Coordinated Motor Output in the Hindlimb of the 7-Day Chick Embryo
(neuroembryology/neurophysiology/neural specificity/neuroethology)

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ABSTRACT Electromyographic recordings from individual identified ankle muscles of the 7-day chick embryo (stage 31) were used to determine the organization of motor output at a developmental stage shortly after the onset of spontaneous motility in the leg. During spontaneous motility of the embryo, the electromyographic recordings from the gastrocnemius, peroneus, and tibialis muscles displayed bursts of motor unit activity which alternated with periods of little or no activity. Since control of skeletal muscle in the chick embryo is neurogenic rather than myogenic, these findings imply that the motorneurons to a given muscle are driven by a common source. Since flexor and extensor muscles are activated at different times, different central connections to flexor and extensor motoneurons must be present in the central nervous system of the 7-day embryo. Moreover, since inhibition is known to play an important role in the selective activation of agonist and antagonist muscles, the present results suggest that functional inhibitory synapses may be present in the lumbosacral central nervous system at this stage of development. The basic pattern of muscle activation observed in the 7-day embryo is similar to that seen in older embryos. Since these patterns appear prior to the time at which motor responses to sensory stimulation of the leg can be demonstrated, it is likely that the neural pattern-generating circuits for selective activation of muscles are established in the central nervous system without reliance on functional reflexes.

Direct observations of motility in embryos of higher vertebrates give the impression that their overt behavior is unorganized (1). For instance, the movements of wings and legs in chick embryos do not appear to be coordinated with each other until near hatching time, and the jerky movements of the legs do not give the impression of coordinated muscle activity. However, behavioral observations have severe limitations; the actual state of neural organization at a given embryonic stage can be assessed only by electrophysiological methods. The present study was undertaken to examine the neural organization of motor systems in the specific case of very early leg movements of the chick embryo using the method of electromyographic (EMG) recordings. The 7- to 7.5-day embryo (stage 31, Fig. 1) was chosen because this stage is close to the inception of overt motility in the immature leg. It was possible to place suction electrodes on individual identified muscles of the hindlimb in the intact embryo during spontaneous movements in situ and to obtain EMG recordings for prolonged periods. The experimental design was to record simultaneously from two synergists or from two antagonists operating on the same joint, in order to answer the question of whether or not the motor system is organized at that stage. In fact, we observed coactivation of synergists and phase lag in the activation of antagonists. These positive results permit inferences concerning the existence of central neuronal organization in the spinal motor system. It is of special interest that sensory feedback through reflex circuits is not yet completed in the 7-day embryo.

Overt motility in the leg begins with weak spontaneous flexions and extensions at stages 28+ and 29 (6 days; refs. 2 and 3); they are performed in conjunction with body movements, but not during every activity phase. A day later (stage 31), the movements are more pronounced. At that stage the different parts of the leg are discernible; the toes are demarcated but still part of a toe plate (Fig. 1). The cartilage anlagen (except for phalanges) are formed and the articulations of femur, tibia, and fibula are almost completed. The individual muscles have just become separated, myotubes are formed, and cross striation in muscle fibers has become visible during the preceding day. The nerve pattern is nearly completed and nerve fibers have contacted the myotubes.

MATERIALS AND METHODS

White Leghorn chicken eggs obtained from a local hatchery were incubated in a forced-draft incubator maintained at 37 ± 1°C and 60% humidity. Eggs were removed from the

Abbreviation: EMG, electromyographic.

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FIG. 1. Photograph of a 7-day stage 31 (4) chick embryo. Crown to rump length: 17 mm.
stored on magnetic tape with a Teac 3340 recorder. The signals were played back on an oscilloscope and filmed with a kymograph camera. Recordings were made from 11 embryos, of which six provided unambiguous evidence of patterned motor output. In five cases, the results were ambiguous, possibly due to inaccurate electrode placement or muscle damage.

For a quantitative measure of the temporal sequence of activation of the pair of synergist muscles, gastrocnemius and peroneus, phase relationships with respect to onset of EMG bursts were analyzed. Each EMG record was divided into 100-msec intervals, and the average frequency of motor unit activity in each interval was determined by counting spikes according to the method of Bergström (6). The onset of an EMG burst was defined as the beginning of the first interval in which the average frequency was 40 Hz and was followed by one or more intervals of at least 40 Hz activity.

