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Neural stem cells generate neurons *in vitro* which mimic their *in vivo* molecular identity and axon/dendrite morphology

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Abstract (147 words)

Neuronal morphology is determined by intrinsic and extrinsic cues, but their relative importance is poorly understood. Mammalian neuronal cultures typically assay axon/dendrite regenerative ability; rarely have cultures extended from neural stem cells to mature neurons. *Drosophila* is an excellent model organism to study neurodevelopment, from neural stem cell (neuroblast; NB) formation through axon/dendrite polarization. Previously, *Drosophila* cultures have been difficult to maintain. Here, we describe a protocol that allows long-term growth of embryo NB-derived neurons. We show that cultured neurons (1) develop from identified NBs; (2) proliferate at similar rates to *in vivo* neurons; (3) polarize to form axons and dendrites; (4) sort axonal and dendritic cargoes; (5) express transgenes marking identified interneurons and motor neurons; and (6) retain aspects of their *in vivo* morphology. We conclude that *Drosophila* neuronal cultures are useful for identifying intrinsic and extrinsic cues that specify neuronal identity, morphology and subcellular processes.

Main

Neurons reproducibly generate diverse morphologies that underlie their connectivity and function. Understanding how neurons establish unique

morphological characteristics is necessary to reveal how specific synaptic connections are assembled and maintained, which may provide useful insights into the mechanisms of neuronal repair following injury or disease.

Our current understanding of how neurons undergo morphogenesis comprises a series of cell-intrinsic and cell-extrinsic cues, although the latter has been studied significantly more than the former^{1,2}. Gradients of morphogens have been shown to regulate neurite guidance and axon termination under varied conditions and in multiple organisms^{3–5}. It is well-documented that cell-extrinsic cues are regulators of neurite morphology and synapse selection; however, two neurons can develop within the same space, exposed to the same extracellular signals, yet exhibit different morphologies and connectivities. This suggests that cell-intrinsic factors also guide neuronal morphogenesis.

While the extracellular environment surrounding neurons provide cues for neurite outgrowth, axon pathfinding, and synapse formation, there is less understanding of the extent to which cell-intrinsic programming influences morphological and connective characteristics. Recent work within the *Drosophila* ventral nerve cord (VNC) has shown that neuronal molecular identity established during neuroblast (NB; *Drosophila* neural stem cells) lineage development is critical for establishing unique morphological characteristics of neurons like axon projection, dendrite arborization, and specific synaptic partner choice^{6–10}. These findings support a significant contribution of molecular identity in establishing neuronal morphology, but we do not yet understand the relative contributions of cell-extrinsic and cell-intrinsic cues in establishing neuronal morphology. Nor does the field currently utilize a system that allows us to seamlessly move between *in vivo* and *in vitro* techniques within the same organism in a robust enough way to parse how cell-intrinsic and cell-extrinsic cues contribute to neuronal morphology and synapse development. Specifically, the extent to which molecular identity confers neuronal morphology and synapse selection is an area of research that could leverage an expanded toolkit¹¹. The relative importance of extrinsic cues can be determined by eliminating them, e.g. with dissociated neurons cultured *in vitro*, and assaying for loss of specific aspects of neuronal morphology.

71 Neuron primary cultures of murine and rat tissue are already widely
72 used¹². These methods have been crucial in understanding the sub-cellular
73 architecture of neurons and neuronal polarization. However, the expense of
74 maintaining animal colonies¹³ paired with a lack of neuron-type specificity
75 are weaknesses that have limited progress^{14,15}. Furthermore, most
76 neuronal cultures are initiated from disruption and re-growth of mature
77 neurons, rather than assaying them from birth to maturation. Here we
78 overcome these limitations using *Drosophila*, where neurons can be
79 cultured from the NB stage to the mature neuron stage. A combination of a
80 rich genetic toolbox¹⁶ and well-characterized connectomes spanning
81 multiple life stages^{17,18} presents a unique opportunity to parse contributions
82 of cell-intrinsic versus cell-extrinsic cues driving development of individual
83 neuronal morphology.

84 Primary culture of *Drosophila* neurons is not a novel idea. Early
85 studies characterized NB asymmetric cell division and showed that NBs
86 could generate their stereotyped “temporal cascade” of sequential gene
87 expression when cultured in isolation^{19,20}. More recently, differentiated
88 embryonic and larval fly neuron cultures²¹ have been used to study
89 microtubule regulation^{22,23}, dynamics^{24–26}, axon outgrowth²⁷, and neuronal
90 cargo trafficking²⁸. While these studies have provided data about neuronal
91 cytoskeletal regulation, they lacked the specificity of investigating an
92 identified neural subtypes or individual neuron identity; nor do they extend
93 cultures over multiple days. By using driver lines to label individual
94 neurons, paired with knowledge of their synapse specificity, we can use
95 embryonic fly neuron cultures to ask questions about acquisition of unique
96 morphology of a specific neuron, or synapse specificity in the absence of
97 intact cell-extrinsic guidance cues. Crucially, the process of embryo
98 dissociation removes NBs from their *in vivo* environment before
99 differentiation, depriving them of patterned cell-extrinsic cues and resulting
100 in the development of contact-naïve neurons born from neural stem cells *in*
101 *vitro*.

102 Here, we describe a protocol to isolate and culture embryonic
103 *Drosophila* NBs as they generate their neuronal progeny, introducing them
104 to *in vitro* conditions prior to the initial establishment of axons and dendrites
105 (Figure 1). We validate that specific populations and neurons in culture can

be labeled using binary genetic driver strategies. We validate that these neurons are represented in culture at similar proportions to *in vivo*. We show that NBs differentiate at rates comparable to those *in vivo* and can form polarized compartments with appropriately sorted axon cargo. Most excitingly, we found that neurons from specific populations maintain molecular markers of their identity, indicating that molecular and morphological identity is not changed by removal from their native environment. We therefore propose that use of fly neuron cultures established during NB proliferation and differentiation stages provides a system in which we can characterize cell-intrinsic guides to *Drosophila* neuronal development and offers a unique opportunity to robustly combine *in vivo* and *in vitro* experiments within the same system.

Results

Neuronal differentiation occurs normally from neuroblast to neuron during *in vitro* culture

Within the *Drosophila* VNC, the formation and proliferation of NBs have been well-characterized. NBs delaminate from the neuroectoderm beginning at embryonic stage 9, and start dividing to generate their lineage during stage 11²⁹. A robust marker for NB identity is Worniu (Wor) a bHLH member of the Snail family³⁰. NBs divide asymmetrically to self-renew and produce an intermediate neural progenitor called a ganglion mother cell (GMC); each GMC typically divides once to generate two post-mitotic neurons. Thus, NBs produce a clone of neurons. A robust marker for neurons is Elav (Embryonic Lethal Abnormal Vision), a conserved neuronal marker in both flies and mammals^{31,32}. Each NB generates a unique lineage based on the spatial factors present at NB formation, named by row and column (e.g. NB5-2 is located in the fifth row, second column of NBs)³³.

