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Mechanisms and significance of reduced activity and responsiveness in resting frog tadpoles

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Summary

Hatchling *Xenopus laevis* tadpoles spend most of their time attached to objects or the water surface by mucus secreted by a gland on the head. While attached, swimming activity and responsiveness to swim-initiating stimuli are reduced over long periods of time. We have investigated the mechanisms and significance of this apparent long-term inhibition. In behavioural experiments we show, firstly, that innervation of the cement gland and $GABA_A$-mediated inhibition are necessary for attachment to reduce responsiveness, and secondly, that denervation of the cement gland increases tadpole activity and increases their predation by damselfly nymphs (Zygoptera). To investigate the neuronal pathway from the cement gland to $GABA_A$ inhibition, we have devised an immobilized, inverted tadpole preparation where a weight attached to the mucus simulates the force as it hangs. Simulated attachment reduces responsiveness and spontaneous fictive swimming activity. We have recorded the activity and responses of trigeminal neurons innervating the cement gland. They are spontaneously active and simulating attachment results in a sustained increase in this activity. We propose that hanging from a mucus strand increases firing in cement gland afferents. This leads to tonic GABA inhibition that reduces tadpole activity and responses, and leads to fewer attacks by predators.

Key words: GABA, *Xenopus*, tadpole, trigeminal, immobility, cement gland, tonic inhibition.

Introduction

In certain behavioural states like sleep, levels of motor activity and responsiveness are reduced. Such states may make animals less conspicuous or function to conserve energy. They can occur with a circadian rhythm, e.g. honey bee (Kaiser and Steiner-Kaiser, 1983), cockroach (Tobler and Neuner-Jehle, 1992), larval zebrafish (Zhdanova et al., 2001) and mammals (Glenn and Dement, 1981). Alternatively, they depend on external stimulation, for example when sucker attachment inhibits swimming in the leech (Gray et al., 1938), or foot contact inhibits flight in insects (Pringle, 1974; Krämer and Markl, 1978). The most widely documented state of reduced responsiveness is induced by restraint or postural inversion. Given many names, including animal hypnosis, tonic immobility and thanatosis (Gallup, 1974), this state is found in many different species, e.g. crayfish (Krasne and Wine, 1975), stick insect (Bässler, 1983), cricket (Nishino and Sakai, 1996), locust (Faisal and Matheson, 2001), toad (Gargaglioni et al., 2001) and guinea pig (Monassi et al., 1997). Knowledge of the neuronal basis of such states of reduced motor activity and responsiveness is limited. Could some of them depend on tonic inhibition within the nervous system?

To investigate the neuronal basis of a state-dependent reduction in responsiveness we chose the hatchling tadpole of *Xenopus laevis*. These young animals spend 99% of their time at rest, hanging from a mucus strand secreted by a cement gland on the front of the head. While attached in this way to objects in the water or the surface meniscus, tadpoles are less responsive to trunk skin touch (Boothby and Roberts, 1992a) and dimming that excites the pineal eye (Jamieson and Roberts, 2000). Finally, spontaneous swimming does not occur during attachment, but is seen in unattached tadpoles (Jamieson and Roberts, 2000). The simplicity of both the behaviour and the nervous system of the hatchling *Xenopus* tadpole has been the impetus for its use in the study of the neuronal control of locomotion (for a review, see Roberts et al., 1997). In immobilised tadpoles fictive swimming can be recorded from the motor nerves with no requirement for anaesthesia. The sensory stimuli, which start and stop swimming in the behaving tadpole, are also effective in the fictive preparation, and the neuronal pathways through which they act have been partially characterized (for a review, see Roberts, 1997). Touch-sensitive neurons innervate the tail, trunk and head skin and their activation can initiate swimming (Clarke et al., 1984; Roberts and Sillar, 1990; Roberts, 1980). Swimming can also be initiated when dimming excites pineal ganglion cells (Jamieson and Roberts, 1999). Significantly for our study of reduced responsiveness, pressure on the head skin or cement gland can stop swimming. These tissues are

innervated by trigeminal sensory neurons, which fire in response to pressure and excite GABAergic midhindbrain reticulospinal neurons (MHRs). These, in turn, produce $GABA_A$-mediated IPSPs (inhibitory postsynaptic potentials) in rhythmic spinal neurons and cause swimming activity to stop (Roberts, 1980; Boothby and Roberts, 1992a,b; Perrins et al., 2002; Li et al., 2003). Could these same pathways be responsible for long-term reductions in responsiveness?

Our aim was to use behavioural experiments to examine the reduction in responsiveness to skin touch and to dimming, when freely behaving tadpoles hang attached by a strand of mucus. We tested whether the sensory innervation of the cement gland and GABAergic inhibition are necessary for attachment to reduce responsiveness and spontaneous activity. Since reduced responsiveness and activity during attachment may make tadpoles less obvious to predators, we looked at the effect of cement gland denervation on the predation of tadpoles by one of their most important natural predators, Odonate nymphs. To investigate the neuronal basis of reduced activity and responses, we simulated cement gland attachment in an immobilised preparation where recordings of afferent activity from the cement gland could be made over extended periods. Our aim was to define the sensory input needed to induce and maintain the state of reduced responsiveness during attachment.

Preliminary accounts of this work have been presented (Lambert and Roberts, 2000a,b).

Materials and methods

All experiments were carried out on stage 37/38 *Xenopus laevis* Daudin tadpoles (Nieuwkoop and Faber, 1956), at room temperature, 20±2°C. For dissection, tadpoles were anaesthetized in 0.1% MS-222 (3-amino-benzoic acid ethyl ester; Sigma, St Louis, MO, USA) in saline, of the following composition: NaCl, 115 mmol l^{-1}; KCl, 3 mmol l^{-1}; $CaCl_2$, 2 mmol l^{-1}; $NaHCO_3$, 2.4 mmol l^{-1}; $MgCl_2$, 1 mmol l^{-1}; Hepes, 10 mmol l^{-1}; adjusted to pH 7.4 with 5 mol l^{-1} NaOH. Usually tadpoles were then pinned onto a rotatable Sylgard® block in a small bath. Swimming was initiated by stroking the tadpole's skin using a hand-held mounted mouse whisker (15 μm tip diameter). Dissection was carried out using mounted finely etched tungsten microneedles and fine forceps.

Behaviour

Experiments on responsiveness were carried out in a darkroom in circular glass dishes 7 cm in diameter and filled to a depth of 3 cm with dechlorinated tapwater or saline. A piece of 0.3 mm diameter wire fixed to the side of the dish was submerged below the surface of the water. Using a wire entangled in their cement gland mucus, tadpoles could be placed on the bottom of the dish (unattached) or attached to the wire *via* the mucus (attached; Fig. 1A). Unless stated otherwise, responses to stimuli were tested 1 min after tadpoles were placed in position.

To test the effect of attachment on responsiveness to touch stimuli, individual tadpoles were stroked across the base of the tail or on the head (Fig. 1B) while viewing through a binocular microscope. Each tadpole was tested only once.

To test the effect of attachment on responsiveness to dimming, two halogen lights (Philips PAR20, 50 W) 50 cm from the dish were used. One was set to full brightness and the other dimmed to minimal brightness. Four tadpoles at a time were placed in the dish in either the unattached or attached position. To stimulate, the bright light was switched off so the light dimmed from ~4800 to ~180 Lux. The light was switched on again after 5 s. Responses were considered to be initiated by the dimming if they occurred within those 5 s. Each tadpole was tested only once.

Mandibular nerve lesioning

Under anaesthetic, skin on the side of the head overlying the distal end of the mandibular nerve was opened, enabling the nerve to be seen. The nerve was then severed using two tungsten needles (Fig. 1C). The tadpole was re-pinned on its other side and the process repeated. As a control, a sham operation involved making a lesion of tissue just caudal to the nerve. The tadpole was allowed to recover for 25 min in saline prior to tests on behaviour (in saline). Testing for responsiveness began after 5 min adaptation to the light conditions (one halogen light at 50 cm, ~4600 Lux). For each tadpole, unattached and attached tests alternated and a total of five tests were done in each state.

