Fast Locomotor Burst Generation in Late Stage Embryonic Motility

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Bradley NS, Ryu YU, Lin J. Fast locomotor burst generation in late stage embryonic motility. J Neurophysiol 99: 1733–1742, 2008. First published February 13, 2008; doi:10.1152/jn.01393.2007. We examined muscle burst patterns and burst frequencies for a distinct form of repetitive leg movement recently identified in chick embryos at embryonic day (E)18 that had not been previously studied. The aim was to determine if burst frequencies during repetitive leg movements were indicative of a rhythm burst generator and if maturing muscle afferent mechanisms could modulate the rhythm. Electromyographic recordings synchronized with video were performed in ovo during spontaneous movement at E15, E18, and E20. Multiple leg muscles were rhythmically active during repetitive leg movements at E18 and E20. Rhythmic activity was present at E15 but less well formed. The ankle dorsi flexor, tibialis anterior, was the most reliably rhythmic muscle because extensor muscles frequently dropped out. Tibialis anterior burst frequencies ranged from 1 to 12 Hz, similar to frequencies during fast locomotor burst generation in lamprey. The distribution in burst frequencies at E18 was greatest at lower frequencies and similar to locomotor data in hatchlings. Relative distributions were more variable at E20 and shifted toward faster frequencies. The shell wall anterior to the leg was removed in some experiments to determine if environmental constraints associated with growth contributed to frequency distributions. Wall removal had minimal impact at E18. E20 embryos extended their foot outside the egg, during which faster frequencies were observed. Our findings provide evidence that embryonic motility in chick may be controlled by a fast locomotor burst generator by E15 and that modulation by proprioceptors may emerge between E18 and E20.

I NT R O D U C T I O N

Body movements generated during embryogenesis are collectively referred to as embryonic motility. It begins embryonic day (E)3 in the chick and by E6 includes flexion and extension of the leg (Bekoff 1976). Throughout most of development, movements look random, jerky and small in amplitude, a pattern also referred to as type I motility (Hamburger and Oppenheimer 1967). Yet by E9, leg movements are produced by alternating flexor/extensor muscle activity and coupled joint rotations at the hip, knee, and ankle at frequencies of 0.2–2 Hz (Bekoff 1976; Bradley and Bekoff 1990; Chambers et al. 1995). The muscle patterns and coupled rotations of hip and knee persist up through hatching, suggesting they are controlled by a pattern generating network (Bekoff 1976, 1992; Bradley 1999, 2001; Bradley et al. 2005; Sharp et al. 1999).

Spinal networks are sufficient to produce the leg movements (Hamburger et al. 1965, 1966; O’Donovan and Landmesser 1987). Early motility appears to be generated by excitatory properties of immature spinal neurons that are initially cholinergic, then glutamatergic by E8–E10 (Hanson and Landmesser 2003; Milner and Landmesser 1999; O’Donovan and Chub 1997; O’Donovan et al. 1994). It is thought the spontaneous build up of excitation within a recurrently connected network of neurons initiates a bout of motility, and activity-dependent depression then slowly builds and terminates the bout (O’Donovan and Chub 1997; Tabak et al. 2000). Excitatory network dynamics continue up to at least E13 and possibly as late as at E15 (Hanson and Landmesser 2003; O’Donovan 1989; O’Donovan and Chub 1997).