To determine whether NBs *in vitro* differentiate at a comparable rate to *in vivo*, stage 11 embryos were dissociated and cultured for 30 minutes, 1 hour, 2 hours, 6h hours, and 12 hours (correlating to embryonic stages 11, 12, 14, 15, and 17, respectively)²⁹. Fixed cells were stained for the NB marker Wor and the neuronal marker Elav, along with the anti-alpha tubulin

(Dm1 α) to show general cell morphology. Each time point was analyzed to determine the proportion of NBs and neurons (Figure 2A-F). Wor was expressed in every analyzed cell at the 30 minute time point, and none expressed the neuronal marker Elav, consistent with the new-born NB5-2 lineage having just started proliferation (Figure 2A, F). At 1 hour, fewer cells express Wor, while 25% express Elav indicating differentiation has begun (Figure 2B, F). Eventually, by 12h (correlating to stage 17 *in vivo*) none of the cells were Wor+ and all expressed the neuronal marker Elav (Figure 2E, F). *In vitro*, cells begin to develop short processes by 1 hour, and most cells had developed a neurite with the average length of 9.457 μ m (Figure 2B, G, H). By 12 hours, all cells had developed neurites with an average length of 38.494 μ m, varying widely from cell to cell (Figure 2G, H). This is similar timing to neuronal process formation *in vivo*^{34,35}. These data show that NBs differentiate and develop *in vitro* on a comparable timeline to *in vivo*.

Specific neuronal populations can be labeled *in vitro* using the Gal4/UAS genetic labeling system

A major weakness of mammalian neuronal tissue culture techniques is difficulty identifying rare neuronal sub-types in isolated cultures. Utilizing fly genetics in combination with cell culture is a promising technique to study specific neuronal subtypes and individual identifiable neurons. To determine whether neurons expressing transgenes in specific NB lineages can be generated at similar ratios *in vitro* as they exist *in vivo*, we assayed for expression of NB5-2 neuronal progeny. We can identify NB5-2 and its lineage due to the existence of a split-Gal4 line which drives expression of a *UAS-GFP* transgene in NB5-2 and its clone of interneurons^{8,9}. Quantification of the GFP+ neurons in the NB5-2 shows it produces approximately 24 interneurons per hemisegment (Figure 3B-D) by stage 14 *in vivo*⁸. NB5-2 Gal4 is expressed at the intersection of Wg and Vnd spatial transcription factors (Figure 3A). This line has been well-characterized *in vivo*^{8,9}, making it an excellent transgenic line to test for expression *in vitro*.

Embryos expressing GFP under control of the NB5-2 Gal4 (*NB5-2-Gal4 UAS-GFP*) were dissociated and cultured at 25°C for 24 hours,

equivalent to early L1 larva. To assess whether neurons in culture are labeled at a similar rate to *in vivo*, samples were stained for GFP to mark the NB5-2 progeny; tubulin (Dm1 α) to determine the outline of unlabeled neurons; and Elav to mark neuronal nuclei (Figure 3E-E"). Three cultures were generated from 30 embryos. Samples were imaged in non-overlapping wide fields of view with 20x magnification across 3 coverslips (Figure 3E). Images were analyzed to count the number of Elav+ and Elav+ GFP+ cells per frame (Figure 3E, F). *In vivo*, approximately 4.1% of neurons expressed GFP per brain (Figure 3B-D, G) (n = 5 brains). *In vitro*, 5% of assayed neurons expressed GFP (n = 25 images) (Figure 3E, F). These data show that NB5-2 derived neurons express the lineage marker NB5-2-Gal4 UAS-GFP at comparable ratios *in vivo* and *in vitro*. We conclude that identified neurons develop at similar proportions *in vivo* and *in vitro*.

Axon and dendrite compartments are established during *in vitro* culture

Mammalian neurons re-establish axon and dendrites after mature neuron dissociation and culture³⁶, but whether they can form proper axon and dendrite domains *de novo* when cultures are initiated at the stem cell phase, and neurite outgrowth occurs for the first time *in vitro* is unknown. Here we determine whether *Drosophila* NBs differentiating *in vitro* can generate axon-dendrite polarity when they are formed *de novo* in culture. NB5-2-Gal4 transgenic embryos were dissociated prior to axon/dendrite formation and cultured *in vitro* for 24h. This timepoint is correlated to L1 larval stage, when NBs have all differentiated and the mature Elav+ neurons all have neurites (Figure 2). Cultures were stained for GFP (to identify NB5-2 derived neurons), an epitope on the dendrite marker DenMark³⁷, and the pre-synaptic marker Bruchpilot (Brp)³⁸ (enriched in axonal compartments) (Figure 4A-A"). We observed uniform distribution of GFP across neuronal membranes, as expected. We also found that DenMark only labeled neuronal cell bodies, proximal neurites, and small patches within more distal neurite areas (Figure 4A-A"), consistent with labeling restricted to soma and dendrites. To determine whether Brp puncta were correctly localizing primarily in axonal areas we calculated the relative area of myr-GFP alone (axonal) and DenMark-labeled regions of each cell

and determined that 24h cultures had axon and dendritic regions that occupy roughly equal cell areas (Figure 4D). We then analyzed the percentage of Brp puncta that localized within axonal versus dendritic compartments and found a significant number of presynapses correctly localize within axonal compartments (Figure 4A'-A'', E).

By utilizing the L1 larval *Drosophila* annotated electron microscopy volume in CATMAID³⁹ (Figure 4B), in which NB5-2 derived interneurons have been fully annotated within multiple hemisegments, we can quantify the number of pre- and postsynapses *in vivo*. Importantly, the number of pre-synapses on NB5-2-derived neurons was indistinguishable between *in vivo* and *in vitro* (n=18 and 17 cells, respectively) (Figure 4G). To assess whether annotated presynaptic puncta might be located in dendritic regions, each presynapse was analyzed to determine if it was within 3 synapse lengths of a postsynapse (an example of a single NB5-2 lineage neuron, Saaghi 5, shown in Figure 4C-C''). The vast majority of presynapses in annotated NB5-2 neurons *in vivo* localize within axonal compartments (Figure 4F). Taken together, we conclude that *Drosophila* neurons forming neurites *in vitro* have the intrinsic ability to form polarized compartments and accurately sort axonal and dendritic cargo in the absence of cell-extrinsic cues.

Identified motor neurons maintain their molecular and morphological identity during *in vitro* culture

After determining that embryonic *Drosophila* neurons can be specifically labeled *in vitro* and have the intrinsic ability to polarize and sort cargo to their appropriate compartments in the absence of extracellular cues, we wanted to determine whether neurons developed *in vitro* maintain markers of their molecular identity. For this, we utilized the best characterized motor neurons in the *Drosophila* embryo, the U motor neurons^{7,40}. There are the five U motor neurons in each VNC hemisegment, which are born sequentially from NB7-1. Each U neuron expresses the transcription factor Even Skipped (Eve), and individual U neurons can be distinguished by a set of molecular markers: U1 (Hb+), U2 (Hb+ Zfh2+), U3 (Zfh2+), U4 (Zfh2+ Runt+), and U5 (Cas+ Runt+) (Figure

5A)⁷. Thus, *in vivo* there are equal numbers of all five U1-U5 motor neurons (20% each).

