Development 135, 3707-3717 (2008) doi:10.1242/dev.026773

The development of motor coordination in *Drosophila* embryos

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We used non-invasive muscle imaging to study the onset of motor activity and emergence of coordinated movement in *Drosophila* embryos. Earliest movements are myogenic, and neurally controlled muscle contractions first appear with the onset of bursting activity 17 hours after egg laying. Initial episodes of activity are poorly organised and coordinated crawling sequences only begin to appear after a further hour of bursting. Thus, network performance improves during this first period of activity. The embryo continues to exhibit bursts of crawling-like sequences until shortly before hatching, while other reflexes also mature. Bursting does not begin as a reflex response to sensory input but appears to reflect the onset of spontaneous activity in the motor network. It does not require GABA-mediated transmission, and, by using a light-activated channel to excite the network, we demonstrate activity-dependent depression that may cause burst termination.

KEY WORDS: *Drosophila*, Embryo, Movement, Muscle, Coordination

INTRODUCTION

Although the only output of the nervous system is movement, we know remarkably little about how animal embryos organise and perfect their movements as their nervous systems mature and begin to function. This is in contrast to our understanding of the input side of the brain, where decades of research have revealed many of the mechanisms that underlie the formation and tuning of the well-ordered sensory maps that constitute the interface between the external world and the central network.

At the heart of the output machinery are central pattern generating circuits whose outputs drive stereotyped patterns of movement such as swimming, walking and breathing (Grillner et al., 2005). These rhythmic patterns form a large part of the repertoire of animal movements. Part of the difficulty in studying how such circuits develop and begin to function lies in the fact that they are embedded within the central nervous system and do not have the explicit, often two-dimensional, organisation that characterises the anatomy of many sensory maps. To this anatomical problem are added technical difficulties inherent in attempting to make a quantitative assessment of the maturing functional properties of such circuits (such as coordination between multiple different neurons) in the developing embryonic nervous system.

However, there are instances, such as the spinal cord in *Xenopus* and zebrafish, where numbers of cells are small and the complete set of different cell classes required for early movements can be defined (Bernhardt et al., 1990; Brustein et al., 2003; Hale et al., 2001; Roberts, 1990; Roberts et al., 1998). This catalogue, in combination with paired cell recordings in *Xenopus* allows a very complete picture to be built up of how these cells operate in the motor network that generates swimming (Li et al., 2007; Li et al., 2004). In both cases, swimming emerges from earlier spontaneous movements driven by motor outputs that begin shortly after functional endplates are formed on the myotomes of the trunk (Kuwada et al., 1990; Saint-Amant and Drapeau, 1998; van Mier et al., 1989). Initial outputs in zebrafish appear to depend on periodic depolarisation of electrically coupled neurons in the early spinal network (Roberts and Perrins, 1995; Saint-Amant and Drapeau, 2000; Saint-Amant and Drapeau, 2001; Soffe and Roberts, 1982). These early outputs are interesting because they raise the obvious issue of whether such precocious network activity is incidental to the sequential assembly of motor circuitry or required for its normal development. Activity is also periodic and organised into bursts in early spinal networks of chick and mouse (Landmesser and O'Donovan, 1984; Suzue, 1996); recent work with the mouse indicates that acetylcholine-mediated transmission during this phase is essential to the development of normal patterns of rhythmic output from central pattern generators controlling limb movements (Myers et al., 2005).

Clearly therefore, many motor networks become active before they are required to generate patterned movements that contribute to normal behaviour and a key issue is whether this activity is part of a necessary developmental step in which functional properties of circuitry are validated and adjusted to ensure optimal performance. We have chosen to use *Drosophila* embryos as models to study the development of locomotor circuitry and coordinated movement. A major advantage of *Drosophila* is that we can use non-invasive techniques to monitor the outputs of the motor circuitry and to manipulate the network activity as it develops. To do this, we need to define the basic features of motor development and to show whether, as in other organisms, normal development includes a phase of early activity in which the network becomes periodically active before it is required for mature patterns of behaviour.

Here, we report the use of a novel, non-invasive method to study the onset of motor activity in *Drosophila*. Using this method, we investigate the beginnings of function in the neural network and the subsequent appearance of coordinated movement. A previous study has suggested that neural control of motor activity develops continuously and gradually from the earliest embryonic muscle twitches to the well-orchestrated peristaltic waves that are characteristic of larval crawling and that this reflects the progressive maturation and increasing complexity of underlying motor circuitry (Pereanu et al., 2007). However, our experimental analysis shows that this is not the case and that the earliest movements in *Drosophila*

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Accepted 26 September 2008

are myogenic in origin. In fact, body wall muscles responsible for locomotion only come under neural control late in embryogenesis. Our analysis defines exactly when this neural control of movement begins and shows that early motor output is organised into periodic bursts of activity. We also show that this activity is not triggered as a reflex response to developing sensory inputs but probably results from spontaneous activity that starts as electrical properties of neurons in the central network mature. We also find that the onset of neural control is accompanied by a phase of gradual improvement in behavioural performance from muscle contractions that are uncoordinated to recognisable locomotor motifs and the later emergence of complete sequences that resemble larval crawling.

MATERIALS AND METHODS

Flies

Wild type is Oregon-R. Flies carrying GFP traps *G203* and *ZCL2144*, *UAS-TNT-G*, *UAS-TNT-VIF*, *UAS-grim*, *UAS-ChR2, PO163-GAL4, elav-GAL4* and *GluRIII* were recombined and crossed to generate embryos of the following genotypes: (1) *w;G203;ZCL2144*, (2) *elav-GAL4/+;G203/G203,UAS-TNT-G;ZCL2144*, (3) *elav-GAL4/+;G203/G203,UAS-TNT-VIF;ZCL2144*, (4) *elav-GAL4/+;G203/G203,UAS-grim;ZCL2144*, (5) *w;G203,GluRIII;ZCL2144*, (6) *w;UAS-TNT-G,G203/G203;ZCL2144/PO163-GAL4,ZCL2144*, (7) *w;UAS-TNT-VIF,G203/G203;ZCL2144/PO163-GAL4,ZCL2144*, and (8) *elav-GAL4/+;UAS-ChR2/+;UAS-ChR2/+*. Embryos were raised at 25°C.

Imaging

Embryos carrying muscle markers *G203* and *ZCL2144* were placed in saline between a gas permeable membrane (BioFolie, Grenier) and a cover-glass and imaged in real time using a Hamamatsu ORCA-ER camera on a Leica DM IRBE confocal microscope with a Yokagawa CSU-10 scanner and a 10× objective. Movies captured at 5 frames/second using the Perkin Elmer Temporal Module image analysis system were analysed frame by frame in QuickTime and muscle contraction on and offsets were documented in Microsoft Excel.

Visualising and recording embryonic movements before 16 hours AEL

To assess when movement begins, embryos were selected immediately after the formation of the second midgut constriction [13 hours after egg laying (AEL)]. Dechorionated embryos were placed on slides and covered with a layer of halocarbon oil to prevent dehydration. Five-minute video recordings of the ventral surface were captured on digital tape every 15 minutes, using a Leica M420 microscope, JVC TK-C1380 video camera and Sony DSR-309 digital videocassette recorder. Denticle band movements in late embryos (>18.5 hours AEL) were also recorded in this way.

Testing embryonic reflex responses

To investigate the capacity of the embryo to produce larval-like behaviours prior to hatching, we pierced the vitelline membrane of dechorionated embryos with a glass needle to allow the animal to hatch prematurely. We tested the touch response in wild-type embryos hatched prematurely 18.5-20.5 hours AEL. Each embryo was allowed to emerge completely from the vitelline membrane before testing (around 5 minutes) and then stroked gently on the anterior segments using an eyelash. Each embryo was tested 10 times, with at least 30 seconds between each trial (to allow for recovery and to prevent adaptation). To assess the ability of prematurely hatched embryos to self-right, we gently rolled the newly hatched embryo (18.5-20.5 hours AEL) onto its dorsal surface with forceps and measured the time taken for the embryo to right itself. Each embryo was tested three times, with at least 2 minutes recovery time between each trial.

