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Skin impulse excitation of spinal sensory neurons in developing *Xenopus laevis* (Daudin) tadpoles

Lisa J. James* and Stephen R. Soffe†

School of Biological Sciences, University of Bristol, Bristol BS8 1UG, UK

*Present address: School of Physiology and Pharmacology, University of Bristol, Bristol BS8 1TD, UK

†Author for correspondence (s.r.soffe@bristol.ac.uk)

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SUMMARY

Responses to gentle touch in young *Xenopus* tadpoles are mediated by spinal cord sensory Rohon–Beard neurons. Tadpoles also respond to noxious stimuli that elicit 'skin impulses', which propagate between epithelial cells over the whole body surface, somehow entering the CNS to generate a response. After hatching (~48 h post-fertilization), skin impulse signals enter the CNS only via cranial nerves, but previous evidence suggested the possibility of direct entry to the spinal cord before this (~24 h). We have used behavioural and electrophysiological methods to explore the developmental pattern of skin impulse entry into the spinal cord and the involvement of Rohon–Beard neurons. Lesioning confirmed that skin impulse signals can directly enter the spinal cord in young embryos, but access decreases over ~12 h and disappears soon after hatching. Electrical recordings from central Rohon–Beard axons in young embryos showed firing in response to skin impulses. However, unit recordings from Rohon–Beard somata showed that individuals that responded to touch within a characteristic, localised receptive field did not fire to skin impulses, whereas others from similar locations responded reliably. Developmental loss of skin impulse access to the spinal cord mirrored the known spread of sensitivity to gentle touch as the peripheral mechanosensory endings of Rohon–Beard neurons mature. Together, these results suggest that Rohon–Beard neurons respond to skin impulses only while immature, providing a transitory route for skin impulses to excite the CNS. In this way, Rohon–Beard neurons would mediate responses first to noxious and then to localised, gentle touch stimuli as the neurons developed.

Key words: epithelial impulse, nociception, growth cone.

INTRODUCTION

When they hatch, amphibian tadpoles can produce behaviour that enables them to survive the start of free-swimming larval life. This depends on their ability to detect external stimuli and respond appropriately. Swimming in *Xenopus laevis* tadpoles can be initiated by a range of photic and mechanical stimuli (Roberts, 1978; Kahn et al., 1982; Foster and Roberts, 1982; Clarke et al., 1984). Mechanosensory Rohon–Beard neurons innervate the skin of the trunk and tail and are excited by a light stroke (Roberts and Hayes, 1977). The central axons of Rohon–Beard neurons transmit excitation via sensory interneurons (Sillar and Roberts, 1988; Li et al., 2003) to the central neurons that generate and coordinate motor responses (Roberts, 2000; Roberts et al., 2010). The skin of *Xenopus* tadpoles is electrically excitable and generates long, cardiac-like impulses in response to noxious stimuli such as pokes (Roberts, 1969). These skin impulses spread out in all directions from any stimulated point, eventually exciting the central nervous system (CNS) and, if the animal has the neuromuscular capacity, evoking a behavioural response (Roberts and Stirling, 1971). Skin impulses have also been recorded in other developing anurans [*Rana* and *Bufo* (Roberts and Hayes, 1977)] and some urodeles [*Cynops orientalis* (Fan and Dai, 1962; Sun and Dai, 1982); *C. pyrroghaster* (Sato et al., 1981)], although not others [e.g. *Triturus* (Roberts and Clarke, 1983)], where they also elicit behavioural responses. It has long been realised that, if a skin impulse is to evoke a behavioural response, there should be an afferent pathway from the electrical signal in the epithelium to the CNS (Roberts, 1971; Ito, 1986). However, despite nearly 50 years having elapsed since the first unambiguous demonstration of conducted impulses in amphibian skin (Chuang and Dai, 1961; Fan and Dai, 1962), it remains unknown how this transmission of electrical activity from non-neural to neural tissue occurs.

In *Xenopus*, the skin impulse provides early sensitivity to noxious stimuli over the entire body surface. This sensitivity first becomes evident behaviourally at around 24 h of age [developmental stage 24 (Nieuwkoop and Faber, 1956)], once muscular responsiveness has developed (Roberts and Smyth, 1974), although action potentials are generated in epidermal cells from stage 19 (Spencer, 1974). They can be recorded up to at least stage 41 (Roberts and Stirling, 1971). It was initially suggested that skin impulses could act by directly exciting the peripheral processes of skin sensory Rohon–Beard neurons (Roberts, 1971). Although this seemed the most likely pathway, later evidence suggested that it was not the means of action; at around the time of hatching (stage 37/38), skin impulses were shown not to excite Rohon–Beard neurons (Roberts and Hayes, 1977; Clarke et al., 1984). High spinalisation at this developmental stage prevented skin impulses from initiating swimming, suggesting that their access to the CNS is cranial. Lesioning then showed that one trigeminal nerve provides a sufficient access pathway to allow the initiation of swimming; the olfactory tract might provide another (Roberts, 1996). Importantly, however, lesion experiments on younger animals (stage 27 or less) originally suggested that excitation from the skin impulse could apparently enter the CNS via the spinal cord early in development (Roberts, 1971). This evidence suggested that access of skin impulse signals to the CNS might change during development and raised again the possibility that Rohon–Beard neurons present a suitable pathway.

This study re-addresses the nature of the skin impulses in *Xenopus* tadpoles and particularly their access to the CNS. We consider their role, allowing embryos to respond to noxious stimuli early in development, in relation to the skin sensitivity to light touch provided by Rohon–Beard mechanosensory neurons. We first chart a developmental change in access of skin impulse signals to the CNS and then explore the route by which they enter the spinal cord in younger embryos. In doing so, we provide evidence that skin impulse access occurs via immature Rohon–Beard sensory neurons prior to their developing mature mechanosensory properties.

MATERIALS AND METHODS

All experiments were performed on *Xenopus laevis* (Daudin) tadpoles between developmental stages 28 and 37/38 (Nieuwkoop and Faber, 1956) at room temperature (20–24°C) in saline (ionic composition in $mmol\,l^{-1}$: 115 NaCl, 2.4 $NaHCO_3$, 3.0 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 10 Hepes) adjusted to pH 7.4 with $5\,mol\,l^{-1}$ NaOH. Where necessary, the youngest animals were released from their egg membranes using fine forceps.

Behaviour

All lesions were generated with mounted etched tungsten microneedles, fine forceps and a small mounted piece of razor blade. Lesions were generated in a plastic Sylgard-lined dish containing a dilute solution of anaesthetic (0.1% MS-222, Sigma-Aldrich, Dorset, UK), and the animals were allowed to recover in saline for at least 5 min before behavioural testing. Operations were performed under a dissecting microscope. Strokes to the skin were delivered using a hand-held, mounted gerbil hair (~15 μm tip diameter). Pokes were delivered using a thicker, less flexible hair cut off to a blunt tip.

Electrophysiology

Tadpoles were immobilised in $10\,\mu mol\,l^{-1}$ α-bungarotoxin (Sigma-Aldrich, Dorset, UK) in saline for 30 min, then allowed to recover in saline for at least 5 min. Immobilised animals were pinned through the notochord to a rotatable Sylgard platform. Saline was perfused through the experimental dish at a rate of $\sim 2\,ml\,min^{-1}$.