For experiments testing spontaneous activity and predation, the anaesthetized tadpole was placed in a groove in the Sylgard base of a small dish and secured with a V-shaped pin around the neck region. A 1 mm wide, chisel-like blade made from a piece of razorblade was pressed down through the tadpole in a single motion until it cut into the Sylgard layer below. To cut the mandibular nerve innervating the cement gland, the blade was positioned parallel to the proximal surface of the cement gland. To perform a control cut, the blade was positioned level with the caudal edge of the eye and the most dorsal edge of the cement gland and in a dorso-ventral direction. After surgery tadpoles were placed in saline for five minutes and then into 50:50 saline:aerated tapwater for 2 h to allow the wound to heal.

Predation

Damselfly (Zygoptera) nymphs, 1–1.5 cm (measured from the head to the base of the lamellae), were collected from a drainage canal near Bristol and identified (Brooks, 1997) as mostly *Ischnura elegans* and *Coenagrion puella*, but with some *Platycnemus pennipes* and *Coenagrion pulchellum*. Nymphs were kept under a 12 h:12 h light:dark cycle, fed on a diet of *Daphnia*, starved for 3 days prior to trials and used only once. For testing, a nymph was transferred to a white circular plastic dish (6.5 cm diameter) of aerated tapwater in natural daylight. The nymph was given 30 min to acclimatise before a tadpole was introduced to the dish. A video recorder (Sony, Tokyo, Japan) placed 50 cm above recorded the activity in six dishes simultaneously.

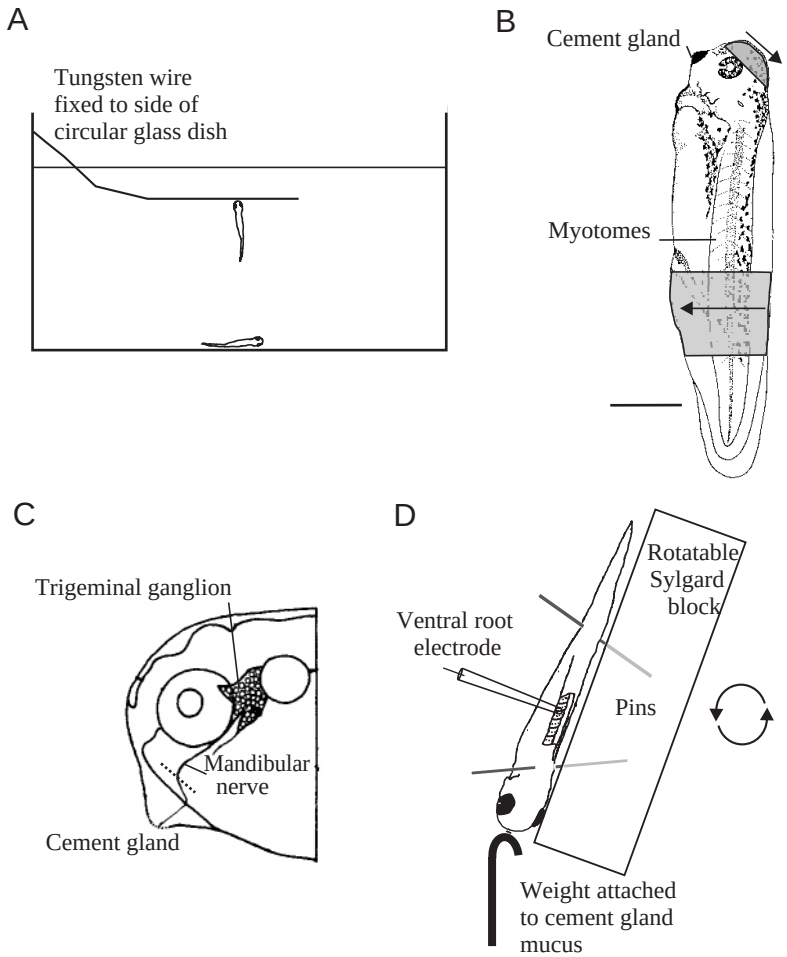

Fig. 1. Diagrams of experimental setups. (A) Cross section of the dish used in behavioural experiments. Tadpoles could be unattached on the bottom of the dish, or attached by their cement gland mucus to a piece of wire, which was fixed to the side of the dish. (B) Scale diagram of the *Xenopus* tadpole to show the location of strokes to the head and tail used in behavioural and electrophysiological experiments (shaded areas indicate region and arrows indicate direction). Scale bar, 1 mm. (C) Diagram of the head of the tadpole showing the mandibular nerve of the trigeminal ganglion innervating the cement gland. The site of the lesion is shown by a dotted line. (Modified from Roberts and Blight, 1975.) (D) Simulating cement gland attachment. The immobilised tadpole was pinned to a Sylgard block that was rotated so that the tadpole pointed downwards. A weight attached to the cement gland mucus was able to hang freely. An extracellular suction electrode on an intermyotomal cleft monitored fictive swimming activity from a motor nerve. Arrows show the direction of rotation of the block.

Behavioural pharmacology

In order to test the effect of drugs, the skin overlying the dorsal fin and the myotomes on the right-side was removed from the level of the 1st to the 9th post-otic myotome (see Fig. 1B). This dissection was also carried out on control animals. Following dissection, animals were given 10 min to recover in saline and a further 5 min in the experimental dish and lighting conditions. Drugs were dissolved in the saline in the dish. Responsiveness to dimming or touch was tested as above.

Drugs used were bicuculline methochloride, CGP-35348 ({3-aminopropyl}{diethoxymethyl}phosphinic acid), SR-95531 (6-imino-3-{4-methoxyphenyl}-1{6H}-pyridazinebutanoic acid hydrobromide) (all from Tocris, Avonmouth, UK), L-NAME (*N*Ψ-Nitro-L-arginine methyl ester hydrochloride) and D-NAME (*N*Ψ-nitro-D-arginine methyl ester hydrochloride) (both from Sigma).

Electrophysiology

Anaesthetised tadpoles were pinned onto a rotatable Sylgard block in a bath perfused with saline, slit along the dorsal fin, and then transferred to α-bungarotoxin (10 μmol l^{-1} in saline; Sigma) for up to 20 min. After immobilisation, tadpoles were returned to the bath and re-pinned with their right side up and their rostro–caudal axis perpendicular to the axis of rotation of the Sylgard block (Fig. 4D). The skin overlying the dorsal fin and the right myotomes was removed from the level of the 4th to the 9th post-otic myotome (when testing responsiveness) or 1st to the 9th post-otic myotome (when recording trigeminal activity). The preparation was illuminated by a halogen light source and light pipe, or by a green LED (Farnell, Wetherby, UK) with peak emission of 525nm, which is close to the wavelength of maximum sensitivity of the pineal eye (520 nm; Foster and Roberts, 1982). A minimum period of 15 min after dissection was allowed for recovery prior to recording. To record ventral root activity a glass suction electrode (60 μm diameter tip) was placed over an intermyotomal cleft. To record trigeminal activity, the skin, eye and meninges overlying the right trigeminal ganglion were removed. A 60 or 30 μm diameter glass suction electrode was placed on the ventral lobe of the exposed trigeminal ganglion to record multiple- or single-unit activity respectively (Fig. 4A).

To simulate cement gland attachment, the block was rotated so the rostro–caudal axis of the tadpole pointed downwards at an angle of about 70° from vertical. A length of silver wire (0.1 mg mass) equivalent to that of a typical tadpole in water (0.09 mg; volume 2.32 mm^3, density 1.04 g cm^{-3}; Roberts et al., 2000) could be attached to the cement gland mucus and then allowed to hang freely (Fig. 1D).

Responsiveness was tested by giving stimuli at intervals of 2 min. Any fictive swimming was stopped within 20 s by pressing on the head skin with a hand-held mounted hair. Three stimuli with nothing attached to the cement gland mucus were followed by three stimuli with the weight attached, and this was repeated. The stimulus used was dimming of an LED (500 ms, from ~8000 to either ~5000 or ~150 Lux).

Chemicals used were 2 mmol l^{-1} kynurenic acid, 10 μmol l^{-1} α-bungarotoxin, 0.1% 3-aminobenzoic acid ethyl ester (MS-222) and 100 μmol l^{-1} cadmium chloride (all Sigma). Chemicals were either bath applied or delivered through a multi-barrelled microperfusion system. Fine polyethylene tubes led to a single nozzle with a tip diameter of approximately 120 μm (for kynurenate) or 60 μm (for MS-222). One barrel contained the chemical dissolved in saline, another contained a solution of Fast Green in saline and another barrel contained control saline, which was applied between drug applications to prevent perfusion artefacts. During bath application of 100 μmol l^{-1} $CdCl_2$, the skin and meninges overlying the hindbrain and midbrain were removed to improve access.

