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RESEARCH ARTICLE

Spontaneous locomotor activity in late-stage chicken embryos is modified by stretch of leg muscles

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ABSTRACT

Chicks initiate bilateral alternating steps several days before hatching and adaptively walk within hours of hatching, but emergence of precocious walking skills is not well understood. One of our aims was to determine whether interactions between environment and movement experience prior to hatching are instrumental in establishing precocious motor skills. However, physiological evidence of proprioceptor development in the chick has yet to be established; thus, one goal of this study was to determine when in embryogenesis proprioception circuits can code changes in muscle length. A second goal was to determine whether proprioception circuits can modulate leg muscle activity during repetitive limb movements for stepping (RLMs). We hypothesized that proprioception circuits code changes in muscle length and/or tension, and modulate locomotor circuits producing RLMs in anticipation of adaptive locomotion at hatching. To this end, leg muscle activity and kinematics were recorded in embryos during normal posture and after fitting one ankle with a restraint that supported the limb in an atypical posture. We tested the hypotheses by comparing leg muscle activity during spontaneous RLMs in control posture and ankle extension restraint. The results indicated that proprioceptors detect changes in muscle length and/or muscle tension 3 days before hatching. Ankle extension restraint produced autogenic excitation of the ankle flexor and reciprocal inhibition of the ankle extensor. Restraint also modified knee extensor activity during RLMs 1 day before hatching. We consider the strengths and limitations of these results and propose that proprioception contributes to precocious locomotor development during the final 3 days before hatching.

KEY WORDS: Proprioception, Autogenic excitation, Reciprocal inhibition, Electromyography, Restraint, Tibialis anterior, Gastrocnemius

INTRODUCTION

Chicks are precocious walkers. Within hours of hatching they can be trained to walk down darkened corridors (Sindhurakar and Bradley, 2010) and to negotiate dynamic postural challenges (Rácz et al., 2011). Hatchlings also modify leg muscle activity with changes in stepping conditions (Johnston and Bekoff, 1996; Muir and Gowri, 2005). These precocious locomotor abilities are evidence that proprioceptive afferents can accurately code the state of the leg for controlling the center of mass during stance and locomotion at hatching. During the final week *in ovo* prior to hatching, chicks begin producing repetitive limb movements (RLMs) having muscle patterns comparable to stepping during locomotor behaviors post-hatching (Bradley et al., 2008; Ryu and Bradley, 2009). Therefore, it is plausible that proprioceptive coding of limb posture and movement is established prior to hatching, and that proprioception may influence both embryonic behavior and preparations for precocious postnatal locomotion, a notion first proposed several decades ago (Narayanan and Malloy, 1974). Nonetheless, studies have commonly emphasized the central control of embryonic motility (Bekoff, 2001; Muir, 2000), while the functional status of proprioception in the normal developing embryo and its role in establishing precocious postnatal locomotor skill remain to be fully understood.

Several studies have charted the anatomical development of proprioceptor end organs and their spinal circuits but little is known about the physiological or behavioral function of the proprioceptive system during embryogenesis in the chick. The primary afferent synapse with motor neurons is established early in embryogenesis and precedes peripheral development. The proximal portion of primary sensory fibers reaches motor neurons by E7–E8 (Davis et al., 1989), at which time monosynaptic excitatory potentials can be evoked (Lee et al., 1988); and the axon collaterals may extend within the dorsal funiculus, branching over many spinal segments by E10 before retracting into adult-like distributions by E17 (Eide and Glover, 1995). The peripheral segment of sensory axons contacts undifferentiated intrafusal fibers by E13, forms end plates by E14 and induces differentiation of 5–6 intrafusal fibers by E15, while afferent fibers are still unmyelinated (Maier, 1992; Maier, 1993). By E17, intrafusal fiber counts appear to be complete in leg muscles, capsular structures are clearly defined and annulospiral endings are well differentiated. By E18, the relative expression of fetal and neonatal myosin heavy chain proteins has begun to decline (Maier, 1992; Maier, 1993). Thus, anatomical evidence suggests that spindle receptors may be functional by E17–E18, though differentiation continues beyond hatching (Maier, 1992). Descending motor pathways are also established within this time frame, reaching lumbar levels by E5–E7, and are well established by E14 (Okado and Oppenheim, 1985), but their impact on gamma motor neurons remains to be elucidated. In addition, development of cutaneous sensory function begins very early and may serve a proprioceptive function before muscle afferents (Scott, 1982). Cutaneous afferents are responsive to skin contact and trigger limb movement by E6–E7 (Scott, 1982), and display graded slow or rapid adapting properties by E17 (Koltzenburg and Lewin, 1997). However, it has yet to be established when and to what extent proprioceptive afferents can code changes in joint position or muscle length during embryogenesis.

A number of early studies appeared to indicate that sensory input had minimal or no impact on embryonic movements, except possibly hatching (Bekoff, 2001; Muir, 2000). The available evidence also suggested that movement experience during

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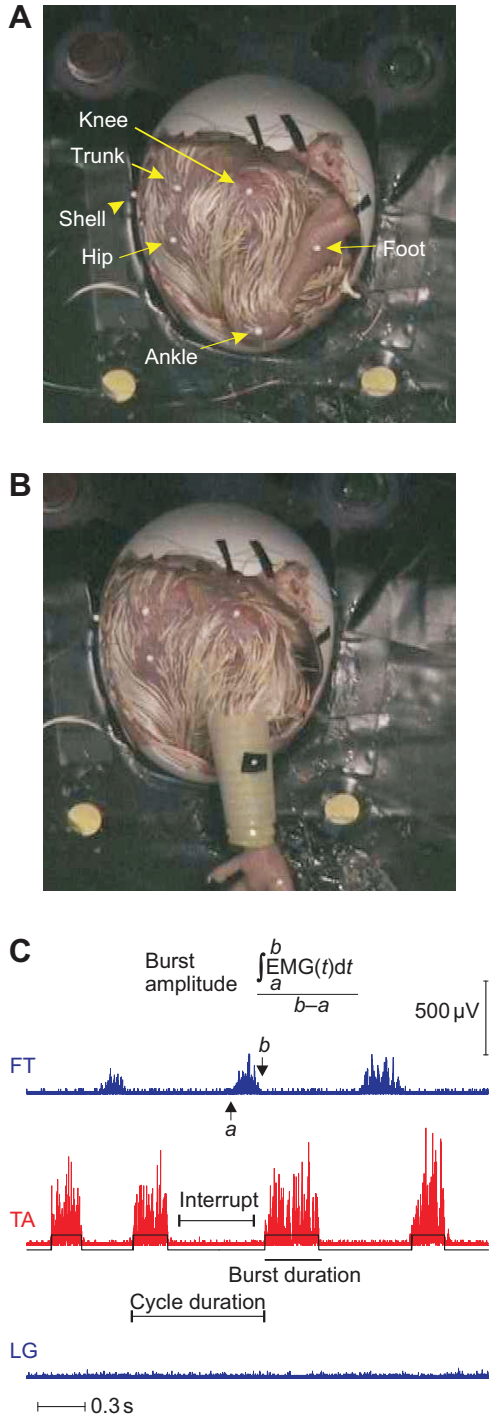

Fig. 1. Leg postural conditions and EMG burst detection methods. (A) Some of the shell was removed to prepare the leg for video and EMG recording while also retaining the leg within the egg during the control phase of the experiment. During the control posture, the ankle was maintained in marked dorsiflexion and movement was potentially constrained by the shell wall. However, it did not appear that leg movement was actively constrained by the shell wall because the foot rarely appeared to contact the wall either during rest or movement. (B) At the end of the control phase, the leg was lifted out of the egg and a lightweight splint was applied. During the experimental phase, the splint constrained the ankle in extreme extension. The splint also extended the hip and knee to varying degrees (Table 1). White markers, indicated by arrows (A), approximated the location of leg joints as well as a shell reference (reference origin) for off-line digitizing and kinematic data processing. (C) EMG burst detection was computer-automated to capture sequences of repetitive muscle activity. Burst detection was implemented using three parameters: burst threshold (2–3× signal during EMG inactivity, not shown), burst duration (20–1000 ms) and interburst interrupt (≥20 ms). Cycle duration measured the interval between consecutive burst onsets. The integrated area between burst onset (*a*) and offset (*b*) estimated burst amplitude. FT, femorotibialis; TA, tibialis anterior; LG, lateral gastrocnemius.

