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Development of Hindlimb Locomotor Activity in the Bullfrog (*Rana catesbeiana*) Studied in vitro

Abstract. *The isolated central nervous system of the bullfrog larva (tadpole) is a valuable model system for studying the development of central motor control because the neural activity for locomotion is expressed in vitro. Patterned synaptic activation of immature hindlimb motoneurons is present before the bones and muscles of the hindlimb differentiate, and it develops against the background of the tadpole's functionally mature motor program for tail oscillations. This activation of hindlimb motoneurons later produces patterned bursting that underlies coordinated stepping and frog kicks.*

The frog occupies the evolutionary niche between aquatic and terrestrial vertebrates, a phylogenetic position that is reflected in the frog's locomotor development. The larval frog (tadpole) swims by means of tail oscillations, whereas after metamorphosis, the frog walks, hops, and swims using the hindlimbs either alternately (stepping) or synchronously (frog kicks). These two patterns of hindlimb coordination are the basis of the locomotor gaits used by all terrestrial quadrupeds (1). In this report we describe the developmental changes in central nervous system (CNS) motor activity that are associated with the frog's transition from aquatic to terrestrial locomotion at metamorphosis.

We have previously shown, using this in vitro CNS preparation, that tail beating in the tadpole is controlled by an endogenous locomotor program distributed throughout the spinal cord and expressed only by the axons of motoneurons that innervate the axial swimming musculature (2). This program produces alternate contractions of the axial musculature on the left and right sides of the body and tail and coordinates intersegmental activity of the muscles (2). Regulation of tail-beat rate and modification

of intersegmental coordination result from sensory interactions with the central locomotor program (3).

Motoneurons innervating the axial

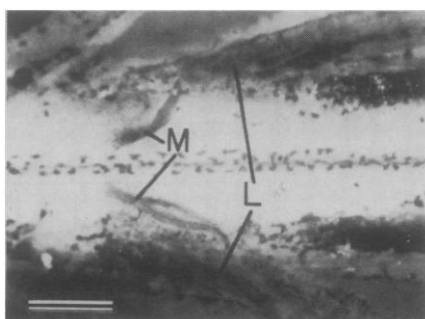


Fig. 1. Ventral spinal cord showing the medial and lateral rootlets that compose the ventral roots of the lumbar enlargement of the tadpole. The medial rootlets (M) contain only axons of primary motoneurons and innervate axial muscles that produce tail beating in the tadpole. The lateral rootlets (L) are composed of axons of lateral motor column motoneurons that innervate muscles of the hindlimbs. These two rootlets are normally enclosed in a common meningeal sheath, but can be dissected free of each other to be studied independently in electrophysiological experiments. The medial rootlets have been stained with toluidine blue for clarity. Calibration bar, 500 μ m.

swimming musculature are found at all levels of the spinal cord and are anatomically distinct from the lumbar motoneurons that innervate the hindlimbs (4, 5). The former (primary motoneurons) are born during embryonic stages, whereas hindlimb motoneurons that form the lateral motor column (LMC) proliferate and differentiate during larval life (5). In the lumbar enlargement, axons of these two populations of motoneurons exit the spinal cord in discrete rootlets that combine to form the ventral roots (2, 4). The medial rootlets that contain axons of primary motoneurons can be dissected free of the lateral rootlets that contain axons of hindlimb motoneurons (Fig. 1), and the electrophysiological activity of motoneurons subserving tail beating or hindlimb locomotor behavior can be studied independently (2).

We examined the development of the pattern generator for interlimb coordination of hindlimb locomotor activity and its relationship to the existing motor program for tail beating. We simultaneously recorded spontaneous activity of the medial and lateral rootlets of the ninth ventral roots in 101 isolated CNS preparations maintained in oxygenated Ringer solution (2, 3). Axons of primary motoneurons in the medial rootlets exhibited episodes of patterned bursting (2) until metamorphosis, consistent with the continued use of the tail for locomotion (Fig. 2, A to C).

