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Metamodulation of a Spinal Locomotor Network by Nitric Oxide

D. L. McLean and K. T. Sillar

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Nitric Oxide Selectively Tunes Inhibitory Synapses to Modulate Vertebrate Locomotion

D. L. McLean and K. T. Sillar

J. Neurosci., May 15, 2002; 22 (10): 4175-4184.

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Development and Role of GABA_A Receptor-Mediated Synaptic Potentials During Swimming in Postembryonic *Xenopus laevis* Tadpoles

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Reith, Carolyn A. and Keith T. Sillar. Development and role of GABA_A receptor-mediated synaptic potentials during swimming in postembryonic *Xenopus laevis* tadpoles. *J. Neurophysiol.* 82: 3175–3187, 1999. We have investigated the contribution of GABA_A receptor activation to swimming in *Xenopus* tadpoles during the first day of postembryonic development. Around the time of hatching stage (37/8), bicuculline (10–50 μM) causes a decrease in swim episode duration and cycle period, suggesting that GABA_A receptor activation influences embryonic swimming. Twenty-four hours later, at stage 42, GABA_A receptor activation plays a more pronounced role in modulating larval swimming activity. Bicuculline causes short, intense swim episodes with increased burst durations and decreased cycle periods and rostrocaudal delays. Conversely, the allosteric agonist, 5β-pregnan-3α-ol-20-one (1–10 μM) or the uptake inhibitor, nipe-cotic acid (200 μM) cause slow swimming with reduced burst durations and increased cycle periods. These effects appear to be mainly the result of GABA release from the spinal terminals of midhindbrain reticulospinal neurons but may also involve spinal GABAergic neurons. Intracellular recordings were made using KCl electrodes to reverse the sign and enhance the amplitude of chloride-dependent inhibitory postsynaptic potentials (IPSPs). Recordings from larval motoneurons in the presence of strychnine (1–5 μM), to block glycinergic IPSPs, provided no evidence for any GABAergic component to midcycle inhibition. GABA potentials were observed during episodes, but they were not phase-locked to the swimming rhythm. Bicuculline (10–50 μM) abolished these sporadic potentials and caused an apparent decrease in the level of tonic depolarization during swimming activity and an increase in spike height. Finally, in most larval preparations, GABA potentials were observed at the termination of swimming. In combination with the other evidence, our data suggest that midhindbrain reticulospinal neurons become involved in an intrinsic pathway that can prematurely terminate swim episodes. Thus during the first day of larval development, endogenous activation of GABA_A receptors plays an increasingly important role in modulating locomotion, and GABAergic neurons become involved in an intrinsic descending pathway for terminating swim episodes.

INTRODUCTION

Although much is known about glycine's role in mediating reciprocal inhibition between antagonistic spinal motor pools in a range of vertebrates (e.g., Alford and Williams 1987; Buchanan 1982; Cohen and Harris-Warrick 1984; Dale 1985; Soffe 1987), the contribution of GABA to rhythm generation is less clear. Nevertheless, the widespread distribution of GABA throughout the brain and spinal cord suggests important func-

tional roles for GABAergic transmission during locomotor activity. In the lamprey, GABA has been implicated in modulating burst rate and intersegmental coordination, with GABA_B receptors affecting the magnitude and GABA_A receptors reducing the variability of the phase lag between segmental oscillators (Tégner et al. 1993). In the neonatal rat, both GABA_A and GABA_B receptor blockade increases swimming frequency, but only GABA_A receptor activation affects burst amplitude (Cazalets et al. 1994). More recent experiments on the neonatal rat preparation showed that either strychnine or bicuculline could abolish ipsilateral flexor/extensor alternation and disrupt left-right coordination, suggesting that the coactivation of GABA_A and glycine receptors may be important in coordinating activity between antagonistic motor pools (Cowley and Schmidt 1995).

During fictive swimming in immobilized *Xenopus* embryos (stage 37/8) (Nieuwkoop and Faber 1956), ventral root activity consists of simple biphasic impulses that alternate across the body and progress caudally with a brief intersegmental delay (for review, see Roberts 1990). The left/right alternation appears to be mediated solely by glycinergic spinal commissural interneurons (Dale 1985; Soffe 1987). There is no evidence that GABA_A receptor activation contributes significantly to rhythm generation at this early stage because fictive swimming is apparently unaffected by either bicuculline (40 μM) or curare (100 μM), two GABA_A receptor antagonists in this preparation (Soffe 1987). However, motoneurons do possess GABA_A receptors (Soffe 1987) and GABA-immunoreactive neurons are present in the embryonic brain and spinal cord (Roberts et al. 1987). So far, a role for only one population has been described. Gentle pressure applied to the rostral cement gland causes swimming to prematurely terminate, coincident with a train of bicuculline-sensitive potentials recorded intracellularly in embryonic motoneurons (Boothby and Roberts 1992a). Cement gland afferents are thought to activate GABAergic midhindbrain reticulospinal (mhr) neurons whose axons descend and terminate within the spinal cord (Boothby and Roberts 1992b). GABA_B receptor agonists have been shown to alter spike threshold and presynaptically modulate the strength of reciprocal glycinergic inhibition, but the circumstances under which these effects are brought into play during swimming are unclear (Wall and Dale 1993).

Twenty-four hours later (at stage 42) fictive swimming activity is more complex and flexible (Sillar et al. 1991, 1992). Given that GABA receptors play an important role during locomotor activity in other vertebrate preparations (Cazalets et

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al. 1994; Tégnier et al. 1993), it is conceivable that GABAergic neurons may become involved in the control of larval swimming. Thus in the present study we focused on the possible contribution made by GABA_A receptor activation to swimming at larval stage 42 compared with embryonic stage 37/8. Aspects of this work have been published previously in abstract form (Reith and Sillar 1995).

METHODS

Experiments were carried out on stage 37/8 embryos and stage 42 larvae of the South African clawed frog, *Xenopus laevis* (Nieuwkoop and Faber 1956), which were obtained by induced breeding following injection of human chorionic gonadotrophin (1,000 U/ml, Sigma) into pairs of adults from a laboratory colony. Tadpoles were immobilized in the neuromuscular blocking agent, α -bungarotoxin (1.25 μ M, Sigma) and then transferred to a preparation bath (~2 ml volume) containing frog Ringer of the following ion composition (in mM): 115 NaCl, 2.5 KCl, 2.5 NaHCO₃, 10 HEPES, 1 MgCl₂, and 2 or 4 CaCl₂ (2 mM for extracellular experiments and 4 mM for intracellular experiments); buffered to pH 7.4 with 1 N NaOH. The saline was continuously recirculated, being gravity fed from a stock bottle containing 100 ml of saline and returned by means of a peristaltic pump.

The animals were secured on their right side through the notocord, using fine pins etched from tungsten wire, to the silicone elastomer (Sylgard) surface of a rotating Perspex table located in the preparation bath. After removal of the flank skin, on the left side, from around the level of the anus to the otic capsule, extracellular recordings of ventral root activity were made by placing glass suction electrodes (~50 μ M tip opening) in the exposed intermyotomal clefts wherein lie the axons of spinal motoneurons. The position of each electrode was noted as its distance in clefts numbered sequentially from the otic capsule. Fictive swimming activity was initiated either by dimming the illumination or by applying brief (0.5–1 ms) current pulses (using a Digitimer DS2 isolated stimulator) to the tail skin via a glass suction electrode. In three experiments a second stimulating electrode was positioned on the cement gland of embryo preparations, and similar stimuli were used to terminate swimming (cf. Boothby and Roberts 1992a). For intracellular recordings, a rostral section of myotomes was removed, using tungsten needles, to reveal the underlying spinal cord. Recordings were then made using glass microelectrodes pulled on a Campden Instruments moving coil microelectrode puller (model 753) or a P2000 laser puller (Sutter Instruments) from filamented borosilicate glass capillary tubes (1 mm OD). The electrodes were filled with 2 M KCl and had resistances of 80–130 M Ω . KCl electrodes were chosen because the leakage of chloride ions into the interior of these small neurons causes chloride-dependent inhibitory postsynaptic potentials (IPSPs) to be massively reversed in sign so that they become large and depolarizing. This greatly facilitates the detection and analysis of GABAergic and glycinergic IPSPs. In addition, depolarizing GABA IPSPs can be readily distinguished from glycinergic ones on the basis of their duration and their sensitivity to selective agonists and antagonists (Reith and Sillar 1997). Penetrations were made in the ventral portion of the spinal cord using capacity overcompensation. The recorded cells were rhythmically active during fictive swimming and were assumed to be motoneurons because the ventral portion of the cord is known to consist almost entirely of cells of that type (Roberts and Clarke 1982). Electrophysiological data were recorded and stored onto video tape using a VR100B digital recorder (Instrutech, New York, NY) and analyzed off-line either manually or using the "Spike 2" analysis software package (CED, Cambridge, UK).

Pharmacological agents were bath applied to the perfusate by adding known quantities to the stock bottle to achieve the desired final concentration. The drugs used in this study were strychnine (1–5 μ M, Sigma), bicuculline (10–50 μ M, Sigma), and 5 β -pregnan-3 α -ol-20-one (5 β 3 α ; 1–5 μ M, Sigma). The duration of fictive swim episodes

was averaged over five consecutive episodes evoked at 5-min intervals recorded under each experimental condition from five similar experiments. For each experiment, measurements were made of rostral and caudal burst durations; cycle period, which was measured from the onset of one burst to the onset of the next and rostrocaudal delay, measured from the start of the rostral burst to the start of the caudal burst. The first 500 ms of activity in each episode was ignored to avoid possible influences arising directly from sensory stimulation. Averages were calculated, for each parameter, from a total of 60 measurements (20 measurements from each of 3 different episodes under each condition). In the case of spinalization experiments, where episodes were <20 cycles long, more episodes were measured to obtain a total of 60 cycles. Averages are given as means \pm SE.

