J. exp. Biol. **161**, 25–41 (1991)
Printed in Great Britain © *The Company of Biologists Limited 1991*

THE INTACT CENTRAL NERVOUS SYSTEM OF THE NEWBORN OPOSSUM IN LONG-TERM CULTURE: FINE STRUCTURE AND GABA-MEDIATED INHIBITION OF ELECTRICAL ACTIVITY

BY R. R. STEWART[1,*], D.-J. ZOU[1], J. M. TREHERNE[1], K. MØLLGÅRD[2], N. R. SAUNDERS[3] AND J. G. NICHOLLS[1]

[1]*Department of Pharmacology, Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland,*
[2]*Department of Clinical Neurological Sciences, University of Southampton, South Block, Southampton General Hospital, Southampton SO9 4XY, UK* and [3]*Institute of Medical Anatomy, The Panum Institute, Blegdamsvej 3, DK-2200, Copenhagen, Denmark*

Accepted 17 June 1991

Summary

1. The entire central nervous system (CNS) of the newly born, South American opossum (*Monodelphis domestica*) was isolated and maintained in basal medium, Eagle's (BME) with 0.2% foetal calf serum and antibiotics. Isolated CNS preparations remained electrically excitable for up to 10 days. The fine structure of the spinal cord was normal after 5 days in culture: axons, synapses, dendrites and glia were virtually unchanged. Signs of degeneration were evident only in dorsal areas of the spinal cord, which had been denervated by removal of the dorsal root ganglia during dissection.

2. Amino acid transmitters such as glycine, glutamate, *N*-methyl-D-aspartate (NMDA) and gamma-aminobutyric acid (GABA), applied to the bathing fluid, rapidly and reversibly inhibited synaptic transmission in cervical segments of the spinal cord. GABA (10–100 μmol l^{-1}) produced a dose-dependent reduction in the magnitude of ventral root responses evoked by dorsal root stimulation. GABA also inhibited synaptically activated compound action potentials produced by spinal cord stimulation. Dose–response curves for GABA obtained in different preparations were highly reproducible.

3. Both $GABA_A$ and $GABA_B$ receptors were reversibly activated by selective agonists and inhibited by specific antagonists. The actions of GABA were potentiated by benzodiazepines, competitively antagonised by bicuculline (a selective $GABA_A$ antagonist) and mimicked by muscimol (a $GABA_A$ agonist).

*Present address: CNRS Laboratoire d'Etude des Régulations Physiologiques, 23 Rue Bequerel, F-67087 Strasbourg, France.

Key words: opossum, development, central nervous system, receptors, GABA, spinal reflexes, *Monodelphis domestica*.

Baclofen (a specific $GABA_B$ agonist) also inhibited electrical activity and was competitively antagonised by the $GABA_B$ antagonist, CGP 35348.

4. After 5 days of culture in BME or minimal essential medium (MEM), GABA dose–response curves were unchanged from those observed immediately after removal of the CNS. The inhibitory potency of baclofen was also unaffected by culture in BME. By contrast, after 5 days of culture in MEM, baclofen no longer inhibited electrical activity. This difference between BME and MEM could be attributed to the higher content of L-histidine in MEM. Thus, addition of $150\,\mu mol\,l^{-1}$ L-histidine to BME produced similar results to culture in MEM: the inhibitory action of baclofen was virtually abolished after 3–5 days. L-Histidine had no effect on freshly dissected preparations. Chronic application of L-histidine did not affect glycine or glutamate responses after 5 days. Addition of D-histidine or other amino acids, such as arginine, to BME did not abolish the responses to baclofen.

5. These results show that the isolated CNS of the newborn opossum survives well in long-term culture and that it provides a useful preparation to study receptor development and plasticity of an intact mammalian CNS *in vitro*. The rapid and reversible penetration of agonists and antagonists into the preparation also allows for pharmacological manipulation of the neuronal environment.

Introduction

During development and maturation of the mammalian nervous system, transmitter receptors become distributed over appropriate regions of specified cells. The numbers of receptors and their properties depend not only on the stage of development but also on levels of activity and pharmacological agents. The developmental stages and sites at which transmitters and receptors appear in the CNS can be studied using autoradiography and homogenate binding assays. It is difficult to test receptors for the development of a functional response to their transmitters in living embryos, owing to complications of anaesthesia, inadequate access to the area of interest and poor penetration of drugs. Mammalian *in vitro* brain slices (Andersen, 1981) have greatly facilitated the investigation of synaptic mechanisms, circuitry and pharmacology. The thickness of the slice, however, cannot usually exceed $700\,\mu m$, so as to allow an adequate supply of oxygen (Andersen, 1981). Slices from neonatal brains have also been maintained in long-term culture (Gähwiler, 1984). These slices retain morphology analogous to that in the intact CNS, with neurones forming a monolayer of cells. A disadvantage of all slice preparations is that neuronal connections coming in and out of the plane of the slice are severed. More complete CNS preparations have been obtained using neonatal rat spinal cord *in vitro* (Otsuka and Konishi, 1974); this preparation was extended to include the brain stem as well (Onimaru and Homma, 1987). As with invertebrates, it has been possible to isolate part of the CNS together with effector organs, such as ribs and intercostal muscles, which continue to show spontaneous rhythmical activity (Suzue, 1984). Drugs can be applied at known concentrations, but the dissections are difficult and the survival time is relatively short, usually a few hours.

The ability to maintain an entire mammalian CNS in long-term culture with all central connections intact would, therefore, provide a preparation with certain advantages. The intact CNS of *Monodelphis domestica* can now be removed 2–5 days after birth (Nicholls *et al.* 1990). At this stage, the opossum has only an embryonic forebrain (two-layered) and no cerebellum, corresponding to a 14-day rat embryo (Saunders *et al.* 1989). Its eyes, ears and hindlimbs are also at an early stage of development. This immaturity, coupled with the small size of the intact CNS (approximately 1.5 cm long, with a spinal cord 0.5 mm in diameter), allows adequate access for oxygen and nutrients and, presumably, contributes to the robust nature of the preparation. The intact CNS is suitable for *in vitro* studies and can, for example, survive in Krebs' fluid for more than 4 days, continuing to produce action potentials upon stimulation. This is remarkable, since Krebs' fluid contains only salts and glucose. Even after 4 days in these minimal conditions, blast cells continue to divide, as determined by labelling with bromodeoxyuridine (Nicholls *et al.* 1990). That the preparation is functionally decorticate and devoid of conscious sensation is evident from the absence of all centres higher than the medulla.