To determine if U motor neurons can generate their molecular identity having been born and differentiated from NBs *in vitro*, embryonic cultures from flies expressing GFP under the control of the CQ-Gal4⁴⁰ (specifically expressed in the five U motor neurons) were prepared and fixed after 24 hours *in vitro* (when all neurons have produced neurites; Figures 2, 3). We performed two experiments; one to assay the fate of early-born U1/U2 neurons (Figure 5B-C,G), and one to assay the fate of late-born U3-U5 neurons (Figure 5D-F, H). When early-born markers were used (Hb, Zfh2), we found that among Eve⁺ marked neurons, an average of 24.1% were Hb⁺ identified as putative U1, 17.6% were Hb⁺ Zfh2⁺ identified as putative U2, and 58.1% were Zfh2⁺ accounting for the remaining U3-5 (Figure 5B-C) showing no significant difference to wild-type *in vivo* molecular markers (Figure 5G). Similarly, the late-born marker (Runt, Cas) experiment revealed that among Eve⁺ marked cells, 20.3% were Runt⁺ and Cas⁺ identified as putative U5, 17.7% were Runt⁺ and identified as U4. The remaining 62% were Eve⁺ only, accounting for the remaining U1-U3 neurons (Figure 5D-F) showing no significant difference to wild-type *in vivo* molecular markers (Figure 5H).

Interestingly, U1 and U2 are bipolar neurons *in vivo*, with two primary neurites extending one neurite contralaterally and the other ipsilaterally to dorsal muscles, while U3-5 are unipolar neurons, with a single primary neurite bifurcating and extending only ipsilateral projections⁷. *In vitro*, we find that putative U1 and U2 cells usually presented with bipolar morphology (Figure 5B-C, J; arrowheads), while most U3-5 cells extended only one primary neurite (Figure 5D-F, K; arrowhead). Together, our results show that embryonically isolated *Drosophila* NBs can generate and maintain the identity of motor neurons, and the proper formation of uni- and bi-polar axon morphology.

Discussion

In this study, we adapted *Drosophila* hanging drop neural cultures^{21,41,42} to determine the extent to which NBs generate neurons *in vitro* that retain aspects of their *in vivo* developmental programs, identity,

and morphology. We confirmed^{19,43} and further characterized the finding that embryonically derived NBs differentiate and develop neurites *in vitro* on a timeline similar to *in vivo*. Previous work assessing cell-intrinsic neuronal programming focused on NB asymmetric division *in vitro*²⁰, and on showing that NBs maintain the intrinsic ability to progress their *in vivo* transcription factor cascade¹⁹. Until this point, embryonic fly neural tissue culture has not assessed the extent to which neuronal identity and morphology are maintained if NBs differentiate and develop *in vitro*, outside their native environment.

While rodent-derived primary embryonic cultures are more structurally similar to human cells, lack of genetic access means that specific neuron subtypes can't be labeled *in vitro*, closing off an entire toolkit of methods used to study intracellular mechanisms and sub-cellular organization. Indeed, this method in conjunction with fly connectomes creates the capability to probe cell-intrinsic drivers of synapse specificity between known synaptic partners *in vitro*. Mammalian culture systems rely on electroporation, transfection, and viral transduction to manipulate gene expression⁴⁴, and often cannot control which neural subtypes within their culture are affected. Use of binary gene expression systems to label neurons cultured *in vitro* results in the seamless manipulation of specific sub-populations of neurons *in vitro*.

We sought to determine whether specific neuron lineages could be accurately represented within a culture. Mammalian tissue culture systems lack routine genetic access or the body of knowledge to label specific lineages in culture. Previous applications of *Drosophila* neuron cultures used transgenes that labeled all neurons (Elav-Gal4 or nSyb-Gal4)^{23,24,27,41,45–49}. One study specifically labeled Mushroom Body gamma neurons but isolated them from pupae 5 hours after head eversion^{50,51}, when terminal differentiation is already well underway⁵². While it was reported these pupal-derived neurons maintained some aspects of their *in vivo* morphology, this could be due to their *in vivo* development prior to dissociation.

We obtained evidence that ratios of specific populations of neurons can be accurately represented in relation to the entire neuron population *in vitro*. We used the NB5-2 lineage to test this *in vitro*, and quantified

comparable ratios of NB5-2-derived neurons within a total neuronal population at time matched intervals. This ratio may be heavily impacted by cultured cell density²⁰. Failure to appropriately generate neurons within a lineage would result in lower levels of specific neurons represented in cultures compared to *in vivo*. Optimization to determine cultured cell densities to best represent individual populations of neurons in culture may prove useful. This approach may reveal that specific neuron subtypes are more viable in culture.

It stands to reason that cell density factors heavily into NB-to-neuron development²⁰; too few surrounding cells in proximity to a cultured NB will likely result in failure to generate neurons. While we found that 10 embryos per 50 μ L droplet results in the successful differentiation of NBs, it might prove useful to optimize for lower cell concentrations, if keeping neurons out of contact is required for an experiment, which are easier to assess than densely packed ones.

Additionally, while there is ample evidence that mammalian neurons have the intrinsic capability to form polarized compartments with accurately sorted cargos *in vitro*^{12,36}, it's important to remember that these foundational studies were performed on terminally differentiated cells allowed to regrow neurites after initially developing *in vivo*. The data showed here are contact-naïve neurons that developed entirely from precursors *in vitro*. Mechanisms of polarity establishment have been characterized in some populations of *Drosophila* neurons *in vivo*^{22,53}, and polarity establishment of pupal mushroom body neurons *in vitro*⁵⁰. To our knowledge, work presented here is the first reported instance of embryonic *Drosophila* neurons establishing neuronal polarity in the absence of their native extracellular environment. Indeed, in addition to forming dendritic and axonal compartments *in vitro*, we showed that NB5-2 lineage neurons can accurately generate and sort presynaptic cargo *in vitro* at rates comparable to *in vivo*. This result suggests that *Drosophila* embryonic neuron cultures can be used to study mechanisms of polarity establishment, cargo sorting, and sub-cellular axon- and dendrite-specific structures in specific neuron types and lineages.

Finally, we show evidence that U1-U5, a population of larval motor neurons derived from the NB7-1 lineage retain both their molecular identity

and gross morphological characteristics even when they are born outside of their native environment. Previous work showed that transcription factor cascades within NBs are not reliant on cell-extrinsic signals and can advance *in vitro*¹⁹. Here, this population was chosen for this analysis because of its extensive characterization^{7,11,40,54,55} and stereotyped marker gene expression⁷, giving us the opportunity to compare *in vitro* data to *in vivo* datasets. Excitingly, across experiments that probed sets of early- and late-born marker genes, we observed population-level fidelity of marker gene expression compared to ratios seen *in vivo*. This validates previous findings that NB lineage formation progresses exclusively through cell-intrinsic mechanisms^{19,56}. Furthermore, while recent studies have begun to elucidate the connection between molecular identity and morphology⁸⁻¹⁰, the *in vitro* maintenance of bipolar or unipolar morphology among U1-2 or U3-5 respectively, adds evidence that molecular identity plays a significant role in the establishment of neuronal morphology even in the absence of extracellular neurite guidance cues.

