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Contrasting developmental axon regrowth and neurite sprouting of *Drosophila* mushroom body neurons reveals shared and unique molecular mechanisms

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Abstract

The molecular mechanisms regulating intrinsic axon growth potential during development or following injury remain largely unknown despite their vast importance. Here we have established a neurite sprouting assay of primary cultured mushroom body (MB) neurons. We used the MARCM technique to both mark and manipulate MB neurons, enabling us to quantify the sprouting abilities of single WT and mutant neurons originating from flies at different developmental stages. Sprouting of dissociated MB neurons was dependent on *wnd*, the DLK ortholog, a conserved gene that is required for axon regeneration. Next, and as expected, we found that the sprouting ability of adult MB neurons was significantly decreased. In contrast, and to our surprise, we found that pupal-derived neurons exhibit increased sprouting compared to neurons derived from larvae, suggesting the existence of an elevated growth potential state. We then contrasted the molecular requirements of neurite sprouting to developmental axon regrowth of MB neurons, a process that we have previously shown requires the nuclear receptor UNF acting via the TOR pathway. Strikingly, we found that while TOR was required for neurite sprouting, UNF was not. In contrast, we found that PTEN inhibits sprouting in adult neurons, suggesting that TOR is regulated by the PI3K/PTEN pathway during sprouting and by UNF during developmental regrowth. Interestingly, the PI3K pathway as well as Wnd were not required for developmental regrowth nor for initial axon outgrowth suggesting that axon growth during circuit formation, remodelling and regeneration share some molecular components but differ in others.

Keywords

Axon Regeneration; Neurite Sprouting; Developmental axon regrowth; TOR; Primary Culture

Introduction

A fundamental and largely unanswered question in neuroscience is what regulates the intrinsic growth potential of axons and dendrites during development and following injury. It is well established that adult central nerve system (CNS) neurons, in contrast to young neurons or neurons from the peripheral nerve system (PNS), fail to regenerate following injury (Liu et al., 2011) and this failure is critical in pathological conditions such as spinal

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cord injury. Axon regeneration in the CNS is thought to be limited by extrinsic inhibitory signals (David and Aguayo, 1981) as well as by a low intrinsic growth potential. Indeed, the axon growth potential of purified mammalian retinal ganglion cells (RGCs) decreases with age (Goldberg et al., 2002), and remarkably, young neurons extend axons even when plated on inhibitory growth conditions (Buchser et al., 2012), suggesting that successful axon regeneration depends on a crosstalk between the extrinsic environment and the intrinsic growth potential. In the classical preconditioning experiment that proved the existence of a genetic program governing the intrinsic growth ability, Neumann and Woolf (1999) used dorsal root ganglia (DRG) axons because they possess two axons, one projecting to the PNS and one that projects to the CNS. While the peripheral axons readily undergo regeneration following injury, the CNS axon does not. Remarkably, however, when they first lesioned the PNS axon ('preconditioning lesion'), the CNS axon was able to undergo regeneration. Since the preconditioning lesion must occur prior to the CNS lesion, this suggests that the existence of a genetic program that activates and elevates the intrinsic growth state of the CNS axon (Neumann and Woolf, 1999). Moreover, recent studies have highlighted specific genes that are down- or up-regulated during development and affect growth (Moore et al., 2009).

Over the years, many studies were conducted in an attempt to uncover the genetic program underlying axon intrinsic growth potential (Blackmore, 2012). One common dogma in this field is that axon regeneration likely utilizes the same machinery that is used during circuit formation since in both cases axons are being extended. Therefore, many studies focused on attempting to 'rejuvenate' adult CNS axons in order to induce regeneration following injury (Filbin, 2006). However, recent studies using different model systems have shown that axon growth during circuit formation and during regeneration are not identical (Blizzard et al., 2007).

The *Drosophila melanogaster* mushroom body (MB) is a unique system to study various stages of axon growth due to the fact that it undergoes stereotypic neuronal remodelling during metamorphosis. The MB is comprised of three types sequentially-born neurons, out of which only the γ -neurons undergo remodelling, in which they prune their axons up to a specific branch-point and later regrow to an adult-specific, medial lobe (Lee and Luo, 2001). Therefore, the MB enables a unique comparison of initial axon outgrowth and developmental axon regrowth.

We have recently shown that *unf* (*unfulfilled*; also known as Hr51 - hormone receptor at 51, or Nr2e3) is cell autonomously required for developmental axon regrowth of MB γ neurons following pruning but not for initial axon outgrowth of any MB neuron type (Yaniv et al., 2012). We have also shown that UNF regulates developmental regrowth, at least in part, via the target of rapamycin (TOR) pathway. TOR is a serine-threonine kinase that regulates, among other processes, cell growth, cell proliferation cell survival and protein translation (Hay and Sonenberg, 2004) and was specifically shown to promote axon regeneration following injury in mammals (Park et al., 2008). Therefore, we were surprised to find that, similarly to UNF, TOR is required for developmental regrowth but not for initial axon outgrowth suggesting that developmental axon regrowth is not only distinct from initial axon

outgrowth but also shares some molecular mechanisms with axon regeneration following injury.