RESULTS

Comparison of the effects of bicuculline on swimming at stage 37/8 and stage 42 activity

In *Xenopus* embryos (stage 37/8, Fig. 1A1) and larvae (stage 42, Fig. 1B1), episodes of sustained swimming activity lasting many seconds are initiated following a brief electrical stimulus to the tail skin. At each stage the coordination of swimming activity is similar, but larval swimming comprises much longer, more complex ventral root bursts than those seen in the embryo (Sillar et al. 1991). We compared the effects of bicuculline (10–50 μ M) on four parameters of fictive swimming (episode length, rostral and caudal burst duration, cycle period, and rostrocaudal delay) at these two developmental stages. At both stages, bicuculline caused a reversible reduction in the duration of swim episodes evoked in response to stimulation of the skin (Fig. 1, A2 and B2). Figure 1, A3 and B3, compares the effect of 50 μ M bicuculline on episode lengths in five consecutive episodes from five different embryo and five different larval experiments. Under control conditions the average episode length was similar at each stage: 40.1 \pm 7.1 s and 40.6 \pm 7.8 s (mean \pm SE) for the embryonic and larval preparations, respectively. In the presence of 50 μ M bicuculline, the reduction in episode duration was much larger in larval preparations than in embryos. In embryos, episodes decreased by ~50% to 21.7 \pm 6.2 s, whereas the larval episodes in the presence of bicuculline were reduced to only 3.4 \pm 0.8 s, ~8% of control durations. The effects of bicuculline were reversible because returning to control saline increased the length of episodes again to 49.8 \pm 13.6 s for embryo and 17.1 \pm 3.2 s for larval preparations. These data suggest that endogenous activation of GABA_A receptors has a marked influence on the duration of swim episodes, with the effect becoming much more pronounced by stage 42.

Bicuculline had only a relatively small effect on other parameters of embryonic swimming activity ($n = 5$). Excerpts of swimming activity before and after the addition of bicuculline are shown for one typical experiment in Fig. 2A, where the only obvious change is a small decrease in cycle periods (Fig. 2, A1 cf. A2). The data graphed in Fig. 2A3 show that cycle periods were indeed significantly ($P < 0.001$) reduced from 53.9 \pm 0.8 ms to 48.5 \pm 0.6 ms, 10 min after the bath application of 50 μ M bicuculline, whereas burst durations and rostrocaudal delays were not significantly affected (t -test, $P > 0.01$, not shown). Thus at stage 37/8, the frequency of fictive swimming was the only other parameter to be affected significantly after GABA_A receptor blockade in each of five experiments (t -test, $P < 0.01$). When the data from these five

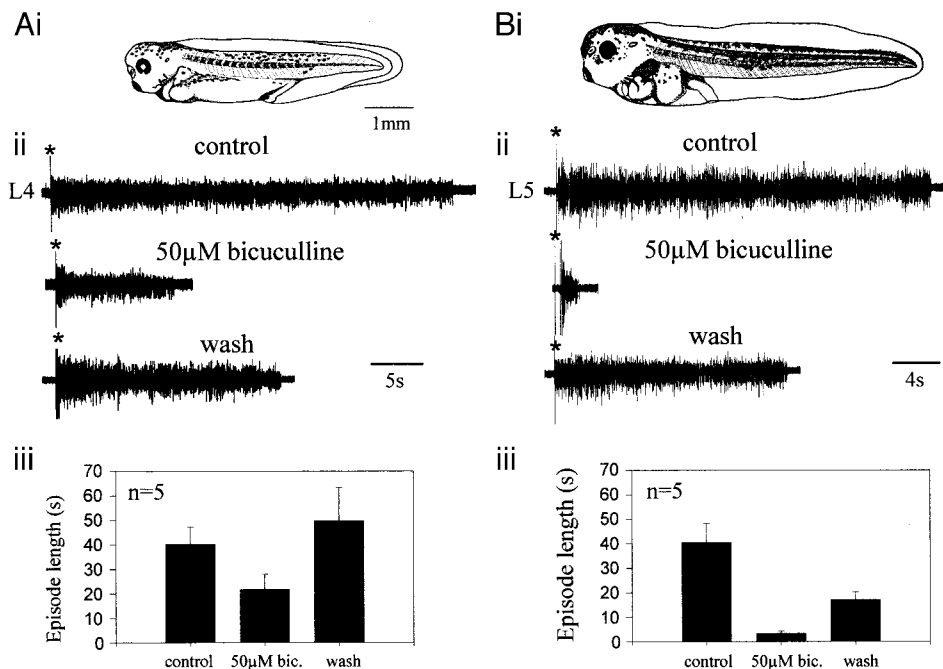


FIG. 1. Effects of bicuculline on swim episode length in embryos and larvae. *A1*: the late embryo, stage 37/8. *A2*: bicuculline caused a reversible decrease in the length of embryonic swim episodes. *A3*: graph of average episode length calculated from 25 embryonic episodes from 5 preparations at each stage in which episodes decreased from 40.1 ± 7.1 s under control to 21.7 ± 6.2 s in the presence of bicuculline and returned to 49.8 ± 13.6 s after returning to control saline. *B1*: the larva, stage 42. *B2*: bicuculline causes a more marked decrease in larval swim episodes that are partially reversed following wash in control saline. *B3*: 50 μ M bicuculline caused a decrease in the duration of larval episodes from 40.6 ± 7.8 s to 3.4 ± 0.8 s, which were increased again to 17.1 ± 3.2 s after washing off the antagonist.

experiments are pooled, embryo cycle periods decreased on average by 9.7% (range 7.0–12.6%). This change, accompanied with the decrease in swim episodes suggests that GABA_A receptor activation plays some role in controlling the output of the central pattern generator for embryonic swimming. More specifically, the fact that blockade of GABA_A receptor-mediated inhibition reduced the duration of swim episodes implies that the maintenance of rhythmic activity is somehow facilitated by endogenous GABAergic influences.

In contrast to the situation just described for embryos, by larval stage 42, the marked decrease in swim episodes by bicuculline (Fig. 1, *B2* and *B3*) is accompanied by significant changes in all parameters of the swimming motor pattern. The example in Fig. 2*B* clearly shows that bath application of 50 μ M bicuculline results in fast, intense activity. Like the embryo, cycle periods decreased, from 52.8 ± 0.6 ms to 45.6 ± 1.1 ms (Fig. 2*B3*). This decrease in cycle periods under bicuculline was significant in 10 of 11 preparations examined (*t*-tests; $P < 0.01$) where on average cycle period were reduced by 11.6% (range 5.5–27.8%). In addition, rostral burst durations increased by 15.6% from 14.7 ± 0.4 ms to 17.0 ± 0.5 ms (Fig. 2*C1*), whereas caudal burst durations also increased by >20%, from 12.8 ± 0.3 ms to 15.6 ± 0.4 ms (Fig. 2*C2*). In pooled data from 9 of 11 experiments where bicuculline significantly increased burst durations the average effect was 18% (range 6.3–54%). In addition, rostrocaudal delays decreased from 8.0 ± 0.2 ms to 4.9 ± 0.3 ms under bicuculline (Fig. 2*C3*). In contrast to the situation in embryos, rostrocaudal delays in larvae vary with cycle periods so that long cycle periods are accompanied by long delays and vice versa (Tunstall and Sillar 1993). Hence the decrease in delays under bicuculline could be produced simply as a consequence of the decrease in cycle periods. Figure 2*D* shows plots of rostrocaudal delays against cycle periods, over whole episodes before and during GABA_A receptor blockade. It can be seen that bicuculline causes short cycle periods that are associated with rostrocaudal delays shorter than those measured under control

conditions. However, in the range of cycle periods that overlap in control conditions and under bicuculline (40–80 ms), delays under bicuculline declined as a proportion of cycle period from $13.8 \pm 0.4\%$ ($n = 104$ cycles) to $10.7 \pm 0.6\%$ ($n = 47$ cycles). Thus GABA may affect intersegmental coordination in ways other than purely through changing cycle periods.

The preceding results suggest that the endogenous activation of GABA_A receptors has a profound influence on larval swimming activity by directly affecting the excitability of the central pattern generator. If this is the case and the results are not due to some nonspecific effect of bicuculline, then agents that potentiate GABA_A receptor activation would be expected to have the opposite effect to bicuculline on larval activity. We therefore used the neurosteroid, 5 β -pregnan-3 α -ol-20-one (5 β 3 α), a potent allosteric modulator of the GABA_A receptor. We have previously shown that 5 β 3 α is a specific agonist at the GABA_A receptor in *Xenopus* tadpoles, which enhances responses to endogenous GABA release in this preparation (Reith and Sillar 1997). 5 β 3 α (1–10 μ M) had consistent and significant effects on larval ventral root activity ($n = 5$), which are illustrated in Fig. 3. 5 β 3 α (5 μ M) caused a decrease in episode length as shown in the example in Fig. 3*A*, where episode length was reduced from 48.0 ± 15.0 s (*A1*) to 16.1 ± 6.4 s (*A2*). Subsequent application of 20 μ M bicuculline further reduced episodes to an average of 4.3 ± 1.4 s (*A3*). Thus like the experiments described earlier for bicuculline alone (i.e., Fig. 1), bicuculline in the presence of steroid still caused episodes to decrease in duration rather than reversing the effect of the steroid (see DISCUSSION). In contrast to the antagonist, however, 5 β 3 α caused the swimming pattern to become slow and weak (Fig. 3, *B2* cf. *B1*) an effect that was reversed by the subsequent bath application of bicuculline (not shown). The steroid significantly ($P < 0.001$) decreased the average rostral burst duration from 15.0 ± 0.4 ms to 8.3 ± 0.3 ms (Fig. 3*C1*) and the average caudal burst duration from 11.4 ± 0.3 ms to 8.6 ± 0.2 ms (Fig. 3*C2*). The frequency of swimming was also significantly reduced, with average cycle periods increasing

