Systematic analysis of *C. elegans* embryo behavior reveals a stereotyped developmental progression and a rhythmic pattern of behavioral quiescence

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Abstract

Systematic analysis of rich behavioral recordings is being used to uncover how circuits encode complex behaviors. Here we apply the approach to embryos. What are the first embryonic behaviors and how do they evolve as early neurodevelopment ensues? To address these questions, we present a systematic description of behavioral maturation for *Caenorhabditis elegans* embryos. Posture libraries were derived from a genetically encoded motion capture suit imaged with light-sheet microscopy and annotated using custom semi-automated tracking software (Multiple Hypothesis Hypergraph Tracking; MHHT). Analysis of cell trajectories, postures, and behavioral motifs revealed a stereotyped developmental progression. Early movement is dominated by flipping between dorsal and ventral coiling, which gradually slows into a period of reduced motility. Late-stage embryos exhibit sinusoidal waves of dorsoventral bends, prolonged bouts of directed motion, and a rhythmic pattern of pausing, which we designate slow wave twitch (SWT). Synaptic transmission is required for late-stage motion but not for early flipping or the intervening inactive phase. A high-throughput behavioral assay and calcium imaging revealed that SWT is elicited by the rhythmic activity of a quiescence-promoting neuron (RIS). Similar periodic quiescent states are seen prenatally in divergent animals and may play an important role in promoting normal developmental outcomes.

Introduction

The set of synaptic connections assembled during development form a physical substrate for brain activity, behavior, and cognition. Diverse model systems have been deployed to identify the molecular components essential for initiating this process, from neuronal differentiation to synaptogenesis. In parallel, postnatal behaviors have been studied from genes to cells to circuits. By comparison, few datasets bridge these lines of inquiry, i.e., probe the emergence of functional neural circuitry. New principles are likely to be discovered through focused analysis of nascent neuronal circuits and the behaviors they elicit. Indeed, the structural remodeling of synapses peaks early and becomes less dynamic with age and the maturation of some circuitry is restricted to specific stages (critical periods) early in development (Hensch, 2004).

In 1695, Antonie van Leeuwenhoek expressed his amazement at first seeing motility in unborn mussels, writing that the graceful rotations were beyond comprehension. Careful observation has since uncovered details of embryonic behavior across phylogeny (Preyer, 1885). Studies of the chick embryo were particularly influential, where it was established that rhythmic muscle contractions were mediated by stereotyped spontaneous activity in neuromuscular circuitry (Hamburger, 1963). Behavioral classification has traditionally relied on expert observers; however, new tools for data-driven analysis of rich behavioral recordings are accelerating progress in ethology and systems neuroscience (Berman, 2018; Brown & de Bivort, 2018; Datta et al., 2019; Pereira et al., 2020). Applying this approach to embryos has the potential to answer several questions about early brain development. What are the first behaviors embryos exhibit? Is there a stereotyped developmental progression for these

behaviors? Is this progression dictated by the timing of ongoing neurodevelopment (i.e. circuit wiring)? Do embryonic circuits encode behaviors by mechanisms distinct from those found in mature, post-natal circuits?

To address these questions, here we build a comprehensive picture of embryonic behavior in the microscopic roundworm *Caenorhabditis elegans*. *C. elegans* offers several advantages for investigating the emergence of functional neuronal circuitry and behavior. Embryos are small (~50x30x30 μm^3) and transparent, enabling in vivo imaging of cell migration, neurite growth, synaptogenesis, neuronal activity, and behavior during the mere ~14 hours from fertilization to hatching (Shah et al., 2017; Barnes et al., 2020; Heiman & Shaham, 2009; Fan et al., 2019; Christensen et al., 2015; Moyle et al., 2020; McDonald et al., 2020; Sengupta et al., 2020; Ardiel et al., 2017; Hall & Hedgecock, 1991; Atakan et al., 2020; Bayer et al., 2021). *C. elegans* boasts a sophisticated molecular genetic toolkit, a complete neuronal wiring diagram, and a comprehensive atlas of single cell gene expression across embryogenesis (White et al., 1986; Cook et al., 2019; Witvliet et al., 2021; Packer et al., 2019). *C. elegans* has been a powerful system for analyzing circuits and behavior; however, virtually all such studies have focussed on post-embryonic stages, including “cradle to grave” behavioral recordings (Stern et al., 2017; Churgin et al., 2017). In embryos, cell movement has been fully tracked from the first cell division up to the first muscle contraction, which occurs at about 430 minutes post-fertilization (mpf; Bao et al., 2006; Giurumescu et al., 2012; Kumar et al., 2016). Tracking movement beyond this point is complicated by the embryo’s rapid transitions between entangled body postures. At hatching, the 222-cell larval nervous system already supports directed locomotion and even learning and memory (Hong et al., 2017). Thus, a functional

motor program emerges over what is arguably the least documented 6 hours of the worm's life (430-800 mpf).

It is currently not known when the nervous system begins generating behavior. Pre-synaptic proteins are first localized in the worm's "brain", a large circumferential bundle of axons called the nerve ring, less than an hour before the first muscle contraction (McDonald et al. 2020). However, these pre-synaptic proteins exhibit high diffusional mobility at this stage (McDonald et al. 2020) and it is unclear when nerve ring synapses become functional. Mutants lacking essential muscle genes arrest as two-fold embryos, indicating that muscle contractions are required for elongation of the embryo's body (Williams & Waterston, 1994). Elongation is unaffected in mutants deficient for neuronal function, implying that initial muscle contractions do not require neuronal input. In a developmental series of electron micrographs, pre-synaptic vesicle clusters at body wall neuromuscular junctions are first observed at ~550 mpf (Durbin, 1987). In late-stage embryos, backward locomotion correlates with activity of backward command interneurons (Ardiel et al., 2017), suggesting the existence of a functional motor program prior to hatching. Here we documented all postural transitions for the final 4.5+ hours of embryogenesis (530 mpf to hatching). We find that embryos exhibit a stereotyped pattern of behavioral maturation, including a period of rhythmic quiescence. This rhythmic quiescence is elicited by the RIS neuron, which promotes sleep-like behaviors in larvae and adults (Turek et al., 2013; Wu et al., 2018). Rhythmic sleep-like states are a highly conserved feature of prenatal behavior (Balaban et al., 2012; Poreanu et al., 2007; Okai et al., 1992; Szeto & Hinman, 1985; Corner, 1977). *C. elegans* offers a powerful system for investigating this phenomenon from genes to cells to circuits.