Light stimulation in embryos expressing ChR2

Parental flies were fed yeast paste containing all-trans retinal (100 μM) for 2 days prior to collection of embryos carrying ChR2. ChR2 embryos were raised in darkness prior to experiments. Light pulses (20 mseconds, minimum required for contractile responses in late embryos expressing ChR2) of specific wavelengths were delivered at 1 Hz (to avoid synaptic run down) using a stimulator (Master-8, A.M.P.I.) to control the interlock on the acousto-optic tuneable filter of a 488 nm laser (Melles-Griot, 534-A-A03). Embryos were imaged without activating ChR2, by illuminating with long-pass filtered visible light [Thorlabs long-pass filter (>550 nm)], on an Olympus BX51 WI microscope (×10 objective).

RESULTS

Drosophila larvae crawl by means of sequential muscle contractions that pass forwards or backwards along the body axis

The *Drosophila* larva moves over the substrate by peristaltic crawling. Even before hatching, embryos execute forwards and backwards waves of peristalsis and a study of the development of these movements has recently been reported (Pereanu et al., 2007). These authors suggest that a backward wave of peristalsis leads to forward movement, but this is not the case. A forward crawl requires a forward wave of contraction in the abdominal segments, and is initiated in the abdomen by contraction of the most posterior segments (A8/9) (Dixit et al., 2008). Thereafter, the posterior end of the animal attaches to the substrate, and a wave of contractions advances anteriorly as each segment (A7-A1) is transiently lifted from the substrate, pulled forwards and then lowered. Each abdominal segment engages with the substrate through an anterior belt of cuticular denticles that act as anchorage points as neighbouring segments move forwards in the peristaltic wave. As contractions begin in the abdomen, the head and thorax are extended forwards and anchored by the mouth hooks. A backward crawl reverses the peristaltic wave of contraction from A1 to A8/9, and is initiated by a backwards contraction of the head and thorax.

A method for quantitative analysis of coordinated movements in intact embryos

We tracked the development of embryonic movement by monitoring contractions of individual muscles non-invasively. Our method uses fly strains carrying green fluorescent protein (GFP) traps in proteins (Morin et al., 2001) expressed at the Z-lines of somatic muscles. At 25°C embryogenesis in *Drosophila* lasts 21 hours and embryos begin to perform coordinated, crawling-like movements at about 18 hours after egg laying (AEL) (Pereanu et al., 2007). To study development of these movements, we combined two GFP trap lines (*w;G203;ZCL2144*), which allowed us to image muscles from 16 hours AEL onwards using spinning disc confocal microscopy. For imaging, we released embryos from the egg case into saline and sandwiched them between a cover glass and gas permeable membrane. Embryos treated in this way from 16 hours AEL develop normally and patterns of movement parallel those seen in embryos retained within the vitelline membrane. Embryos left for many hours in sandwich preparations develop into normal larvae that crawl on agar plates, feed and continue to develop (n>20).

We find that, in principle, the contraction-relaxation cycle of every muscle can be recorded in animals moving freely over a substrate (Fig. 1) providing a precise, non-invasive method for monitoring motor outputs during behaviour. For our study, we focussed on the segmentally repeated ventral longitudinal muscles that form a major part of the larval musculature used in crawling. We recorded images of these muscles at five frames per second to obtain precise records of their contractions throughout the period during which coordinated movements first develop. This continuous record of muscle activity in many segments allows for a detailed description of the sequence of motor development and for quantitative comparisons between control and genetically manipulated animals.

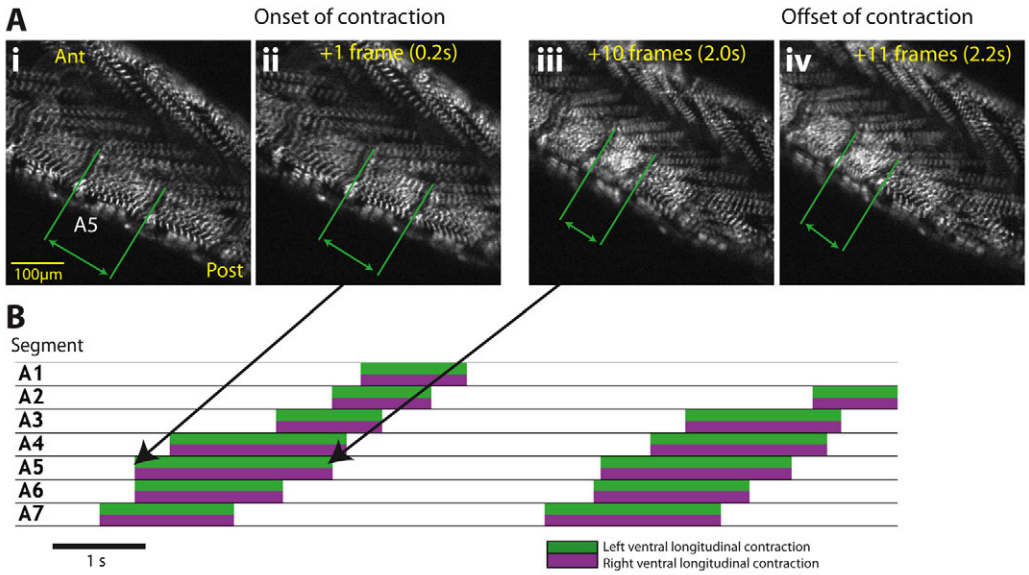


Fig. 1. Recording and analysing muscle contractions in freely moving *Drosophila* larvae. (**A**) Four images from a movie of a first-instar larva during forward crawling (a wave of muscle contractions that propagate from posterior to anterior, propelling the larva forward). Images are enlarged and selected to show onset of contraction (i,ii) and beginning of relaxation (iii,iv) in muscles of segment A5, during the sequence. (**B**) Entire sequence (two waves of forward peristalsis showing all abdominal segments) analysed frame-by-frame, with onsets and offsets of ventral longitudinal muscle contractions on either side of the animal in segments A1-7 documented.

The normal sequence of behavioural development in *Drosophila* embryos

We first describe the development of movement and the acquisition of simple reflex behaviours. These descriptions refine and augment earlier accounts (Pereanu et al., 2007) and are a prerequisite for our experimental investigation of the mechanisms that underlie the emergence of coordinated patterns of motor output in the embryo.