The later half of embryonic development is intriguing as the chick advances to hatching and precocious walking (Jacobson and Hollyday 1982; Muir et al. 1996). Persistence of timing irregularities in EMG (Bekoff 1976) and kinematics at later ages (Bradley et al. 2005; Sharp et al. 1999) have been difficult to reconcile with expectations of a locomotor pattern generator, leaving open the question of whether it controls embryonic motility (Bradley 1999; Bradley et al. 2005). The chick is a biomechanical system, and its relationship to the in ovo environment is under constant change, raising the possibility that physical constraints might also contribute to irregular attributes of motility (Bradley et al. 2005). We recently reported a distinct form of spontaneous repetitive limb movement at E18 not observed in younger animals that may advance progress on these issues (Bradley and Jahng 2003; Bradley et al. 2005). The leg movements and electromyogram (EMG) began abruptly and appeared ballistic; they were faster and more rhythmic than repetitive movements at earlier ages; at times, they resembled tremor or clonus; and they could be sustained for many cycles within an estimated frequency range of 2–10 Hz. Bekoff (1976) briefly noted EMG bursts at E19 consistent with our recordings; convulsive-like movements noted by Oppenheim (1972) also may be related. We were intrigued by the observation that the frequency range appeared to be equivalent to the combined frequencies for walking, swimming, and astepping in hatchlings (Johnston and Bekoff 1996). Additionally, the burst frequencies for the repetitive movements appeared to be within the range identified as the fast locomotor frequency in lamprey swimming (Cangiano and Grillner 2003), a network extensively characterized and that may provide insights into the control of these movements.

Thus in this study, we conducted the first focused examination of these distinct repetitive limb movements (RLMs) in embryos between E15 and E20 because available evidence suggested the movements likely arise within these ages. The goal was to determine if late stage limb movements are indicative of a rhythm burst generator and if maturing muscle afferent mechanisms can modulate the rhythm. We describe patterns of rhythmic activity in multiple leg muscles at E18 and E20 and...
emerging rhythmic activity at E15. Experiments are also presented that explored the possible impact of environmental constraints associated with body growth late in embryonic development. We discuss burst features of RLMs that collectively suggest embryonic motility in the chick during the later stages of development is controlled by a fast locomotor burst generator and that modulation by proprioceptors may emerge in the final days prior to hatching.

METHODS

Fertile Leghorn chicken eggs were incubated in a force draft, humidified incubator under standard conditions until experimenta- tion at E15, E18, or E20. Eggs were maintained in moist, heated chambers (37.5°C) during preparation and recording. Pulse rate and behavioral activity were monitored to verify optimal behavioral status. Staging criteria by Hamburger and Hamilton (1951; reprinted 1992) were used to verify embryonic age. All procedures were approved by the University Institutional Animal Care and Use Committee.

EMG and video recordings

Egg shell was removed to create a window over the embryo, and membranes were deflected to obtain a sagittal view of the wing and leg. Four muscles of the ipsilateral leg and wing were implanted with bipolar fine wire electrodes (50 μm silver) for EMG recording. Muscles implanted were selected from the following: sartorius (SA), hip flexor; femorotibialis (FT), knee extensor; tibialis anterior (TA), ankle dorsiflexor; extensor digitorum longus (EDL), ankle and digit dorsiflexor; lateral gastrocnemius (LG), ankle extensor; latissimus dorsi (LD), shoulder retractor; trapezius (TR), scapular retractor. The exposed leg was also marked for digitizing limb movements by inserting modified minimin pins at six locations (lower thoracic spine, hip, knee, ankle, foot). In a portion of experiments, the shell wall was more extensively removed anterior to the foot so that the leg would be free to extend beyond the egg during spontaneous movements (foot-free condition).

Embryos were transferred to a humidified and shielded Styrofoam chamber with a clear Plexiglas lid placed on a grounded anti-vibration table. A probe attached to a force transducer was placed in contact with the thigh to monitor body movement (Bradley and Jahng 2003). EMG, force, and video were synchronized during continuous recording and stored to disk (Datapac 2K2, Run Technologies). EMG signals were band-pass filtered (100–1 kHz), amplified (×2,000), and computer sampled at 4 kHz. Force output was amplified (×20,000), low-pass filtered (30 Hz) and computer sampled at 500 Hz. At the end of the experiment, after euthanizing the embryo, location of electrode tips was verified by dissection.

