Age-Related Changes and Condition-Dependent Modifications in Distribution of Limb Movements During Embryonic Motility

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INTRODUCTION

Although they credit Visintini and Levi-Montalcini (1939) as the first to recognize the periodicity of motility in chick embryos, Hamburger and Balaban (1963) provided the first analyses. Their analyses indicated that beginning embryonic day (E) 3.5, movement occurred in “cycles,” each cycle consisting of an “activity phase” lasting 5–15 s and containing 2–10 movements, followed by an “inactivity phase” of 30–60 s. Findings indicated that the duration of activity and inactivity was variable, and the lengths of the two phases within a cycle were unrelated, yielding an irregular periodicity. Over age, the activity phase lengthened and the inactivity phase shortened until activity was nearly continuous at E13 (Hamburger 1963) but remained cyclic through to E17 (Hamburger et al. 1965). Hamburger (1963) proposed that two mechanisms controlled motility, one turned it on and another turned it off. Based on studies of chronic spinal embryos, he and his colleagues argued that the structure of episodic motility (activity plus inactivity phases) sat within the spinal cord but that the brain and possibly other components (muscle, proprioception) contributed to the control of motility with increasing age (Hamburger et al. 1965).

Subsequent studies of spinal embryos revisited the notion of two separate control mechanisms and suggested that repetitive movements within an activity phase were controlled by local spinal circuitry but that supraspinal and propriospinal inputs regulated the temporal distribution of activity by E9 (Bradley and Bekoff 1992; Oppenheim 1975). In the absence of descending input, activity phase duration and cycle times for repetitive limb movements were shortened to the point of producing tonic seizure-like activity by E15. The precise regulatory mechanisms are not known, but anatomic and physiologic studies suggest both descending and afferent inputs can potentially impact motility during the latter two-thirds of embryonic development. Reticulospinal pathways reach the lumbar-sacral spinal cord by E5 (Okado and Oppenheim 1985) and make synaptic contacts by E6–7 (Shiga et al. 1991). Electrical stimulation of the ventral pontine and medullary reticular formation or bath application of N-methyl-D-aspartate (NMDA) to the brain stem can evoke motor neuron responses in the lumbar-sacral cord by E6 (Sholomenko and O’Donovan 1995), and embryos are responsive to cutaneous and proprioceptive stimuli by E7 (Oppenheim 1972).

Studies in the acute isolated spinal cord have greatly advanced understanding of the endogenous spinal control of motility. O’Donovan and colleagues demonstrated that the repetitive bursts of motor neurons responsible for limb move-
ments (O’Donovan and Landmesser 1987) are accompanied by waves of neural activity beginning ventrolaterally and spreading dorsomedially across the spinal cord (O’Donovan et al. 1994). Because periodic initiation of rhythmic activity persisted following an array of ablations (Ho and O’Donovan 1993), and recovered in the presence of either excitatory or inhibitory pharmacological blockades (Chub and O’Donovan 1998), they proposed that both the periodic initiation of activity and rhythmic excitation during activity are emergent products of population dynamics (O’Donovan and Chub 1997). They proposed that the periodic initiation of activity is the product of recovery from (long-lasting) activity-dependent depression of transmitter release within ventrolateral neurons (see also Fedirchuk et al. 1999); whereas the duration of rhythmic activity is a function of the number of neurons recruited by recurrent excitation and the time to recover from short-lasting synaptic depression (O’Donovan 1999). A mathematical model of these elements was recently published (Tabak et al. 2000).

A focus of work in our lab is to determine if movement-dependent experience shapes attributes of motility in preparation for adaptive posthatching behaviors, for sensory inputs can potentially impact motility during the second to third embryonic week prior to hatching at E21. Primary afferent synaptic efficacy is established by E7.5 (Davis et al. 1989; Lee et al. 1988), muscle afferent innervation is established by E11–E13 (Maier 1992, 1993), cutaneous coding of movement may begin as early as E7 (Koltzenburg and Lewin 1997; Oppenheim 1972; Scott 1982), and by E20 proprioceptive input arising from flexion initiates hatching (Bekoff and Kauer 1982; Bekoff and Sabichi 1987). Because recent studies indicated that the distribution of activity is extremely variable with increasing age (Ganley and Bradley 1999; Rose et al. 1998), the purpose of this study was to determine the normal within-embryo variability for motility between E9 and E18. Also, because activity distribution can be altered by a reduction in buoyancy at E9 (Bradley 1997; Chambers et al. 1995), a second purpose of this study was to determine if the reduction in buoyancy impacts activity distribution at later ages. The distribution of activity was also examined following application of an ankle restraint or chronic spinal transection so as to compare data across preparations to gain further insight into the neural control of episodic behavior in the intact embryo. Preliminary findings were recently published (Bradley 2000).