To pinpoint the onset of muscle contractions, we made video recordings of embryos from a stage when they are still clearly immobile (13 hours AEL, when the second midgut constriction has just formed). Embryonic movements begin about 14 hours AEL as isolated brief muscle twitches (see Movie 1 in the supplementary material). As development proceeds, twitches become stronger and more frequent, and begin to involve multiple segments. These multi-segmental events often involve simultaneous contractions of many muscles on one side, causing embryos to roll from side to side within the vitelline membrane – a characteristic embryonic movement that continues until about 17 hours AEL. By 16 hours AEL, GFP expression in the Z-lines is sufficiently strong that live confocal imaging can be used to film the pattern of muscle contractions that drives these early patterns of movement. As shown in Fig. 2, muscle contractions on one side of the animal propagate rapidly across many segments, from anterior-to-posterior or posterior-to-anterior. Within the vitelline membrane, this causes the embryo to roll,

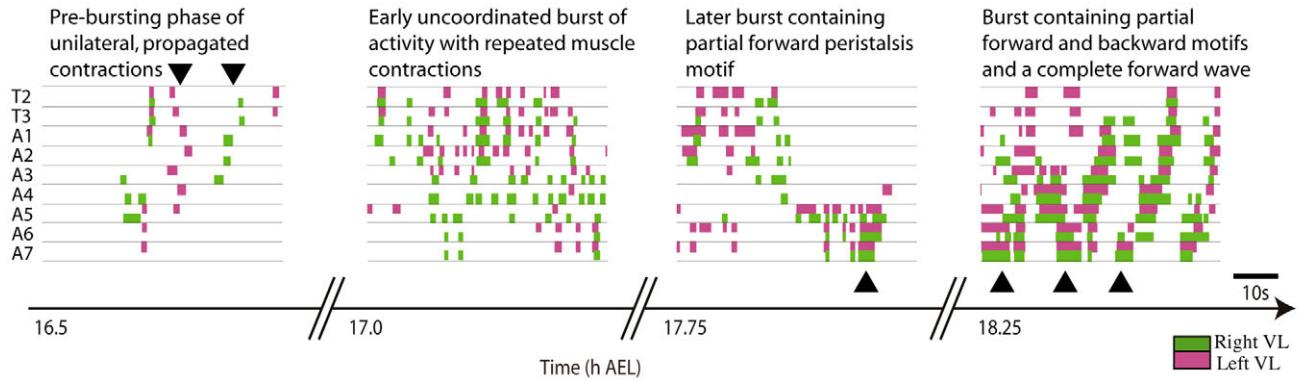


Fig. 2. Gradual development of coordinated sequences. Example data from continuous recording of movements in a single embryo 16-19 hours AEL, using muscle imaging. Before 17 hours AEL, muscle contractions occur as isolated twitches and unilateral rapidly propagated sequences across segments (arrowheads), which in intact embryos cause rolling within the vitelline membrane. At 17 hours AEL, there is a burst of activity (brief repeated muscle contractions occurring asynchronously in all abdominal segments, with little side-to-side coordination). Later bursts of activity contain motifs resembling elements of forward or backward crawling, in that muscles on left and right sides contract together, and, in comparison with earlier stages, contractions are relatively prolonged (arrowhead). At 18.25 hours AEL, embryos begin to perform sequences resembling complete waves of larval crawling, together with partial waves typically seen slightly earlier in development (arrowheads).

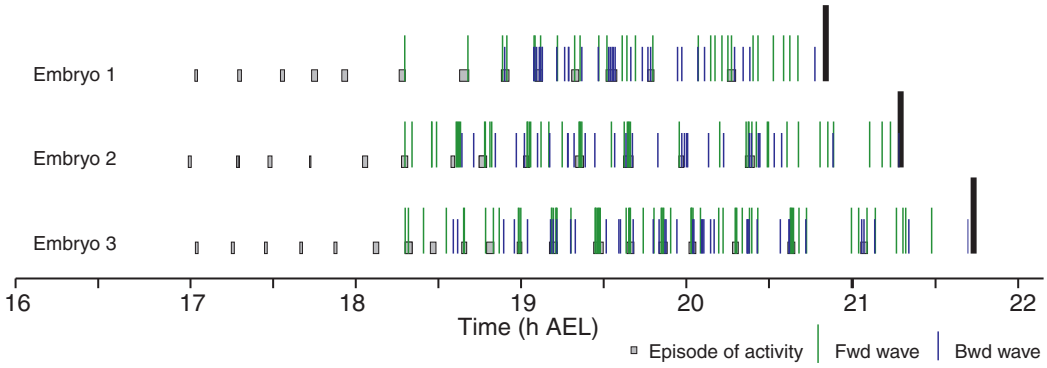


Fig. 3. Locomotor development in wild-type *Drosophila* embryos. Movements recorded from three embryos imaged from 16 hours AEL until hatching (around 21 hours AEL) using muscle imaging. From 17 hours AEL, muscle activity is episodic: sustained (30 seconds–2 minutes) bursts of muscle contraction are separated by longer periods of relative quiescence. The first complete wave of forward peristalsis occurs at ~18.25 hours AEL and the traces are aligned at this point. Black bars indicate hatching.

whereas an embryo removed from the egg case simply flexes from side to side as the muscles contract (see Movie 2 in the supplementary material).

At 17 hours AEL, we identified a striking change in patterns of muscle contraction (Fig. 2). Embryos at this stage show a burst of activity consisting of multiple contractions in many muscles on both sides of the animal. This episode is disorganised – no parts of the burst bear any resemblance to peristaltic crawling and there are no recognisable or reiterated sequences. Immediately after this burst, there follows a period of about 1 hour during which activity is markedly reduced. Occasional isolated contractions that occur typically involve only one or a few segments, and there is no evidence of coordination between the two sides (see Movie 3 in the supplementary material). However, this 'quiet period' is regularly punctuated by short episodes of increased activity lasting 1-2 minutes and occurring on average every 12 minutes (n=7, s.e.m.=0.3 minutes) (Fig. 3). During these episodes, sequences resembling incomplete forward peristalsis are observed (Fig. 2; see Movie 4 in the supplementary material), which become more obvious towards 18 hours AEL. Very reliably, just over 1 hour after the first burst of activity, we observe the first complete wave of contractions resembling those seen in forward peristalsis. In this first sequence, a wave of contraction propagates from posterior to anterior through the abdominal segments (A8-A1), with at least a partial coincidence of contraction on each side of the segments involved (Fig. 2; see Movie 5 in the supplementary material).

Although sequences resembling peristaltic crawling now occur, embryos do not hatch and crawl until some 3 hours later at 21 hours AEL. We made records of contractile activity throughout these final 3 hours of embryogenesis, which reveal that periodic bursts of activity continue until ~30 minutes before hatching (Fig. 3). Backward peristaltic waves consistently appear after the first few waves of forward peristalsis and embryos perform complete sequences of forward and backward peristaltic contractions, interspersed with incomplete, partial waves and other contraction patterns. Shortly before hatching, bursts and partial waves cease. Instead, there are now occasional complete sequences of forward and backward peristalsis that culminate in specialised movements that break the vitelline membrane (Siekhaus and Fuller, 1999), at which point the larva crawls out over the substrate and begins to exhibit mature patterns of behaviour.

To show whether normal patterns of behaviour can begin with the onset of peristaltic contractions, or whether, as we expected, there would be a progressive acquisition of more mature patterns of movement as development continued, we hatched embryos prematurely at intervals after the first onset of peristaltic sequences, by releasing them from the vitelline membrane. Such embryos will move over an agar surface and we tested them for their ability to perform two characteristic patterns of larval behaviour: the reflex response to touch and the 'righting' response. Larvae respond to

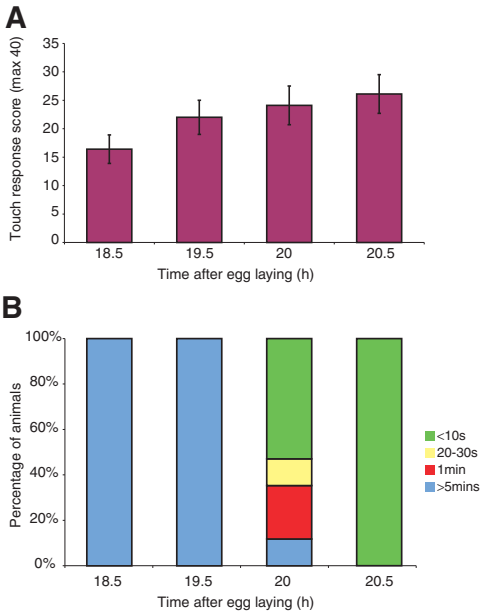


Fig. 4. Acquisition of larval reflexes in prematurely hatched *Drosophila* embryos. (**A**) Touch response. Average touch response scores are shown (and s.e.m.). Each embryo was tested 10 times (giving a score out of 40) and 10 embryos were tested at each developmental age. Embryos were selected at tracheal filling (18.5 hours AEL) and aged on agar plates at 25°C, then prematurely hatched just before testing. (**B**) Acquisition of self-righting. Embryos were rolled upside down onto their dorsal surface, and time to self-right recorded. Embryos were tested three times and 10 embryos were tested at each developmental age (embryos at different developmental stages were selected as in A).