Vertebrate primary culture systems have relied on using differentiated neural tissue from embryonic or postnatal animals^{57,58}. These primary neuron cultures are composed of cells who have already undergone differentiation, extended and polarized neurites, and begun forming synaptic connections. Dissociation cleaves existing neurites, but evidence exists of cellular memory mechanisms that may influence development of previously-developed neurons⁵⁹. Use of embryonic *Drosophila* tissue as starting material results in contact-naïve neurons developing *in vitro* for the first time, allowing us to assess development in the complete absence of cell-extrinsic cues. Shifting to this paradigm to re-examine morphogenesis *in vitro* may provide insights easily overlooked when using previously developed tissue for initial cell cultures⁵⁰.

Our new data combined with previously published work provides evidence that differentiation and development of naïve neurons *in vitro* results in neurons with similar molecular identities and morphologies, but not a perfect replica of *in vivo* morphology. We suspect this may be due to multiple factors: Primarily, we acknowledge that neural development is both a product of a cell's molecular identity and a cell's interaction with its local environment^{10,60,61}. Directional gradients play a large role in the driving of

neurite migration through attractive and repulsive cues^{4,62}. Indeed, the ability to distinguish cell-intrinsic from cell-extrinsic developmental cues relies on their ‘scrambled’ nature in this system. This scrambling of the extracellular environment itself can present an opportunity for inaccurate neuronal development *in vitro*. Ultimately, we envision this technique to be utilized primarily not as a standalone method, but in concert with *in vivo* studies, behavioral assays, and large datasets from RNA sequencing, electron microscopy, or proteomics studies.

Increasingly, scientists recognize that a critical aspect of understanding how a brain is built is to decode how cell-intrinsic cues driven by genetic expression mechanisms give rise to neuronal identity and guide neuronal diversity. We propose that these data support a fly neuronal culture system is an acceptable paradigm in which to study cell-intrinsic mechanisms of neuronal identity establishment and morphogenesis and may prove useful in the dissection of cell-intrinsic versus cell-extrinsic cues guiding neuronal development.

Methods

Preparation of materials for hanging drop culture setup

We used cell culture chambers designed and made with Computer-Aided Manufacturing using Autodesk Fusion 360. We used polyether ketone (PEEK) plastic for its chemical compatibility and rigid capability to hold tolerances and heat. The parts were designed, arrayed, and machined by Oregon Fabrication and Design (OFAD), our resident fabrication and machine shop, on a CNC mill with a vacuum table (Datron M8 Cube). Tissue culture chambers made in this manner are autoclavable and reusable. After use, chambers are soaked in 100% Acetone for 1 hour to aid in removal of vacuum grease and media residue from the chamber. Chambers were then wiped down with low-residue napkins, stored in an autoclave-safe container, and autoclaved before each use. Sterilized chambers are kept in containers until use.

Incomplete Schneider’s Insect Media (SIM++) is defined as Schneider’s Insect Media with 20% heat-inactivated Fetal Bovine Serum and 10 ug/mL Insulin added. SIM++ was filter-sterilized with a 0.22 micron

420 filter and stored in 2 mL frozen aliquots, which can be stored at -20°C for
421 up to 1 year. Before use, SIM++ is defrosted, supplemented with 10 U
422 Penicillin/Streptomycin, and re-filtered. The resulting SIM+++ can be used
423 for up to 2 weeks. To prepare 200 mL Incomplete Dispersion Buffer (I-DB),
424 100 mg phenylthiourea and 3 mL Pen/Strep were added to 197 mL Hank's
425 Balanced Salt Solution (HBSS, Gibco), sterilized with a 0.22 µm filter, and
426 frozen in 10 mL aliquots. Frozen I-DB can be stored at -20°C for up to 1
427 year. Before use, aliquots are defrosted and 0.035g Dispase II and 0.007g
428 Collagenase type V are added. Dissociation buffer was incubated in a 37°C
429 water bath for 30 minutes before it was filter-sterilized with a 0.22 µm filter,
430 resulting in Complete Dissociation Buffer (C-DB). C-DB can be used for 2
431 weeks and stored at 4°C.

432 Concanavalin A (ConA)-coated coverslips were prepared by first
433 laying out 22x22 mm 1.5 coverslips on a clean, stable surface. 200 µL of
434 30 µg/mL ConA suspended in sterile deionized water was placed into the
435 center of each coverslip. The working area was covered to prevent dust
436 accumulation, and the coverslips were incubated at room temperature for 2
437 hours. ConA solution was removed from coverslips, and the area was
438 again covered to allow the coverslips to dry at room temperature overnight.
439 The side of the coverslip coated in ConA was marked by writing "up" in the
440 corner of the coverslip, and the coverslips were stored ConA-side up in a
441 10 cm plate. Before use, coverslips were sterilized in a laminar flow hood
442 using UV light for 30 minutes. Coverslips can be stored at room
443 temperature for up to 6 months.

444 **Embryo collection, staging, and preparation**

446 First, crosses resulting in genotypes of interest were set up. To
447 collect embryos, we either (a) allowed the flies to lay eggs for 1 hour and
448 aged the resultant embryos for 5 hours at 25°C, or (b) collected embryos
449 overnight, and picked stage 11 embryos out of the resulting collection.
450 Next, embryos were prepared for dissociation by removing their chorion by
451 soaking the embryos in 100% bleach for 4 minutes, followed by thorough
452 flushing with deionized water until no bleach smell remained.

453 To ensure that all embryos used for culture setup are stage 11,
454 embryos were visualized under 488 nm light on a stereoscope or dissecting

microscope. The yolk is autofluorescent, forming the distinctive shape of a flip-flop shoe ([Figure 1](#)). Using a paintbrush or fine-tipped straight forceps, 10 embryos per genotype per culture were collected into 200 μ L of SIM+++. Our standard is to set up 3 biological replicates per culture (e.g. 30 embryos per genotype in 200 μ L SIM+++). After dechoriation, embryos are fragile and may burst easily, so must be handled with care. We ensured all embryos sank to the bottom of the media before proceeding with the dissociation protocol by centrifuging samples for 30 seconds at 1000 rpm.

Embryo dissociation and establishment of hanging drop neuron cultures

SIM+++ was removed and replaced with 200 μ L (or equal volume to collection SIM++) of 70% ethanol to sterilize embryos. Embryos are fully submerged in ethanol by centrifugation for 30 seconds at 1000 rpm. For optimal culture health, were not submerged in ethanol for longer than 5 minutes. All steps after this were performed in a laminar flow hood to maintain sterility.