Extracellular recordings from the skin, spinal cord or motoneuron axons in the intermyotomal clefts were all made using glass suction electrodes. To record the skin impulse, an electrode (60 μm diameter) was placed on the outside of the skin. To record from the cut rostral surface of the whole spinal cord, the most rostral myotomes were first removed to expose the spinal cord, which was then severed at the level of the third post-otic myotome. Activity from the whole spinal cord was recorded with a 60 μm diameter electrode, which was bent to an angle of 90 deg close to the tip. It was used to suck up the exposed end of the spinal cord. For more-local recording from the dorsal part of the cut surface of the cord, a smaller (40 μm diameter) electrode was used in the same way to suck up the end of only the dorsal part of the spinal cord. To record from Rohon–Beard neuron somata, the dorsal fin was split over the rostral spinal cord, and the pigment cells and any loose tissue lying over the exposed nervous system gently cleared away using fine, etched tungsten needles. An electrode (20 μm diameter) was placed onto the top of the spinal cord, just to one side of the midline, and gentle suction applied. Ventral root recordings were made using electrodes (~60 μm diameter) applied to intermyotomal clefts after first removing the overlying skin with tungsten needles.

Data were collected using Signal version 2.16 software through a CED 1401 Plus (Cambridge Electronics Design, Cambridge, UK), at a sampling rate of 5 or 10 kHz. Electrical stimuli (single 0.1–0.5 ms current pulses, 0–20 μA) were applied through a suction electrode on the skin. As for behavioural experiments, mechanical stimuli were delivered to the skin using fine mounted hairs (strokes) or stiffer, blunt hairs (pokes).

Drugs were applied by addition to the circulating saline. The following were used: kynurenate (Sigma-Aldrich, Dorset, UK) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Tocris, Bristol, UK).

Statistics

Data were analysed using Minitab (versions 13 and 14). The statistical tests used are stated in the Results. The outcome of tests was regarded as significant where $P<0.05$. Unless stated otherwise, measurements are expressed as means ± s.d.

RESULTS

Selective activation of responses to skin stimulation

Soon after hatching, at developmental stages 35/36–37/38, a stroke to the trunk skin of *Xenopus* tadpoles initiates swimming by directly exciting the free nerve endings of skin sensory Rohon–Beard neurons (Roberts and Hayes, 1977). A poke to the skin also initiates swimming. This might also be through direct stimulation of Rohon–Beard neurites (Roberts and Smyth, 1974). However, a poke also produces a skin impulse that spreads through the skin of the embryo and enters the CNS as a signal that can initiate a motor response, usually swimming (Roberts and Stirling, 1971). A skin impulse is not normally produced by a stroke alone. In 19 tadpoles at stages 35/36–37/38, a horizontal lesion was made through the tail and caudal trunk (Fig. 1A,B) to sever neurites that extend ventrally from the Rohon–Beard cells in the spinal cord (Roberts, 1971; Taylor and Roberts, 1983). The region of the skin ventral to the cut was therefore no longer innervated by Rohon–Beard free nerve endings. These tadpoles still responded to a stroke or poke to the innervated skin dorsal to the cut but no longer responded to a stroke to the denervated skin below the cut. Because there is a limited caudal spread of Rohon–Beard peripheral neurites (Roberts and Hayes, 1977), stimuli below the cut were applied at least 0.5 mm caudal to the rostral extent of the cut to ensure that the skin stimulated was denervated. In contrast to strokes, tadpoles still responded to a poke to the skin below the cut. This can occur because the skin in the area below the cut is still able to generate a skin impulse, which can then spread through the intact skin, enter the CNS and initiate a motor response (Roberts, 1971). Selective activation of responses with or without a skin impulse can therefore be produced by, respectively, a poke below or a stroke above a horizontal cut through the tail and caudal trunk.

Complete cranial transection caudal to the otic capsule (Fig. 1A,B, arrowhead), following a horizontal cut through the tail and caudal trunk, produced tadpoles that no longer responded to a skin impulse produced by a poke to the denervated skin below a horizontal cut. They were, however, still able to respond to a stroke above the cut. This effect of cranial transection confirmed the findings of Roberts (Roberts, 1996), who reported that, at this stage of development, the trigeminal nerves, or in some cases the olfactory nerves, are required for skin impulse signals elicited by a poke to enter the CNS and evoke a response. Therefore, although the poke stimuli would have been expected to evoke skin impulses, they were unable to access and therefore influence the CNS. The continued effectiveness of a stroke applied above the horizontal cut showed that the motor circuitry necessary to generate a response was not impaired. It is important to note that a poke above the cut can still evoke a response by directly stimulating the free nerve endings of intact Rohon–Beard

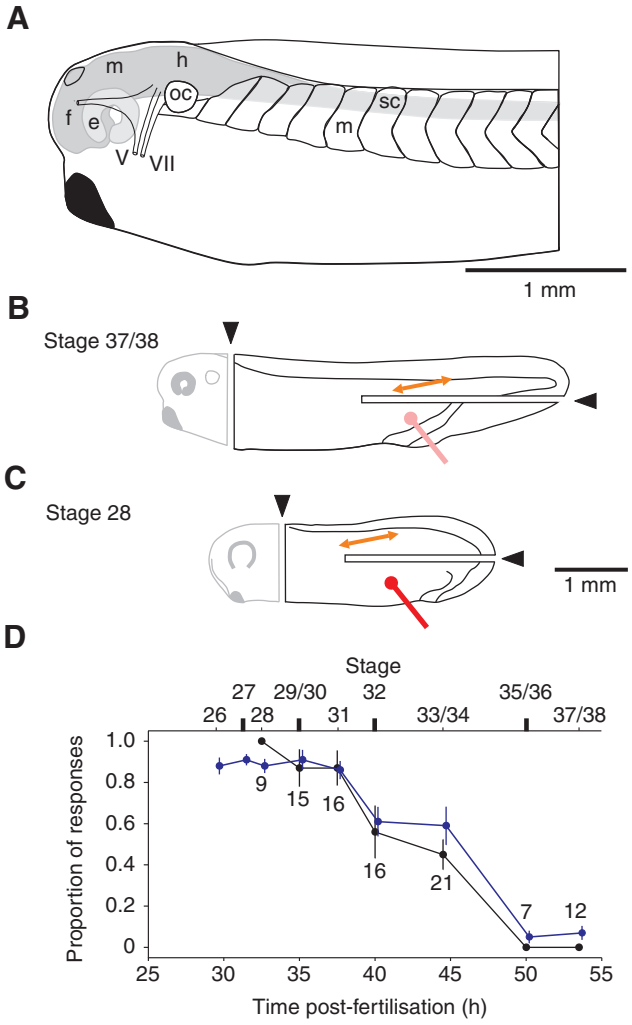

Fig. 1. Responsiveness to skin-impulse-generating stimuli. (A) Stage 37/38 tadpole showing the position of trigeminal (V) and facial (VII) cranial roots, the otic capsule (oc) and eye (e). Forebrain (f), midbrain (m), hindbrain (h), spinal cord (sc) and overlying myotomes (m) are indicated. (B) Following a horizontal lesion to cut ventrally projecting Rohon–Beard sensory neurites (horizontal arrowhead), embryos can respond to a poke below the cut but to a stroke (orange arrow) only above the cut. Following additional cranial transection just caudal to the otic capsule (vertical arrowhead), stage 37/38 tadpoles lose the ability to respond to pokes below the cut (pink round-headed arrow). (C) Stage 28 embryos, lesioned and stimulated as in B, still respond to pokes below a horizontal cut (red round-headed arrow) even after a cranial lesion caudal to the otic capsule. (D) Following cranial transection, the proportion of responses by animals to pokes below a horizontal cut decreases through development. The two lines represent two separate studies (blue: *N*=20 tadpoles per stage; black: *N* values indicated on the graph).

neurites and therefore directly exciting the CNS (caudal hindbrain and spinal cord). It is for this reason that the horizontal cut is required to provide a region where a poke will evoke a skin impulse selectively.

