THE STOPPING RESPONSE OF *XENOPUS LAEVIS* EMBRYOS: PHARMACOLOGY AND INTRACELLULAR PHYSIOLOGY OF RHYTHMIC SPINAL NEURONES AND HINDBRAIN NEURONES

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Summary

1. *Xenopus laevis* embryos stop swimming in response to pressure on the cement gland. This behaviour and 'fictive' stopping are blocked by bicuculline ($10 \mu mol l^{-1}$), tubocurarine ($110 \mu mol l^{-1}$) and kynurenic acid ($0.5 mmol l^{-1}$).

2. Intracellular recordings from spinal neurones active during swimming have shown that pressure on the cement gland evokes compound, chloride-dependent inhibitory postsynaptic potentials (IPSPs). These are blocked by bicuculline, tubocurarine and kynurenic acid, but are unaffected by strychnine ($2 \mu mol l^{-1}$).

3. When the cement gland is pressed, trigeminal ganglion activity precedes both the IPSPs and the termination of 'fictive' swimming activity recorded in rhythmic spinal neurones. The trigeminal discharge is unaffected by the antagonists bicuculline, tubocurarine, kynurenic acid and strychnine.

4. Intracellular recordings from the hindbrain have revealed neurones that are normally silent, but rhythmically inhibited during 'fictive' swimming. In these neurones pressure on the cement gland evokes depolarising potentials, often with one or more spikes.

5. We propose that the stopping response depends on the excitation of pressure-sensitive trigeminal receptors which innervate the cement gland. These release an excitatory amino acid to excite brainstem GABAergic reticulospinal neurones, which inhibit spinal neurones to turn off the central pattern generator for swimming. There may also be a less direct pathway.

Introduction

In a number of animals locomotor rhythms can be terminated as a result of a specific mechanical stimulus (Fraenkel, 1932; Gray and Sand, 1936; Gray *et al.* 1938; Lissmann, 1947; Chadwick, 1953; Dingle, 1961; Gray, 1968; Weevers, 1971; Pringle, 1974; Arshavsky *et al.* 1985; Satterlie *et al.* 1985). Little work has been done on the neural pathways responsible for these responses, and nothing is known of their pharmacology.

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Swimming in the *Xenopus* embryo stops as a result of the deformation of pressure-sensitive trigeminal neurites (movement-detector neurones) that inner-vate the cement gland and head skin (Roberts and Blight, 1975; Roberts, 1980). Such a stimulus occurs when the animal swims into an obstruction. In a previous paper (Boothby and Roberts, 1992) we concluded (1) that these trigeminal neurones form the afferent pathway of the stopping response; (2) that their axons in the hindbrain could directly excite inhibitory interneurones, which are probably GABAergic and project to both sides of the spinal cord; and (3) that the afferents could excite excitatory interneurones with axons that cross the hindbrain to activate the inhibitory interneurones. These proposals were open to pharmaco-logical testing.

Where the nature of primary somatosensory neurones has been studied, all such cells appear to be excitatory (Eccles, 1964; Jessell *et al.* 1986). Thus, one might expect the trigeminal neurones of *Xenopus* to be excitatory, like the Rohon-Beard neurones that innervate the trunk skin (Clarke *et al.* 1984) and release an excitatory amino acid (Sillar and Roberts, 1988*b*). It is not surprising, therefore, that immunostaining for the inhibitory transmitters glycine (Dale *et al.* 1986) and GABA (Roberts *et al.* 1987) has not revealed any inhibitory neurones in the trigeminal ganglia. The proposed excitatory interneurones of the hindbrain could be analogous to the second-order sensory dorsolateral interneurones of the spinal cord, which relay excitation from the Rohon-Beard neurones to the neurones of the central pattern generator (CPG) by the release of an excitatory amino acid (Clarke and Roberts, 1984; Sillar and Roberts, 1988*a*,*b*; Roberts and Sillar, 1990). One might therefore expect both the trigeminal neurones and the putative excitatory hindbrain interneurones to release an excitatory amino acid.

Immunostaining for glycine and GABA in the hindbrain has revealed a number of neurone classes. These include the glycinergic commissural interneurones (Roberts *et al.* 1988) and the GABAergic midhindbrain reticulospinal neurones, vestibular complex commissural neurones and rostral hindbrain commissural neurones (Roberts *et al.* 1987). At least the first of these neurone classes projects to the spinal cord and could, therefore, be responsible for inhibiting the pattern generator for swimming.

To investigate the above possibilities we have used neurotransmitter antagonists specific to excitatory amino acids, glycine, and GABA. We have also made intracellular recordings from spinal neurones and hindbrain neurones to seek direct evidence for inhibitory and excitatory inputs, respectively, following mechanical distortion of the cement gland, the stimulus that normally specifically stops swimming (Roberts and Blight, 1975). Some of the results have been reported in preliminary form (Boothby, 1988; Boothby and Roberts, 1988).

Materials and methods

Embryos and larvae of *Xenopus laevis* (at stage 37/38: Nieuwkoop and Faber, 1956) were reared from eggs obtained from a captive breeding colony. When

necessary, animals were removed from their egg membranes with fine forceps. All experiments were carried out at room temperature (18–22 °C) in a dish containing tapwater (behavioural observations only) or perfused with saline (ionic composition: NaCl, $115 \, \text{mmol} \, l^{-1}$; NaHCO$_3$, $15 \, \text{mmol} \, l^{-1}$; KCl, $2.5 \, \text{mmol} \, l^{-1}$; CaCl$_2$, $2 \, \text{mmol} \, l^{-1}$; MgCl$_2$, $0.5 \, \text{mmol} \, l^{-1}$) adjusted to pH 7.4 by bubbling with 95 % O$_2$:5 % CO$_2$. Drugs were applied by switching the perfusate to saline with the drug added. The following pharmacological agents were used: bicuculline methiodide, kynurenic acid, strychnine sulphate and *d*-tubocurarine chloride (all from Sigma).

Animals were prepared for behavioural observations and extracellular recordings as described previously (Boothby and Roberts, 1992). All dissection was carried out on animals anaesthetised in MS-222 (Sigma). Immobilisation was in $1.25 \times 10^{-5} \, \text{mol} \, l^{-1}$ α-bungarotoxin (Sigma). Electrical and tactile stimuli were administered as before. Gentle pressure stimulation of the cement gland or head skin, by a sinusoidally moving loop of tungsten wire, are both effective methods of terminating swimming, but the former is more reliable (Boothby and Roberts, 1992) and was therefore used routinely. However, for each set of experiments, trials using head stimulation were conducted to look for any differences in effect. In all cases, stimuli to the head produced qualitatively the same result as stimuli to the cement gland.

To measure the reliability of stopping behaviour in the presence of neurotransmitter antagonists the dorsal fin and skin overlying the brain were first removed to improve drug access. After recovery (at least 15 min) the embryos were placed in normal saline and were either left to move freely or held at the level of the gills between etched tungsten micropins. Swimming was initiated by dimming the illumination or by a gentle stroke to the trunk with a gerbil hair. Embryos then stopped swimming when they bumped into the side of the dish or when given a gentle pressure stimulus to the cement gland. After checking that the response was reliable, animals were transferred to saline containing the test antagonist and left for 20 min. The reliability of the stopping response was then reassessed. Finally, the embryos were returned to normal saline for up to 40 min for recovery and the response was tested again.

The 'fictive' stopping response was tested similarly. In both behavioural and 'fictive' experiments the response was considered reliable if it occurred in 80 % or more of a minimum of seven trials (Boothby and Roberts, 1992).