EMG analyses

EMG and the force trace of body movement were reviewed to identify all samples of repetitive muscle bursting in ≥1 EMG channel accompanied by repetitive leg movement. These sequences were then examined in the synchronized video file to verify that each EMG sample was accompanied by repetitive limb movement. EMG samples having four or more bursts that appeared to be similarly spaced in time were retained for analyses. EMG signals were then duplicated and rectified for dual signal display to analyze burst patterns. EMG burst onsets and offsets were automatically detected (Datapac 2K2), based on predefined burst criteria that we found through trial and error could be optimally applied in nearly all cases: burst threshold (2–3 times baseline amplitude), burst duration (20–400 ms), and interburst interrup- (20 ms). Cycle period duration was defined as the time between consecutive burst onsets (ms), and burst frequency (1/ cycle period duration) was calculated in cycles per second (Hz).

Statistical analyses of age and group effects were completed using the Student’s t-test and Bonferroni correction for multiple comparisons. Pearson correlations were used in linear trend analyses examining potential age-related changes in burst patterns. Significance was set at P < 0.05. Numerical summaries of data report group means ± SD. Data binned by frequency (Hz) for plotting were sorted as follows: 1 Hz (0–1 Hz), 2 Hz (1–2 Hz), etc.

RESULTS

Results represent the findings of 69 experiments that typi- cally produced ≥3–4 h of continuous recording at E15, E18, or E20. A total of 3,232 leg RLMs were identified, revealing a broad range of muscle activity patterns during spontaneous movement. At some point in most E18 and E20 experiments, rhythmic bursting was observed concurrently in all flexor and extensor muscles recorded (Fig. 1, A and D). Usually the pattern was sustained for three to four bursts, but occasionally the pattern was sustained for many bursts. For example, in Fig. 1A from an E20 control experiment, the two flexors (SA, TA) were synchronously active for 12 bursts and alternated with activity in two extensor muscles (FT, LG). The sample was preceded by 70 s of slower rhythmic SA and TA bursting and intermittent extensor bursting (not shown), and the sample was followed by another 10 s of bursting in all muscles at a slightly faster frequency than shown. It was common for only flexor synergists to burst rhythmically (Fig. 1, B and E). In such cases, activity in extensor muscles was either irregular or absent (Fig. 1, B and E). In Fig. 1B, for example, the flexors TA and SA were rhythmically co-active throughout the RLMs, low-amplitude FT bursting between initial flexor bursts eroded into irregular bursting, and LG activity was barely detectable. In many instances, LG was active before or after the RLMs, or briefly at the start, then dropped out (Fig. 1C). Occasionally extensor muscles where rhythmically active while flexors dropped out (not shown). Experiments included RLMs that paired knee extensor FT and ankle flexor TA for a portion of the RLMs. It was also common for large amplitude TA bursts to dominate the EMG pattern (Fig. 1C). Although E15 embryos generated many movement sequences, EMG for 14 experiments, totaling 38 h of recording, yielded few sequences of rhythmic bursting in at least one muscle; Fig. 1F is representative of the best samples.

The frequency of EMG bursting during RLMs appeared to vary from 1 to 8 Hz or more within experiments and to vary also across consecutive cycles within RLMs. In Fig. 1B, for example, TA bursting increased from 1 to 4 Hz over the first 10 cycles; a progressive slowing of burst frequency across cycles was also common (Fig. 1D). By far the most consistent observation during RLMs was the repetitive large amplitude bursting of the ankle flexor, TA (Fig. 1, B and C). Rhythmic TA bursting was the most reliable feature of RLMs at E18 and E20 during control and experimental conditions, making it the most amenable to a set of measurement criteria. The EDL was inadvertently implanted in one E20 embryo, and because the data were consistent with TA results at E20, the embryo was included in comparisons.