To better understand the relationship between developmental axon regrowth and regeneration following injury, we have established a system that harnesses the genetic power of *Drosophila* to assess the sprouting potential of dissociated primary-cultured WT and mutant MB neurons. Using this culture system we explore here the sprouting characteristics of dissociated MB neurons as a function of age as well as begin to dissect the molecular mechanisms underlying sprouting. We found that while developmental axon regrowth and sprouting following cell dissociation share some molecular mechanisms, they differ in others, suggesting that they involve distinct genetic components.

Materials and Methods

Drosophila strains

All flies were reared at 25°C and grown on standard corn media. *InR*³¹ and *PTEN*¹¹⁷ were provided by Dr. Ernest Hafen (Brogiolo et al., 2001), *wnd*³ was provided by Dr. Catherin Collins (Collins et al., 2006) *akt1*^{q1} was generated by (Staveley et al., 1998) and provided by Dr. Stephen M. Cohen (Hietakangas and Cohen, 2007) *unr*^{ΔL04325} and *TOR*^{LL04239} MARCM flies were previously described (Yaniv et al., 2012).

Mosaic Analysis

MB Neuroblast clones were generated at 24h after egg laying (AEL) by crossing each mutant to its appropriate MARCM ready fly (Luo, 2007). For all chromosome arms, the MB specific-Gal4-201Y driver was used. Newly hatched larva (NHL) were heat shocked for 40-60 min at 37°C and grown at 25°C on standard corn media.

Sprouting assay

Flies were dissected on ice in a sterile Ringers solution and the brains were transferred into ice-cold cell dissociation solution (Sigma, c1544). Brains were incubated with 10mg/ml collagenase (Sigma, c0130), dissolved in cell dissociation solution at 29°C for 30-45 minutes (L3 and pupal brains-30 min, adult brains - 45 min). Next, the brains were centrifuged in 300Xg at 4°C for 6 minutes. The supernatant was discarded and the disintegrated brains were washed with cell dissociation solution. Finally, the cells were resuspended in the culture media which consisted of standard Schneider's medium (Biological industries) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and antibiotics ([1:100], 100XAntibiotic-Antimycotic, Gibco), mechanically dissociated by pipetting up and down until no aggregates were observed and then kept on ice. The cells were plated on a glass bottom 96-well plate (MatTek, catalog P966-1.5-5-F Case) coated with L-poly-lysine (0.01% solution, Sigma, P4832) and cultured in culturing medium in an incubator at 25°C. When indicated, rapamycin ([100nM], Sigma, R8781) was added. Images were taken using Zeiss LSM 710 confocal microscope, and processed with ImageJ (NIH). Total neurites length or longest neurite per cell were measured using the ImageJ plugin - simple neurite tracer (Longair et al., 2011).

Statistical analysis

For longest neurite per cell quantification, statistical analysis was performed by a two-way ANOVA (Figure 4) or by a standard t-test (Figures 3, 5-6). For total neurites length per cell quantification, statistical analysis was performed by a standard t-test.

Immunostaining and imaging

Flies were dissected on ice in Ringers solution, fixed with 4% PFA for 20 minutes. All washes and staining incubations were performed in PBT (PB+0.3% Triton). Brains were then stained with rat- α -mCD8 (Invitrogen MCD0800 1:200) and mouse- α -FasII (Developmental studies hybridoma bank (DSHB) 1D4 1:50) as primary antibodies. Alexa488- α -Rat (Invitrogen, 1:300) and Alexa647- α -mouse (Invitrogen, 1:300) were used as secondary antibodies. All stained brains were imaged by confocal microscopy using Zeiss LSM 710. Images were processed with the ImageJ (NIH).

Results

Neurite sprouting of dissociated cultured MARCM clones enables single cell analysis of WT and mutant neurons

In order to elucidate the intrinsic mechanisms regulating axon growth following injury of MB neurons, we decided to examine their neurite sprouting ability following dissociation. We therefore dissected *Drosophila* brains in which MB neurons express a GFP reporter at different developmental stages and plated the dissociated cells to visualise sprouting (Figure 1A). During this dissociation process the neurons lose their projections most likely due to mechanical injury, similar to severe axotomy (Figure 1B₂-D₂).

We used several methods in order to label the MB neurons, and to visualise sprouting in culture. First, we labeled MB neurons by expressing a membrane-bound GFP (mCD8:GFP) driven by the Gal4-201Y driver, which is expressed in post mitotic γ neurons and in a subset of the later-born α/β neurons (Yang et al., 1995) (Figure 1B₁). During the process of dissociation (day 0 in vitro - DIV 0) the cells lost their projections (Figure 1B₂). Following plating, the cells began to sprout neurites, which grew over time (Figure 1B₃₋₄). While these dissociated neurons grew well in culture, the density of the labeled neurites resulted in a dense mesh of neurons and neurites, which precluded quantitative analyses. To generate more sparse labeling, we generated MARCM neuroblast clones expressing mCD8:GFP driven by the Gal4-201Y driver (Figure 1C). Although this indeed resulted in much fewer GFP⁺ neurites, they were still too dense to perform quantitative analyses. Eventually, to achieve a single neurite resolution, we generated Gal4-201Y labeled MB MARCM neuroblast clones, dissected the brains and mixed them with unlabeled brains harboring the same genotype, resulting in very sparse labeling of an otherwise dense culture which allowed monitoring the growth of single axons (Figure 1D, supplementary Figure S1). Keeping the culture at the high density was important as we found that low-density cultures were not as viable and had delayed neurite sprouting and growth (data not shown). Using a primary culture of MB neurons that was generated from MARCM clones therefore allows single neuron resolution analyses and characterization of the sprouting ability of WT as well as mutant MB neurons.

