OCHI and N. SATO, Expl. Brain Res. *4*, 43 (1967).
[25] D. R. CURTIS and J. M. CRAWFORD, A. Rev. Pharmac. *9*, 209 (1969).
[26] D. R. CURTIS, A. W. DUGGAN, D. FELIX and G. A. R. JOHNSTON, Brain Res. *32*, 69 (1971).
[27] G. TEN BRUGGENCATE and I. ENGBERG, Brain Res. *25*, 431 (1971).
[28] L. L. IVERSEN and M. J. NEAL, J. Neurochem. *15*, 1141 (1968).
[29] J. M. HOPKIN and M. J. NEAL, Br. J. Pharmac. *40*, 136P (1968).
[30] M. J. NEAL and H. G. PICKLES, Nature, Lond. *223*, 679 (1969).
[31] Å. LJUNGDAHL and T. HÖKFELT, in preparation.

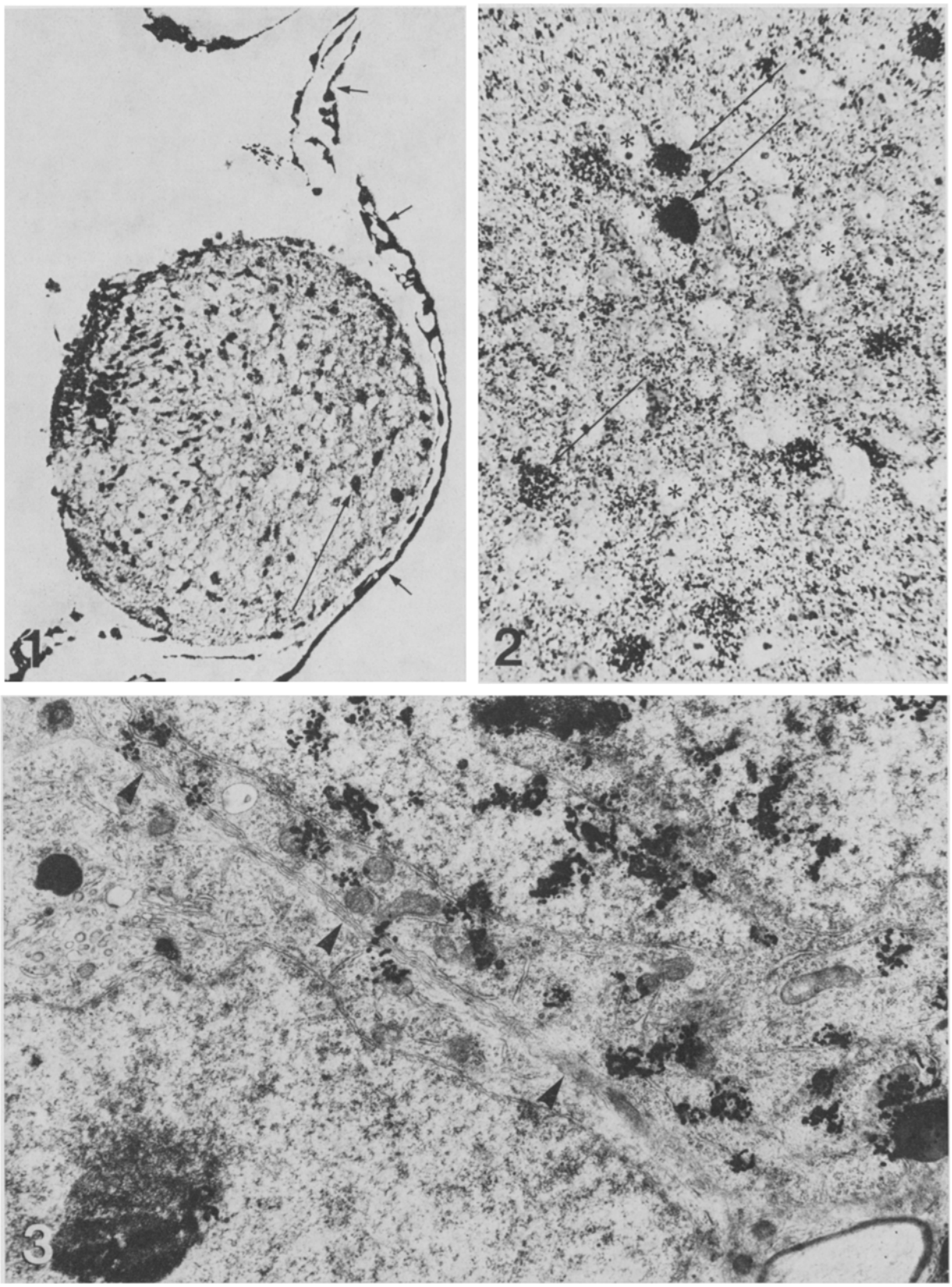

Fig. 1. Light microscopic autoradiograph of rat spinal cord culture (26 days in vitro) incubated with (^{3}H)-GABA ($10^{-6}M$, 5 min). Many cells (long arrow) in the explant are covered by grains. Note the heavy accumulation over the neuritic-meningeal outgrowth zone (short arrows). ×160. Fig. 2. Light microscopic autoradiograph, same tissue and treatment as in Figure 1. Several cell bodies are heavily labelled (arrows) whereas the majority of cells (asterisks) are covered only by few grains. × 640. Fig. 3. Electron microscopic autoradiograph of rat spinal cord culture (26 days in vitro) incubated with (^{3}H)-glycine ($5\times10^{-6}M$, 15 min). Parts of 2 neurones are seen, arrow indicating cell border. The upper cell is covered by many grains, whereas the lower one is almost completely free of label. ×17,000.

conditions. In preliminary experiments using perchloric acid extraction[32], about 6% of the isotope was associated with acid insoluble material in cultures incubated with (^{3}H(-glycine ($10^{-5}M$, 15 min). It is therefore possible that protein-bound glycine may contribute to the autoradiographic pattern, although only to a limited extent.

It has previously been speculated that the uptake of GABA and glycine as revealed in autoradiographic studies may occur in those neurones utilizing these amino acids as neurotransmitters[1–9]. Although the neuronal uptake of these amino acids clearly occurs only in a limited number of neurones of the explant – indicating specificity of the uptake mechanisms – it is at present not possible to decide whether glycine and GABA are taken up in the same or in separate