These results established methods to stimulate selectively motor activity either by direct mechanosensory stimulation of the free nerve endings of Rohon–Beard neurites (stroke above the cut) or by the skin impulse (poke below the cut). They also confirmed that only signals by the former route have access to the nervous system caudal to the otic capsule in hatchling (stage 35/36–37/38) tadpoles.

Development of skin impulse responsiveness

We next examined whether access of skin impulse stimuli to the nervous system changes during development. The results of an earlier study suggested that, in very young *Xenopus* embryos (stage 26), skin impulse signals might enter the caudal spinal cord directly (Roberts, 1971) and therefore not require cranial access. To test this, we blocked the cranial routes for skin impulse access to the nervous system by cranial transection caudal to the otic capsules. We then selectively evoked skin impulses by poking the skin of the ventral tail below a horizontal cut. The responsiveness of animals to pokes in this ventral region was then monitored between stages 28 (Fig. 1C) and 37/38 (Fig. 1B). Those that responded reliably (four or five times out of five) were counted as positive for skin impulse signal entry to the CNS not requiring a cranial route. In each case, we confirmed the ability of embryos to produce a motor response by a stroke to the trunk skin. Behavioural responses ranged from a single bend or twitch, through repeated unilateral or bilateral bends, to sustained swimming.

We found a clear developmental change (Fig. 1D). The proportion of cranially transected embryos that responded to pokes in the ventral region decreased with age (increasing developmental stage; total *N*=302 tadpoles). As described above, at stages 35/36 and 37/38, embryos almost never responded to skin impulse stimulation. By contrast, most embryos tested up to stage 31 responded. Between stages 31 and 35/36, a period of about 12 h, the numbers responding fell sharply. This failure of cranial transection at the level of the otic capsules to eliminate responses reliably in embryos at stages before 35/36 suggested that skin impulse signals in these younger animals are able to enter the nervous system by some other route that is caudal to the level of the otic capsules.

Locating access of skin impulse excitation to the post-otic CNS

Various lesions were used to determine whether a route for entry of skin impulse signals in young animals was localised or distributed along the CNS. Stage 31 embryos were used because they show distinct and reliable behavioural responses to stimuli and are experimentally robust. The majority of embryos at stage 31 still responded to poke-elicited skin impulses following cranial transection caudal to the otic capsules (Fig. 1D). To explore possible routes for entry, complete transection of the tail or trunk or removal of areas of the skin in a ring around the circumference of the animal was then used to limit the spread of skin impulses peripherally (Fig. 2), and more-localised lesions were used to block neuronal pathways centrally.

Following post-otic cranial transection (Fig. 2B), progressive removal of caudal regions of the tail and trunk failed to prevent motor responses to skin impulse stimuli and therefore their access to the CNS (Fig. 2C). At its most extreme, this showed that a length of intact skin over only the rostral trunk and caudal hindbrain (~0.5 mm long) and connected ventrally to the site of stimulation provided a sufficient access route for skin impulse stimuli to evoke a response. The importance of this remaining rostral region was then tested by removing the skin in a ring at this level (Fig. 2D). Reliable responses were still found in three out of three embryos. Skin rings at a mid-trunk level that limited possible access to only the caudal part of the trunk and tail also failed to abolish responses to skin impulses evoked caudally. This responsiveness was only abolished by additionally transecting the spinal cord in the middle of the skin ring and thereby cutting central pathways to the motor circuitry rostral to the skin ring (Fig. 2E). In these latter cases, responses could still be evoked by stimuli rostral to the skin ring.

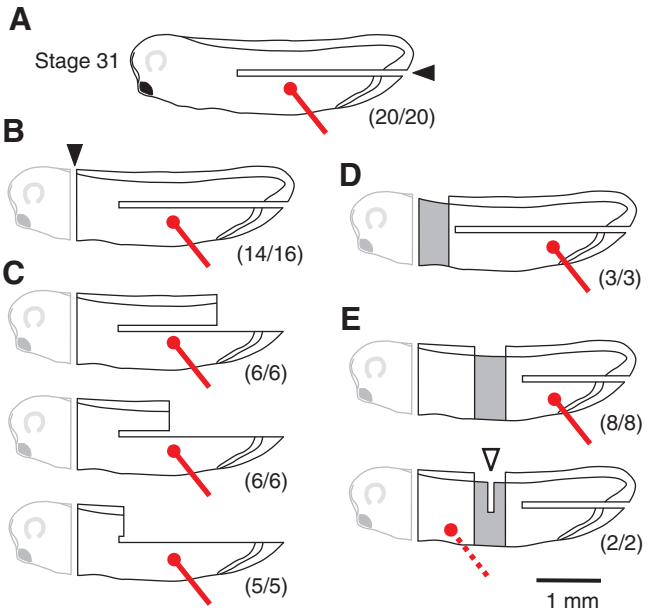

Fig. 2. Locating possible access routes for skin impulse signals to the CNS in stage 31 tadpoles. In each case, numbers indicate responsiveness (tadpoles responding/tadpoles tested). Single pokes to the skin (round-headed arrow) below a horizontal lesion through the tail and caudal trunk (A) and after additional cranial transection caudal to the otic capsule (B) reliably evoked a motor response. (C) Animals continued to respond reliably after further vertical lesions were made to remove increasing lengths of tail and trunk. (D) Single pokes below a horizontal lesion reliably evoked a response after removal of a complete ring of skin (shaded) spanning the level of the caudal hindbrain and rostral spinal cord. (E) Responses were still evoked by a poke caudal to a complete skin ring at the mid-trunk level but were abolished when a vertical cut was used to transect the spinal cord at the level of the skin ring (open arrowhead). Pokes rostral to the skin ring still evoked a response (dashed round-headed arrow)

As well as showing a need for continuity in the CNS for a response following a stimulus caudal to a skin ring, this also shows that the skin impulse does not produce motor responses by directly eliciting muscle contraction [as also concluded by Roberts (Roberts, 1971)]. Together, these results show that, at stage 31, skin impulse stimuli can elicit a behavioural response provided that they have access to the CNS anywhere along its length caudal to the otic capsules. A small region (~0.5 mm in length) is sufficient for entry, rather than a specific longitudinal location.

Skin impulse access to the spinal cord in cranially transected embryos

In order for tadpoles to produce a motor response to a skin impulse, the CNS must be activated by the skin impulse. More specifically, neurons in the CNS including the spinal cord must be made to fire by skin impulses. We confirmed this activation by making electrical recordings from the spinal cord in young embryos at stages 31 (N=5) and 32 (N=5) immobilised by α-bungarotoxin. As shown above, skin impulse signals in tadpoles at this stage of development appear to have direct access to the spinal cord. A suction electrode was used to record from the whole, rostral, cut surface of the spinal cord, transected at approximately the level of the third post-otic cleft. Following this rostral transection, embryos were horizontally lesioned along the tail and caudal trunk (as above) to allow electrical stimulation of the skin with or without directly exciting Rohon–Beard neurites (Fig. 3A). The presence or absence of skin impulses in response to stimuli was confirmed using a suction electrode applied to the skin.