METHODS

Video data were obtained from leghorn chicken embryos incubated for experiments at E9, E10, E12, E15, or E18. Age was verified using established staging criteria (Hamburger and Hamilton 1951). The samples included embryos drawn from an array of earlier and more recent studies representing five experimental preparations: embryos prepared as controls for electromyographic (EMG) and/or kinematic studies (control); embryos that did not experience EMG or kinematic procedures (control II); embryos prepared for kinematic study during an acute reduction in buoyancy (ARB); embryos prepared for kinematic study during mechanical constraint of ankle movement (AR); and embryos prepared for kinematic study after chronic spinal transection (spinal). Table 1 summarizes the number of experiments per preparation and age and indicates those embryos from published studies whose data were re-analyzed for inclusion in this study. All procedures were approved by the University Institutional Animal Care and Use Committee.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Average,*</th>
<th>Total (n)</th>
<th>E9†</th>
<th>E10†</th>
<th>E12†</th>
<th>E15†</th>
<th>E18†</th>
</tr>
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<tbody>
<tr>
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<td>12</td>
<td></td>
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<td>12</td>
<td>12</td>
</tr>
<tr>
<td>ARB</td>
<td>55</td>
<td>36</td>
<td>7</td>
<td></td>
<td>6</td>
<td>13</td>
<td>10</td>
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<tr>
<td>AR</td>
<td>40</td>
<td>20</td>
<td>11</td>
<td>(9)</td>
<td></td>
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<tr>
<td>Spinal</td>
<td>41</td>
<td>41</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses indicate embryos drawn from Bradley (1999) (control) and Bradley and Sebelski (2000) (AR) for re-analysis. * Average duration of recording per experimental group. † Number of experiments per experimental group and age.

Experiments were conducted in ovo and the eggs were maintained in a temperature-controlled chamber (38°C) throughout preparation and recording. A window was placed in the shell to expose the embryo, and in all groups, except control II, membranes were cut (for EMG and/or kinematic preparation). In ARB experiments, 5–7 ml of amniotic fluid was extracted at all ages except at E18; ARB experiments were not performed at E18 due to difficulty extracting more than 2 ml of fluid. In AR experiments, a rigid ankle restraint was secured to the right lower leg at E9 or E12; space limitations as body size increased prohibited application of the appliance at older ages. Spinal experiments were conducted at E9 after removing three to four segments of spinal cord between somites 18 and 22 at E2. Completeness of thoracic transections was confirmed by 10-μm serial sections after block silver stain (Shimizu et al. 1990). Spinal experiments were not performed at later ages due to difficulty maintaining viability. Pulse rate and rhythm were monitored throughout experiments and total exposure was limited to 90 min to optimize behavioral data (Chambers et al. 1995).