In contrast to the unchanging pattern of bursting in primary motoneurons, we found that the activity of hindlimb motoneurons changes dramatically during larval development. Prior to stage VIII (6), activity in the lateral rootlets increased with the onset of medial rootlet bursting, but showed little evidence of patterned bursting. Slow potentials recorded from the axons of hindlimb motoneurons, however, revealed unmistakable rhythmicity by stage III (Fig. 2A), indicating that at least some of the hindlimb motoneurons are receiving patterned synaptic inputs at that time. Between stages VIII and XIV, spike activity gradually increased near the peaks of the slow potentials so that by stage XIV a clear pattern of alternating burst activity was evident (Fig. 2B). We determined phase relationships between bursts in different nerves by dividing the latency to burst onset by the total cycle period, using the onset of medial rootlet bursts as reference points. Both the alternating bursts and the rhythmic depolarizations that preceded were phase-shifted 180° from the homolateral medial rootlet bursts [mean phase \pm standard error = $0.50 \pm .01$, $N = 93$] and always occurred at the same fre-

quency as the medial rootlet bursts. The difference in phase between homolateral medial and lateral rootlet bursting eliminates the possibility that activity attributed to hindlimb motoneurons was an artifact of activity in primary motoneurons. Synchronous bursting of the left and right lateral rootlets was seen only in tadpoles at stage XVII or beyond and was frequency- and phase-locked to medial rootlet bursts (homolateral and heterolateral mean phase, 0.08 ± 0.02 , $N = 19$; Fig. 2C). These two modes of lateral rootlet bursting parallel the two modes of hindlimb locomotor behavior—stepping and frog kicks.

We used the isolated CNS with the peripheral nerves to the hindlimb intact to compare patterned burst activity of the seventh ventral root (to monitor activity of the motor program for tail oscillations), the crural nerve (innervating hip flexors), profundus anterior (innervating hip flexors and knee extensors of the anterior thigh), and profundus posterior (innervating hip extensors and knee flexors of the posterior thigh) of the ipsilateral hindlimb. Bursting in the seventh ventral root and crural nerve showed that hip flexion would occur in virtual synchrony with ipsilateral trunk flexion (mean phase, 0.07 ± 0.01 , $N = 16$). Anterior and posterior profundus nerves each innervate muscles that act in opposite ways on hip and knee joints (7). Thus, some of the axons constituting

each nerve will be active during the extension phase of stepping and others during the flexion phase. Nonetheless, bursts alternating with primary motoneuron activity could often be distinguished on the basis of their size and shape from bursts synchronous with primary motoneuron activity (Fig. 2D). The mean phase for bursts nearly synchronous with ipsilateral primary motoneuron activity was $0.11 (\pm 0.01, N = 16)$ in both hindlimb nerves and $0.46 (\pm 0.02, N = 16)$ for bursts occurring in the interburst interval of primary motoneurons. Videotape analyses show that stepping is the only rhythmic hindlimb behavior exhibited *in vitro* by preparations consisting of the isolated CNS with the hindlimbs, caudal trunk, and their motor innervation intact. Stepping occurs only in conjunction with operation of the central motor program for tail oscillations, and limb flexion is nearly synchronous with ipsilateral trunk flexion (8). Thus, the peripheral nerve activity shown in Fig. 2D is probably the output of a pattern generator for alternate extension and flexion of the hindlimb during stepping.

We calculated the proportion of preparations that spontaneously exhibited each of the different burst patterns throughout larval development (Fig. 2E); the motor program for tail oscillations was reliably active from the very earliest stages to stage XII, when the tail is rapidly degenerating. The burst activity

that apparently underlies hindlimb stepping emerged next, reaching a peak at stage XV and then declining. Last to develop was the neural activity that apparently underlies frog kicks, which reached a peak between stages XVI and XX. That all types of spontaneous activity declined during metamorphic climax is consistent with our failure to find spontaneous patterned activity in the isolated CNS of the adult bullfrog and with the decline in activity during late embryonic development in other species (9). This decline could result from further maturation of intrinsic spinal cord circuitry, from suppression of spontaneous activity by the maturing brain, or from both (9, 10).

