

# **N-METHYL-D-ASPARTATE (NMDA) AND NON-NMDA (METABOTROPIC) TYPE GLUTAMATE RECEPTORS MODULATE THE MEMBRANE POTENTIAL OF THE SCHWANN CELL OF THE SQUID GIANT NERVE FIBRE**

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## **Summary**

L-Glutamate application can produce three different responses in the membrane potential of the Schwann cell of the tropical squid, *Sepioteuthis sepioidea*, which appear to be mediated by three pharmacologically distinct classes of receptor. A class of non-NMDA-type receptors, with some similarities to metabotropic glutamate receptors, mediates the development of a rapid and long-lasting hyperpolarization. Two pharmacologically distinct classes of NMDA-type receptor are present. One mediates the development of a slow depolarization accompanied by a long-lasting change in responsiveness of the Schwann cell. The second produces rapid depolarizing responses during the period of this changed responsiveness. All three types of receptor can be activated by dipeptides containing excitatory amino acids.

## **Introduction**

The amino acid glutamate plays an important role in intercellular signalling in the nervous system. It functions as the major excitatory neurotransmitter in the vertebrate central nervous system (Monaghan *et al.* 1989) and is the leading candidate for the excitatory neuromuscular transmitter in a range of invertebrates (Duce, 1988). However, evidence is also increasing that glutamate may play a key role in neuronal–glial signalling in both vertebrate and invertebrate nervous systems (see Barres, 1989). It has been known for some time that glutamate is able to depolarize cultured astrocytes (Bowman and Kimelberg, 1984; Kettenmann and Schachner, 1985), but more recently there has been a discussion as to whether this effect is due to the activation of an electrogenic glutamate uptake mechanism or to a direct activation of glutamate receptors (Brew and Attwell, 1987; Barbour *et al.* 1988; Schwartz and Tachibana, 1990; Sarantis and Attwell, 1990; Wyllie *et al.* 1991).

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It has also been suggested that glutamate receptor activation underlies the effects of glutamate in a number of invertebrate glial preparations, including those of the adaxonal Schwann cells of the squid giant axon (Villegas, 1978*a,b*; Lieberman *et al.* 1989; Evans *et al.* 1991*a*) and those of the neuropile glia of the leech segmental ganglion (Ballanyi *et al.* 1989; Dörner *et al.* 1990). In the majority of the above studies the glutamate receptors present on the glial cells have been demonstrated to be of the quisqualate/kainate (non-NMDA) subtype. However, in the adaxonal Schwann cells of the giant axon of the tropical squid *Sepioteuthis sepioidea* evidence has been presented that both NMDA- and non-NMDA-subtype glutamate receptors are present (Evans *et al.* 1991*a*).

In this paper we report further details of the pharmacological properties of the three different subtypes of glutamate receptor found on the Schwann cells of the tropical squid and discuss their possible functional roles.

### Materials and methods

Giant nerve fibres with a diameter of 300–400  $\mu\text{m}$  were dissected in sea water from the hindmost stellar nerve of the squid *Sepioteuthis sepioidea*. Giant axons with their surrounding Schwann cell sheaths were then isolated and cleaned of adhering bundles of small nerve fibres by dissection in artificial sea water (see below).

Electrophysiological techniques were as described previously and involved the successive measurements of electrical potentials of a series of Schwann cells by brief impalements from inside the axon (Villegas, 1972, 1973, 1975; and see Lieberman *et al.* 1989). All experiments were carried out at room temperature (20–22°C). In experiments where the degree of hyperpolarization of the Schwann cell membrane by a given drug has been quantified, it was calculated as the difference between the mean measurement of the control resting potential in the 2 min period immediately before drug application (averaged over 5–10 penetrations of individual Schwann cells) and the mean measurement of membrane potential in the 2 min period immediately after the end of the drug pulse (averaged over 5–10 penetrations of individual Schwann cells). The results are given as the mean hyperpolarization induced by a drug (in  $\text{mV} \pm \text{s.e.}$ ) of the results obtained from at least four axons. A blind protocol was used in which the investigator sampling the Schwann cell membrane potentials did not know the identity of the test pulses being applied to the preparation. All the experiments shown were repeated at least three times in the format shown.

Drugs superfused over the surface of the preparation were dissolved in artificial sea water containing 442  $\text{mmol l}^{-1}$  NaCl, 10  $\text{mmol l}^{-1}$  KCl, 11  $\text{mmol l}^{-1}$   $\text{CaCl}_2$ , 45  $\text{mmol l}^{-1}$   $\text{MgCl}_2$  and 10  $\text{mmol l}^{-1}$  Tris–Cl buffer (pH 8.0). All the superfused solutions were continuously bubbled with a mixture of 95 %  $\text{O}_2$  and 5 %  $\text{CO}_2$ .

All drugs were obtained from the Sigma Chemical Co., except for glutamylglutamate which was obtained from Peninsula Laboratories Inc., and (IS,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), cis-(dicarboxyl)-2,4-methanoglutamic acid (cis-DMG) and L-(+)-2-amino-3-phosphonopropionic acid (L-AP3), which were obtained from Tocris Neuramin.

## Results

### *Types of glutamate response*

The membrane potential of the Schwann cell of the giant axon of the tropical squid *Sepioteuthis sepioidea* can produce three different responses to the application of pulses of L-glutamate (Villegas, 1981), which appear to be mediated by three pharmacologically distinct classes of receptor (Evans *et al.* 1991a). They include a rapidly induced and long-lasting hyperpolarization of the membrane potential of the Schwann cell in response to a short 1 min pulse of L-glutamate, a biphasic response consisting of a rapid hyperpolarization followed by a slow depolarization to a long, 10 min, pulse and a rapid depolarization in response to a short pulse of glutamate following the induction of the slow depolarization.

### *The rapid hyperpolarizing response*

The rapidly induced, long-lasting hyperpolarization has previously been shown to be due to the glutamate-induced release of acetylcholine from the Schwann cells themselves; this feeds back onto nicotinic cholinergic receptors on the Schwann cell to promote the hyperpolarization (Villegas, 1972, 1973, 1975; Lieberman *et al.* 1989). This response was more sensitive to (IS,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (trans-ACPD), the selective agonist for metabotropic glutamate receptors (Sugiyama *et al.* 1987, 1989), than to the other agonists tested. It had a threshold between  $10^{-11}$  and  $2 \times 10^{-11}$  mol l<sup>-1</sup> and a half-maximal response in the region of  $10^{-10}$  mol l<sup>-1</sup> (Fig. 1A,B). In contrast, the amino acid L-glutamate itself had a threshold between  $2 \times 10^{-10}$  and  $5 \times 10^{-10}$  mol l<sup>-1</sup> and a half-maximal response in the region of  $5 \times 10^{-10}$  mol l<sup>-1</sup> (Fig. 1A). However, the reduced effectiveness of L-glutamate could be due to the activity of an active uptake system for this amino acid in this preparation (see Evans *et al.* 1992). Dose-response curves revealed that the response was more sensitive to quisqualate and kainate than to N-methyl-D-aspartate (NMDA). This indicated that the response was likely to be mediated by a metabotropic-type glutamate receptor rather than a quisqualate/kainate-type glutamate receptor as previously suggested (Lieberman *et al.* 1989; Evans *et al.* 1991a). In contrast to previous observations in another species of squid, *Alloteuthis subulata* (Lieberman *et al.* 1989), L-aspartate was not found to be a very potent agonist of this response in *Sepioteuthis sepioidea*. A 1 min pulse of  $10^{-5}$  mol l<sup>-1</sup> L-aspartate gave a hyperpolarization of only  $5.8 \pm 0.4$  mV ( $N=3$ ). The response was stereoselective for the L form of glutamate, as the D form gave no response at concentrations up to  $10^{-7}$  mol l<sup>-1</sup> (Fig. 1A), agreeing with previous work (Villegas, 1981).