In this paper, we have further exploited this preparation: we have devised favourable culture conditions, examined structural survival by electron microscopy and investigated the GABA-mediated inhibition of electrical activity in the spinal cord *in vitro*. The fine structure of the spinal cord appeared normal after 5 days in culture; a full description of other CNS areas will appear in detail elsewhere (K. Møllgård, unpublished observations). The actions of GABA on freshly dissected preparations were similar to those observed in the adult CNS of other mammalian species (Bormann, 1988). Responses to bath application of drugs were rapid, reversible and reproducible. These properties have allowed us to show that $GABA_B$ receptor responses can be modulated by L-histidine during long-term culture.

Materials and methods

Removal and long-term culture of the CNS

The breeding colony of South American, grey opossums (*Monodelphis domestica* Saunders *et al.* 1989) was maintained as described previously (Saunders *et al.* 1989; Nicholls *et al.* 1990). Opossum pups were removed from their mothers 3 days after birth and anaesthetized by cooling on ice. This procedure provides a highly effective method of anaesthesia for new-born marsupials, which cannot thermoregulate. The entire CNS was then removed (Nicholls *et al.* 1990), while immersed in basal medium, Eagle's, which contained Earle's salts but not L-glutamine (BME, Gibco, Life Technologies Ltd, Scotland, UK). The BME was continuously gassed with O_2/CO_2 (95 %:5 %). Culturing involved placing the CNS in 15 ml of BME, containing 0.2 % foetal calf serum (Gibco) and 0.1 mg ml^{-1} gentamycin sulphate (Essex Chemicals AG, Lucerne, CH) at 23 °C, bubbled with O_2/CO_2 (95 %:5 %). Two other media (Gibco) were tested: minimum essential medium

(MEM) and Leibovitz-15 (L-15). All the amino acids, whether added to the culture medium or acutely applied to the preparation, were obtained from Sigma (St Louis, MO, USA).

Electron microscopy

The entire, isolated CNS from 3-day-old animals or from 3-day-old animals cultured for 5 days was fixed for 4–6 h in a solution of 1.5 % glutaraldehyde and 1.5 % paraformaldehyde in 0.1 mol l^{-1} cacodylate buffer (pH 7.4). The CNS was rinsed overnight in the same buffer and dissected into appropriate tissue blocks. The specimens were then postfixed in 2 % OsO_4, contrasted *en bloc* with a solution of 1 % uranyl acetate for 1 h at room temperature, dehydrated and embedded in Epon. All fixation and dehydration steps were performed at 4 °C. Survey sections, 1 μm thick, were stained with Toluidine Blue and examined with the light microscope. Thin sections, 30–40 nm thick, were cut on an LKB ultratome Nova II, picked up on Formvar-coated copper grids and contrasted with uranyl acetate and lead citrate.

Electrical recordings

Recordings were obtained from both acute and cultured preparations. The brain was separated from the spinal cord just caudal to the brain stem and suction electrodes (containing BME) were placed on each end of the cord. Electrical signals were amplified by a low-noise differential amplifier (Almost Perfect Electronics, Basel) and displayed on an oscilloscope and chart recorder. Signals were either stored on magnetic tape (Hewlett-Packard 3964A, CA, USA) or digitised using a VR-100 digital recorder (Instrutech Corp., Mineola, NY, USA). Supramaximal electrical stimuli were applied to the preparation every 10 s. Suction electrodes for stimulating or recording from dorsal or ventral roots were made with a conventional electrode puller from 25 μl measuring micropipettes (Clay Adams, Accu-Fill 90). Pulled electrodes were scored near their tips with a diamond scribe, the tips broken off, and the ends of the pipettes were fire-polished to a diameter of 30–40 μm. To test the effects of different media, amino acids and amino acid derivatives on electrical excitability, Krebs' fluid (composition in Nicholls *et al.* 1990) was exchanged for a test medium (L-15, MEM or BME) or for Krebs' fluid containing the relevant amino acid. In later experiments, test substances were applied directly to BME, which was bubbled continuously with O_2/CO_2 (95 %:5 %). Baclofen and tetrodotoxin were obtained from Sigma (MO, USA) and CGP 35348 was a generous gift from Dr H.-R. Olpe, Ciba-Geigy AG, Basel. Midazolam was kindly donated by Professor W. Haefely, Hoffman-La Roche AG, Basel. The values quoted for the half-maximal inhibition produced by GABA and for the Hill slope were obtained by fitting the data to the Hill equation by weighted non-linear regression analysis using GraphPad InPlot (GraphPad Software, San Diego, USA). When data points are plotted as means, the error bars represent the standard error of that mean.

Results

Choice of culture medium

Three different culture media (L-15, MEM and BME) were tested for their ability to influence spontaneous and evoked electrical activity and to promote long-term survival of the isolated CNS. In acutely isolated preparations, changing the bathing fluid from Krebs' solution to L-15 produced immediate suppression of all electrical activity in the spinal cord. As L-15 contains higher concentrations of amino acids than the other media, each of the amino acids was then applied to the preparations bathed in Krebs' fluid at the concentration present in L-15. Some amino acid derivatives were also tested. The amino acids glycine, glutamate and aspartate as well as the amino acid derivatives *N*-methyl-D-aspartate (NMDA) and gamma-aminobutyric acid (GABA) were all found to influence electrical excitability. Less potent or without effect at the concentrations found in L-15 were arginine, glutamine, histamine, histidine, isoleucine, methionine, phenylalanine, serine, threonine, tyrosine and valine. From this profile it appeared that two media, BME and MEM, could in principle be suitable culture solutions. After maintenance of the isolated CNS in BME or MEM for 1–7 days, the medium was changed to Krebs' fluid and the spinal cord tested for electrical excitability. For reasons described in the section on $GABA_B$-mediated responses, BME proved to be better than other media tested.

Survival of the intact CNS in long-term culture

Freshly dissected and cultured spinal cords were compared histologically under the light and electron microscopes. After 5 days of culture in BME, 1 μm survey sections from thoracic segments of the opossum spinal cord showed that there were surprisingly few degenerating cells in ventral areas of the section (Fig. 1A,B). Moreover, cells in the ventral horn showed no signs of chromatolysis even though all ventral roots had been cut. It is clear, however, that degenerating cells were present in dorsal areas of the cultured spinal cord. This was to be expected since the dorsal root ganglia had been removed during dissection. The health of the cultured preparation was further illustrated by the numerous dividing blast cells evident around the central canal (Fig. 1B). A comparison of the electron micrographs taken from the ventral areas shown in Fig. 1A,B, demonstrates that it is difficult to distinguish between the preparation fixed immediately after dissection and that fixed after 5 days of culture (Fig. 2A,B). In both representative sections, axons, dendrites, synapses, extracellular space and radial glia appear similar. Particularly striking are the absence of myelin and the small numbers of glial cells.