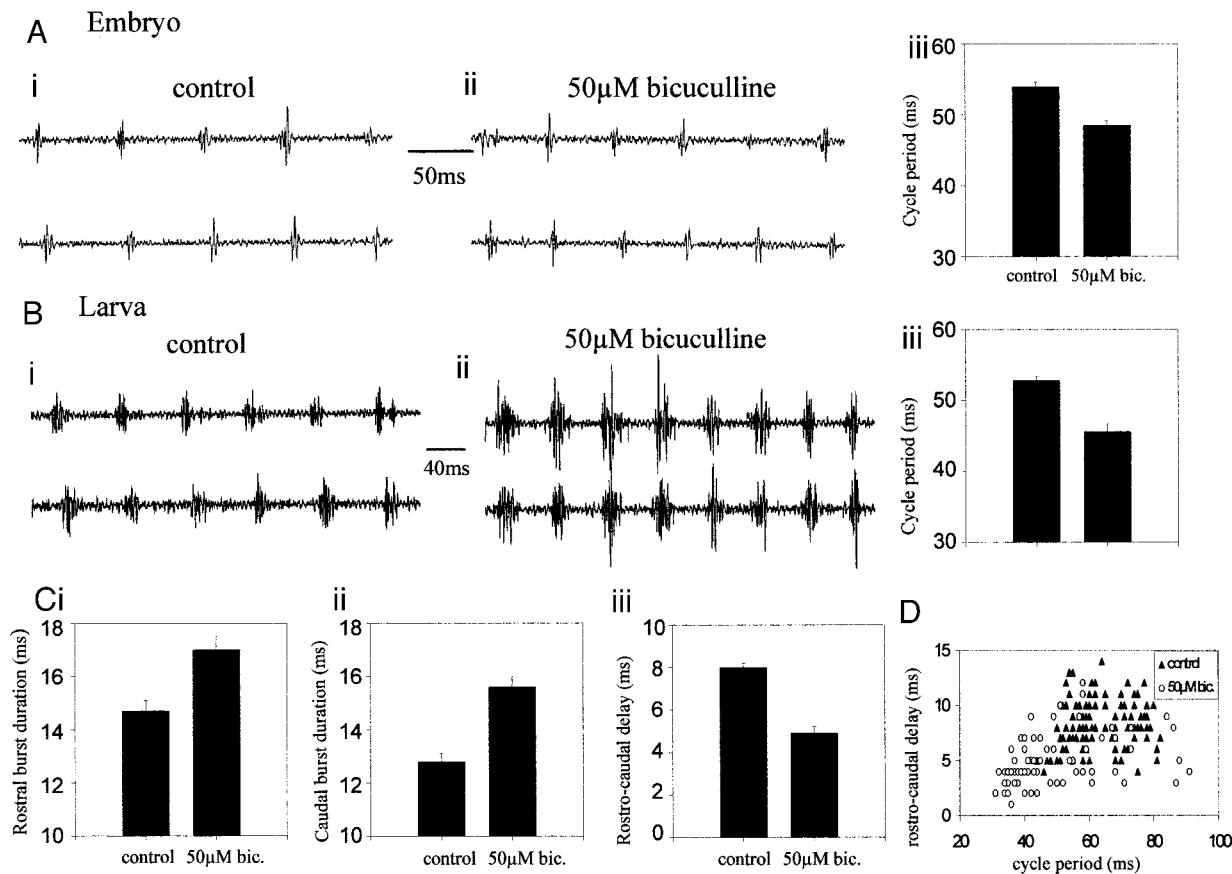


FIG. 2. Effects of bicuculline on embryonic and larval ventral root activity. *A*: excerpts of ventral root activity before (1) and after (2) the addition of 50 μM bicuculline. The activity appears little affected by the antagonist apart from a slight decrease in cycle period. *A3*: graphed data averaged from 20 measurements from 3 different episodes of activity under each condition confirms that cycle period decreased significantly from 53.9 ± 6.5 ms in control to 48.5 ± 5.4 ms in the presence of 50 μM bicuculline ($P < 0.01$). *B1* and *B2*: by stage 42, 50 μM bicuculline obviously increases the intensity of ventral root activity. *B3*: cycle period was again decreased from 52.8 ± 0.6 ms in control to 45.6 ± 1.1 ms in 50 μM bicuculline. *C*: in addition, rostral burst duration increased from 14.7 ± 0.4 ms to 17.0 ± 0.5 ms (*C1*), caudal burst duration also increased from 12.8 ± 0.3 ms to 15.6 ± 0.4 ms (*C2*), and rostrocaudal delay decreased from 8.0 ± 0.2 ms to 4.9 ± 0.3 ms (*C3*). All of these changes occurring as a result of the antagonist were highly significant (*t*-test, $P < 0.01$). *D*: plot of rostrocaudal delay against cycle period before (\blacktriangle) and after (\circ) the bath application of 50 μM bicuculline, indicating that rostrocaudal delay is decreased, partly as a result of the decrease in cycle period and partly via a direct action.

from 49.6 ± 0.7 ms to 67.8 ± 0.7 ms (Fig. 3C3). This was accompanied by an increase in the magnitude of the rostrocaudal delays from 10.4 ± 0.3 ms to 20.0 ± 0.2 ms (Fig. 3C4). Plots of delay against cycle period showed that, although shorter cycle periods with shorter delays were only seen under control conditions, at similar cycle periods, the steroid still increased delays to a greater extent than those seen under control (Fig. 3D). Again, as suggested by bicuculline experiments described in Fig. 2, delays appear to be directly affected by GABA neurotransmission. Similar experiments were also carried out with the GABA uptake inhibitor, nipecotic acid (200 μM). Figure 3, *E1* and *E2*, shows that nipecotic acid (200 μM) produces a slow, weak swimming rhythm, similar to that recorded in the presence of steroid ($n = 5$). Moreover, in the majority of experiments (3 of 5), nipecotic acid, like $5\beta\text{3}\alpha$, reduced episode lengths (not illustrated).

Because the actions of bicuculline, nipecotic acid, and $5\beta\text{3}\alpha$ all rely on an endogenous source of GABA to exert their effects, the main conclusion from these initial ventral root experiments is that by stage 42, an intrinsic GABAergic system is capable of significantly influencing the output of the central

pattern generator for swimming. The main effect of GABA_A receptor activation is to reduce the frequency, intensity, and duration of swimming, although, paradoxically, the block of GABA_A receptors also produces short swim episodes (see DISCUSSION).

Effects of bicuculline and $5\beta\text{3}\alpha$ in the presence of strychnine

To determine more precisely the role of synaptic inhibition mediated by GABA_A receptor activation alone during rhythmic swimming activity, the next set of experiments was carried out after first blocking glycinergic inhibition with strychnine. Blocking both types of inhibition (with 1–5 μM strychnine and 20–50 μM bicuculline) revealed another developmental difference between embryonic and larval preparations. Figure 4 shows an example of the effects of simultaneously blocking glycine and GABA_A receptors at each stage. Following bath application of 5 μM strychnine to the embryo, episode length was largely unaffected, although there was a slight increase in swimming frequency (Fig. 4, *A2* cf. *A1* and *A5*). Subsequent bath application of 50 μM bicuculline in the presence of