Intracellular recordings were made using microelectrodes filled with $3 \, \text{mol} \, l^{-1}$ potassium acetate or $2 \, \text{mol} \, l^{-1}$ potassium chloride (115–180 MΩ and 50–100 MΩ resistances, respectively). To record from spinal neurones that were rhythmically active during 'fictive' swimming, a length of spinal cord was exposed by removing the dorsal three-quarters of the overlying myotomes (post-otic myotomes 0–6) on the recording side. Spinal neurones were most reliably penetrated superficially in the ventral quarter of the cord. Marking studies have shown this region to contain mostly motor neurones (Roberts and Clarke, 1982; Soffe and Roberts, 1982*a*). In more dorsal positions, motor neurones are uncommon and any neurones

rhythmically active during 'fictive' swimming are likely to be premotor CPG interneurones (Clarke and Roberts, 1984; for a review, see Roberts, 1990). To record hindbrain neurone activity, the hindbrain was exposed by removing the dorsal half of the rostral myotomes (post-otic myotomes 0–2) on the recording side and the meninges overlying the hindbrain. Data were recorded conventionally, displayed on a digital storage oscilloscope and stored on magnetic tape.

Throughout this paper N is used to indicate the number of neurones from which a particular result was obtained.

Results

Pharmacology of the stopping response

To ascertain the probable identity of the neurotransmitters involved in the stopping response, the reliability of the response was tested in the presence of different neurotransmitter antagonists. Both the behavioural and 'fictive' stopping responses were monitored, the latter using extracellular ventral root recordings and intracellular rhythmic spinal neurone recordings (see Materials and methods and below).

In normal saline, stopping behaviour was 89 % reliable (20 trials each in 24 animals) and 'fictive' stopping was 94 % reliable (at least nine trials each in 46 animals). After transfer to normal saline for recovery following antagonist applications, the stopping response was always more than 80 % reliable (same number of trials as above).

Excitation

The trigeminal neurones, and any excitatory hindbrain interneurones involved in the pathway, are likely to release an excitatory amino acid. We tested the effect of 0.5 mmol l^{-1} kynurenic acid, an excitatory amino acid antagonist, on the reliability of the stopping response. This was without effect on swimming, but blocked the stopping response both in behavioural (8 % reliable in 20 trials each in six animals) and 'fictive' (6 % reliable in at least nine trials each in eight animals) experiments (Fig. 1A). We therefore concluded that an excitatory amino acid is involved at the primary sensory synapses and/or elsewhere in the stopping response pathway.

Inhibition

GABA release could be responsible for inhibiting the CPG interneurones and motor neurones following cement gland stimulation. Bicuculline is an effective GABA antagonist both in *Xenopus* (Soffe, 1987) and in other vertebrate systems (Curtis *et al.* 1970). We therefore tested its effect on the stopping response. At a concentration of $10 \mu \text{mol l}^{-1}$ it dramatically reduced the reliability of the response in both behavioural (3 % reliable in 20 trials each in six animals) and 'fictive' (6 % reliable in at least nine trials each in 14 animals) experiments (Fig. 1B). Bixby and Spitzer (1982) showed that, in *Xenopus*, tubocurarine blocks responses of Rohon-

Beard neurones to the application of GABA, implicating tubocurarine as another GABA antagonist. We therefore tested its effect on the stopping response. 110 μmol l^{-1} tubocurarine reduced the reliability of stopping in both behavioural (11 % reliable in 20 trials each in six animals) and 'fictive' (7 % reliable in at least eight trials each in 13 animals) experiments (Fig. 1C). We concluded that the stopping response pathway involves GABA.

Dose–response relationships

Since kynurenic acid, bicuculline and tubocurarine all clearly block the stopping response, we investigated the effect of changing their concentration on the reliability of the 'fictive' response. The concentration ranges tested were 0.1–1.5 mmol l^{-1} kynurenic acid (at least nine trials in each of 6–8 animals at each concentration), 1–40 μmol l^{-1} bicuculline (at least eight trials in each of six animals at each concentration) and 10–300 μmol l^{-1} tubocurarine (at least seven trials in each of four animals at each concentration). For each antagonist a dose–response plot (using log concentration) gave a sigmoid curve (Fig. 2). Bicuculline was more potent than tubocurarine and kynurenic acid, but all three had a significant effect on the stopping response.

Effect of cement gland stimulation on rhythmic spinal neurones

The stopping response pathway could 'turn off' swimming in three ways: by inhibiting neurones of the central pattern generator and thereby depriving the motor neurones of their excitatory drive; by inhibiting the motor neurones directly; or by inhibiting both the CPG interneurones and motor neurones. We tested these possibilities by recording intracellularly from spinal neurones that were rhythmically active in the typical pattern during 'fictive' swimming (Roberts and Kahn, 1982; Figs 1, 3Ai,Di).

When recordings were made from ventral rhythmic neurones (putative motor neurones: see Materials and methods) their resting potentials lay in the range -53 to -92 mV (mean -74 mV; $N=21$) and their spike amplitudes were between 20 and 50 mV above the resting potential, consistent with those recorded previously (Roberts and Kahn, 1982; Soffe, 1990). As expected, gentle pressure stimulation of the cement gland reliably terminated 'fictive' swimming recorded extracellularly from the ventral root and intracellularly from rhythmic spinal neurones (Figs 1 and 3Ai). Occasionally inhibitory postsynaptic potentials (IPSPs) were seen at the end of 'fictive' swimming episodes in intracellular recordings (Fig. 3Ai), but more usually they were not apparent (Figs 1 and 5A). In contrast, when the embryo was at rest an identical stimulus normally evoked clear hyperpolarising compound IPSPs (Fig. 3Aii). With increasing stimulus strength, leading to progressively greater deformation of the cement gland, the number of IPSPs and their size and duration increased ($N=5$; Fig. 3B). Electrical stimulation of the cement gland also evoked similar IPSPs ($N=3$; Fig. 3C), the latency of which lay in the range 30–37 ms (mean 34 ms from nine IPSPs). Minimum latencies of responses to mechanical stimuli, measured from the beginning of the movement of the loop of
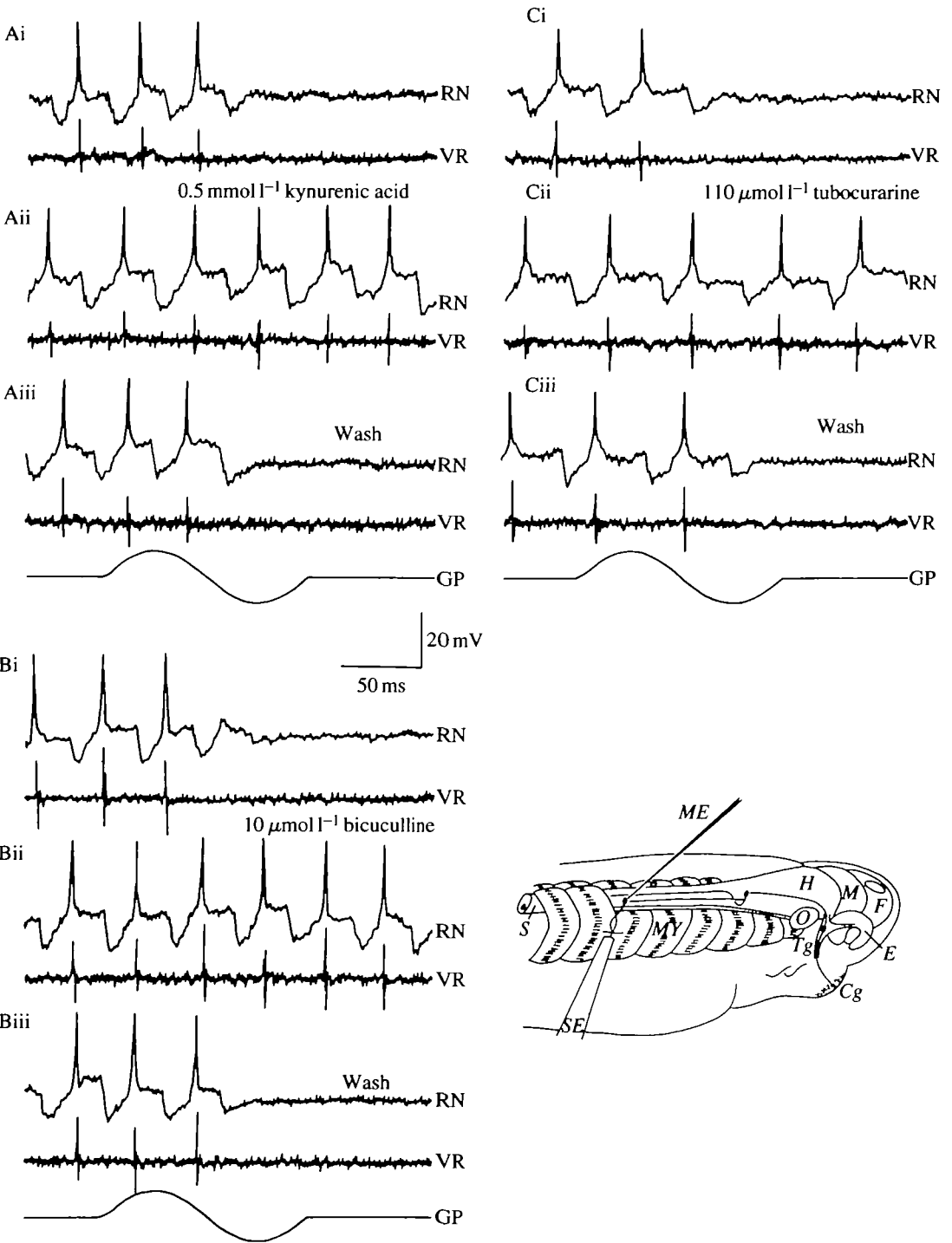
Fig. 1