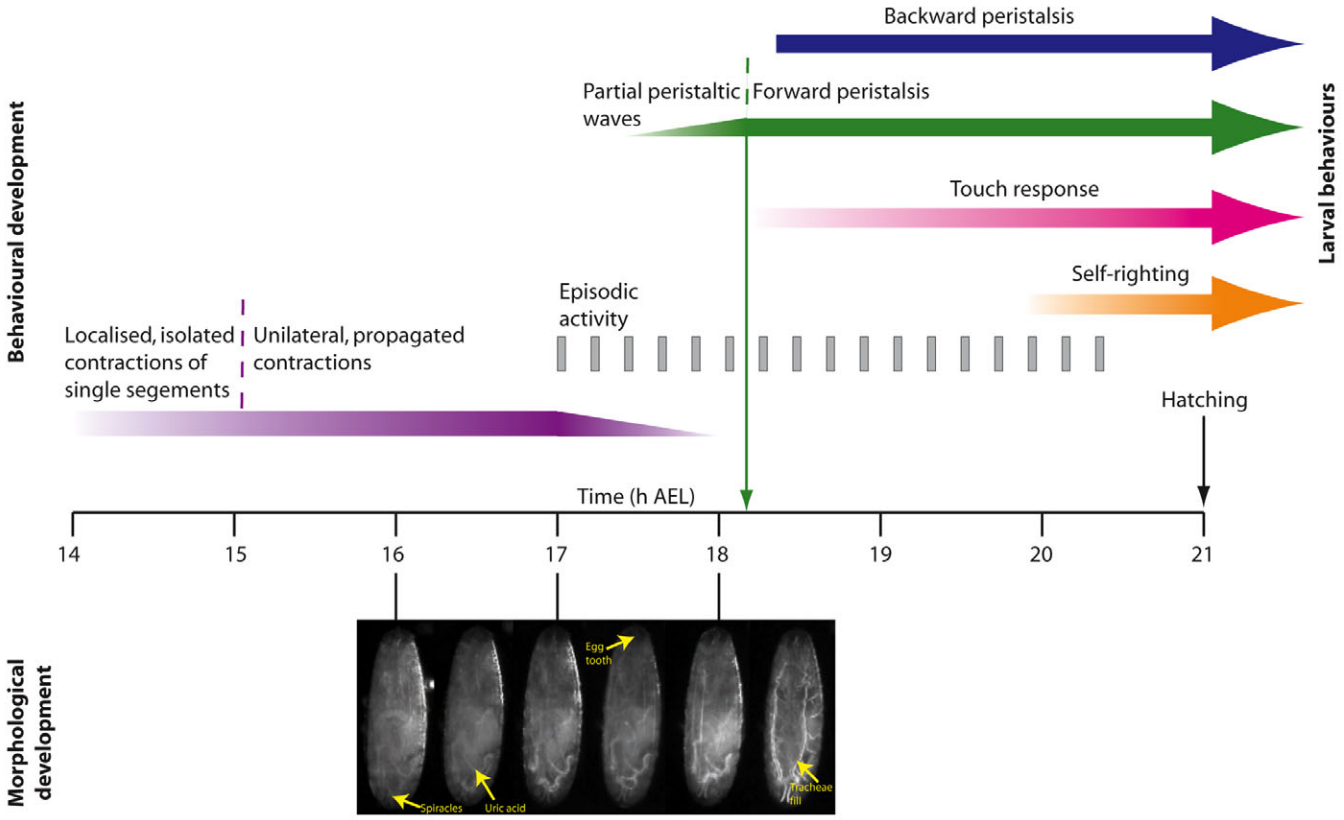


Fig. 5. Timeline of behavioural and morphological development. Timeline showing major changes in contraction patterns identified by muscle imaging, and onset of larval-like reflexes. Morphological development 16–18.5 hours AEL [roughly corresponding to stages 17b-d of Pereanu et al. (Pereanu et al., 2007)]. At 16.0 hours AEL, spiracles are detected as dorsal papillae on the terminal segment. At 16.5 hours, uric acid is detected in the Malpighian tubules, becoming strong by 17.0 hours. At 17.5 hours the median tooth tip becomes faintly visible anteriorly. By 18.0 hours the tooth is clear but tracheae have not filled. At 18.5 hours, tracheae fill.

anterior touch in a characteristic fashion, incorporating one or more of the following movements: head withdrawal, head turning and reverse peristaltic waves (Kernan et al., 1994). The righting response is seen when a larva is placed upside down on its dorsal surface and consists of a sequence of movements that rapidly turn it the right way up with its ventral surface in contact with the substrate.

The touch response was tested by using an eyelash to stroke the anterior segments of wild-type embryos hatched prematurely 18.5-20.5 hours AEL. The response was scored as follows: no response=0, hesitation=1, withdraws anterior or turns=2, single reverse contractile wave=3, multiple reverse contractile waves=4 (Kernan et al., 1994). We find that the response increases steadily, indicating that changes in sensorimotor processing are occurring as the embryo develops (Fig. 4A). Although there is almost always some response to touch, even at 18.5 hours AEL, and embryos at this stage sometimes display backward peristaltic waves spontaneously, indicating that the separate elements of the complete reflex are present, the full response of a backwards wave of contractions rarely occurs. We conclude that sensory inputs only become fully integrated with circuits controlling motor output during later stages of embryogenesis, so that increasingly mature motor responses are triggered.

To assess the ability of prematurely hatched embryos to self-right, we gently inverted prematurely hatched embryos onto their dorsal surface with forceps and measured the time that these embryos required to right themselves. When mature larvae are deliberately rolled onto their dorsal surface, they contract their muscles strongly, in a circumferential sequence that throws the body into a curve and rolls them back rapidly onto their ventral surface (≪10 seconds). At 18.5 hours AEL, embryos are incapable of self-righting, even when left for >20 minutes (Fig. 4B). Embryos first become capable of self-righting 20 hours AEL, shortly before hatching. Even at this late stage, the response is often slow in comparison with mature larvae.

The complete sequence, from the earliest movements, through the onset of coordinated sequences of contraction, and the later integration of touch and righting reflexes is shown in Fig. 5.

The onset of bursting represents a transition from myogenic movements to muscle contraction that is under neural control

The earliest contractions seen in the embryo, that is the isolated twitches from 14 hours AEL and the later unilateral waves of contraction, occur well before neurons have developed the electrical properties that allow them to generate propagated action potentials (17 h AEL) (Baines and Bate, 1998) (A. Nair, PhD thesis, University of Cambridge, 2005). It seems therefore that early muscle contractions in the *Drosophila* embryo are not driven by the firing of motoneurons, but are likely to be myogenic in origin.