Results

Building embryo posture libraries

To model posture, we tracked the location of the paired skin cells that run along the left and right sides of the *C. elegans* embryo, hereafter designated seam cells. GFP-expressing seam cell nuclei were imaged by light-sheet fluorescence microscopy, which allows rapid optical sectioning with minimal photodamage. With single view imaging (iSPIM; Wu et al., 2011; Kumar et al., 2015), we recorded volumes at 3 Hz for more than 4.5 hours without delaying the timing of the Q/V5 cell division (~695-725 mpf) or hatching (<820 mpf; Fig. 1a). Following image acquisition, all seam cell nuclei were tracked across more than 50,000 image volumes (>1 million links per embryo; Fig. 1b, Movie 1). To optimize nucleus detection, we compared several segmentation methods in manually annotated image volumes. The best performance was obtained using a 3D convolutional neural network (Supplementary Note).

We developed Multiple Hypothesis Hypergraph Tracking (MHHT) to link seam cells across image volumes. Due to the embryo's anatomy, seam cell movements are correlated and distances between neighboring cells are constrained (Fig. 1c, Supp. Fig. S1). MHHT leverages these interdependencies to facilitate semi-automated tracking that is robust to imperfect detections. In Multiple Hypothesis Tracking (MHT), the top K assignments for minimizing frame-to-frame movement are evaluated over N frames (Reid, 1979; Cox & Hingorani, 1996). MHHT extends MHT by using empirically derived covariances to assign costs to competing cell assignment hypotheses based on their deviation from expected motion patterns. Using a depth-first search, MHHT identifies the cell assignment hypothesis that has the lowest cost accrued over N frames, disregarding branches that generate physically impossible body

contortions (i.e., body segments passing through one another; Fig. 1d). For a ground truth dataset in which all seam cell detections were confirmed by expert observers, MHHT failed to correctly assign at least one seam cell in only 1.46% of 51,553 image volumes (for $K=5$, $N=3$), a 27.2% reduction in error rate compared to using global nearest neighbor tracking (GNN, i.e., $K=1$, $N=1$). Error rates increase considerably for non-curated datasets with missing or false cell detections. Using our best detection method (3D U-Net; Cicek et al., 2016), MHHT failed to correctly assign at least one seam cell in 6.52% of 51,553 image volumes (for $K=25$, $N=2$), a 14.2% reduction in error rate compared to GNN. MHHT notifies users when a cost threshold is crossed, allowing for manual editing of cell assignments before resuming automated tracking. A detailed description of MHHT is provided in Supplementary Note and the code is freely available at <https://github.com/lauziere/MHHT>. Collectively, these methods (iSPIM, 3D U-Net segmentation, and MHHT) comprise a semi-automated pipeline for documenting embryonic posture. We generated a comprehensive catalogue of postural transitions for three control embryos, recording the position of all seam cells three times per second from 530 mpf to hatch (>4.5 hours). These 150,000+ annotated image volumes (available on FigShare, <https://figshare.com/s/6d498d74f98cca447d41>) create a new resource for the systematic analysis of embryo behavior.

Eigen-embryos compactly describe posture and behavioural motifs

Adult worms crawling on a 2D agar surface explore a low dimensional postural space. Stephens et al. (2008) found that over 90% of variance in dorsoventral bending along the midline could be accounted for with just 4 principal components (PCs), which they termed

“eigenworms”. To determine if embryo postures could be similarly described, we used seam cell positions to fit each side of the body with a natural cubic spline and then computed dorsoventral bending angles between adjacent seam cells (totalling 18 bends in each volume; Fig. 2a, b). Four PCs captured over 88% of the variance in this metric (Fig. 2c, d). The eigenvectors of these four components (termed eigen-embryos) were stereotyped between animals, with PC1 for example capturing ventral or dorsal coiling and PC2 describing postures with opposing anterior and posterior bends (Fig. 2e). In this framework, any embryonic posture can be described by a linear combination of eigen-embryos. PC2 and PC3 approximate sinusoids with a phase difference of 90° (a quadrature pair), which can be combined to generate travelling waves. The contribution of eigen-embryos shifted consistently across development, with less variance accounted for by PC1, as PC2 and PC3 gained prominence (Fig. 2d). Consistent with behavioral maturation, the developmental shift towards PC2 and PC3 better aligns eigen-embryos with eigenworms, for which the top two PCs jointly approximate a quadrature pair (Stephens et al., 2008). The relatively limited dimensionality of dorsoventral bending observed in both adults and embryos is likely a consequence of muscle anatomy, with electrically coupled muscle bundles running ventrally and dorsally along the body (White et al., 1986).

Owing to the bilateral symmetry of seam cells, we could not assign left versus right identity and consequently could not distinguish dorsal from ventral bending. Given the differential neuronal input to dorsal and ventral muscles (White et al., 1978; Hallam & Jin, 1998), we wondered whether embryos developed a systematic asymmetry in postures. Indeed, a coiling bias was apparent early versus late in all three embryos, as indicated by the tails of the

PC1 amplitude distribution (Supp. Fig. S2a). To determine whether this bias was consistently dorsal or ventral, we acquired a supplemental dataset of 16 embryos in which additional fluorescent markers were imaged at higher spatial resolution. In these image volumes, left and right seam cell nuclei could be unambiguously distinguished by manual inspection. The low temporal resolution of this supplemental dataset (5 min intervals) precluded behavioural tracking; however, the postures exhibited by these embryos were described by a similar set of eigen-embryos (Supp. Fig. S2c) and revealed a dorsal coil bias in the final hours of embryogenesis (Supp. Fig. S2b). By assuming a late-stage dorsal coil bias we were able to assign left and right identity to seam cells in the three embryos recorded at 3 Hz.