Stimuli were first applied dorsal to the horizontal cut (Fig. 3A). At low stimulus intensities, there were often brief (14.3±15.2 ms; range 3.6–44.3 ms) spinal cord firing responses starting at short latency (6.1±2.0 ms; asterisk in Fig. 3B). As no skin impulse was evoked, these responses were due to the direct stimulation of Rohon–Beard sensory neurites in the skin. When the stimulus intensity was increased, the short-latency activity from direct Rohon–Beard stimulation remained (latency 5.3±1.2 ms; asterisk in Fig. 3C) and was joined by a longer latency (30.2±12.2 ms; range 15.2–52.1 ms; N=10) and longer duration (239±227.7 ms; range 42.6–645.9 ms) firing activity. This coincided with the appearance of a skin impulse on the skin electrode record (latency ~30.8±8.5 ms; Fig. 3C), making it probable that this longer latency spinal cord activity was the result of the skin impulse exciting the nervous system.

Stimuli were then applied ventral to the horizontal cut to selectively evoke skin impulses alone. Stimuli just below the skin impulse threshold evoked no activity in the spinal cord (Fig. 3D). Increasing the stimulus intensity above the skin impulse threshold produced activity in the spinal cord at a relatively long latency (34.9±12.2 ms; N=9) that was not significantly different to the later response to stimuli applied dorsal to the horizontal cut (two-sample t-test: t=0.74, P=0.48; Fig. 3E). In no case following stimulation ventral to the horizontal cut was there a short-latency response attributable to direct stimulation of Rohon–Beard neurites.

The difference in latency between the brief discharge attributed to direct Rohon–Beard stimulation and the discharge associated with skin impulse activity can be explained by the signal pathways involved. Skin impulses spread through the skin in all directions from the stimulus site. Assuming the most direct conduction pathway from the stimulus site to the skin recording electrode (1.4±0.4 mm), the latency to the initial peak of the recorded skin impulse (35.7±6.0 ms; N=6) gives it a conduction velocity of ~40 mm s^{-1} [similar to values estimated previously for a range of stages between 31 and 41: 50–110 mm s^{-1} (Roberts and Stirling, 1971); 40–70 mm s^{-1} (Alpert et al., 2007)]. This is much slower than the value of 200 mm s^{-1} estimated for Rohon–Beard peripheral neurites (Clarke et al., 1984). The greater latency to skin-impulse-evoked discharge recorded in the spinal cord is therefore consistent with a significant part of the conduction pathway to the CNS being through the skin.

Overall, 20 animals tested at stage 31 and 12 animals at stage 32 showed spinal cord activity associated with a skin impulse. These results demonstrate that, in embryos at this developmental stage, the skin impulse can directly excite activity in the spinal cord.

Recording population activity from presumed Rohon–Beard neurons

The failure of previous recordings from Rohon–Beard neurons at stage 37/38 to show any response to skin impulses (Roberts and Hayes, 1977; Clarke et al., 1984) is perhaps not surprising given the evidence for loss of skin impulse entry to the spinal cord by this stage (cf. Roberts, 1996). However, Rohon–Beard neurons are the principal trunk-skin sensory neurons with peripheral neurites whose free nerve endings penetrate the skin. These neurons therefore remain strong candidates for mediating entry in the younger embryos where we have shown that skin impulses can directly excite activity in the spinal cord. We therefore attempted to record activity specifically in these neurons in response to stimuli that evoked a

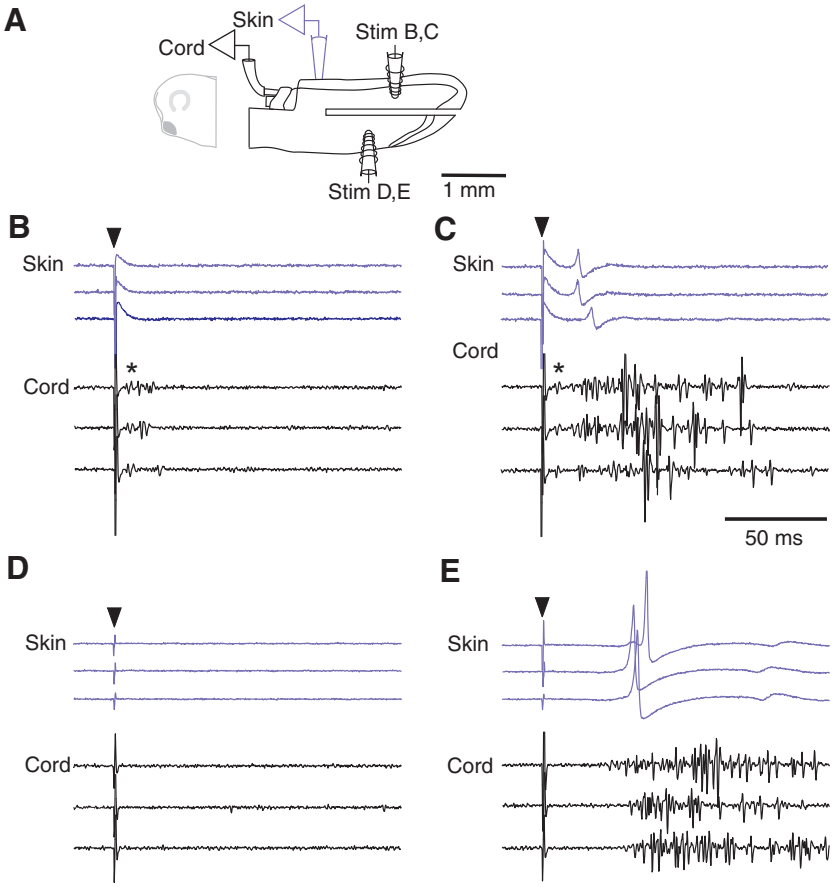

Fig. 3. Spinal cord responses to electrical stimulation of the skin. (A) The experimental preparation using a stage 31 animal that has been cranially transected and horizontally lesioned. To record spinal activity, a suction electrode has been attached to the whole cut end of the rostral spinal cord (cord) following removal of some skin and rostral myotomes. Skin impulses are monitored with a second suction electrode (skin) attached to the skin. A stimulating suction electrode (Stim) is attached above or below the horizontal lesion. (B) Three stacked responses recorded from the skin (blue) and cord (black) to single stimuli (at arrowhead) above the horizontal lesion. In each case, a stimulus just below the threshold for a skin impulse evokes brief discharge at relatively short latency in the spinal cord (*). (C) Above the skin impulse threshold, stimuli at the same site evoke short-latency discharge and additional longer latency, longer duration discharge. (D) Stimuli below the horizontal lesion and just below the skin impulse threshold evoke no response in the spinal cord. (E) Stimuli above the skin impulse threshold at the same site evoke only the longer latency, longer duration discharge. Note that the latencies to the skin impulse and spinal cord discharge are both longer at this second stimulus site than in C. The increased amplitude of the skin impulse is the result of increased suction applied to the recording electrode.

skin impulse. We again made recordings from the cut rostral end of the spinal cord in animals at stage 31 (Fig. 4A), but in this case a smaller suction electrode (diameter 40 μm) was applied to only the dorsolateral region where the ascending central axons of Rohon–Beard axons run (Clarke et al., 1984). In addition, as glutamate is the fast neurotransmitter released by Rohon–Beard neurons (Sillar and Roberts, 1988; Li et al., 2003), we used glutamate antagonists (the broad-spectrum antagonist kynurenate or the competitive AMPA/kainate receptor antagonist CNQX) to block synaptic excitation from Rohon–Beard neurons to prevent them exciting additional firing in post-synaptic neurons.