embryonic development did not contribute in forward reference to later adaptive behaviors (Haverkamp and Oppenheim, 1986). Neither deafferentation nor immobilization appeared to alter the development of motility (Hamburger et al., 1966; Narayanan and Malloy, 1974), and in *Xenopus* or *Ambystoma* embryos, immobilization had only transient impact on locomotor development (Haverkamp, 1986; Haverkamp and Oppenheim, 1986). Perturbation studies in the second embryonic week in chicks found modest indicators of sensory impact, but these studies could not sort out mechanical versus neural effects (Bradley, 1997; Bradley, 1999; Bradley and Sebelski, 2000; Sharp et al., 1999). Nonetheless, chicks deafferented as embryos failed to hatch or walk after assisted hatching (Narayanan and Malloy, 1974), whereas chicks deafferented after hatching produced most features of bilateral rhythmic stepping (Bekoff et al., 1987). These findings suggested to us that late embryonic and postnatal development may be shaped by embryonic sensorimotor activity. In our first attempt to test proprioceptive function, we proposed that the shell wall imposed mechanical constraints minimizing movement variability, thereby masking potential proprioceptive modulation. So we reduced the mechanical constraint of one leg by removing the adjacent shell wall. E20 embryos at least occasionally extended the leg beyond the egg, but we found no fundamental change in the RLM EMG pattern (Ryu and Bradley, 2009). Yet, after shell removal, knee extensor EMG during RLMs appeared to be more variable and high frequency RLM cycles appeared to be more common, suggesting to us that proprioceptive function could be at least intermittently driven by destabilized limb movements.

The question of proprioceptor function prior to the onset of walking is important because if proprioceptive circuits can code movements *in ovo*, then they may provide activity-dependent input to both reflex and spinal locomotor circuits and aid refinement of motor commands important for precocious motor skill. Such a role has been shown for refinement of descending motor circuits and forelimb reaching skill in the kitten (Martin et al., 2004; Martin et al., 2005). In this study we sought to more effectively drive and reveal proprioceptor function in *Gallus gallus* (Linnaeus 1758) during normal spontaneous movements by constraining the leg in a posture significantly different from the control posture *in ovo*. One goal of our study was to clearly establish when proprioceptive circuits code changes in limb posture or muscle length. Based on our earlier work, we hypothesized that proprioceptive circuits code changes in muscle length and/or tension during self-generated movement prior to hatching. The second goal was to determine whether an atypical postural constraint would induce significantly different RLM muscle patterns. We hypothesized that the locomotor-related circuits producing RLMs in intact late-stage embryos are modulated by proprioceptive inputs prior to the onset of adaptive locomotion at hatching.

RESULTS

Impact of the splint on resting leg posture and movement

In total, 4023 sequences of rhythmic bursting in one or more muscles were identified and included in the following analyses of

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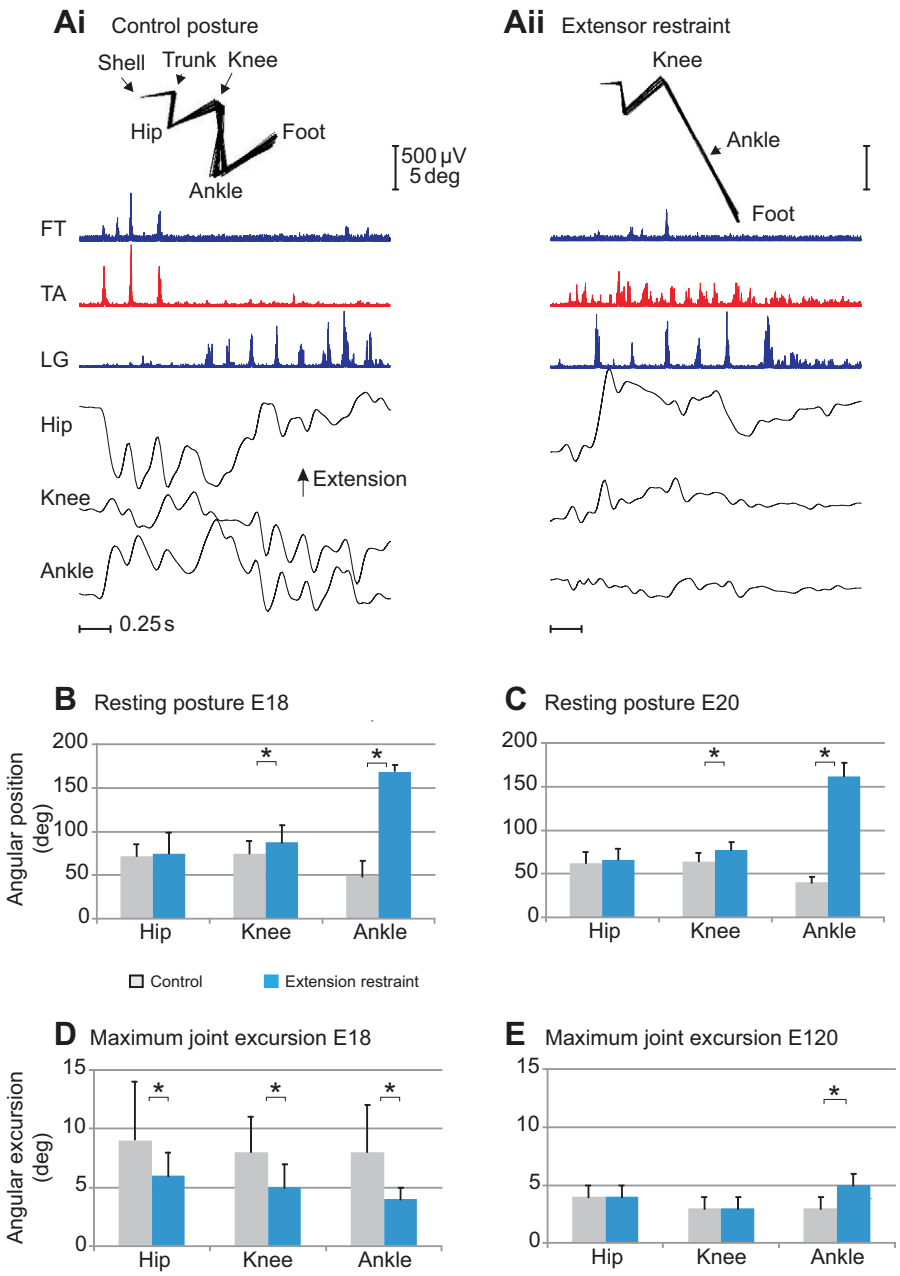

Fig. 2. Kinematic effects of the ankle splint on resting leg posture and movement during repetitive limb movements (RLMs). (A) Two plots from the same experiment illustrate RLM kinematics and EMG activity at E18 during control (Ai) and extension restraint (Aii). Line traces at the top of Ai and Aii are consecutive, superimposed leg stick images (plot resolution 30 Hz) that illustrate the hip, knee and ankle joint angle excursions during RLMs in control posture and extension restraint. The corresponding time series plots beneath the EMG traces indicate that maximum angular excursion ranges were ~10 deg (knee) to 15 deg (hip, ankle) during control posture and that motions were diminished during extension restraint. (B–E) Bar graphs indicate the group means and standard deviations for kinematic data at E18 (B,D) and E20 (C,E). Joint markers could not be tracked in three experiments, thus graphs summarize kinematic measures for a mean of 73 RLM sequences per embryo at E18 (N=10 embryos) and 147 sequences per embryo at E20 (N=11 embryos). (B) The splint significantly altered resting leg posture at E18 by extending the knee (P<0.005) and ankle (P<0.003). (C) The splint also altered leg posture at E20, increasing extension at the knee (P<0.002) and ankle (P<0.002). (D) The effects of the splint on maximum joint angular excursion range during RLMs were small (3–5 deg); but at E18, maximum excursion range was reduced at the hip (P<0.05); knee (P<0.006) and ankle (P<0.003). (E) Ankle excursions for RLMs at E20 were slightly greater during splint application than during control posture (P<0.014). Asterisks indicate significant within-subject differences (see Table 1 for details).