The spinal cord of intact CNS preparations cultured in BME continued to be electrically excitable. After 5 or even 10 days in culture, compound action potentials of normal amplitude and time course were still evoked by electrical stimulation. For example, stimulation of the caudal end of an acutely isolated CNS

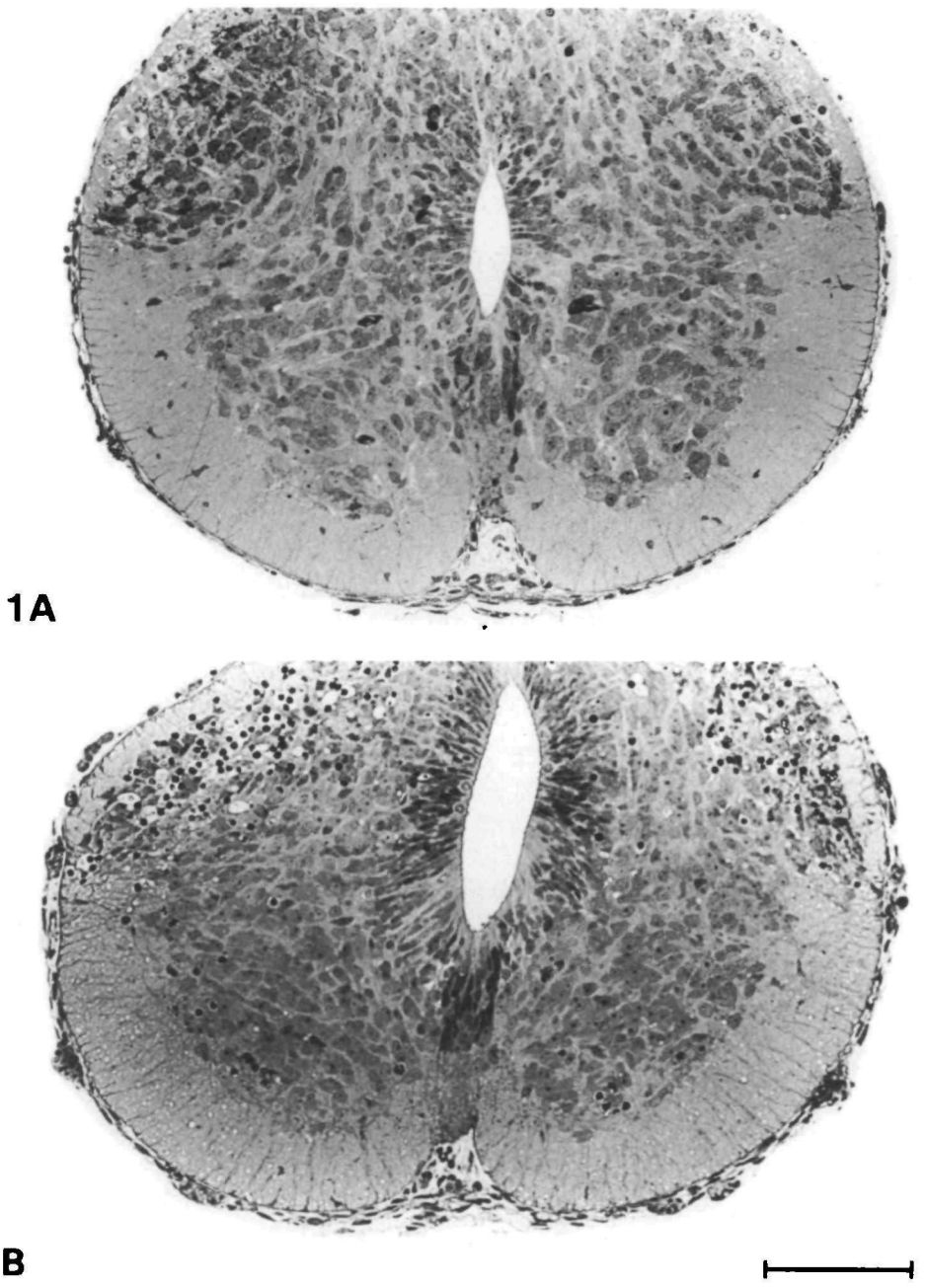

Fig. 1. (A) A 1 μm Toluidine-Blue-stained transverse section through a thoracic segment of the opossum spinal cord fixed immediately after removal from a 3-day-old animal. (B) A similar micrograph also from a thoracic area of a 3-day-old CNS fixed after culture for 5 days in BME, 0.2 % foetal calf serum and antibiotics. Scale bar, 100 μm.

or a preparation cultured for 10 days allowed similar compound action potentials to be recorded in the thoracic region (Fig. 3A,B).

These results clearly demonstrated that intact preparations could be maintained in a healthy state for several days, even though electrical activity (Hansen, 1985) and fine structure are susceptible to hypoxia and lack of nutrients.

GABA-mediated inhibition of electrical activity

Gamma-aminobutyric acid (GABA), applied to the bathing fluid, inhibited reflexes in the cervical segments of the spinal cord. The ventral root responses, evoked by dorsal root stimulation, were inhibited by GABA in a dose-dependent manner (Fig. 4A,B). GABA also reduced the amplitude of compound action potentials recorded at one end of the spinal cord when stimulated at the other (Fig. 5A). The inhibitory action of GABA was rapid, achieving a maximum in about 10–20 s. The inhibition was only partially sustained in the presence of GABA (Fig. 5B), presumably as a result of desensitisation of the response or localised GABA uptake. The effect was fully reversed 2 min after washing; this allowed doses to be applied regularly every 10 min. Preparations were not fatigued by this dosage cycle or by prolonged, repetitive stimulation for several hours. The inhibitory actions of GABA remained constant over these periods (Fig. 5B). The concentration of GABA producing a 50 % reduction in maximum response (EC_{50}) was 54 μmol l^{-1} (Fig. 5C). GABA was consistently a less potent inhibitor of compound action potentials in the spinal cord than of the dorsal–ventral root reflex. Whether this reflects the severing of the dorsal and ventral roots or represents a physiological difference *in vivo* is not yet clear. Nevertheless, the range of GABA concentrations suppressing electrical activity in the spinal cord agrees with the potency of GABA-mediated effects on central neurones in other species (Bormann, 1988). The fitted Hill slope (0.98) of the data suggests that the mechanism of action does not depend on positive cooperativity. In all the graphs shown here, the effects of GABA were assessed by measuring the amplitudes of compound action potentials; comparable measurements of integrated areas under compound action potential traces produced virtually identical dose–response curves.

A striking and consistent result was that even after 5 days in BME, the sensitivity of the preparation to GABA was not significantly altered compared with that of preparations tested immediately after dissection (Fig. 5C).