Ethanol was replaced with 200 μ L (or equal volume to ethanol) of fresh SIM+++ to wash off remaining ethanol. The tube was gently tapped to ensure all embryos were coated in SIM+++, and embryos were collected to the bottom of the tube again by centrifuging the sample for 30 seconds at 1000 rpm. SIM+++ was removed from the embryos and replaced with 200 μ L (or equal volume to SIM++) of Complete Dissociation Media (1x Hank's Balanced Salt Solution, 500 μ g/mL phenylthiourea, 15 Units Penicillin/Streptomycin), sterile-filtered (0.22 μ m) and stored in frozen 10 mL aliquots; defrosted, 0.035g Dispase II and 0.007g Collagenase type V added, incubate at 37°C for 30 minutes, and then re-filter-sterilized before use).

Using a sterile micro-pestle, embryos were ground in Dispersion Media until no visible pieces of tissue remained, and the sample was homogenously cloudy. The homogenized embryos were incubated at 37°C in a water bath, allowing chemical dissociation to proceed for 5 minutes. The sample was returned to the hood and 200 μ L SIM+++ was added to halt chemical digestion. The dissociated cells were mixed by pipetting up

and down several times with the pipette used to add the SIM+++. Dissociated cells were collected by centrifugation for 5 minutes at 1000 rpm, with a small visible pellet forming at the bottom of the tube.

The centrifuged tube was returned to the hood, and the supernatant was removed without disturbing the pellet. Per culture to be established, 50 μ L SIM+++ was added to the pellet (e.g. if using 30 embryos for 3 cultures, 150 μ L SIM+++ added to the pellet). The pellet was resuspended by pipetting with a p200 pipette set to 100 μ L until the media was homogenously cloudy. Culture Chambers were prepared by applying a thin line of vacuum grease to the square surface's perimeter surrounding the circular chamber well, where the coverslip will fit in snugly. We used a 3 mL syringe fitted with an 18 $\frac{1}{2}$ gauge needle shortened with wire cutters for this step. The vacuum grease adheres the coverslip to the chamber. This preparation can be done in the hood during the chemical digestion and centrifugation steps and set aside until needed.

The cell suspension was distributed in 50 μ L droplets to the center of a culture chamber (Figure 1). The droplet should not spread significantly after deposition to ensure contact with the coverslip. Using curved forceps, a UV-sterilized, ConA-coated coverslip was placed with the labeled ConA-side facing down onto the droplet, ensuring the droplet contacted the coverslip. Once coverslips are aligned within the chamber, they were adhered by gently pressing down along the edges. The chambers were placed coverslip-down in a 25°C incubator to initiate cell adhesion. After 2 hours, cells are adhered to the coverslip. Culture chambers were inverted after 2 hours *in vitro* so that the coverslips were facing up, and the cultured cells are growing in hanging droplets (illustrated in Figure 1). Failure to do so will result in flattened cells with membranous extensions that do not resemble their *in vivo* appearance.

Fixation and sample storage:

After desired culture time (we have successfully cultured viable neurons for as little as 30 minutes and up to time-matched adult life; two weeks *in vitro*), chambers were removed from the incubator. Coverslips were removed from their chambers and placed culture-side up in a labeled dish that accommodates a 22x22 mm coverslip. The center of the

coverslips were flooded with fixation buffer (4% paraformaldehyde in 1x PBS) out to the vacuum grease perimeter. Samples were incubated in fix for 20 minutes. Fixation buffer was removed and immediately replaced with 1x PBS to wash.

While submerged in PBS, the side of curved forceps was used to gently scrape the edges of the coverslip and remove as much vacuum grease as possible, being careful to remove any large clumps. After vacuum grease removal, PBS was removed replaced with the same volume of fresh PBS. Samples were twice washed with gentle shaking at room temperature for 5 minutes. The second was replaced with fresh PBS with 100 μ L 70% ethanol added per 1 mL PBS to discourage post-fixation contamination. Coverslips were stored at least 24 hours before staining to allow fixation-induced autofluorescence to fade. Stored coverslips were used within 2 months after fixation.

Immunofluorescence staining and imaging

Cells were permeabilized with 0.5% TritonX diluted in PBS for 10 minutes at room temperature. After permeabilization, samples were incubated with blocking buffer (PBS, 0.1% TritonX, 1% Bovine Serum Albumin) at room temperature with gentle shaking for 30-60 minutes to minimize non-specific antibody binding. Block Buffer was replaced with primary antibodies diluted in 1x PBS. Samples were incubated with gentle shaking overnight at 4°C. Primary antibody was removed and replaced with 1x PBS, and samples were 'rinsed' by gently agitating the sample at the bench for 20 seconds two times. After the 'rinse' steps, samples were washed twice with PBS with gentle shaking at room temperature for 5 minutes. After the second wash, samples were treated with secondary antibodies diluted in 1x PBS. Samples were covered to protect from light and incubated with gentle shaking at room temperature for 2 hours. After secondary antibody incubation, the rinse and wash steps performed between primary and secondary antibody incubations were repeated. After the final wash, samples were again flooded with PBS before gently blotting excess PBS from samples and mounting in FluorSave mounting media. Samples were allowed` to dry for at least 3 hours while protected from light to ensure the coverslip is strongly adhered to the slide before imaging.