The termination of a burst was defined as the end of an interval with average frequency of 40 Hz if that interval was followed by more than one interval with average frequency less than 40 Hz. The phase of the peroneus with respect to gastrocnemius (Per/Gast) was defined as the latency divided by the period. The period (T) of one cycle of gastrocnemius activity was defined as the time between the onset of two successive gastrocnemius bursts within an activity period. The latency (L) was defined as the time from the onset of a gastrocnemius burst to the onset of the subsequent peroneus burst (see inset, Fig. 3). According to this definition, a phase equal to zero or one means that the two muscles begin their activity at the same time. Other values of phase indicate that the muscles are activated at different times. The phase values were grouped into 10 bins of 0.1 phase each, and a histogram was plotted to show the distribution of phase in activity periods recorded from two embryos (Fig. 3A).

The same procedure was followed for determining the phase relationships for the pair of antagonist muscles, gastrocnemius and tibialis. As described above, the period (T) of gastrocnemius was used. The latency (L) was defined as the time from the onset of a gastrocnemius burst to the onset of the subsequent tibialis burst. Phase of tibialis with respect to gastrocnemius (Tib/Gast) was defined as latency divided by period. A phase histogram was plotted from data obtained from three different embryos (Fig. 3B).

The leg was stimulated either by stroking the dorsal or plantar surface of the foot with a glass needle drawn out to a long flexible tip, or by a quick passive movement either of the whole leg, or of the foot alone.

RESULTS

EMG recordings (Fig. 2) obtained during spontaneous motility from identified muscles in a 7-day (stage 31) chick embryo show that each muscle is activated by bursts of motor unit discharge. The bursts in different muscles occur at specific times relative to one another. The recordings made from the two ankle extensors, gastrocnemius and peroneus, reveal that the onset of activity in these synergists tends to occur at the same time (Fig. 2A). In contrast, recordings made from the ankle flexor, tibialis, and the ankle extensor, gastrocnemius, reveal that the onset of activity in these antagonists tends to occur at different times (Fig. 2B).

A quantitative analysis of the pattern of activation, based on phase relationships (Fig. 3), shows that activation of
peroneus with respect to activation of gastrocnemius tends to occur near phase zero or one (Fig. 3A). In contrast, the phase histogram of tibialis activation measured relative to gastrocnemius activation shows that tibialis tends to be activated at phases intermediate between zero and one (Fig. 3B).

The histograms confirm the impression gained from visual inspection of the EMG records.

The EMG recording technique was also used to test whether sensory stimulation of the leg of the 7-day embryo was capable of reflexly activating motor units. There is considerable behavioral evidence that sensory stimulation will not evoke a reliable behavioral response in the hindlimb of the 7-day (stage 31) chick embryo (2, 7–9). It is possible that in the stage 31 embryo sensory stimulation did activate the discharge of some motor units, but that the resulting contraction was too weak to be detected by visual observation of overt behavior. Therefore, EMG recordings were made from gastrocnemius and tibialis muscles during gentle stroking of the leg and during rapid passive flexions or extensions of the ankle joint at that stage. The stimulation was performed during an inactivity period so that any change in motoneuron discharge frequency could be readily detected. No motoneuron discharge was evoked by the stimulation. In embryos at later stages of development, stimulation led to distinct movements and, therefore, necessarily involved motoneuron discharge.

**DISCUSSION**

The following inferences can be drawn concerning the organization of the central nervous system are based on earlier evidence that contractions of skeletal muscle in the early chick embryo are neurogenic and not myogenic (10–12), and it may be added that if in 7-day embryos the sciatic nerve is cut, both EMG activity and contractions in muscles distal to the cut are abolished (Bekoff, in preparation).

The present experiments were designed to explore the degree of central organization existing in the embryonic spinal cord near the onset of motility. The first slight spontaneous movements in the three major segments of the leg are observed at 6 days (stage 28+) and 29; refs. 2 and 3). By stimulating the ventral roots, Morris and Landmesser (13) have shown that functional neuromuscular contacts are present at 5–6 days of incubation, and there is a high degree of selectivity in nerve outgrowth and synapse formation. Histological observations show that at 7 days practically all muscles are established and individual muscles receive nerve branches (3). From all these data one can infer that at 7 days there already exist in the lumbar lateral motor column distinct motor pools that are specified for particular muscles.