EMG and video recordings during spontaneous activity *in ovo* at E15, E18 and E20. The splint altered leg resting posture (Fig. 1A,B) and had mixed effects on movement during RLMs. Kinematic effects are summarized in Fig. 2 and Table 1. Kinematic plots are shown for an RLM during control (Fig. 2Ai) and ankle extension restraint (Fig. 2Aii) in an E18 experiment. The stick plots illustrate that the splint extended the ankle and the knee, but did not alter hip angle. The kinematics time series indicate that the splint attenuated

Table 1. Group means and s.d. for leg resting posture and movement during control and ankle extension restraint

	Angular position prior to movement				Maximum angular excursion range			
Joint	Control (deg)	ER (deg)	Z	P<	Control (deg)	ER (deg)	Z	P<
E18 (N=10)*								
Hip	72±14	75±24	−0.408	0.4	9±5	6±2	−1.696	0.05
Knee	75±14	88±19	−2.655	0.005	8±3	5±2	−2.53	0.006
Ankle	49±18	168±8	−2.805	0.003	8±4	4±1	−2.814	0.003
E20 (N=11)*								
Hip	62±13	66±13	−0.979	0.2	4±1	4±1	−0.181	0.5
Knee	64±9	78±9	−2.937	0.002	3±1	3±1	−1.354	0.09
Ankle	40±7	161±16	−2.937	0.002	3±1	5±1	−2.209	0.014

ER, extension restraint. Z, P: one-tailed, Wilcoxon signed ranks test.
*Number of repetitiive limb movements (RLMs) digitized per embryo: 53–113 for E18, 39–273 for E20.

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rotations of all three leg joints. Within-subject analyses indicated that the splint significantly extended the ankle and knee but had no effect on hip angle at either E18 (Fig. 2B) or E20 (Fig. 2C). On average, the splint extended the ankle ~125 deg beyond the control posture and extended the knee 10 deg. In E18 embryos, the splint also reduced maximum angular excursion range at all three joints an average of 3–4 deg (Fig. 2D). However, in E20 embryos, maximum excursion ranges were similar at the hip and knee, and slightly greater at the ankle during extension restraint (Fig. 2E; Table 1). Thus, kinematic data indicated that both the shell and the splint imposed restraints on ankle excursions. Leg posture appeared to be similarly altered by the splint at E15; however, kinematic methods could not be reliably applied and maintained during ankle extension restraint (see Materials and methods).

General impact of ankle extension restraint on EMG activity

The splint significantly extended the ankle and knee joints. Thus, muscles crossing the ankle anteriorly (i.e. tibialis anterior, TA) were stretched, whereas muscles crossing the ankle posteriorly (lateral gastrocnemius, LG) or knee anteriorly (femorotibialis, FT) were slackened. The effect of extension restraint was readily observed in EMG traces of RLM sequences at E20, and also detected at E18, but had no impact at E15. Plots for two RLM sequences from the same experiment are shown in Fig. 3 and illustrate the more common effects of ankle restraint at E20. During the control RLM sequence (Fig. 3A), ankle dorsiflexor (TA) burst durations were brief and most bursts were relatively low to moderate in amplitude. TA bursts were accompanied by repetitive bursting of the knee extensor (FT) and/or ankle extensor (LG). During the more intense bursting toward the end of the sequence, the EMG activity was accompanied by similarly timed rotations at the hip, knee and ankle joints. However, there were occasions when the FT or LG was repetitively active and the TA remained silent or minimally active (Fig. 2Ai). After the ankle was secured in the splint, there was an apparent increase in TA activity and a decrease or complete drop out of LG and FT activity (Fig. 3B). Increased TA activity was also observed at E18, but extensor activity was more likely to persist (Fig. 2Aii).

Significant effects of ankle extension restraint on ankle flexor EMG

Ankle extension restraint significantly altered ankle flexor burst parameters at both E20 and E18, but had no effect on RLM EMG at E15. The impact of extension restraint on TA EMG at E20 is illustrated by data for one experiment in Fig. 4, and is summarized for all age groups in Fig. 5. In Fig. 4, TA parameters are plotted for each burst in order of occurrence across consecutive RLMs for the three conditions: control, extension restraint (ER) and post-restraint control (PC). On average, TA burst duration, cycle duration and burst amplitude (Fig. 4A–C, respectively) increased during extension restraint. During post-restraint control, TA burst duration and amplitude returned to control values. However, TA cycle duration did not return to the control range (Fig. 4B), a trend observed in 3 of 6 experiments that included a post-restraint control at E20 and 2 of 4 experiments at E18 (Fig. 5A). As indicated by asterisks in Fig. 5A, Wilcoxon signed ranks tests indicated that the increases in TA parameters during restraint were significant for experiments at E20 (N=12, Bonferroni P<0.01). TA burst duration nearly doubled from control to extension restraint (control 67±10 ms, ER 114±31 ms, Z=–2.936, P<0.002), cycle duration increased 32% (control 197±68 ms, ER 260±103 ms; Z=–2.756, P<0.004) and burst amplitude more than doubled (control 1.6±0.5 mV s, ER 3.6±1.6 mV s; Z=–3.059, P<0.002). At E18 (N=12), ankle splint

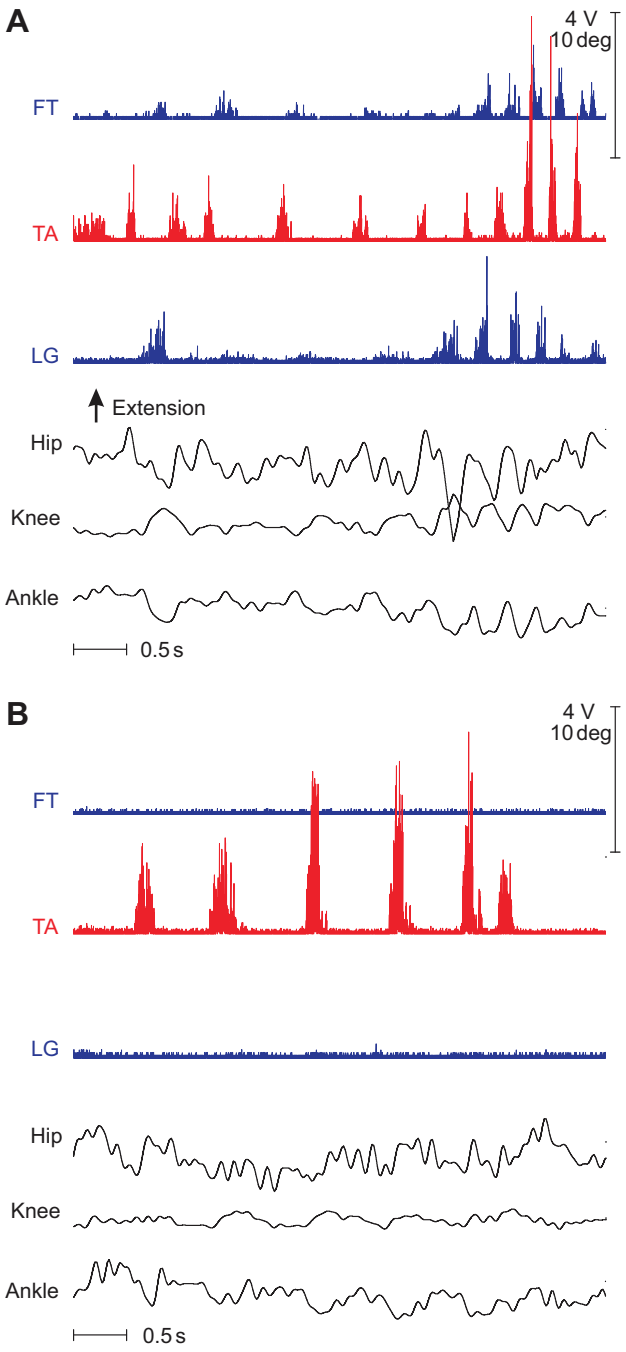

Fig. 3. Impact of extension restraint on EMG activity during RLMs at E20. Leg EMG and joint excursions are plotted for two RLMs from the same experiment during control (A) and ankle restraint (B). (A) During control, ankle flexor (TA) EMG was rhythmically active and intermittently accompanied by bursts in both the ankle extensor (LG) and knee extensor (FT). (B) During extension restraint, the TA was rhythmically active but bursts were longer in duration and larger in amplitude. In addition, FT and LG activity dropped out. Maximum joint excursion range was not different between conditions.

effects were more modest but still significant for TA burst duration (control 67±10 ms, ER 77±16 ms; Z=–2.673, P<0.005) and burst amplitude (control 1.1±0.4 mV s, ER 1.4±0.5 mV s; Z=–2.903, P<0.003). However, TA cycle duration tended to shorten during extension restraint at E18 (control 227±31 ms, ER 211±35 ms, Z=–2.040, P<0.021) but TA cycles during control RLMs also tended