As described above, stimuli applied above a horizontal cut but below the skin impulse threshold evoked brief firing at a short latency in all animals tested (5.1±0.2 ms; N=28; Fig. 4B). This was consistent with direct stimulation of peripheral Rohon–Beard neurites. The shorter duration of firing (compare with Fig. 3B) was consistent with the more restricted position of the recording electrode and blockage of activity in post-synaptic neurons by the glutamate antagonists. Above the skin impulse threshold (Fig. 4C), the short-latency response remained (5.4±1.0 ms), but there was an additional response at longer latency (35.4±10.3 ms) and of longer duration (22.8±12.1 ms; range 14.6–40.8 ms). As described above, we interpret this longer latency activity as a response to the skin impulse. In this case, however, it should represent activity only in neurons responding directly to the skin impulse – presumed to be sensory Rohon–Beard neurons. Following a skin impulse, later discharge in these Rohon–Beard neurons was less extended than the equivalent firing recorded from the whole cord, again consistent with the more restricted recording position and the glutamate antagonists. However, it was still more extended than the initial firing resulting from direct Rohon–Beard stimulation. Individual units could not be recognised, but it is likely that the response was the asynchronous firing of many separate neurons. This long response was presumably a consequence of the longer duration of each skin impulse [~100 ms (Roberts and Stirling, 1971)] and might increase the likelihood of swimming after a brief skin-impulse-generating stimulus.

When stimuli were applied ventral to the horizontal tail cut, to selectively activate skin impulses alone, there was no central response at stimulus levels below the skin impulse threshold (Fig. 4D). However, stimuli above the skin impulse threshold showed central responses in all animals tested (Fig. 4E; 10 stage 31/32 embryos in 1 mmol l^{-1} kynurenate and 18 in 10 μmol l^{-1} CNQX). Again, these responses were of longer latency than those to direct Rohon–Beard neurite stimulation (31.7±6.8 ms compared with 5.1±0.2 ms), and of longer duration (25.4±16.52 ms; range 4.3–55.1 ms).

The latency to the start of the skin impulse recorded from the skin and the central response recorded in the spinal cord both varied somewhat in each animal (Fig. 5A). If the activity we were recording, presumed to be from Rohon–Beard neurons, was evoked by the skin impulse then, where this happened, we should expect the two latencies to vary in parallel. We examined records from four animals at stage 32. In each case, significant correlation between the two latencies (Fig. 5B) supported our proposal that the central activity was a direct response to the skin impulses. If our recordings were from the axons of Rohon–Beard neurons then the results suggest that Rohon–Beard neurons of these younger animals can respond to skin impulses.

Evidence for responses by individual Rohon–Beard neurons

To try to confirm the suggestion that Rohon–Beard neurons in younger tadpoles respond to skin impulses, whereas those of

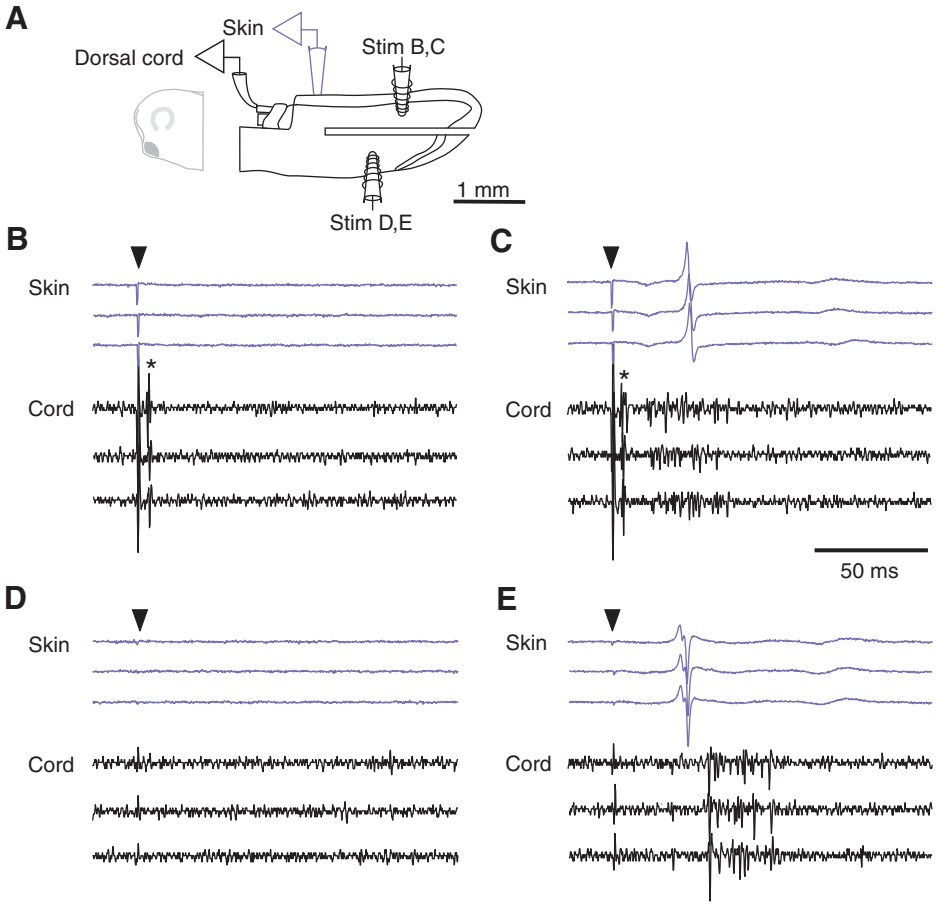

Fig. 4. Responses of presumed Rohon–Beard neurons to electrical stimulation of the skin. (A) Recordings were made as in Fig. 3 but from just the dorsal region of the cut end of the rostral spinal cord (dorsal cord). Experiments were performed in 10 μmol l^{-1} CNQX. (B) Stimuli applied above a horizontal lesion, below the threshold for a skin impulse, evoke brief discharge at short latency (*). (C) Above the skin impulse threshold, the initial short-latency response is followed by longer duration discharge. (D) Stimuli applied below the horizontal lesion no longer evoke a short-latency discharge but, above the skin impulse threshold (E), they still evoke a longer latency, longer duration discharge.

hatchling tadpoles do not, we made single-unit recordings from individual Rohon–Beard neurons at different developmental stages. To record from Rohon–Beard neurons, a small suction electrode was applied to the top of the spinal cord, just to one side of the midline in a position to contact Rohon–Beard somata (Fig. 6A,E) (Hughes, 1957). Once the electrode was recording spike activity characteristic of Rohon–Beard neurons in response to hand-delivered strokes to the skin, the receptive field of that particular Rohon–Beard neuron was mapped (cf. Roberts and Hayes, 1977; Clarke et al., 1984). Stroke and poke stimuli were then applied to the skin within and outside the defined receptive field.

Recordings from older animals (stages 35/36–37/38, N=7) confirmed earlier findings that Rohon–Beard neurons do not respond to skin impulses at these stages. All neurons fired constant-amplitude spikes to strokes within a clear receptive field (Fig. 6A,B). In response to a poke within the receptive field, small bursts of impulses could still be evoked. However, these could occur before, during or after the skin impulse (visible as an artefact on the same records, shaded in Fig. 6C), making it unlikely that the skin impulse was responsible. This was confirmed by pokes outside the receptive field, which elicited skin impulses but failed to elicit Rohon–Beard spikes (Fig. 6D). It is therefore likely that the relatively slow manual pokes within the receptive field stimulated Rohon–Beard neurites directly during the depression of the skin.