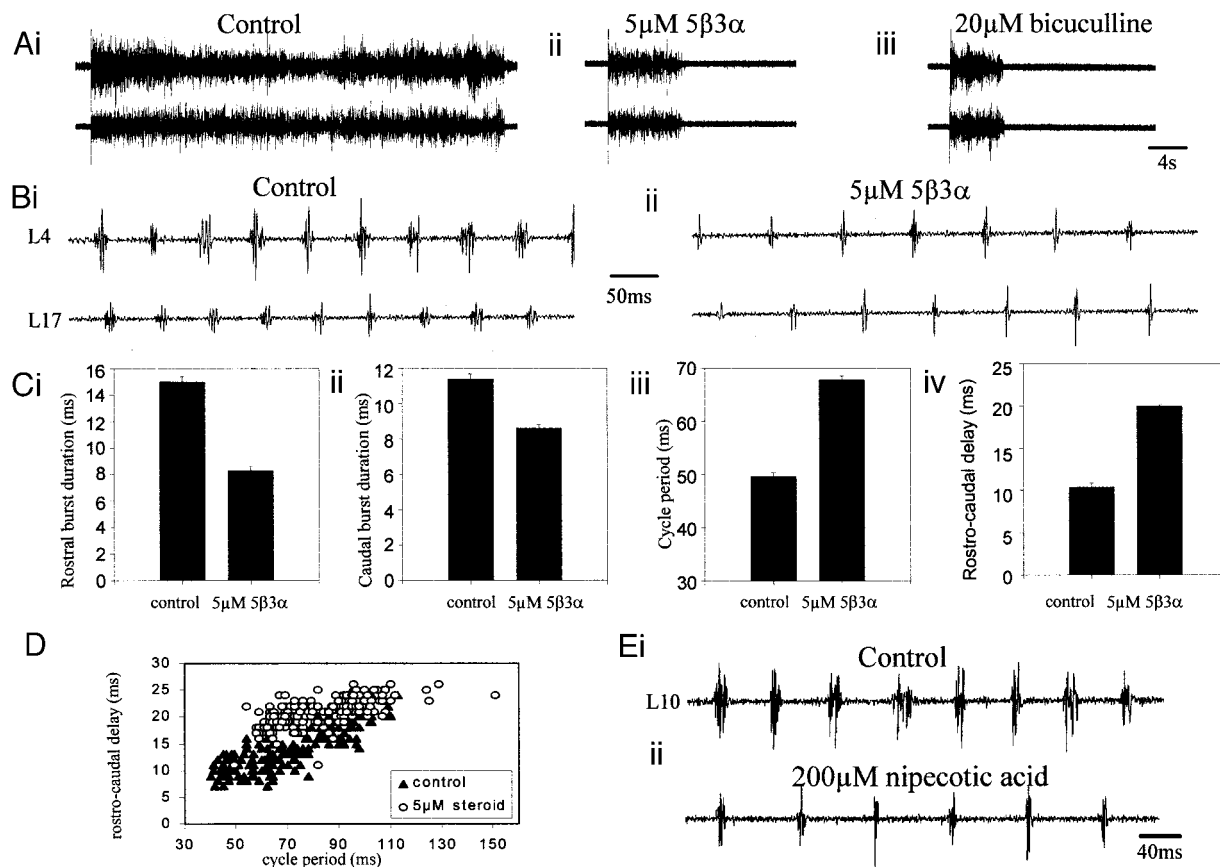


FIG. 3. Effects of 5 β 3 α on larval ventral root activity. *A1–A3*: bath application of 5 μ M 5 β 3 α markedly reduced episode length, which then remained short in the presence of 20 μ M bicuculline. *B1* and *B2*: bath application of 5 μ M 5 β 3 α caused the swimming pattern to become slow and weak. *C1*: rostral burst duration decreased from 15.0 ± 0.4 ms to 8.3 ± 0.3 ms. *C2*: caudal burst duration decreased from an average of 11.4 ± 0.3 ms to 8.6 ± 0.2 ms. *C3*: cycle period increased from 49.6 ± 0.7 ms to 67.8 ± 0.7 ms. *C4*: rostrocaudal delay also increased from 10.4 ± 0.3 ms to 20.0 ± 0.2 ms. (All of these changes were significant, $P < 0.01$). *D*: plot of rostrocaudal delay against cycle period showing that part of the effect of the steroid on delay is mediated through the increase in cycle period but that there is also a direct effect. *E1* and *E2*: the GABA uptake inhibitor, nipecotic acid (200 μ M) also causes a slow activity that is also comprised of decreased ventral root bursts. Note, data illustrated in *A*, *B–D*, and *E* are from 3 different preparations.

strychnine elicited an intense nonrhythmic burst before the onset of each swim episode (Fig. 4*A3*). This suggests that GABA_A receptors are normally activated in response to skin stimulation. The sustained swimming activity that followed the initial nonrhythmic burst showed a further increase in frequency (Fig. 4, *A3* and *A5*). The effects of strychnine are not so easily reversible as those of bicuculline, but returning to control saline did abolish the burst of activity at the beginning of the episode and reduce the swim frequency (Fig. 4, *A4* and *A5*).

By stage 42, a very different picture emerges as shown in Fig. 4*B*. Following the bath application of either strychnine alone (1–5 μ M, Fig. 4*B2*) or bicuculline (20–50 μ M, e.g., Fig. 2*B2*), rhythmic ventral root activity is still recorded. Like bicuculline, strychnine causes an increase in burst durations and a decrease in cycle periods (Fig. 4, *B2* cf. *B1* and *B5*) but with an increase rather than a decrease in episode length (Fig. 4*B1* cf. Fig. 1*B2*). However, following the coapplication of bicuculline and strychnine (*B3*) essentially all rhythmic activity is abolished, and only an intense nonrhythmic burst is normally recorded on the ventral roots. In a few cases this nonrhythmic burst terminated with a few cycles of rhythm (e.g., arrowed in Fig. 4*B3*). Although the activity immediately after electrical stimulation of the skin is nonrhythmic, a rostrocaudal delay,

like that seen during swimming, was still observed (not shown), indicating that the motor system is not engaging in fictive struggling; this alternative motor pattern is characterized by a marked increase in burst durations but is accompanied by a reversal of the intersegmental delay to caudorostral (Soffe 1991). Rhythmic activity returned following wash in control saline (4, *B4* and *B5*). Thus by stage 42, a certain amount of inhibition seems to be required for the central pattern generator to produce rhythmic ventral root activity.

Enhancing GABA inhibition, in the presence of strychnine, with 5 β 3 α , resulted in similar effects to those already reported for the steroid alone (see above). Figure 5 shows an example of the consistent effects of the steroid in the absence of glycinergic inhibition ($n = 5$). Application of 5 β 3 α in the presence of strychnine weakens swimming (Figs. 5, *A3* cf. *A2* and *A1*). Although 2 μ M strychnine increased rostral burst durations from 13.8 ± 0.3 ms to 20.7 ± 0.6 ms, 5 μ M 5 β 3 α in the presence of strychnine decreased average burst durations to 12.1 ± 0.4 ms (Fig. 5*B1*). Similarly, caudal burst durations increased from 10.1 ± 0.3 ms to 15.2 ± 0.5 ms under strychnine and decreased to 8.3 ± 0.2 ms after the bath application of 5 β 3 α (Fig. 5*B2*). This decrease in the intensity of bursts by the steroid was paralleled by a slowing of the rhythm because

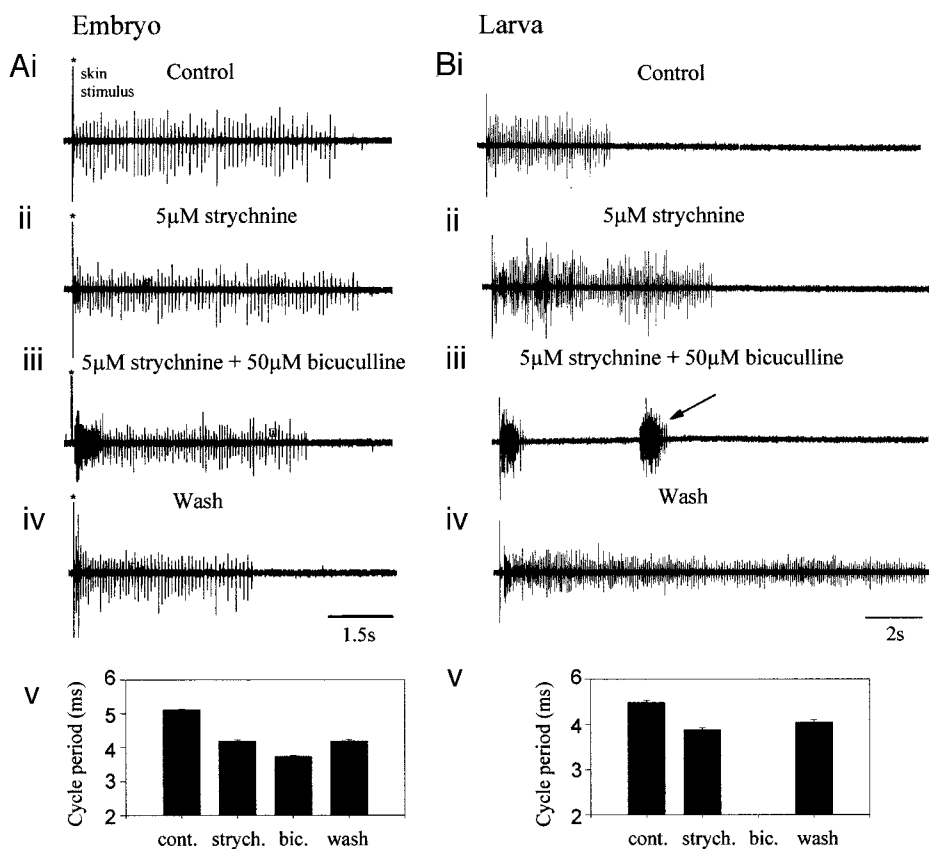


FIG. 4. Effect of blocking inhibition on embryonic and larval rhythm generation. *A1*: episode of control embryonic ventral root activity. *A2*: 5 μM strychnine has little effect on the episode length but causes a slight increase in swim frequency. *A3*: subsequent addition of 50 μM bicuculline causes a burst of intense activity at the beginning of the episode, thought to be due to the actions of bicuculline on sensory pathways, after which swimming activity is sustained with a further increase in frequency. *A4*: returning to control saline abolished the sensory effect of bicuculline, and the frequency of the ventral root activity is at least partially decreased (see text). *A5*: measurements of cycle periods confirmed that they decreased from 51.1 ± 0.2 ms in control, to 41.9 ± 0.4 ms in the presence of 5 μM strychnine, and to 37.4 ± 0.3 ms in the presence of strychnine and 50 μM bicuculline. Cycle periods were at least partly reversed in wash saline to 41.9 ± 0.5 ms (see text). *B1*: control larval swim episode following a skin stimulus. *B2*: ventral root activity is sustained at a higher frequency in the presence of 5 μM strychnine. *B3*: coapplication of 50 μM bicuculline generally abolishes rhythmic ventral root activity both in response to a skin stimulus and during spontaneous activity. On some occasions, however, cycles of swimming activity were recorded following the end of the burst (at arrow). *B4*: shortly after returning to control saline, rhythmic ventral root activity resumes. *B5*: histogram showing that cycle period decreased from 44.8 ± 0.5 ms in control to 38.8 ± 0.4 ms in 5 μM strychnine. Coapplication of bicuculline abolished swimming, which then resumed with a cycle period of 40.4 ± 0.6 ms after washing into control saline.

cycle periods increased from 42.0 ± 0.6 ms in strychnine to 53.2 ± 0.5 ms in the presence of both agents (Fig. 5*B3*). Rostrocaudal delays similarly increased from 14.7 ± 0.3 ms to 18.6 ± 0.3 ms (Fig. 5*B4*). These effects of the steroid in the presence of strychnine are consistent with there being a prominent GABA_A receptor-mediated input during fictive swimming, which helps to maintain swimming activity even in the absence of glycinergic inhibition. The next topic to be investigated addresses the location of the GABAergic neurons that influence swimming.