To test this idea directly, and to define the precise moment in embryogenesis when the nervous system first generates a motor output, we compared the developing patterns of movement in normal embryos with those that occur in embryos where evoked synaptic transmission has been blocked by the expression of tetanus toxin (using the pan neuronal driver *elav-Gal4*) (Sweeney et al., 1995). We reasoned that myogenic movements would be unaffected

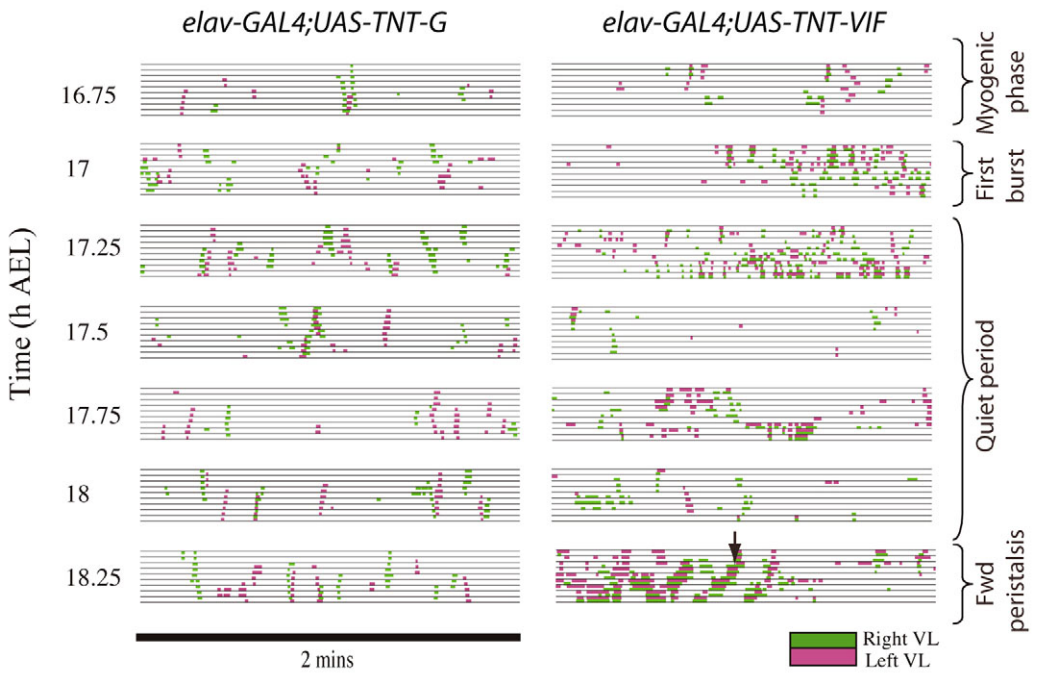


Fig. 6. Patterns of muscle contraction in embryos with and without synaptic transmission. (Left) Muscle contractions recorded at 15-minute intervals from an embryo that lacks all evoked synaptic transmission through pan-neuronal expression of active tetanus toxin (*elav-GAL4;UAS-TNT-G*). (Right) Similar recording made from a control embryo expressing inactive tetanus toxin (*elav-GAL4;UAS-TNT-VIF*). For each genotype, four embryos were recorded and analysed. Similar data were obtained in each case, but for clarity, results from single embryos are shown. Arrow indicates the first complete wave of forward peristalsis.

by blocking synaptic transmission and therefore common to the two classes of embryos, whereas neurally controlled muscle contractions would be seen only in embryos with functional synapses.

These experiments (Fig. 6) show that early muscle twitches and the unilateral multisegmental contractions that cause rolling movements, occur equally in embryos with and without synaptic transmission. These movements are therefore myogenic in origin. Contractile activity in the two classes of embryos diverges first with the onset of bursting patterns of contraction. There are no such bursts in embryos that lack synaptic transmission, whereas they begin at 17 hours AEL in embryos that express an inactive form of tetanus toxin, as in the wild type. These bursts require synaptic transmission and are therefore neurally controlled. More importantly, as the first such burst represents the earliest point in embryogenesis at which the pattern of muscle contractions in normal embryos diverges from that seen when all synaptic transmission is blocked, we conclude that the first burst is caused by the earliest motor outputs generated by the embryonic nervous system.

We were interested to see whether spontaneous, i.e. non-evoked release of neural transmitter (glutamate) at the neuromuscular junction might contribute to early embryonic movement. To test this, we performed two experiments. First, we analysed the movements of embryos in which presynaptic terminals were removed from the muscles by expressing the cell death gene *grim* (Wing et al., 1998) ectopically in all neurons, so that they, and their axons, degenerated during embryogenesis. There is no bursting activity in these embryos but myogenic contractions persist at a frequency that is not significantly different from that seen when evoked synaptic transmission is blocked (Fig. 7). This suggests that spontaneous release of transmitter does not make a major contribution to myogenic activity. In the second set of experiments, we analysed movement in embryos homozygous for a null mutation in the muscle-specific subunit of the glutamate receptor (GluRIII) (Marrus et al., 2004). Again, there is no sign of bursting activity and characteristically myogenic contractions persist (Fig. 7). However, in the absence of the receptor, the frequency of these contractions is significantly reduced in comparison with denervated embryos (*elav-GAL4;UAS-grim*) and embryos without synaptic transmission (*elav-Gal4; UAS-TNT-G*). This sensitivity of myogenic movement to the removal of the receptor may indicate that non-neuronal sources of glutamate such as the haemolymph (Chen et al., 1968) can contribute to embryonic muscle contractions.

The onset of motor activity is not triggered by sensory input

We next asked why the motor network begins to burst at a particular point in development. One possibility is that the network fires as a reflex response to rising levels of activity in sensory neurons as they mature (Sanes et al., 2006). Sensory activity could either be spontaneous or evoked in response to myogenic contractions occurring prior to the first burst. To show whether this is indeed the case, we raised embryos in which sensory input was blocked by the selective expression of tetanus toxin in sensory neurons using the *P0163-Gal4* driver (Hummel et al., 2000; Suster and Bate, 2002). Expression of PO163 begins at stage 12 in the precursors of sense organs, well before any movement begins (Wolf and Schuh, 2000) and such embryos are completely insensitive to mechanical stimulation (Suster and Bate, 2002). Remarkably, despite the loss of all sensory input, these embryos made a normal transition on schedule from myogenic to bursting, neurally controlled contractile activity (Fig. 8). We conclude that early activity of the motor network is an autonomous property of the central network that does not require input from the sensory system. However, the frequency of subsequent bursts is reduced in embryos that lack sensory input (3.0±0.3 bursts/hour compared with 4.6±0.3 bursts/hour in controls, n=5 in each group, Student's t-test, P=0.004), and although coordinated sequences resembling peristalsis are eventually generated [as expected from earlier work (Suster and Bate, 2002)], they are considerably delayed (74±13 minutes, P<0.0005).

Bursts of activity are not a feature of spontaneously active motoneurons, but are network derived

As an alternative to reflex triggering of motor output in the embryo, bursting activity might be a network phenomenon, which arises as embryonic neurons mature and become spontaneously active. In this