Using differential interference contrast (DIC) microscopy and manual visual inspection, Hall & Hedgecock (1991) published one of the few reports on *C. elegans* embryonic behavior. They describe embryo motion as flips about the anteroposterior axis that decline in frequency ahead of the emergence of the “larval locomotor pattern”. This developmental progression was readily apparent in posture space. Flips about the anteroposterior axis were seen as oscillating PC1 amplitudes, which signifies alternating dorsal and ventral coiling (Fig. 2f; Supp. Fig. S2d, e). The “larval locomotor pattern” was best captured by PC2 and PC3 amplitudes, for which phase-shifted cycling describes dorsoventral bend propagation along the body axis, reminiscent of sinuous crawling (Fig. 2f; Supp. Fig. S2f). Thus, the posture library captures a developmental progression from immature (dorsoventral flipping) to mature (sinusoidal crawling) embryo behavior.

Assessing speed, direction of motion, and bout durations

Mean squared displacement (MSD) is commonly used to derive descriptive metrics and categorize motion of particles and cells. Consistent with confinement, MSD of embryonic seam cell nuclei saturated in minutes, with the highest plateaus (corresponding to the anteriormost cells) approximating the square of the mean radius of the egg (Fig. 3a). Thus, the embryo's anterior end explores the full eggshell volume. A "diffusion coefficient" (D) was estimated by linear fit over 0 to 10 s time lags (30 frames), revealing greater motility for anterior than posterior seam cells (Fig. 3b). We also observed a consistent developmental shift, with the least motility around 600 mpf (Fig. 3c). Fig. 3d highlights the developmental transitions of an anterior (H1) and posterior (V6) seam cell. As predicted from their MSD (Fig. 3a), H1 visits the full extent of the eggshell, while V6 is more confined. Note that while the "diffusion coefficients" for the H1 nucleus are virtually identical at 530 and 750 mpf, the trajectories reveal distinct modes of motion, consistent with the different postures found at these time points (Fig. 2d).

To further characterize embryo movement, we considered speed and directional persistence. For embryo speed, we measured displacement of left-right seam cell pair midpoints (H1 to V5) over 1 s (3 frames), and then calculated a weighted average based on the straight-line length of the body at that segment and volume (Fig. 3e). Embryo speed varied with age, mirroring the developmental shift in seam cell diffusion coefficients described above (Fig. 3c, e). The coefficient of variation in embryo speed highlights the extent to which movement becomes increasingly punctuated in the final hours of embryogenesis (Fig. 3f), with both big displacements and periods of relative inactivity (Fig. 3e). To estimate directional persistence, we scored and timed movements along the anteroposterior axis (see θ in Fig. 3d). Forward and backward movement were defined as $\bar{\theta}$ less than 45° or greater than 135° , respectively (Fig.

3e). Persistence in both directions increased over the final two hours of embryogenesis (Fig. 3g). In summary, our analysis reveals a consistent developmental progression in embryo behavior. Immature embryos flip between dorsal and ventral coiling with near continual motion, while mature embryos show increased directional persistence and pausing, with the transition from immature to mature marked by a period of relative inactivity.

Synaptic signalling is required for mature motion, but not dorsoventral flipping

With a bias for dorsal coiling, sinuous crawling, and increasingly directed movement, late-stage embryos exhibit several potential signatures of neuronal influence. To establish a role for synaptic signalling, we analyzed *unc-13(s69)* mutants, which have a nearly complete block in synaptic vesicle fusion (Richmond et al., 1999). These mutants are essentially paralyzed after hatching. In late-stage embryos (750 mpf), *unc-13* mutant movement was also strongly impaired, as seen in shorter cell trajectories and smaller diffusion coefficients (Fig. 4a). By contrast, at 530 mpf, seam cell diffusion coefficients for *unc-13* mutants were indistinguishable from controls (Fig. 4b). To search for more subtle defects, we considered the most salient behavior at this stage, i.e., flips about the anteroposterior axis. At 530 mpf, control and *unc-13* mutants were flipping between fully dorsally and fully ventrally coiled postures 3 to 4 times per minute, with 75% of the transitions executed in under 10s (Fig. 4c). Flips can be considered translations along the PC1 axis (Fig. 2f, Supp. Fig. S2d, e) and sub-categorized based on PC2 and PC3 amplitudes during the transition, in this case by the larger absolute mean (PC2 or PC3) and the sign of that mean (- or +). Considering 4 motifs (PC2-, PC2+, PC3-, and PC3+), we found that trajectories through posture space were similarly distributed in control and *unc-13* mutants. For

example, ventral to dorsal flips most often initiated in the tail, with intermediate postures having posterior dorsal bending (i.e., the PC2- motif; Fig. 4d). Fig. 4e summarizes the prevalence of each flip motif. Note that dorsal to ventral flips were also more likely to initiate in the tail (i.e., the PC2+ motif; Fig. 4e). This was unexpected, as locomotion after hatching (and in older embryos; Supp. Fig. S2f) is dominated by bends propagating from head to tail, likely requiring neuronal coordination. These results suggest that motion in immature embryos is largely independent of UNC-13-mediated synaptic transmission, although an immature form of synaptic transmission (not requiring UNC-13) cannot be ruled out.

A high-throughput brightfield assay for embryonic twitching

To accelerate mutant analysis, we devised a high throughput assay to quantify the twitching structure of late-stage embryogenesis. Brightfield images of up to 60 embryos were acquired at 1 Hz, with frame-to-frame changes in pixel intensity used as a proxy for movement (Fig. 5a, Movie 2). The resulting twitch profiles were highly stereotyped, exhibiting three salient phases: an immature active phase, followed by a period of relative inactivity, followed by an active phase characterized by a broadened distribution of movement magnitudes (Fig 5b, Supp. Fig. S3a). Thus, the motion profile observed in the brightfield assay resembles that derived from seam cell tracking. The similarity in the developmental progression of behavior obtained by these disparate methods confirms that the iSPIM imaging regime was non-invasive and validates the use of the high-throughput brightfield assay to screen for mutants altering embryo behavior.