Neurite sprouting in culture is a highly dynamic process

Using the method we developed, we then examined the dynamics of the sprouting process in culture. We live-imaged WT MB clones derived from L3-larvae for 12 hours, between days 0-2 following dissociation. Following plating, most of the cells start to sprout neurites that grow within few minutes time (Figure 2A, supplementary Figure S2). Over time, one to few neurites became more elongated compared to others. The growing neurites were highly dynamic: we observed processes such as neurite elongation, pathfinding and retraction in the time scale of hours and minutes (Figure 2B, supplementary Figure S3). Overall, live-imaging of neurite sprouting may shed light on dynamic processes required for growth, such as actin and microtubule assembly, axon determination and synapse formation.

Neurite sprouting following dissociation resembles axonal regeneration following injury

Next, we wanted to examine whether our assay of neurite sprouting of dissociated neurons resembled axon regeneration following injury. As a proof of concept, we decided to test whether perturbation of a protein that is known to be required for axon regeneration following injury affects sprouting in our system. *wallenda* (*wnd*), the ortholog of the Dual Leucine Zipper Kinase (*DLK*) is a gene that encodes for an evolutionary conserved mitogen activated protein Kinase Kinase Kinase (MAPKKK). While the worm *DLK* activates p38, in *Drosophila* and in mammals it predominantly activates JNK. Despite these differences, *Wnd* and *DLK* were shown to be important for axon regeneration in worms, flies and mammals (Xiong et al., 2010; Lu et al., 2014). We therefore tested the sprouting ability of *wnd^Δ* MB clones derived from L3-larvae. We dissociated the brains containing MARCM clones and diluted them with brains not containing clones as described above. To quantify the results, we took images of labeled cells, measured and compared the length of the neurites several days following dissociation. We measured both the longest neurite per cell as well as the total neurite length (see materials and methods). *wnd^Δ* neurons sprout neurites following dissociation, however these neurites were significantly shorter than WT neurites, as the longest *wnd^Δ* neurite was 3 times shorter throughout the experiment (Figure 3). The quantification of the longest neurite was comparable to the summation of the all neurites (Fig.3 C compared to D) and therefore due to simplicity we continued to use the longest neurite as readout of the sprouting potential of these neurons throughout the manuscript. These results confirm that *wnd^Δ* plays a role in neurite sprouting following cell dissociation, and suggest that the sprouting assay resembles axon regeneration following injury at least to some degree.

Pupal-derived MB neurons exhibit elevated neurite growth potential

In mammals, it was shown that axon growth potential decreases with age (Goldberg et al., 2002). Using an *ex-vivo* brain culture system Ayaz et al. have previously shown that *Drosophila* small lateral neurons ventral (sLNv) fail to undergo regeneration following laser axotomy while the same neurons in larvae are able to form a growth cone and sprout new neurites (Ayaz et al., 2008). We therefore wanted to further explore the intrinsic growth potential of WT MB neurons as a function of age in our sprouting system. We chose to compare the sprouting ability of neurons derived from four distinct time points in development: (1) L3-larvae, before metamorphosis; (2) neurons derived from pupae aged 6h

after puparium formation (APF), the onset of MB γ neuron pruning; (3) neurons derived from 24h APF pupae, the onset of MB γ neuron developmental axon regrowth; (4) neurons derived from young adult flies. Over 3 days of experiment, WT MB neurons derived from L3 larvae indeed sprouted neurites, which elongated over time (Figure 4A). The most significant increase in L3-derived MB neurites length occurred between day 1-2 following dissociation (Figure 4E). As expected, neurons isolated from adult flies exhibited reduced axon growth potential compared to L3-derived neurites (Figure 4D,E). However, to our surprise, neurons derived from the intermediate pupal-stages - 6h APF pupae (Figure 4B) or 24h APF pupae (Figure 4C) sprouted neurites that became significantly longer than those of L3-derived neurons (Figure 4E), suggesting that MB neurons exhibit elevated axon growth potential during metamorphosis. Taken together, our results suggest that while sprouting potential dramatically decreases in adult flies, sprouting potential surprisingly increases during metamorphosis.

TOR is required for efficient neurite sprouting

We have previously shown that UNF regulates developmental axon regrowth, at least in part via the TOR pathway (Yaniv et al., 2012). TOR is a serine-threonine kinase that plays a pivotal role in regeneration following injury in mammals - while deletion of the mTOR pathway inhibitor phosphatase and tension homolog (PTEN) in adult RGCs results in robust axon sprouting following optic nerve injury in mice, pharmacological inhibition of mTOR represses regeneration (Park et al., 2008). We therefore set out to uncover the role of TOR signaling pathway in sprouting following cell dissociation of MB neurons. We hypothesized that TOR would be required to promote sprouting following dissociation, as it does following injury, and therefore tested the sprouting ability of *TOR^{LL04239}* neurons derived from L3-larvae. Indeed, *TOR^{LL04239}* neurons sprouted neurites that were significantly shorter than WT throughout the culturing procedure (Figure 5, compare B to A, quantified in C). To further validate the role of TOR in sprouting, we treated WT MB neurons derived from L3 larvae with rapamycin, an inhibitor of TOR. Over 3 days of experiment, the rapamycin treated neurons sprouted significantly shorter neurites than control (Figure 5D). We concluded that the TOR pathway is required for efficient sprouting of dissociated cultured MB neurons, consistent with its' pro-regenerative effects.