Video recordings and analyses

Video recordings were continuous over the duration of an experiment. Video was collected at 30 frames/s and stored on VHS tape with a SMPTE time code for subsequent analyses. VHS recordings were reviewed frame-by-frame to determine the SMPTE code at onset and offset of all activity in the ipsilateral wing and/or leg (the right side is typically presented if the egg is not rolled in the 24 h preceding an experiment). No distinction in onset and offset was made between wing and leg movements in intact embryos; but in spinal embryos, the wing and leg were separately analyzed. Contralateral limbs were not considered because they could not be reliably analyzed. Any displacement, regardless of how brief, was defined as activity (Fig. 1). Inactivity less than 10.0 s was treated as part of the activity preceding and following it. Inactivity exceeding 10.0 s was defined as a pause. Combined, activity and the subsequent pause formed an episode, and episode duration was equivalent to the time between consecutive onsets in activity. Activity duration, pause duration, episode duration, and percent activity were calculated for each episode, and subject means were generated for within- and between-group comparisons. Standard deviations and coefficients of variation were calculated within subject and averaged within group to examination parameter variability. Linear regression statistics were performed to examine the relationships between variables. A total of 165 video recordings, ranging from 30 to 60 min in duration, were reviewed to determine the distribution of activity (Table 1). All videos were analyzed at least twice and nearly all videos were analyzed by two reviewers. Two- and one-way ANOVAs were used to test for significant main effects at the level of P < 0.05. In two-way ANOVAs, the group mean was substituted for one E9 missing cell. One-way ANOVA and one-tailed Student t-tests were used for post hoc comparisons, and the Bonferroni correction (P = 0.05/number of comparisons) was used to adjust the level of significance.
and represents the episodic initiation of motility. Duration is equivalent to the interval between consecutive onsets in activity, of the subsequent pause (p1) equaled an episode (e1). Note that episode activity peaking at E12 and dropping to lowest levels at E18 decreased in episode duration between E9 and E18, the percent episode duration decreased primarily between E9 and E12, and continued a downward trend in smaller increments between E12 and E15, and decreased between E15 and E18 (Fig. 3). One-way ANOVAs also indicated that linear regression statistics exhibited significant age-related trends (Table 2). The strength of co-variation for activity and episode duration increased between E9 and E12 then decreased slightly during E12–E18. Activity varied most closely with episode duration at E9 and decreased between E9 and E18. Activity duration did not vary significantly with the subsequent pause at any age and only modestly with the preceding pause at E9 (Table 2).

Episode variability, or variability in periodic onset of activity, ranged 30–50 s across ages. Nonetheless, the coefficient of variation indicated episode variability was nearly 54% of mean episode duration at E9 and increased to nearly 90% at E18 (Fig. 4C). Both within-subject standard deviations and coefficients of variation indicated episode variability were primarily attributable to age-related increases in activity duration variability (Fig. 4, A and C). Pause duration variability, in contrast, progressively decreased with age, the coefficient of variation remaining unchanged, suggesting that pause variability had minimal impact on episode variability after E9.

**Acute reduced buoyancy enhanced age-related changes in distribution of motility**

Age-related trends were also significant and even more apparent in ARB than in control embryos (Fig. 3). Activity duration progressively increased with age as pause duration progressively decreased, thus percent activity also increased with age. An age-related trend for episode duration fell short of significance (P < 0.08). ANOVA comparisons indicated that the strength of co-variation between activity and episode duration also increased with age while the relationship between pause and episode duration decreased (Table 3). Variability of activity and episode duration also increased with age (Fig. 4, B and C).

ANOVA comparisons indicated that activity duration was longer, pause duration shorter, and percent of activity greater for ARB than control embryos (Fig. 3). Post hoc comparisons for each of the four parameters were significant at E15 (Student t-test, Bonferroni correction, P < 0.0125), and percent activity was significantly greater at all ages except at E10. Group differences between ARB and control embryos were also apparent in individual recordings. For example, ARB plots typically included the longest activity durations per age and contained fewer episodes per unit time than control plots due to the overall increase in activity duration (Fig. 5). Further, the longer activity durations and shorter pause durations in ARB, as compared with control embryos, also appeared to account for the significantly stronger linear relationship between activity and episode duration as well as a weaker relationship between pause and episode duration (Table 3). An ANOVA comparison indicated activity duration variability was also greater in ARB than control embryos (Fig. 4B) and at E15 in specific (Bonferroni correction P < 0.0125). However, when subject standard deviations were normalized to their respective means, relative variability for ARB and control embryos was similar (Fig. 4C).

**Ankle restraint lengthened pause and episode duration**

Due to inclusion of activity lasting less than 10 s, temporal measures were substantially less than previously reported for AR embryos, though age-related trends persisted (Bradley and...
Sebelski 2000), and most parameters differed significantly from those for control embryos. One-way ANOVAs for age indicated that activity duration did not vary between E9 (28 s) and E12 (29 s), whereas pause duration decreased (from 93 to 37 s), episode duration decreased (from 120 to 65 s), and percent activity increased (from 24 to 40%). One-way ANOVA comparisons for AR and control embryos indicated that both activity duration and its relationship to episode duration were similar between groups (Fig. 6). However, pause and episode duration were significantly longer and percent activity was significantly less for AR than control embryos; all post hoc Student t-tests were significant at E9 but fell short of the Bonferroni correction ($P < 0.025$) at E12. Nonetheless, as illustrated in Fig. 6, pause and episode duration varied more closely in AR than control embryos, linear coefficients averaging 0.95 ± 0.06 at E9, 0.77 ± 0.15 at E12, and post hoc tests were significant at both ages. Additionally, activity duration was typically less variable and pause duration more variable in AR than control embryos; post hoc tests were significant at E9. Also, because brief movements were rarely noted in the earlier study, instances of activity less than 10.0 s were tabulated, and ANOVA comparisons confirmed that AR produced fewer brief movements (4, E9; 6, E12) than control conditions (6, E9; 9, E12). Post hoc comparisons (Bonferroni, $P < 0.025$) were significant at E12 and fell just short of significant at E9 ($P < 0.026$).