In the frequency domain, we detected an *unc-13* dependent signal in the wild-type scalogram: a 20-40 mHz feature in the final 1-2 hours ahead of hatching (Fig. 5c, d, Supp. Fig. S3b). We call this feature “slow wave twitch” or SWT. In *unc-13* mutants, peak SWT power ratios were markedly diminished compared to wild-type (Fig. 5e) and failed to localize to a specific developmental stage (Fig. 5d). Thus, SWT represents a rhythmic embryonic behavior with a period of 25-50 seconds that depends on synaptic transmission.

To probe the neuronal underpinnings of SWT, we evaluated mutants perturbing specific neurotransmitters. Peak SWT power ratios were unaffected by loss of the vesicular transporters for biogenic amines (CAT-1/VMAT), glutamate (EAT-4/VGLUT), or GABA (UNC-47/VGAT). By contrast, peak SWT power ratios but were strongly diminished in mutants lacking the vesicular transporter for acetylcholine (UNC-17/VACHT; Fig. 5e), indicating that cholinergic transmission plays a prominent role in generating SWT. Body wall muscles express two classes of nicotinic acetylcholine receptors: homopentameric ACR-16 receptors and heteropentameric levamisole receptors containing UNC-29 subunits (Richmond & Jorgensen, 1999; Touroutine et al., 2005). Mutants lacking UNC-29 had significantly reduced peak power ratios in the SWT frequency band, a defect that was rescued by a transgene expressing UNC-29 in muscle (Fig. 5e). By contrast, peak SWT power ratios were unaffected in *acr-16* mutants (Fig. 5e). Thus, SWT requires cholinergic signalling through UNC-29-containing receptors in muscle. Peak SWT power ratios were also strongly diminished in mutants lacking neuropeptide processing enzymes (EGL-3/PC2 and EGL-21/CPE; Fig. 5e), implicating neuropeptide signalling in SWT.

SWT is mediated by FLP-11 release from RIS

The timing of SWT coincides with an increased propensity for pausing (Fig 3e); consequently, we hypothesized that periodic quiescent bouts were generating signal in the 20-40 mHz frequency band. Neuropeptides released from RIS and/or ALA are known to elicit behavioural quiescence in larvae and adults (Turek et al., 2013; Turek et al., 2016; Van Buskirk et al. 2007; Nath et al., 2016). We investigated whether either of these neurons is required for SWT by analyzing mutants lacking RIS (*aptf-1/AP-2*) or ALA (*ceh-17/PHOX2A*) function (Turek et al. 2013; Pujol et al., 2000; Van Buskirk et al. 2007). Consistent with a role for RIS, we found that peak SWT power ratios were markedly reduced in *aptf-1* mutants but were unaffected in *ceh-17* mutants (Fig. 6a). The somnogenic neuropeptides released from RIS are encoded by the *flp-11* gene (Turek et al., 2016). While their developmental progression was superficially normal in terms of gross movement magnitude, the *flp-11* mutant scalogram lacked an SWT signature (Fig. 6b). Indeed, peak SWT power ratios in *flp-11* mutants were severely diminished compared to wild-type (Fig. 6a). These data suggest that late-stage movement is structured by periodic release of FLP-11 from RIS, specifically increasing power in the 20-40 mHz frequency band.

To relate RIS signalling and behavior, we simultaneously monitored RIS motion and activity with a genetically encoded calcium sensor, GCaMP6f (Chen et al., 2013), fused to a calcium insensitive fluorophore, mCherry. Tracking GCaMP/mCherry ratios in the RIS cell body in embryos aged at least 645 mpf (Fig. 6c, d), we observed RIS calcium transients at a rate approximating 20-40 mHz, similar to the SWT frequency band (Fig. 6e). Furthermore, RIS calcium transients correlated with behavioral slowing (Fig. 6g). In mutants lacking FLP-11 neuropeptides, RIS activity was unaltered; however, behavioural quiescence was substantially reduced (Fig. 6e,f), as was the negative correlation of RIS calcium transients with motion (Fig.

6g). These results suggest that a prominent feature of late-stage embryonic behavior is a rhythmic pattern of quiescence elicited by FLP-11 release from RIS.

Discussion

Here we describe a systematic analysis of behavioral maturation in *C. elegans* embryos. Our analysis leads to three principal conclusions. First, embryos follow a stereotyped behavioural trajectory: early flips between dorsal and ventral coiling (most often initiated in the tail) slowing into a period of reduced motility, followed by emergence of mature motion (including sinuous crawling and rhythmic quiescent bouts). Second, synaptic transmission is required for late-stage movement, but not for early dorsoventral flipping or the intervening inactive period. Third, the bouts of behavioral quiescence punctuating late-stage movement are elicited by FLP-11 release from RIS neurons.

Flipping between dorsal and ventral coiling dominates early embryo behavior. This behavioral motif is not obviously affected by loss of *unc-13*, implying it does not depend on synaptic transmission. Flipping could be mediated by intrinsic oscillatory activity in muscles or by neuronal signaling that persists in the absence of UNC-13 (e.g. gap junctions or non-conventional synaptic transmitter release). It will be interesting to determine what drives immature flipping and whether this early behavior is required in some way for the subsequent emergence of mature behaviors. Flipping gradually slows into a period of relative inactivity. This slowdown is apparently not neuronally evoked, as it requires neither neuropeptide processing enzymes nor UNC-13. Slowing does coincide with a shift in the forces defining body morphology, from a squeeze generated by contraction of circumferential actin bundles of the

epidermal cytoskeleton to containment within a tough, yet flexible extracellular cuticle (Priess & Hirsh, 1986). This structural transition could impact behavior; however, preliminary calcium imaging experiments suggest that at least part of the slowdown can be attributed to reduced muscle activity, rather than simply a structural constraint limiting motion. A shift from cytoskeletal to exoskeletal control of morphology is likely re-iterated at each larval molt and could contribute to molt-associated lethargus quiescence (Raizen et al., 2008). The brightfield motion assay promises to be a useful screening tool to further investigate this phenomenon.