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Figures

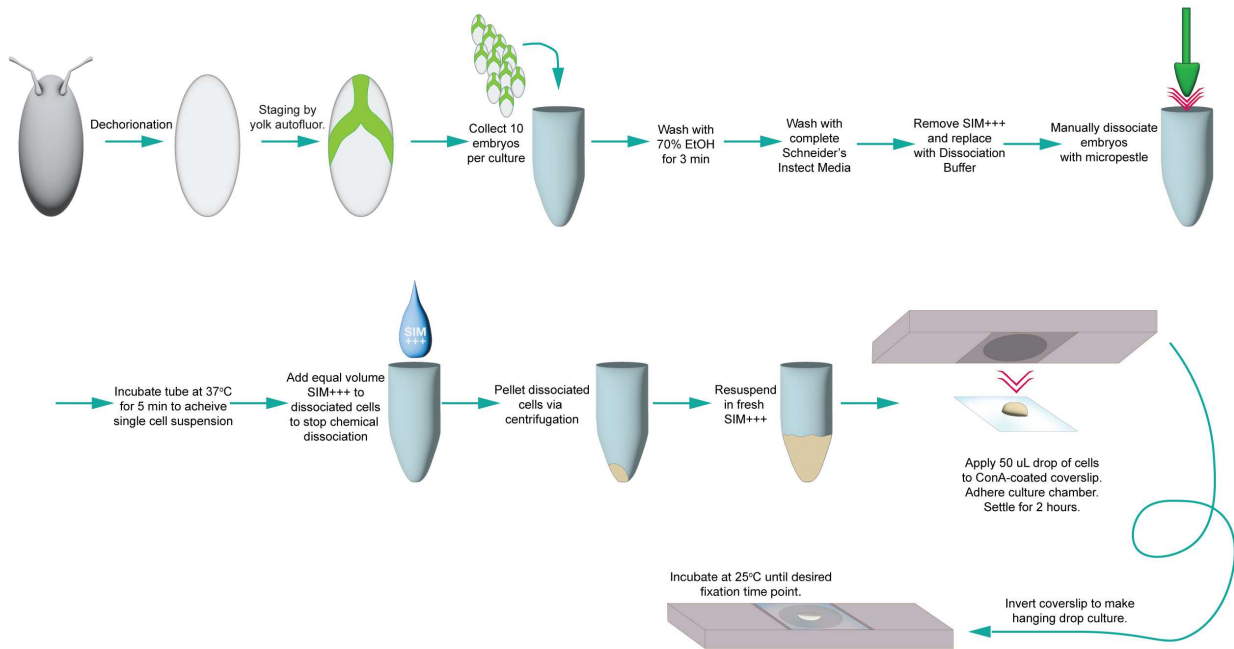


Figure 1. A schematic for establishing *Drosophila* embryonic *in vitro*

cultures. Briefly, stage 11 embryos are collected and dechorionated. Stage 11 embryos are identified via the flip-flop pattern formed by yolk autofluorescence in 488 nm light. 10 embryos are collected per culture and are washed briefly with 70% ethanol to sterilize. The sterilized embryos are moved to a laminar flow hood, then washed with complete Schneider's Insect Media (SIM+++), before embryos are manually dissociated via micro pestle grinding in Dissociation Buffer. The tube is incubated for 5 minutes at 37°C to allow enzymatic digestion of intercellular contacts, and dissociation is halted by addition of an equal volume of SIM++. Cells are then pelleted and resuspended in 50 µl of SIM+++ per culture. 50 µl droplets are added to the well of tissue culture chambers, and a ConA-coated coverslip is adhered to the chamber with vacuum grease. Cells are allowed to settle and adhere to the ConA-coated surface for 2 hours before the chamber is inverted, resulting in hanging droplet cultures. See methods for details.

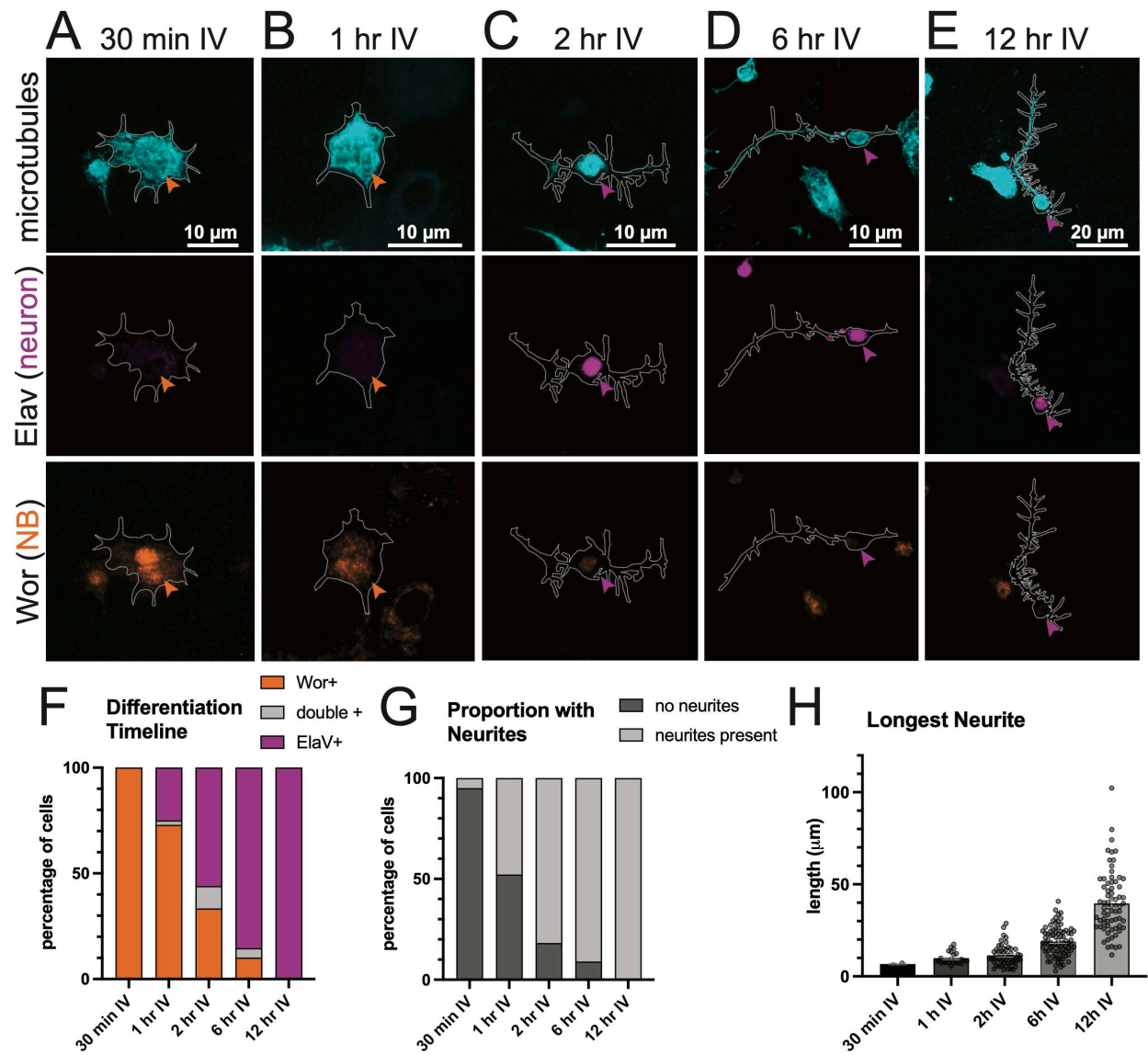


Figure 2. Neuronal differentiation occurs normally from neuroblast to neuron during *in vitro* culture.

(A-F) Dissociated embryos for the indicated time *in vitro* (IV) progress from all expressing the NB marker Worniu to all expressing the neuronal marker Elav. The microtubule marker shows all cells in culture. Arrowhead, cell body.

(G) Cultured cells gradually form neurites.

(H) Cultured cells gradually form longer neurites.