Fig. 1. The effect of antagonists on the reliability of the 'fictive' stopping response. The diagram shows the preparation for intracellular recording (*Cg*, cement gland; *E*, eye; *F*, forebrain; *H*, hindbrain; *M*, midbrain; *ME*, microelectrode; *MY*, myotomes; *O*, otic capsule; *S*, spinal cord; *SE*, suction electrode; *Tg*, trigeminal ganglion). The traces show 'fictive' swimming activity recorded extracellularly from the ventral root (VR) and intracellularly from rhythmic spinal neurones (RN). A–C each show (i) the stopping response in normal saline, (ii) a second trial in the presence of the test antagonist and (iii) a final trial after recovery in normal saline. (A) $0.5 \, \text{mmol} \, l^{-1}$ kynurenic acid, (B) $10 \, \mu\text{mol} \, l^{-1}$ bicuculline or (C) $110 \, \mu\text{mol} \, l^{-1}$ tubocurarine blocked the response. The lowest trace (GP) indicates the timing of the gentle pressure stimulus to the cement gland (downward slope is pressure) for all three trials in each group of records. Calibration: 50 ms, 20 mV (intracellular).
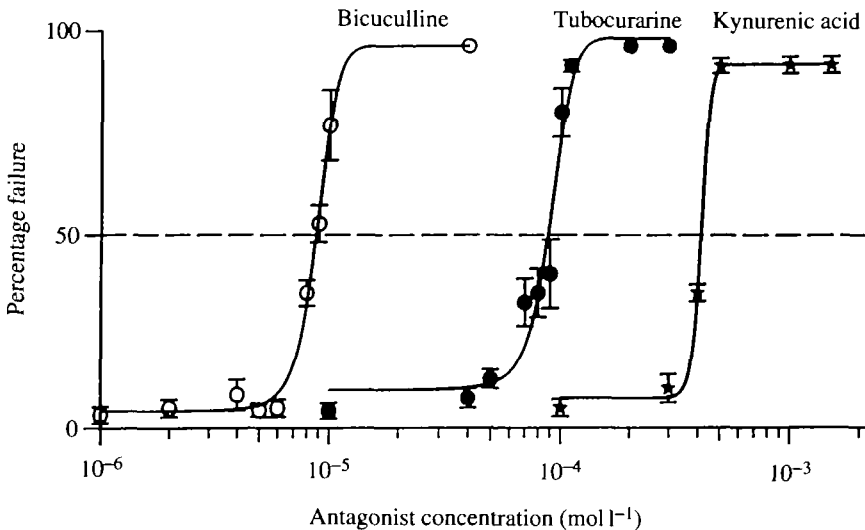

Fig. 2. Effects of antagonist concentration on the reliability of the 'fictive' stopping response. The graph shows the percentage failure of the 'fictive' stopping response, plotted as a function of the concentration of the antagonist. In each case a characteristic sigmoid curve was produced. Bars show standard errors. Sigmoid curves were fitted by FigP computer software.

tungsten wire towards the cement gland, were more variable (range 30–43 ms, mean 37 ms from 46 IPSPs).

Recordings were also made from more dorsal rhythmic neurones (putative CPG interneurones: see Materials and methods; $N=7$). 'Fictive' swimming activity, the stopping response and the IPSPs evoked by stimulating the cement gland at rest were indistinguishable from those recorded from putative motor neurones (Fig. 3D). This suggested that the CPG interneurones and motor neurones receive the same inhibitory synaptic inputs when the stopping response is evoked.
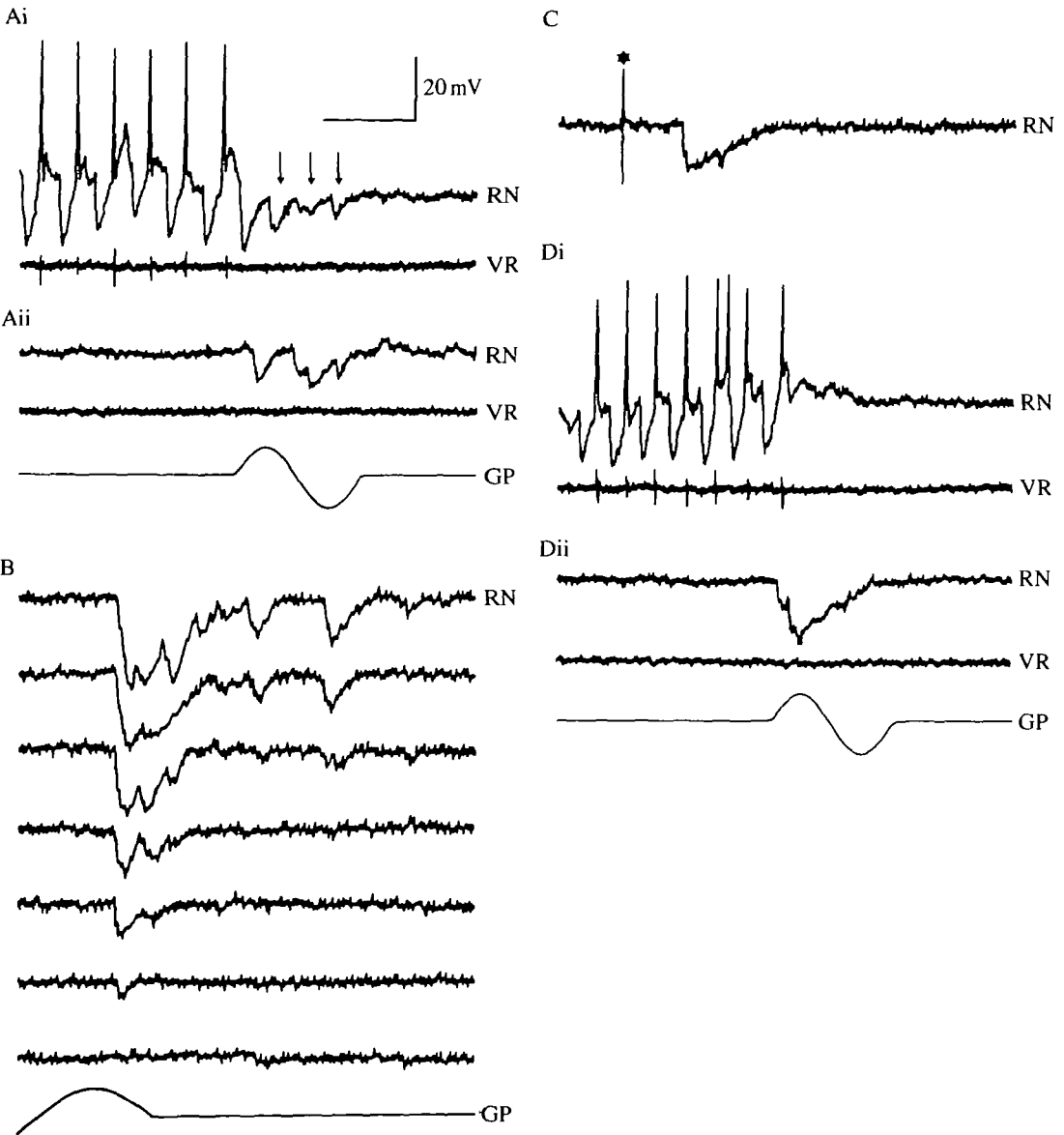
Fig. 3