Electrical activity was amplified and then sent *via* a CED1401*plus* digital interface (Cambridge Electronic Design, CED, Cambridge, UK) to a personal computer where it was monitored, stored and analysed off-line using Spike2 or Signal software (CED), which could also be used to control the LED. A controlled pull on the cement gland mucus was produced using a loop of tungsten wire mounted *via* a lever to a loudspeaker cone driven by the CED 1401*plus* digital interface.

Individual trigeminal units, identified on the basis of spike shape, were discriminated off-line, using the Spike2 template matching function. For analysis of single unit activity, 5 min of unattached and attached activity were used. Firing rate was calculated as a single value: total number of spikes/total time period. Interspike interval (ISI) histograms were constructed with a bin width of 100 ms. The coefficients of variation (CV) of the ISIs were calculated (standard deviation ISI/mean ISI) to describe the regularity of firing. Autocorrelograms were constructed from 5 min of activity with 200 or more spikes (bin width 100 ms).

Light levels were measured using an ISO-TECH ILM 350 (RS Components, Corby, UK).

Statistical tests used are stated in the Results and were carried out using Minitab (version 10.51) and Excel. All values are means ± standard deviation (S.D.). Non-parametric statistics were used when data failed to meet criteria of normality.

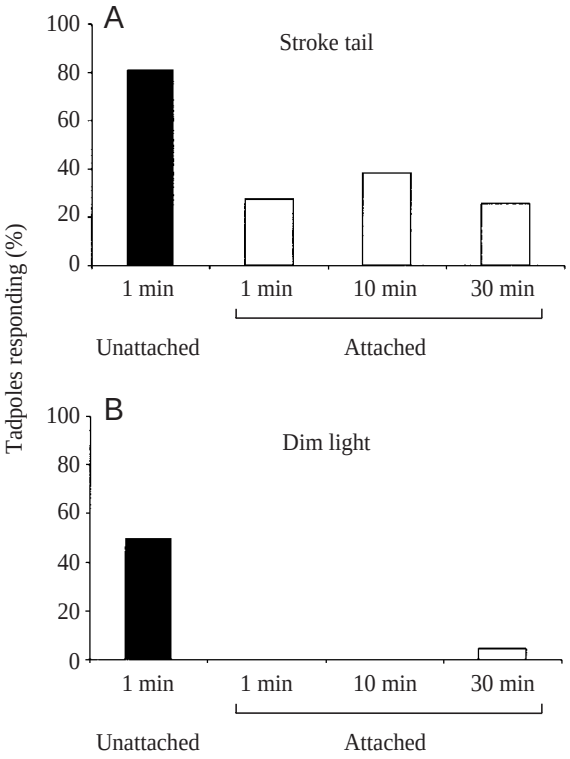

Fig. 2. Cement gland attachment reduces responses to touch and light dimming. Percentage of tadpoles responding to stimulation when unattached (black bars), and at increasing times after attachment (1, 10 and 30 min, white bars). (A) Responses to a tail stroke are significantly reduced in all attached groups compared to unattached (P<0.01 for all, χ^2=43, 27 and 44, d.f.=1, α corrected for multiple tests using Dunn–Sidak method). (B) Responses to dimming are also reduced during attachment. With no responses after 1 and 10 min, attached responses are grouped and there is a significant reduction from unattached (P<0.001, χ^2=36.3, d.f.=1).

Results

Is reduced responsiveness during attachment long-lasting?

Reduced responsiveness is not just a short-term effect of attachment but can last for periods of attachment up to at least 30 min (Fig. 2). When tested with a tail stroke after 1 min, 81% of unattached tadpoles (N=75) responded but significantly fewer responded after hanging by their cement glands for 1 min (N=70), 10 min (N=63) or 30 min (N=64) (Fig. 2A). Using a dimming stimulus, fewer unattached tadpoles responded after 1 min (N=24) but responses were again significantly reduced during attachment; 0% (N=24) responded to dimming after both 1 and 10 min and only 4% (N=24) after 30 min (Fig. 2B).

We also tested the responses of tadpoles to head skin touch. 13 out of 22 tadpoles swam or flexed the trunk in response to a stroke to the head skin when unattached, whereas 1 out of 16 responded after hanging, attached by their cement glands, for 1 min (P<0.001, χ^2=11.09, d.f.=1). Attachment therefore significantly reduced responses to head skin stimulation.

Does reduced responsiveness depend on innervation of the cement gland?

Cutting through the trigeminal mandibular nerves on each side of the body removed the effect of cement gland attachment on responsiveness to tail strokes. In tadpoles with both mandibular nerves cut, responsiveness was no longer reduced by attachment (unattached, 84±21% responded; attached, 82±26%; P=0.787, W=9.0, N=10, Wilcoxon matched-pairs signed-ranks test). Sham-operated tadpoles were still significantly less responsive to tail strokes when attached (unattached, 72±17% responded; attached, 20±13%; P=0.006, W=55, N=10, Wilcoxon matched-pairs signed-ranks test).

Since cutting the trigeminal innervation of the cement gland abolishes the reduced responsiveness during attachment, we conclude that trigeminal sensory innervation is necessary for attachment to influence responsiveness.

Table 1. *The effect of inhibitory antagonists on the ability of cement gland attachment to reduce responsiveness of freely behaving tadpoles to dimming*

	Responsiveness (%)						
	Control			Antagonist			
Antagonist	Unattached	Attached	Reduction	Unattached	Attached	Reduction	Difference
Bicuculline (20 μmol l^{-1})	78±39	3±12	75±38**	90±29	60±42	30±36*	45**
SR-95531 (20 μmol l^{-1})	93±18	48±37	45±33**	100	100	0	45**
Bicuculline (20 μmol l^{-1})+CGP-35348 (200 μmol l^{-1})	90±16	8±18	82±20**	95±17	72±31	23±27*	59**
CGP-35348 (200 μmol l^{-1})	98±6	38±38	60±40**	88±20	42±38	46±37**	14
L-NAME (10 mmol l^{-1})	88±24	13±18	75±23*	83±33	13±28	70±37*	5

Difference = $\text{reduction}_{\text{control}} - \text{reduction}_{\text{antagonist}}$.

Values are means ± S.D. N=12 tadpoles were tested for each treatment except L-NAME (8 tadpoles).

Significance of reduction was tested by the Wilcoxon matched-pairs signed-ranks test; significance of the difference in reduction between control and antagonist by the Wilcoxon Rank Sum test. *P<0.05, **P<0.01.

Does reduced responsiveness depend on GABAergic inhibition?

If the reduced responsiveness of attached tadpoles is due to the activity of the stopping pathway, then $GABA_A$-mediated, tonic or long-lasting inhibition of rhythmic spinal neurons will be necessary for attachment to reduce responsiveness (see Introduction). The effects of GABA antagonists on responsiveness during cement gland attachment were therefore tested in freely behaving tadpoles. Each tadpole received five stimuli while unattached and five while attached by its cement gland, with unattached and attached tests alternating. Removal of a section of skin over the trunk muscles allowed bath-applied drug access to the nervous system.

Dimming was used as a stimulus in most tests because it can be controlled precisely. Tadpoles from the same batch of eggs were tested in control saline (N=12) or saline containing an antagonist (N=12). The effect of antagonists was measured as the difference between the reduction in responsiveness produced by attachment in control medium (con) and the reduction produced in the presence of an antagonist (ant; difference=$\text{reduction}_{\text{con}}$–$\text{reduction}_{\text{ant}}$). This difference was significant in the presence of $GABA_A$ antagonists (Table 1): bicuculline (20 μmol l^{-1}; P=0.0072), SR-95531 (20 μmol l^{-1}; P=0), and bicuculline (20 μmol l^{-1}) together with the $GABA_B$ antagonist CGP-35348 (200 μmol l^{-1}; P=0.0002). In contrast, the $GABA_B$ antagonist CGP-35348 (200 μmol l^{-1}) alone produced no significant difference (P=0.4079). Swimming can also be initiated by a stroke across the base of the tail with a hand-held whisker. Attachment normally reduced responsiveness to this touch stimulus from 90±10% to 32±18% (P=0.003; not shown), but in the presence of $GABA_A$ antagonist SR-95531 (20 μmol l^{-1}) the reduction was insignificant, from 88±18% to 85±21% (P=0.371; N=12, Wilcoxon matched-pairs signed-ranks test). These results suggest that the reduced responsiveness during attachment depends on tonic inhibition acting primarily through $GABA_A$ receptors.