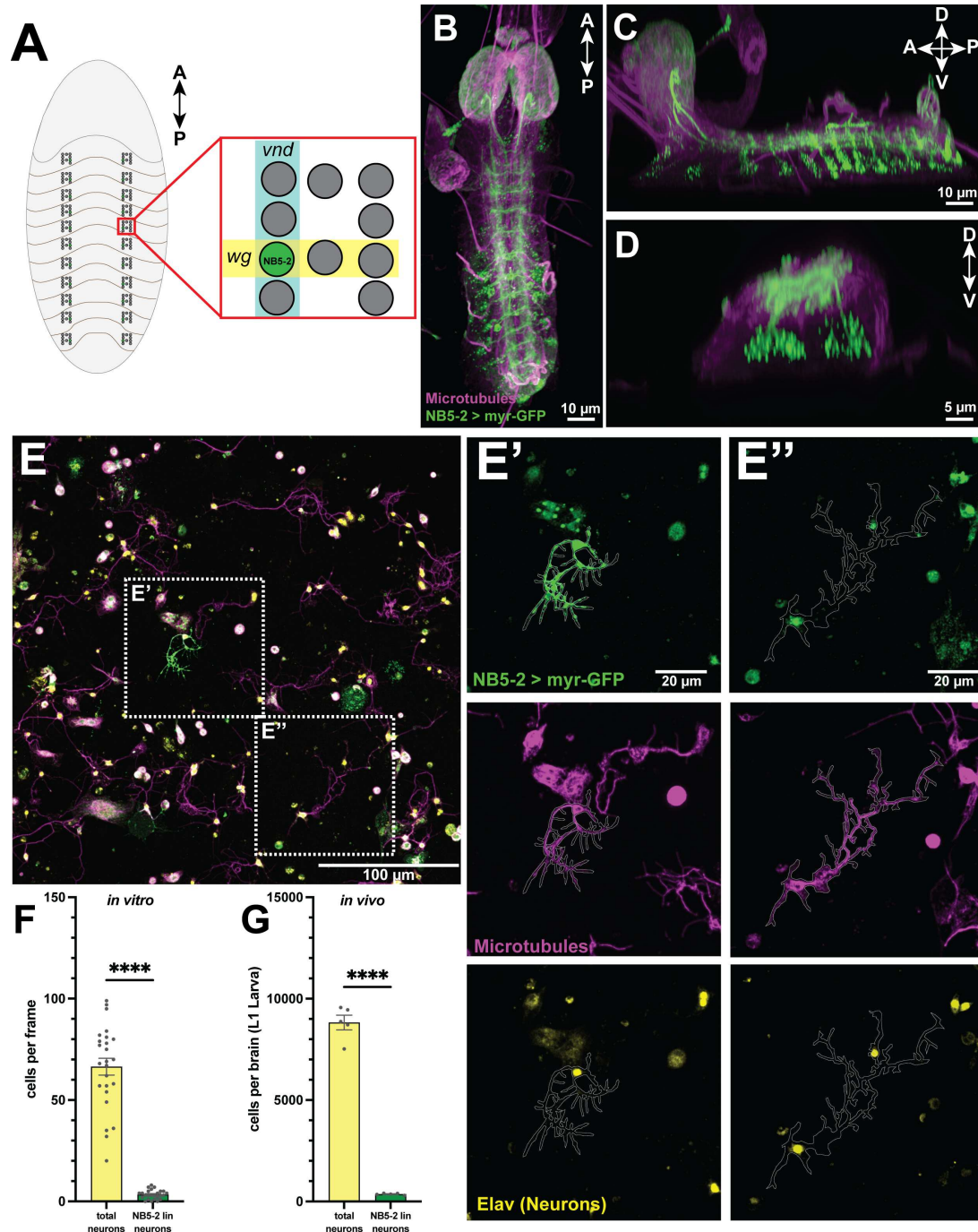
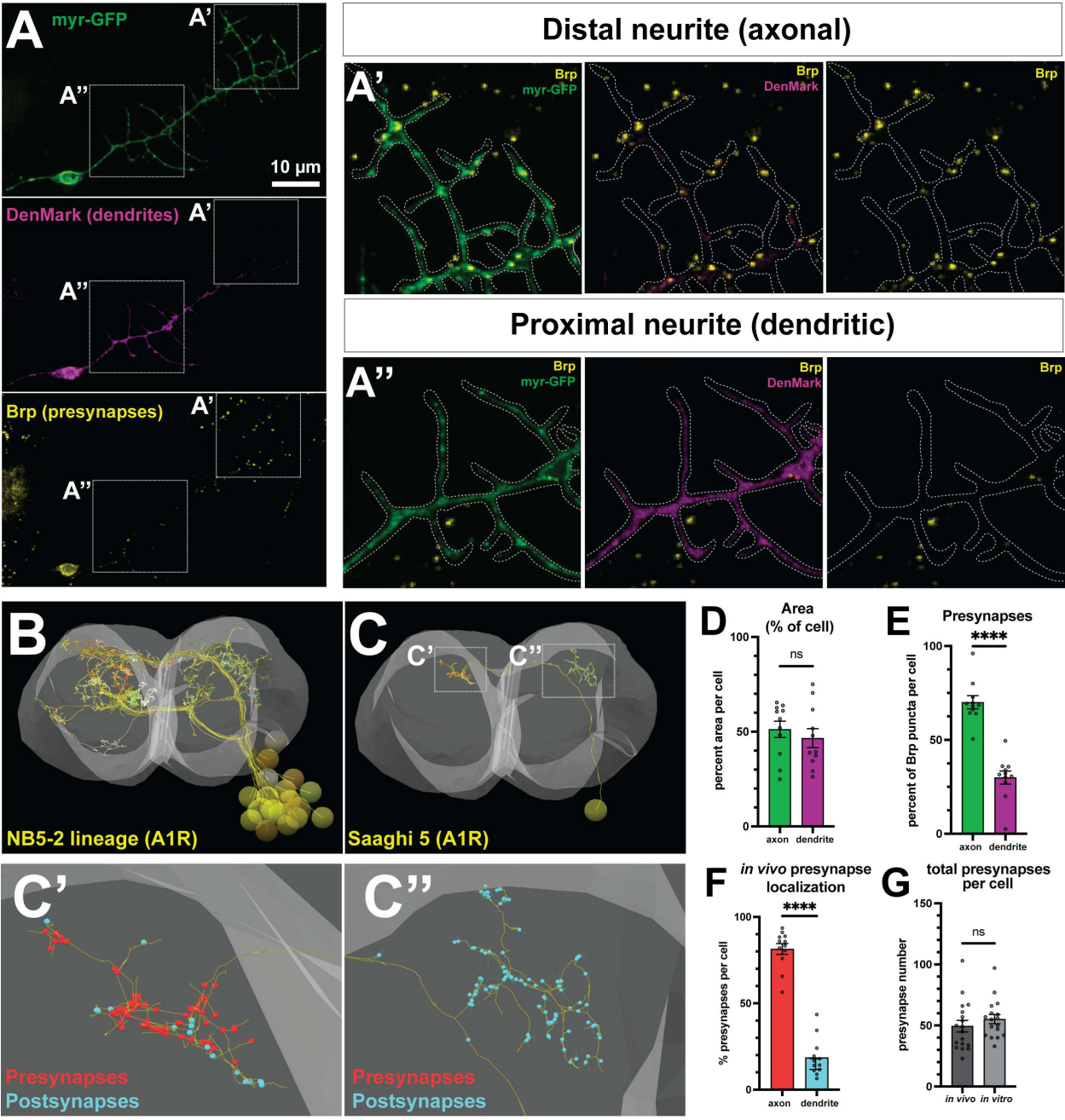


Figure 3. Transgenic reporter lines are expressed normally during *in vitro* culture.