neurone populations. The studies of IVERSEN and BLOOM[7] on spinal cord homogenates, however, show that the two amino acids are selectively taken up in separate nerve endings. These data would thus favour the view of two spinal cord neurone populations, each characterized by a different inhibitory amino acid transmitter. These two neurone populations could thus represent the morphological correlate to spinal inhibitory neurones postulated in neurophysiological studies[21, 22, 26, 33].

Uptake of amino acid neurotransmitters into glial cells has previously been demonstrated with autoradiography[2, 3, 5, 10, 34, 35], and it is also evident from the present results. The intense labelling of glial cells may indicate an efficient uptake mechanism, which could be of physiological importance for the transmitter inactivation[36].

Zusammenfassung. Autoradiographische Untersuchungen haben gezeigt, dass ^{3}H-Glycin und ^{3}H-GABA in kultivierte Neurone und Gliazellen des Rückenmarks der Ratte aufgenommen werden. Aus unseren licht- und elektronenmikroskopischen Befunden geht hervor, dass die Nervengewebskultur ein geeignetes Versuchsmodell für Aufnahme und zelluläre Lokalisation von Überträgersubstanzen ist.

E. HÖSLI, Å. LJUNGDAHL, T. HÖKFELT and L. HÖSLI

Department of Neurophysiology, Neurological Clinic of the University of Basel, CH-4051 Basel (Switzerland); and Department of Histology, Karolinska Institutet, S-104 01 Stockholm 60 (Sweden),
22 August 1972.

[32] We would like to thank Dr. H. P. VON HAHN and Miss V. STEINMANN for carrying out the perchloric acid extractions.
[33] L. HÖSLI, P. F. ANDRÈS and E. HÖSLI, Brain Res. *34*, 399 (1971).
[34] I. R. FAEDER and M. M. SALPETER, J. Cell Biol. *46*, 300 (1970).
[35] P. M. ORKAND and E. A. KRAVITZ, J. Cell Biol. *49*, 75 (1971).
[36] Acknowledgments: This study was supported by grants No. B72-14X-2887-3B and No. B72-14-P-3662-02B) from the Swedish Medical Research Council and by grants from Karolinska Institutets Forskningsfonder, Ollie och Elof Ericssons Stiftelse, Stiftelsen Margarethahemmet and from Magn. Bergvalls Stiftelse. The skillful technical assistance of Mrs. W. HIORT and Miss A.-C. SWENSON is gratefully acknowledged.