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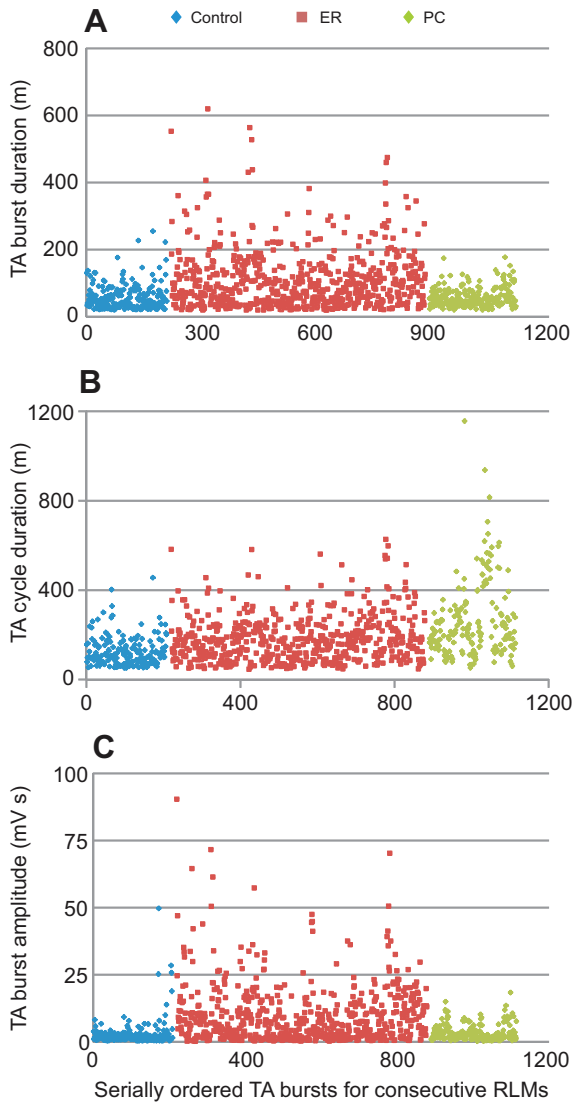

Fig. 4. Variations in TA burst parameters between postures during an E20 experiment. Each of three burst parameters are plotted for the same TA bursts serially ordered across consecutive RLMs during control (blue), extension restraint (ER, red) and post-restraint control (PC, green). (A) Mean TA burst duration (±s.d.) increased from 57±39 ms (control) to 108±86 ms (ER) then returned to 57±29 ms (PC). (B) Mean TA cycle duration increased from 128±67 ms (control) to 186±118 ms (ER) and increased further to 267±198 ms (PC). (C) TA burst amplitude sharply increased from a mean of 1.3±2.4 mV s (control) to 4.7±5.4 mV s (ER), then dropped to 1.7±1.5 mV s (PC).

to be longer than at E20 or E15. At E15 (N=10), TA burst and cycle durations during both postural conditions were similar to E20 control values (control burst duration 65±13 ms, cycle duration 188±28 ms; ER burst duration 67±14 ms, cycle duration 196±22 ms). TA burst amplitude at E15 did not vary between conditions and was approximately one-third of the amplitude at E20 and half the amplitude at E18 (control, ER: 0.5±0.1 mV s).

The magnitude of the effect on TA burst parameters imposed by extension restraint increased with age (Fig. 5B). The Kruskal–Wallis test indicated that the effects were significant for TA burst duration (χ^2=13.874, d.f.=2, P<0.001), cycle duration (χ^2=14.952, d.f.=2, P<0.001) and burst amplitude (χ^2=19.566, d.f.=2, P<0.001). *Post hoc* Mann–Whitney U-tests were significant for 4 of 6 comparisons (Bonferroni P<0.009) numerically identified in Fig. 5B (see legend for details). TA burst amplitude for control posture also increased with age (χ^2=22.477, d.f.=2, P<0.001, and *post hoc* results indicated that the incremental increases were significant for E15 to E18 (U=4.0, Z=–3.693, P<0.001) and E18 to E20 (U=30.0, Z=–2.425, P<0.008), Bonferroni correction P<0.025.

The relationship between TA burst duration and cycle duration also varied between postural conditions. Regression plots for representative experiments at each age are shown in Fig. 6. Both slopes and regression coefficients (R^2) for regression analyses indicated that during control posture, the relationship between burst and cycle duration was poorly defined at all three ages (Table 2). However, during extension restraint, the relationship was significantly strengthened at both E20 and E18 (Table 2).

Effects of extensor restraint on EMG activity of the ankle extensor

During extension restraint at E20, we frequently observed that EMG activity for extensor muscles decreased or dropped out (Fig. 3B). Given the evidence for homonymous motor neuron excitation during stretch of the TA at E20 and E18, we examined RLM bursts in the primary antagonist muscle, LG, and the extensor synergist, FT, for evidence of reciprocal inhibition. To determine whether LG was more likely to drop out during RLMs while the TA was lengthened by extension restraint, we divided the number of LG bursts by the number of TA bursts for all RLMs generated during control (control ratio) and compared this with the relative burst counts during extensor restraint (ER ratio). Control and ER ratios for LG are plotted in Fig. 7. LG participation significantly decreased or dropped out completely during extension restraint at E20 (Fig. 7A), and a similar trend was observed at E18 (Fig. 7B), but not at E15 (Fig. 7C). At E20 (N=10), the control ratio was 0.58±0.46 and the ER ratio was 0.12±0.11 (Wilcoxon signed ranks tests, Z=–2.192, P<0.02). The relative participation of LG was much greater at E18 than at E20 during both postural conditions. The control ratio exceeded 1.0 in 6 of 11 experiments at E18 (Fig. 7B) and averaged 1.44±1.36; still, during TA stretch, the ER ratio dropped by half to 0.72±0.5 (Z=–1.601, P<0.055). At E15 (N=9), LG and TA burst counts were similar for RLMs between postural conditions (control 0.34±0.27, ER 0.4±0.36). Further, LG burst parameters did not vary between conditions (Table 3). At E20, burst duration increased during extension restraint (exceeding the control mean + s.d.) in three experiments, decreased in one, and remained unchanged in five. During extension restraint at E18, LG burst duration and amplitude increased in three experiments and did not vary in eight. At E15, LG burst duration and amplitude increased in one experiment and did not vary in nine.

Effects of ankle restraint on EMG activity of the knee extensor

There were also many instances when the FT dropped out during extension restraint (Fig. 3B), as summarized in Fig. 8. At E20 (Fig. 8A), the ratio of FT and TA burst counts averaged 0.72±0.57 in control and 0.39±0.47 in extensor restraint (Wilcoxon signed ranks tests, Z=–1.632, P<0.053). At E18 (Fig. 8B), FT participation averaged 1.03±1.36 in control and dropped to 0.47±0.51 in extensor restraint (Z=–2.001, P<0.03). On average, FT burst amplitude decreased by 14% at E20 during extension restraint, but FT burst duration did not differ significantly between conditions at either E20 or E18 (Table 4).

We knew from earlier studies that at E20, FT burst onset can occur in the first half of a TA cycle (Fig. 3A) or in the second half (Fig. 1C), or it can burst twice, early and late in the TA cycle (first