(A) Cartoon showing the formation of the NB5-2-Gal4 specific transgene used to express GFP in all cells of the NB5-2 lineage.

(B-D) NB5-2 derived neurons expressing GFP in dorsal view, anterior up (B); lateral view, anterior up (C); and posterior view, dorsal up (D). (E-E'') *In vitro* cultures from NB5-2-Gal4 UAS-GFP embryos. (E) 20x magnification field of view; boxes show zoomed in portions inset shown in E' and E'' (F-G) Quantifications showing that NB5-2-Gal4 UAS-GFP labels an equal percentage of neurons *in vitro* as *in vivo*; *in vivo* numbers derived from GFP+ neurons among Elav+ nuclei in L1 larval brains (n=5).



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Figure 4. Axon and dendrite compartments are established during *in vitro* culture.

(A-A'') A GFP+ NB5-2 lineage neuron differentiated *in vitro* shows polarized distribution of the dendritic marker DenMark³⁷ to the proximal presumptive dendrite domain (A') and the distal presumptive presynaptic marker Bruchpilot (Brp)³⁸ (A'').

(B-C'') NB5-2 neuronal lineage annotated in a TEM volume^{18,39} with pre- and post-synaptic domains shown (C-C'').

(D-G) *In vivo* quantifications performed using annotated NB5-2 neurons within the L1 larval TEM volume. Quantifications show that while NB5-2 lineage neurons grown *in vitro* maintain relatively equal areas of dendritic and axonal regions (D), presynaptic Brp puncta are significantly more likely to localize within axonal compartments than dendritic (E). *In vivo*, localization of presynapses within axonal and dendritic regions were quantified by assessing the localization of annotated presynapses in relation to annotated postsynapses, being designated a dendritic presynapse if within 3 synapse lengths of a postsynaptic puncta on the same neurite cable (F). Between *in vivo* and *in vitro* samples, the number of counted presynaptic puncta per cell were not significantly different (G).

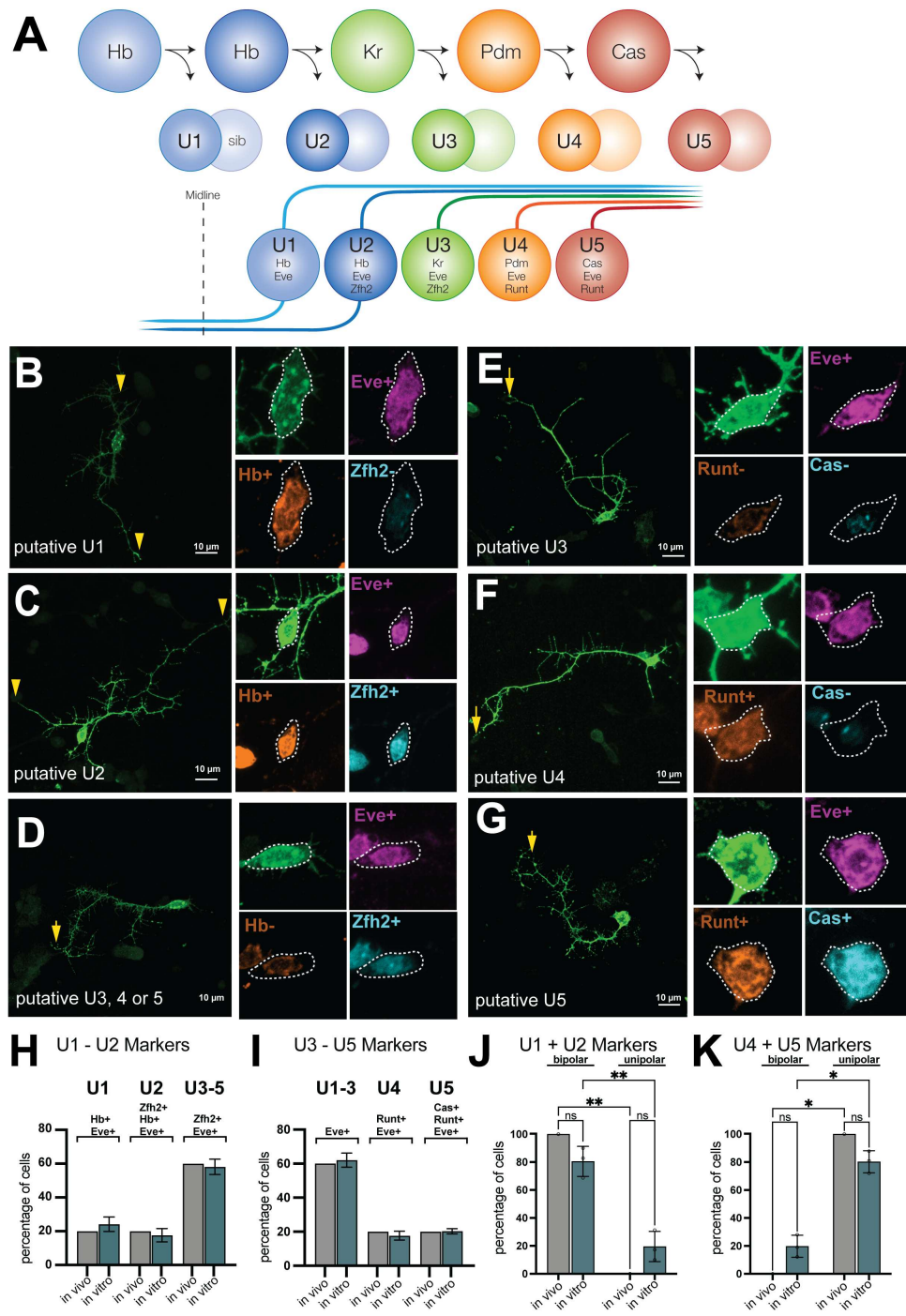


Figure 5. Identified motor neurons establish their molecular and morphological identity during *in vitro* culture.

(A) Schematic showing the molecular markers that distinguish the U1-U5 motor neurons; the early-born U1-U2 have bipolar neurites, whereas the

later-born U3-U5 have unipolar neurites. The Eve-negative VO neuron born between U3 and U4⁷ is not shown for clarity.

(B-C) Molecular markers expressed by the early-born U1 and U2. Note the bipolar neurites (arrowheads).

(D-G) Molecular markers expressed by the late-born U3-U5. Note the unipolar neurites (arrowhead).

(H-I) The percentage of Eve⁺ CQ-Gal4-labeled neurons expressing marker genes to distinguish U1 or U2 (Hb, Zfh2) or U4 or U5 (Runt, Cas) were calculated across 3 biological replicates, and the average (teal) was plotted against the reported percentages of U motor neurons expressing those markers *in vivo*⁷ (gray).

(J-K) The percentage of *in vitro* U motor neurons expressing U1 or U2 (J) and U4 or U5 (K) were analyzed to determine the percentage with bipolar versus unipolar morphology across 3 biological replicates (teal), which was plotted against the reported⁷ percentages of bipolar versus unipolar neurons of the same type *in vivo* (gray).

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