We then repeated these recordings with 14 younger embryos, at developmental stages shown previously to be able to respond to direct input of skin impulse signals to the spinal cord (two at stages 33/34 and 13 at stages 31/32; Fig. 1D). Recordings had not previously been made from Rohon–Beard neurons in these younger animals. Surprisingly, the results were the same as for the older tadpoles: all responded to strokes within the receptive field (Fig. 6E,F) and also to pokes within the receptive field that elicited a skin impulse (Fig. 6G), but none responded to pokes delivered outside the receptive field (Fig. 6H). This result was in conflict with our recordings from presumed Rohon–Beard axons (Fig. 4) that showed clear responses to skin impulses. It seemed unlikely that the recordings of group activity made dorsally from the cut rostral end of the spinal cord were from neurons other than Rohon–Beard neurons. We therefore considered the possibility that there are some Rohon–Beard neurons, perhaps immature neurons, that do respond to skin impulses but would not be identified by showing sensitivity to a light stroke within a localised receptive field.

We made further recordings from the dorsal surface of the spinal cord following transection of the whole body at the rostral end of the spinal cord (Fig. 7A). For convenience of recording, a slightly different method was used to allow specific stimulation of skin impulses. Instead of the previous horizontal cut, a vertical cut was made right through the dorsal half of the caudal trunk to sever the skin on both sides, together with the spinal cord and thus denervate the skin behind the cut. A suction electrode was placed caudal to this cut to allow selective stimulation of skin impulses, whose presence was monitored using additional suction electrodes applied to the skin close to the stimulus site and the recording site. In these recordings, we did not first try to define a receptive field but simply looked for a response to a skin impulse. Recordings were made from 16 animals between stages 29/30 and 32. In response to evoked skin impulses, large spikes were recorded from nine presumed Rohon–Beard neurons in eight of these animals (Fig. 7B); most (five of nine) were from the youngest animals (stage 29/30). Five of the nine recordings were made in saline containing the glutamate

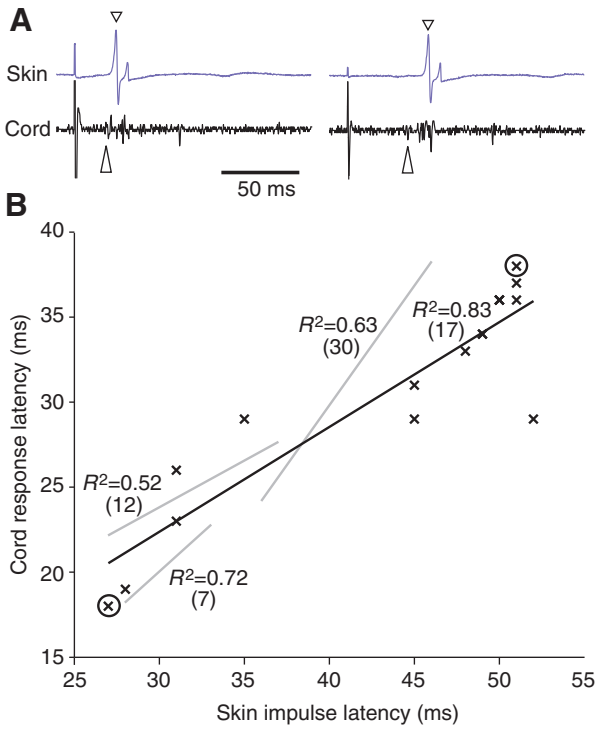

Fig. 5. Temporal relationship between the skin impulse and discharge in presumed Rohon–Beard neurons. Following skin stimulation, the latencies of responses recorded from presumed Rohon–Beard neurons in the spinal cord co-vary with latencies of skin impulses recorded from the skin. (A) Recordings (circled in B) of shorter (left) and longer (right) latency skin impulses (blue traces) and dorsal spinal cord responses (black traces); recorded as in Fig. 4 from an animal at stage 31. Arrowheads indicate where latency measurements were made: the peak of the skin impulse and the start of spinal cord discharge. (B) Spinal cord response latencies from the same recording (crosses) increase significantly with skin impulse latency (black line; $P<0.0001$). Circled points are illustrated in A. Regression lines are also shown for responses from three additional stage 31 animals (grey). The R^2 values are indicated (grey lines all $P<0.05$); the number of responses is shown in brackets.

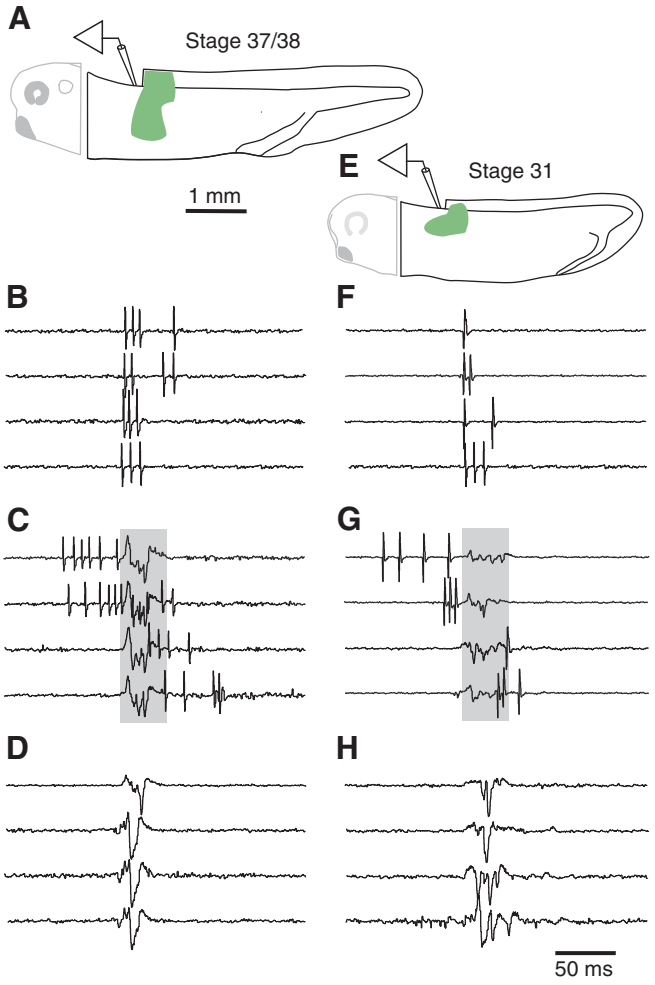

Fig. 6. Mature Rohon–Beard neurons do not respond to skin impulses. Single-unit recordings from Rohon–Beard neurons following mechanical stimulation of the skin. (A) Experimental preparation that allows recording of a single Rohon–Beard cell at the dorsal surface of the spinal cord of a stage 37/38 animal following cranial transection. The receptive field mapped for an individual neuron in response to stroking the skin is indicated (green shading). (B) Single-unit records from the same Rohon–Beard neuron following strokes within the receptive field. (C) Pokes inside the receptive field generate skin impulses (seen as artefacts on the single-unit recording: grey bar) and also single-unit responses. Note that the Rohon–Beard spikes can follow or precede the skin impulse. (D) Pokes outside the receptive field for the same neuron generate skin impulses but no Rohon–Beard spikes. (E–H) As for A–D but for a single Rohon–Beard neuron in a stage 31 animal.