The conclusion that the rapid hyperpolarizing response to short pulses (1 min) of L-glutamate was mediated via a non-NMDA subtype of glutamate receptor was supported by pharmacological blocking experiments. Fig. 2A shows that the rapid hyperpolarizations of the Schwann cell membrane induced either by applying a 1 min pulse of  $10^{-8}$  mol l<sup>-1</sup> L-glutamate or by firing the giant axon at 100 Hz for 1 min were both blocked by D,L-2-amino-4-phosphonobutyric acid (APB or AP4) ( $10^{-6}$  mol l<sup>-1</sup>). APB is a potent blocking agent of non-NMDA-type glutamate receptors in squid Schwann cells (Lieberman *et al.* 1989; Evans *et al.* 1991a) and in several cephalopod muscles (Bone and

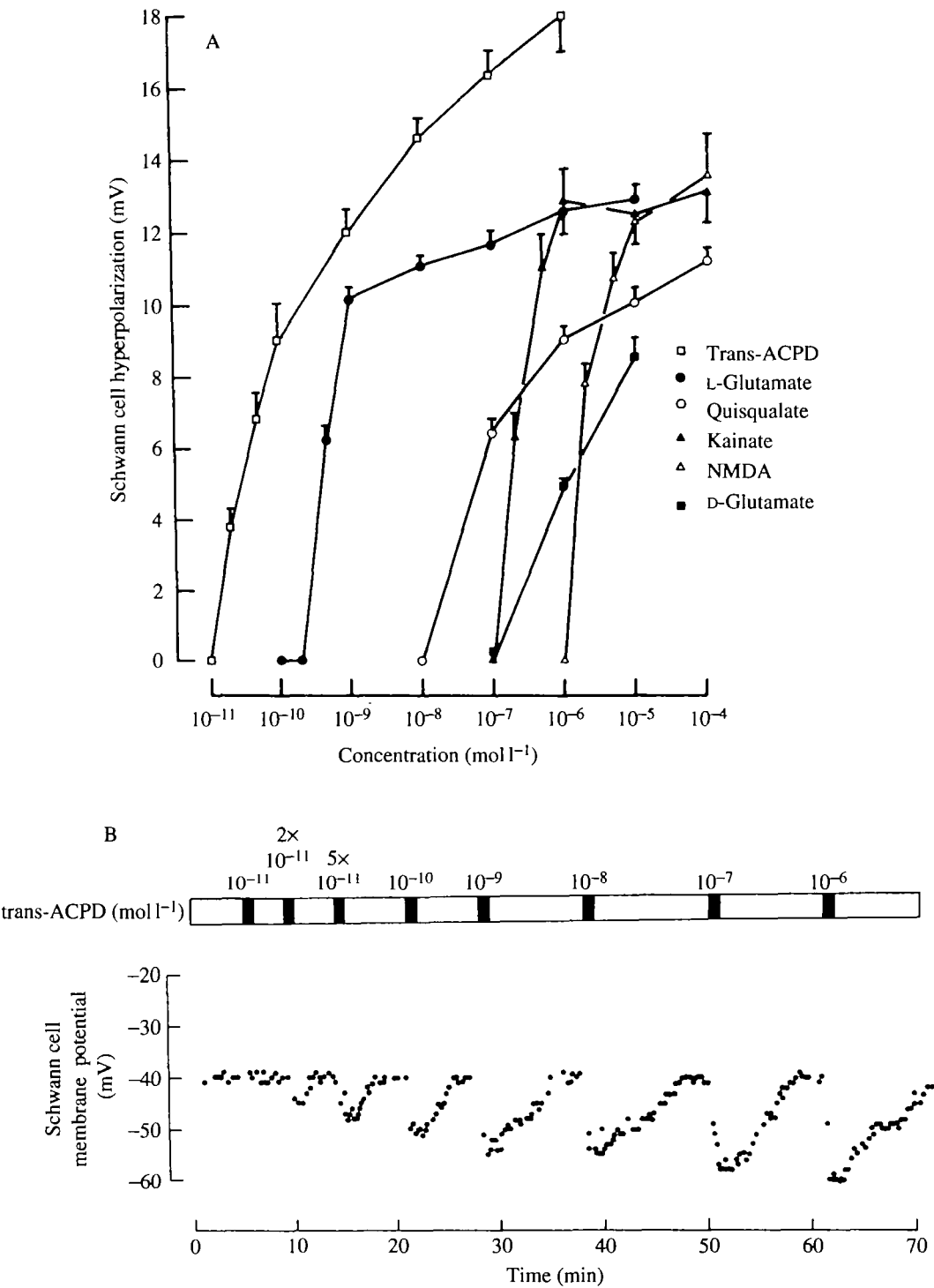


Fig. 1

Fig. 1. (A) Dose-response curves for the hyperpolarizing effects of 1 min pulses of trans-ACPD (open squares), L-glutamate (filled circles), quisqualate (open circles), kainate (filled triangles), NMDA (open triangles) and D-glutamate (filled squares) on the Schwann cell membrane. The values represent the difference  $\pm$  S.E. between the mean membrane potential in the 2 min period before agonist application and the mean membrane potential in the 2 min period after the end of the 1 min pulse of agonist. Each solution was tested on at least four nerve fibres. (B) Effect of 1 min pulses of various concentrations of trans-ACPD (filled bars) on the Schwann cell membrane potential. Each point represents the potential difference recorded in a different Schwann cell.

Howarth, 1980; Florey *et al.* 1985), whereas it acts as an agonist at a specific subtype of glutamate receptor in vertebrates. The hyperpolarizing responses in the squid Schwann cell were not blocked by D,L-2-amino-5-phosphonovaleric acid (APV or AP5) ( $10^{-6}$  mol l $^{-1}$ ), a selective NMDA receptor antagonist (Fig. 2A). Another antagonist of non-NMDA-type glutamate receptors, L-glutamic acid diethyl ester (GDEE) did not block either hyperpolarizing effect when used at a concentration of  $10^{-6}$  mol l $^{-1}$ , but when used at  $10^{-4}$  mol l $^{-1}$  it produced a partial block of the effect of a 1 min pulse of  $10^{-8}$  mol l $^{-1}$  L-glutamate and a complete block of the rapid hyperpolarization induced by firing the giant axon at 100 Hz for 1 min (Fig. 2B). The non-NMDA nature of this subtype of glutamate receptor is further supported by experiments in which 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) ( $10^{-6}$  mol l $^{-1}$ ) blocked the hyperpolarizing effects both of a 1 min pulse of  $10^{-8}$  mol l $^{-1}$  L-glutamate and of firing the giant axon at 100 Hz for 1 min (Fig. 2C). The suggestion that the rapid hyperpolarization induced by short pulses of L-glutamate is mediated by the activation of a metabotropic-like glutamate receptor subtype is further confirmed by the finding that the response was blocked by L(+)-2-amino-3-phosphonopropionic acid (L-AP3) (Fig. 2D), a potent and selective antagonist of the metabotropic glutamate receptor in vertebrate tissues (Irving *et al.* 1990; Schoepp *et al.* 1990).

The specificity of the blocking agents for the rapid hyperpolarizations induced by glutamate and by firing the giant axon at 100 Hz for 1 min was tested by comparing their capacity to block the actions of a range of neurotransmitter substances previously described as being capable of modulating the membrane potential of the Schwann cell of the squid giant axon (see Evans *et al.* 1986, 1990; Reale *et al.* 1986; Villegas, 1981, 1984). One minute pulses of  $10^{-6}$  mol l $^{-1}$  APV,  $10^{-6}$  mol l $^{-1}$  APB,  $10^{-4}$  mol l $^{-1}$  GDEE or  $10^{-6}$  mol l $^{-1}$  CNQX did not reduce the size of the Schwann cell hyperpolarization induced by 1 min pulses of  $10^{-7}$  mol l $^{-1}$  carbachol,  $10^{-6}$  mol l $^{-1}$  D,L-octopamine,  $10^{-9}$  mol l $^{-1}$  vasoactive intestinal peptide (VIP) or  $10^{-9}$  mol l $^{-1}$  substance P (data not shown). Thus, the blocking agents used are specific for the receptors mediating the effects of glutamate in this preparation.

### *The slow depolarization*

The biphasic response of the Schwann cell membrane potential to a prolonged pulse of L-glutamate can be separated into an initial rapid hyperpolarization, which has the same pharmacological characteristics as the rapid hyperpolarization, induced by a 1 min pulse of L-glutamate (see Evans *et al.* 1991a), and a slower depolarization. The slower