Pharmacology and properties of IPSPs

Since kynurenic acid, bicuculline and tubocurarine block the stopping response, one would expect the IPSPs evoked in rhythmic spinal neurones by cement gland stimulation to be affected in the same way. We therefore tested the effects of these antagonists on IPSPs evoked by cement gland stimulation. As predicted, the IPSPs were either abolished or significantly reduced in size by $0.5 \, \text{mmol} \, l^{-1}$ kynurenic acid ($N=4$; Fig. 4A), $10 \, \mu\text{mol} \, l^{-1}$ bicuculline ($N=4$; Fig. 4B) or $110 \, \mu\text{mol} \, l^{-1}$

Fig. 3. The effect of cement gland stimulation on rhythmic spinal neurones. The traces show extracellular recordings from the ventral roots (VR) and intracellular recordings from rhythmic spinal neurones (RN). (A) In a ventral rhythmic spinal neurone (i) 'fictive' swimming was terminated by a cement gland stimulus (which, in this example, also evoked clear IPSPs at the end of the episode (arrows); cf. Figs 1 and 5A) and, (ii) at rest, a compound IPSP was evoked by another cement gland stimulus. (B) As the strength of the pressure stimulus was increased (bottom to top), the size, number and duration of the IPSPs increased. (C) Electrical stimulation of the cement gland (star indicates artefact of 0.5 ms current pulse) evoked an IPSP at a latency of about 34 ms. (D) In a more dorsal rhythmic spinal neurone, the responses to cement gland stimulation (i) during swimming and (ii) at rest were similar to those normally evoked in more ventral rhythmic spinal neurones. GP, gentle pressure stimulus trace (upward slope is pressure). Only one such trace is shown for each group of records. Calibration: 100 ms (A and D), 50 ms (B and C); 20 mV.

tubocurarine (N=2; Fig. 4C), providing further evidence that an excitatory amino acid and GABA are involved in the stopping response. Lower concentrations of these antagonists reduced the size, number and duration of the IPSPs without abolishing them (N=3; Fig. 4B).

The glycine antagonist, strychnine, blocks glycinergic IPSPs from the embryo spinal cord commissural interneurones in *Xenopus* (Dale, 1985), which are responsible for reciprocal inhibition in the central pattern generator (Soffe *et al.* 1984; Dale, 1985). Commissural interneurones extend into the hindbrain, so their descending axons might release glycine onto the CPG interneurones and motor neurones in response to cement gland stimulation and thereby stop swimming. We therefore tested the reliability of the IPSPs during the application of strychnine. At a concentration of $2\,\mu\text{mol}\,l^{-1}$, which is sufficient to block mid-cycle inhibition during 'fictive' swimming (Dale, 1985; Soffe, 1987), strychnine was without effect on the IPSPs evoked in rhythmic spinal neurones at rest by cement gland stimulation (N=4; Fig. 4D). We concluded that glycine is not involved in the stopping response pathway.

The IPSPs in the motor neurones of the lamprey (Kahn, 1982) and of *Xenopus* embryos (Roberts and Kahn, 1982; Soffe, 1987) that occur phasically during 'fictive' swimming are chloride-dependent. We tested the IPSPs evoked by cement gland stimulation for this property by recording from ventral rhythmic neurones with microelectrodes filled with $2\,\text{mol}\,l^{-1}$ KCl. In each case the IPSPs were reversed, indicating their chloride-dependence (N=3; Fig. 4E).