Studies focusing on the mammalian ortholog of UNF, photoreceptor specific nuclear receptor (PNR), suggest that it represses the expression of tuberous sclerosis 1 (Tsc1), which in turn alleviates its repression on the TOR pathway (Park et al., 2008). Indeed, in epistatic experiments *in vivo*, we have shown that overexpressing activated TOR pathway components suppress the UNF regrowth defect (Yaniv et al., 2012). In order to delineate the role of UNF in sprouting following cell dissociation, we analyzed the sprouting ability of WT and *unf^{LL04325}* neurons derived from L3-larvae. To our surprise, we could not detect any significant difference in the length of WT and *unf^{LL04325}* neurites (Fig 5E, quantified in G), suggesting that while UNF is necessary for axon regrowth following pruning, it is not required for sprouting of larval neurons.

Since developmental regrowth of MB γ neurons occurs during metamorphosis, we suspected that comparing the sprouting ability of neurons derived from L3-larvae might not

reflect the *in-vivo* activity of UNF. Therefore, we compared the sprouting following dissociation of *unf^{LL04325}* and control neurons derived from 24h APF pupae. Similar to the results obtained from WT neurons (Figure 4C), *unf^{LL04325}* neurons derived from 24h APF pupae sprout relatively long neurites, which were comparable in length to WT 24h APF pupae-derived neurons (Figure 5F, quantified in H). Therefore, we conclude that despite its role in developmental regrowth of MB γ neurons, UNF does not seem to play a role in sprouting following cell dissociation.

Although activated via UNF to promote developmental regrowth, the canonical pathway regulating TOR is the Insulin receptor (*InR*) - Phosphoinositide 3-kinase (*PI3K*) - *Akt* (*InR*-*PI3K*-*Akt*) pathway, which is inhibited by PTEN (Hay and Sonenberg, 2004). We therefore decided to test whether perturbing the *InR*-*PI3K*-*Akt* pathway affects neurite sprouting potential. First, using the MARCM technique, we generated and isolated MB γ neurons that were homozygous mutant for *akt1^{q1}*. As expected, larval derived *akt1^{q1}* MB γ neurons exhibited reduced sprouting ability compared to control neurons (Figure 6 A-C). Next we wanted to determine whether deleting *PTEN* in MB neurons boosts the low sprouting ability of neurons derived from adult flies, similar to what was shown in mammalian RGCs (Park et al., 2008). Indeed, as early as one day following dissociation, *PTEN¹¹⁷* neurites were significantly longer than WT (Figure 6 D-F), although the increase was moderate. Taken together, this data suggest that during sprouting following injury, TOR is regulated via the canonical *InR*-*PI3K*-*Akt* pathway and not via UNF.

***wallenda* and *InR*/*PI3K*/*Akt* are not required for developmental regrowth of MB neurons**

Our previous study has suggested that developmental regrowth is distinct from initial axon outgrowth but shares similarities with axon regeneration. The fact that UNF is not required for neurite sprouting suggests that while sprouting following cell dissociation and developmental regrowth of MB neurons share some of the same key players, they differ in others. We therefore set out to examine whether other genes, which are important for regeneration are also required *in-vivo* for developmental MB regrowth. As mentioned, the MB γ -neurons undergo stereotypical neuronal remodelling during metamorphosis: during the early larval stages, MB γ neurons extend a bifurcated axon that projects to the medial and dorsal lobes (Figure 7A₁). At the onset of metamorphosis, in time-coordinated fashion, γ neurons prune their axons up to a specific branch-point as well as their dendrites (Figure 7A₂). Later during development, γ axons regrow to an adult-specific, medial lobe (Figure 7A₃₋₄) (Lee and Luo, 2001). Accordingly, we dissected adult brains containing WT, *akt1^{q1}*, *InR³¹*, *wnc⁸* and *PTEN¹¹⁷* MARCM neuroblast clones and analyzed them for adult γ lobe occupancy. As controls, we dissected brains containing *TOR^{LL04239}* and *unf^{LL04325}* MARCM MB neuroblast clones which indeed exhibited developmental regrowth defects as previously reported (Figure 7C-D). In contrast, *akt1^{q1}*, *InR³¹* and *PTEN¹¹⁷* clones seemed normal and fully occupied the adult specific γ lobe (Figure 7F-H) similar to WT clones (Figure 7B). In addition, *wnc⁸* clones also presented normal developmental regrowth phenotype (Figure 7E), similar to WT. Because PTEN is an inhibitor of the TOR pathway, we expect that *PTEN* mutant neurons will exhibit increased growth potential. We therefore decided to test whether *PTEN¹¹⁷* MARCM neuroblast clones exhibit earlier or accelerated axon growth during development. When observed at 24h APF, the time when MB γ axons

normally initiate developmental axon regrowth, *PTEN*^{l17} γ neurons looked identical to WT neurons (Figure 7 I-J). Thus we conclude that despite their role in axon sprouting and regeneration, the InR/PI3K/Akt pathway or Wnd do not play a role in neither initial axon outgrowth nor developmental regrowth.