Nitric oxide (NO) can facilitate $GABA_A$ inhibition in *Xenopus* tadpoles (McLean and Sillar, 2000b). McLean and Sillar (2000a) suggested that the GABAergic neurons of the stopping pathway (MHRs) also release NO in older tadpoles. If NO is released together with GABA in the stage 37/38 tadpole, this may amplify the inhibition of spinal neurons during attachment. Using the same protocol as with GABA antagonists (see above), we tested for an effect of the NO-synthase inhibitor, L-NAME, on the ability of attachment to reduce responsiveness. In these experiments, drug access was improved by running a tungsten needle between the myotomes and the dorsal spinal cord in the region where the skin had been removed. An inactive form of NAME, D-NAME, was used as a control. L-NAME (10 mmol l^{-1}) had no significant effect on the responsiveness reducing effect of attachment (P=0.956, see Table 1).

Does activity increase when the cement gland is dennervated?

If $GABA_A$-mediated inhibition is activated while tadpoles hang from their mucus strand, this might be expected to reduce spontaneous movements. At stage 37/38, *Xenopus* tadpoles hang attached for 99% of their time and do not move while attached (Jamieson and Roberts, 2000). We therefore compared the activity of sham-operated tadpoles with others where the cement gland was denervated by cutting the maxillary nerve. Tadpoles were placed in a dish of dechlorinated tapwater and allowed 5 min to acclimatise. Activity was then observed and all flexions and swimming movements timed to the nearest second for a total of 30 min.

Movement of control tadpoles occurred only when they were not attached, either lying on the bottom of the dish or when the cement gland mucus strand had broken. They then swam briefly, stopping as soon as they contacted the side of the dish or the water surface. In contrast, denervated tadpoles could start and continue swimming while attached. When swimming freely, they often did not stop on contact with the side of the dish but swam along it for prolonged periods. Over a 30 min period control tadpoles made, on average, no movements (median=0, interquartile range=2, N=44) whereas denervated tadpoles made 2 movements (median=2, interquartile range=1, N=44). This increase in the number of movements, from 0 to 4 $\text{tadpole}^{-1}\ h^{-1}$, was significant ($W$=2409.5, P=0.0002; Wilcoxon rank sum test). The duration of

activity also increased significantly in denervated tadpoles, from 0 to 8 s tadpole^{-1} h^{-1} (W=2424.0, P=0.001; data not shown).

Does predation increase when the cement gland is denervated?

What is the significance of the normal inhibition of tadpole responses and activity while they hang attached? One possibility is that tadpoles that do not move will be less likely to be detected and eaten by predators. Odonate nymphs are important predators of many larval anuran species (Lawler, 1989; Skelly and Werner, 1990; Chovanec, 1992) and have been observed in the same ponds as *Xenopus* tadpoles in South Africa (Alan Roberts, personal observation). We therefore used damselfly nymphs (Zygoptera) to compare the predation of control tadpoles to those with denervated cement glands. Although the species used in this study are native to Europe and therefore not a natural predator of *Xenopus laevis*, preliminary studies showed that they catch and eat *Xenopus* tadpoles, especially if the tadpoles move.

We placed individual control or denervated tadpoles with a single nymph and made video recordings to monitor attacks. During a 2 h observation period, 28 out of 44 control tadpoles were eaten and this number increased significantly to 36 out of 44 in denervated tadpoles (χ^2=3.77, d.f.=1, P<0.05). This suggests that one selective advantage of the reduction in responsiveness and spontaneous activity that occurs when tadpoles are attached might be to reduce predation.

Does simulated attachment reduce responses in immobilised tadpoles?

To investigate the neuronal pathways and mechanisms for reduced responsiveness during attachment we devised a new tadpole preparation. Immobilised tadpoles were pinned head down and a weight was attached to the cement gland mucus strand to simulate the attached state (Fig. 1D). Responsiveness was tested using dimming stimuli, which are more controllable and repeatable than touch. Fictive swimming responses were monitored by recording activity in the motor nerves going to the trunk muscles (Fig. 3A).

Before testing, the threshold dim level was first determined for one tadpole in each of the two batches of tadpoles tested. The lower level of the dimming was reduced until 4 or 5 out of 6 dims initiated fictive swimming. This threshold level was assumed to be similar for all tadpoles in the batch being tested. Weight attachment significantly reduced the percentage of fictive swimming responses to a threshold dim from 78±25% of dims in the unattached state to 23±21% in the weight-attached state (Fig. 3B; P<0.001, W=136.0, N=16, Wilcoxon matched-pairs signed-ranks test).

Does weight attachment reduce spontaneous swimming in immobilised tadpoles?

As in the freely behaving tadpoles (Jamieson and Roberts, 2000) spontaneous fictive swimming activity was present in immobilized tadpoles. The occurrence of spontaneous fictive swimming was recorded over 5 min with no weight attached, followed by 5 min with a weight attached to the cement gland.

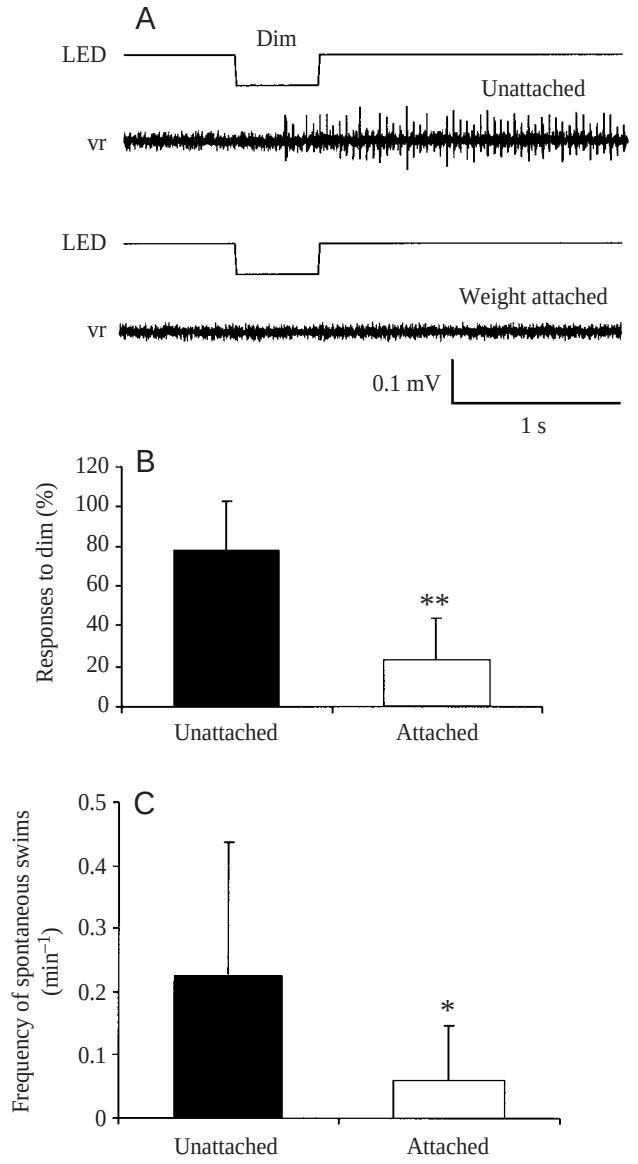

Fig. 3. Simulated cement gland attachment reduces responses to dimming and spontaneous swimming of immobilised tadpoles. A suction electrode on the motor nerve or ventral root (vr) records fictive swimming. (A) Examples of fictive responses to a dim (LED). When unattached, rhythmic bursts in the vr indicate that swimming is initiated, but with the weight attached there is no response. (B) Percentage of dims initiating fictive swimming in the unattached (black bar) and weight attached (white bar) states. Values are means + S.D. (N=16). Weight attachment significantly reduced the % of responses to dimming (**P<0.001). (C) The frequency of spontaneous fictive swims measured over 10 min in the unattached (black bar) and weight attached (white bar) states (means + S.D., N=12). Weight attachment significantly reduces the number of spontaneous swims (*P=0.009).

This was repeated, giving a total of 10 min for each state. Each 5 min recording began 1 min after either attaching or removing the weight. All spontaneous swims were stopped within 20 s by pressing the head skin with a hand-held mounted hair.