Monitoring spontaneous motor output to identified muscles permits a further step in the analysis of central circuitry. We have observed in the 7-day embryo that synergistic muscles tend to be activated simultaneously, while antagonistic muscles tend to be activated with phase lag. Two inferences can be drawn. First, since many axons from a particular motor pool are coactivated, it is likely that the members of a pool have common input from a presynaptic interneuronal center. There may also be electrical coupling among the neurons of the motor pool. Second, since antagonistic muscles are activated with phase lag, it is likely that there exist at least two distinct interneuronal centers controlling the several motor neuron pools. Moreover, these centers must be linked with each other and with motor neuron pools in an orderly fashion, involving both excitatory and inhibitory synapses. This assumption is based on findings in other systems that selective activation of agonists and antagonists is generated by a network using both excitatory and inhibitory synapses (14–16). While it may be possible to explain a phase lag between antagonists in terms of excitatory synapses and "fatigue" of selected elements, such an explanation would require additional assumptions for which no data exist. On the other hand, Oppenheim et al. (17) have found spherical and flattened vesicle-containing synapses in the ventral lumbar cord of 7-day embryos, and suggested the existence of excitatory and inhibitory synapses at this early stage. The essential point is that coactivation of synergists and phase lag of antagonists are observed at a stage shortly after the inception of spontaneous leg motility.

While some of the basic elements of the neural circuitry necessary for later integrated behavior, such as hatching or walking, apparently are present at this early stage, the system is far from perfect. The EMG recordings (Fig. 2) show that the alternation of antagonists is quite imprecise, with partial overlap in some phases. A study of EMG recordings in subsequent stages (Bekoff, in preparation) gives a picture of progressive refinement of the patterns of muscle activation. So far, the investigation has been confined to intralimb muscle coordination. It will be of interest to study the origin of integration of movements in both hindlimbs which is essential for the hatching process and later locomotion.

The central organization that we infer was not predictable.
either from observations of overt behavior (1, 2) or from the existing electrophysiological recordings from the lumbar spinal cord (12, 18). The latter have shown that patterns of polyneuronal bursts are closely correlated with behavioral activity periods. The neural recordings monitored a population of unidentified neurons in the ventral spinal cord; this methodology did not permit recognition of temporal patterning of neuronal subunits serving specific muscles. Our EMG recordings did, in fact, reveal such a patterning. There is no discrepancy between the spinal cord recordings and the EMG recordings presented by us. The difference between the results is a function of the higher resolving power of the EMG recordings in the early embryo and may be explained by the fact that the electrodes used by Provine and Ripley (12, 18) for the central nervous system recordings simultaneously sampled neuronal discharges from cells with different phase relationships.

The neural organization that we postulate seems to have been built into the central nervous system prior to the completion of reflex circuits. Preyer (7) discovered a pre-reflexogenic motility period in the chick embryo. While the first movements (neck muscle contractions) begin at 3.5 days, exteroceptive stimulation of the first region to become sensitive (the head) does not elicit behavioral responses before 7.5 days (19). Stimulation of the thigh elicits the first weak responses at 7.5–8 days (8, 9), that is, shortly after our EMG recordings were made. However, the possibility that sensory neuron discharge occurs already in stages preceding overt responsiveness to exteroceptive stimuli cannot be excluded. Light microscope studies of the formation of reflex arcs are equally inconclusive. Silver impregnation studies indicate an ingrowth of dorsal root collaterals into the gray matter between 6 and 7 days (20, 21). Serial electron micrographs identifying the developmental stage and the region at which the reflex circuits are closed are not yet available for the chick embryo. Nevertheless, it seems safe to assume that at 7–7.5 days (stage 31) the synaptic closure of the reflex circuits in the lumbar sacral cord is, at best, only beginning. This suggests that the neuronal organization that exists at this stage has developed independently of sensory input. Deafferentation experiments of the hindlimb (9, 22) are consistent with this view in that they have demonstrated a normal morphological differentiation of the lateral motor column and of ventral roots, and typical spontaneous embryonic motility, in the absence of sensory ganglia. Observations on the ontogeny of coordinated leg movements in amphibians (23), of flight and calling song in crickets (24), of flight in locusts (25), and of the origin of motor control in lobster swimmerets (26, 27) have led to the same conclusion: sensory input is not required for the development of coordinated motor output.

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