**Thoracic spinal transection altered the distribution of motility caudal to the lesion**

Thoracic spinal transection selectively reduced the absolute and relative activity duration of leg motility compared with both wing motility in spinal embryos and motility in control embryos. ANOVA comparisons indicated activity duration, episode duration, and percent activity were significantly less for spinal leg than wing motility, whereas pause duration did not differ between limbs (Fig. 7). Also, as illustrated in Fig. 8, linear regressions for activity and episode duration did not differ significantly between the leg ($R = 0.76 ± 0.1$) and wing ($0.66 ± 0.13$). However, regression statistics for pause and episode duration were significantly stronger for the leg ($R = 0.93 ± 0.02$) than wing ($0.83 ± 0.06$). Coefficients of variation were significantly greater by 25–44% for activity, pause, and episode duration in the leg compared with wing.

ANOVA comparisons between spinal leg and control motility were similar to comparisons between spinal leg and wing (Bonferroni correction, $P < 0.025$). Activity duration and percent activity were significantly less for spinal leg than control motility, but pause duration was similar between groups (Fig. 7). Also, the linear regressions for activity and episode duration were similar, but regression statistics for pause and episode duration were significantly stronger for spinal leg than control motility (Fig. 8). Finally, coefficients of variation were significantly greater by 38–47% for activity.
pause, and episode durations in spinal leg. Parameters for spinal wing did not differ from those for control embryos.

**DISCUSSION**

**Age-specific transformations in temporal distribution of limb movements**

The distribution of motility was first described nearly 40 yr ago, and despite differences in methodology, control embryos exhibited age-related trends similar to those first reported by Hamburger et al. (1965). Activity increased E9–E12 and decreased E15–E18; pause duration decreased primarily between E9 and E12, followed by smaller incremental decreases E12–E18; and percent activity peaked E12–E15, then dropped to lowest levels E18. [However, Bollweg and Sparber (1999), using electrophysiological methods, reported increases in activity between E12 and E18.] Overall, control embryos appeared less active than observed by Hamburger and colleagues (Hamburger et al. 1965; Oppenheim 1975). However, given the strong agreement between control and control II data, the lower activity levels are likely due to longer samples and/or our use of video to review past events (rather than direct observation of ongoing events). It is possible the video image obscures subtle visual perceptions; nonetheless, video permits multiple reviews for greater consistency and sensitivity in statistical comparisons.

Results of this study also revealed that activity and pause duration exhibited different relationships to the periodicity of motility over age (Fig. 9A). Activity duration closely co-varied with episode duration at E9, and the strength of the relationship increased with age. Pause duration, conversely, varied closely with episode duration at E9, but the relationship dropped off precipitously with age. There was also a modest relationship between activity duration and the preceding pause at E9, but this too dropped off with age, and as first noted by Hamburger and Balaban (1963), no relationship was found between activity duration and the subsequent pause at any age. Finally, age-related trends also indicated that variability in the episodic distribution of activity was primarily attributable to activity duration.

**Periodicity is a variable attribute of motility**

Results are consistent with studies in both chick and rat, indicating that the periodic initiation of motility is more variable than stereotypic (Hamburger and Balaban 1963; Narayan et al. 1971). In our study, episode duration ranges exceeded 100 s, and standard deviations of 30–50 s were typical at all ages. Variable periodicity is observed in other immature motor systems, such as the lobster stomatogastric ganglion, but