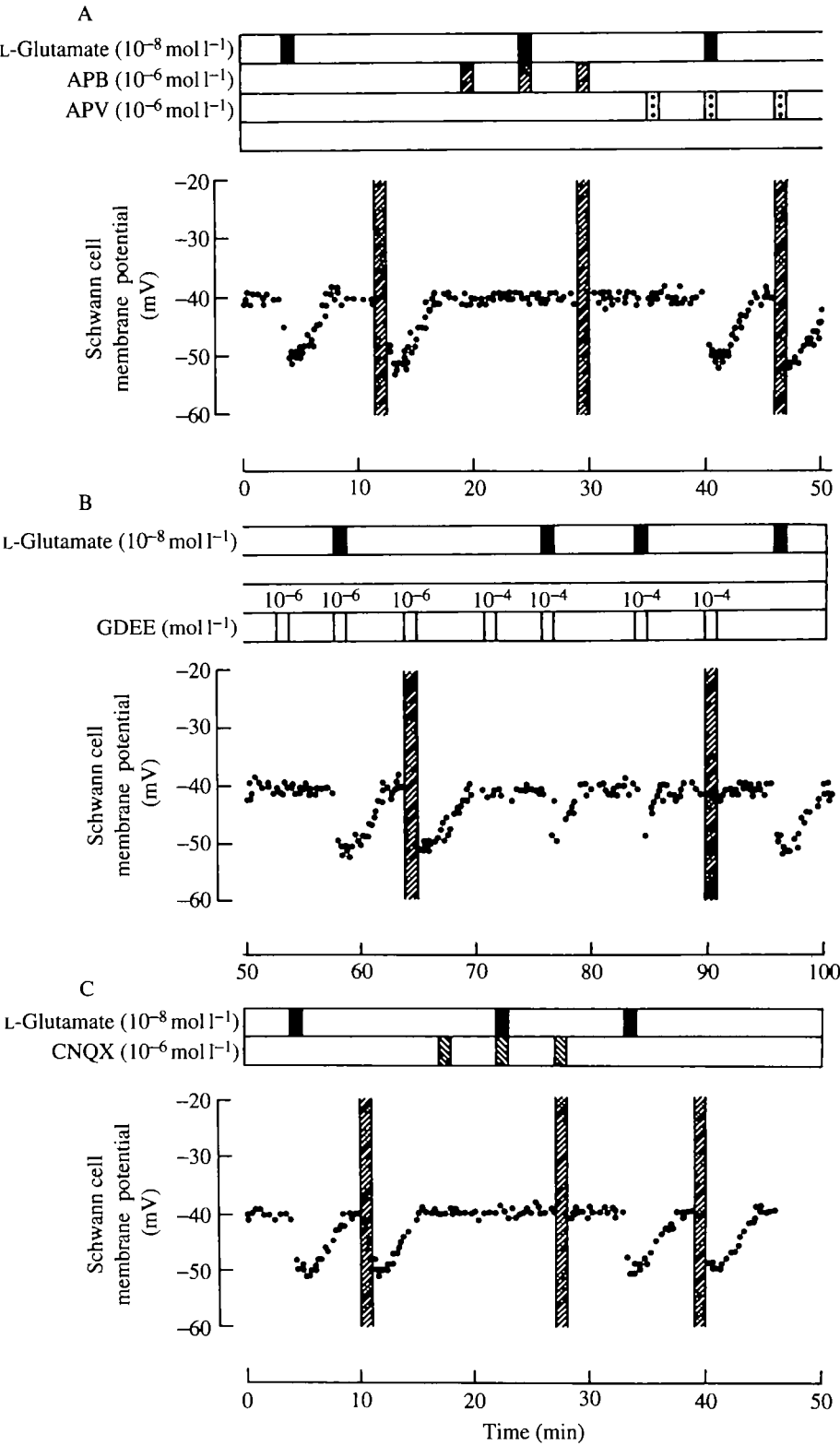


Fig. 2A–C

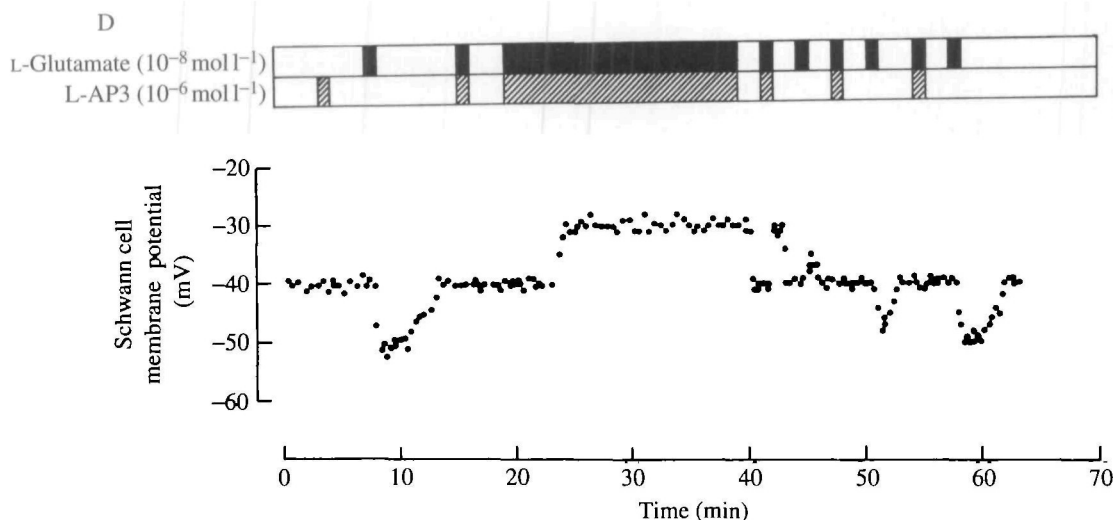


Fig. 2. The blocking actions of 1 min pulses of (A) APB ( $10^{-6}$  mol  $l^{-1}$ ) (hatched bar) and APV ( $10^{-6}$  mol  $l^{-1}$ ) (stippled bar); (B) GDEE ( $10^{-6}$  and  $10^{-4}$  mol  $l^{-1}$ ) (open bar); and (C) CNQX ( $10^{-6}$  mol  $l^{-1}$ ) (hatched bar) on the hyperpolarizations of the Schwann cell membrane initiated by the application of 1 min pulses of  $10^{-8}$  mol  $l^{-1}$  L-glutamate (filled bars) and by stimulation of the giant axon at 100 Hz for 1 min (vertical hatched bars). (D) L-AP3 ( $10^{-6}$  mol  $l^{-1}$ ) (hatched bars) selectively blocks the rapid hyperpolarization induced by a 1 min pulse of  $10^{-8}$  mol  $l^{-1}$  L-glutamate (filled bar) but not the slow depolarization induced by a prolonged pulse of L-glutamate or the rapid depolarizations subsequently induced by short pulses of L-glutamate. The records in B are a continuation of the experiment shown in A, whilst those in C and D were obtained in a separate preparation. Each point represents the potential difference recorded in a different Schwann cell.

depolarization had a threshold between  $10^{-9}$  and  $10^{-8}$  mol  $l^{-1}$  L-glutamate (Fig. 3A) and after its induction responses to short (1 min) pulses of L-glutamate became depolarizing. Previous pharmacological studies indicate that the slow depolarization is mediated by NMDA-type glutamate receptors (Evans *et al.* 1991a). The response was blocked by APV ( $10^{-6}$  mol  $l^{-1}$ ), but not by APB ( $10^{-6}$  mol  $l^{-1}$ ), L-AP3 ( $10^{-6}$  mol  $l^{-1}$ ) (Fig. 2D) and CNQX ( $10^{-6}$  mol  $l^{-1}$ ) (Fig. 3B), and it is mimicked by the application of NMDA with a threshold occurring between  $5 \times 10^{-10}$  and  $10^{-9}$  mol  $l^{-1}$  (Evans *et al.* 1991a). It is also mimicked by the application of cis-(dicarboxyl)-2,4-methanoglutamic acid (cis-DMG) (Fig. 3C) ( $10^{-8}$  mol  $l^{-1}$ ), one of the most potent and selective synthetic agonists of NMDA receptors (Lanthorn *et al.* 1990; Allan *et al.* 1990), which is itself only a poor agonist (threshold between  $10^{-8}$  and  $10^{-7}$  mol  $l^{-1}$ ) of the rapid hyperpolarizations induced by glutamate in the squid Schwann cell preparation. It was not initiated by the application of 10 min pulses of quisqualate, kainate (Evans *et al.* 1991a) or L-aspartate up to a concentration of  $10^{-5}$  mol  $l^{-1}$  or by trans-ACPD up to a concentration of  $10^{-7}$  mol  $l^{-1}$  (Fig. 3D).

#### The fast depolarization

We have previously shown that the fast depolarization induced by a short (1 min) pulse