Possible role for the midhindbrain reticulospinal neurons during larval swimming activity

Of the eight populations of neurons that show GABA immunoreactivity at stage 37/8 (Roberts et al. 1987), two populations are located in the spinal cord, three populations in the hindbrain, and the remaining three populations are located in the mid- and forebrain. The most caudal of the hindbrain neurons are the mhr neurons that lie ~ 200 μm caudal to the otic capsule. The vestibular complex neurons are located at the level of the otic capsule, and the third, rostral hindbrain population lie just rostral to the entry of the trigeminal nerve. Immunocytochemical studies at larval stage 42 indicate that the same populations of GABAergic neurons are present, and no new populations have developed compared with the situation at stage 37/8 (Reith 1996). Transecting the brain at the level of the otic capsule ("transected" preparation, Fig. 6*A*) should therefore leave only the mhr neurons and two populations of spinal GABA neurons intact. The essential components of the rhythm generator for swimming are located in the spinal cord,

so at this level of transection, larval preparations can still sustain episodes of swimming activity. Figure 6*B* shows activity from a transected preparation before and after the addition of bicuculline. Bicuculline (50 μM) caused the usual reversible decrease in episode length, which in the example in Fig. 6*B* decreased from an average of 21.9 ± 3.0 s in control to 1.0 ± 0.01 s in the presence of bicuculline and then increased again to 8.3 ± 0.5 s after returning to control saline. The histograms in Fig. 6*C* also confirm that the antagonist still causes the typical increase in burst durations (Fig. 6, *C1* and *C2*) and decrease in cycle periods and rostrocaudal delays (Fig. 6, *C3* and *C4*). These data suggest that the population of neurons that plays a role in modulating larval swimming is located below the level of the otic capsule, and therefore either the mhr or the spinal populations (or both) are involved.

Spinalizing preparations at the level of the fifth postotic myotome ("spinalized" preparation, Fig. 6*A*) removes the influence of all but the two populations of spinal GABAergic neurons. At this level of spinalization, however, the preparation can only produce very short bouts of swimming presumably because a proportion of the descending excitatory interneuron pool has also been removed (cf. Roberts and Alford 1986). Hence, in these experiments, low doses (10–20 μM) of *N*-methyl-D-aspartate (NMDA), insufficient to trigger swimming on their own, were applied to raise the levels of excitation and thus increase episode durations following skin stimulation. Bicuculline was found to have only small and inconsistent effects on burst durations and rostrocaudal delay that were often not reversible. As in the intact and transected preparations, however, there was a tendency for the length of episodes

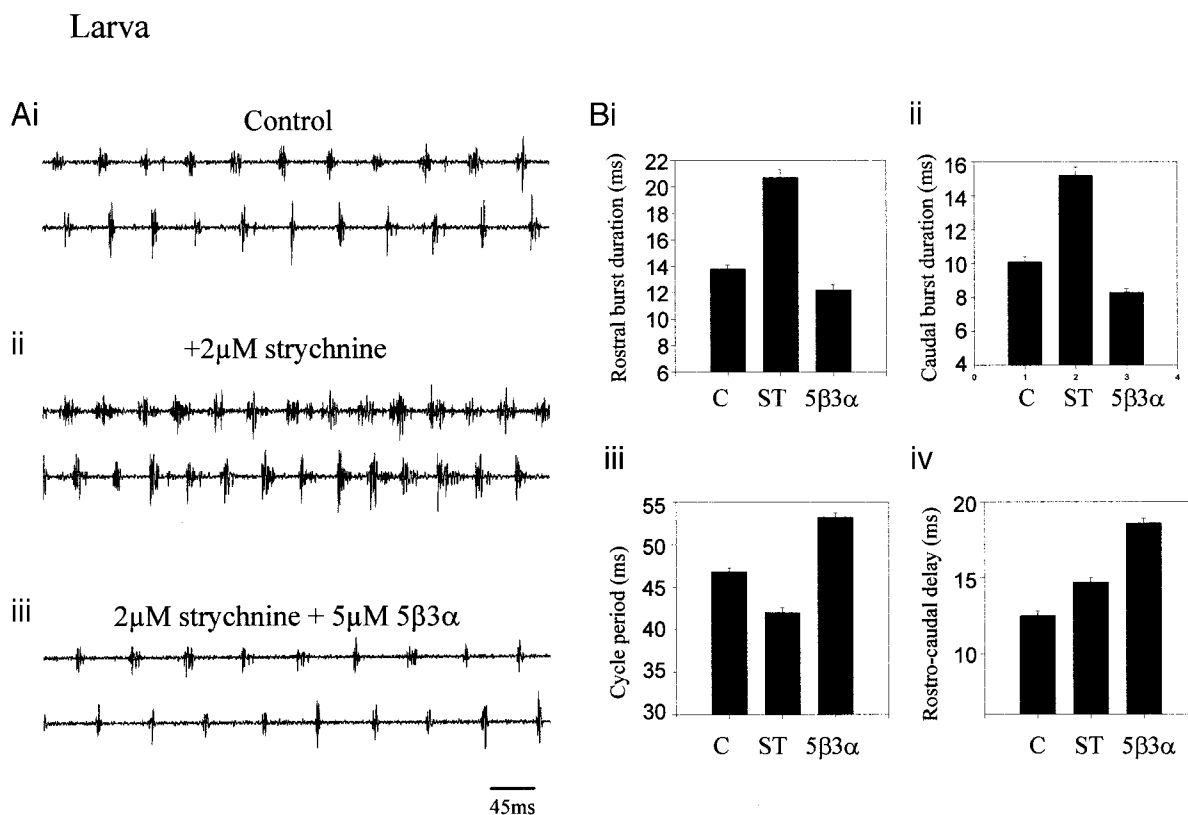


FIG. 5. Effects of 5 β 3 α in the presence of strychnine. *A*: the intensity and frequency of larval ventral root activity (*I*) is enhanced by the bath application of 2 μ M strychnine (2) and then decreased again after the bath application of 5 μ M 5 β 3 α (3). *B*: graphed data of the significant changes occurring as a result of the bath application of strychnine and 5 β 3 α . *B1*: rostral burst duration averaged 13.8 \pm 0.3 ms in control, 20.7 \pm 0.6 ms in the presence of strychnine, and 8.3 \pm 0.2 ms in the presence of strychnine and steroid. *B2*: similarly caudal burst duration changed from 10.1 \pm 0.3 ms in control, to 15.2 \pm 0.5 ms in strychnine, and to 8.3 \pm 0.2 ms in the presence of 5 β 3 α . *B3*: cycle period was decreased from 46.8 \pm 0.4 ms to 42.0 \pm 0.6 ms by strychnine and increased to 53.2 \pm 0.5 ms by 5 β 3 α . *B4*: rostrocaudal delay increased in strychnine from 12.5 \pm 0.3 ms to 14.7 \pm 0.3 ms and then further increased by 5 β 3 α to 18.6 \pm 0.3 ms.

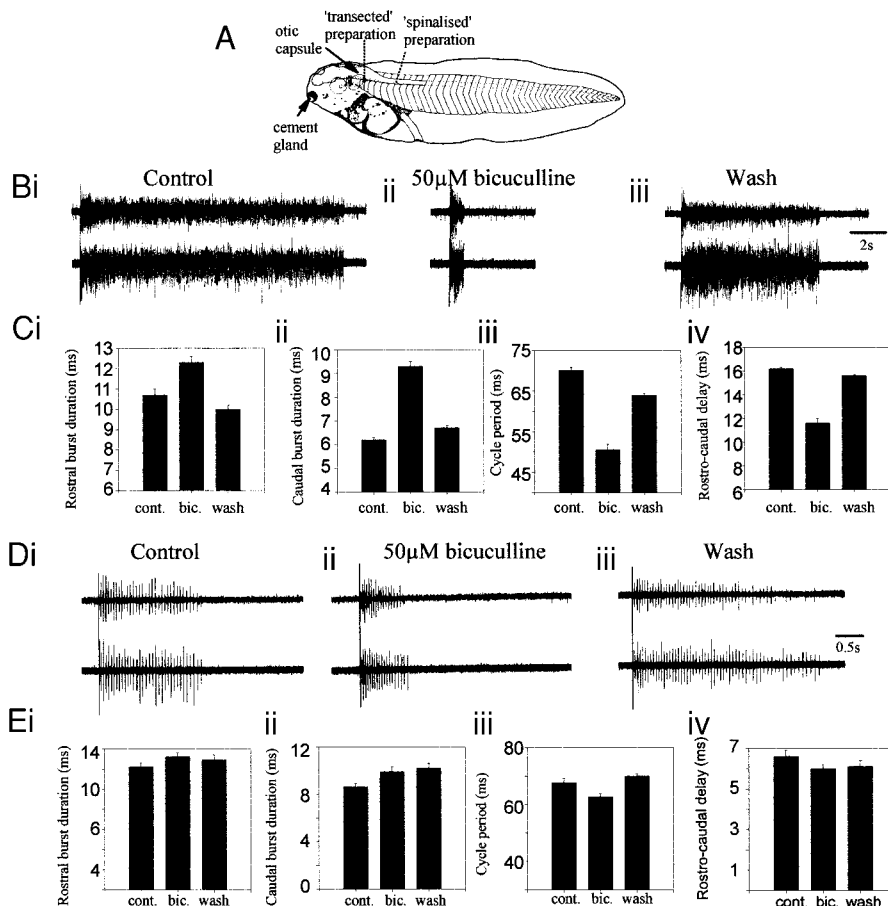
to be reversibly reduced by bicuculline (5 of 7 experiments). An example of the effect on episode length is shown in Fig. 6*D*, where bicuculline was washed on to the preparation twice (only the 1st application is shown) and on each occasion caused a decrease in episode length. The average episode length in control was 2.2 \pm 0.1 s, reduced to 1.1 \pm 0.05 s in 50 μ M bicuculline and then increased to 3.7 \pm 0.7 s after returning to control saline. Reapplying the bicuculline reduced episode lengths again to 1.3 \pm 0.07 s (not illustrated). The other most consistent change in the ventral root activity of the spinalized animal was a small but significant decrease in cycle period (5 of 7 experiments), which decreased from 67.8 \pm 1.4 ms in control to 62.7 \pm 1.2 ms in 50 μ M bicuculline and increased again to 70.0 \pm 0.9 ms after washing off the bicuculline (Fig. 6*E3*). However, although the decrease in cycle period was significant, it was not as marked as the effect of bicuculline on the swimming frequency of intact or otic capsule transected preparations (Figs. 2*B* and 6*C3*). The other parameters of swimming were not consistently or significantly changed by bicuculline in the spinalized preparation (Fig. 6, *E1*, *E2*, and *E4*). These results suggest that there is only a small contribution to swimming from the spinal GABA populations, although if these neurons were normally excited by descending systems then this will be removed following spinalization. It is notable that episode length and cycle period are the same two