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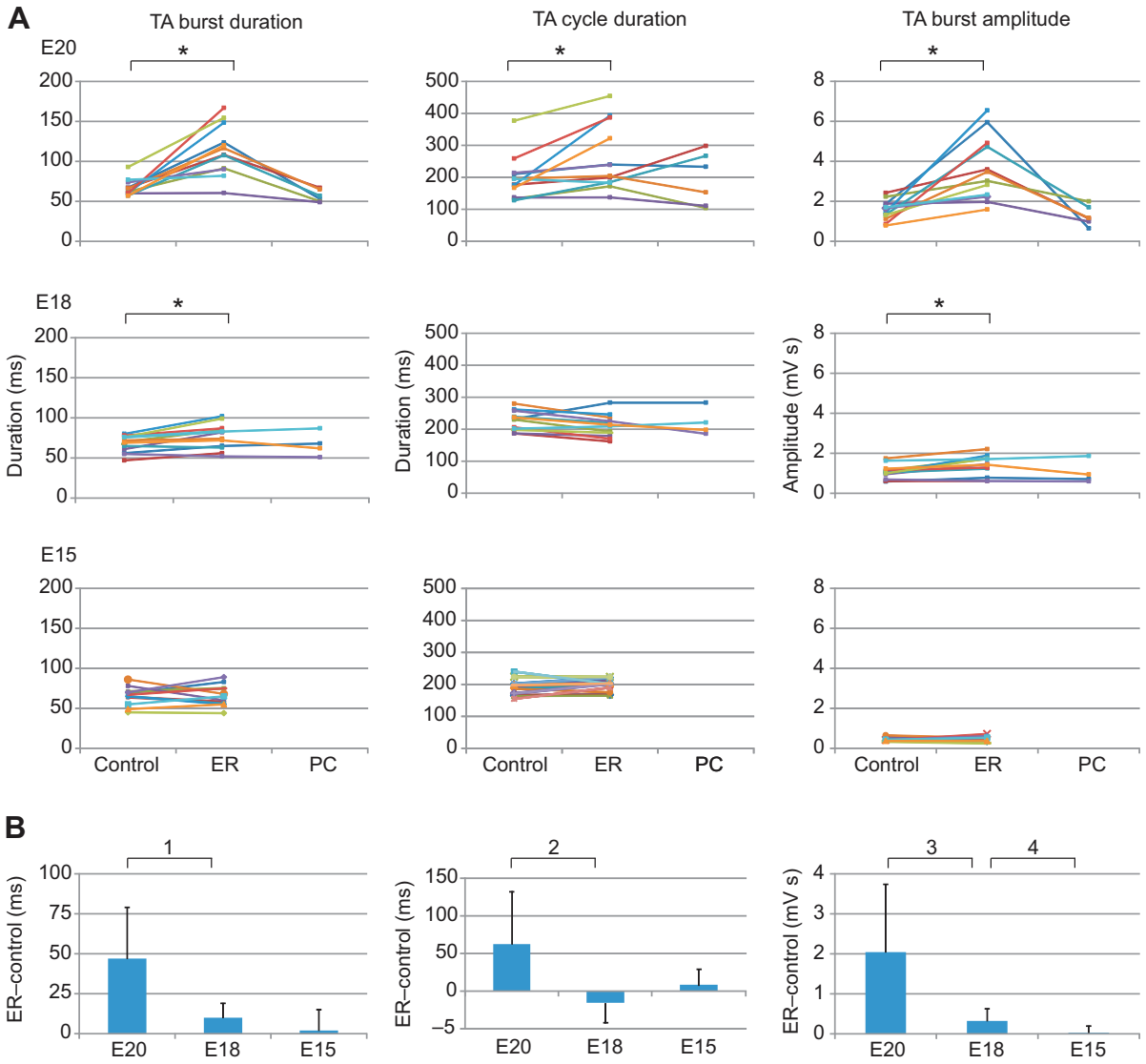

Fig. 5. Mean within-embryo and between-group comparisons of TA burst parameters. Analyses for TA burst parameters are ordered by column; left to right: burst duration, cycle duration and burst amplitude. (A) Line plots identify within-embryo means for burst parameters during control, ankle restraint and post-restraint control at E20 (*N*=12), E18 (*N*=12) and E15 (*N*=10). E20 experiments averaged 273 control and 380 ER bursts. E18 experiments averaged 276 control and 237 ER bursts. E15 experiments averaged 251 control and 328 ER bursts. Asterisks identify significant differences between control and extension restraint within group based on one-tailed, Wilcoxon signed ranks tests and Bonferroni corrections ($P<0.01$); statistical details are specified in the Results and are only summarized here. During extension restraint at E20, TA burst duration increased ($P<0.002$), cycle duration increased ($P<0.004$) and burst amplitude increased ($P<0.002$). During extension restraint E18, TA burst duration increased ($P<0.005$) and burst amplitude increased ($P<0.003$), but the decrease in TA cycle duration fell short of significant ($P<0.021$). (B) Plots identify group means (±s.d.) for the relative effect of extension restraint on TA burst parameters, i.e. the difference between extension restraint and control posture, and the results of between-group comparisons. Kruskal–Wallis tests for comparisons across age were significant (see Results). Mann–Whitney *U*-tests for *post hoc* comparisons indicated the effects of extension restraint were greatest at E20 and least at E15. Significant *post hoc* comparisons after Bonferroni correction ($P<0.009$) are numbered: $^{1}U=21$, $Z=-2.948$, $P<0.002$, $^{2}U=11$, $Z=-3.522$, $P<0.001$, $^{3}U=16$, $Z=-3.233$, $P<0.001$, $^{4}U=23$, $Z=-2.44$, $P<0.008$.

cycle of Fig. 2Ai). These variations in pattern might indicate that FT bursts are sensory modulated, so we asked whether extension restraint at E20 altered FT burst parameters. The splint placed the knee, as well as the ankle, in more extension. Also, the splint effectively increased the lever arm formed by the lower leg and acting at the knee. Thus, it is likely that the splint altered motion-dependent dynamics at the knee and proprioceptive feedback to the knee extensor motor pool. As indicated in Table 5, there was a significant bias for early onset (FT1) compared with late onset (FT2) during control posture, whereas FT1 and FT2 bursts were equally distributed during extension restraint. Further, the R^2 coefficient for regression analyses (Table 5) indicated that the scaling between FT1 burst duration and TA cycle duration was stronger during extensor restraint, while the scaling was equally strong for FT2 during the two postural conditions.

DISCUSSION

In this study we hypothesized that proprioceptive circuits code changes in limb posture or muscle length and modulate circuits producing RLMs prior to hatching, because chicks walk adaptively within hours of hatching (Muir, 2000; Sindhurakar and Bradley, 2010). We tested these hypotheses by applying a restraint to impose

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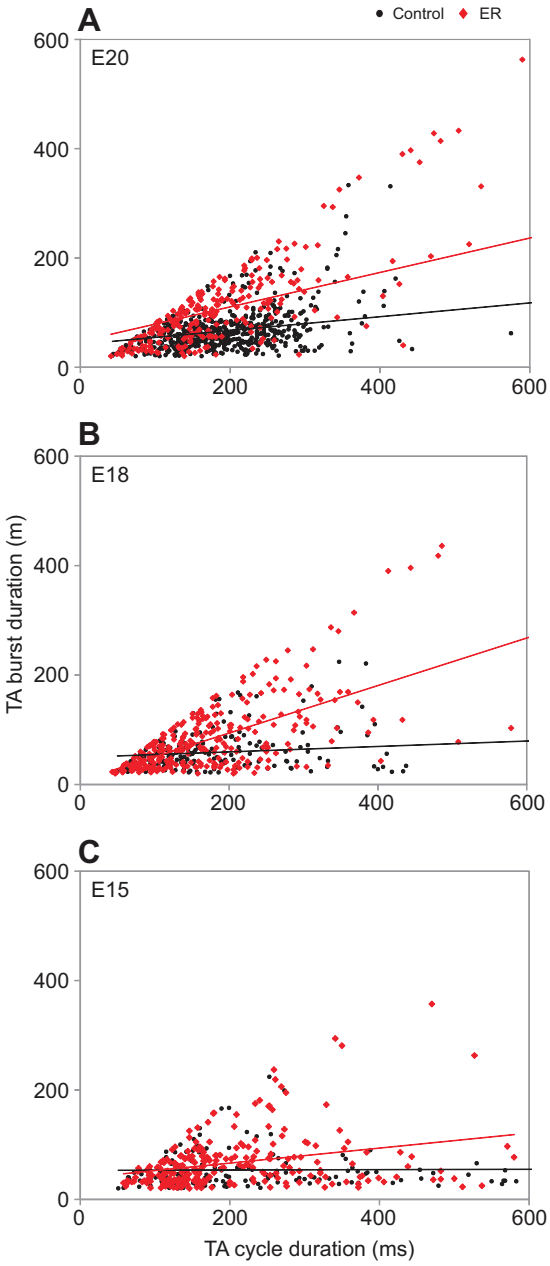

Fig. 6. Examples of the relationship between TA burst duration and TA cycle duration. TA burst duration is plotted relative to the concurrent TA cycle duration for all RLM cycles during control and extension restraint (ER) for three experiments (A–C). Regression slopes for Pearson correlations are also plotted for control posture (black) and ankle restraint (red). A small number of data points exceeding one or both axes were cropped for visually optimal and uniform presentation of parameter ranges, but were included in the regressions: six points in A, three in B and four in C, equally divided between control posture and extension restraint. (A) E20 experiment. Control: 678 TA cycles, slope=0.13, R^2=0.10; ER: 316 cycles, slope=0.32, R^2=0.36. (B) E18 experiment. Control: 229 cycles, slope=0.05, R^2=0.03; ER: 342 cycles, slope=0.43, R^2=0.39. (C) E15 experiment. Control: 185 cycles, slope=0.003, R^2=0.001; ER: 314 cycles, slope=0.14, R^2=0.09.

an atypical limb posture during spontaneously generated RLMs. The restraint significantly extended both the ankle and the knee. The resulting changes in RLM burst parameters supported both hypotheses and provided evidence that proprioceptors provide input to the spinal cord about limb posture and/or muscle length at least 3 days before hatching. The results also indicated that proprioceptive input can alter both the rhythm and pattern of RLMs at least 1 day before hatching.