RLMs exhibited a broad frequency range

Automated burst analyses of rectified recordings at E18 and E20 detected a total of 21,819 TA bursts and indicated that TA
cycles mostly fell between 1 and 12 Hz. An example of the TA cycle period distribution for all RLMs identified during a single E18 control experiment is shown in Fig. 2A. In this example, the initial analysis of 358 TA cycles produced a frequency range of 1–19 Hz with a skewed distribution around a peak of 4 Hz. Using three a priori criteria for burst detection (burst threshold, burst duration, and interburst interval), we found that analyses often missed brief TA bursts clearly in rhythm with surrounding bursts and sometimes recognized small spurious bursts within otherwise rhythmic burst sequences. Also many RLMs included several irregularly timed TA bursts, especially at the start or end of a sequence, such as seen in the final three to four TA bursts of Fig. 1C. Because we wished to determine whether the temporal structure of rhythmic EMG during RLMs is similar to those for locomotor behaviors, we narrowed our focus to the most stable samples of repetitive activity. Thus only the portion of the RLMs in which the frequencies for three or more consecutive TA cycles yielded a

FIG. 1. Typical examples of electromyographic (EMG) and force recordings during spontaneous repetitive leg movements in 6 embryos. A: all flexor [sartorius (SA), tibialis anterior (TA)] and extensor [femorotibialis (FT), lateral gastrocnemius (LG)] muscles were active in this 10-s segment from an embryonic day (E) 20 embryo. Burst frequency averaged 1.8 Hz over 10 TA bursts. A force probe (F) was placed in contact with the hip to track body displacements and was used as an indicator of limb movements during review of EMG records. B: sustained bursting was observed in the flexor synergists in the absence of well-formed extensor activity in this 10-s record of an E20 repetitive limb movements (RLMs). Burst frequency averaged 3.2 Hz over 18 TA bursts. C: the TA was often the only muscle exhibiting robust rhythmic activity, as in this 5-s recording of an E20 RLMs. Average frequency for the 16 consecutive TA bursts between arrows was 9.1 Hz. D: alternating bursts of TA, LG, and eventually FT were rhythmically active in this 10-s segment from an E18 embryo. Average frequency for the 15 bursts between \( \downarrow \) was 2.9 Hz. The SA implant was lost in this experiment. E: rhythmic bursting of the flexor synergy is sustained in the absence of well-formed extensor activity and low-amplitude SA bursting in this 10 s record at E18. F: rhythmic activity was sustained in only 1 of 3 muscles implanted in a 5-s record at E15. LG bursts averaged \( \sim 5 \) Hz. EMG and force gain \( \times 2 \) (A–C), \( \times 4 \), (D–F). Vertical calibration 0.25 mV.
SD $\leq$ 1 Hz were retained. The effect of editing the E18 data in Fig. 2A is shown in Fig. 2B. Note that editing retracted the upper end of the frequency distribution (15–19 Hz) but retained burst frequencies between 2 and 9 Hz and the peak frequency of 4 Hz. In control experiments, the editing procedure retained 42% (5,246) of all TA cycles and 64% (1,147) of all RLMs. Within embryo (E18 and E20), editing retained 33% of all RLMs, but those retaining fewer than 10 RLMs were excluded from further analyses (7 embryos).

RLMs were more difficult to distinguish at E15 due to infrequent repetitive bursting in even a single muscle. In addition, many sequences of rhythmic or semi-rhythmic EMG were accompanied by little or no leg movement. We examined the latter sequences as well, speculating that they might be a developmental precursor to the more distinct RLMs at E18. In total, 596 sequences were identified in 13 of 14 embryos; however, 5 embryos produced no TA sequences, and 6 embryos produce only 1–4 sequences. In total, 59 sequences contained TA bursting that met criteria for inclusion, and half the sample came from a single E15 embryo.