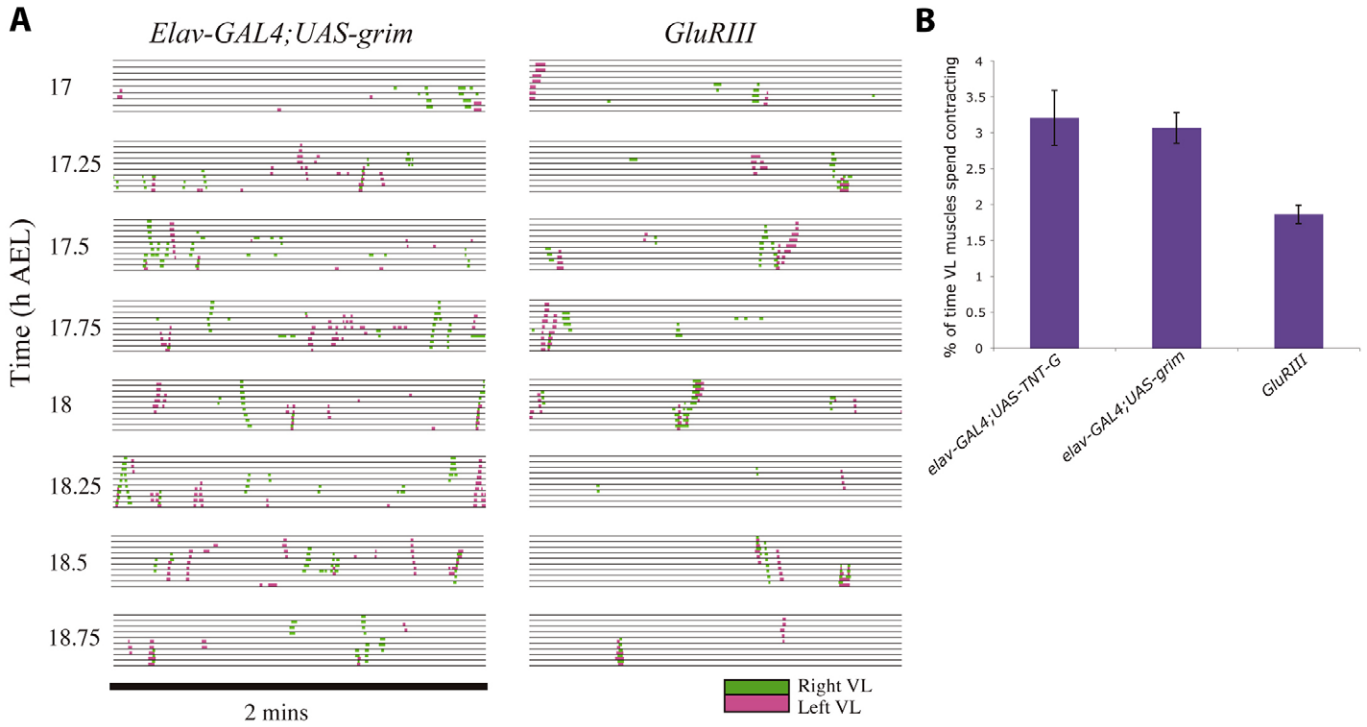


Fig. 7. Disrupting glutamate-mediated transmission at the NMJ reduces myogenic movements in the embryo. (**A**) Representative data for an embryo expressing *grim* panneuronally (*elav-GAL4;UAS-grim*) and an embryo homozygous for a mutation in an essential glutamate receptor subunit GluRIII. Unilateral waves of contraction occur in both, persisting for several hours. (**B**) Mean proportion of time each VL muscle spent in contraction 17-18 hours AEL is calculated for each genotype (n=4 for each genotype, with four or five 2-minute traces analysed for each embryo, error bars indicate s.e.m.). Embryos mutant for GluRIII show a lower frequency of contractions than embryos in which transmission at the NMJ is blocked presynaptically (through expression of TNT-G), and embryos lacking presynaptic terminals (through expression of *grim*).

view the interconnectedness of immature neurons might be sufficient to trigger repeated firing (resulting in a burst of activity) through recurrent excitation as levels of spontaneous firing rise to some threshold level.

To show whether embryonic bursting activity is autonomous to the motoneurons, or requires presynaptic drive from the motor network, we deprived motoneurons of their inputs by blocking evoked synaptic transmission from their presynaptic partners. As all presynaptic input to embryonic motoneurons in *Drosophila* is acetylcholine mediated (Baines et al., 2001) (also Richard Baines, personal communication), we targeted tetanus toxin to these neurons using the *Cha-Gal4* driver (Salvaterra and Kitamoto, 2001). These embryos show no bursting activity and we conclude that bursts are driven by activity in the central network.

Interestingly, however, at 17 hours AEL, the frequency of muscle contractions when motoneurons are simply deprived of their inputs is greater than that seen when all synaptic transmission, including that from motoneurons, is blocked [in embryos that are *Cha-GAL4;UAS-TNT-G*, each ventral longitudinal (VL) muscle contracts 5.4% of the time (s.e.m.=0.3%, n=4), whereas in embryos that are *elav-GAL4;UAS-TNT-G*, each VL muscle contracts on average just 3.3% of the time (s.e.m.=0.3%, n=4), Student's t-test, P=0.002] (see Fig. S1 in the supplementary material). This suggests that neurons such as motoneurons do indeed become spontaneously active at the stage when normal bursting would begin. However, this spontaneous activity is not itself bursting.

Bursts of activity induce network depression, with slow kinetics of recovery

If bursting activity truly depends on spontaneous activity reaching a threshold value, we reasoned that this might be detectable by comparing the number of muscle contractions occurring just before and just after each burst. Although activity levels before and after bursts were highly variable, it is clear from our quantitative analysis that a period of heightened spontaneous activity precedes each episode, and that levels of activity are much reduced after an episode (Fig. 9A, part i). Continuous analyses of muscle contractions occurring after a burst of activity show a steady increase in the frequency of contractions before the next burst occurs (Fig. 9A, parts ii, iii).

The reduction in spontaneous activity after each episode could represent the effect of an intrinsic activity-dependent depression of neuronal firing or synaptic transmission. We tested this hypothesis directly by recording the embryonic response to stimulation at defined intervals after a burst. To stimulate neurons without the need for direct access with electrodes, we expressed channel rhodopsin 2 (ChR2) in all neurons using *elav-GAL4* (Schroll et al., 2006). ChR2 is a light-activated cation-selective ion channel from the green alga *Chlamydomonas reinhardtii*. In the presence of all-trans retinal (an essential co-factor), neurons expressing ChR2 fire action potentials in response to a light stimulus of the appropriate wavelength (488 nm). We used long-pass filtered visible light (>550 nm), which does not activate ChR2, to identify bursts of activity occurring naturally in such embryos between 17.5 and 18.5 hours AEL. At varying intervals after the end of a burst (5, 6, 7, 8,