parameters that are affected by bicuculline at stage 37/8, suggesting that embryo swimming might be influenced by spinal GABAergic neurons.

The main conclusion, therefore from these spinalization experiments is that by stage 42, an important GABA input to the spinal swimming circuitry comes from a population of neurons located in the caudal hindbrain, most probably the mhr neurons described by Roberts et al. (1987).

GABAergic input during rhythm generation

Thus far we have described a developmental increase in the contribution of GABAergic transmission to swimming during early postembryonic development. The next question we addressed was when during swimming are GABA_A receptors activated? Intracellular recordings from ventrally positioned spinal neurons, presumed motoneurons, were made to determine the timing of GABAergic IPSPs. To facilitate this goal, glycinergic transmission was first blocked with strychnine, and 2 M KCl was used as the microelectrode electrolyte because this has the effect of reversing and enhancing chloride dependent potentials, including GABA-mediated IPSPs (Reith and Sillar 1997). Figure 7 shows an episode of swimming recorded from a larval motoneuron (*top traces*, MN) with an accompanying ventral root monitor (*bottom traces*, vr) before strychnine



nine application (A1). The expanded trace (A2) shows more clearly the alternating pattern of on-cycle excitation, leading to action potentials, in time with the ipsilateral ventral root, and depolarizing (reversed) midcycle inhibition, occurring when neurons on the opposite side of the cord are active. The phase of the midcycle glycinergic inhibition was measured as a marker to determine whether or not a midcycle component remained after the bath application of strychnine. Measurements were made of the time from the peak of the on-cycle spike to the start of the midcycle IPSP divided by the time to the peak of the subsequent on-cycle spike (i.e., y/x , Fig. 7A2). The onset of the IPSPs was chosen because this will closely reflect the timing of the action potentials in contralateral rhythmic neurons. The plot of midcycle IPSP phase values over a range of cycle periods (Fig. 7A2) shows little variation in the onset of the strychnine-sensitive IPSPs that occurred at phase values of ~ 0.5 over a whole episode of rhythmic activity. One feature of swimming that was present in control but becomes more apparent in the presence of strychnine (Fig. 7B1) is that, although midcycle inhibition is largely abolished, sporadic depolarizing potentials occur in some cycles of activity. When these potentials occur, they are relatively long in duration and cause a decrease in input resistance, as evidenced by the decrease in spike height (asterisks in Fig. 7B1). These potentials were considered to be GABAergic because of 1) their long duration compared with glycinergic IPSPs (Reith and Sillar 1997), 2) their duration was enhanced by $5\beta 3\alpha$, and 3) they were blocked by bicuculline (see Fig. 8). Episodes of swim-

ming, recorded in the presence of strychnine, were analyzed to determine whether these GABA potentials occurred at any particular phase of the swim cycle. The expanded traces in Fig. 7B2 show excerpts of activity from four parts of an episode in which there are 1) no apparent underlying GABA potentials, 2) GABA potentials that appear to fall midcycle and GABA potentials occurring in either 3) the falling or 4) the rising phase of the on-cycle excitation. This suggests that the GABA potentials are variable in their timing within a cycle of activity, and indeed the phase plot, measured from six episodes under strychnine, confirms that GABA potentials can occur at any point during a cycle (Fig. 7B3). Note, however, that GABAergic IPSPs occurring on-cycle, during the excitatory phase of swimming, would be excluded from this type of analysis (see DISCUSSION). It seems likely that the mhr neurons are responsible for generating these GABAergic IPSPs, although the possibility that they are produced by spinal neurons normally activated by descending excitatory pathways cannot be completely discounted.

Termination of swimming by GABAergic inhibition

The idea that GABAergic mhr neurons become involved in the descending control of larval fictive swimming is supported by the fact that episodes of larval swimming can terminate with a barrage of depolarizing IPSPs in motoneurons. These IPSPs were observed in at least some and occasionally in all episodes recorded in two-thirds of larval preparations (24 of 36; e.g.,

FIG. 6. Effects of bicuculline on the ventral root activity of "transected" and spinalized larvae. A: to determine the location of the population of GABAergic neurons that exert their effects on the swimming pattern generator, the CNS of larval preparations was cut at 2 different levels. Transection at the level of the otic capsule ("transected" preparation) removes all but the midhindbrain reticulospinal and 2 spinal populations of GABAergic neurons. Spinalization at the level of the 5th postotic myotome ("spinalized" preparation) ensures that only the spinal populations of GABAergic neurons can still influence swimming activity. B1–B3: bicuculline reversibly decreases the length of swim episodes of transected preparations. C1: under control conditions, average rostral burst duration was 10.7 ± 0.3 ms; it was increased by 50μ M bicuculline to 12.3 ± 0.3 ms and then decreased to 10.0 ± 0.2 ms after returning to control saline. C2: caudal burst duration was similarly increased from 6.2 ± 0.1 ms to 9.3 ± 0.2 ms by 50μ M bicuculline and then decreased to 6.7 ± 0.1 ms after washing off the bicuculline. C3: cycle period averaged 70.1 ± 0.7 ms, 50.6 ± 1.3 ms, and 63.9 ± 0.5 ms under control, 50μ M bicuculline and wash, respectively. C4: average rostrocaudal delay in control saline was 16.2 ± 0.1 ms; it then decreased to 11.6 ± 0.4 ms in the presence of 50μ M bicuculline and was increased again to 15.6 ± 0.1 ms in wash saline. All of these changes were significant (t -test, $P < 0.01$). D1–D3: in the majority of spinalized larval preparations, in the presence of 10 – 20μ M NMDA (see text), 50μ M bicuculline caused a reversible decrease in episode length. E1, E2, and E4: neither burst durations or rostrocaudal delays were significantly changed in the presence of 50μ M bicuculline (t -test, $P > 0.01$). E3: cycle period decreased significantly from 67.8 ± 1.4 ms to 62.7 ± 1.2 ms by 50μ M bicuculline and then increased to 70.0 ± 0.9 ms after washing off the antagonist (t -test, $P < 0.01$).