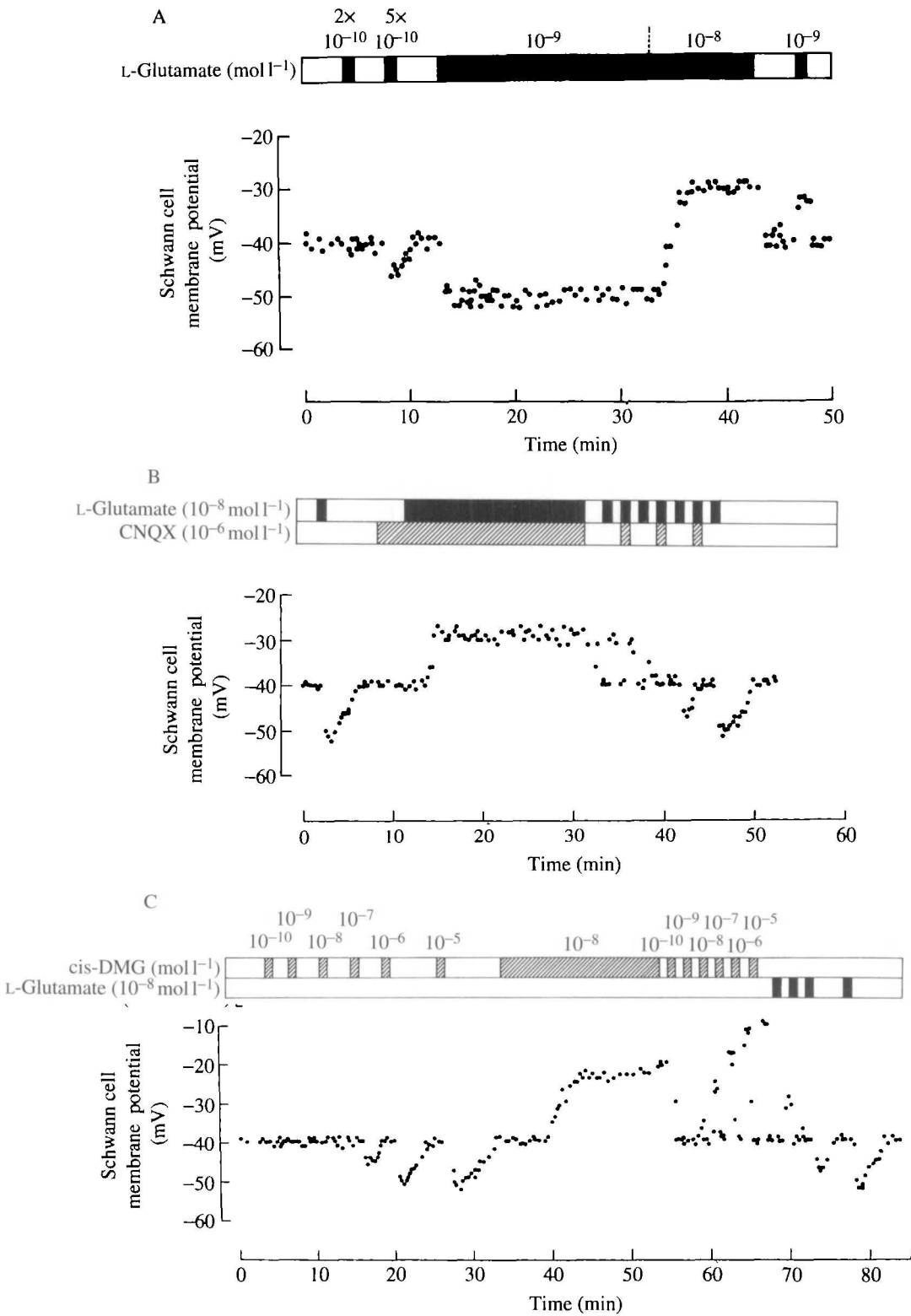


Fig. 3A-C



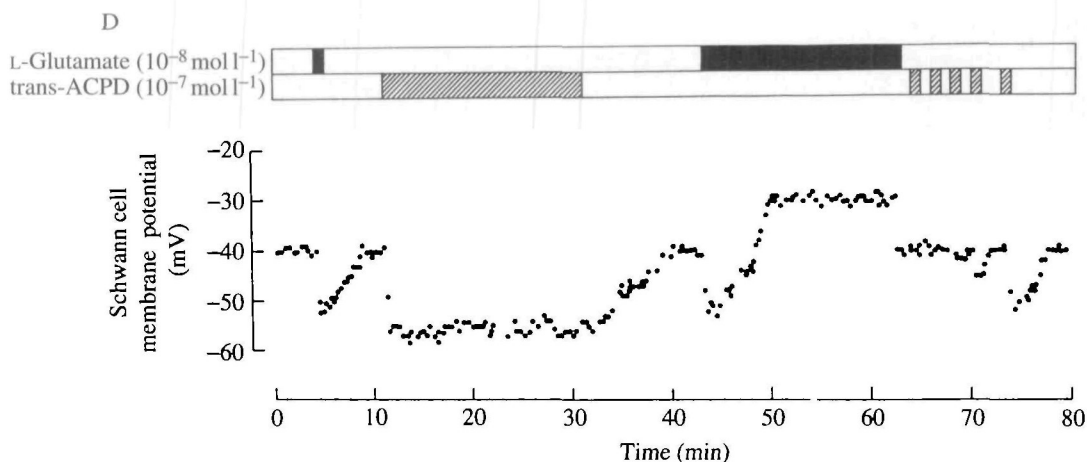


Fig. 3. (A) Threshold determinations of the effects of short 1 min pulses and prolonged pulses of L-glutamate (filled bars) on the Schwann cell membrane potential. Note that, after the induction of the slow depolarization by  $10^{-8} \text{ mol l}^{-1}$  L-glutamate, the response to a short 1 min pulse of  $10^{-9} \text{ mol l}^{-1}$  is now a depolarization. (B) CNQX ( $10^{-6} \text{ mol l}^{-1}$ ) (hatched bars) blocks the initial hyperpolarizing effect but not the slow depolarization due to a prolonged 20 min pulse of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (filled bar). After the slow depolarization, CNQX does not block the rapid depolarizations induced by 1 min pulses of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) but does block the initiation of the slow hyperpolarizing responses when they reappear. (C) Effect of 1 min pulses of various concentrations of cis-DMG (hatched bars) on the Schwann cell membrane potential before and after exposure of the preparation to a prolonged pulse of cis-DMG ( $10^{-8} \text{ mol l}^{-1}$ ). This prolonged pulse produces a slow depolarization but no hyperpolarization of the membrane potential. After the slow depolarization, the effects of 1 min pulses of cis-DMG and  $10^{-8} \text{ mol l}^{-1}$  L-glutamate (filled bars) are transiently depolarizing but revert to rapid hyperpolarizing responses after a delay. (D) A prolonged pulse of trans-ACPD ( $10^{-7} \text{ mol l}^{-1}$ ) (hatched bar) does not initiate a slow depolarizing response and short 1 min pulses of trans-ACPD ( $10^{-7} \text{ mol l}^{-1}$ ) do not initiate rapid depolarizations after a slow depolarization initiated by a prolonged pulse of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ), but initiate slow hyperpolarizations after a delay of 7 or 8 min. Each point represents the potential difference recorded in a different Schwann cell.

of L-glutamate given after a prolonged 10 min pulse of L-glutamate has a threshold between  $10^{-9}$  and  $10^{-8} \text{ mol l}^{-1}$ , and that such fast depolarizations can also be initiated by 1 min pulses of L-glutamate after a slow depolarization induced by NMDA at concentrations of  $10^{-9} \text{ mol l}^{-1}$  and above (Evans *et al.* 1991a). Specificity studies (Fig. 4A) indicated that this rapid depolarization could also be induced by 1 min pulses of  $10^{-5} \text{ mol l}^{-1}$  NMDA, as well as similar 1 min pulses of L-glutamate but not by 1 min pulses of quisqualate or kainate up to a concentration of  $10^{-5} \text{ mol l}^{-1}$  or by 1 min pulses of trans-ACPD up to a concentration of  $10^{-7} \text{ mol l}^{-1}$  (Fig. 3D). Interestingly a 20 min pulse of  $2 \times 10^{-9} \text{ mol l}^{-1}$  L-glutamate (Fig. 4B) did not induce a slow depolarization of the Schwann cell membrane above the resting membrane potential. It only initiated a transient hyperpolarization (cf. prolonged hyperpolarization induced by a 20 min pulse of  $10^{-9} \text{ mol l}^{-1}$  L-glutamate in Fig. 3A) and the Schwann cell membrane potential returned to its resting value after 5 min. Nevertheless, short 1 min pulses of NMDA given after this glutamate pulse still induced a rapid depolarizing response with the same threshold