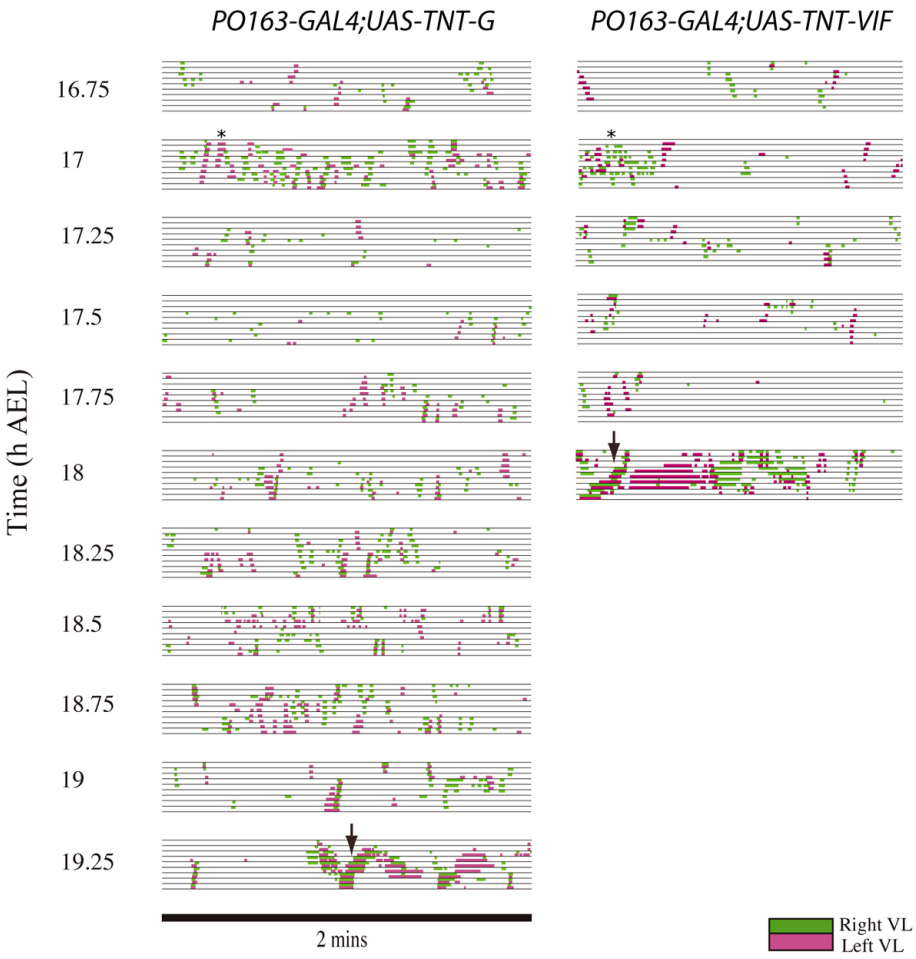


Fig. 8. Patterns of muscle contraction with and without sensory input. (Left) Muscle contractions recorded at 15-minute intervals from an embryo without sensory input (*PO163-GAL4;UAS-TNT-G*). (Right) A similar recording made from control embryo with normal synaptic transmission from sensory neurons (*PO163-GAL4;UAS-TNT-VIF*). For each genotype, five embryos were recorded and analysed, but results from single representative embryos are shown. In embryos with no sensory input, a burst of muscle contractions occurred 17 hours AEL, as in controls. However, the first properly coordinated sequences are delayed in embryos that lack sensory input (arrows).

9 and 10 minutes), we stimulated with a series of 488 nm light pulses of increasing duration (25, 50, 100, 200 and 400 mseconds separated by 30 second intervals). We recorded the length of the minimum pulse required to evoke a vigorous response (contraction within 15 seconds of stimulus, lasting over 15 seconds and involving most, if not all, segments, i.e. resembling a naturally occurring burst). Any embryo performing a burst before the stimulation protocol was applied was excluded. These responses were then translated into a score (response to 25 mseconds scores 5, 50 mseconds scores 4, 100 mseconds scores 3, 200 mseconds scores 2, 400 mseconds scores 1 and no response to any of the pulses scores 0). Embryos were tested only once because the stimulation protocol could produce synaptic rundown, and this in turn could affect the outcome of subsequent trials. Embryos were pulsed with longer wavelengths of light (568 nm and 638 nm), which do not activate ChR2, to obtain control response scores (vigorous movements occurring soon after one of the test pulses, but which are naturally occurring rather than stimulated by ChR2 opening, i.e. the rate of false positives).

We found that there were virtually no vigorous responses to stimuli delivered after a 5-minute delay, although small contractile events may occur at any time between bursts (Fig. 9B). It is not clear whether these small events are the result of stimulation or simply spontaneous contractions. However, they do indicate that muscles can contract soon after a burst and that the site of depression must therefore be upstream of the contractile apparatus. Responses were elicited to increasingly brief pulses as the interval from the previous burst of activity increased. The fact that much stronger stimulation (a longer test pulse) is required to excite the network soon after an episode, when compared with later, supports the view that network depression (with slow kinetics of recovery) follows episodes of activity. Although we cannot rule out the possibility that the site of this depression is the neuromuscular junction, this seems unlikely given that electrophysiological experiments demonstrate that recovery of the neuromuscular junction from depression following repetitive stimulation occurs within 1 minute in embryos 16 hours AEL (Broadie and Bate, 1993). Naturally occurring inter-burst intervals in wild-type embryos (dechorionated but not devitellinised) are on average 12.0 minutes long (and show a tight distribution, s.e.m.=0.3 minutes, n=7). Interestingly, the shortest interval that we recorded between spontaneous bursts was 6.6 minutes, and this is very similar to the shortest interval we found before a second burst of activity could be triggered by stimulation.

Inhibitory neurotransmission is not required for early episodic activity in *Drosophila* embryos

The activity-dependent depression that operates in the embryonic nervous system could conceivably be mediated by the recruitment of inhibitory neurons. We examined embryos in which fast inhibitory neurotransmission through GABA receptors is disrupted by a mutation in *Rdl*, a gene coding for a subunit of a GABA-gated Cl^- ion channel (ffrench-Constant, 1993; Lee et al., 2003). *Rdl* is expressed in the embryonic CNS and embryos that are homozygous for the Rdl^1 allele lack detectable *Rdl* transcripts, do not hatch and die around 24 hours AEL (Stilwell et al., 1995).

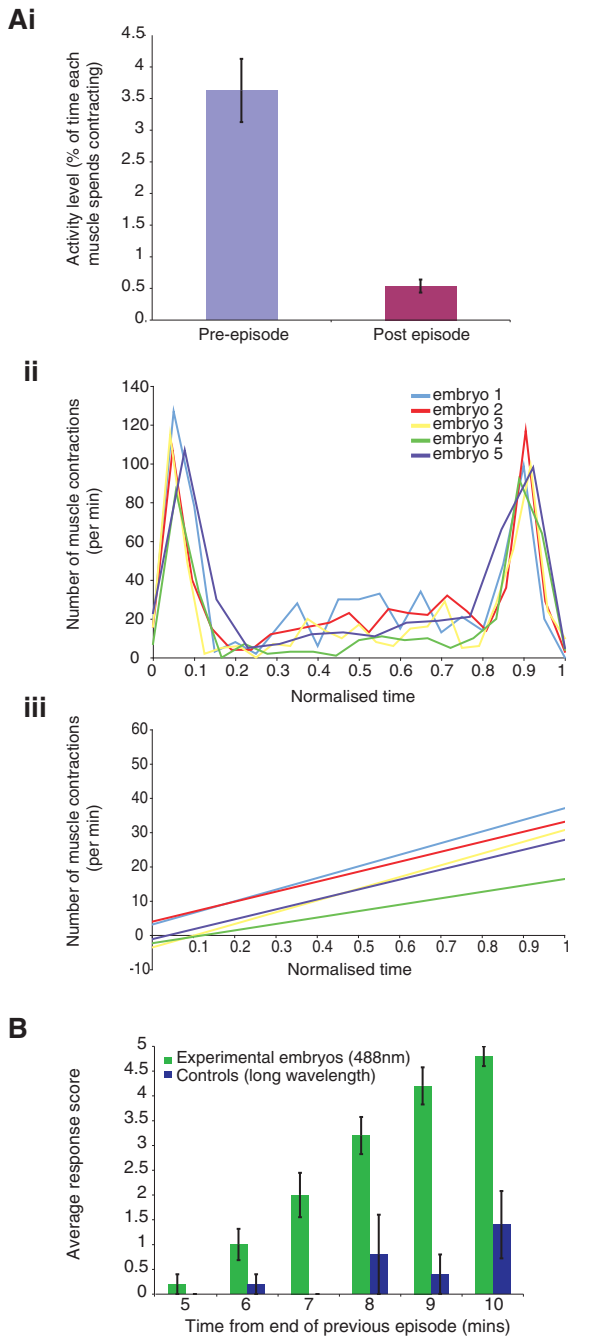


Fig. 9. Activity-dependent depression during bursting motor output. (**A**) (i) Mean level of activity before and after bursts in wild-type embryos (*n*=3, 15 pre-burst and 15 post-burst sequences) (error bars=s.e.m.). (ii) Number of muscle contractions (in 1-minute time bins) from start of second burst until the end of third burst in five embryos. Data are normalised so that each cycle duration=1. (iii) Regression lines for number of contractions per minute during inter-burst interval. (**B**) Activity-dependent depression in embryos expressing channel rhodopsin 2 in all neurons (*elav-GAL4;UAS-ChR2*). Average response scores are shown for different intervals from the end of a naturally occurring burst of activity (*n*=5 at each time interval and error bars indicate s.e.m.). Response scores correspond to shortest stimulus pulse producing a long-lasting (over 15 seconds) vigorous contractile response (response to 25 mseconds scores 5, 50 mseconds 4, 100 mseconds 3, 200 mseconds 2, 400 mseconds 1, no response to any pulse 0). Stimuli: 488 nm light and control wavelengths that do not activate ChR2 (568 and 638 nm).