We identify SWT as a new form of behavioral quiescence, occurring during embryogenesis. Osmotic stress and reduced insulin signalling cause behavioral quiescence around hatching, which is accompanied by developmental arrest (Bayer et al., 2021). The salt and insulin regulated quiescence and SWT quiescence occur at a similar stage of embryonic development and both require FLP-11, suggesting that these two forms of quiescence are mechanistically related. Other forms of *C. elegans* behavioral quiescence have been described during larval molts (lethargus; Raizen et al., 2008) and in response to nutrient excess (satiety; You et al., 2008), cellular stress (e.g., heat shock; Hill et al., 2014), starvation (Skora et al., 2018; Wu et al., 2018), and physical restraint (Gonzales et al., 2019). Behavioral quiescence has been shown to exhibit sleep-like properties: reversibility, reduced sensitivity to external stimuli, homeostatic regulation, and a stereotyped posture (Raizen et al., 2008; Choi et al., 2013; Iwanir et al., 2013; Cho et al., 2014; Tramm et al., 2014; Nagy et al., 2014; Schwarz & Bringmann, 2017). Quiescent states can also share deep molecular conservation with sleep in other systems. For example, regulation by both LIN-42/PERIOD (Konopka & Benzer, 1971; Hardin et al., 1990; Sun et al., 1997; Tei et al., 1997; Monsalve et al., 2011) and AP-2 transcription factors

(Mani et al., 2005; Turek et al., 2013; Kucherenko et al., 2016; Hu et al., 2020). Among the different quiescent states, SWT shares several properties with lethargus quiescence. First, both are elicited by RIS release of FLP-11 (Fig 6; Turek et al., 2016). Second, lethargus quiescence coincides with molting (i.e. cuticle replacement), while SWT coincides with the synthesis of the first cuticle. Indeed, the embryonic transcriptional profile from the end of gastrulation to hatching correlates with oscillating gene expression across each larval stage (Meeuse et al., 2020). Together, these similarities suggest that SWT may be akin to the developmentally timed sleep associated with each larval molt.

An embryonic quiescent state has several potentially important implications. First, if quiescence is required for normal embryonic development, that could help explain why sleep is ubiquitously conserved. Second, sleep disruption is a common co-morbid symptom in neurodevelopmental disorders, including Autism spectrum disorders (ASD; Wintler et al., 2020). Could disrupted prenatal sleep be a risk factor for ASD? ASD linked genes exhibit peak co-expression in mid-fetal neurons (Parikshak et al., 2013; Willsey et al., 2013; Satterstrom et al., 2020), which has been interpreted to suggest that ASD results from disruption of some aspect of fetal brain development. The precise fetal defects associated with ASD have not been identified, perhaps because the specific circuits exhibiting peak ASD gene co-expression have not been identified. Interestingly, we find that embryonic RIS is among the neurons most enriched for expression of ASD linked gene orthologs (Supp. Fig. S4). Based on these results, we speculate that an important function of ASD linked genes is to promote embryonic sleep behaviors.

How might SWT relate to early brain development? Correlated spontaneous activity is essential for the refinement and maturation of developing neuronal circuitry in a variety of contexts (Kirkby et al., 2013). For example, behavioral maturation of *Drosophila* is dependent on patterned spontaneous neuronal activity in embryos (Crisp et al., 2011; Carreira-Rosario et al., 2021). Could RIS be influencing network connectivity by broadly structuring neuronal activity? Embryonic quiescence may also be a mechanism to preserve energy stores. In this scenario, disrupting sleep could predispose embryos to metabolic stress. Indeed, transcriptional stress responses are associated with sleep disruption in worms, flies, and mice (Sanders et al., 2017; Shaw et al., 2000; Cirelli et al., 2006; Terao et al., 2003; Naidoo et al., 2005). It will be interesting to see whether prenatal metabolic stress alters early brain development.

Despite the presence of many important neurodevelopmental benchmarks, behavior during the final hours of *C. elegans* embryogenesis is largely unexplored. The embryonic posture library described here will provide a resource to test new hypotheses about how (and when) embryonic circuits assemble. Semi-automated tracking with MHHT should accelerate expansion of this resource, with the discovery of new embryonic behaviors providing an entry point to study nascent neuronal circuitry. The potential for recording pan-neuronal activity in unanesthetized embryos executing well-defined behaviors (Ardiel et al., 2017) as a stereotyped connectome emerges (Witvliet et al., 2021), uniquely positions *C. elegans* to investigate fundamental aspects of early brain development.

Methods

Strains and reagents

Animals were maintained on nematode growth medium (NGM) seeded with *Escherichia coli* (OP50) as described in Brenner (1974). The posture library was built with strain JR667 *unc-119(e2498::Tc1); wls51[SCMp::GFP + unc-119(+)]*, which expresses GFP in hypodermal seam cell nuclei. For the supplemental postures described in Supp. Fig. S2b, c, *wls51* was combined with epidermal actin marker, *mclS50[lin-26p::vab-10(actin-binding domain)::GFP + myo-2p::GFP]* and one of the following mCherry transgenes: *stls10131[elt-7p::H1-wCherry + unc-119(+)]* (SLS20), *stls10501[ceh-36p::H1-wCherry::his-24p::mCherry + unc-119(+)]* (SLS21), *stls10691[ref-1p::H1-wCherry + unc-119(+)]* (SLS22), *nuSi187[pat-10p::NLS-GCaMP::mCherry-NLS]* (SLS23), *stls10138[tbx-38p::H1-wCherry; unc-119(+)]* (SLS24), or *stls10808[nob-1p::H1-wCherry; unc-119(+)]* (SLS25).