A major component of GABA-mediated inhibition of compound action potential activity was mediated by $GABA_A$ receptors. Bicuculline competitively antagonised the actions of GABA, since the dose–response curve was shifted to the right in a parallel manner (Fig. 6A). A similar parallel shift, however, would have been observed through non-competitive interaction if the $GABA_A$ receptors had a large receptor reserve. A non-competitive interaction seems unlikely, however, as bicuculline is a competitive antagonist at micromolar concentrations in the CNS of other mammals (e.g. Curtis *et al.* 1970). Physiological inhibitory actions occurring during reflex activity were also mediated by $GABA_A$ receptors:

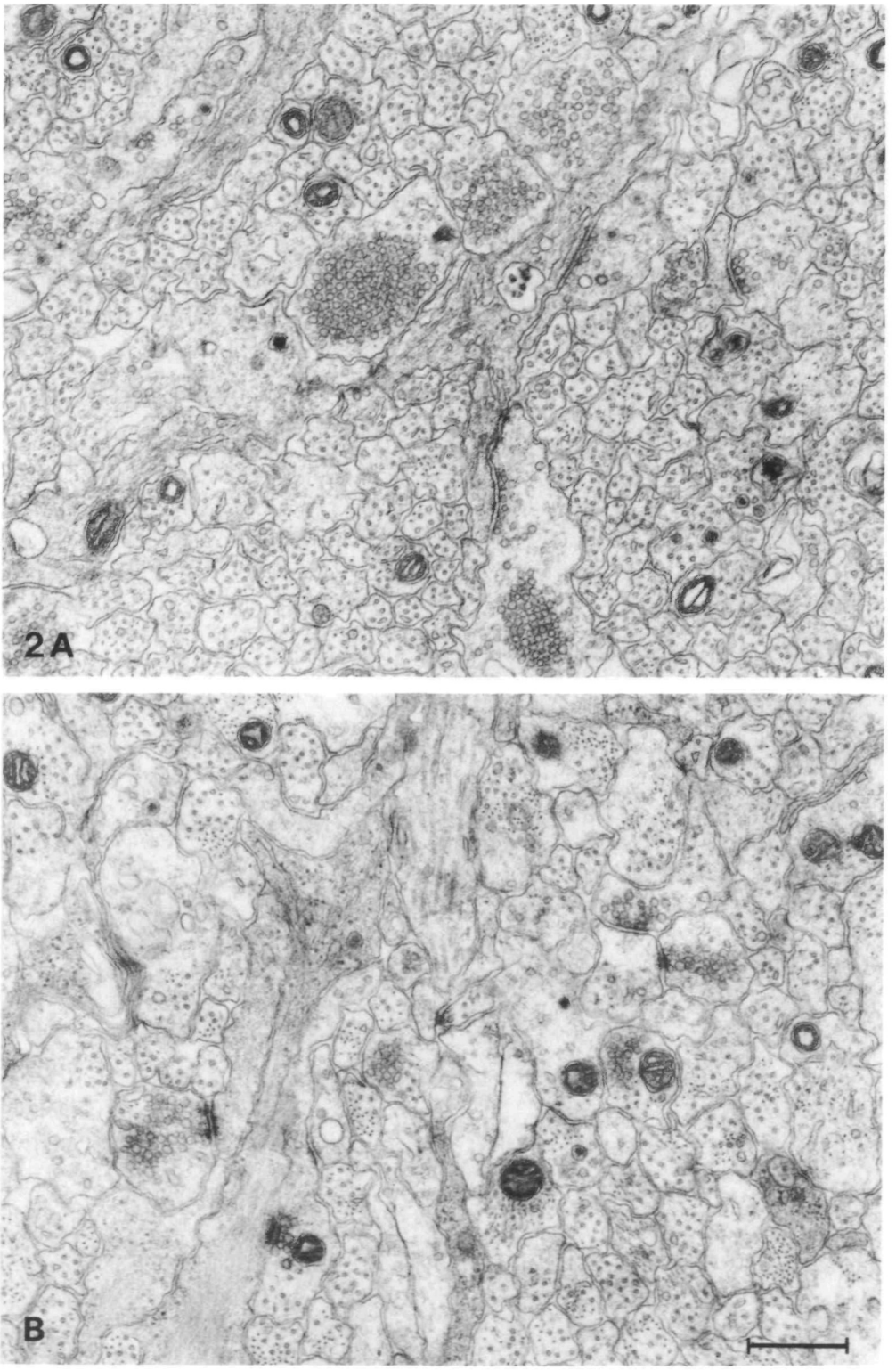
2A
B

Fig. 2. (A) Electron micrograph from a representative ventral region of a transverse section through a thoracic segment of the opossum spinal cord fixed immediately after removal from the animal. (B) A similar micrograph also from a ventral spinal cord fixed after 5 days of culture in BME, 0.2% foetal calf serum and antibiotics. Both preparations have strikingly similar dendrites, synapses and radial glia as well as an absence of myelin. Scale bar, 0.5 μm.

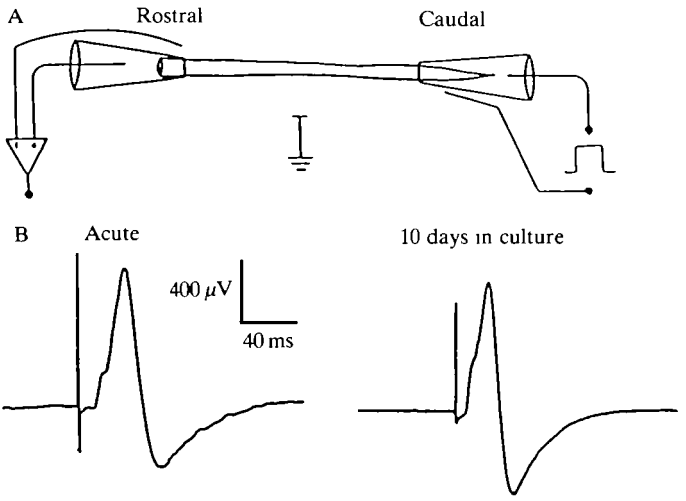

Fig. 3. (A) Suction electrode recording arrangement: a stimulating electrode is placed over the caudal end of the spinal cord and a recording electrode over the rostral end, from which the midbrain has been removed, immediately before recording. (B) Compound action potentials evoked in the spinal cord by electrical stimulation. The left-hand trace was obtained from a freshly dissected preparation and that on the right from a preparation that had been cultured in BME for 10 days.

application of bicuculline to preparations increased the amplitude of spontaneous ventral root discharges (Fig. 6B). If bicuculline was left in contact with the preparation for several minutes or hours, spontaneous bursts of electrical activity appeared several minutes after washing off the drug. They occurred rhythmically every 10–20 s (Fig. 6B) and were reminiscent of bursts of motoneurone discharges that occur during convulsant activity produced by bicuculline injections *in vivo* (Curtis *et al.* 1970). These actions of bicuculline suggest that endogenously released GABA is acting as a transmitter in this neonatal spinal cord.