Discussion

Culturing of primary dissociated neurons is a highly useful tool to elucidate the molecular mechanisms underlying neuronal growth (McKeon et al., 1991). *D. melanogaster* offers a powerful genetic toolkit (del Valle Rodriguez et al., 2012) that is highly beneficial in deciphering the complex genetic mechanisms involved in axonal intrinsic growth potential, and can thus complement mammalian models. *Drosophila* primary neuronal cultures were demonstrated by others to study various cell processes such as the cellular response to the steroid hormone 20-hydroxyecdysone (E20) (Kraft et al., 1998), and characterisation of the neuronal network (Saad et al., 2012). In our study we harnessed the power of *Drosophila* genetics to establish a system that enables the analysis of neurite sprouting of WT or mutant neurons in up to a single cell resolution. To achieve this, we cultured neurons that were dissociated from brains containing MARCM clones of the appropriate genotypes. While in this study we have analysed the growth of mushroom body (MB) neurons, one can culture any neuron which can be robustly labeled. Finally, this culturing system is amenable to pharmacological manipulations, such as rapamycin treatment, and also enables live imaging that could be performed using various cellular reporters to study the cellular mechanisms of axon growth.

Using this approach we have shown that MB neurons are viable following dissociation in culture, can be maintained for over one week (data not shown), sprout neurites that extend over time and eventually create what seems to be new connections between cells. In order to label MB neurons we used a Gal4 driver that is relatively specific to the MB, Gal4-201Y, which labels the γ neurons but also a late-born subpopulation of the α/β neurons. Since we mainly examined larval cultures, the pupal-derived α/β neurons were not present. Using MARCM neuroblast clones has two main advantages: first, it helps ‘clean’ the Gal4 reporter as it only positively labels clones whose generation is temporally regulated by the heat shock activation of the flippase; second, it enables the generation of a genetically modified clone which can be homozygous mutant or express a specific transgene in an otherwise heterozygous unlabeled environment. Overall, we believe that this neurite sprouting assay thus offers a promising platform for studying the intrinsic growth potential and other cell biological aspects of primary cultured neurons.

In this work we suggest that axon growth potential is developmentally regulated. Similar to mammals, we and others (Ayaz et al., 2008) have shown that the growth potential of adult neurons is greatly diminished and evolutionary conserved. To our surprise however, we found that neurons dissociated from pupal brains exhibit increased sprouting abilities, suggesting the existence of a heightened growth potential program during metamorphosis. This enhancement can be attributed to the fact that during metamorphosis, the nervous system exhibits increased plasticity and the neurons are more primed to undergo

degenerative and regenerative events. The molecular nature of this developmentally regulated 'elevated state' of axon growth potential is of great interest to the field.

Unique features of the primary culture of dissociated *Drosophila* neurons

Primary and established cell lines have been extremely useful in mammalian research in a wide variety of topics ranging from basic cell biology, cancer biology and neuronal development. While cell lines are easy to grow, they have a tendency to acquire mutations and the comparison between different cell lines is often problematic. Primary cell purification has allowed the efficient culturing of some neurons, such as hippocampal and dorsal root ganglia (DRG) neurons (e.g. Seijffers et al., 2006; Zou et al., 2009; Yamamoto et al., 2012; Peterson et al., 2014), but not all neuronal types. In addition, while manipulation of these cultures using siRNA is common and easy, the rapid generation of primary cultures of neurons harboring a specific mutation is more difficult and labor intensive. Additionally, while knocking down the expression of specific genes using RNAi is often problematic due to off targets effects (e.g. Mishra et al., 2008; Baek et al., 2014) and due to varying expression levels of the RNAi's, mutant analysis is much more conclusive. Therefore, a direct comparison between processes assayed in culture and physiological processes occurring *in vivo* is often problematic.

In contrast to the well-studied mammalian culture systems, *Drosophila* primary cell culture is much less well established and includes many unknown parameters (Kraft et al., 1998; Saad et al., 2012). For example, the exact concentration of primary *Drosophila* neurons that will allow for rapid and efficient axon growth has not been fully determined. In fact, we found that high density of neurons supports faster axon growth. The primary culture system that we have established here harnesses the awesome genetic power of the fly to support a dense culture with sparse neuronal labeling of WT or genetically manipulated disease. Using our system we have grown MB neurons but using other cell type specific Gal4 drivers should allow the isolation of any neuronal type. Finally, our study is a demonstration that by using this primary culture system, in combination with mosaic analyses *in vivo*, we can compare the requirement of specific genes in two cellular contexts while using the *same cell type* and the *exact same allele*. We believe this is a crucial advantage of our system as it allows us to compare 'oranges with oranges and apples with apples'.