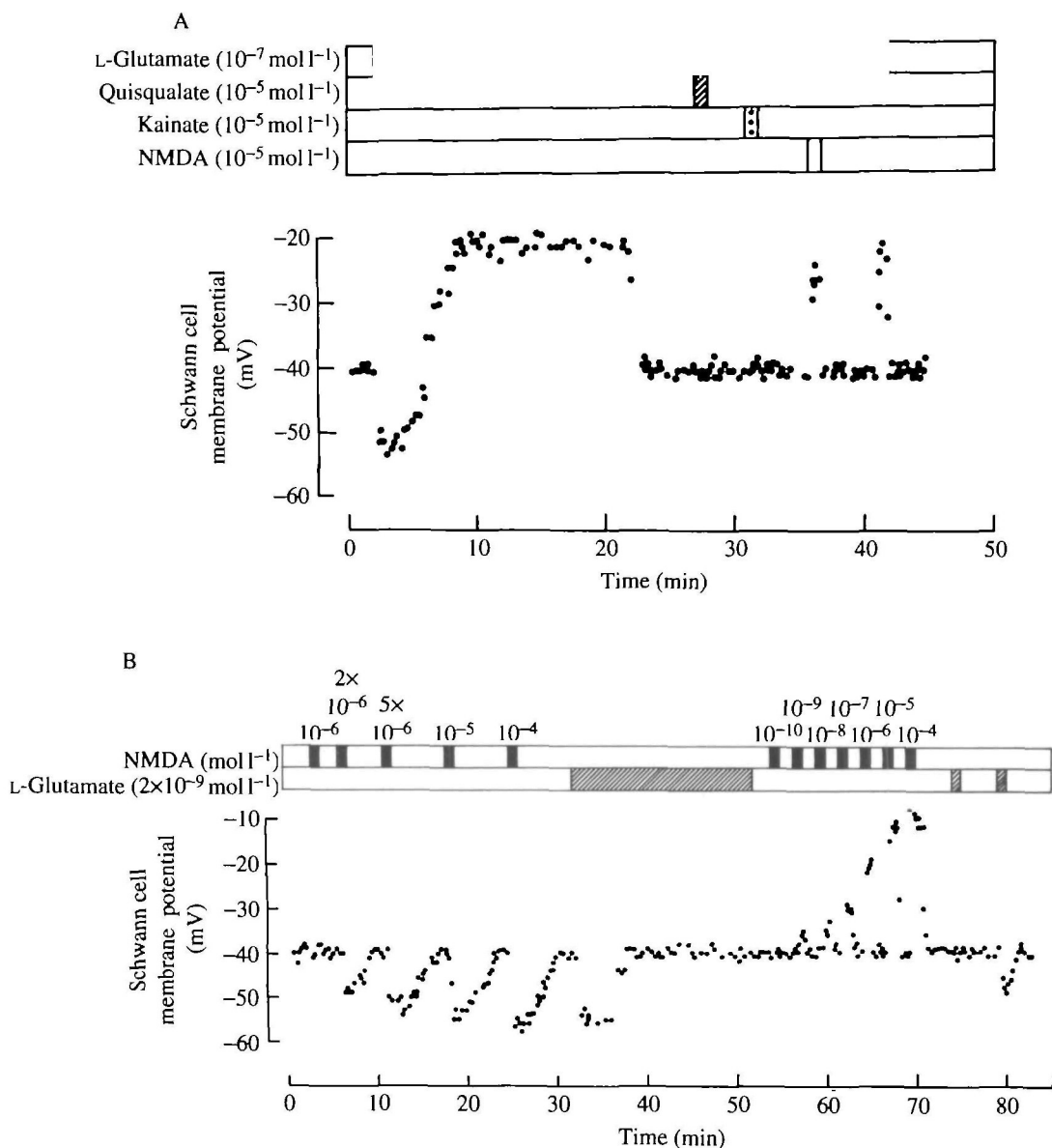


Fig. 4. (A) Specificity studies on the rapid depolarization of the Schwann cell membrane induced by 1 min pulses of quisqualate ( $10^{-5}$  mol l $^{-1}$ ) (hatched bar), kainate ( $10^{-5}$  mol l $^{-1}$ ) (stippled bar), NMDA ( $10^{-5}$  mol l $^{-1}$ ) (open bar) and L-glutamate ( $10^{-7}$  mol l $^{-1}$ ) (filled bar) after the induction of a slow depolarization by a prolonged pulse of L-glutamate (filled bar). (B) Effect of 1 min pulses of various concentrations of NMDA (filled bar) on the Schwann cell membrane potential before and after the exposure of the preparation to a prolonged pulse of L-glutamate ( $2 \times 10^{-9}$  mol l $^{-1}$ ) (hatched bar). This prolonged pulse of glutamate produced a transient hyperpolarization but no slow depolarization above the resting potential of the Schwann cells. Nevertheless, the properties of the Schwann cell have still been modulated. Each point represents the potential difference recorded in a different Schwann cell.

(between  $10^{-10}$  and  $10^{-9}$  mol l<sup>-1</sup>), which is almost three orders of magnitude lower than that needed to produce a hyperpolarizing response before the application of the prolonged glutamate pulse. This indicated that a prolonged slow depolarization of the Schwann cells above their resting potential was not a necessary prerequisite for inducing a rapid depolarizing response. *cis*-DMG is also a potent activator of this class of NMDA receptor on the squid Schwann cell, with a threshold between  $10^{-10}$  and  $10^{-9}$  mol l<sup>-1</sup> (Fig. 3C).

The receptors mediating the rapid depolarizing response appear to be pharmacologically distinct from the receptors mediating the other responses to glutamate in this preparation. Although the response could be induced by NMDA, it did not appear to be blocked by  $10^{-6}$  mol l<sup>-1</sup> APV (Fig. 5A),  $10^{-6}$  mol l<sup>-1</sup> L-AP3 (Fig. 2D) or  $10^{-6}$  mol l<sup>-1</sup> CNQX (Fig. 3B) and was only slightly reduced by  $10^{-6}$  mol l<sup>-1</sup> APB, a concentration that completely blocked the hyperpolarizing responses induced by a 1 min pulse of L-glutamate (Fig. 5B). However, if the 1 min pulse of  $10^{-8}$  mol l<sup>-1</sup> L-glutamate was given in the presence of  $10^{-6}$  mol l<sup>-1</sup> GDEE, the rapid depolarizing response was irreversibly blocked and could not be induced by a subsequent 1 min pulse of L-glutamate given alone (Fig. 5C). Further, if the 20 min long pulse of L-glutamate was given in the presence of  $10^{-6}$  mol l<sup>-1</sup> GDEE, although the initial hyperpolarization and slow depolarization appeared to be normal, no rapid depolarizing responses could be initiated by subsequent 1 min pulses of L-glutamate given at the end of the long glutamate pulse (Fig. 5D). Instead, there was initially no response to the short glutamate pulses and after a delay the normal hyperpolarizing responses returned.

#### *The actions of dipeptides containing excitatory amino acids*

In a number of instances it has been suggested that endogenous dipeptides containing the excitatory amino acids L-glutamate and L-aspartate act directly at the same sites as L-glutamate in vertebrate nervous tissue (see Zaczek *et al.* 1983; Westbrook *et al.* 1986). However, it has been pointed out that many of the actions of *N*-acetylaspartylglutamate may be produced by a rapid extracellular enzymatic cleavage to release neuroactive L-glutamate (see Moffett *et al.* 1990) or by contamination of synthetic *N*-acetylaspartylglutamate with potassium ions (Whittemore and Koerner, 1989). In the present study we have examined the ability of a range of dipeptides containing these amino acids to mimic the various effects of L-glutamate on the membrane potential of the Schwann cell of the squid giant axon.

A survey of the hyperpolarizing actions of a range of dipeptides tested as 1 min pulses at a concentration of  $10^{-8}$  mol l<sup>-1</sup> on the membrane potential of the Schwann cell is shown in Table 1. The most effective dipeptide tested was *N*-acetylaspartylglutamate, a naturally occurring dipeptide in vertebrate nervous tissue. The response of the Schwann cell to 1 min pulses of *N*-acetylaspartylglutamate was dose-responsive (Fig. 6). *N*-Acetylaspartylglutamate was more potent at inducing this response than L-glutamate itself (Figs 6, 7A,B). The other dipeptides tested, including  $\beta$ -aspartylglycine, an endogenous molluscan dipeptide (McCaman and Stetzler, 1985), produced smaller rapid hyperpolarizations of the Schwann cell membrane.

*N*-Acetylaspartylglutamate was also able to mimic the effects of a long pulse of L-glutamate in that concentrations of  $10^{-8}$  mol l<sup>-1</sup> and above produced a slow depolarization