Further evidence of normal $GABA_A$ receptor properties in this neonatal CNS was provided by the effects of bath-applied benzodiazepines (Fig. 7A). Midazolam (20 μmol l^{-1}) increased the sensitivity of the preparation to bath-applied GABA. Maximal effects were observed several hours after the initial exposure to midazolam and reversal upon washing was also slow. Muscimol (0.1–10 μmol l^{-1}), a selective $GABA_A$ agonist, inhibited electrical activity in a dose-dependent manner and was more potent than GABA (results not illustrated).

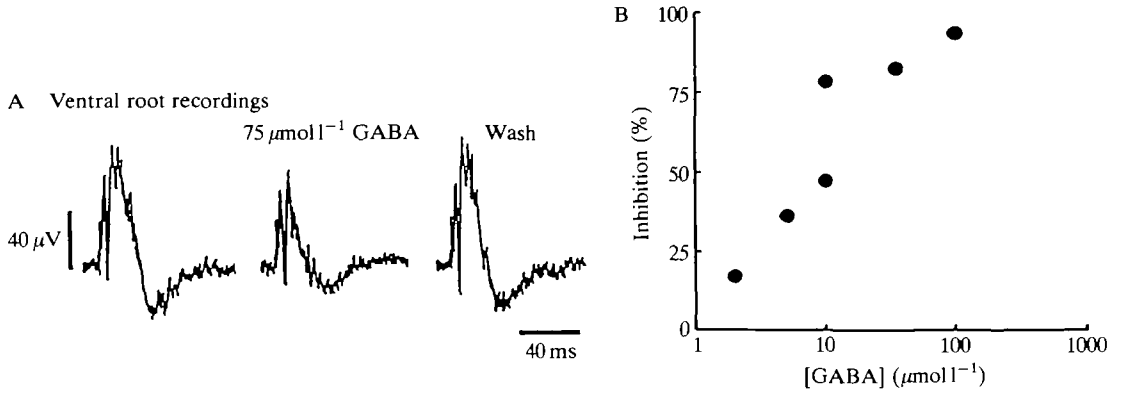

Fig. 4. (A) Ventral root responses elicited by dorsal root stimulation: traces were obtained with suction electrodes on the dorsal and ventral roots. GABA (75 μmol l^{-1}) reversibly reduced the amplitude of the response. (B) Dose–response curve for the GABA-mediated inhibition of ventral root responses, taken from a single experiment. GABA was found to have a similar potency in five further experiments.

$GABA_B$-mediated responses

Activation of $GABA_B$ receptors by baclofen, a selective $GABA_B$ agonist (Bowery, 1989), also inhibited electrical activity (Fig. 7B). The extent of inhibition produced by $GABA_B$ receptor stimulation was less than that for $GABA_A$ receptors: a maximal dose of baclofen never abolished electrical activity completely. The effects of baclofen were competitively antagonised by the selective antagonist CGP 35348 (Olpe *et al.* 1990). This provides further evidence for involvement of a $GABA_B$ receptor component.

Glycine also reduced the amplitude of the evoked compound action potentials (Fig. 8), but desensitisation was more rapid and more pronounced than with muscimol or baclofen. *N*-Methyl-D-aspartate (NMDA) produced a small excitatory effect in the first few seconds followed by a strong inhibition (Fig. 8). The actions of bath-applied glutamate were similar to those of NMDA (data not shown). These amino acids were not tested in detail, but they were used to assess the relative specificity of GABA in inhibiting electrical activity.

Sensitivity of the $GABA_B$ responses to amino acids in the culture medium

After 5 days of culture in BME, the sensitivity to baclofen was almost the same as in acute preparations. By contrast, in MEM responses to baclofen were virtually abolished (Fig. 9A), while those to GABA were unchanged. To identify the component in MEM responsible for the loss of $GABA_B$ activity, preparations were cultured in BME with different supplements of amino acids. It was found that 5 days of culture in BME with a supplement of 150 μmol l^{-1} L-histidine (thus raising the concentration from 50 μmol l^{-1} to that in MEM) resulted in a drastic reduction in the sensitivity of the preparation to baclofen (Fig. 9B). Supplements of L-histidine at lower concentrations (50 μmol l^{-1}) produced smaller depressions

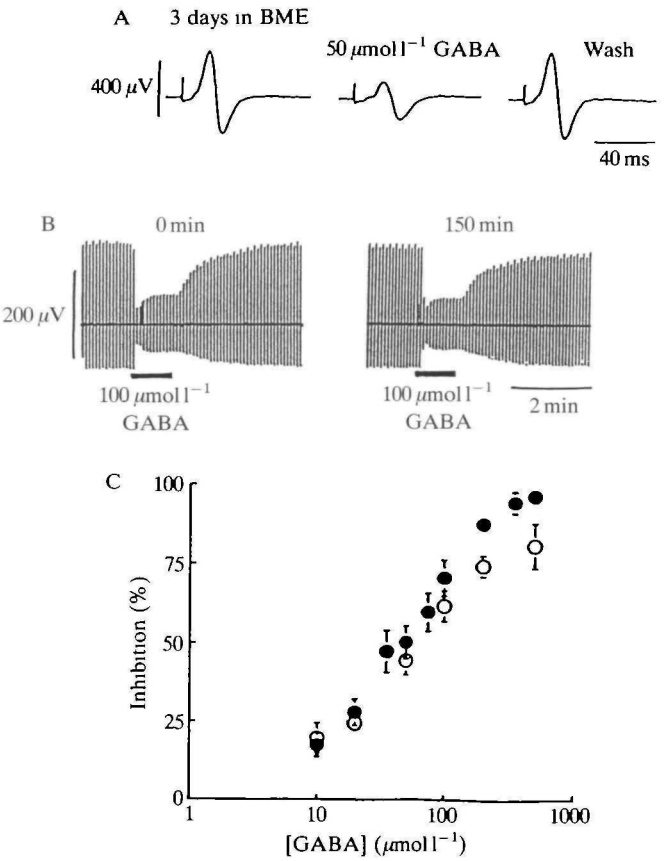

Fig. 5. (A) Compound action potentials in the spinal cord. Bath-applied GABA (50 μmol l^{-1}) reversibly decreased the amplitude of these responses in a preparation that had been cultured in BME for 3 days. (B) Constancy of GABA-mediated inhibition of electrical activity. Regular compound action potential traces (resulting from stimulation every 10 s) are shown on a slower time-base than in A. GABA (100 μmol l^{-1}) was applied to the same preparation at 10-min intervals: the extent of the decrease in amplitude of the compound action potentials and the duration of the inhibition were very similar after 150 min. (C) Dose–response curve for GABA-mediated inhibition. Note that the mean responses of 14 freshly dissected preparations (●) did not change after 5 days in culture (○, means±S.E.M. of 12 preparations).

of the baclofen response (data not shown). The action of L-histidine was stereospecific: addition of 150 μmol l^{-1} D-histidine or arginine to BME did not produce any obvious depression of the baclofen responses (Fig. 9B).