The following mutant alleles were used in this study: *unc-13(s69)*, *unc-47(e307)*, *unc-17(e245)*, *acr-16(ok789)*, *unc-29(x29)*, *glr-1(n2461)*, *cat-1(e1111)*, *egl-3(n150)*, *egl-21(n476)*, *ceh-17(np1)*, *aptf-1(gk794)*, *aptf-1(tm3287)*, and *flp-11(tm2706)*.

For rescuing *unc-29* expression in muscle, plasmid KP#4524 was generated by inserting the *pat-10* promoter (550 bp), the SV40 nuclear localization sequence, mCherry with introns, the EGL-13 nuclear localization sequence, the *gpd-2* 3' UTR with SL2 acceptor site, *unc-29* cDNA, and the *unc-54* 3' UTR between the SpeI and AgeI sites in pCFJ910.

For calcium imaging in RIS, plasmid KP#4525 was generated by inserting the *flp-11* promoter (3159 bp), GCaMP6f with introns, a flexible linker (GSSTSG(AP)₇ASEF), mCherry with introns, and the *unc-54* 3' UTR between the SbfI and StuI sites in pCFJ910.

The miniMOS method (Frøkjær-Jensen et al., 2014) was used to integrate KP#4524 and KP#4525, generating single copy insertions: *nuTi233*[*pat-10p*::NLS::mCherry::SL2::UNC-29] and *nuTi580*[*flp-11p*::GCaMP6f::mCherry].

Embryonic age was determined by measuring the time elapsed since twitch onset (which was defined as 430 mpf).

Posture library

Sample preparation: Gravid adults were dissected to release embryos, which were transferred to a patch of poly-L-lysine (1 mg/mL) in a diSPIM imaging chamber filled with M9 buffer. Embryo orientation was set to minimize Z-steps, i.e. objective translation along the embryo's minor axis. Prior to fluorescence imaging, brightfield recordings were acquired to stage the embryos.

Microscope: On a diSPIM (Kumar et al., 2015), a pair of perpendicular water-dipping, long-working distance objectives (40x, 0.8 NA) were used for brightfield and fluorescence imaging. Acquisition was controlled using Micro-Manager's diSPIM plugin (<https://micro-manager.org/>). Note that although the diSPIM can collect two orthogonal views, only a single view was used to build posture libraries, i.e. iSPIM mode. Sweeping a 488 nm laser beam (measured <1 μ W after the objective), a two-dimensional MEMS mirror in the diSPIM scanhead generated the light-sheet and defined the image volume. To maximize speed, the camera (pco.edge 4.2 or ORCA-Flash 4.0) was oriented such that the chip readout direction was along the embryo's minor axis. The imaging objective was translated in step with the light-sheet, generating 36 planes at a spacing of 1.2 μ m. Exposure time per plane was 4 ms and volumes

were acquired at 3 Hz. To reduce file size, the camera was set to 4x binning, resulting in 16-bit images with 0.65 μm pixels. See Duncan et al. (2019) for detailed protocols on imaging embryos with the diSPIM.

For the supplemental postures described in Supp. Fig. S2b, c, diSPIM acquisition parameters were optimized for spatial resolution rather than speed. Specifically, diSPIM mode was used to acquire image volumes from perpendicular objectives with no pixel binning at the camera. The two orthogonal views were then computationally fused to generate isotopically resolved image volumes (Wu et al., 2013; Guo et al., 2020). Laser power was at least an order of magnitude greater than for the volumes acquired at 3 Hz (i.e., $> 30 \mu\text{W}$, measured after the objective). The 561 nm laser line was scanned simultaneously, with dual-color image splitters separating detection at the camera (W-VIEW GEMINI, Hamamatsu). Volumes were acquired every 5 minutes from pre-twitching until hatching, with each image volume comprising 50 planes at a spacing of 1 μm . Exposure time per plane was 5 ms, with volumes acquired in < 900 ms.

Analysis:

Image volumes were segmented using a large kernel 3D U-Net trained on an average of the Dice coefficient and binary cross-entropy loss functions. See Supplementary Note for additional details on image segmentation. In an early volume with good detections, seam cells were manually positioned along the body axis by observing movement over several frames. From this seed volume, cell position was tracked across time using MHHT. See Supplementary Note and <https://github.com/lauziere/MHHT> for complete details on MHHT.

Left and right seam cells were fit with natural cubic splines using MATLAB's 'cscvn' function. DV bends were then computed from the midpoint (mL/R) along the spline connecting adjacent seam cells and the corresponding midpoint (mR/L) on the opposite side of the body (Fig. 2a). For example, for the DV bend between H0L and H1L:

$\vec{v}_1$ connects mL to H0L;

$\vec{v}_2$ connects mR to mL;

$\vec{v}_3$ connects mL to H1L; and

$$\text{DV bend} = \arctan2([(\vec{v}_1 \times \vec{v}_2) \times (\vec{v}_2 \times \vec{v}_3)] \cdot (\vec{v}_2 / |\vec{v}_2|), [\vec{v}_1 \times \vec{v}_2] \cdot [\vec{v}_2 \times \vec{v}_3])$$

The MATLAB class 'msdanalyzer' was used to evaluate mean square displacement (Tarantino et al., 2014).

Brightfield motion assay

Sample preparation: Several (≥ 4) gravid adults per strain were dissected directly in an M9 filled glass bottom microwell imaging dish (MatTek Corp., P35G-1.5-20-C). Up to 60 pre-twitching embryos were arrayed on a patch of poly-L-lysine (0.1 mg/mL) using water currents generated with a worm pick. Worm remains and unwanted embryos were removed with a micropipette and the dish was lidded.

Microscope: Embryo arrays were recorded on an inverted microscope (Zeiss, Axiovert 100) with a 5x, 0.25 NA objective. A CCD camera (CoolSNAP HQ2) generated 16-bit images with a pixel size of 1.29 μm . Transmitted light power was adjusted to get background pixel counts of ~ 500 from a 2 ms exposure. Images were acquired at 1 Hz using Micro-Manager control software.