Relationship between rhythmic neurone and trigeminal ganglion neurone activity

Anatomical and physiological work (Roberts and Blight, 1975; Davies *et al.* 1982) suggested that the trigeminal neurones involved in the stopping response pathway are primary sensory neurones. If this were the case, the antagonists that block the stopping response should be without effect on the trigeminal response to cement gland stimulation. Extracellular recordings from the trigeminal ganglion and intracellular recordings from rhythmic spinal neurones were therefore made
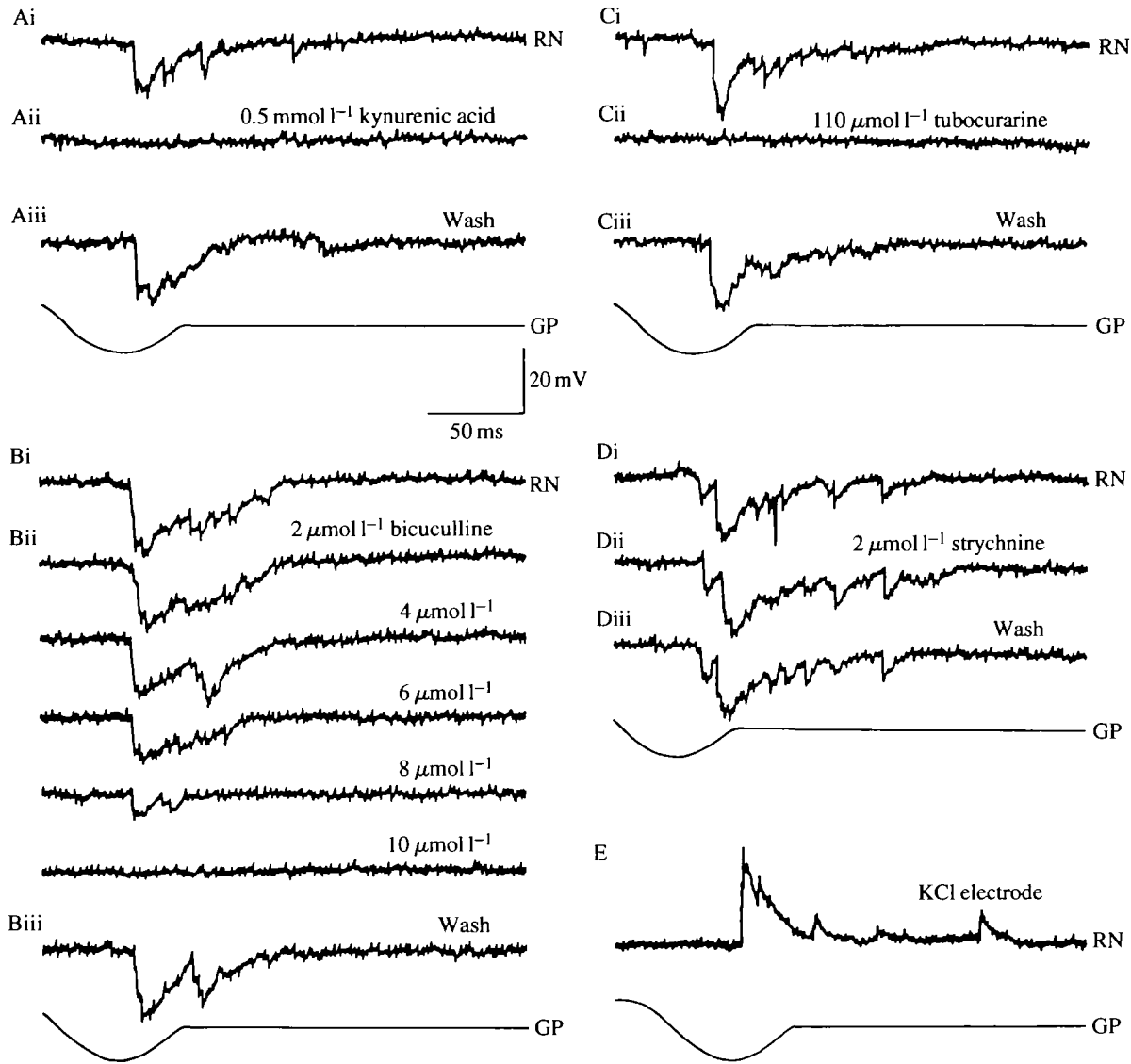
Fig. 4. The effect of antagonists on the IPSPs of ventral rhythmic spinal neurones. The traces show intracellular recordings from rhythmic spinal neurones (RN). A–D show (i) an IPSP in normal saline, (ii) one or more subsequent trials after the application of the test antagonist and (iii) a final trial after recovery in normal saline. (A) $0.5\,\text{mmol}\,l^{-1}$ kynurenic acid, (B) 2–$10\,\mu\text{mol}\,l^{-1}$ bicuculline or (C) $110\,\mu\text{mol}\,l^{-1}$ tubocurarine blocked the IPSPs. (D) $2\,\mu\text{mol}\,l^{-1}$ strychnine was without effect. (E) The IPSPs were reversed when recorded with a KCl microelectrode. GP, gentle pressure stimulus trace (downward slope is pressure). Only one such trace is shown for each group of records.
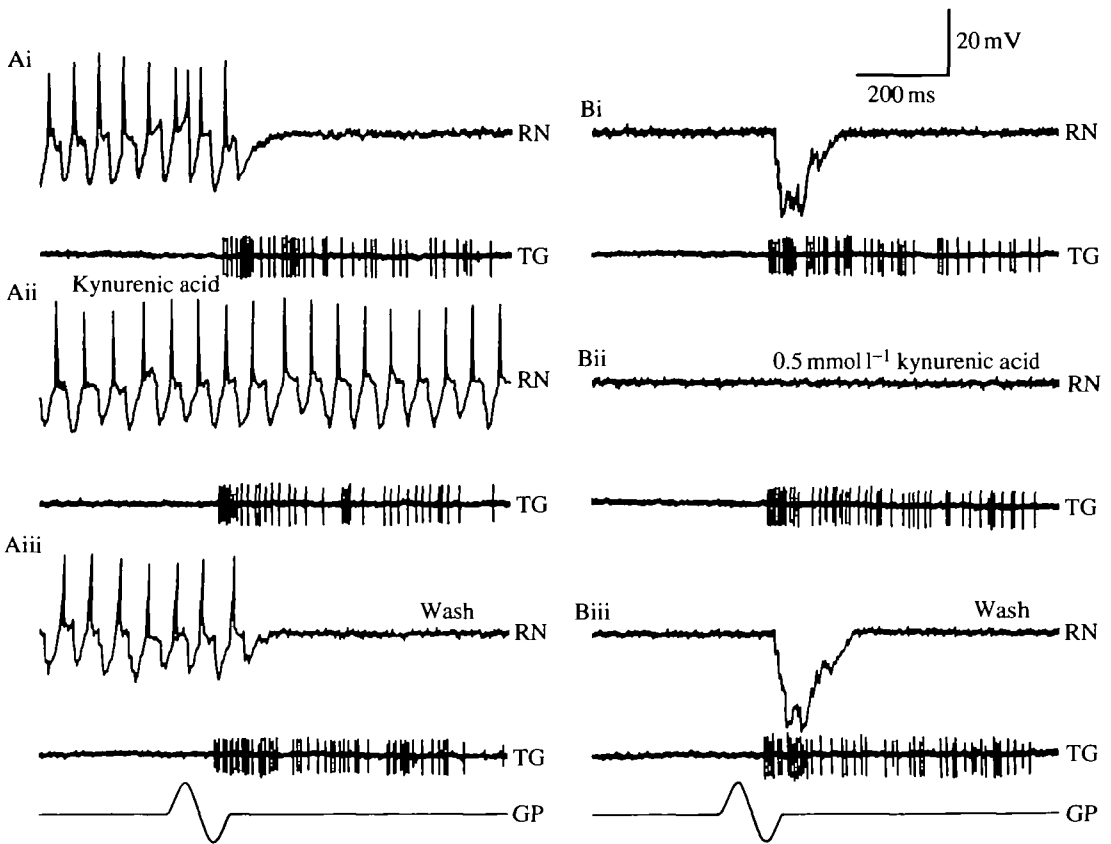
Fig. 5. Trigeminal ganglion activity and the stopping response. The traces show extracellular recordings from the trigeminal ganglion (TG) and intracellular recordings from rhythmic spinal neurones (RN). In response to gentle pressure stimulation of the cement gland, the trigeminal ganglion activity increased and (Ai) 'fictive' swimming ceased or, (Bi) at rest, IPSPs were evoked within one cycle period of the onset of this increase. A and B each show (i) the stopping response or production of IPSPs in normal saline, (ii) a second trial after the application of $0.5\,\text{mmol}\,l^{-1}$ kynurenic acid ('fictive' stopping or IPSPs recorded from spinal neurones are abolished but trigeminal ganglion activity is left intact), (iii) a final trial after recovery in normal saline. GP, gentle pressure stimulus trace (downward slope is pressure). Only one such trace is shown for each group of records.

simultaneously $(N=5)$. We then tested the effect of antagonists on trigeminal ganglion activity during the stopping response.

In response to gentle pressure stimulation of the cement gland, the firing frequency of units recorded from the trigeminal ganglion increases (Boothby and Roberts, 1992; Fig. 5A). The termination of 'fictive' swimming (Fig. 5A) or the appearance of IPSPs (Fig. 5B) always occurred within one cycle period of the onset of this increased discharge. The application of $0.5\,\text{mmol}\,l^{-1}$ kynurenic acid (Fig. 5), $10\,\mu\text{mol}\,l^{-1}$ bicuculline, $110\,\mu\text{mol}\,l^{-1}$ tubocurarine or $2\,\mu\text{mol}\,l^{-1}$ strych-

nine was without effect on the trigeminal activity ($N=3$). This confirmed that any GABA or excitatory amino acid synapses involved in the pathway are 'downstream' from the trigeminal ganglia, and is compatible with the trigeminal neurones being first-order sensory neurones.

Effect of cement gland stimulation on hindbrain neurones

If trigeminal neurones, excited by pressure on the cement gland, release an excitatory amino acid, there must be at least one class of hindbrain interneurone between the trigeminal neurones and the spinal cord. These would be excited by the trigeminal neurones and, as a result, would inhibit the CPG interneurones and motor neurones, thereby terminating swimming. If this were the case one might expect to be able to record from hindbrain neurones that receive excitation when the stopping response is evoked. We tested this by recording intracellularly from hindbrain neurones.