**TABLE 2. Average within-embryo correlation coefficients (R) for control embryos**

<table>
<thead>
<tr>
<th>Motility Parameter</th>
<th>E9</th>
<th>E10</th>
<th>E12</th>
<th>E15</th>
<th>E18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity/episode*</td>
<td>0.73</td>
<td>0.80</td>
<td>0.91</td>
<td>0.87</td>
<td>0.85</td>
</tr>
<tr>
<td>Pause/episode*</td>
<td>0.82</td>
<td>0.53</td>
<td>0.42</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>Pause/activity†</td>
<td>0.28</td>
<td>0.21</td>
<td>0.19</td>
<td>0.22</td>
<td>0.16</td>
</tr>
<tr>
<td>Previous pause/activity†</td>
<td>0.41</td>
<td>0.30</td>
<td>0.16</td>
<td>0.14</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Values are means ± SD. * Varied significantly with age. † Pause/activity = p/a; previous pause = p/a², Fig. 1.
variability decreases with maturation of circuit elements (Richards et al. 1999). Periodic motility presents a very different developmental profile. The periodic initiation of activity was most stable at E9 and became more variable with age.

O’Donovan and Chub (1997) proposed that periodic initiation of motility is an emergent product of population dynamics attributable to immature neuronal properties. In their model, based on the isolated spinal cord, activity is the product of recurrent excitation and is dependent on the number and strength of functional spinal synapses. The termination of activity and intervening pause are the product of synaptic depression within the network. The close co-variations for both

FIG. 5. Activity and pause durations are plotted for consecutive episodes and relative to the concurrent episode duration for 4 ARB embryos. Plots adhere to the same conventions described for Fig. 2.
activity and pause duration with episode duration at E9 in control embryos in our study is consistent with their model and suggests that the two parameters contributed equally to the episodic distribution of activity at E9. Also, activity duration modestly co-varied with the preceding pause duration in control E9 embryos, suggesting that there was some shared element of control underlying the two parameters that might account for the more stable episode duration at younger ages. Tabak and O’Donovan (1998) also found that activity duration co-varied with the preceding pause in the isolated spinal cord and proposed that synaptic depression regulates the strength of recurrent excitation. That is, the longer time spent in recovery from depression, the greater number of functional excitatory synapses available to support the next activity phase. Thus based on their model (O’Donovan 1999; Tabak et al. 2000), “slow” synaptic depression appears to be the critical mechanism underlying the periodic distribution of the activity at younger ages. The mechanisms responsible for synaptic depression of the spinal network are not currently known, but presynaptic transmitter depletion and postsynaptic receptor desensitization have been proposed (Fedirchuk et al. 1999). Given the rapid decrease in pause duration after E9 in intact embryos, we propose that the increased episode variability with age may be partially attributable to resolution of synaptic depression, yielding a network more ready to respond to developing extra-network sources (Fig. 9B).

Age-related transformations in the distribution of motility

Age-related changes in the distribution of motility likely reflect both the maturation-dependent changes in spinal network behavior and increasing influence of extra-spinal projections. At E9, the overall similarity in periodic activation of motility in spinal leg, spinal wing, and control data, i.e., the co-variation of both activity and pause duration with episode duration, supports the view first posed by Hamburger (1963) and later Oppenheim (1975) that motility distribution in the intact E9 embryo is primarily controlled by local spinal circuits. However, the reduction in both absolute and relative duration of activity in spinal leg, compared with both spinal wing and control data, suggests that recurrent excitability of spinal circuitry is somewhat dependent on extra-spinal inputs by E9. Further, the extended pauses observed in the isolated cord (2–30 min, Tabak et al. 2000; 12 min, Fedirchuk et al. 1999), compared with longest pauses in intact embryos (11–

**FIG. 6.** Activity and pause durations are plotted for consecutive episodes and relative to the concurrent episode duration for 2 AR embryos. Plots adhere to the same conventions described for Fig. 2.

**FIG. 7.** Average motility parameters for spinal leg and wing movements compared with control motility. Columns represent between-subject averages and standard deviations. ***, significant differences between spinal leg and spinal wing and between spinal leg and control parameters.
70 s), suggest that network depression is also influenced by extra-network circuitry, such as descending and/or afferent pathways, by E9. However, similarities in pause duration across spinal leg, spinal wing and control raise the possibility that the prolonged depression observed in the isolated cord is influenced by additional variables unique to the preparation.