Age effects

There was a small but significant shift toward higher TA burst frequencies from E18 to E20. At E18, the relative distribution of TA burst frequencies for 15 of 16 embryos largely fell between 2 and 6 Hz (Fig. 3A). At E20, in contrast, the distribution was more broadly dispersed between 2 and 11 Hz (Fig. 3B). Analyses for peak, average and median frequency yielded nearly identical trends and indicated that TA cycle frequency increased from E18 to E20. The average frequency shifted from 4.0 ± 1.0 Hz at E18 to 5.4 ± 1.9 Hz at E20, and the difference was significant ($P < 0.006$). The distribution of TA burst frequencies for the E15 embryo having the largest data set resembled that for E18 embryos, with 79% of cycles falling between 3 and 7 Hz (vertical bars, Fig. 3C).

Given the small TA sample, we examined rhythmic sequences in LG, FT, and LD, representing different subsets of embryos. Pooled across embryos, burst frequencies for all muscles fell between 2 and 12 Hz (line plots, Fig. 3C), and averages ranged from 4.2 to 4.8 Hz.

Sequences of stable TA bursting ($\pm$ 1 Hz) were typically brief, averaging four to five cycles at E18 and E20. However, all embryos generated stable sequences two or more times longer than the average length as illustrated by two experiments summarized in Fig. 4, A and B. Each vertical bar in Fig. 4, A and B, represents one RLM in order of occurrence from left to right, and bar height indicates the number of TA cycles per RLMs. In the E18 experiment (Fig. 4A), longest stable sequences of 5–10 cycles were variably distributed across $\geq$ 4 h of continuous recording. The longest stable sequences observed at E18 were composed of 16 consecutive cycles (not shown). In the E20 experiment (Fig. 4B), stable RLMs sequences were generated over $\geq$ 10 h and included sequences of 16 to 27 consecutive cycles, some of the longest recorded. In pooled TA data at E15, 54 of 59 TA sequences were three to four cycles long and the longest was eight cycles.
During E18 experiments, our impression was that longer bouts of stable TA bursting might be associated with a restricted range or preferred frequency similar to those for posthatching locomotor behaviors. In Fig. 4A, for example, the dotted black line, referenced to the right axis, indicates that the mean TA burst frequency hovered around 2–4 Hz during longer RLMs sequences. Faster frequencies (6–11 Hz) were only observed during shortest sequences. To further examine this trend, RLMs were re-ordered from shortest sequences to longest, and then within this sorting, sequences were ordered by TA burst frequency. To illustrate, E18 data in Fig. 4A are re-ordered and plotted in Fig. 4C. Data for the E20 experiment in Fig. 4B is re-ordered in D. Plots revealed two key points of interest. One, during brief sequences of three to four cycles, a broad frequency range (1–12 Hz) was expressed in all embryos. Two, during longer sequences, frequency range was more constrained and the range differed between ages. At E18, longer sequences were associated with the lower end of the frequency range (1–4 Hz). In Fig. 4C, for example, a TA burst frequency of ~1–2 Hz was observed during longest sequences.
of 8–10 cycles. At E20, in contrast, frequencies of 1–4 Hz were seldom observed, and sequences of five cycles or longer were associated with the upper end of the frequency range. In Fig. 4D, for example, the frequency range of 8–12 Hz was observed during sequences of 12–27 cycles.

**Foot-free frequency at E20**

We considered that the upward shift in RLMs frequencies at E20 might be partially attributed to increasing spatial constraints imposed on limb posture and movement during the final days of growth in ovo, and so we removed the shell wall anterior to the exposed leg and foot (foot-free). Following shell removal, all E20 embryos ($n = 11$) spontaneously extended the foot outside the egg for at least a portion of the recording, such that 74% of foot-free RLMs (496 RLMs) were produced while the foot was outside the egg. In contrast, E18 embryos ($n = 9$) exhibited little or no modification in leg posture after shell removal, and only 3% (13 RLMs) were generated while the foot partially extended outside the egg. Regardless of foot position, fewer TA cycles exhibited rhythmic stability under foot-free conditions (32 ± 11%) compared with control (42 ± 14%; $P < 0.003$), and only 13 of 21 embryos retained 10 RLMs or more with a SD ≤1 Hz after editing (6 embryos at E18, 7 at E20).