Zur Feinstruktur früher Veränderungen an Rattenhirngewebe nach Injektion von Methylnitrosoharnstoff

Bestimmte N-Nitrosoverbindungen erzeugen nach Untersuchungen von DRUCKREY et al.[1, 2] und JÄNISCH et al.[3] bei wiederholter Applikation Tumoren des Zentralnervensystems von Versuchstieren. Obwohl umfangreiche Untersuchungen an solchen experimentellen Geschwülsten vorliegen, wurde bisher noch nicht über präblastomatöse Veränderungen am Gehirn nach Einwirkung neurotroper Resorptivkanzerogene berichtet. Degenerative Veränderungen an perivaskulären Zellen und an Gliazellen, die innerhalb 24 h nach Applikationen einer einmaligen hohen Dosis Äthylnitrosoharnstoff auftraten, beobachtete LANTOS[4]. Wir haben mit Hilfe einer geeigneten Präparationsmethode Veränderungen an Rattengehirnen nach wiederholter Applikation von Methylnitrosoharnstoff (MNH) untersucht und berichten über erste Ergebnisse.

Material und Methode. 130–150 g schweren Wistarratten wurden in 4wöchigem Abstand 30 mg/kg Körpergewicht MNH i.p. injiziert. 12 Tage nach der ersten Injektion sowie 8 und 14 Tage nach der zweiten Injektion Perfusionsfixation des Gehirns, Einbettung von drei ca. 2 mm dicken Frontalscheiben des ganzen Gehirns je Versuchstier in Epon, dreidimensionale Zielpräparation mit Anfertigung von lichtmikroskopischen Übersichtsschnitten, Semidünnschnitten und Ultradünnschnitten (DIETZ und WIEGAND[5]).

Befunde. 12 Tage nach der ersten Injektion sowie 8 und 14 Tage nach der zweiten Injektion von MNH finden sich lichtmikroskopisch erkennbare perivaskuläre Zellproliferationen (Figur 1, Inset). Gehirne von Kontrolltieren, die nach der gleichen Methode präpariert wurden, zeigen keine derartigen Veränderungen. Elektronenmikroskopisch lassen sich die proliferierenden Zellen als Adventitiazellen kleiner Gefässe identifizieren (Figur 1). Sie sind gegen die Gefässendothelien durch eine Basalmembran abgegrenzt. Der perivaskuläre Raum ist erweitert und in der ganzen Zirkumferenz der Gefässe von proliferierenden Adventitiazellen ausgefüllt. Durch eine zweite äussere Basalmembran wird er gegen die erheblich geschwollenen perivaskulären Astrozytenfortsätze begrenzt. Bei den nach der zweiten Injektion untersuchten Tieren finden sich Lücken in dieser äusseren Basalmembran (Figur 2). Adventitiazellen, welche offenbar durch solche Basalmembranlücken in den Bereich des Hirngewebes gelangen, zeigen die gleiche Proliferationstendenz wie die Zellen im perivaskulären Raum. Sie breiten sich unter Verdrängung der Hirngewebsstrukturen konzentrisch um die Gefässe aus, besitzen aber keine insbesondere gegen das Neuropil abgrenzende Basalmembran mehr. Der Grundcharakter der Zellen im perivaskulären Raum und in der Peripherie der Proliferationsherde unterscheidet sich nicht. Von Einzelzelle zu Einzelzelle finden sich jedoch beträchtliche Variationen des Organellengehaltes des Zytoplasmas. Die Mehrzahl der Zellen mit langgestreckten, vielfach untereinander verschlungenen Fortsätzen enthält wenig RER und Ribosomen und gelegentlich feine intrazytoplasmatische Filamente. Weiterhin findet man Zellen mit einem ausserordentlich hohen Ribosomengehalt, andere mit ei-

[1] H. DRUCKREY, S. IVANCOVIC und R. PREUSSMANN, Naturwissenschaften *51*, 144 (1964).
[2] H. DRUCKREY, S. IVANCOVIC und R. PREUSSMANN, Z. Krebsforsch. *66*, 389 (1965).
[3] W. JÄNISCH, D. SCHREIBER, R. STENGEL und V. STEFFEN, Exp. Path. *1*, 243 (1967).
[4] P. L. LANTOS, Experientia *27*, 1322 (1971).
[5] W. DIETZ und K. WIEGAND, Zentbl. allg. Path. *115*, 22 (1972).