Age-related changes in distribution of motility beyond E12 appear to be primarily attributable to maturational processes governing the excitatory drive of spinal circuits. One, pause duration substantially decreased with age, suggesting that network depression has limited impact on the initiation and duration of activity during the second half of embryonic development. Two, activity duration variability increased with age as the range in activity duration lengthened and included the increasing incidence of very brief movements (Fig. 2). Thus it is likely that the variability is due to drive of the spinal network for motility by multiple sources of excitation that are yet immature. Some excitatory drive is likely from intra-spinal

FIG. 8. Activity and pause durations are plotted for consecutive episodes and relative to the concurrent episode duration for wing and leg movements in 1 spinal embryo. Plots adhere to the same conventions described for Fig. 2.

FIG. 9. Motility parameters for the distribution of motility varied across age (AGE) in both control and ARB embryos and across conditions within age for ARB, AR and spinal preparations as summarized for key findings (A). Increases in activity duration were associated with increases in the co-variation between activity and episode duration, suggesting activity duration largely determined the episodic distribution of motility with increasing age. Conversely, decreases in pause duration were associated with decreases in the co-variation between pause and episode duration, suggesting that pause duration had little impact on the distribution of motility after E9. The co-variation between activity and pause duration was generally poor, except for comparisons at E9. A generalized model to account for these findings, plus the age-related increases in the variable episodic distribution of motility, is proposed (B). Episodic distribution of motility is largely determined by spinal network properties up to E9; spinal network excitability provides the drive for generating motility (activity) and network synaptic depression imposes the pauses in activity (solid circle with dashed line) to produce episodic behavior. Orderly recruitment of limb muscle synergists (flexors; extensors) and their reciprocal activation during intact behavior at E9 (Bradley and Bekoff 1990) suggests that spinal network activity may recruit elements of the half-centers, commonly thought to control limb stepping motions for locomotion (Orlovsky et al. 1999). Spinal network excitability appears to be increasingly dependent on excitatory drive (solid line) from brain stem centers, such as the medullary reticular formation (mRF), and afferent pathways (dashed line to left) beginning as early as E9; E15 appears to be the oldest age network excitability is sufficient to generate motility spontaneously (O'Donovan 1989). The increasing variability in episodic distribution of motility beyond E9 may be attributed in part to excitatory input via descending brain stem pathways, such as the mRF, pontine reticulospinal, and vestibulospinal tracts, as brain stem centers and projections are refined. Other brain stem centers may exert mixed modulatory effects on spinal network circuitry (dotted lines). These modulatory inputs are proposed to arise from centers undergoing substantial maturation in the week prior to hatching, including those controlling rhythmic respiratory movements (RR) and circadian rhythms (CR). Somatosensory inputs may also have mixed effects over the later stages of development (see DISCUSSION for details).
and/or afferent sources, for activity phases lengthened between E11 and E17 in chronic spinal embryos (Hamburger et al. 1965). Nonetheless the chronic spinal embryos exhibited a reduction in activity duration, compared with age-matched controls, leading Hamburger and colleagues to argue that brain input also provided an excitatory drive to spinal motility circuits. The precise pathways driving motility circuits are not known, but electrical stimulation of medullary gigantocellular neurons can trigger motor activity and application of NMDA to the brain stem can decrease pause duration by several minutes in the isolated brain stem-spinal cord by E6 (Sholomenko and O’Donovan 1995), suggesting that descending pathways are potentially available to provide some excitatory drive to spinal networks soon thereafter. It also appears that dopaminergic pathways may begin to impact activity distribution between E13 and E16 (Chub 1991; Sedlack 1992). Additionally, age-related increases in very brief movements observed in the present study appeared to accompany respiratory-like chest movements, raising the possibility that motility circuits are also driven by the developing respiratory center. Finally, Hamburger et al. (1965) speculated that proprioceptive reflexes might also lengthen activity phases, and our findings in ARB embryos provide evidence for this view, as discussed in the following text.