The actions of L-histidine on baclofen responses developed slowly over days. During the first day in culture, responses to baclofen remained practically unchanged in the presence of 150 μmol l^{-1} L-histidine added to BME. Exposure to 150 μmol l^{-1} L-histidine for 3 days was less effective than 5 days of exposure in reducing baclofen responses. Long-term culture with L-histidine did not abolish

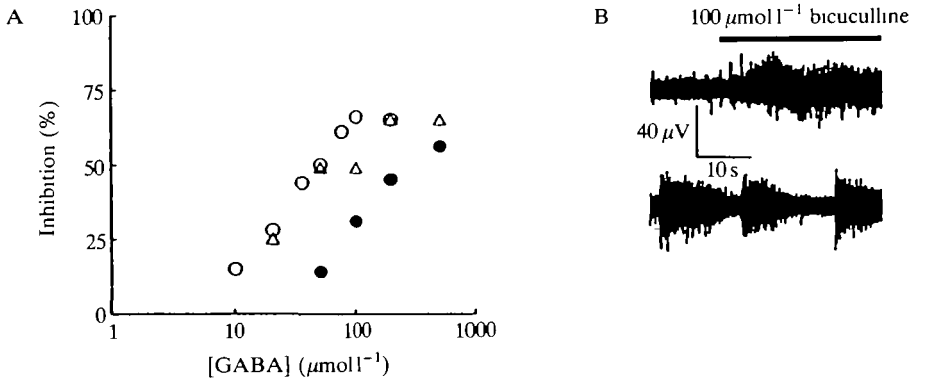

Fig. 6. (A) The effect of bicuculline on GABA-mediated inhibition of compound action potentials. The control curve for GABA (○) was shifted to the right in a parallel fashion in the presence of 10 μmol l^{-1} bicuculline (●). The action of bicuculline was reversible: on washing, GABA sensitivity returned to normal (△). The response measured was the reduction in transmission of the compound action potential through the spinal cord elicited by electrical stimulation. Similar antagonism by bicuculline was observed in four further experiments. (B) Increases in spontaneous electrical discharge from ventral roots following application of 100 μmol l^{-1} bicuculline (upper trace). In this experiment rhythmical, burst-like activity was observed several minutes after washing out the bicuculline (lower trace).

inhibitory responses produced by glycine, glutamate, NMDA, GABA or muscimol (data not shown).

Discussion

Our results show that the neonatal opossum CNS survives well in culture and that it maintains electrical excitability and normal responses to neurotransmitters for several days. The ultrastructure of the CNS in culture shows that preparations remain healthy for at least 5 days. Even though the ventral roots had been cut, chromatolysis (Carpenter, 1976) was not apparent and dividing cells were still abundant around the central canal. There was evidence of degenerating cells in dorsal areas, presumably as a result of severing the dorsal roots. Studies by K. Møllgård (unpublished data) indicate that, although some development continues, the CNS of an 8-day-old animal is more mature than that of a 3-day-old preparation maintained in culture for 5 days. BME is the best medium we have tested so far for the prolonged culture of the preparation, although it must represent a far from optimal medium: with only 0.2 % foetal calf serum the levels of proteins, growth factors, hormones and vitamins are minimal. Cerebrospinal fluid at this stage of CNS development *in vivo* contains a total protein concentration equivalent to 5–10 % foetal calf serum (Dziegielewska *et al.* 1989). Presumably enrichment as well as temperature adjustment will provide an even more favourable environment for survival and development. Even so, the viability of the preparation in long-term culture has been surprisingly good. The retention

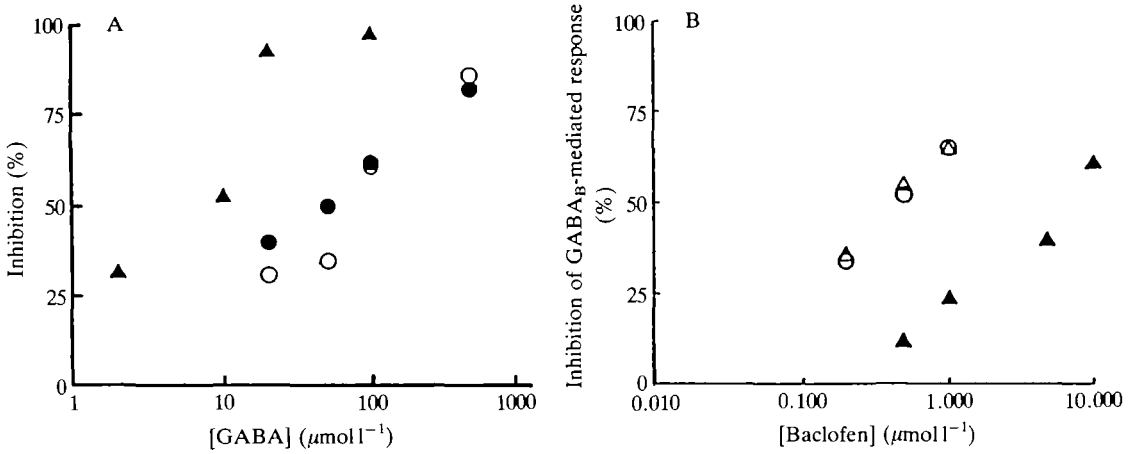

Fig. 7. (A) Midazolam potentiation of GABA-mediated inhibition of compound action potentials. The control curve for GABA (●) was shifted to the left in the presence of 20 μmol l^{-1} midazolam (▲) after exposure to the compound for 2 h. This action of midazolam was reversible, since on washing for 3 h GABA sensitivity returned to normal (○). The response measured was the reduction in transmission of the compound action potential through the spinal cord elicited by electrical stimulation. These effects of midazolam were observed on two further preparations. (B) Effect of CGP 35348, a selective $GABA_B$ antagonist, on $GABA_B$-mediated inhibitory responses. These results were obtained from a single, representative CNS preparation, immediately after removal from the animal. The graph shows dose–response curves to baclofen (a $GABA_B$ agonist) in the absence (○) and presence (▲) of CGP 35348. The antagonism was reversible on washing (△). The response measured was the reduction in transmission of the compound action potential through the spinal cord elicited by electrical stimulation. GCP 35348 was found to antagonise the actions of baclofen in three further preparations.

of electrical activity in culture for up to 10 days, the $GABA_A$- and $GABA_B$-mediated responses after 5 days, as well as the excellent preservation of structure in electron micrographs, show that, even using the present culture conditions, the preparation is suitable for studies on long-term receptor regulation and development in an intact CNS *in vitro*.