Proprioception established by E18

Our findings provide the first clear, reliable evidence that proprioceptors can implement both autogenic excitation and reciprocal inhibition during self-generated behavior in the intact chick embryo. Extension restraint significantly enhanced TA burst parameters during spontaneous RLMs at E18 and E20. Extension restraint increased TA burst duration, TA burst amplitude and the scaling of TA burst duration relative to cycle duration. Although we did not measure TA muscle length, it is reasonable to assume that the 100–110 deg increase in ankle extension altered the working length of extrafusal and intrafusal muscle fibers, as observed under similar constraint but smaller ankle ranges in the cat (Hyngstrom et al., 2007). Ia afferents were likely responsible for autogenic excitation of TA motor units given that the central synapse between primary afferents and motor neurons is functional by E7–E8 in the chick (Davis et al., 1989; Lee et al., 1988). Autogenic mapping of Ia afferents to motor neurons is also highly selective by the end of embryogenesis in the mouse (Wang et al., 2007). Further, it is likely that autogenic excitation was enhanced by the resisted shortening of repetitively contracting TA fibers and contributed to modulations in TA EMG at E18 and E20. In E15 experiments, however, no autogenic excitation was detected, indicating that intrafusal fibers were not effective length transducers, corroborating anatomical evidence that their differentiation is not complete until E17 (Maier, 1993).

Enhanced TA recruitment during ankle restraint was accompanied by diminished recruitment of LG, the functional antagonist of TA, strongly indicative of reciprocal inhibition, and additional evidence that proprioceptor transduction was robust during self-generated movements at least 3 days before hatching. LG participation in RLM cycles was reduced ~50% (E18) to 79% (E20) during extension restraint, and when bursts were generated at E20, amplitude was reduced ~29%, collectively indicating that drive to ankle extensor motor neurons was lower during extensor restraint. Our findings during intact behavior complement and extend reports of reciprocal inhibition in surgically reduced neonatal experiments. In mouse, glycine-mediated reciprocal inhibition of antagonist motor neurons can be weakly evoked by electrical activation of Ia afferents at P0 (day of birth) and by muscle tap P8 (Wang et al., 2008). Tendon taps can also elicit reciprocal inhibition in EMG recordings from human neonates (McDonough et al., 2001). In the adult decerebrate cat, robot-controlled ankle extensions within a positional context somewhat similar to our extensor restraint induced LG motor neuron inhibition in phase with TA stretch (Hyngstrom et al., 2007). Voltage clamp recordings indicated that the inhibition was produced by reduced persistent inward sodium currents in LG motor neurons. The inhibition was mediated by Ia inhibitory interneurons activated by TA stretch, because LG inhibition was lost after TA tenotomy. Thus, we conclude that by E18 in the chick, muscle spindles are sufficiently mature to code stretch and drive primary afferent modulation of Ia inhibitory circuits during self-generated movement, and their impact becomes more robust between E18 and E20. However, stretch-activated inhibition was never absolute; when LG was active, burst amplitude was reduced but burst duration did not significantly vary from the range for control RLMs. We presume that inhibition was incomplete because the embryos were neurologically intact and movements were self-generated. However, LG deletions were also common during control RLMs, and could have been produced by prolonged reciprocal inhibition during TA bursting and/or immature spinal or descending pathways. In rat,

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Table 2. Group means and s.d. for linear regression analyses comparing the relationship between TA burst duration and TA cycle duration during control and ankle extension restraint

	Slope	*Z*	*P*<	R^2	*Z*	*P*<
E20 (*N*=12)						
Control	0.16±0.10			0.14±0.10		
ER	0.25±0.11	−2.197	0.015	0.30±0.11	−2.746	0.004
E18 (*N*=12)						
Control	0.08±0.10			0.07±0.09		
ER	0.23±0.19	−2.668	0.005	0.19±0.17	−2.432	0.008
E15 (*N*=8)						
Control	0.19±0.15			0.16±0.13		
ER	0.21±0.16	−0.56	0.3	0.19±0.15	−0.7	0.3

P: one-tailed, Wilcoxon signed ranks test, Bonferroni correction *P*<0.01.

membrane properties of LG motor neurons and their descending drive are slower to mature (Brocard et al., 1999). Also, during sustained depolarization, they are less able to fire repetitively and fire at slower frequencies than TA motor neurons (Vinay et al., 2000).

Extensor restraint at E20 appeared to exert similar, albeit weaker, inhibitory trends during recruitment of the knee extensor, FT.

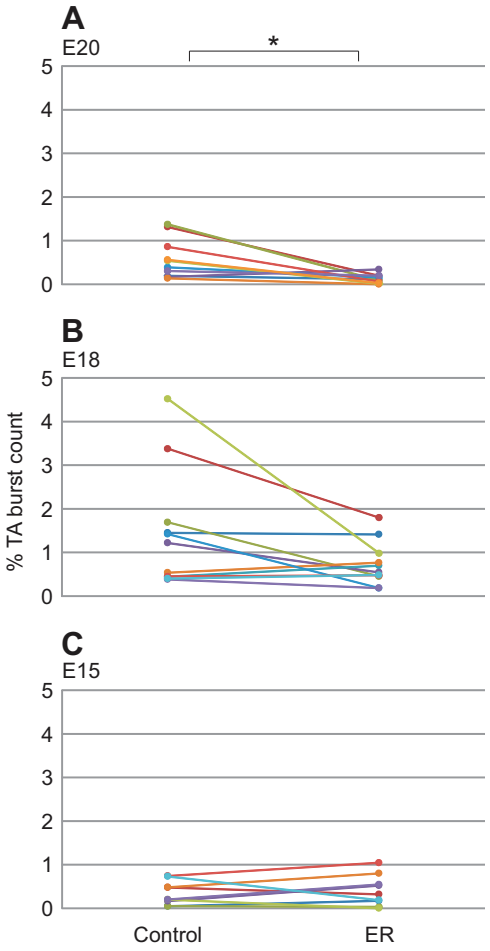

Fig. 7. Participation of the ankle extensor muscle, LG, during RLMs in control posture and extension restraint. Participation of LG activity was quantified by the ratio of total LG burst number relative to total TA burst number in the same condition. LG participation is plotted for each experiment per age group: (A) E20, *N*=10; (B) E18, *N*=11; and (C) E15, *N*=9. The reduction in LG participation during extension restraint was significant (asterisk) at E20 (*P*<0.02) and fell just short of significant at E18 (*P*<0.055); at E15 it was not significant (*P*<0.3) (see Results for details).

Participation tended to be less frequent during extensor restraint at both E18 and E20. At E20, when active, FT burst amplitude was reduced, even as burst duration and cycle duration lengthened in concert with TA parameters. The weaker restraint effects on FT parameters, compared with LG, are consistent with evidence that primary afferents selectively inhibit the motor pools of muscles acting as antagonists at the same joint in the cat (Nichols et al., 1999) and neonatal mouse (Wang et al., 2008). Thus, reduced FT recruitment was not likely produced by TA Ia inhibitory circuits. The FT variably functions as a hip flexor and knee extensor (Jacobson and Hollyday, 1982a; Jacobson and Hollyday, 1982b; Johnston and Bekoff, 1996; Ryu and Bradley, 2009), thus it is possible that there were mixed effects of excitation and inhibition acting at the FT motor pool. For example, the restraint placed the knee in greater extension (less FT stretch), possibly reducing the overall level of autogenic excitation within the FT motor pool. Yet the mechanical effects of ankle restraint may have also introduced excitatory synaptic effects, because the restraint effectively lengthened the lever arm formed by the lower leg (compare Fig. 2Ai and 2Aii), increasing the total limb load acting at the knee. Under our conditions, it is possible that the increased load at the knee resisted intrafusal fiber shortening during alpha-gamma co-activation and Ia excitation of FT motor neurons. Unfortunately, there is limited information on Golgi tendon organs in birds and none on their development or function to speculate as to their possible contributions (Maier, 1998).