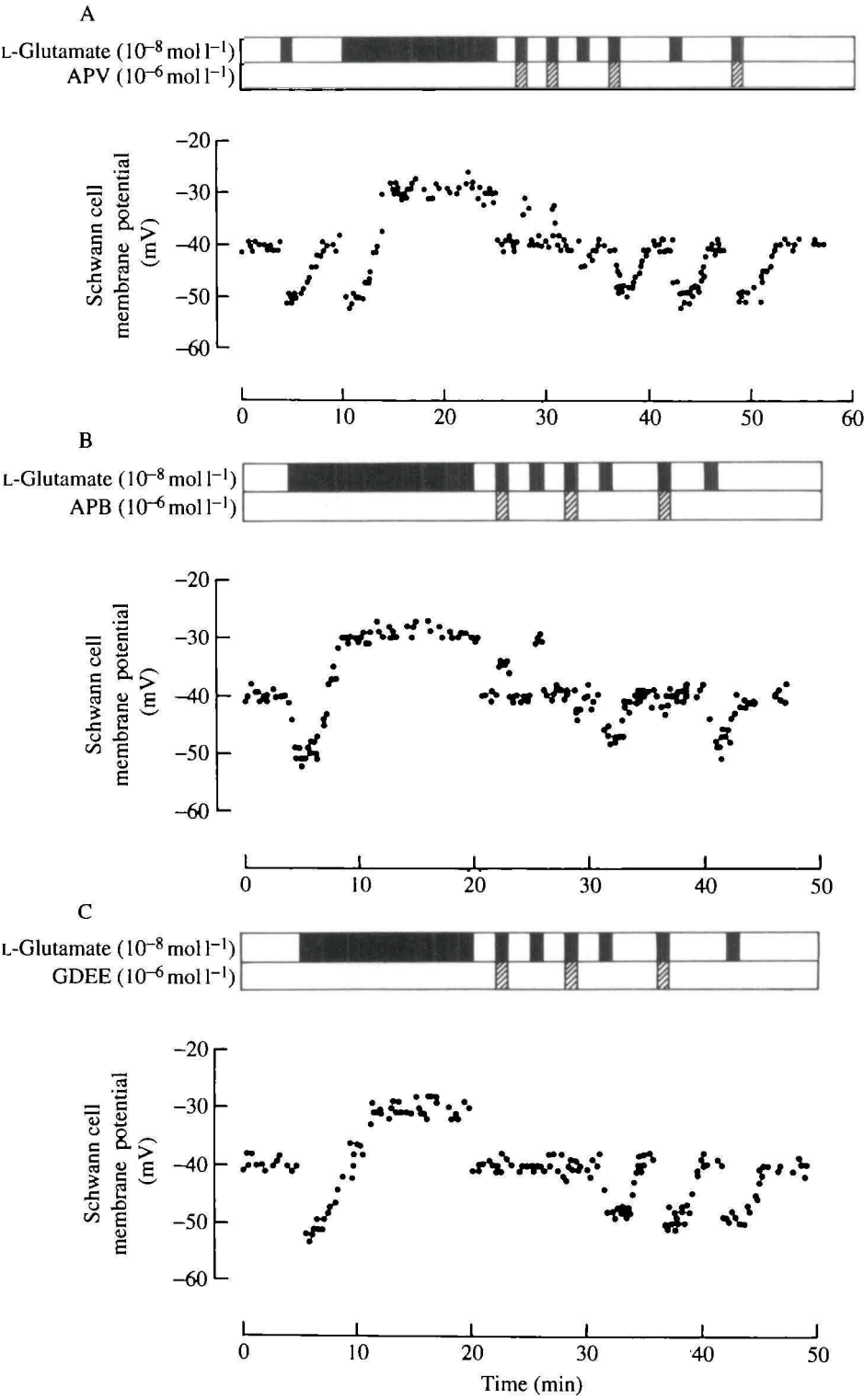


Fig. 5A-C

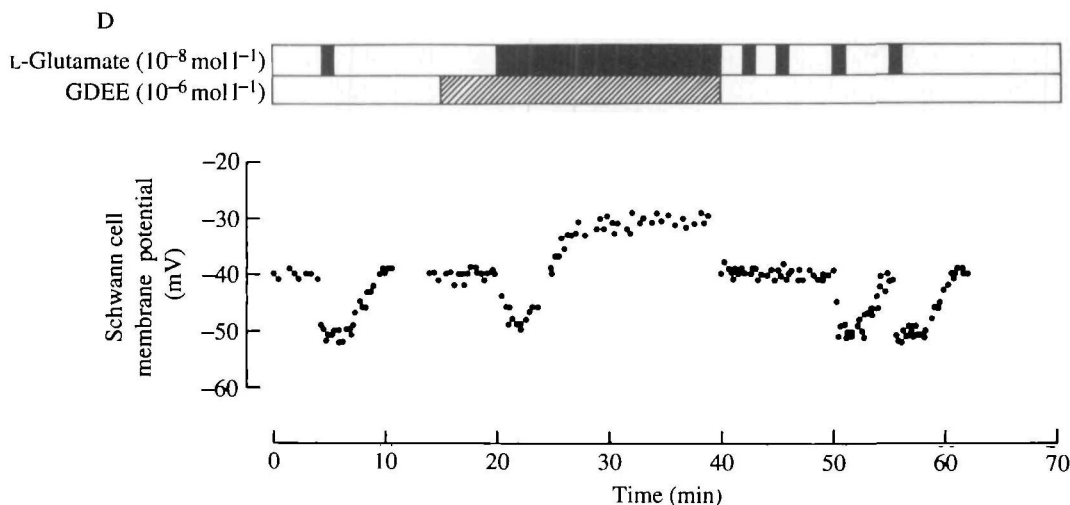


Fig. 5. (A) APV (1 min pulses,  $10^{-6} \text{ mol l}^{-1}$ ) (hatched bars) does not block the transient depolarizing effect or subsequent hyperpolarizing effects of 1 min pulses of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (filled bars) given after the induction of a slow depolarization of the Schwann cell membrane by exposure to a prolonged pulse of L-glutamate (filled bar). (B) APB (1 min pulses,  $10^{-6} \text{ mol l}^{-1}$ ) (hatched bars) partially blocks the transient depolarization and completely blocks the subsequent hyperpolarizing effects of 1 min pulses of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (filled bar) given after the induction of a slow depolarization of the Schwann cell membrane by exposure to a prolonged pulse of L-glutamate (filled bar). (C) GDEE (1 min pulses,  $10^{-6} \text{ mol l}^{-1}$ ) (hatched bars) completely blocks the transient depolarization but not the subsequent hyperpolarizing effects of 1 min pulses of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (filled bar) given after the induction of a slow depolarization of the Schwann cell membrane by exposure to a prolonged pulse of L-glutamate (filled bar). (D) A prolonged pulse of GDEE ( $10^{-6} \text{ mol l}^{-1}$ ) (hatched bar) does not block the rapid hyperpolarization and subsequent slow depolarization of the Schwann cell membrane induced by a prolonged pulse of L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (filled bar), but the subsequent transient depolarizing responses are blocked. Each point represents the potential difference recorded in a different Schwann cell.

after the initial rapid hyperpolarization (Fig. 7B). Furthermore, short pulses of *N*-acetylaspartylglutamate were also capable of initiating a rapid depolarization of the Schwann cell membrane after the induction of a slow depolarization by a prolonged pulse of *N*-acetylaspartylglutamate (Fig. 7B) or L-glutamate ( $10^{-8} \text{ mol l}^{-1}$ ) (Fig. 6B). This effect has a threshold between  $10^{-11}$  and  $10^{-10} \text{ mol l}^{-1}$  *N*-acetylaspartylglutamate (Fig. 6B). None of the other dipeptides tested was capable of inducing the rapid depolarization up to a concentration of  $10^{-8} \text{ mol l}^{-1}$  (Fig. 7B).

### Discussion

Pharmacological parallels with glutamate receptor subtypes in many vertebrate preparations (Foster and Fagg, 1984; Watkins and Olverman, 1987; Monaghan *et al.* 1989) indicate that the three types of glutamate response observed in the adaxonal Schwann cells of the giant axon of the tropical squid are likely to be mediated *via* the activation of three distinct subtypes of glutamate receptor rather than by the activation of

an electrogenic glutamate uptake system. However, the pharmacological properties of the squid Schwann cell glutamate receptors do not correspond exactly with those of any of the different subclasses of vertebrate glutamate receptor currently identified.

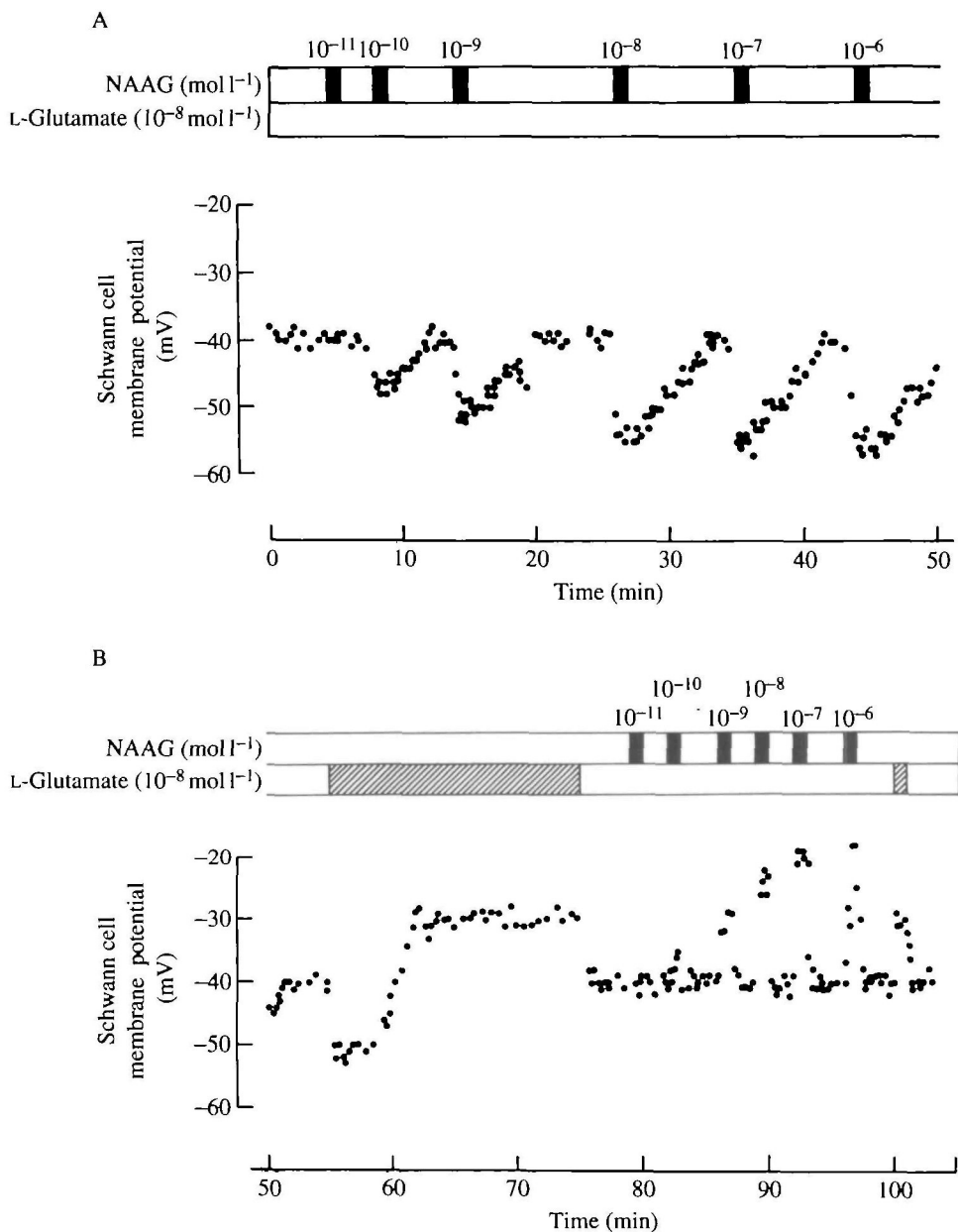


Fig. 6. Effect of 1 min pulses of various concentrations of *N*-acetylaspartylglutamate (NAAG) (filled bars) on the Schwann cell membrane potential (A) before and (B) after the induction of a slow depolarization by exposure of the preparation to a prolonged pulse of L-glutamate (hatched bar). Each point represents the potential difference recorded in a different Schwann cell.