The explanation for the remarkably long survival of the opossum CNS *in vitro* is unknown. It will be important to determine whether it is a uniquely marsupial property, perhaps related to the survival of young born at such an early stage of CNS development. It may be that the survival of these preparations is an extension of the ability of eutherian foetuses to develop under conditions of low arterial oxygen tension. The explanation of the prolonged survival of the opossum CNS *in vitro* has implications for the understanding of the deleterious effects of hypoxia on the mammalian CNS.

The reduction in the $GABA_B$ response after 5 days of culture in MEM was shown to result from the high concentration of L-histidine. The mechanism by which the $GABA_B$-mediated response is inhibited by L-histidine, but not other

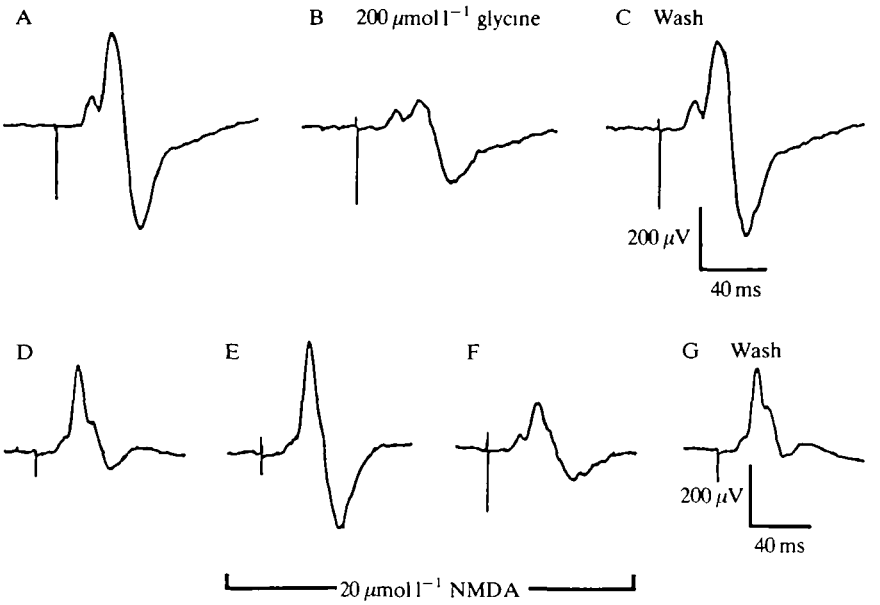

Fig. 8. (A–C) Inhibition of electrical activity by glycine. (A) Control stimulus-evoked compound action potential; (B) response in the presence of 200 μmol l^{-1} glycine applied directly to the bath; (C) response after washing. (D–G) Inhibition of electrical activity by NMDA after an initial potentiation. (D) Control stimulus-evoked compound action potential; (E) initial response in the presence of 20 μmol l^{-1} NMDA, within 1 min of its application to the bath; (F) later response in the presence of 20 μmol l^{-1} NMDA, 2–3 min after application; (G) response after washing.

amino acids, is not clear. Three mechanisms affecting $GABA_B$ receptors can be suggested: (1) chronic exposure to L-histidine could kill a population of neurones containing $GABA_B$ receptors. To accommodate this hypothesis, the $GABA_A$ and $GABA_B$ receptors would have to be distributed on different neurones, since the dose–response curve to GABA was not significantly altered during culture with L-histidine. Moreover, culture with L-histidine did not produce massive cell death visible by light microscopy (K. Møllgård, unpublished observations). (2) L-Histidine could cause the $GABA_B$ receptor to uncouple from a G-protein-linked effector molecule, e.g. an ion channel or second messenger system. Such desensitisation mechanisms typically occur rapidly, not over several days in culture, and are usually rapidly reversible, certainly over the time course of a typical experiment (e.g. 2–4 h). No such rapid reversibility was observed in the experiments reported here. It would be interesting to test for the reversibility of the L-histidine effect over several days, since a recovery of the baclofen response after prolonged washing would argue against the first mechanism. It is entirely possible that long-term exposure to L-histidine influences cellular processes downstream from the receptor/G-protein complex (e.g. a direct effect on ion channels or second messenger systems). This might correlate with the relatively

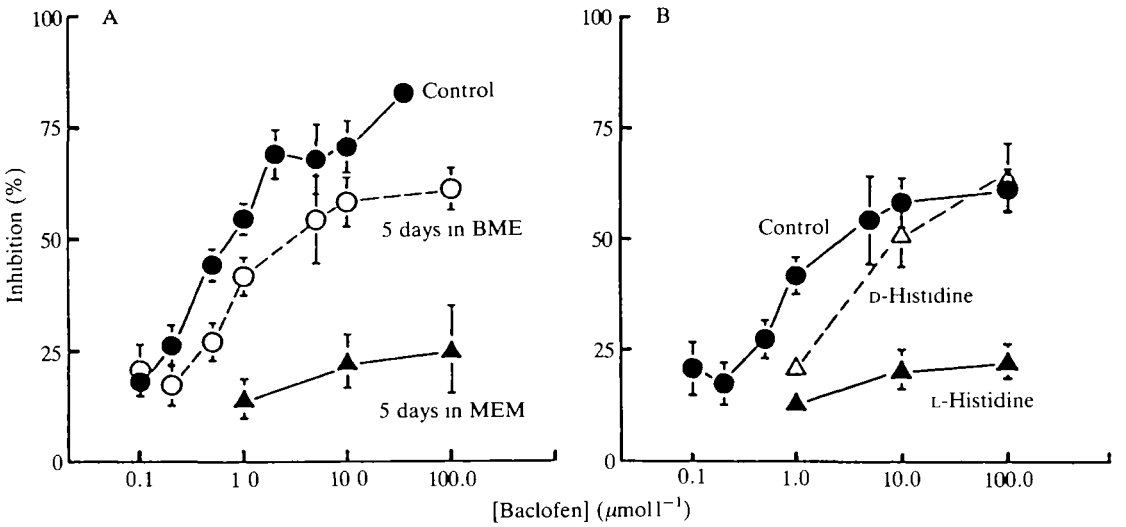

Fig. 9. (A) Culture in MEM for 5 days produced a decrease in the $GABA_B$-mediated inhibition of the response. The control curve (●) represents the mean inhibitory effect of baclofen on compound action potentials from seven acute preparations. Twelve preparations cultured in BME for 5 days (○) were almost as responsive to baclofen as control preparations. When six preparations were cultured under similar conditions but in MEM, the inhibition produced by baclofen was virtually abolished (▲). (B) Culture in BME with L-histidine also decreased the $GABA_B$ response. Culturing for 5 days with a supplement of L-histidine (150 μmol l^{-1}) added to BME virtually abolished the baclofen response (▲, 12 preparations) when compared with the response obtained after 5 days culture in BME alone (●, 12 preparations). The action of L-histidine was stereospecific, since a supplement of D-histidine (150 μmol l^{-1}) in BME for 5 days (△, six preparations) was considerably less potent than its enantiomer. L-Histidine effects appeared slowly after exposure for more than 1 day. Values are mean±S.E.M.