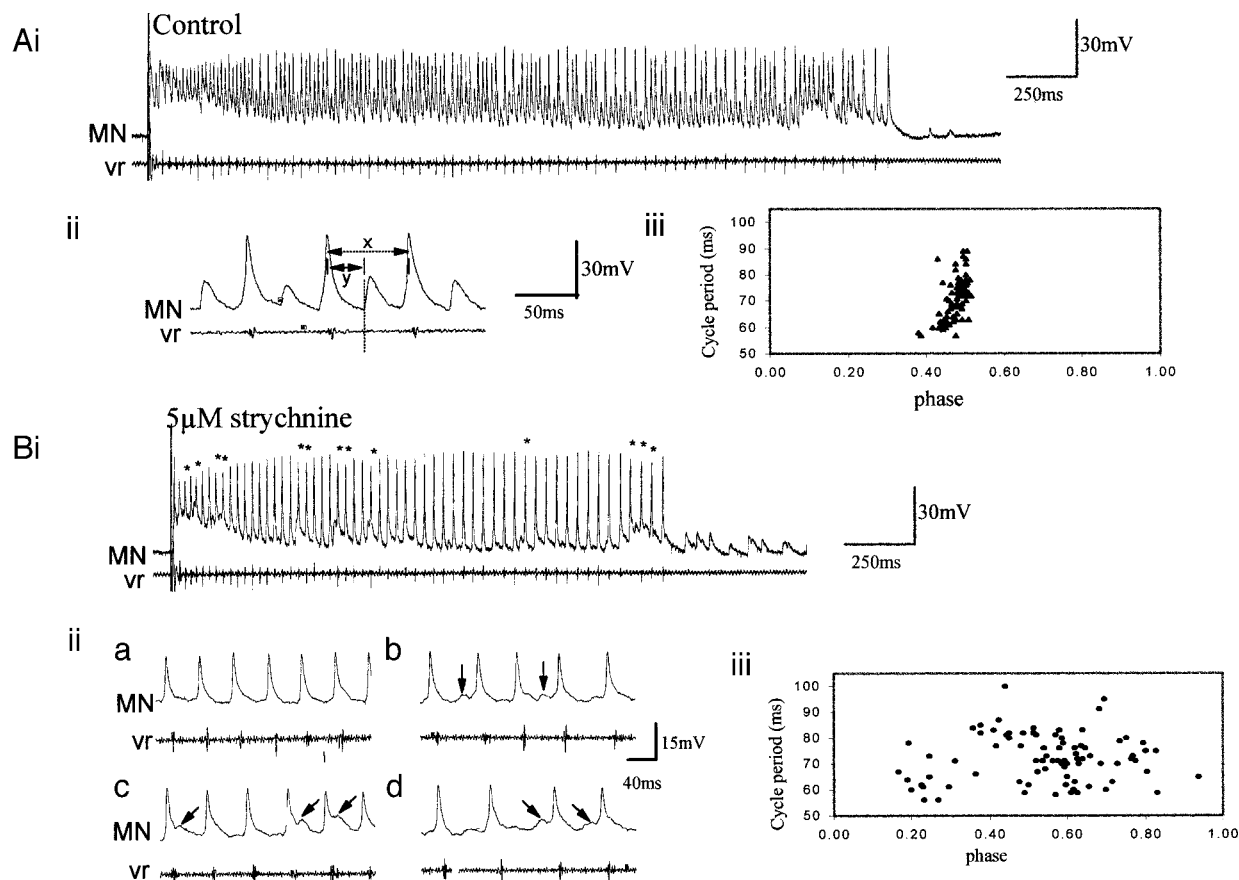


FIG. 7. GABA potentials underlie larval rhythm generation. *A1*: control activity recorded from a larval motoneuron with a KCl-filled microelectrode. *A2*: expanded trace showing the typical pattern of on-cycle excitation, leading to action potentials, followed by midcycle (reversed) inhibition while neurons on the opposite side of the cord are active. The phase of the midcycle inhibition was measured as the onset of the inhibitory postsynaptic potential (IPSP) in relation to the time between the peak of the on-cycle spike (i.e., y/x). *A3*: plot of cycle period against phase of midcycle inhibition showing that there is little variation in the onset of the midcycle inhibition measured over a whole episode. *B1*: in the presence of $1 \mu\text{M}$ strychnine, midcycle inhibition is abolished, but underlying GABA potentials (see text) are observed at several points in the episode (at asterisk). *B2*: excerpts of rhythmic activity during which *a*) there was no obvious midcycle component, *b*) GABA potentials appeared to occur midcycle, *c*) GABA IPSPs occurred on the falling phase of the on-cycle excitation, and *d*) GABA IPSPs occurred on the rising phase of the on-cycle excitation. *B3*: the plot of cycle period against phase shows clearly the random occurrence of GABA IPSPs throughout 6 episodes of larval swimming under strychnine.

Fig. 8, *B1* and *B2*). This phenomenon has never been documented in recordings from embryonic motoneurons (e.g., Reith and Sillar 1997; Roberts et al. 1984; Sillar and Roberts 1993) and was not observed in 14 preparations examined in the present study either where embryonic swimming episodes end with the membrane potential smoothly declining to its resting level (Fig. 8*A1*). Interestingly, however, the self-termination of larval swimming episodes (Fig. 8, *B1* and *B2*) closely resembles the termination of embryonic swimming following cement gland stimulation (Fig. 8*A2*) (see Boothby and Roberts 1992a; Reith and Sillar 1997), in that trains of depolarizing IPSPs are evident. We sought confirmation that the potentials coinciding with the end of the majority of larval swim episodes result from GABA_A receptor-mediated inhibition. First, they are long compared with glycinergic IPSPs (Reith and Sillar 1997), and second, they are still observed at the end of episodes recorded in the presence of $5 \mu\text{M}$ strychnine (Fig. 8*C1*, $n = 18$). Third, $10 \mu\text{M}$ $5\beta 3\alpha$ massively enhanced the duration of the potentials at the termination of swimming to the point that they summated, greatly increasing the time for the membrane potential to return to control levels (Fig. 8*C2*, $n = 4$). Fourth, bicuculline

completely abolished these IPSPs (Fig. 8*C3*, $n = 12$). These observations further support the idea that GABA_A receptor-mediated potentials in spinal neurons are used to terminate larval swim episodes.

DISCUSSION

The results presented in this paper show that between stage 37/8 and 42, the endogenous synaptic activation of GABA_A receptors plays an increasingly important role in controlling rhythmic swimming activity in *Xenopus* tadpoles. Over the first 24 h of larval development, the effect of GABA_A receptor blockade changes from affecting only swim duration and frequency to modulating all swimming parameters.

Paradoxically, both removing and enhancing GABAergic inhibition decreases the duration of swim episodes, indicating that GABA transmission also plays a role in maintaining rhythm generation. Presumably, any reduction in inhibition will lead to higher levels of excitability in the network that might prevent or prematurely terminate activity. This could occur if, for example, the increased excitability triggered active

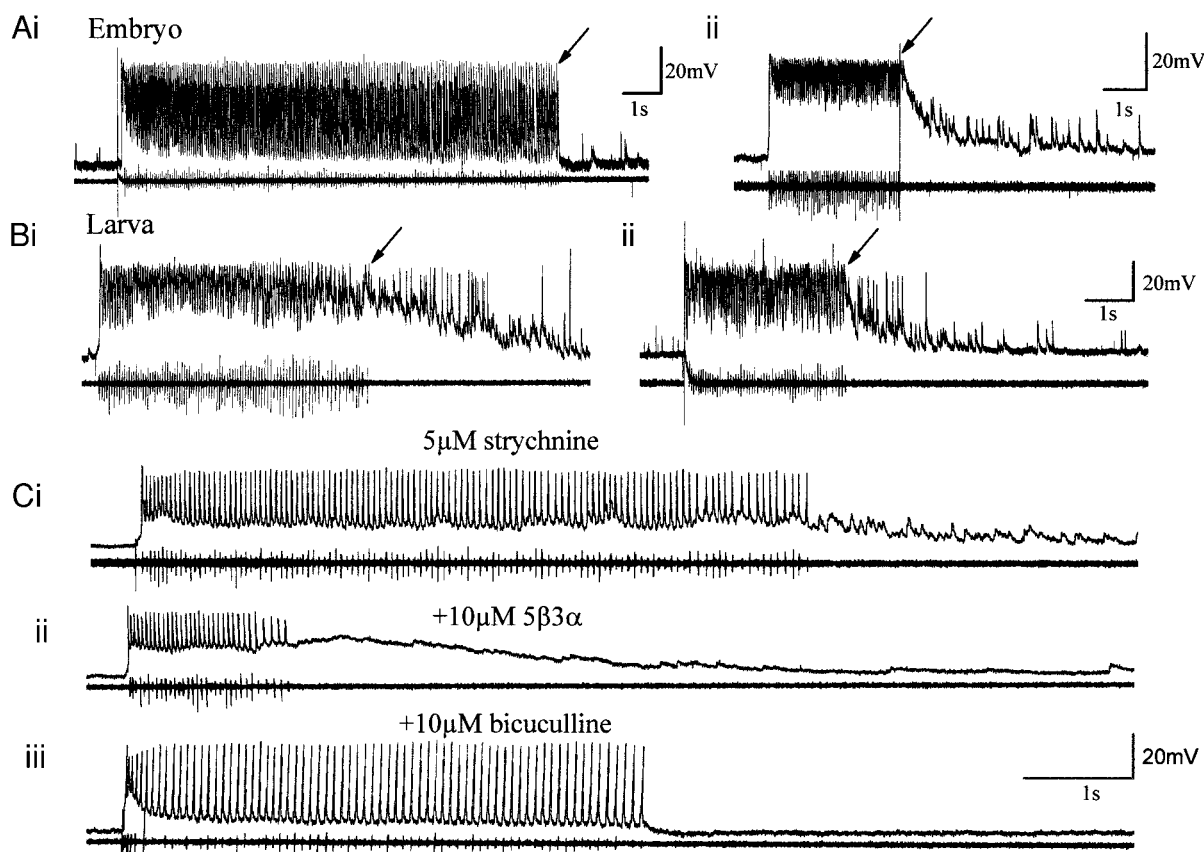


FIG. 8. Larval episodes terminate with GABA potentials. *A1*: intracellular recording from an embryo motoneuron showing rhythmic activity terminating with no obvious synaptic input. *A2*: following electrical stimulation of the cement gland, episodes are terminated prematurely with a train of potentials (see Reith and Sillar 1997). *B1* and *B2*: at stage 42, the majority of recordings from motoneurons revealed that swim episodes could end with a barrage of IPSPs in the absence of any obvious sensory input. *C1*: the potentials at the termination of larval episodes were not blocked by $5 \mu\text{M}$ strychnine. *C2*: $10 \mu\text{M}$ $5\beta 3\alpha$ enhanced both the apparent tonic depolarization during rhythmic activity and the IPSPs occurring at the termination of swimming, causing them to summate. *C3*: bicuculline decreased the level of tonic inhibition and abolished the potentials at the termination of the episode.

membrane properties in spinal neurons that then lead to strong hyperpolarizing conductances that suppressed network activity. Oscillatory membrane properties are present in spinal cord neurons of *Xenopus* larvae following activation of NMDA receptors, and they are enhanced following block of inhibitory amino acid receptors (Reith et al. 1998; Scrymgeour-Wedderburn et al. 1997). These bistable membrane properties are common in larval neurons but rare in embryonic ones, providing a plausible explanation for differences in the effects of coapplying strychnine and bicuculline in larvae where rhythm generation is abolished and in embryos where rhythm generation persists. Alternatively, it could be argued that bicuculline at high concentrations may be having some direct nonspecific effect, and it has been reported that at higher concentrations bicuculline blocks apamin-sensitive calcium-dependent potassium channels in adult mammalian thalamic reticular neurons (Debarbieux et al. 1998). Because *Xenopus* neurons do possess a calcium-dependent potassium current, albeit with exceptionally slow kinetics and which does not produce a clear slow afterhyperpolarization (Wall and Dale 1995), it is possible that the action of bicuculline at $50 \mu\text{M}$ could be nonspecific (Wall and Dale 1995). However, a shortening of episodes was seen at concentrations as low as $10\text{--}20 \mu\text{M}$, which would not be expected to act nonspecifically. The maximum shortening of episode lengths was above $20 \mu\text{M}$, but the precise concentra-

tion could vary between preparations. The marginally higher concentrations used here compared with previous studies (e.g., Soffe 1987) were probably necessary because for our extracellular experiments the spinal cord was not exposed and drug access problems could become relevant. In addition, using apamin on embryo preparations resulted in an increase in the length of swim episodes (Wall and Dale 1995), and therefore any nonspecific effect on the calcium dependent potassium current by bicuculline would be expected to have the opposite effect to that observed in this present study.