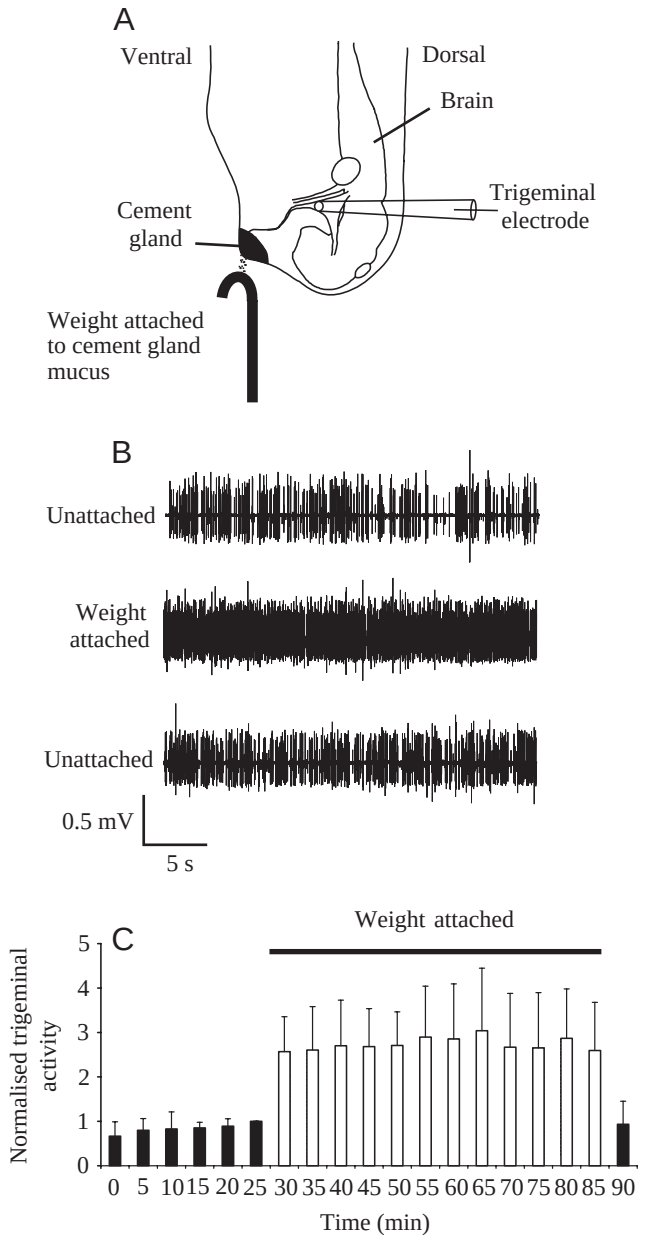

Fig. 4. Multiple-unit activity in trigeminal sensory neurons innervating the cement gland is increased by attaching a weight to the mucus strand. (A) Diagram of the head end of the inverted tadpole preparation. The weight attached to the cement gland mucus simulates attachment. Multi-unit activity is recorded by a suction electrode on the trigeminal ganglion. (B) Examples of a 30 s duration multi-unit recording showing spontaneous spike activity in the unattached state and increased activity when the weight is attached. (C) Combined results from 7 animals showing a sustained increase in trigeminal activity over a 60 min period of weight attachment and a return to control levels after the weight is removed. Activity is measured as the number of spikes in each 30 s period and is normalised relative to that at 25 min for each recording. Values are means + S.D.

The frequency of spontaneous swimming episodes was 0.23±0.21 swims min^{-1} when no weight attached. This frequency was significantly reduced to 0.06±0.09 swims min^{-1} with a weight was attached to the cement gland (Fig. 3C; P=0.009, W=45.0, N=12, Wilcoxon matched-pairs signed-ranks test).

Does simulated attachment result in a sustained increase in trigeminal activity?

Previous studies showed that cement gland mechanosensory neurons are spontaneously active and fire a brief burst of impulses in response to brief distortion of the cement gland (Roberts and Blight, 1975; Boothby and Roberts, 1992a). This activity was only recorded for a few seconds and so could not be used to predict what role cement gland sensory neurons may play over the longer periods of time for which we have demonstrated the effects of attachment. To determine whether cement gland mechanosensory neurons in the trigeminal ganglion could sustain firing during prolonged attachment we hung a weight from the cement gland mucus of immobilised tadpoles in a head-down position and recorded trigeminal activity (Fig. 4A).

Multi-unit activity was recorded from cement gland mechanosensory neurons for 30 s periods at intervals of 5 min. In the unattached state, spontaneous activity was recorded (Fig. 4B). After 25 min, a weight was attached to the cement gland mucus where it hung freely for 1 h before being removed. When the weight was first attached there was a transient peak in activity. Increased activity was then sustained for the whole 60 min period of attachment before returning to the previous unattached level after the weight was removed. The number of spikes in each 30 s recording was counted and activity in 7 animals normalised, by dividing each count by the count at 25 min (immediately before weight attachment) in the same animal (Fig. 4C). Linear regression over the 60 min period of attachment showed no significant relationship between activity and time (P=0.642, F=0.218, r^2=0.002). However, there was a significant increase in activity during simulated attachment (P=0.000, F=22.54, d.f.=2,20, repeated-measures ANOVA after grouping the activity from 0 to 25 min (unattached) and from 30 to 85 min (attached)).

What is the activity of single trigeminal units during attachment?

Multi-unit recording has the advantage that recordings can be maintained for long periods of time. However, it did not allow us to determine whether the same neurons are responsible for spontaneous activity in the unattached state and increased activity in the attached state, or what the firing rates were for individual neurons. When smaller electrodes were used, all individual units (N=20 in 15 tadpoles) showed spontaneous activity, a transient increase in firing frequency when the weight was attached, and a long-term increase in discharge during weight attachment (Fig. 5).

To study the irregular pattern of discharge in the trigeminal sensory units, we recorded 5 min of unattached (spontaneous)

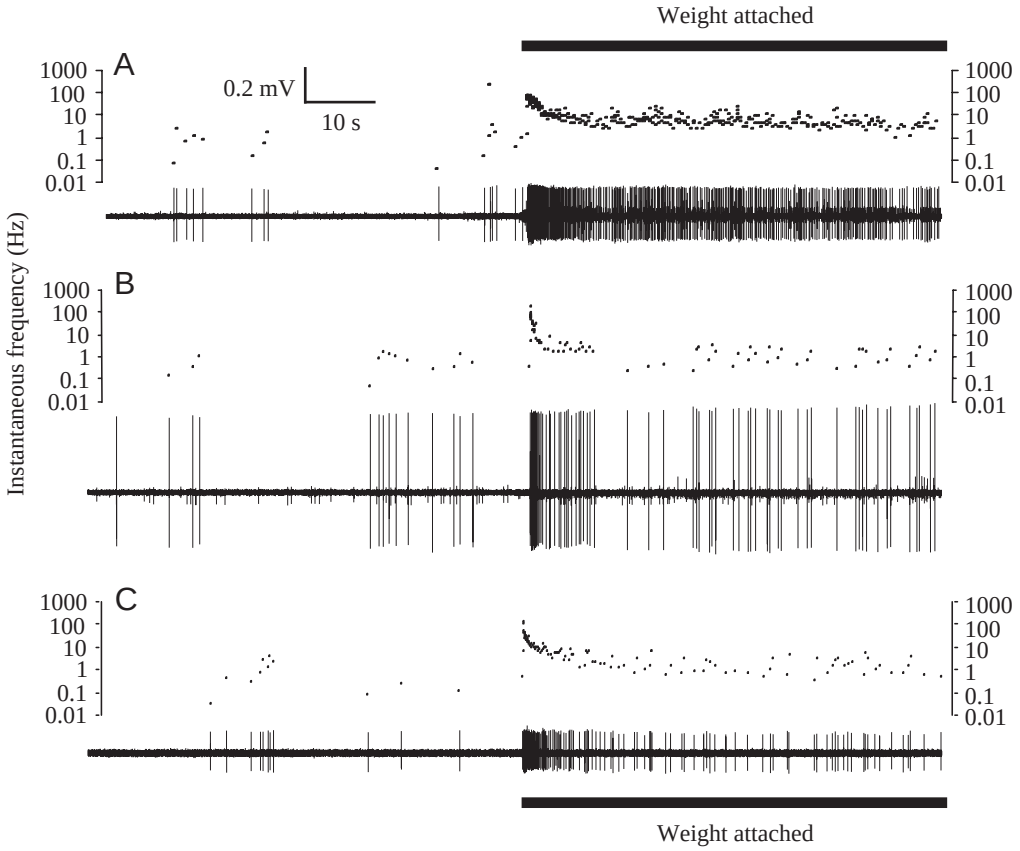

Fig. 5. Spontaneous activity of single trigeminal units and responses to the attachment of a weight to the cement gland mucus. (A-C) recordings from 3 units (lower traces) with the instantaneous frequency plot (logarithmic scale) of each unit shown above the recording. Spontaneous activity is followed by an initial transient response to attachment of the weight before firing levels out to a rate greater than spontaneous activity.