Table 1. Hyperpolarizing actions of dipeptides

Dipeptide	Hyperpolarizing response (mV)	N
N-Acetylasparylglutamate	14.0±0.5	3
L-Glutamate	11.0±0.2	11
Aspartylglutamate	8.5±0.8	4
β-Aspartylglycine	7.1±1.8	4
Glutamylglutamate	4.6±0.9	4
Aspartylaspartate	4.9±0.5	5
L-Aspartate	0	3

Results are expressed as the mean±S.E.M. of the hyperpolarizations produced by the introduction of a 1 min pulse of the dipeptide or control amino acids into the superfusate at a concentration of  $10^{-8}$  mol l<sup>-1</sup>. N, number of observations for each compound.

The glutamate receptors responsible for the release of acetylcholine from the Schwann cells and for the subsequent nicotinic cholinergic receptor-induced long-lasting hyperpolarization (Villegas, 1972, 1973, 1975; Lieberman *et al.* 1989) appear to be of the non-NMDA variety since they are activated by trans-ACPD, L-glutamate, quisqualate and kainate in preference to NMDA. This is supported by the fact that they are blocked by the non-NMDA antagonist CNQX and by high doses of the general non-NMDA antagonist GDEE ( $10^{-4}$  mol l<sup>-1</sup>) (present experiments and Lieberman *et al.* 1989) but not by the specific NMDA receptor antagonist APV ( $10^{-6}$  mol l<sup>-1</sup>). They appear to express some similarities with the metabotropic glutamate receptors (Sugiyama *et al.* 1987, 1988) since they are preferentially activated by trans-ACPD, blocked by L-AP3 (Irving *et al.* 1990; Schoepp *et al.* 1990) and activated by quisqualate but not by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Lieberman *et al.* 1989). The Schwann cell metabotropic glutamate receptors appear to be closely related to the vertebrate metabotropic glutamate receptor encoded by the mGluR2 cDNA clone since they are activated better by trans-ACPD and glutamate than by quisqualate (see Tanabe *et al.* 1992). The receptors also express some differences since they are activated by kainate but not by ibotenate (Lieberman *et al.* 1989) and they are blocked by CNQX. An additional similarity is that, like some of the metabotropic receptors, they are blocked by APB (or AP4), which conventionally acts like an agonist of some vertebrate glutamate receptor subtypes (Monaghan *et al.* 1989) whilst at other glutamate receptors it has not been determined whether it acts as an agonist or an antagonist (Watkins, 1988). APB also blocks the actions of non-NMDA-type glutamate receptors in several cephalopod muscle preparations (Bone and Howarth, 1980; Florey *et al.* 1985). Furthermore, it has been postulated that this class of squid Schwann cell glutamate receptor may be involved in increasing intracellular Ca<sup>2+</sup> levels to mediate the release of acetylcholine (see Evans *et al.* 1991b), but it is not known whether these receptors activate an inositol-trisphosphate-mediated release of intracellular Ca<sup>2+</sup> or an increase in Ca<sup>2+</sup> permeability of the cell membrane. The lack of involvement of an ion-channel-mediated event in the activation of these receptors is suggested in the tropical squid preparation, since no change in

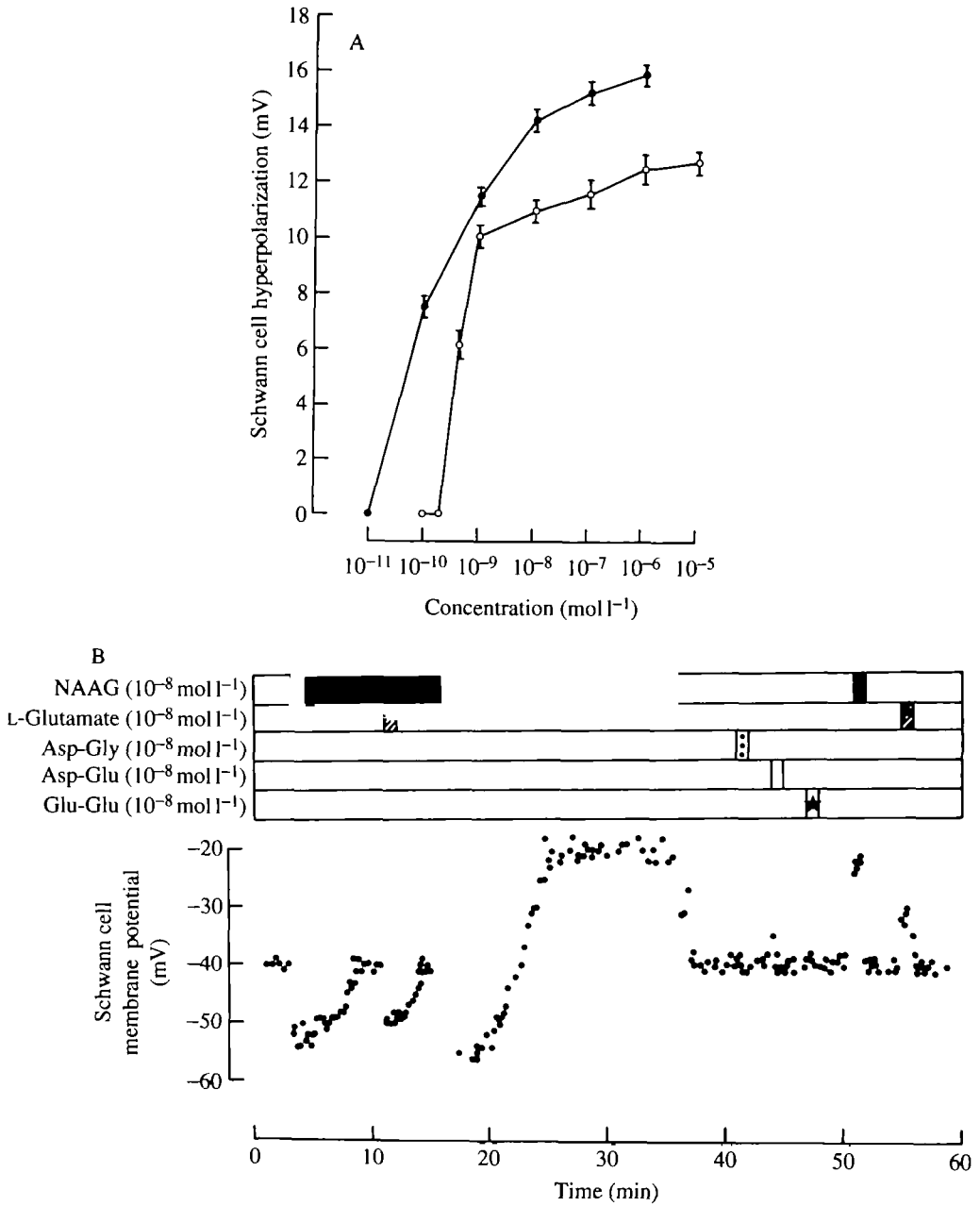


Fig. 7

membrane potential can be observed in response to the application of short pulses of glutamate after blockade of the cholinergic receptors with  $\alpha$ -bungarotoxin (P. D. Evans, V. Reale, R. M. Merzon and J. Villegas, in preparation). However, in another species of squid, *Alloteuthis subulata*, blockade of the cholinergic receptors with tubocurarine reveals the presence of a glutamate-induced depolarization (Lieberman *et al.* 1989). Another difference between the glutamate-receptor-mediated effects in these two species of squid



Fig. 7. (A) Dose-response curve for the hyperpolarizing effects of 1 min pulses of *N*-acetylaspartylglutamate (filled circles) and L-glutamate (open circles) on the Schwann cell membrane. The values represent the differences  $\pm$  s.e. between the mean membrane potential in the 2 min period before agonist application and the mean membrane potential in the 2 min period after the end of the 1 min agonist pulse. Each solution was tested on at least four nerve fibres. (B) Specificity studies on the rapid depolarization of the Schwann cell membrane induced by 1 min pulses of  $10^{-8}$  mol l $^{-1}$ , *N*-acetylaspartylglutamate (NAAG) (filled bar), L-glutamate (hatched bar)  $\beta$ -Asp-Gly (stippled bar), Asp-Glu (open bar) and Glu-Glu (starred bar) after the induction of a slow depolarization by a prolonged pulse of NAAG ( $10^{-8}$  mol l $^{-1}$ ) (filled bar). Each point represents the potential difference recorded in a different Schwann cell.

is that aspartate was a much more powerful agonist of these receptors in *Alloteuthis* than in *Sepioteuthis*. Thus, the receptors mediating this effect on the adaxonal glial cells of the squid may exhibit species differences.