**Mutability of motility parameters**

For some time, we have been interested in whether the in ovo environment and/or resulting movement experiences imposed by it contribute to attributes of motility, and here consider findings across conditions, as summarized in Fig. 9A. In two previous studies, we observed several kinematic changes in wing and leg movements indicative of mechanical dampening plus a reduction in pause duration in E9 ARB embryos (Bradley 1997; Chambers et al. 1995). In this study of E9–E15 ARB embryos, pause duration was again decreased, plus an increase in activity duration was found. Studies of similar mechanical constraints in mammals (oligohydramnios) have reported dampening of select limb movements, but the net level of activity did not appear to be altered (Robinson and Smotherman 1992; Sival 1993). However, it appears that the fluid reduction was coupled with a reduction in total free space in utero; this is thought to have an inhibitory effect on activity during normal late-stage development because rat fetuses exhibit increased levels of activity when freed from uterine constraint (Robinson and Smotherman 1992). In the ARB preparation, on the other hand, total free space appeared to be slightly increased by the fluid extraction, owing to rigid (shell) rather than muscular (uterine) walls and resulted in more extended limb postures compared with control conditions (unpublished observations). Conversely, extreme movement restriction of the ankle in AR embryos lengthened pause duration. Further, AR embryos failed to exhibit the age-related increase in activity duration at E12 and exhibited fewer brief movements than control embryos. It is possible that longer pauses were the result of mechanically dampening brief movements that would have parcelled the pauses into smaller segments. However, because wing movements were not mechanically restrained but remained coupled with leg movements (Bradley and Sebelski 2000), it appears AR conditions exerted a central effect on motility distribution.

Collectively across conditions, the findings suggest that the distribution of activity is largely determined by the spinal network for motility up through approximately E9–E10 and becomes increasing dependent on other sources for excitatory drive as intrinsic network excitability decreases and synaptic depression resolves (Fig. 9B). The shorter activity duration exhibited by spinal embryos suggests descending inputs provide at least some excitatory drive by E9. The progressive increases in activity duration with age in control embryos may be attributed in part to maturation of descending reticulospinal pathways (Glover and Petursdottir 1988). The progressive increases in activity duration variability between E12 and E18 may be partly due to mechanisms controlling the emergence of breathing movements (Akiyama et al. 1999) and circadian rhythms (Akasaka et al. 1995) in the final days before hatching.

We propose that the increasing variability with age is also attributable to maturing somatosensory pathways and changes in the bias of these inputs as the embryo increases in size and the fixed space in ovo imposes an increasingly flexed posture. Given the greater age-related increases in activity duration in ARB compared with control embryos, somatosensory inputs likely begin to enhance the level of excitability in spinal circuits between E9 and E12. However, their effects are likely modest at this time, for elimination of motion-dependent feedback between E10 and E12 does not appear to alter motility as it re-emerges from blockade (Oppenheim et al. 1978). Nonetheless given the increase in pause duration in AR embryos, postural context and/or extent of constraint may also be increasingly important and account for some of the variability in motility distribution during the later half of development. In support of these views, cutaneous afferents may contribute to modest ARB and AR affects at younger ages (Bradley and Sebelski 2000; Koltzenburg and Lewin 1997; Scott 1982), and proprio- spinal afferents may impact parameters by E12–E15 (Maier 1992, 1993), for they initiate vigorous hatching by E20 (Bekoff and Kauer 1982; Bekoff and Sabichi 1987). We speculate that afferent inputs associated with flexion and extension postures may differently impact the control of motility and that the normal reduction in activity during the final days of normal development in ovo may be at least partially attributable to the extreme flexed posture and constrained excursions imposed by the shell wall. Somewhat related views regarding late-stage constraints have been previously raised (for review, see Oppenheim 1973). However, in 5-min observations of E18–E19 embryos, after partial exteriorization of the head and upper body, Oppenheim (1973) did not find a difference in the total number of movements manually counted, suggesting there was neither a decrease in hatching behaviors nor a net increase in activity as observed in exteriorized rat fetuses (Robinson and Smother 1992). Exteriorization of the chick may have introduced confounding effects not observed in the rat, but it is not possible to reconcile the differences here. Nonetheless given the common observation of reduced activity across animals during late gestation as they out-grow their embryonic space (ten Hof et al. 1999), further study into the role of posture on distribution of embryonic movements is warranted.

In sum, age-related changes in the distribution of motility
and selective effects of experimental preparations suggest that activity duration and pause duration operate independent of one another with increasing age. Results also suggest that transformations in the control of pause duration are generally complete by E12, whereas control of activity duration, initially somewhat stereotypic, becomes more variable with age. Activity duration also appears to be influenced by environment-related conditions with increasing age, and this may partially account for the age-related increases in activity duration variability. Supporting ideas first put forward by Hamburger et al. (1965), results suggest that the age-related trends in activity duration are a result changing input weights for descending and afferent sensory contacts at spinal levels. The precise contributions of these extraspinal inputs to the form and distribution of motility over embryonic development remain to be elucidated.

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