Because behavioral use of the hindlimbs does not normally begin until near the end of the 2- to 3-year larval period (8, 11), we were surprised to find that rhythmic slow potentials in hindlimb motoneurons were present as early as stage III. The hindlimbs of a stage III tadpole (the youngest we examined) are primitive buds consisting of undifferentiated mesenchyme, and the peripheral nerves that have just invaded the limb bud appear unorganized (12). At this stage, hindlimb motoneurons are small, tightly packed, and have few somatic synapses (13). Proliferation, migration, differentiation, and death of hindlimb motoneurons proceed until stage XV (14). Thus, the rudiments of the presumptive interlimb

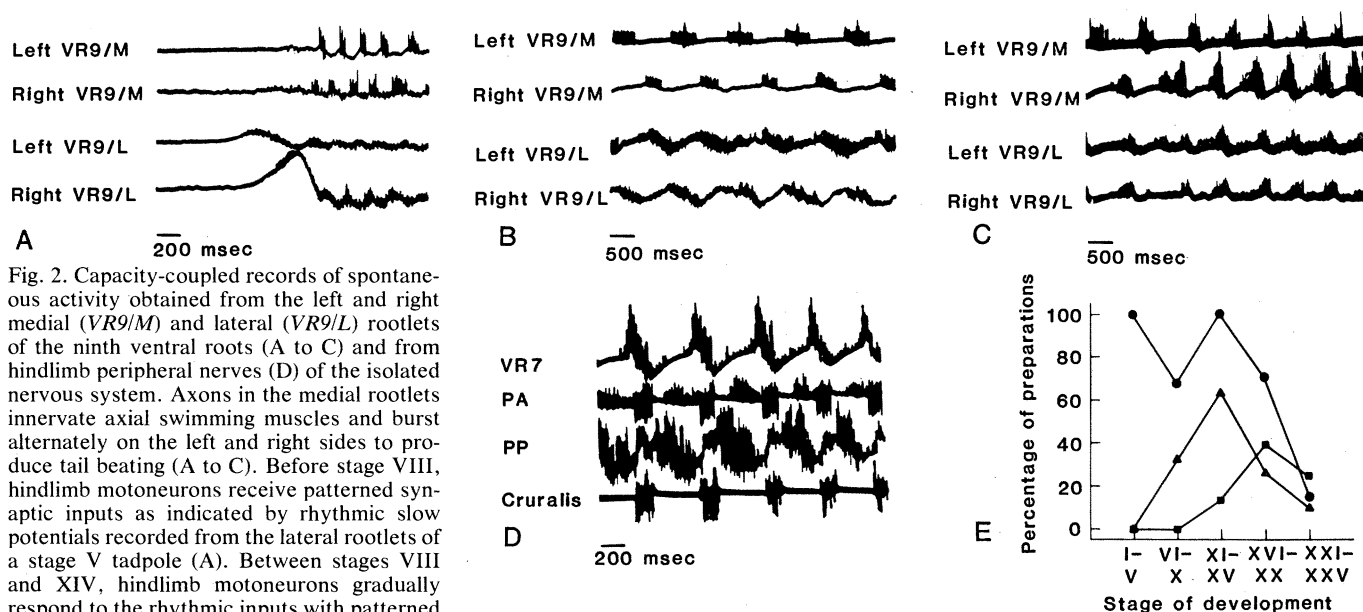


Fig. 2. Capacity-coupled records of spontaneous activity obtained from the left and right medial (VR9/M) and lateral (VR9/L) rootlets of the ninth ventral roots (A to C) and from hindlimb peripheral nerves (D) of the isolated nervous system. Axons in the medial rootlets innervate axial swimming muscles and burst alternately on the left and right sides to produce tail beating (A to C). Before stage VIII, hindlimb motoneurons receive patterned synaptic inputs as indicated by rhythmic slow potentials recorded from the lateral rootlets of a stage V tadpole (A). Between stages VIII and XIV, hindlimb motoneurons gradually respond to the rhythmic inputs with patterned bursts of spike activity (B). In every preparation, the alternating slow potentials and bursts of LMC motoneurons were shifted approximately 180° with respect to homolateral medial rootlet bursts. From stage XVII on, both alternating and bilaterally synchronous bursting were seen (C). Both modes of activity continued to be phase- and frequency-locked to the bursts of primary motoneurons. Parts (B) and (C) were obtained from the same stage XVII preparation. The data of (D), which were obtained from the isolated nervous system of a stage XVII tadpole, show patterned bursting in profundus anterior and profundus posterior (the two sciatic nerve branches of the thigh), cruralis, and the ipsilateral seventh ventral root (VR7). Computation of the proportion of preparations (stages I to V, $N = 5$; VI to X, $N = 21$; XI to XV, $N = 11$; XVI to XX, $N = 33$; XXI to XXV, $N = 20$) that showed patterned bursting in the medial rootlets and alternate and synchronous bursting in the lateral rootlets (E) shows that the motor program for tail oscillations (●) was present at the earliest larval stages, followed by the emergence of neural activity underlying stepping (▲) and finally frog kicks (■). All forms of spontaneous activity declined at metamorphic climax.

motor program are apparent despite the undifferentiated periphery and immaturity of hindlimb motoneurons.