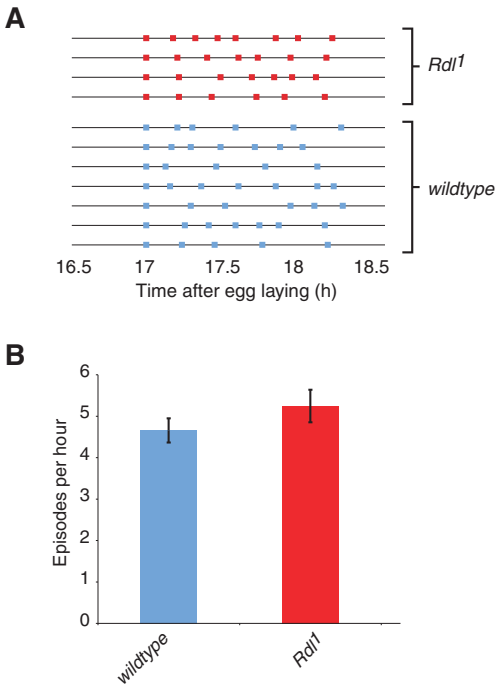
Rdl^{1} embryos show bursting activity from 17 hours AEL. From 17 to 18 hours AEL, the frequency of bursts in these embryos is not significantly different from that seen in embryos with completely intact synaptic transmission (Fig. 10). After 18 hours AEL, the bursts of activity in the mutant embryos lengthen and activity gradually becomes nearly continuous, with rapidly propagated, repeated waves of contraction. Thus, at least initially, GABAergic transmission is not required for the organisation of activity into bursts.

DISCUSSION

We have used a novel method to document the development of behaviour in the embryo of *Drosophila*. We find that there is a sharp transition from early, myogenic patterns of movement to muscle contractions that are driven by outputs from the central nervous system. We can pinpoint exactly when the first outputs of the motor system occur and show that the transition from myogenic to neurally controlled contractions occurs reliably at 17 hours AEL, and that the hallmark of this transition is the onset of bursting activity in the motor network. We regard the bursting activity that we record as particularly interesting. From first principles, we might not have anticipated that function would begin in this way – the first signs of maturing excitability in motorneurons could simply have been a steady increase in the number of random twitches that occur as the spontaneous firing rate increases. Indeed, this is exactly what we observe when motoneurons are isolated from their presynaptic inputs at the stage when bursting normally begins. Interestingly, this is also when embryos with defective glial sheathing begin to manifest a phenotype of continuous rapid twitching, probably reflecting uncontrolled firing of neurons as they first become active (Pereanu et al., 2007; Strigini et al., 2006). However, in normal embryos the earliest motor outputs are clearly driven by the concerted, periodic firing of many neurons in the motor network. This characteristically rhythmic firing, together with the fact that activity begins several hours before it is required for hatching and larval crawling suggests that bursting may be important for the proper maturation of the network and the emergence of coordinated movements.

There is considerable circumstantial evidence to support this idea: rhythmic oscillations occur in different parts of developing vertebrate nervous systems, notably spinal cord (Landmesser and O'Donovan, 1984) and retina (Wong et al., 1993), and in retina this precocious activity is essential for development and refinement of normal patterns of connectivity (Gnuegge et al., 2001; Katz and Shatz, 1996). The stomatogastric network of the embryonic lobster also becomes active early in development before feeding can occur, and spectrographic analysis shows that these rhythms are irregular and may represent immature phases in the development of fully adult outputs (Rehm et al., 2008).

Significantly, we find that output from *Drosophila* network 'improves' during bursting, in that motifs begin to appear in the record of contractions that resemble elements of normal crawling sequences – contractions that are coordinated across the midline with delays between segments. These partial sequences are followed by complete, but still imperfect waves of contractions from which well coordinated forward and later backward peristaltic sequences will develop. The gradual appearance of coordination is certainly consistent with the idea that rhythmic activity drives activity-dependent adjustment and tuning of the developing motor network. An alternative is that bursting begins before all cellular components of the motor circuitry are present and that progressive appearance of more mature contraction patterns simply reflects growth and addition of further essential elements to the motor network. Experiments to distinguish between these two alternatives are now planned.



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We not only find striking similarities between temporal characteristics of episodic activity in *Drosophila* embryos and motor outputs recorded from chick spinal cord, but also analogous network properties. Two properties essential for spontaneous episodic activity in spinal networks are hyperexcitability and cell firing modulated according to recent network history (Fedirchuk et al., 1999). We find evidence for autonomous increases in levels of activity occurring shortly after neurons acquire a mature complement of currents (allowing action potential propagation for the first time), and this increase coincides with the appearance of oscillatory, bursting activity in normal embryos. In addition, GABA expression in embryos is low when motor output first begins (Kuppers et al., 2003), and removing GABAergic transmission has little effect on early bursting activity. The combination of spontaneous firing and low levels of the major inhibitory neurotransmitter could create a 'hyperexcitable' state in the embryonic network. We also find that, as in vertebrates, episodic activity is a network property, rather than a feature of individual neurons.

Spontaneous activity in an interconnected excitatory network, could result in widespread neural activity, and with activity-dependent depression (like that described in chick spinal cord), lead to the onset of slow oscillatory output (Tabak et al., 2001; Tabak et al., 2000). We deduce from patterns of muscle contraction seen, using ChR2 to stimulate the embryonic nervous system, that neuronal firing rates are modulated by the recent history of the network in *Drosophila*. Such a direct demonstration of activity-dependent depression with slow kinetics of recovery has only been achieved in one other developing network – the chick spinal cord. Here, stimulation experiments have revealed both depression of synaptic potentials after an episode of bursting activity, with slow recovery during the inter-episode interval (Fedirchuk et al., 1999) and depression of the whole network, as indicated by briefer duration and lower cycling frequency (indicators of excitability) within evoked episodes that were artificially elicited soon after a spontaneous episode when compared with later in the inter-episode interval (Tabak et al., 2001).

Although episodic activity is characteristic of embryonic movement it is not appropriate to the behaviour of hatched larvae, which perform sustained bouts of forward crawling as they search for food. Not surprisingly, therefore, we find that bursting activity ceases shortly before hatching and this forms part of a sequence of behavioural maturation during the late stages of embryogenesis. Shortly after the first crawling-like movements (18.25 hours AEL), embryos are relatively unresponsive to touch and unable to perform righting reflexes, but by the time bursting ceases and the animal is about to hatch, touch responsiveness has increased markedly and righting reflexes are present. The animal is now ready to emerge and it is likely that specialised movements of hatching and, perhaps, other aspects of behavioural maturation are triggered hormonally, as loss of Amontillado or PHM (proteins required in neuroendocrine biosynthesis) prevents or delays the hatching sequence (Jiang et al., 2000; Siekhaus and Fuller, 1999). We have documented several hours of activity in the embryonic nervous system prior to this sequence, accompanied by progressive acquisition of more mature patterns of behaviour. We now plan to investigate the role of this precocious activity to show whether it is incidental to or essential for the normal development of coordinated behaviour.

We thank Helen Skaer and Matthias Landgraf for their comments. This work was supported by grants from Merck Sharpe and Dohme (S.J.C.), and the Wellcome Trust (075934) (M.B.). M.B. is a Royal Society Research Professor.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/22/3707/DC1

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