We have previously shown that the slow depolarization of squid Schwann cells induced by long pulses of L-glutamate is likely to be mediated *via* the activation of NMDA-type glutamate receptors (Evans *et al.* 1991a). This is the first demonstration of NMDA-type glutamate receptors in any glial cell preparation, although *Xenopus laevis* oocytes injected with mRNA from an astrocytoma cell line (R-111) have recently been shown to acquire a small number of NMDA receptors (Matute *et al.* 1992). This glutamate effect in the squid Schwann cells is blocked specifically by APV ( $10^{-6}$  mol l $^{-1}$ ) but not by APB ( $10^{-6}$  mol l $^{-1}$ ) (Evans *et al.* 1991a), GDEE ( $10^{-6}$  mol l $^{-1}$ ), L-AP3 ( $10^{-6}$  mol l $^{-1}$ ) or CNQX ( $10^{-6}$  mol l $^{-1}$ ) (present study). The present study shows that the L-glutamate-induced slow depolarization has a threshold between  $10^{-9}$  and  $10^{-8}$  mol l $^{-1}$ , whilst NMDA has a lower threshold (between  $5 \times 10^{-10}$  and  $10^{-9}$  mol l $^{-1}$ ) for the induction of this effect (Evans *et al.* 1991a). The effect is also mimicked by cis-DMG ( $10^{-8}$  mol l $^{-1}$ ), a potent agonist of vertebrate NMDA receptors (Lanthorn *et al.* 1990; Allan *et al.* 1990). This receptor in the squid is not activated by quisqualate, kainate (Evans *et al.* 1991a) or L-aspartate up to a concentration of  $10^{-5}$  mol l $^{-1}$  or by trans-ACPD up to a concentration of  $10^{-7}$  mol l $^{-1}$ . Since Ca $^{2+}$  is needed to initiate and maintain this slow depolarization (Villegas, 1978a,b; Evans *et al.* 1991a), as with NMDA receptors in neurones (Malenka *et al.* 1989), the NMDA receptors on the squid Schwann cell also mediate an increase in calcium permeability. It has been postulated that NMDA receptors in vertebrate preparations can only be activated in the presence of low doses of glycine, which acts as a co-agonist (see Thomson, 1989). The squid Schwann cell NMDA receptors may differ from classical vertebrate NMDA receptors in this respect, since glycine is unlikely to accumulate to significant levels in the superfused preparation. Nevertheless, it is not possible at present to rule out an accumulation of glycine in the small spaces between the Schwann cells and the giant axon. However, a vertebrate glycine-insensitive NMDA receptor has recently been found to be expressed in *Xenopus* oocytes after the injection of guinea pig cerebellar mRNA (Sekiguchi *et al.* 1990). Attempts to test directly whether the squid NMDA receptor can be modulated by glycine have been complicated because in some preparations glycine application alone can initiate a very variable change in the membrane potential of the Schwann cell (J. Villegas and R. M. Merzon, unpublished observations). Another apparent difference between the

squid and vertebrate NMDA receptors is that the squid receptor can be activated in artificial sea water containing  $53 \text{ mmol l}^{-1} \text{ Mg}^{2+}$  whereas the vertebrate receptor is blocked in a voltage-sensitive way by  $1 \text{ mmol l}^{-1} \text{ Mg}^{2+}$ . This may represent an adaptation of the squid NMDA receptor to a marine environment.

The fast depolarizing responses to L-glutamate induced after the properties of the Schwann cells have been modified by the activation of the NMDA receptors appear to be mediated by a second class of NMDA receptor. They are specifically activated by NMDA with a threshold between  $10^{-10}$  and  $10^{-9} \text{ mol l}^{-1}$  in contrast to L-glutamate, which had a threshold between  $10^{-9}$  and  $10^{-8} \text{ mol l}^{-1}$  (Evans *et al.* 1991a), and they are not activated by quisqualate or kainate up to  $10^{-5} \text{ mol l}^{-1}$  or by trans-ACPD up to  $10^{-7} \text{ mol l}^{-1}$ . cis-DMG is also a potent activator of these receptors with a threshold between  $10^{-10}$  and  $10^{-9} \text{ mol l}^{-1}$ . However, the properties of these receptors differ from those of the NMDA receptors mediating the slow depolarization since they are not blocked by APV or CNQX, but they are blocked after exposure of the preparation to  $10^{-6} \text{ mol l}^{-1}$  GDEE. They were also partially blocked by APB. A rapid activation of NMDA receptors has also been described at a variety of vertebrate central synapses (Mody and Heinemann, 1987; D'Angelo *et al.* 1990). At present the mechanism that enables this second set of NMDA receptors to become activated and inhibits the activation of the metabotropic glutamate receptors for a prolonged period after the stimulation of the NMDA receptors mediating the slow depolarization is not known, but it seems likely to be related to a prolonged elevation of  $\text{Ca}^{2+}$  concentration in the Schwann cells.

A novel finding of the present investigation is the fact that the dipeptide *N*-acetylasparylglutamate is a potent agonist of all three glutamate receptor types found on the squid Schwann cells. This peptide has previously been shown to be localised selectively in various regions of the vertebrate central nervous system and to be capable of activating only a portion of the glutamate receptors present (Zaczek *et al.* 1983; Moffet *et al.* 1990). Another dipeptide,  $\beta$ -aspartylglycine, also showed a significant ability to activate the glutamate receptors mediating the release of acetylcholine from the Schwann cells but, as with the other dipeptides tested up to  $10^{-8} \text{ mol l}^{-1}$ , it did not activate the receptors responsible for the induction of the rapid depolarization after the induction of a slow depolarization by L-glutamate.  $\beta$ -Aspartylglycine was originally isolated as a dipeptide from the nervous system of the gastropod mollusc *Aplysia californica* (McCaman and Stetzler, 1984, 1985) but its function remains unknown. However, as mentioned above, many of the effects of *N*-acetylasparylglutamate have been suggested to be brought about either by a contamination of the synthetic peptide with high levels of potassium ions (Whittemore and Koerner, 1989) or by a cleavage of the dipeptide by the enzyme *N*-acetylated  $\alpha$ -linked acidic dipeptidase to release L-glutamate as the active factor (see Moffett *et al.* 1990). In the present experiments, contamination with potassium ions resulting in a depolarization of the Schwann cell membrane could perhaps account for the ability of this peptide to mimic the fast and slow depolarizing effects mediated by the NMDA receptors but seems unlikely to be able to account for its ability also to mimic the activation of the metabotropic glutamate receptors. In addition, although L-glutamate could be enzymatically released from several of the peptides used in the present study, it could not be produced from  $\beta$ -aspartylglycine and aspartylaspartate and any aspartate

released from the latter two peptides would seem unlikely to be responsible for their actions owing to the very low sensitivity of this preparation to applied L-aspartate. Further studies on the specificity and activities of any dipeptidases present in the Schwann cell system of the squid are required to resolve the question of whether these dipeptides containing acidic amino acids are acting directly on receptors in this preparation.

The non-NMDA glutamate receptors mediating the release of acetylcholine from the Schwann cells are extremely sensitive to L-glutamate (threshold between  $2 \times 10^{-10}$  and  $5 \times 10^{-10} \text{ mol l}^{-1}$ ) and are likely to be activated by glutamate released non-synaptically from the squid giant axon by stimulation at high frequencies (Lieberman *et al.* 1989; Evans *et al.* 1991a). The activation of these receptors turns on a complex multistep process that amplifies the original signal and leads to an increase in the permeability of the Schwann cell membrane to potassium ions at times when the squid is likely to be using its giant-fibre-mediated escape response pathway (Villegas, 1981; Villegas *et al.* 1988; Evans *et al.* 1991b). One possible function for this system would be to buffer any accumulation of potassium ions in the adaxonal space that might interfere with the conduction of nerve impulses along the giant axons. The NMDA-type glutamate receptors responsible for the induction of the slow depolarization and the subsequent rapid depolarizing responses have higher thresholds for activation by L-glutamate (between  $10^{-9}$  and  $10^{-8} \text{ mol l}^{-1}$ ) and are thus only likely to be activated *in vivo* under extreme conditions of prolonged activity of the giant axon at high frequency. The slow depolarization above the resting membrane potential is not, however, a prerequisite for the induction of the change in responsiveness. Thus, the Schwann cells of the squid giant axon possess NMDA receptors that can lead to long-term changes in responsiveness as do many other neuronal systems (Davies *et al.* 1989; Kauer *et al.* 1988a,b; Malenka *et al.* 1989; Watkins and Collingridge, 1989).

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