When recordings were made from the middle dorsoventral third of the hindbrain (with the brain viewed from the side, as in Fig. 6A), deep in the brain, neurones with activity compatible with an inhibitory rôle in the stopping response were found ($N=9$). These were all situated between the level of the caudal edge of the otic capsules and the level of the first post-otic myotomes (Fig. 6A). Their resting potentials lay in the range $-36\,\text{mV}$ to $-103\,\text{mV}$ (mean $-66\,\text{mV}$; $N=6$). When the embryo was at rest the neurones were silent, but in response to cement gland stimulation one or more depolarising compound excitatory postsynaptic potentials (EPSPs), sometimes with superimposed spikes, were evoked. With increasing stimulus strength the size and duration of these EPSPs increased ($N=3$;

Fig. 6. Hindbrain neurone activity and the effect of cement gland stimulation. (A) Drawing of the rostral central nervous system to show the positions of successful recordings from hindbrain neurones. Filled circles, cells excited by cement gland stimulation; open circles, rhythmic neurones. Shading indicates the area in which midhindbrain reticulospinal neurones are located (Roberts *et al.* 1987). F, forebrain; H, hindbrain; M, midbrain; 1–5, myotomes; O, otic capsule; P, pineal body; S, spinal cord; Tg, trigeminal ganglion. The traces in B–G show extracellular recordings from the ventral root (VR) and intracellular recordings from hindbrain neurones (HB). (B) When the animal was at rest, cement gland stimulation evoked a compound EPSP in a hindbrain neurone. As the strength of the pressure stimulus was increased (bottom to top) the size and duration of the response increased. Note that pulling on the mucus secreted from the cement gland also evoked a depolarisation (at the arrowheads). (C) Electrical stimulation of the cement gland (star indicates artefact of 0.5 ms current pulse) evoked an EPSP at a latency of 25 ms. (D) As a result of dimming the lights (at the arrow) 'fictive' swimming was evoked (recorded from the ventral roots) and the hindbrain neurone received rhythmic inhibition. (E) Injection of constant depolarising current (0.3 nA: marked by a bar) increased the amplitude of the IPSPs. (F) Gentle pressure stimulation of the cement gland, which was successful in terminating 'fictive' swimming, stopped the rhythmic inhibition and the hindbrain neurone was slightly depolarised (at the arrowhead). (G) In another test in the same neurone, a pair of spikes followed the termination of the rhythmic inhibition. GP, gentle pressure stimulus trace (upward slope is pull; downward slope is pressure). Only one such trace is shown for the group of records in B.

Fig. 6B). Electrical stimulation of the cement gland also evoked EPSPs (N=3; Fig. 6C), the latency of which lay in the range 23–28 ms (mean 25 ms from 10 EPSPs). As before, measurements of latency using mechanical stimuli were more variable (range 28–45 ms, mean 34 ms from 19 EPSPs). When current was injected into these neurones spikes were often evoked, but these did not trigger any ventral
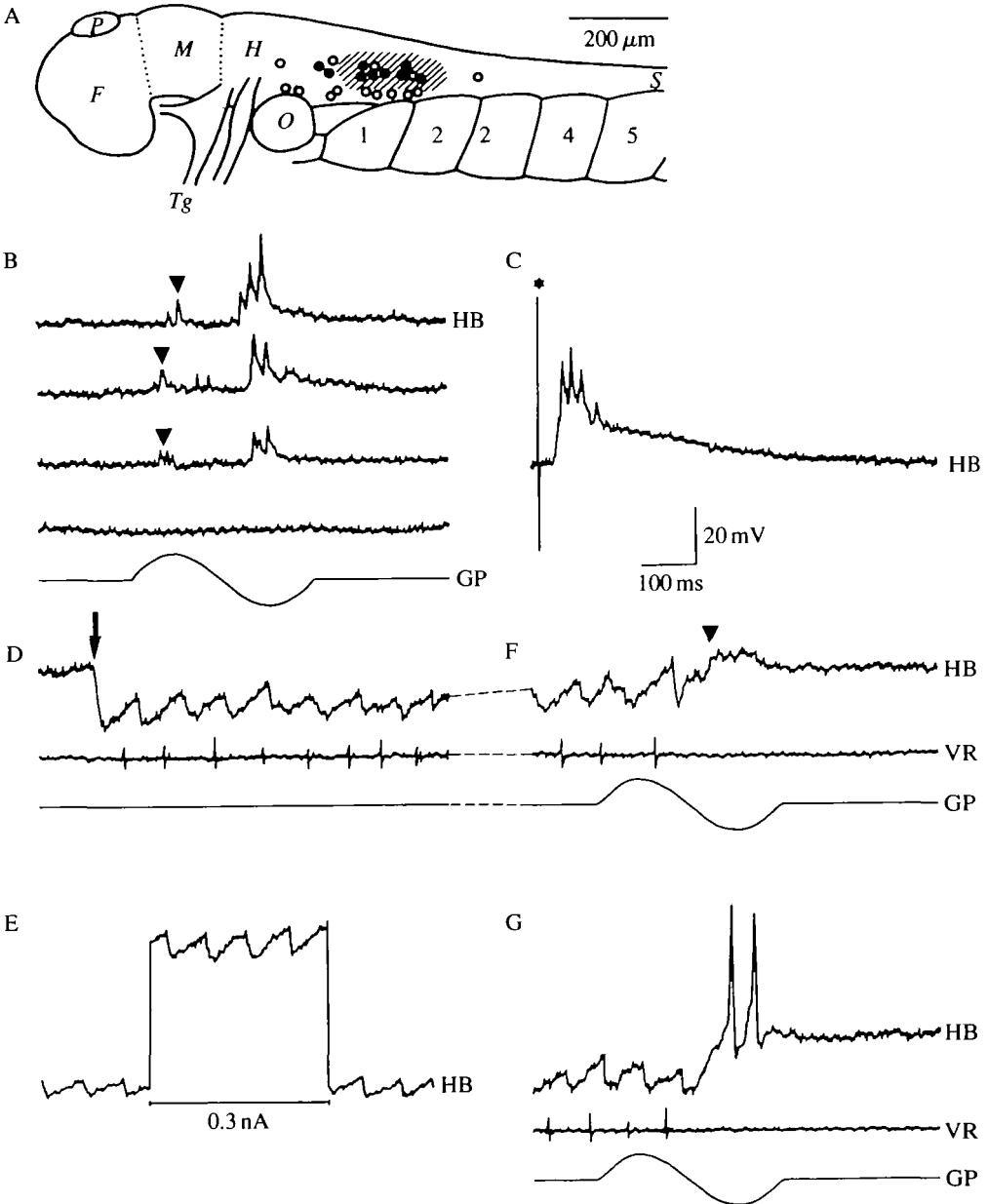

Fig. 6

root activity and were without any observable effect when given during 'fictive' swimming. The neurones were not excited by swim-initiating stimuli (either dimming the illumination or a gentle stroke to the trunk with a gerbil hair).

During 'fictive' swimming the neurones were rhythmically inhibited, the IPSPs occurring out of phase with the ipsilateral ventral root spikes (Fig. 6D). When constant depolarising current (0.3 nA) was injected during 'fictive' swimming, there were no signs of spike-like potentials in phase with the ipsilateral ventral roots, as would be expected in CPG interneurones and motor neurones; the only effect was an increase in the amplitude of the IPSPs (Fig. 6E). In response to cement gland stimulation, the rhythmic inhibition stopped in time with 'fictive' swimming recorded from the ventral root (Fig. 6F) and was often followed by one or more depolarising potentials, sometimes with spikes (Fig. 6G).

Other types of neuronal activity were recorded in the hindbrain, the most common of which resembled that characteristic of rhythmic spinal neurones (Roberts and Kahn, 1982). These neurones were penetrated superficially usually in the ventral half of the hindbrain ($N=14$; open circles in Fig. 6A). They were presumed to be the hindbrain equivalents of the CPG interneurones or motor neurones of the spinal cord.

Discussion

GABA-mediated IPSPs

The IPSPs evoked in spinal rhythmic neurones as a result of gentle pressure stimulation of the cement gland are the first GABAergic IPSPs to have been described in *Xenopus*. The only other IPSPs studied in detail in this animal are the glycinergic mid-cycle IPSPs that occur during 'fictive' swimming, also recorded from rhythmic spinal neurones (Dale, 1985; Soffe, 1987). The two types of IPSP are in many respects similar. Both are compound events, suggesting that they are the result of collective inhibitory input from a group of presynaptic interneurones. While the duration and amplitude of the mid-cycle IPSPs are around 20–30 ms and 15 mV, respectively (Soffe, 1987), those of the IPSPs evoked by cement gland stimulation are far more variable and depend on the type of stimulus. The strength and duration of the pressure stimulus to the cement gland will determine the number of primary sensory neurones activated. Furthermore, if more than one pathway from the trigeminal sensory neurones to the site of inhibition in the spinal cord exists, as is suggested from this and earlier work (Boothby and Roberts, 1992), this could also lead to the variable time course of the compound IPSP. The component potentials that make up the compound IPSPs are similar in shape to the mid-cycle IPSPs, with a fast rise time and a slow fall time (Fig. 4).