Analysis: Pixel intensities were extracted from a box (21x21 pixels) centered on each embryo and frame to frame pixel intensity changes larger than threshold (25) were tallied. The onset of sustained twitching was detected as the first tally (smoothed over 30 frames) greater than X pixels (120 of a potential 441). Background intensity for each frame was also extracted from a perimeter around the embryo (61x61 pixels). Hatching was detected as the point at which the mean intensity at the embryo position (smoothed over 30 frames) approximated the mean intensity of the background perimeter (signal-to-background ratio of at least 0.92). Fresh hatchlings occasionally swam into the field of view, registering pixel changes in the embryo and background perimeter. Data were therefore excluded for frames in which the background perimeter registered changes greater than threshold (35) in more than X pixels (40 of a potential 240 when smoothed over 30 frames).

Peak SWT power ratios were computed as follows:

1) For each embryo, 15 min twitch profiles were extracted with a sliding window (200 s step size) from 1 h post twitch until hatching. Missing datapoints were replaced with a reversed duplication of the immediately preceding interval of the same length.

2) Using MATLAB's Fast Fourier transform ('fft') function, each 15 min twitch profile was transformed from the time domain (i.e. pixels changing per second, $y(t)$) to the frequency domain, $Y(f)$ for equally spaced frequency bins from $f_0 = 0$ to $f_{900} = 1$ Hz, i.e. the sampling rate.

3) For each 15 min twitch profile, the proportion of the signal attributed to the SWT frequency band was computed:

$$\text{SWT power ratio} = \frac{\sum_{0.02 \leq f \leq 0.04 \text{ Hz}} |Y(f)|^2}{\sum_{0 < f \leq 0.5 \text{ Hz}} |Y(f)|^2}$$

4) For each embryo, the timing and magnitude of the largest SWT power ratio was recorded.

To estimate a baseline for peak SWT power ratios (i.e., the dashed lines in Figs. 5e, 6a), pairs of neighbouring values were randomly shuffled prior to the discrete Fourier transformation (between steps 1 and 2 above) for a sample of wild-type embryos.

Scalograms were generated using MATLAB's Continuous 1-D wavelet transform ('cwt') function (with 'morse' wavelet).

Calcium imaging

Sample preparation: Several (≥ 15) gravid adults per strain were dissected directly in an M9 filled glass bottom microwell imaging dish (MatTek Corp., P35G-1.5-20-C). Embryos with no more than 4-cells were selected using currents generated with a worm pick and stuck to a patch of poly-L-lysine (0.1 mg/mL) in pairs, aligned end to end along their major axis (a configuration that enabled imaging two embryos per scan). Worm remains and unwanted embryos were removed with a micropipette and the dish was lidded. Prior to calcium imaging, the brightfield assay (see above) was used to time first twitch.

Microscope: At least 215 minutes after the onset of twitching (i.e., ~ 645 mpf), embryos were imaged on an inverted confocal microscope (Nikon, Eclipse Ti2) using a 20x, 0.75 NA objective. Resonant scanning in two directions generated image planes of 1024x256 pixels (0.16 $\mu\text{m}/\text{pixel}$) for two channels (488 and 561 nm lasers) in 33.3 ms. Two embryos were imaged per scan. Volumes were acquired at 1 Hz for 5 min using a piezo stage stepping 7.875 μm between 5 image planes (note that the pinhole was opened to minimize the number of steps required to span the embryos).

Analysis: RIS cell body position and corresponding GCaMP and mCherry intensity were extracted from an image volume cropped around a single embryo. Specifically, following background subtraction (pixel counts-100), each image plane was filtered using a Gaussian smoothing kernel with standard deviation of 5 (MATLAB's 'imgaussfilt' function with 'symmetric' image padding to prevent edge artifacts). The position of the RIS cell body was identified in the mCherry channel as the brightest pixel of the filtered volume. The corresponding intensity in the filtered GCaMP channel was used to compute $\Delta R/R_0 = (R - R_0) / R_0$, where R is the GCaMP/mCherry ratio and R_0 is the ratio at baseline, set as the 10th percentile for each trace. Calcium transients were identified in smoothed traces (moving mean with span 4) using an onset threshold of $\Delta R/R \geq 2$ and an offset threshold of $\Delta R/R < 1.5$.

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547 S.X.; Writing – Original Draft, E.L.A., A.L.; Writing – Review & Editing, J.M.K., H.S.; Supervision,

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Fig. 1. Tracking seams cells with Multiple Hypothesis Hypergraph Tracking (MHHT). a)

Embryonic timeline with imaging window highlighted. mpf: minutes post fertilization. b) XY maximum intensity projection of an embryo expressing GFP in hypodermal seam cell nuclei (left). Segmented (center) and identified seam cells (right). MHHT: Multiple Hypothesis Hypergraph Tracking. Scale bar, 10 μ m. c) Frame-to-frame displacement of V2 along the x-axis of the image correlates with that of other seam cells (top). Frame-to-frame change in the distance between V2 and V3 correlates with neighbouring edge length changes (bottom). See Supp. Fig. S1 for full correlation matrices. d) A cell assignment search tree with width $K=2$ and depth $N=3$. Physically impossible body contortions (self-intersections) are pruned (scissors). The path accruing the least cost is highlighted (outlined circles).

Fig 2. Eigen-embryos compactly describe behavioral maturation. a) Schematic depicting the definition of a dorsoventral (DV) bend. Top: Adjacent seam cell nuclei positions (colored balls) are fit with a natural cubic spline (black line), open circles denote the midpoint between seam cell nuclei. Bottom: View looking down $\vec{v}_2$ to highlight a dorsal DV bend on one side of the body. Curved red arrow indicates DV bend angle. b) DV bends along the left and right sides of the body ('+' and 'o'; top), with the corresponding posture model (bottom). Seam cell nuclei are fit with a natural cubic spline from H0 (blue) to tail (yellow). c) Fraction of the total variance captured by reconstructing postures using 1 through all 18 principal components. d) Fraction of the total variance captured by the first 4 principal components as a function of age. e) Eigen-embryos, i.e. eigenvectors for the first 4 PCs, derived from the 3 individual embryos in grey and from pooled datasets in color. Right: sample postures drawn from the 95th percentile of

amplitudes for the corresponding PC. f) An embryo's PC1 amplitude from 530 mpf until hatch (top). DV bend profiles over highlighted 20 min windows with corresponding PC2 and PC3 amplitudes (bottom).