The distribution of TA burst frequencies after removing the shell wall at E18 (Fig. 5A) did not differ from control data (Fig. 3A). The average foot-free frequency was 3.7 ± 0.8 Hz. The broader frequency distribution at E20 (Fig. 3B) was also observed after shell removal (Fig. 5B), but the distribution shifted further upward 1–2 Hz (average frequency 6.5 ± 1.7 Hz). In nine experiments, both control and foot-free data were obtained and yielded similar results; examples are shown in Fig. 5, C and D. In the E20 experiment (Fig. 5D), TA burst frequency during control RLMs was 2.5 ± 1.7 Hz and increased to 4.3 ± 2.9 Hz after shell removal. An upward shift was observed in 4 of 6 embryos at E20, but in only 1 of 3 at E18, the others shifting downward (Fig. 5C). Although the upward shift at E20 was observed in average, median, and peak frequencies, comparisons fell short of significant ($P < 0.06–0.11$), possibly due to less well-defined frequency distributions after shell removal (Fig. 5B). Within-subject SDs increased from 2.0 Hz (control) to 2.7 Hz ($P < 0.02$) and peak frequency represented less of the total distribution, decreasing from 28 ± 6% (Fig. 3B) to 22 ± 6% of cycles after shell removal ($P < 0.04$).

**TA burst duration**

TA burst duration did not vary with age, condition, or TA cycle period. Note in Fig. 1D, for example, that TA burst durations did not lengthen as TA cycle period increased. Burst durations averaged 62–70 ms, with average deviations of 38–50 ms at E18–E20. Similar averages were also observed at E15. Linear trend analyses for TA burst duration and cycle period were performed for all embryos retaining ≥10 RLMs after editing ($n = 47$). A typical result is shown in Fig. 6. The
correlation coefficient ($R^2$) fell substantially <0.2 in 44 of 47 analyses, suggesting burst duration and cycle period did not linearly co-vary either by age or condition.

**DISCUSSION**

**RLMs share the frequency range of fast locomotor burst generation**

RLMs generated during spontaneous embryonic motility at E15, E18, and E20 expressed burst frequencies of 1–12 Hz during stable sequences of rhythmic activity. Cangiano and Grillner (2003) described the range of 2–12 Hz during fictive swimming in the lamprey hemicord as the “fast locomotor burst rhythm,” a range slightly greater than frequencies (1–8 Hz) during intact swimming (Orlovsky et al. 1999). The shared frequency range suggests that the chick also expresses the fast locomotor rhythm during embryonic motility in ovo. In support of this view, the lower portion of the RLMs frequency range was equivalent to cycle periods for walking and swimming in 1- to 3-day-old hatchlings, and the higher RLMs range during stable sequences of rhythmic activity.
was equivalent to shortest cycle periods during astepping (Johnston and Bekoff 1996). Also, TA burst duration, notably consistent across all three ages, was not modified even when the limb moved into an expanded workspace after removal of the shell wall anterior to the foot. TA correlations with cycle period (Fig. 6) were similar to those reported for walking in hatchlings, although modest positive correlations were reported for swimming and walking (Johnston and Bekoff 1996). TA burst duration does not typically follow changes in locomotor cycle duration (Grillner 1981) but can vary with cycle duration when a mechanical load is applied to ankle dorsiflexors during stepping (Musselman and Yang 2007). Thus TA burst frequency and duration during RLMs appeared to be controlled in a manner consistent with muscle activity controlled by a locomotor burst generator.