Similarities and differences between distinct axonal growth types

In this work we have shown that whereas TOR is important for both developmental regrowth and neurite sprouting following cell dissociation of MB γ neurons, UNF is required solely for developmental regrowth. These results suggest that in response to an insult, TOR might become activated by a different upstream effector to promote sprouting. One of the main pathways known to activate TOR is the InR-PI3K-Akt pathway (Hay and Sonenberg, 2004). Indeed, we found that PTEN, which is a known antagonist of PI3K activity and therefore inhibits TOR activation, repressed sprouting following dissociation in neurons obtained from adult brains. Moreover, neither InR or Akt are required for developmental axon regrowth, which suggests that TOR is regulated by UNF to promote developmental regrowth and the InR-PI3K-Akt pathway during neurite sprouting. Finally, we have shown that *wnd* (*DLK*), which has a conserved role in regeneration following injury, also affects neurite sprouting of

dissociated MB neurons. Despite its pivotal role in regeneration and sprouting, *wnd* does not play a role in developmental regrowth of MB neurons, suggesting that these two mechanisms are distinct. Moreover, *InR*, *PI3K*, *akt* or *wnd* as well as *TOR* and *unf* are not required for initial axon outgrowth, since neurons harbouring mutations in these genes display a normal axon outgrowth phenotype *in vivo*. Taken together, our findings strongly support the existence of at least three distinct axon growth programs: initial axon outgrowth during circuit formation, developmental axon regrowth during circuit remodelling and axon regeneration following injury. Ultimately, we would like to uncover the genetic program underlining regeneration following injury of MB neurons *in vivo*. However, this task is quite complex due to technical limitations of injury and imaging of neurons within the CNS. While sprouting of dissociated MB neurons might not be identical to regeneration following injury, it seems to share the major known pathways. Therefore using the MB neurons as a model should allow us to compare the molecular requirements for different growth processes using the same neuron and the same genetic perturbations and as such holds the promise to shed light on the shared as well as unique molecular mechanisms for these processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ayaz D, Leyssen M, Koch M, Yan J, Srahna M, Sheeba V, Fogle KJ, Holmes TC, Hassan BA. Axonal injury and regeneration in the adult brain of *Drosophila*. *J Neurosci*. 2008; 28:6010–6021. [PubMed: 18524906]
- Baek ST, Kerjan G, Bielas SL, Lee JE, Fenstermaker AG, Novarino G, Gleeson JG. Off-target effect of doublecortin family shRNA on neuronal migration associated with endogenous microRNA dysregulation. *Neuron*. 2014; 82:1255–1262. [PubMed: 24945770]
- Blackmore MG. Molecular control of axon growth: insights from comparative gene profiling and high-throughput screening. *Int Rev Neurobiol*. 2012; 105:39–70. [PubMed: 23206595]
- Blizzard CA, Haas MA, Vickers JC, Dickson TC. Cellular dynamics underlying regeneration of damaged axons differs from initial axon development. *Eur J Neurosci*. 2007; 26:1100–1108. [PubMed: 17767489]
- Brogio W, Stocker H, Ikeya T, Rintelen F, Fernandez R, Hafen E. An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Curr Biol*. 2001; 11:213–221. [PubMed: 11250149]
- Buchser WJ, Smith RP, Pardinas JR, Haddox CL, Hutson T, Moon L, Hoffman SR, Bixby JL, Lemmon VP. Peripheral nervous system genes expressed in central neurons induce growth on inhibitory substrates. *PLoS One*. 2012; 7:e38101. [PubMed: 22701605]

- Collins CA, Waikar YP, Johnson SL, DiAntonio A. Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron*. 2006; 51:57–69. [PubMed: 16815332]
- David S, Aguayo AJ. Axonal elongation into peripheral nervous system “bridges” after central nervous system injury in adult rats. *Science*. 1981; 214:931–933. [PubMed: 6171034]
- del Valle Rodriguez A, Didiano D, Desplan C. Power tools for gene expression and clonal analysis in *Drosophila*. *Nat Methods*. 2012; 9:47–55.
- Filbin MT. Recapitulate development to promote axonal regeneration: good or bad approach? *Philos Trans R Soc Lond B Biol Sci*. 2006; 361:1565–1574. [PubMed: 16939975]
- Goldberg JL, Klassen MP, Hua Y, Barres BA. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. *Science*. 2002; 296:1860–1864. [PubMed: 12052959]
- Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev*. 2004; 18:1926–1945. [PubMed: 15314020]
- Hietakangas V, Cohen SM. Re-evaluating AKT regulation: role of TOR complex 2 in tissue growth. *Genes Dev*. 2007; 21:632–637. [PubMed: 17369395]
- Kraft R, Levine RB, Restifo LL. The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J Neurosci*. 1998; 18:8886–8899. [PubMed: 9786994]
- Lee T, Luo L. Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *Trends Neurosci*. 2001; 24:251–254. [PubMed: 11311363]
- Liu K, Tedeschi A, Park KK, He Z. Neuronal intrinsic mechanisms of axon regeneration. *Annu Rev Neurosci*. 2011; 34:131–152. [PubMed: 21438684]
- Longair MH, Baker DA, Armstrong JD. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics*. 2011; 27:2453–2454. [PubMed: 21727141]
- Lu Y, Belin S, He Z. Signaling regulations of neuronal regenerative ability. *Curr Opin Neurobiol*. 2014; 27:135–142. [PubMed: 24727245]
- McKeon RJ, Schreiber RC, Rudge JS, Silver J. Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. *J Neurosci*. 1991; 11:3398–3411. [PubMed: 1719160]
- Mishra A, Kner B, Paixao S, Kramer ER, Klein R. The protein dendrite arborization and synapse maturation 1 (*Dasm-1*) is dispensable for dendrite arborization. *Mol Cell Biol*. 2008; 28:2782–2791. [PubMed: 18268009]
- Moore DL, Blackmore MG, Hu Y, Kaestner KH, Bixby JL, Lemmon VP, Goldberg JL. KLF family members regulate intrinsic axon regeneration ability. *Science*. 2009; 326:298–301. [PubMed: 19815778]
- Neumann S, Woolf CJ. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. *Neuron*. 1999; 23:83–91. [PubMed: 10402195]
- Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science*. 2008; 322:963–966. [PubMed: 18988856]
- Peterson AJ, Irvine DR, Heil P. A model of synaptic vesicle-pool depletion and replenishment can account for the interspike interval distributions and nonrenewal properties of spontaneous spike trains of auditory-nerve fibers. *J Neurosci*. 2014; 34:15097–15109. [PubMed: 25378173]
- Saad Y, Anabosi M, Anava S, Nadav G, Yerushalmi Y, Ayali A. Fly neurons in culture: a model for neural development and pathology. *J Mol Histol*. 2012; 43:421–430. [PubMed: 22538481]
- Seijffers R, Allchorne AJ, Woolf CJ. The transcription factor ATF-3 promotes neurite outgrowth. *Mol Cell Neurosci*. 2006; 32:143–154. [PubMed: 16713293]
- Staveley BE, Ruel L, Jin J, Stambolic V, Mastronardi FG, Heitzler P, Woodgett JR, Manoukian AS. Genetic analysis of protein kinase B (AKT) in *Drosophila*. *Curr Biol*. 1998; 8:599–602. [PubMed: 9601646]
- Xiong X, Wang X, Ewanek R, Bhat P, DiAntonio A, Collins CA. Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. *J Cell Biol*. 2010; 191:211–223. [PubMed: 20921142]