activity followed by 5 min of weight-attached activity, excluding the first 30 s after weight attachment to eliminate the transient response (Fig. 6). Linear regression of the summed total number of spikes every 30 s during the 5 min of the attached state showed no significant effect of time on activity (P>0.05) for 16 of the 20 units. We carried out further analysis of these 16 units on the assumption that their activity arose from a stationary process (Perkel et al., 1967). For both unattached and attached activity, ISI histograms had a high variance and were skewed (Fig. 6). When attached, the firing rate was significantly higher (1.17±0.78 Hz) than when unattached (0.28±0.31 Hz; P=0.000, t=5.28, N=16, paired t-test). The high coefficients of variation (CV) for the ISIs show that firing was irregular in both cases. There was no significant difference between the two (CV unattached=1.37±0.40; CV attached=1.15±0.51; P=0.093, W=35.0, N=16, Wilcoxon matched-pairs signed-ranks test). Using units that fired at least 200 spikes during 5 min in the unattached (N=2) or attached (N=11) states, autocorrelograms were constructed. These did not reveal any pattern in the firing other than a decreased probability of firing during a short period (≈200 ms) after each spike.

Mechanisms underlying sustained sensory activity

Previous work has shown that chemical synaptic transmission is not necessary for the transient response of trigeminal mechanosensory neurons to brief stimuli, since it was not abolished either by a high Mg^{2+} concentration in the bathing medium, which blocked all muscular responses (therefore presumably synaptic transmission; Roberts and Blight, 1975), or by kynurenate, an antagonist of glutamate receptors (Boothby and Roberts, 1992b). We found that excitatory glutamatergic synaptic interactions are also not required for sustained firing in the attached state. When 2 mmol l^{-1} kynurenic acid, was microperfused onto the trigeminal ganglion, there was no significant change in either firing rate (control=1.05±0.39 Hz, kynurenic acid= 0.99±0.46 Hz, P=0.752, t=0.33) or CV ISI (control=1.06±0.24, kynurenic acid=1.31±0.51, P=0.176, t=1.57; N=6, paired t-tests), measured over 5 min.

It is unlikely that sustained firing in the attached state was due to repeated transient stimulation by the hanging weight moving in the flow of perfused saline, since stopping the flow produced no significant change in either firing rate (control=1.70±0.88 Hz, no flow=1.35±0.68 Hz, P=0.109, t=2.26) or CV ISI measured over 5 min (control=1.02±0.20, no flow=1.10±0.34, P=0.48, t=0.80; N=4 units from 3 recordings, paired t-tests).

Is sensory activity affected by efferent input?

We tested whether the activity of cement gland mechanosensory neurons was affected by synaptic input from the CNS during fictive swimming activity. A ventral root electrode monitored fictive swimming activity while another extracellular electrode monitored single trigeminal units responding to cement gland stimulation. Dimming was used to

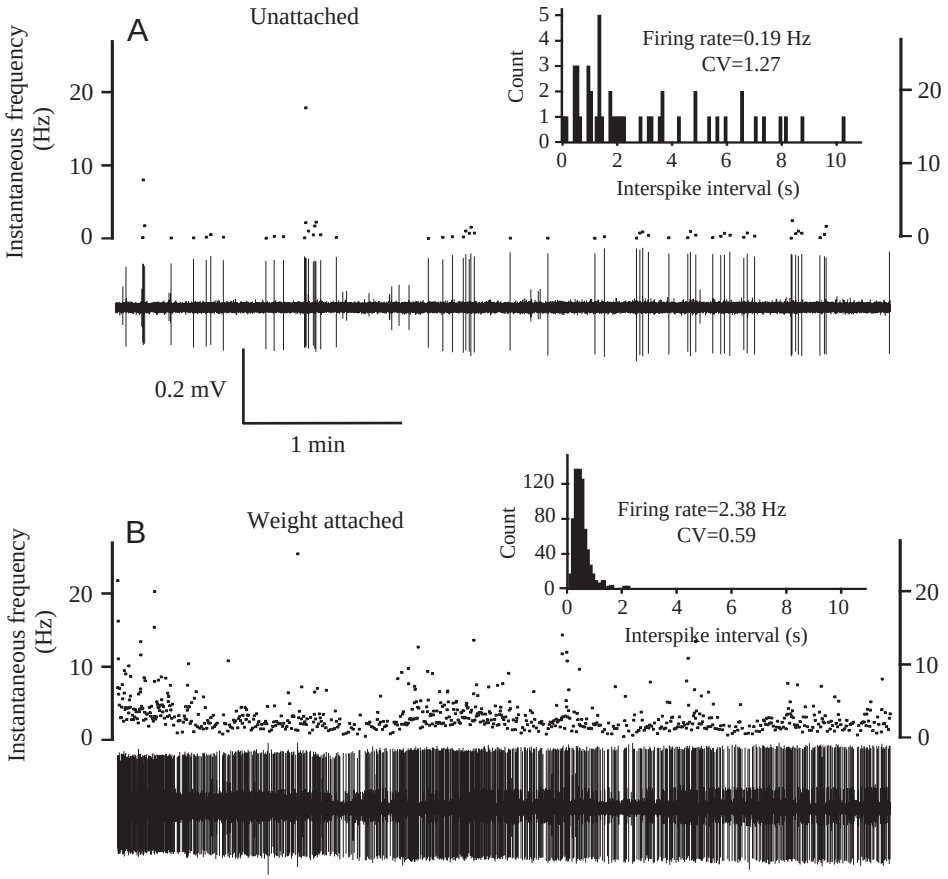

Fig. 6. Extracellular recording from a single trigeminal ganglion unit in the unattached and attached state. The instantaneous frequency plot and interspike interval (ISI) histogram accompanying each trace were constructed from a single unit discriminated on the basis of spike shape. (A) Spontaneous activity in the unattached state. (B) In the weight-attached state, firing rate is increased. For each recording the accompanying ISI histogram is unimodal and skewed. The coefficient of variation (CV=S.D./mean ISI, measure of regularity) values show firing to be irregular. Firing rate over the 5 min period (total number of spikes/total time period) is also shown.

initiate episodes of fictive swimming. The trigeminal units did not respond to the swim-initiating dim itself (*N*=7 units from 5 recordings; Fig. 7). Activity in the unattached state (spontaneous activity) was also not affected by fictive swimming. The firing rates of single units over a period of 30 s before an episode of fictive swimming (0.57±0.46 Hz) were not significantly different from the number in a 30 s period during fictive swimming (0.67±0.50 Hz; *P*=0.41, *t*=0.88, *N*=7 units from five recordings, paired *t*-test).

Increased trigeminal mechanosensory neuron firing during attachment therefore appears to be independent of peripheral synaptic interactions, effects of saline perfusion and efferent inputs during swimming.

What is the origin of spontaneous sensory activity?

Spontaneous firing activity occurred even in the unattached state in all units recorded from the ventral lobe of the trigeminal ganglia, which were excited by attaching a weight to the cement gland mucus. To rule out the possibility that this spontaneous activity is an artefact of suction electrode recording, we used the same technique to make single unit recordings from neurons in the anterior lobe of the trigeminal ganglion, which innervate the head skin and do not show spontaneous activity (Roberts and Blight, 1975). These head skin mechanosensory neurons were excited by broad pressure to the head skin (*N*=5) but showed no spontaneous activity. This indicates that the spontaneous activity of cement gland mechanosensory neurons is unlikely to be an artefact of suction electrode recording.