Proprioceptive inputs modulate locomotor rhythm and pattern prior to hatching

By E20, proprioceptive input from ankle extension restraint also strengthened the relationship between rhythm-related parameters

Table 3. Group means and s.d. for LG burst parameters

	Control	ER	*Z*	*P*<
LG burst duration (ms)				
E20	63±10	70±27	−0.178	0.5
E18	50±8	55±7	−1.959	0.03
E15	60±14	64±15	−1.377	0.09
LG cycle duration (ms)				
E20	215±34	272±89	−1.599	0.06
E18	302±72	273±18	−1.334	0.07
E15	248±80	252±57	−0.561	0.3
LG burst amplitude (mV s)				
E20	1.0±0.3	0.7±0.4	−1.718	0.05
E18	0.7±0.2	0.8±0.2	−0.8	0.3
E15	0.5±0.2	0.5±0.3	−0.51	0.4

LG burst parameters are shown for E20 (*N*=9), E18 (*N*=11) and E15 (*N*=10). *Z*, *P*: one-tailed, Wilcoxon signed ranks test, Bonferroni correction *P*<0.017.

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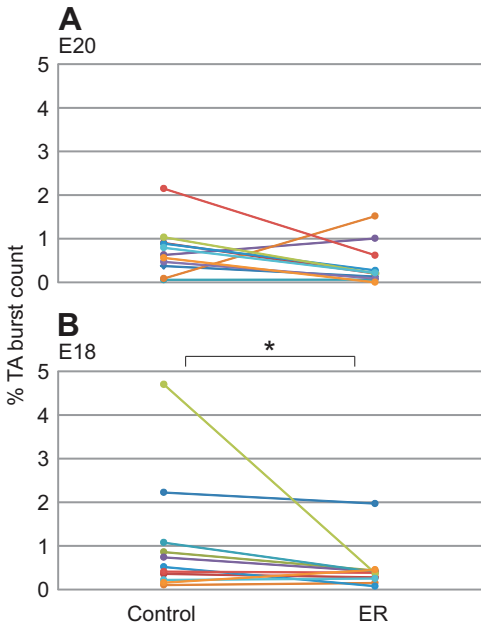

Fig. 8. Participation of the knee extensor muscle, FT, during RLMs in control posture and extension restraint. FT participation decreased during ankle restraint at E20 (A) and E18 (B), *N*=11 per group. The decrease was significant (asterisk) at E18 (*P*<0.03), and fell just short of significant at E20 (*P*<0.053) (see Results for details).

controlled by spinal pattern generating circuits. Increases in TA burst duration were accompanied by increases in TA cycle duration, strengthening the co-variation between these parameters. This scaling is typical of extensor muscle activity during weight-supported locomotion but not flexor activity in adult (Grillner and Zangger, 1984) and neonatal animals (Bradley and Smith, 1988), and is attributed to autogenic excitation of both muscle spindles and Golgi tendon organs (Donelan and Pearson, 2004; Pearson et al., 1998). However, more recent studies in cat and human have shown that this scaling is not unique to extensor activity. During fictive locomotion in the cat evoked by electrical stimulation of the midbrain locomotor region, changes in cycle duration were attributed to changes in flexor phase duration (Yakovenko et al., 2005). During stepping in human infants, selective loading of leg flexor or extensor muscles lengthened the flexor or extensor phase, respectively, and step cycle duration (Musselman and Yang, 2007). Selective control of each phase is essential to environmentally adaptive locomotion, i.e. adjustment of extensor phase to support body weight, and adjustment of flexor phase to clear environmental barriers or to advance the leg against a resistance. Thus, we conclude that TA responses during extension restraint indicate that proprioceptive function in the embryo is sufficiently developed to modulate locomotor rhythm at least 1 day prior to hatching.

Table 4. Group means and s.d. for FT bursts

	Control	ER	*Z*	*P*<
FT burst duration (mV)				
E20	68±15	72±17	−0.445	0.4
E18	51±7	51±9	−0.178	0.5
FT cycle duration (ms)				
E20	216±62	330±178	−2.223	0.014
E18	270±66	244±43	−1.689	0.05
FT burst amplitude (mV s)				
E20	0.9±0.3	0.8±0.2	−1.956	0.03
E18	0.7±0.2	0.6±0.3	−0.264	0.4

FT burst parameters are shown for E20 (*N*=11) and E18 (*N*=11).
Z, *P*: one-tailed, Wilcoxon signed ranks test, Bonferroni correction *P*<0.017.

Table 5. Group means and s.d. for FT burst onset at E20

	Control	ER	*Z*	*P*<
Burst onset (% of TA cycle)				
FT1	26±5	27±5		
FT2	70±4	71±3		
Distribution of bursts (% of sample)				
FT1	66±8*	51±18	−1.917	0.03
FT2	34±8*	49±18	−1.917	0.03
R^2 regression coefficient: FT burst duration/TA cycle duration				
FT1	23±16	53±28	−2.705	0.004
FT2	86±9	86±6	−0.089	0.5

N=11; *Z*, *P*: one-tailed, Wilcoxon signed ranks tests, Bonferroni correction *P*<0.01.
*Control distribution (FT1, FT2), *Z*=−3.062, *P*<0.002.

The FT is a knee extensor but it also contributes to hip flexion, thus it has a complex EMG pattern that varies across stepping behaviors in post-hatching chicks (Jacobson and Hollyday, 1982a; Johnston and Bekoff, 1996). FT burst pattern also varies during RLMs, and by E20, two different bursts can be identified (FT1, FT2). FT1 begins early in the RLM cycle and overlaps with ankle and hip flexor EMG, while FT2 overlaps with ankle extensor EMG (Ryu and Bradley, 2009). We previously proposed that variations in FT burst generation were evidence that movement-dependent modulation of limb CPG control is established prior to hatching, but our results were only weakly supportive (Ryu and Bradley, 2009). In that study, we removed the shell wall, allowing the leg to extend beyond the egg; however, the RLMs were less rhythmically stable than control RLMs and leg posture was free to vary. FT bursting was variable, possibly due to reduced postural stabilization and variable proprioceptive feedback. Thus, in this study we tested whether FT bursting could be altered by a novel postural restraint that also stabilized leg posture during spontaneous RLMs. The restraint extended the knee and ankle, creating a single, longer leg segment extending from knee to toes, that likely altered mechanical forces acting at the knee. When the FT was active during RLMs, the relative timing of the first and second bursts did not change, evidence that the two-burst FT pattern is centrally generated (Jacobson and Hollyday, 1982b). However, the relative distribution of FT1 and FT2 changed significantly. During control posture, FT1 was more prevalent than FT2 (ratio 2:1), whereas during extension restraint, the two FT bursts were equally expressed, and FT1 burst duration closely varied with cycle duration. Restraint applied to the cat hindlimb during cutaneous evoked paw shaking produced similar shifts in knee extensor activity from an early burst coactive with the TA to a later burst coactive with the LG, a shift not seen after limb deafferentation (Koshland and Smith, 1989a; Koshland and Smith, 1989b). Centrally generated two-burst patterns also occur during fictive locomotion in the neonatal rat, and recruitment varies with the form of network activation, i.e. pharmacologic, electrical currents (Klein and Tresch, 2010). Thus, we interpret our findings as significant evidence that proprioceptive input produced by limb movement can modulate centrally generated RLM patterns prior to hatching.

Role of proprioception prior to hatching

Although ankle extension restraint revealed proprioceptor function, our results do not address whether proprioceptive circuits are normally driven by movement *in ovo* or whether they modulate

embryonic motor control prior to hatching, questions that are important and warrant focused study. Under control conditions, TA burst frequency can vary from 1 to 10 Hz without any apparent variation in postural context or modulation in TA burst duration (Bradley et al., 2008). While these features attest to the central generation of RLMs, they also suggest there is no sensorimotor-related modulation, like that imposed by a changing belt speed during treadmill locomotion, to account for the parameter variations. It is possible, for example, that spindle length and/or muscle proprioceptive circuits may not be tuned to the muscle working ranges of control posture *in ovo*. The frequent low amplitude bursts and deletions in LG activity during control RLMs also raise the question of whether the stretch imposed by marked ankle flexion (Fig. 1A) activates LG proprioceptors, or whether their central synapses are inhibited by other spinal and/or descending pathways. For example, as embryos outgrow egg volume, the shell wall potentially imposes passive mechanical constraints by limiting working ranges for posture and movement (Bradley et al., 2005; Sharp et al., 1999), but it is unclear whether extensive foot contact and limb loading occur during RLMs (Bradley et al., 2008). We have only observed sustained leg–shell contact, accompanied by tonic extensor EMG or extensor–flexor coactivity, during pre-hatching postural extensions associated with body rotation (N.S.B., unpublished). Thus, to more clearly determine the *in ovo* environmental effects on motor control development, future studies need to determine whether proprioceptors are active within normal ranges of posture and movement, and whether they can also modulate central circuits during self-generated movements.