- Yamamoto H, Demura T, Morita M, Banker GA, Tanii T, Nakamura S. Differential neurite outgrowth is required for axon specification by cultured hippocampal neurons. *J Neurochem.* 2012; 123:904–910. [PubMed: 22928776]
- Yang MY, Armstrong JD, Vilinsky I, Strausfeld NJ, Kaiser K. Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron.* 1995; 15:45–54. [PubMed: 7619529]
- Yaniv SP, Issman-Zecharya N, Oren-Suissa M, Podbilewicz B, Schuldiner O. Axon regrowth during development and regeneration following injury share molecular mechanisms. *Curr Biol.* 2012; 22:1774–1782. [PubMed: 22921367]
- Zou H, Ho C, Wong K, Tessier-Lavigne M. Axotomy-induced Smad1 activation promotes axonal growth in adult sensory neurons. *J Neurosci.* 2009; 29:7116–7123. [PubMed: 19494134]

Figure 1. Dissociated *Drosophila* mushroom-body (MB) neurons are viable and sprout neurites in culture

(A) Neurite sprouting assay scheme. (B-D) Representative inverted grayscale confocal Z projections of neurons derived from 3rd instar larvae expressing membrane bound GFP (mCD8-GFP) driven by: (B) Gal4-201Y; (C) Gal4-201Y in MB MARCM neuroblast clones; (D) similar to (C) but mixed with unlabeled brains in a ratio of 2/1 (see schemes B₁-D₁). While axon sprouting was evident in all conditions, only condition (D) allowed a single cell resolution while maintaining a high-density culture that was essential for rapid growth. Images were taken 0-2 days following cell dissociation (days *in vitro* - DIV). Scale bar is 20

micron. Genotypes: (B) *Gal4-201Y, UAS-CD8::GFP/cyo*, (C-D) *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80 /FRTG13, UAS-CD8::GFP, Gal4-201Y*

Figure 2. Neurite Sprouting in culture is a dynamic process.

(A-B) Inverted grayscale confocal Z projections of L3 larvae-derived WT neurons, which were cultured 2 hours (DIV0 - A) or one day (DIV 1 - B) prior to live-imaged at fixed intervals. Dynamic processes including the sprouting of several neurites (A) and retraction of others (B) were observed. Scale bar is 20 micron. Red arrows indicate newly growing or retracting neurites, accordingly. Genotypes: *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80 /FRTG13, UAS-CD8::GFP, Gal4-201Y*

Figure 3. Wallenda (Wnd) is required for efficient sprouting of dissociated MB neurons. (A-B) Representative inverted grayscale confocal Z projections of L3 larvae-derived WT (A) and *wnd^Δ* (B) neurons 1-2 days following cell dissociation. Scale bar is 20 micron. Red arrows indicate the longest neurite per cell. (C-D) Quantification of the longest neurite (C) or total neurites length (D) per cell of L3 larvae-derived WT and *wnd^Δ* neurons 1-3 days following dissociation (days *in vitro* - DIV). Statistical analysis was performed using standard t-test, *** $P < 0.005$, error bars are Standard Error (SE). Genotypes: (A) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP/+; tub-Gal80, FRT2A/FRT2A*. (B) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP/+; tub-Gal80, FRT2A/wnd^Δ, FRT2A, FRT82B*

Figure 4. Neurite sprouting potential depends on developmental stage

(A-D) Representative inverted grayscale confocal Z projections of dissociated primary cultured MB-neurons derived from WT L3 larvae (A), 6h after puparium formation (APF) pupae (B), 24h APF pupae (C) and adult flies (D) 1-3 days following dissociation (days *in vitro* - DIV). Scale bar is 20 micron. Red arrows indicate the longest neurite per cell. (E) Quantification of the longest neurite per cell of the conditions in A-D. Statistical analysis was performed using 2-way ANOVA, ** $P < 0.01$, error bars are SE. Genotype: y, w, *UAS-CD8::GFP*, *hsFlp122*; *FRTG13*, *tub-Gal80*/*FRTG13*, *UAS-CD8::GFP*, *Gal4-201Y*

Figure 5. While TOR is required for efficient axon sprouting, UNF is not.