When recorded with a potassium acetate microelectrode, both types of IPSP are hyperpolarising and they can be increased in amplitude by injecting depolarising current. With KCl microelectrodes both are reversed, indicating their chloride-dependence (Kahn, 1982; Roberts and Kahn, 1982; Soffe, 1987). While the mid-cycle IPSPs are strychnine-sensitive and thus mediated by the release of glycine

(Dale, 1985; Soffe, 1987), those evoked by cement gland stimulation are blocked by bicuculline and tubocurarine, and are therefore the result of GABA release. The chloride dependence of the GABAergic IPSPs and the fact that they are blocked by bicuculline suggest they are mediated by $GABA_a$ rather than $GABA_b$ receptors (Curtis *et al.* 1970). These are presumably the bicuculline-sensitive GABA receptors identified on motor neurones by Soffe (1987), the function of which was at that time unknown.

In a resting embryo the compound IPSPs evoked by cement gland stimulation are usually very clear (Figs 3, 4 and 5). However, while the termination of 'fictive' swimming in a rhythmic neurone in response to cement gland stimulation is evidence of this inhibition, the same compound IPSPs are rarely apparent at the end of swimming episodes, where they might be expected (Figs 1, 3D and 5; cf. Fig. 3A). This discrepancy could be for one or both of the following reasons: (1) the high level of excitation present during swimming, as evident from the overall rise in membrane potential that results from tonic excitatory input during 'fictive' swimming (Roberts and Kahn, 1982; Soffe and Roberts, 1982*b*; Dale and Roberts, 1985), shunts out the inhibition and thereby reduces the hyperpolarisation of the IPSPs; (2) since hindbrain neurones in the stopping response pathway may be inhibited during swimming (Fig. 6D), as are the sensory interneurones in the spinal cord (Sillar and Roberts, 1988*a*,*b*; Roberts and Sillar, 1990), it is possible that cement gland stimulation during swimming is less effective in exciting hindbrain interneurones than it is at rest, and there is a concomitant reduction in the inhibitory input from hindbrain neurones to CPG neurones and motor neurones.

A simple pathway for the stopping response

The trigeminal movement-detector neurones are excited by the deformation of their pressure-sensitive peripheral neurites, which innervate the cement gland and head skin (Roberts, 1975, 1980; Roberts and Blight, 1975), as occurs when the animal swims into an obstruction. This stimulus causes an increase in trigeminal discharge just before the inhibition of rhythmic spinal neurones, which is apparent from the cessation of 'fictive' swimming or, in an embryo at rest, the appearance of IPSPs, which are presumed to underlie the stopping response. All primary somatosensory neurones appear to be excitatory (Eccles, 1964; Jessell *et al.* 1986); thus, it seems reasonable to assume that those of the trigeminal ganglia are also excitatory. To stop swimming, therefore, they must either directly or indirectly excite inhibitory interneurones.

Both the termination of swimming and the IPSPs resulting from cement gland stimulation are blocked by the GABA antagonists bicuculline and tubocurarine. Also, the IPSPs are unaffected by the glycine antagonist strychnine. These results therefore implicate GABA as the inhibitory neurotransmitter in the stopping response. Immunocytochemistry has revealed a number of GABA-immuno-reactive neurone classes in the *Xenopus* central nervous system (CNS) (Dale *et al.* 1987; Roberts *et al.* 1987). It seems highly likely that one or more of these mediates

the stopping response by inhibiting the CPG interneurones and motor neurones in the spinal cord.

The simplest pathway that could be proposed to underlie the stopping response is that in which trigeminal neurones excite GABAergic interneurones, which in turn inhibit the CPG interneurones and motor neurones in the spinal cord. Since the axons of the trigeminal neurones project into the hindbrain but do not extend far into the spinal cord (Hayes and Roberts, 1983), the GABAergic neurones would have to lie within the brain. Earlier lesion studies (Boothby and Roberts, 1992) place further constraints on the possible pathways involved. First, neurones rostral to the hindbrain are not required. Second, the pathway can cross the hindbrain at three levels: the midhindbrain (at the level of the first post-otic myotomes), the rostral midhindbrain (at the level of the otic capsules) and the rostral hindbrain (at the level of the entry of the trigeminal ganglia). There is a discrete population of GABA-immunoreactive neurones at each of these levels: the midhindbrain reticulospinal neurones, the vestibular complex commissural neurones and the rostral hindbrain commissural neurones, respectively (Roberts et al. 1987). Each of these populations could, in theory, receive excitation from trigeminal neurones and mediate the GABAergic inhibition of the rhythmic spinal neurones, thereby causing swimming to stop.

For the above proposal to hold, the GABAergic neurones involved would require an appropriate morphology. Their cell bodies and dendrites would have to be in a suitable position to be contacted by trigeminal axons. Also, since trigeminal axons do not cross the hindbrain (Hayes and Roberts, 1983), the inhibitory neurones would require both ipsilateral and commissural axons, so as to provide both the ipsilateral and crossed stopping response pathways that have been revealed by lesion studies (Boothby and Roberts, 1992).

Only the most caudal group of GABAergic neurones, the midhindbrain reticulospinal neurones (Roberts et al. 1987), have been shown to fulfil all of the above criteria. They lie just caudal to the otic capsules in the ventral two-thirds of the hindbrain. They have both ipsilateral and contralateral descending axons that project into the spinal cord, and ventral and lateral dendrites in a suitable position to make connections with the descending axons of the trigeminal neurones. The midhindbrain reticulospinal neurones appear during developmental stages 25 and 26, while the stopping response is functional, although unreliable, by stage 28 (Boothby and Roberts, 1992). These neurones could, therefore, provide both the ipsilateral pathway and the crossed pathway at the level of the first post-otic myotomes (Fig. 7A). It is unlikely that the other GABAergic groups possess suitable morphology to mediate the stopping response (Roberts et al. 1987).