**RLMs share features common to burst deletions in fictive locomotion and scratch in adult animals**

We selected the ankle dorsiflexor (TA) for study because it generated the most consistently rhythmic activity of those recorded, and bursts were clearly associated with repetitive limb movements. All leg muscles were at times repetitively active during RLMs (Fig. 1, A and D), but extensors often dropped out (LG, Fig. 1C), generated bursts that fell below criteria for burst detection (FT, Fig. 1C; also flexor SA, Fig. 1D), or burst irregularly (FT, Fig. 1B). Instances where activity either dropped out or amplitude was very low appeared to be similar to “deletions” during fictive locomotion and scratching in adult animals. Deletions during fictive rhythmic activity were characterized as intervals when motor pool activity dropped out for one or more cycles (Lafreniere-Roula and McCrea 2005; Stein 2005; Stein and Daniels-McQueen 2002, 2004). The inconsistent participation of extensors (FT or LG) during RLMs appears to be similar to ankle extensor deletions that extended over many cycles during fictive locomotion and scratching in the cat (Lafreniere-Roula and McCrea 2005; their Fig. 4). Deletions appeared to be more common to extensor motor pools than to flexor pools in the cat; we observed a similar bias toward extensor deletions. However, conclusions must be tempered because we selectively examined records exhibiting stable TA rhythms and thus cannot directly address if the TA was similarly prone to deletions.

The persistence of rhythmic TA bursting during RLMs, even as extensor participation varied (Fig. 1, B–D), is also consistent with the modular model of spinal cord rhythogenesis inspired by studies of deletions in adult fictive preparations (Stein 2005). During fictive scratch in the turtle, hip extensor activity would suddenly drop out for one or more cycles, sometimes accompanied by either a knee flexor or knee extensor deletion. During the interval of a deletion, the burst duration of antagonist muscles was typically extended (Stein and Daniels-McQueen 2002, 2004). In the cat, the antagonist muscle was active during some deletions, but there were also instances when antagonist muscles remained silent during the deletion interval and maintained the burst rhythm preceding the deletion (Lafreniere-Roula and McCrea 2005). Persistence of rhythmic activity in one motor pool, while activity dropped out in antagonist pools acting at the same limb segment and/or synergists acting at adjacent limb segments, has provided evidence that each motor neuron pool controlling limb action is capable of functioning as a rhythmogenic burst module (Stein and Daniels-McQueen 2004). Forelimb flexor and extensor motor pools in the mudpuppy also exhibited independent rhythogenesis after they were surgically separated from one another (Cheng et al. 1998). Although the deletions have been studied primarily in fictive preparations, the hip extensor deletion has been observed during scratching motions in the spinal turtle (Stein and Grossman 1980). Our results extend these findings and suggest motor modules can also function independent of reciprocal inhibition in the neurologically intact and developing chick embryo. The brief and isolated rhythmic bursting we observed in leg muscles at E15 may indicate that the modules have just begun to express independent function apart from network population dynamics thought to control motility at earlier ages.

During both fictive locomotion and scratch in the adult cat, deletions tended to occur concurrently in synergists at proximal and distal joints (Lafreniere-Roula and McCrea 2005). We also observed instances when both knee (FT) and ankle (LG) extensors dropped out (Fig. 1E). Yet it was also common for LG to drop out while FT bursting persisted and occasionally for the LG to rhythmically burst while FT was silent (i.e., initial cycles of Fig. 1D). Our study does not address the question of underlying causes for deletions. However, in the lamprey, evidence indicates fast locomotor bursting within a spinal cord hemi-segment emerges from the spread of excitation among a population of interneurons, called EINs, that synapse with motor neurons (Cangiano and Grillner 2005). Intra- and extracellular recordings indicated that EIN and motor neurons fire once per cycle during peak activity and suggested that recurrent excitation among EIN synchronized the EIN and motor pool to produce a single burst in ventral root recordings per locomotor cycle. A motor neuron might then drop out for one or more cycles of fictive swimming as excitability diminished (Cangiano and Grillner 2005). Reduced depolarizations in motor neurons during burst deletions in EMG records suggest fluctuations in network excitability also underlie deletions in the cat spinal cord (Lafreniere-Roula and McCrea 2005). Studies of burst deletions to date have been examined in fictive and spinal preparations; our findings extend these observations and suggest that the waxing and waning of excitability within the burst-generating network is not a phenomenon unique to neurologically reduced preparations. Our data suggest variations in network excitability are typical of RLMs in the neurologically intact embryo and may account for most of the age-related changes we observed between E15 and E20.