How do the results of the present study compare with those obtained in other vertebrate locomotor systems? For the most part such comparisons are made difficult by the fact that rhythm generation in other systems, such as the lamprey and the neonatal rat, is usually induced by activating the network with endogenous excitants such as NMDA. Nevertheless, during fictive swimming evoked by tail skin stimulation in the lamprey, simultaneous block of GABA_A and glycine receptors causes an increase in burst durations at the beginning of an episode to the point where there is no clear interburst interval, as in *Xenopus*. However, in contrast to *Xenopus* larvae, rhythmic activity still persists after the initial burst and an increase in episode length is observed (Alford et al. 1990). The location of the GABAergic systems that impinge on the locomotor network may also explain interspecies differences. The results

of our spinalization and brain stem transection experiments suggest that the main GABA input to *Xenopus* swimming lies between the level of the otic capsule and the fifth postotic cleft, therefore implicating a role for mhr neurons (Boothby and Roberts 1992b; Roberts et al. 1987). In contrast, for both the lamprey (Alford et al. 1990) and the neonatal rat (Cazalets et al. 1994), GABAergic neurons that regulate locomotor activity must be spinal in origin because significant effects of bicuculline are observed in the isolated spinal cord preparation. In the lamprey, immunocytochemical studies indicate that the GABAergic input to swimming derives from a population of multipolar neurons with local arborizations extending only to nearby spinal segments (Brodin et al. 1990). In the neonatal rat preparation it has been established that there are GABAergic projections from the lower brain stem to the spinal cord, which could modulate swimming (Holstege et al. 1991), but electrophysiological evidence supports the view that spinal GABAergic neurons alone can profoundly affect locomotor activity (Cazalets et al. 1994). In this study, although the effects of bicuculline on spinal larval preparations were inconsistent, there was a tendency for episodes to decrease and cycle periods to increase. It is notable that the same two parameters are affected by bicuculline during swimming activity in intact embryonic preparations. Perhaps spinal GABA neurons exert a small influence on episode durations and cycle periods at both stages of development, whereas the mhr neurons only influence swimming at larval stages.

The present study has focused on the role of GABA_A receptors in the control of swimming, but if GABA neurons are active during fictive locomotion as we suggest, then it is likely that GABA_B receptors are also activated and play a role. Indeed, studies on other vertebrates have suggested GABA transmission influences locomotor activity via coactivation of GABA_A and GABA_B receptors, which modulate different aspects of the locomotor rhythm (Cazalets et al. 1994; Tégner et al. 1993). The role of GABA_B receptor activation on *Xenopus* embryonic swimming rhythm has been investigated previously (Wall and Dale 1993). The GABA_B agonist, baclofen, causes a decrease in the reliability of action potentials and in the reliability of midcycle inhibition, both of which contribute to the observed decrease in ventral root amplitude and episode length. However, blockade of these receptors had little effect on swimming, making it unclear whether endogenous GABA_B receptors are normally activated during embryonic swimming (Wall and Dale 1993). Similarly in *Xenopus* larvae, preliminary experiments using the GABA_B antagonist 2-hydroxysaclofen failed to detect any obvious effects on the swimming rhythm (unpublished observations).

When are GABAergic neurons active during swimming, and how does GABA_A receptor activation modulate locomotor rhythm generation? In the lamprey it has been suggested that GABA_A receptor activation is important in the repolarization of spinal neurons at the end of the burst in each cycle (Alford et al. 1990), whereas in the neonatal rat, there is evidence that both GABA_A and glycine receptor activation may be important in mediating reciprocal left-right and flexor-extensor coordination (Cowley and Schmidt 1995). In *Xenopus* larvae, GABA_A receptor activation does not appear to contribute to the mid-cycle inhibition, which was completely abolished by strychnine. This finding largely rules out the possibilities that GABAergic neurons are rhythmically active in time with the

swimming rhythm or that glycinergic commissural interneurons co-release GABA, as has been shown recently in rat spinal cord neurons (Jonas et al. 1998). Nevertheless, bicuculline and neurosteroid-sensitive GABA IPSPs occurred sporadically throughout episodes of rhythmic activity. Although these IPSPs were not locked to any particular phase of the swimming rhythm, their frequency of occurrence tended to be higher nearer the starts and ends of swim episodes. Moreover, the GABA IPSPs are relatively long in duration (~200 ms) when compared with the cycle periods attained during fictive swimming (~50–100 ms) so a single IPSP could contribute inhibition lasting more than a single cycle. As a result, successive IPSPs could summate to provide a tonic, low-level, depolarizing inhibition that lasts throughout each episode. In support of this idea, it is notable in Fig. 8C that the neurosteroid enhances the level of tonic depolarization recorded under strychnine, whereas bicuculline reduces it to below control (strychnine) levels.

Our results allow us to propose an important role for descending GABAergic inhibition in the intrinsic termination of larval swimming. In *Xenopus* embryos, activation of the descending GABAergic mhr pathway does not appear to be deployed in terminating swimming unless the cement gland is stimulated. Instead, episodes of embryonic swimming display a slow decline in frequency and then terminate spontaneously. Two mechanisms for the intrinsic termination of embryonic swimming have recently been proposed. First, swimming may terminate due to an increase in the activation of a K_{Ca} current resulting from a buildup of intracellular calcium during an episode. This in turn would decrease the input resistance and membrane time constant of neurons in the locomotor network thereby increasing their firing threshold during an episode to the point where they stop firing and swimming terminates (Wall and Dale 1995). Second, the changing balance between levels of extracellular ATP and adenosine may underlie the rundown of the motor pattern. Early in an episode, levels of extracellular ATP (presumably released from an unknown member of the swimming network), rise, but adenosine levels (formed by the breakdown of ATP by ectonucleotidases) are low. ATP causes a reduction in voltage-gated K⁺ currents, reduces spike threshold, and increases the overall excitability of the network. As the episode proceeds, rising levels of adenosine block voltage-gated Ca²⁺ currents to decrease excitability in the network eventually reaching a level at which swimming can no longer be sustained (Dale and Gilday 1996). From a behavioral viewpoint, both of these mechanisms for terminating embryonic swimming, which are intrinsic properties of the spinal rhythm generating network, could be considered as a last resort. They will ensure that in the absence of any extrinsic signal (cement gland stimulation, for example) swimming will eventually cease, but they are inappropriate for terminating swimming mid-episode. It has been suggested that the termination of swimming in embryos could result from a buildup in the potency of inhibitory transmission (Wall and Dale 1995), although there is currently no evidence for such a mechanism. However, our evidence suggests larval swimming can be terminated by a transient increase in GABA release from mhr neurons. In two-thirds of preparations, the ends of episodes of swimming activity coincide with a barrage of GABA IPSPs, similar to those observed at the termination of embryonic swim episodes following stimulation of the cement gland (Fig. 9A2) (Reith and Sillar 1997). The mhr neurons in

embryos are known to be rhythmically inhibited in time with the swimming pattern (Boothby and Roberts 1992a). Cement gland stimulation causes sufficient excitation of the mhr neurons that they overcome the inhibition, fire a burst of action potentials, and so trigger the end of an episode of swimming. However, the reliability of the cement gland stopping pathway steadily declines from stage 37/8 onward and completely disappears by stage 45 (Boothby and Roberts 1992b). By this time the cement gland itself has completely degenerated. Thus the mhr neurons are in the process of being disconnected from their excitatory afferent input from the cement gland. This situation raises the important question of the fate of the mhr neurons during larval development. Numerous hypothetical possibilities relate to the general problem of what happens to a set of neurons when their function at one stage of development disappears at later stages. First, the neurons could degenerate. Second, the neurons could be retained for a different function. Third, the neurons could be deployed in the same role but become activated via new or different routes. For the mhr neurons we propose that the third possibility applies. Immunocytochemical studies indicate that the mhr neurons are present at stage 42 in similar numbers to stage 37/8 (Reith 1996), and the electrophysiological experiments of the present study suggest that they continue to function in the same role, namely in the termination of locomotor activity. The important distinction between the function of the mhr neurons at the two stages is that early in development they are activated exclusively by afferents of the cement gland, whereas at later stages they are activated by alternative central pathways that have yet to be discovered. This developmental transition in the function of the descending GABA pathway provides the tadpole with a method of terminating swimming "at will." Because embryos spend much of their time hanging motionless from their cement glands while larval animals become free swimming, the change in the function of the mhr neurons may be more appropriate to the larval lifestyle.

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