(A-B) Representative inverted grayscale confocal Z projections of L3 larvae-derived dissociated WT (A) and *TOR^{LL04239}* (B) MB neurons, 1-2 days following dissociation (days *in vitro* - DIV). Scale bar is 20 micron. Red arrows indicate the longest neurite per cell. (C-D) Quantification of the longest neurite per cell of the conditions in A and B (C), and of L3-derived WT neurons either treated or untreated with rapamycin (100nM) (D). Statistical analysis was performed using standard t-test, error bars are SE, * $P < 0.05$, ** $P < 0.01$. (E-F) Representative inverted grayscale confocal Z projections of L3 larvae-derived (E) or 24h

APF-derived (F) cultured WT (E₁-F₁) and *unt^{ΔL04325}* (E₂-F₂) MB neurons, two days following cell dissociation. Scale bar is 20 micron. Red arrows indicate the longest neurite per cell. (G-H) Quantification of the longest neurite per cell of L3 larvae-derived (G) and 24h APF-derived (H) MB neurons. Statistical analysis was performed using a standard t-test, error bars are SE, n.s - not significant. Genotypes: (A) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y/FRT40A, FRTG13, cn, bw*. (B) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y/TOR^{LL04239}, FRT40A*. (E) *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80/FRTG13, UAS-CD8::GFP, Gal4-201Y*. (F) *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80/FRTG13, UAS-CD8::GFP, Gal4-201Y, unt^{ΔL04325}*

Figure 6. The PI3K pathway is required for sprouting of dissociated adult MB neurons
 (A-B) Representative inverted grayscale confocal Z projections of L3-derived cultured WT (A) and *akt1^{q1}* MB neurons (B) 1-2 days following dissociation (days *in vitro* - DIV). Scale bar is 10 micron. Red arrows indicate the longest neurite per cell. (C) Quantification of the longest neurite per cell in A and B. Statistical analysis was performed using a standard t-test, error bars are SE, n.s. not significant, * $P < 0.05$. (D-E) Representative inverted grayscale confocal Z projections of adult-derived cultured WT (D) and *PTEN^{l17}* MB neurons (E) 1-2 days following dissociation. Scale bar is 10 micron. (F) Quantification of the longest neurite

per cell on D and E. Statistical analysis was performed using a standard t-test, error bars are SE, *P<0.05. Genotypes: (A) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP; FRT82B, tub-Gal80 / FRT82B*. (B) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP; FRT82B, tub-Gal80/FRT82B, akt1^{q1}*. (D) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y/FRT40A, FRTG13, cn, bw*. (E) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y, /FRT40A, PTEN¹¹⁷*.

Figure 7. Wallenda and the InR-PI3K-Akt pathway are not required for developmental axon regrowth of MB γ neurons.

(A) Schematic representation of MB γ neuron developmental remodelling: At the late larval stage, γ neurons extend a bifurcated axon that projects to both the dorsal (d) and medial (m) lobes. p-peduncle, den-dendrites. At the pupal stage, 18h After puparium formation (APF), γ neurons have pruned their larval-specific branches. At 24h APF γ neurons start to re-extend their axons to eventually form an adult specific medial lobe. (B-G) Representative confocal Z projections of adult WT (B), *TOR^{LL04239}* (C), *unf^{LL04325}* (D), *wnd³* (E), *akt1⁹¹* (F), *InR³¹* (G) and *PTEN¹¹⁷* (H) MB neuroblast MARCM clones. Magenta represents anti-

FasII staining. Green is mCD8:GFP, driven by Gal4-210Y. (I-J) Representative confocal Z projections of 24h APF WT (I) and *PTEN*¹¹⁷ (J) MB neuroblast MARCM clones. Grayscale is mCD8:GFP, driven by Gal4-210Y. Genotypes: (B) *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80 / FRTG13, UAS-CD8::GFP, Gal4-201Y*. (C) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y, UAS-CD8::GFP / TOR^{LL04239}, FRT40A*. (D) *y, w, UAS-CD8::GFP, hsFlp122; FRTG13, tub-Gal80 / FRTG13, UAS-CD8::GFP, Gal4-201Y, un^{ΔL04325}*. (E) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP; wnc³, FRT2A / tub-Gal80, FRT2A* (F) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP; FRT82B, tub-Gal80 / FRT82B, akt1^{q1}*. (G) *y, w, UAS-CD8::GFP, hsFlp122; Gal4-201Y, UAS-CD8::GFP; FRT82B, tub-Gal80 / FRT82B, InR³¹*. (H, J) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y, / PTEN¹¹⁷, FRT40A* (I) *y, w, UAS-CD8::GFP, hsFlp122; tub-Gal80, FRT40A, Gal4-201Y / FRT40A, FRTG13, cn, bw*.