slow reversibility of the L-histidine effect. (3) L-Histidine could cause a down regulation in $GABA_B$ receptors. This hypothesis could be tested directly by radioligand binding assays for $GABA_B$ receptors (Bowery, 1989). Complications, however, would arise if there were a substantial reserve of $GABA_B$ receptors, as correlating changes in binding capacity (or affinity) with functional responses would prove difficult. Further difficulties could include a high proportion of non-specific binding to the limited amount of CNS material available for these assays (H. Bittiger, unpublished observations).

How L-histidine might influence any of the suggested mechanisms is unclear. One possibility is that L-histidine does not act directly, but is transported into neurones and converted to the neurotransmitter histamine by histidine decarboxylase. This suggestion is consistent with the inactivity of D-histidine, which is a poor substrate for this enzyme (Schwartz *et al.* 1991). It is also possible that D-histidine is not easily transported into neurones. Once formed, histamine could then act through any of the three classes of histamine receptor in the CNS, to influence any

of the potential mechanisms outlined above. Furthermore, $GABA_B$ receptors have been shown to inhibit histamine-H_1-receptor-induced inositol phosphate formation in tissue slices of rat cerebral cortex (Crawford and Young, 1988). This clearly demonstrates that these receptors can be functionally coupled and are probably co-localised on the same cells. We are currently investigating the ability of histamine to mimic the actions of L-histidine. Furthermore, descending histaminergic fibres projecting into the spinal cord of adult rodents have been discovered (reviewed by Schwartz *et al.* 1991). It would be interesting to determine whether these fibres exist in the new-born opossum spinal cord and to assess their possible involvement in the suppression of the baclofen response. Detailed investigations into mechanism will be attempted with extracellular recordings of single units from the spinal cord.

Our results demonstrate that the newborn opossum CNS in culture is a highly favourable preparation for studying receptor regulation and eventually, we hope, receptor and synaptic development *in vitro*.

We thank Erich Hardman, Marlies Kunz and Urs Berglas for their excellent work in maintaining the colony of *Monodelphis*. Special thanks are due to our colleague W. B. Adams for his essential help with the electronics, experimental design and reading of the manuscript. We are extremely grateful to Dr Hans-Rudolf Olpe for helpful discussions on $GABA_B$ receptors and for providing CGP 35348. Professor W. Haefely kindly provided us with midazolam and made valuable suggestions, as did Dr H. Bittiger. J.M.T. is a Biozentrum Fellow. Supported by grant no. 31-3004790 from the Swiss Nationalfonds.

References

ANDERSEN, P. (1981). Brain slices – a neurobiological tool of increasing usefulness. *Trends Neurosci.* **4**, 53–56.

BORMANN, J. (1988). Electrophysiology of $GABA_A$ and $GABA_B$ receptor subtypes. *Trends Neurosci.* **11**(3), 112–116.

BOWERY, N. (1989). $GABA_B$ receptors and their significance in mammalian pharmacology. *Trends Pharmac.* **10**, 410–417.

CARPENTER, M. B. (1976). *Human Neuroanatomy*, 7th edition, pp. 107–110. Baltimore: Wilkins & Wilkins Co.

CRAWFORD, M. L. A. AND YOUNG, J. M. (1988). $GABA_B$ receptor-mediated inhibition of histamine H_1-receptor-induced inositol phosphate formation in slices of rat cerebral cortex. *J. Neurochem.* **51**, 1441–1447.

CURTIS, D. R., DUGGAN, A. W., FELIX, D. AND JOHNSTON, G. A. R. (1970). GABA, bicuculline and central inhibition. *Nature* **226**, 1222–1224.

DZIEGIELEWSKA, K. M., HABGOOD, M., JONES, S. E., READER, M. AND SAUNDERS, N. R. (1989). Proteins in cerebrospinal fluid and plasma of postnatal *Monodelphis domestica* (grey short-tailed opossum). *Comp. Biochem. Physiol.* **92**B, 569–576.

GÄHWILER, B. H. (1984). Slice cultures of cerebellar, hippocampal and hypothalamic tissue. *Experientia* **40**, 235–308.

HANSEN, A. J. (1985). Effect of anoxia on ion distribution in the brain. *Physiol Rev.* **65**, 101–148.

NICHOLLS, J. G., STEWART, R. R., ERULKAR, S. D. AND SAUNDERS, N. R. (1990). Reflexes, fictive

respiration and cell division in the brain and spinal cord of the newborn opossum, *Monodelphis domestica*, isolated and maintained *in vitro*. *J. exp. Biol.* **152**, 1–15.

OLPE, H.-R., KARLSSON, G., POZZA, M. F., BRUGGER, F., STEINMANN, M., VAN RIEZEN, H., FAGG, G., HALL, R. G., FROESTL, W. AND BITTIGER, H. (1990). CGP 35348: a centrally active blocker of $GABA_B$ receptors. *Eur. J. Pharmac.* **187**, 27–38.

ONIMARU, M. AND HOMMA, I. (1987). Respiratory rhythm generator neurons in medulla of brainstem–spinal cord preparation of newborn rat. *Brain Res.* **403**, 380–384.

OTSUKA, M. AND KONISHI, S. (1974). Electrophysiology of mammalian spinal cord *in vitro*. *Nature* **252**, 733–734.

SAUNDERS, N. R., ADAM, E., READER, M. AND MØLLGÅRD, K. (1989). *Monodelphis domestica* (grey, short-tailed opossum): an accessible model for studies of early neocortical development. *Anat. Embryol.* **173**, 81–94.

SCHWARTZ, J.-C., ARRANG, J.-M., GARBARG, M., POLLARD, H. AND RUAT, M. (1991). Histaminergic transmission in the mammalian brain. *Physiol. Rev.* **71**, 1–51.

SUZUE, T. (1984). Respiratory rhythm generation in the *in vitro* brain stem–spinal cord preparation of the neonatal rat. *J. Physiol., Lond.* **354**, 173–183.