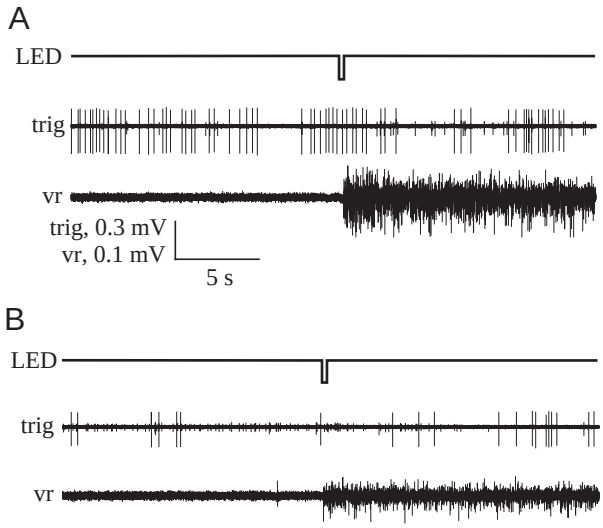

Fig. 7. Cement gland mechanosensory neuron unattached activity is not influenced by dimming (LED trace moves down), or fictive swimming. (A,B) Examples from two tadpoles. Spontaneous activity was recorded by an electrode on the trigeminal ganglion (trig; vertical scale bar, 0.3 mV) and a ventral root electrode monitored fictive swimming activity (vr; vertical scale bar, 0.1 mV).

Spontaneous activity in cement gland mechanosensory

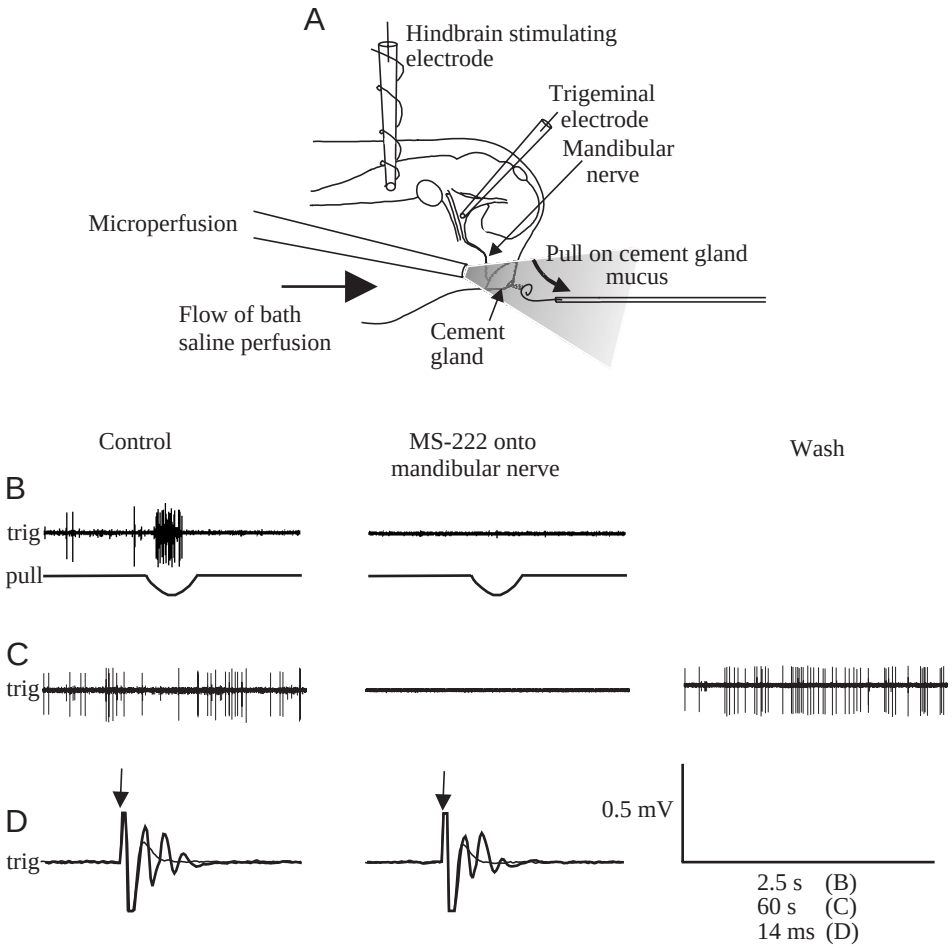

Fig. 8. Blocking impulses generated in receptor endings with anaesthetic abolishes the spontaneous activity of cement gland mechanosensory neurons. (A) Diagram of the preparation. An electrode on the trigeminal ganglion recorded multi-unit activity. A microperfusion nozzle was positioned so the microperfusate flowed over the distal end of the mandibular nerve and the cement gland. The flow of the bath perfusion ensured the microperfusate was washed away and did not contact the trigeminal ganglion. Microperfusion of anaesthetic (0.1% MS-222) onto the distal mandibular nerve as it innervates the cement gland produced the following effects. (B) MS-222 abolished the trigeminal response (trig) to pulling the cement gland mucus. Tungsten wire mounted in a glass capillary is attached by a lever to a loudspeaker cone, the input to which is shown (pull). The tungsten wire pulls on the cement gland mucus when the line (pull) moves down. Horizontal scale bar, 2.5 s (C) MS-222 abolished spontaneous activity. Horizontal scale bar, 60 s. (D) MS-222 did not abolish the antidromic spikes produced by a 300 μs stimulus pulse to the hindbrain (at arrows). Subthreshold stimulation (thin line) is shown together with a response (thick line). After washing off MS-222 by switching microperfusion to saline, spontaneous activity returned. Horizontal scale bar, 14 ms.

neurons does not depend on chemical synaptic input from the CNS as it persisted when 100 μmol l^{-1} $CdCl_2$ was bath-applied to the whole animal. 100 μmol l^{-1} $CdCl_2$ can be presumed to block chemically mediated neurotransmission (cf. Perrins and Roberts, 1995; Zhao et al., 1998) and, after application, fictive swimming failed to occur in response to dimming. The firing rate of individual units over 5 min of unattached activity in control saline (0.53±0.43 Hz) was not significantly different from that over 5 min, beginning 5 min after bath application of 100 μmol l^{-1} $CdCl_2$ (0.64±0.37 Hz; P=0.14, t=1.69, N=7 units from 5 recordings, paired t-test).

Does spontaneous activity arise in the peripheral sensory endings or within the somata lying in the trigeminal ganglion? The mandibular nerve is approximately 400 μm long and connects the trigeminal ganglion to the cement gland. This separation allowed local perfusion of anaesthetic to block any impulses generated in the receptor endings in the cement gland without affecting the ability of the somata to fire impulses (Fig. 8A). Solutions were microperfused onto the distal mandibular nerve and cement gland. The flow of the bath saline perfusion washed the microperfusate away and, by using the dye Fast Green, it could be seen that the flow was restricted to the distal end of the mandibular nerve and did not contact the trigeminal ganglion. During microperfusion of control saline, cement gland mechanosensory neurons responded to pulling on the cement gland mucus by firing a burst of impulses (Fig. 8B). The neurons were also spontaneously active (Fig. 8C). Current pulses (100 or 300 μs) delivered to the hindbrain at the level of the 5th/6th rhombomere using a glass suction electrode (100 μm diameter tip) were able to backfire the trigeminal neurons (Fig. 8D) as their axons descend to the caudal hindbrain (Hayes and Roberts, 1983). Switching to microperfusion of 0.1% MS-222 abolished both the response to pulls on the cement gland mucus and spontaneous activity (Fig. 8B,C), but current pulses to the hindbrain were still able to backfire the neurons (Fig. 8D). After returning to control saline, spontaneous activity returned. These results suggest that spontaneous activity arises in the peripheral receptor endings of the sensory neurons.

Discussion

We have shown that the reduced responsiveness produced by cement gland attachment is a long-term effect, present

at least 30 min after attachment begins. Presumably responsiveness is reduced while attachment lasts, and sometimes this may not be for very long. When not disturbed, the tadpole's cement gland mucus breaks on average every 17 min and tadpoles then swim and reattach (Jamieson and Roberts, 2000). Our behavioural experiments show that the trigeminal innervation of the cement gland is necessary for attachment to reduce responsiveness and spontaneous activity. We can therefore confidently identify the population of trigeminal sensory neurons that provide the afferent input needed because only one identified class of mechanosensory neuron innervates the cement gland (Hayes and Roberts, 1983).