antagonist 10 μmol l^{-1} CNQX, supporting the identification of the recorded neurons as primary sensory neurons and not neurons post-synaptic to them (as described above). In most cases (six of nine), neurons fired only once to each skin impulse; in the remaining cases, they occasionally fired two impulses (Fig. 7C). Although not investigated systematically, the reliability of response was variable. In most cases (seven of nine), spikes occurred following many or all skin impulses, including short trains of skin impulses at intervals of 500 ms (Fig. 7D). In the remaining two of nine cases, however, spikes only occurred occasionally. In summary, these results show that, contrary to previous recordings from single Rohon–Beard neurons (Fig. 6) but in line with our recordings from axons in the dorsal part of the cut rostral end of the spinal cord (Fig. 4), some individual neurons at stages between 29/30 and 32 can respond reliably to skin impulses. Because of their position along the dorsal surface of the spinal cord, we can be confident that the recordings were from Rohon–Beard neurons (see Discussion). It is important to note that we cannot be completely confident that the recorded neurons did not have any receptive field. However, as mature Rohon–Beard neurons showing a clear receptive field to strokes never responded to skin impulses [here or previously (Roberts and Hayes, 1977; Clarke et al., 1984)], we suggest that those individual neurons that did respond were immature Rohon–Beard neurons.

DISCUSSION

Summary of main findings

During the second day of development, young *Xenopus* show a significant change in the way they detect trunk skin stimuli. The sensitivity to gentle stimuli (strokes) seen over an increasing proportion of the trunk and tail skin and conferred by the developing peripheral free nerve endings of sensory Rohon–Beard neurons (Roberts and Smyth, 1974; Roberts and Hayes, 1977) is overlain by responsiveness to strong stimuli (pokes) mediated by propagating skin impulses. At the time of hatching, skin impulse signals must

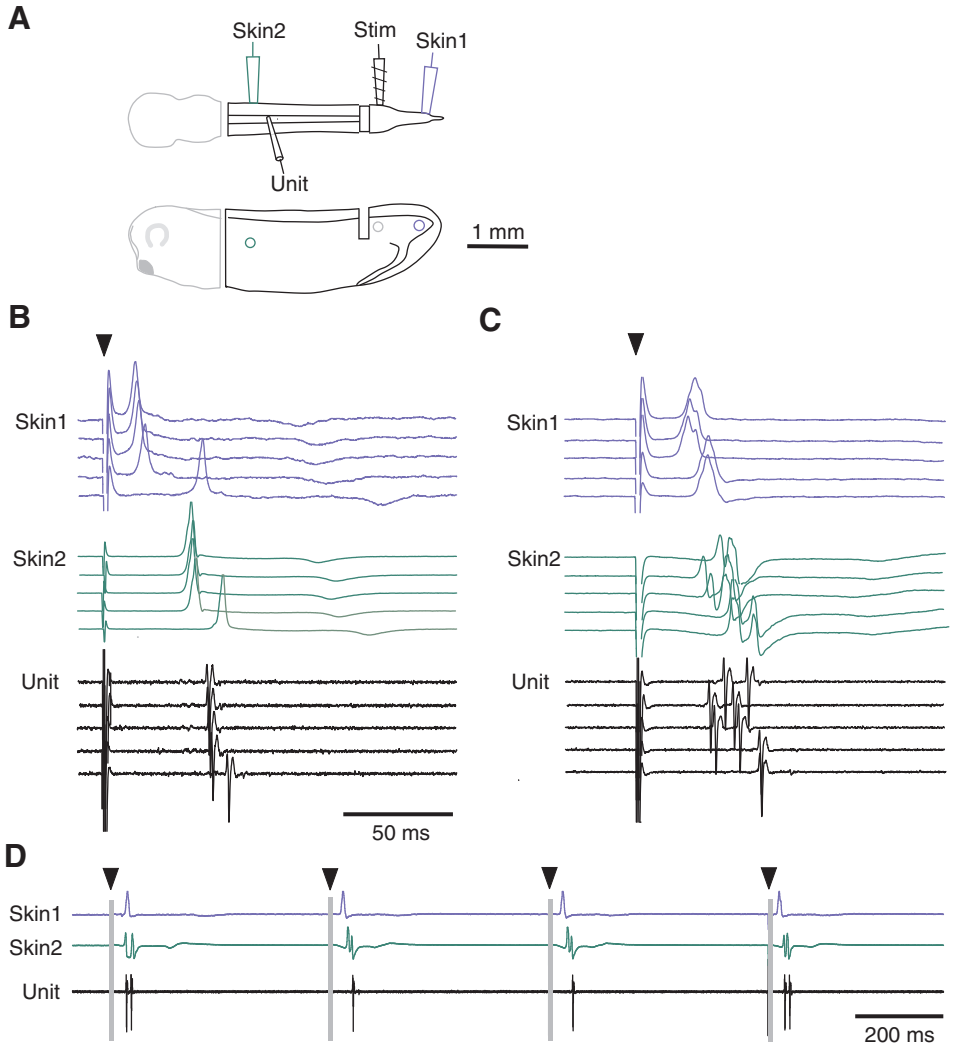

Fig. 7. Presumed immature Rohon–Beard neurons can respond to skin impulses. (A) The experimental preparation used to make recordings of single units from the dorsal surface of the spinal cord in stage 31 animals. Electrical stimuli (stim) are applied caudal to a vertical lesion that transects the spinal cord and denervates the skin caudal to the cut, allowing skin impulses to be evoked selectively. Skin impulses are monitored close to the stimulus site (blue, skin1) and close to the recording site (green, skin2). The saline contains 10 μmol l^{-1} CNQX. (B) Single spikes (*) in response to each stimulus (arrowhead). Note that the latency is relatively long and matches that of the skin impulses recorded close by (green). (C) Single or pairs of spikes evoked by single stimuli in a different animal. The latency is again similar to the skin impulse recorded close by. (D) Single or pairs of spikes reliably following a series of skin impulses.

enter the CNS through cranial roots to initiate a motor response (Roberts, 1996). However, extending a previous observation (Roberts, 1971), we have now demonstrated that, earlier in development, skin impulse signals can directly enter the CNS throughout the length of the spinal cord. This direct access of skin impulse signals to the spinal cord is then lost quite rapidly over a period of ~12 h, between stages 31 and 35/36. Our results support a longstanding suggestion that the Rohon–Beard neurons provide a route for entry of skin impulse signals (Roberts, 1971) but also explain why previous recordings from Rohon–Beard neurons failed to support this (Roberts and Hayes, 1977; Clarke et al., 1984). By stage 37/38, skin impulse signals do not directly excite the spinal cord, and so it is not surprising that mature Rohon–Beard neurons do not respond to them. We have now shown that, earlier in development, mature Rohon–Beard neurons that have developed sensitivity to gentle mechanical stimuli still do not respond to skin impulses, even though these can directly excite the spinal cord. We suggest that entry of skin impulse signals to the CNS is via immature Rohon–Beard neurons, before they have formed their free nerve endings in the skin and developed their characteristic, mature sensitivity to gentle mechanical stimuli.