Fig 3. Transition to punctuated and directed movement ahead of hatching. a) Mean square displacement (MSD) for each seam cell nucleus of an embryo, 530 to 770 mpf. Dotted line depicts predicted unconfined Brownian motion for the H1 seam cell. b) Mean diffusion coefficient (D) for each seam cell pair. X-axis label colored as in a). Three individual embryos in grey, mean +/- SEM in black. c) Diffusion coefficients for all cells computed over 20 min bins. Individual embryos in grey, mean +/- SEM in black. d) Tracks of an H1 and V6 seam cell over 20 minutes at indicated age (left). Darker tracks indicate larger movements ('v'), color-code shows direction of motion along the anteroposterior axis ('θ'; see schematic representation of 'v' and 'θ' at right). e) Embryo speed on a log-scale to highlight late stage pausing (top). Higher magnification views over highlighted 5 min intervals (bottom), with labels for the direction of motion and pausing (pauses defined as embryo speed < 0.5 μm/s). f) Coefficient of variation in embryo speed for 20 min bins. Individual embryos in grey, mean +/- SEM in black. g) Mean duration of forward, backward, and pausing bouts for 20 min bins. Individual embryos are represented as fainter lines with mean +/- SEM in bold.

Fig 4. *unc-13* mutants have a late-stage motility defect. a) 10 min tracks of an H1 seam cell (left) and mean diffusion coefficients of seam cell pairs (right; mean +/- SEM) at 750 mpf for control (WT, n=3) and *unc-13* mutants (n=3). Scale bar, 10 μm. b) Same as a) for 530 mpf (WT,

n=3; *unc-13*, n=2). c) Duration of transitions between fully dorsally and fully ventrally coiled postures from 530-540 mpf. d) An embryo's transitions from ventral to dorsal coiling through posture space from 530-540 mpf (note that only transitions executed in ≤ 10 seconds are plotted). Flips were categorized into four motifs based on the mean amplitude of PC2 and PC3 during the transition (PC2+, PC2-, PC3+, and PC3-). Arrowheads and dots denote the start and end of the flip, respectively, i.e., the last fully ventrally coiled posture to the first fully dorsally coiled posture. e) Summary of coiling transition trajectories in posture space at 530 mpf, with arrowhead sizes indicating the frequency of occurrence. Color-code matches d). Schematized worms are shown as black squiggles with ventral side down and red dot at head.

Fig. 5. A brightfield assay for screening mutants reveals the 'slow wave twitch' (SWT) as a behavioral signature dependent on cholinergic and peptidergic signalling. a) 30 embryos arrayed for the brightfield assay (left). Scale bar, 100 μm . Zoomed images (scale bar, 10 μm) over 3 consecutive frames of the embryo highlighted in the array, with difference images shown below (right). Sample trace depicts the proportion of pixels changing at twitch onset. b) Brightfield assay summary for wild-type (WT; n = 24) and *unc-13* mutants (n = 23), with each line corresponding to an embryo aligned at twitch onset and color-coded by the proportion of pixels changing intensity (smoothed over 10s). c) Difference between wild-type and *unc-13* mutant scalograms (see Supp. Fig. S3b for mean scalograms). Note that the feature in the 20-40 mHz frequency band at ~ 660 to 720 mpf (arrow) represents the 'slow wave twitch' (SWT). d) Timing of peak relative power in the SWT frequency band (20-40 mHz). Mean $\pm$ SEM, with each dot corresponding to an embryo. e) Peak relative power in the SWT frequency band in

several synaptic signalling mutants. Mean $\pm$ SEM, with each dot corresponding to an embryo.

One-way ANOVA and Tukey's honestly significant difference criterion were used to compare strains. Asterisks denote strains statistically distinguishable from wild-type at $p < 0.001$. The dashed line shows the average peak SWT power ratio derived from shuffled wild-type data.

Fig. 6. SWT is mediated by FLP-11 neuropeptides released from RIS. a) Left: Proportion of pixels changing during the 15 min window over which the SWT power ratio peaked. Right: Peak relative power in the SWT frequency band (20-40 mHz). Mean $\pm$ SEM, with each dot corresponding to an embryo. The dashed line denotes the average peak SWT power ratio derived from shuffled data. Alleles for *aptf-1* refer to *gk794* and *tm3287*. b) Top: Brightfield assay summary for wild-type (WT) and *flp-11* mutants, with each line corresponding to an embryo aligned at twitch onset and color-coded by the proportion of pixels changing intensity (smoothed over 10s). Bottom: Wild-type and *flp-11* scalograms. Note the SWT signature in WT (arrow) is missing in *flp-11*. c) A single slice from an image volume of an embryo expressing GCaMP (top) fused to mCherry (bottom) in RIS. Time stamp in seconds and scale bar, 10 μ m. d) Representative traces of RIS movement (above) and activity (below), as measured by normalized GCaMP/mCherry ratios at the cell body. Grey bands denote calcium transient width at half max. Green bar spans the period depicted in c). e) Frequency of calcium transients and quiescent bouts (quiescence defined as speed $\leq 0.5 \mu$ m/s for at least 3 s). f) Duration of calcium transients (peak width at half max) and quiescent bouts. Mean $\pm$ SEM, with each dot corresponding to an embryo. One-way ANOVA and Tukey's honestly significant difference

criterion were used to compare strains. Asterisks denote statistically distinguishable groups at $p < 0.001$. g) Cross correlation between fluorophore intensity and RIS speed.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.