What sensory information comes from the cement gland of the *Xenopus* tadpole while it hangs at rest from mucus secreted by the gland? We have demonstrated that trigeminal cement gland mechanosensory neurons show sustained impulse activity at a frequency near 1 Hz during simulated attachment. Although it was known that these sensory neurons fire in response to an increase in cement gland mucus tension (Roberts and Blight, 1975), it was not known whether they could sustain firing over long periods. Multi-unit recordings show increased activity during simulated attachment, and this is sustained for at least 60 min. We are therefore confident that cement gland sensory afferents can sustain low levels of increased activity throughout most periods of attachment in the freely behaving tadpole. One of the surprises of our study is that during the first day of the tadpole's life these mechanoreceptor afferents are probably active nearly continuously.

Although there are many examples of behavioural states of reduced activity and responsiveness that are induced or maintained by an external stimulus (Gray et al., 1938; Kramer and Markl, 1978; Krasne and Wine, 1975; Nishino and Sakai, 1996; Faisal and Matheson, 2001), the current study is, we believe, the first example where the sensory neurons detecting the external stimulus and thus evoking the reduction in responsiveness, have been identified. This has allowed us to define the pattern of sensory activity that results in reduced responsiveness. The simple behaviour and nervous system of the hatchling *Xenopus* tadpole has been the key to this. Responsiveness is reduced while tadpoles are attached by their cement glands and this behavioural state can be simulated in our immobilised preparation.

The mechanoreceptors that innervate the cement gland may serve two functions. Firstly, by firing a short burst of activity, they inform the tadpole that it has contacted support and should stop swimming (Boothby and Roberts, 1992a). Secondly, by firing continuously at low levels (~ 1 Hz) during attachment, we have shown that they give the tadpole continuous confirmation that it is hanging attached to its mucus strand. These separate functions could be served by two populations of trigeminal sensory neurons. However, our evidence does not support this proposal. In contrast, the activity of single units suggests that trigeminal cement gland mechanosensory neurons form a homogenous population. They all have irregular spontaneous activity; fire a transient burst of impulses when a weight is initially attached so cement gland mucus tension is increased, and show a sustained increase in firing rate while the weight remains attached. Our experiments indicate that the receptor terminals of trigeminal sensory neurons lying in the cement gland (Roberts and Blight, 1975) are excited directly by tension in the mucus strand secreted by the gland.

The responsiveness reducing effect of attachment was abolished by the potent $GABA_A$ antagonist SR-95531 (Heaulme et al., 1986), and significantly reduced by the $GABA_A$ antagonist bicuculline. Attachment still had a significant effect on responsiveness in 20 μmol l^{-1} bicuculline, probably because it was not blocking all $GABA_A$ receptors. The effectiveness of SR-95531 makes it unlikely that the effects of bicuculline are due to the known non-GABA-receptor-mediated effect of bicuculline, which can also block calcium-activated potassium channels (Seutin and Johnson, 1999). We conclude that $GABA_A$ inhibition is necessary for the reduction in responsiveness during attachment. It is also necessary for the stopping response, where the cement gland is transiently stimulated when the freely swimming tadpole contacts an obstruction (Boothby and Roberts, 1992b). In response to pulling on the cement gland mucus or pressing the cement gland, trigeminal mechanosensory neurons fire a burst of impulses (Roberts and Blight, 1975; Boothby and Roberts 1992a). This leads to the termination of swimming activity: the stopping response. Present evidence suggests that the axons of these trigeminal sensory neurons release glutamate to excite inhibitory reticulospinal neurons that, in turn, inhibit rhythmic spinal neurons *via* $GABA_A$-receptors (Boothby and Roberts, 1992b; Perrins et al., 2002; Li et al., 2003). Our conclusion that $GABA_A$-mediated inhibition is necessary for attachment to reduce responsiveness supports the proposal that the same GABAergic reticulospinal neurons are responsible both for stopping swimming activity when the head first contacts support (Perrins et al., 2002), and for reducing responsiveness during attachment.

Tonic inhibition mediated by $GABA_B$ receptors occurs in other systems (Hao et al., 1994; Lin et al., 1996). Since *Xenopus* tadpole spinal neurons also have $GABA_B$ receptors (Wall and Dale, 1993, 1994), they could contribute to the long-term inhibitory effects of attachment by raising the firing threshold of spinal neurons. However, we found that blocking $GABA_B$-receptors with 200 μmol l^{-1} CGP-35348 (an antagonist known to be effective in *Xenopus* tadpoles; Wall and Dale, 1993) had no significant effect on the responsiveness-reducing effect of attachment.

Our evidence suggests that $GABA_A$ inhibition plays a major role in reducing responsiveness when tadpoles are attached, but the possibility that other neurotransmitters are also involved cannot be excluded. Nitric oxide (NO) is present in the tadpole hindbrain (McLean and Sillar, 2001; Lopez and Gonzalez, 2002) and may facilitate GABAergic IPSPs (McLean and Sillar, 2000b, 2002). However, at stage 37/38, a role for NO in facilitating tonic inhibition was not supported by our observation that blocking NO production with L-NAME failed

to have an effect on the ability of attachment to reduce responsiveness. Neurons containing 5-HT are present in the raphe nucleus of stage 37/38 tadpoles (Van Mier et al., 1986) and 5-HT has been shown to block initiation of swimming by both skin stimulation and dimming (Sillar and Simmers, 1994; Jamieson, 1997). 5-HT inhibition of responsiveness to skin stimulation involves presynaptic inhibition of primary afferent neurons (Sillar and Simmers, 1994) and an increase in their firing threshold (Sun and Dale, 1997). This presynaptic action of 5-HT contrasts with the postsynaptic action of GABAergic inhibition on spinal CPG neurons during the stopping response (Li et al., 2003) and, as we now propose, during attachment. 5-HT is known to have effects on CPG neurons but these are excitatory, increasing the duration and intensity of motor bursts in swimming episodes through presynaptic inhibition of glycine release from interneurons within the CPG (McDearmid et al., 1997). The possible involvement of 5-HT in the inhibitory effects of attachment remains to be investigated.

Significance of tonic inhibition during attachment

When *Xenopus* tadpoles are placed in a small dish with a naïve damselfly nymph, the tadpoles that move appear to be the ones that are attacked and eaten. The advantages of keeping still were made clear by Skelly (1994), who showed that anaesthetized tadpoles of *Rana sylvatica* were less likely than active tadpoles to be attacked by dragonfly nymphs (*Anax junius*). We have confirmed that during the first day after hatching *Xenopus* tadpoles are very immobile, spending more than 99% of their time attached and hanging from mucus (Jamieson and Roberts, 2000). At this stage of development (stage 37/38) there is no fitness cost in keeping still as the tadpoles have no mouth and do not start to feed until about 2 days later (Nieuwkoop and Faber, 1956). This contrasts with older tadpoles that need to move in order to feed but then face increased risk of predation, for example by dragonfly nymphs (Lawler, 1989; Chovanec, 1992). Such investigations on other tadpoles suggested that tonic inhibition during attachment in the hatchling *Xenopus* tadpole could be significant in reducing activity and as a consequence, reducing predation. In preliminary experiments, we found that when the cement gland is removed so the tadpoles cannot attach with mucus, they move significantly more, and significantly more are eaten when they are placed with damselfly nymphs (A. Roberts, E. Pariser and P. Lemon, unpublished observations). The experiment we report here shows that denervation of the cement gland has similar effects even though the tadpoles can still attach and hang from mucus secreted by the cement gland. This denervation specifically interrupts the pathway that normally activates GABAergic inhibition when the cement gland is stimulated (Boothby and Roberts, 1992a,b; Perrins et al., 2002).

Our interpretation of our behavioural experiments is that tension in the cement gland mucus during attachment leads to tonic $GABA_A$ inhibition. This inhibition can reduce predation by reducing the activity of the tadpoles. Reduced activity may also help the tadpole to conserve its energy reserves. It was quite unexpected that reduced activity appears to result from continuous, tonic, sensory stimulation and continuous, tonic inhibition during attachment. This means that for its first day out of the egg the tadpole is tonically inhibited for 99% of the time. Is this long-term tonic inhibition an unusual feature of the hatchling tadpole, or could it be more widespread? Unfortunately, few cases have been studied in sufficient detail, so data is lacking, but in the crayfish, tonic inhibition that leads to reduced responsiveness has only been reported under particular behavioural conditions such as during feeding or physical restraint (Vu and Krasne, 1993).

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