Parallel changes during development of skin sensitivity

Xenopus embryos first respond to pokes by generating skin impulses just after the end of their first day post-fertilisation (around stages 24 or 25) (Roberts and Smyth, 1974). At this stage, peripheral neurites of Rohon–Beard neurons and their terminal growth cones first appear beneath the skin in the region over the rostral myotomes (Roberts and Taylor, 1982). From a peak at around stage 26, the number of growth cones gradually decreases (Roberts and Taylor, 1982) and sensitivity to light touch (strokes) extends over the whole of the body surface (Roberts and Smyth, 1974). The spreading area of sensitivity to light touch shows where the ends of the Rohon–Beard neurites have matured to form mechanosensory free nerve endings, replacing the earlier growth cones that have guided neurite extension; the delay between loss of growth cones and appearance of sensitivity to strokes is ~3–5 h (Roberts and Taylor, 1982). By the end of this period of development (stage 35/36), the whole body surface has become sensitive to light touch, which means that it is unlikely that there are now significant numbers of growth cones present. The period of development during which direct access of skin impulse signals to the spinal cord is lost (stages 31 to 35/36) parallels the stages during which sensitivity to light touch reaches the whole body surface and therefore during which the last Rohon–Beard growth cones have disappeared [compare fig. 5 of Roberts and Smyth (Roberts and Smyth, 1974) and fig. 3 of Roberts and Taylor (Roberts and Taylor, 1982)]. We speculate that there is a direct causal relationship between these events: that younger animals can respond to direct entry of skin impulse signals to the spinal cord because they have sufficient immature Rohon–Beard

neurons, with peripheral neurites that are still growing; and that they lose this ability once the peripheral neurites have lost their growth cones and formed their mature free nerve endings. It is important to note that ascending and descending sensory Rohon–Beard central axons extend from stage 22 (Taylor and Roberts, 1983), so there is already a potential central route for distribution of Rohon–Beard sensory signals to trigger a motor response.

Do immature Rohon–Beard neurons provide access for skin impulse signals?

Recordings from the spinal cord in young animals in which there is still access for skin impulse signals to the spinal cord have provided direct evidence that Rohon–Beard neurons can respond to skin impulses. Recordings made from the dorsal region of the rostral end of the cut spinal cord, presumed to be from ascending Rohon–Beard axons, and from the dorsal surface of the spinal cord, presumed to be from Rohon–Beard somata, both showed spikes following skin impulses evoked either by pokes or brief electrical stimuli. Can we be confident that these recordings were indeed from Rohon–Beard neurons? Previously, their identity in extracellular recordings was argued on the basis of dorsal location and the presence of a localised, peripheral receptive field to stroke stimuli (Roberts and Hayes, 1977). Subsequently, the identity of such neurons was confirmed anatomically using intracellular horseradish peroxidase (HRP) injection (Clarke et al., 1984). However, dorsal neurons defined by a localised response to strokes do not respond to skin impulses (see Fig. 6); sensitivity to strokes is therefore not an appropriate method for identifying the neurons that respond to skin impulses. A very dorsal location remains a sufficient basis for identification. For completeness, an alternative possibility is that the recordings were from a group of neurons previously called extra-medullary cells (Hughes, 1957). It is unclear whether these are actually a distinct neuronal class or whether, as seems likely, they are simply a form of extra-spinal Rohon–Beard neuron. They closely resemble Rohon–Beard neurons in anatomy and pattern of neurite outgrowth and differ only in having generally larger somata that have migrated to positions outside the spinal cord. In fact, as argued previously by Roberts and Hayes (Roberts and Hayes, 1977), our recordings are unlikely to have been from these extra-medullary cells as: they are relatively few in number; they are located somewhat dorsolateral to the spinal cord; and they are likely to have been removed when the dorsal surface of the cord was cleared before electrode placement. We conclude therefore that our single-unit recordings from the dorsal surface of the spinal cord were from immature Rohon–Beard neurons. By extension, we conclude that the recordings from the dorsal surface of the cut spinal cord were from the central ascending axons of Rohon–Beard neurons. Our use of glutamate receptor antagonists makes us confident that these axon recordings were from sensory neurons and not from their post-synaptic followers.

Growth cones as possible sites for transduction

An important question the results raise is how transduction of electrical signals occurs between skin cells and Rohon–Beard neurons – between non-neural epithelium and sensory neurites projecting to the CNS. The striking parallel in timing between the developmental loss of direct skin impulse signal access to the spinal cord and the spread over the body surface of sensitivity to gentle stimuli has been described above. An important feature of the change in sensitivity is the loss of growth cones from the ends of the growing Rohon–Beard neurites as their free nerve endings differentiate. One possibility this raises is that the large and complex growth cones of immature Rohon–Beard cells (Roberts and Taylor, 1983; Roberts and Patton, 1985) might provide a large surface area of membrane that is sensitive to electrical signals produced by impulses in the overlying epithelium. As the peripheral Rohon–Beard neurites grow, they penetrate the basal lamina of the skin through pre-existing holes. Their subsequent progress between skin cells and the form of their contact with skin cells is not yet known. The free nerve endings of mature Rohon–Beard neurons make intimate contact with invaginations in individual skin cells. However, it seems that these do not provide a functional electrical connection. Given the much larger surface area of growth cones, perhaps field potentials from impulses in overlying skin cells might depolarise growth cones sufficiently to evoke impulses. The direction of growth cone movement is certainly sensitive to electrical fields (McCaig et al., 2002), and this might make them sensitive to larger field potentials produced by epithelial impulses, particularly if they expressed appropriate voltage-gated Na^+ channels (Zhang et al., 1996). Some growth cones are already known to express ion channels, including voltage-gated Na^+ channels, that allow them to generate impulses (Feigenspan et al., 2010). The particular features of immature Rohon–Beard neurons that allow sensitivity to skin impulses remain to be determined.

Behavioural significance of the skin impulse system

Once the Rohon–Beard neurons have completed their innervation of the trunk and tail skin, paralleled by innervation of the head skin by trigeminal touch receptors (Roberts, 1975; Roberts, 1980; Davies et al., 1982), tadpoles have the ability to detect touch over the whole body surface. Before this, skin impulses and their detection by the CNS provide tadpoles with the precocious ability to at least detect and respond to stronger, noxious stimuli, presumably aiding survival. The system has some advantages: the shortest route from the stimulus to the CNS is automatically used, and the conducting system can be damaged considerably without preventing all conduction (Spencer, 1974). An additional benefit we have noted here might be that a skin impulse can extend the effective duration of a brief stimulus, producing an extended period of Rohon–Beard firing. The recordings from dorsal ascending axons suggest that quite large numbers of Rohon–Beard neurons might respond to a single skin impulse and do this over tens of milliseconds. A very brief stimulus can produce Rohon–Beard firing over an extended period and might make the sensory system more sensitive and reliable.

CONCLUSIONS

In summary, our evidence points to immature Rohon–Beard sensory neurons as providing a route early in development by which skin impulse signals enter the CNS of young *Xenopus* tadpoles and subsequently elicit a motor response. The features of these immature neurons and their relationship with the skin that allow transmission of electrical signals between epithelium and neuron remain unknown. The possibility that this occurs via the growth cones of the Rohon–Beard neurons is currently under investigation. This question of transduction is not restricted to tadpoles. In the hydrozoan *Sarsia tubulosa*, epithelial impulses must somehow excite neurons to produce appropriate tentacular movements associated with ‘crumpling’ responses (King and Spencer, 1981). Here too, it is still not known how transmission between epithelium and neuron occurs (Mackie, 2004). Whatever the mechanism involved in the tadpole, we suggest that Rohon–Beard neurons provide a common route for entry into the CNS allowing detection of different mechanical stimuli at the body surface to trigger a behavioural response. Initially, they mediate nonlocalised detection of noxious

stimuli through skin impulses, but later the same neurons provide a more refined, localised detection of weaker touch stimuli – and detection involving skin impulses then occurs entirely by means of the brain.

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