Involvement of a set of interposed excitatory interneurones

If one or both of the rostral two groups of GABAergic hindbrain interneurones are not part of the stopping response pathway, it is necessary to postulate the involvement of a set of excitatory hindbrain interneurones to account for the two rostral crossing pathways inferred from lesion experiments (Boothby and Roberts,
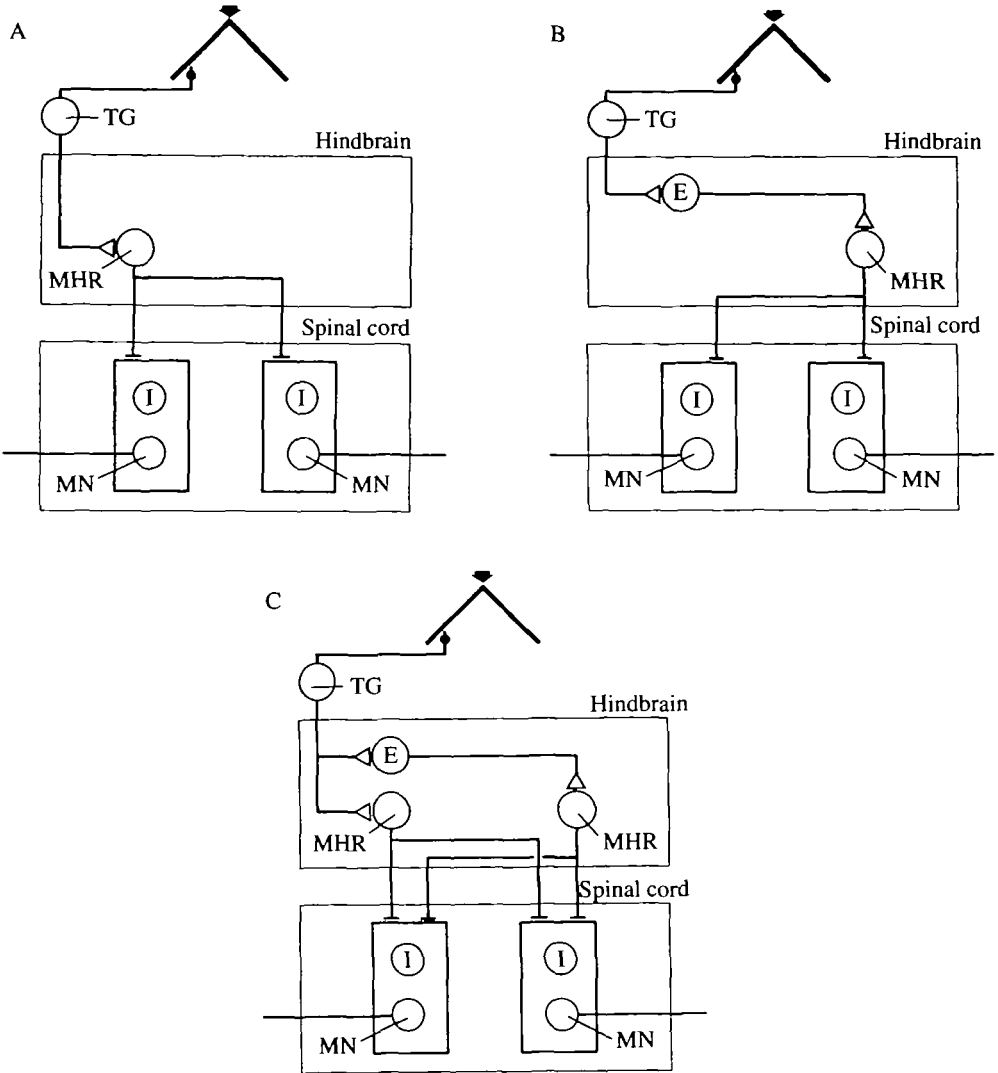
Fig. 7. Hypothetical pathways for the stopping response. A–C show three possible pathways from a single trigeminal ganglion on the left side. The arrow at the top represents a gentle pressure stimulus to the pyramid-shaped cement gland. The two large rectangles represent the hindbrain and spinal cord. Each circle represents a population of neurones and the small rectangles represent the half-centres that together make up the spinal circuitry for swimming. (A) Trigeminal ganglion neurones (TG), which innervate the cement gland directly, excite ipsilateral inhibitory midhind-brain reticulospinal neurones (MHR), which inhibit the CPG interneurones (I) and motor neurones (MN) on both sides of the spinal cord. (B) TG neurones directly excite proposed excitatory hindbrain interneurones (E), which cross to excite contralateral midhindbrain reticulospinal neurones. (C) The two pathways (A and B) combined. Triangles, excitatory synapses; bars, GABAergic synapses.

1992). These excitatory neurones would be interposed between the trigeminal neurones and the remaining GABAergic hindbrain interneurones (Fig. 7B) and, like other second-order sensory neurones (Brodal, 1981), would be expected to have commissural projections. They would provide an analogous route to the dorsolateral commissural neurones of the spinal cord which take excitation from the sensory Rohon-Beard neurones that innervate the trunk skin (Clarke and Roberts, 1984; Sillar and Roberts, 1988a,b; Roberts and Sillar, 1990) to the CPG interneurones on the opposite side (for a review, see Roberts, 1990).

Hindbrain neurone recordings

Intracellular recordings from the hindbrain, broadly in the region of the midhindbrain reticulospinal neurones (Roberts *et al.* 1987; Fig. 6A), have revealed neurones that are excited by cement gland or head skin stimulation and are rhythmically inhibited during 'fictive' swimming. This kind of activity is very similar to that seen in spinal interneurones that are excited by tactile afferents (Roberts and Sillar, 1990), and might therefore be expected in neurones concerned with either the termination or the initiation of swimming, both of which are triggered by tactile head stimulation. Since evoking spikes in individual hindbrain neurones produced neither excitatory nor inhibitory effects on swimming, the identity and rôle of these neurones remain unresolved. However, the following evidence suggests that the neurones recorded could be involved in the stopping response. (1) Gentle deformation of the cement gland, the kind of stimulus used throughout this and previous, related work, has been shown to excite trigeminal movement-detector neurones (which evoke stopping) and not trigeminal rapid-transient neurones (which evoke swimming: Roberts, 1980). For hindbrain recordings the pressure stimulus was particularly slow to avoid losing cell penetrations. (2) Hindbrain neurones were excited not only by pressure stimulation of the cement gland but also by pulling on the mucus secreted by the cement gland (Fig. 6B), which is known to activate movement-detector neurones only (Roberts and Blight, 1975). (3) Swim-initiating stimuli (dimming the illumination or a gentle stroke to the trunk with a gerbil hair) failed to excite these neurones.

The activity described above would be predicted both for the GABAergic inhibitory hindbrain interneurones and for any interposed excitatory interneurones. We therefore conclude that these neurones have at least some of the appropriate properties for a rôle in the stopping response and that they are in the expected location.

A proposal for the stopping response pathway

Taking into account the evidence and uncertainties discussed above, we can propose a pathway for the stopping response that accords with all the experimental data presently available. This hypothesis is illustrated in Fig. 7C. We propose that trigeminal neurones synapse both with a population of rostrocaudally dispersed excitatory interneurones and with the GABAergic midhindbrain reticulospinal

neurones, on the same side of the hindbrain. The excitatory interneurones have contralateral axons crossing at different levels between the mid- and rostral hindbrain, all of which also contact the midhindbrain reticulospinal neurones. Both the primary sensory neurones and the excitatory interneurones release an excitatory amino acid. The axons of the midhindbrain reticulospinal neurones descend to the spinal cord, both ipsilaterally and contralaterally, and inhibit the CPG interneurones and motor neurones by the release of GABA.

Reticulospinal pathways

There are sufficient examples of neurones in the hindbrain contacting spinal cord neurones to make plausible the suggestion that projections from the hindbrain of *Xenopus* could influence the central pattern generator for swimming. The descending projections of reticulospinal neurones are some of the earliest to develop from the brainstem to the spinal cord in all vertebrates studied (e.g. lamprey: Rovainen, 1978, 1979; larval zebrafish: Kimmel, 1982; developing chick: Okado and Oppenheim, 1985; young opossum: Cabana and Martin, 1984; for a review, see ten Donkelaar, 1982). In *Xenopus* they are the first to develop (van Mier and ten Donkelaar, 1984; Nordlander *et al.* 1985; van Mier *et al.* 1986).

Reticulospinal neurones are known to contact both spinal motor neurones (e.g. frog: Cruce, 1974; Shapovalov, 1975; Babalian and Shapovalov, 1984; Babalian and Chmykova, 1987; lamprey: Rovainen, 1974) and premotor interneurones (lamprey: Rovainen, 1974; Buchanan, 1982). It therefore seems likely that they either participate in or impinge upon locomotor circuits. Most of these connections are monosynaptic and excitatory (e.g. cat: Grillner and Lund, 1968; Grillner *et al.* 1970; lamprey: Buchanan *et al.* 1987). There are a few instances, however, where monosynaptic inhibitory connections between brainstem neurones and spinal cord neurones have been demonstrated (cat: Magoun and Rhines, 1946; Llinas and Terzuolo, 1964; rat: Holstege and Bongers, 1990; Holstege, 1991). Further, Holstege (1991) has recently shown one such connection in the rat to be GABAergic. It has been proposed that these inhibitory projections have a general inhibitory rôle, decreasing the level of excitability of the spinal motor neurones (Holstege and Kuypers, 1982). This suggests that there could be close parallels between the descending inhibitory pathways of mammals and those of *Xenopus*. Perhaps the stopping response is broadly analogous to the calming effect of head massage experienced in humans.

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