The early appearance of the pattern generator for coordination between the hindlimbs, activation of this pattern generator only during operation of the locomotor program for tail oscillations, and the rigid phase and frequency locking between bursts of primary and hindlimb motoneurons all suggest that the two pattern generators may be identical or share common neuronal elements. However, the experiments are only suggestive and do not rule out the possibility that the tail and developing hindlimbs are coordinated by separate, discrete mechanisms that are phase coupled and activated by a common source. In either case, the late-developing bilaterally synchronous bursting of the hindlimb motoneurons probably represents a modification or addition to the mechanisms coordinating alternate stepping.

A behavioral parallel to the sequence of electrophysiological activity described here is found in the successive development of axial muscle flexions during swimming, alternate stepping, and then frog kicks (8). The sequential appearance of lateral trunk flexions, lateral trunk flexions accompanied by limb movements, and limb movements without lateral trunk flexions has been amply documented in humans and other species (9, 15). Thus, the results of further experiments with this preparation may provide insights into the organization and early development of locomotion in other vertebrate species.

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Thermoregulation and Mate-Selection in Fowler's Toads?

Fairchild (1) has presented evidence that large male Fowler's toads (*Bufo woodhouseii fowleri*) are more often found in amplexus with females than are small males. While patterns such as this have previously been attributed to male-male competition for mates (2), Fairchild has chosen to explain this pattern as resulting from female choice of larger males. He has supported this assertion with data showing that females more frequently respond to recorded mating calls of large males than to those of small males (3). Thus, he has concluded that the assumed mate selection is based on the quality of calls made by males at the breeding site; the quality of the calls is influenced by both the size and body temperature of the male toad (4). Fairchild further suggests that males thermoregulate to make their calls more attractive to females. Specifically, he hypothesizes that males choose colder microclimates in order to cause their calls to sound like those of a larger male. However, we feel that the evidence presented to support this hypothesis is inadequate. We also believe the assertion that female Fowler's toads select large males with which to mate is not supported by the evidence presented.

Fairchild presented data [table 1 in (1)] to show that larger males were found significantly more often in cold environments. His analysis of the table gave a G statistic of $G(4) = 20.52$, $P < .005$. Using the data reported, we calculate a G statistic of $G(2) = 3.15$, $P > .10$. Thus, while five out of eight large males were found in cold environments, this pattern could have occurred if males randomly selected thermal environments.

A second line of evidence for thermoregulation was that males found in the breeding pond were significantly larger than those on adjacent banks. The breeding pond was found to have a water temperature of 20°C, whereas the air temperature above the bank was 22°C.

Aside from the fact that this modest difference in temperature was determined with a thermometer graduated to only 1°C, the explicit assumption that these measurements of the environment reflect body temperature is not warranted (5). It seems likely that the high thermal conductivity of water would have caused toads swimming in water to assume the temperature of their ambient fluid, but toads on the bank should exchange energy with their environment by convection, radiation, evaporation, and conduction. Thermal radiation to the cold night sky and evaporation easily could have caused these toads to have body temperatures more than 2 degrees below that of the ambient air (5). Thus, without measurements of body temperature, it seems that conclusions concerning behavioral thermoregulation are premature.

Finally, the pattern of large males being found in the breeding pond is consistent with several alternative hypotheses. For example, dominant (larger) males could exclude subdominants from suitable breeding sites which happen to be in the water. Thus, while Fairchild suggests that large males are found in the pond as a result of their "selecting colder calling sites," an alternative is that large males are found in the pond because the pond is the only adequate site for aquatic egg laying (6). This alternative does not require the existence of behavioral thermoregulation to explain males being found in the pond (7). Thus, alternative hypotheses exist that could explain the pattern of male-female associations and distributions of males around the pond, and none of these hypotheses have been tested.

In conclusion, announcement of "the first documented case of behavioral thermoregulation in mate selection by amphibians" seems unwarranted, and the mechanism by which large male Fowler's toads are found in amplexus more often than small males is not resolved.