**Age-related changes in RLMs**

RLMs encompassed a broad frequency range, but the distribution was concentrated within those frequencies observed during posthatching locomotor behaviors (Fig. 3A). Based on pooled cycle period data reported by Johnston and Bekoff (1996; their Fig. 3), step frequencies for hatchlings 1–3 days of age ranged from ~1.4 to 10 Hz with means of 2.8 Hz for walking, 3.2 Hz for swimming, and 5.1 Hz for astepping. Our within-subject mean and peak frequencies for RLMs mostly fell within these ranges at E18, so we anticipated a sharpening around the lower frequencies at E20, and were surprised by the upward shift (Fig. 3B). We considered that increasing mechan-
Both limb posture and burst frequencies at E18 (Fig. 5A). Both limb posture and burst frequencies were impacted at E20 however; embryos extended their limbs into the newly available workspace and generated even faster TA burst frequencies (Fig. 5B). During many of these instances, tremor-like movements were observed at the ankle, suggesting to us that lengthening of dorsiflexors drove proprioceptors and augmented TA burst frequency. It is not known when the muscle afferent system is sufficiently mature to impact behavior in the chicken embryo, but anatomical studies indicate the annulospiral ending innervates the muscle spindle between E11 and E13 (Maier 1992, 1993). Given chicks hatch and walk as early as E20, proprioceptors are probably functional at E20. Also, the finding that 10% fewer TA cycles exhibited stable bursting during RLMs after shell removal at both E18 and E20 may indicate proprioceptors are somewhat functional at E18 and that age-related changes are not solely indicative of network maturation.

The fast locomotor range was also observed in EMG recordings at E15; however, rhythmic bursting was less frequent, almost always limited to a single muscle, and the bursting was only occasionally accompanied by repetitive limb movements. Concurrent rhythmic activity in three to four muscles, as in Fig. 1, A and D, was never observed at E15 even though each muscle could be rhythmically active (Fig. 3C). Leg muscles participate in ensembles of agonist and antagonist activity during motility as early as E9 but at slower rhythms of 0.2–2 Hz (Bradley and Bekoff 1990; Landmesser and O’Donovan 1984). There is considerable evidence that these slow rhythms in younger embryos are governed by a spinal recurrent excitation network and synaptic fatigue (Chub and O’Donovan 1998; O’Donovan and Chub 1997; Tabak et al. 2000). Likewise, evidence indicates recurrent excitation drives the fast locomotor burst generator for the higher end of the burst range emerges in the adult hemicord on removal of contralateral inhibitory connections (Cangiano and Grillner 2003) and pharmacological blockade of glycergic inhibition (Cohen and Harris-Warrick 1984). Collectively, the findings of the preceding studies suggest to us that age-related changes between E15 and E18 are indicative of a recurrent excitatory network that is in transition and/or requires additional input, such as muscle afference or descending input, to sustain spinal network excitability. We are intrigued that expression of the fast locomotor burst generator range at E15 includes higher frequencies associated with ankle tremor observed during foot-free conditions at E20. Further study of these frequencies may provide insights regarding a contribution of central pattern generation in behaviors like clonus or tremor (Beres-Jones et al. 2007).

In sum, our findings demonstrate that by E15 the chick embryo expresses repetitive limb movements in ovo at frequencies of 1–12 Hz and suggest that late stage motility is produced by the fast locomotor burst generator. Shifts in TA bursts toward the high end of the frequency range with age, and after shell wall removal at E20, suggest that proprioceptors may also contribute to the control of RLMs during the final week of embryonic development. EMG during RLMs exhibited features similar to deletions during fictive scratching and locomotion in adult animals and may indicate that modular organization is also a feature of rhythmogenesis in the chick embryo.

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