In conclusion, we propose that proprioception makes important contributions to the transformation of pre-hatching locomotion-related behavior to precocious locomotion post-hatching. In earlier work it was found that chick embryos developed normally after leg deafferentation, but they could not hatch independently, support their body weight or perform rhythmic alternating steps (Narayanan and Malloy, 1974). In contrast, chicks deafferented after hatching could produce rhythmic alternating steps and EMG patterns for stepping that were similar to those of afferent-intact hatchlings (Bekoff et al., 1987). It has also been shown than proprioceptive circuits in the cervical spine contribute to behavioral transformations between *in ovo* motor behaviors and hatching (Bekoff and Kauer, 1982; Bekoff and Trainer, 1979). The transformation from RLMs to locomotion post-hatching is not yet fully understood. RLMs exhibit most of the EMG features for stepping; however, they lack the differential scaling of extensor and flexor burst duration observed during limb loading (Ryu and Bradley, 2009). Nonetheless, our results demonstrate that by constraining the chick embryo's ankle in an atypical posture, the pattern of self-generated RLMs was modified. Sustained stretch of the ankle dorsiflexor activated autogenic excitation and reciprocal inhibition of the ankle antagonist extensor, and modified both the RLM rhythm and pattern. It will be important in future studies to determine in what ways proprioceptors contribute during normal *in ovo* conditions and preparations for precocious locomotion.

MATERIALS AND METHODS

Chicken eggs (*G. gallus*) were obtained from a local hatchery and incubated under standard conditions (37.5°C, 62% humidity). Fertile eggs were transferred at one of three embryonic ages (E15, E18 or E20) to a heated and humidified dish for EMG and kinematic preparations under a stereomicroscope. Age was based on established staging criteria (Hamburger and Hamilton, 1992). Embryos were maintained in a heated and humidified chamber during the experiment. All embryos were recorded under control and experimental conditions (within-subject design). At the end of experiments, embryos were given a lethal dose of pentobarbital. All procedures were approved by the University Animal Care and Use Committee.

Preparation and recording

The egg shell and membranes overlying the lateral surface of the right leg and lower trunk were removed for EMG and kinematic preparation. Only embryos optimally positioned within the egg and requiring minimal handling during preparations were selected for these experiments to minimize trauma and obtain several hours of active behavior. Small incisions were made in the skin, and on average, three leg muscles were implanted with silver bipolar electrodes (wire diameter 50 µm, California Fine Wire Company, Grover Beach, CA, USA). EMG recordings were obtained from a combination of the following muscles: TA (ankle dorsiflexor), extensor digitorum longus (EDL, ankle and toe dorsiflexor), LG (ankle plantiflexor), flexor digitorum profundus (FDP, ankle and toe plantiflexor), FT (knee extensor) and sartorius (SA, hip flexor/knee extensor). Electrode tip locations were verified by dissection at the end of each experiment after the embryo had been killed. In E18 and E20 embryos, markers fabricated from Minutin pins were inserted along the lateral aspect of the leg, approximating the location of the trunk, hip, knee, ankle and foot (Fig. 1A). A marker was placed on the shell as a stable origin for the leg kinematic model (Fig. 2A). Small body size and delicate tissues at E15 were problematic, thus E15 embryos were not prepared for kinematic recording. Detailed descriptions of these methods have been published (Bradley et al., 2005; Chambers et al., 1995; Orosz et al., 1994).

EMG and video were recorded continuously for 4–6 h and automatically stored to disk in 10 min increments (Datapac 2K2, Run Technologies, Mission Viejo, CA, USA). Concurrent EMG and video files were synchronized online by pulses from two independent analog outputs that were stored in the EMG file and were also directed to two LEDs in the video field; one output was automatically activated at the onset of an acquisition file and the other was manually activated at 4–5 min intervals. EMG channels were band pass filtered (100–1000 Hz), amplified (×2000) and digitally sampled at 4 kHz for storage to disk. An overhead video camera captured the sagittal view of the leg and the two LEDs (Fig. 1A). Video was recorded directly to disk (60 pictures s^{-1}).

Ankle extension restraint

All experiments recorded spontaneous leg EMG and kinematics during two postural conditions, the flexed leg position assumed *in ovo*, i.e. control posture (Fig. 1A), and restraint of the ankle in an extended position, i.e. extension restraint (Fig. 1B). Extension restrain was imposed by placing the lower leg and foot in a lightweight splint fabricated from a plastic drinking straw that maintained the ankle joint in an angular position of ~160–170 deg. Activity was first recorded during control posture for 2–3 h and then for 2–3 h during extension restraint. In a few experiments, after removing the splint, we attempted to restore leg control posture (post-restraint control) and record for 1–2 h.

Data analyses

EMG traces and video were reviewed to identify all possible occurrences of repetitive limb movement. Limb movements were typically small in amplitude and not easily visualized in video recordings during ankle extension restraint. Therefore, all EMG sequences of four or more consecutive and similarly spaced bursts in at least one muscle were included in initial analyses, as these methods were previously demonstrated to reliably detect RLM sequences exhibiting locomotor-related EMG activity (Bradley et al., 2008). EMG was rectified to implement automated detection (Datapac 2K2, Run Technologies) of burst onsets and offsets based on pre-defined burst criteria (Fig. 1C): burst threshold (2–3× signal amplitude during EMG inactivity), burst duration (20–1000 ms) and inter-burst interrupt duration (≥20 ms). Only sequences appropriately represented by these automated burst detection parameters were retained for analyses. Consecutive TA burst onsets were used to calculate TA cycle duration. Burst onsets and offsets in all other muscles were also referenced to the concurrent

TA cycle. The integrated area of rectified bursts was calculated to estimate recruitment amplitude in each muscle during all sequences of repetitive activity (Fig. 1C). The relative participation of each extensor muscle was estimated by a ratio of its burst count relative to the TA burst count in the same postural condition.

RLMs identified by EMG were located in the synchronized video recording and automatically digitized (Datapac 22K, Run Technologies) using the leg kinematic model to track leg posture and movement. All kinematic samples were digitized at 60 Hz beginning 1–3 s prior to RLM onset to produce a time series for joint angular position of the hip, knee and ankle. Pilot digitizing indicated that leg segment lengths often varied >10%. Thus, all digitized samples were filtered and joint angles were calculated after correcting for out-of-plane movement using an algorithm (CONVERT) and methods previously established for this purpose (Chambers et al., 1995; Orosz et al., 1994). The angular positions of the hip, knee and ankle in the first digitized picture of each RLM were selected to represent resting leg posture. The maximum angular excursion range at each joint (e.g. maximum angular position minus minimum angular position) was used to estimate the amplitude of leg movement during each RLM.

Burst parameters were averaged within the embryo for control and ankle restraint, but because RLM EMG sample size is often small for younger embryos (Ryu and Bradley, 2009), we set a minimum inclusion requirement of 30 TA bursts per condition, and for LG or FT burst analyses, at least 30 bursts in one of the two conditions. Non-parametric tests were used for statistical comparisons owing to the relatively small number of embryos per group. We anticipated age-dependent effects and used the Wilcoxon signed ranks test for paired within-embryo means, to test for differences between control and ankle extension restraint at each age. Planned significance testing was set at $P<0.05$, one-tailed. However, we treated all EMG burst parameters (i.e. burst duration, cycle duration, burst amplitude, regression slope and correlation coefficient) as interdependent, and used a Bonferroni correction for five burst parameters to adjust the significance level ($P<0.01$). We used the Kruskal–Wallis test to test for a difference in effect of ankle extension restraint across ages, and the Mann–Whitney test for *post hoc* comparisons. Linear regression analyses were used to test for scaling of TA burst duration relative to TA cycle duration only for those samples with >40 bursts due to instability of regressions for small samples. Descriptive statistics report group means ± s.d. for within-embryo means.

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Competing interests
The authors declare no competing financial interests.

Author contributions
N.S.B.: conception, design, data analyses and interpretation of findings, drafting and revising of the article. Y.U.R.: conception, design, data analyses and interpretation of findings, execution of experiments, revising of the article. M.C.Y.: data analyses and interpretation of findings, revising of the article.

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