TITLE

Adult expression of the cell adhesion protein Fasciclin 3 is required for the maintenance of adult olfactory interneurons

RUNNING TITLE

Fas3 is vital to adult neuron survival

Aarya Vaikakkara Chithran^{1,2}, Douglas W. Allan^{2,3} and Timothy P. O'Connor*^{2,3}

¹ Graduate Program in Neuroscience, 3402-2215 Wesbrook Mall, University of British Columbia, Vancouver BC, Canada V6T 1Z3

² Department of Cellular and Physiological Sciences, 2350 Health Sciences Mall, University of British Columbia, Vancouver BC, V6T 1Z3

³ Djavad Mowafaghian Centre for Brain Health, 2215 Wesbrook Mall, University of British Columbia, Vancouver BC V6T 1Z3

*Author for correspondence (<u>timothy.oconnor@ubc.ca</u>)

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SUMMARY STATEMENT

Little is known about the maintenance of adult neural circuits. We show that the continuous expression of Fasciclin 3, a cell adhesion protein involved in axon guidance, is required for neuronal survival in the adult olfactory circuit.

ABSTRACT

The proper functioning of the nervous system is dependent on the establishment and maintenance of intricate networks of neurons that form functional neural circuits. Once neural circuits are assembled during development, a distinct set of molecular programs is likely required to maintain their connectivity throughout the lifetime of the organism. Here, we demonstrate that Fasciclin 3 (Fas3), an axon guidance cell adhesion protein, is necessary for the maintenance of the olfactory circuit in adult *Drosophila*. We utilized the TARGET system to spatiotemporally knockdown *Fas3* in selected populations of adult neurons. Our findings show that *Fas3* knockdown results in the death of olfactory circuit neurons and reduced survival of adults. We also demonstrated that *Fas3* knockdown activates caspase-3 mediated cell death in olfactory local interneurons, which can be rescued by overexpressing p35, an anti-apoptotic protein. This work adds to the growing set of evidence indicating a critical role for axon guidance proteins in the maintenance of neuronal circuits in adults.

INTRODUCTION

The proper functioning of the nervous system is determined by the establishment and maintenance of intricate connections between neurons. During nervous system development, billions of neuronal axons navigate to their targets in response to axon guidance cues in the extracellular environment (Tessier-Lavigne and Goodman, 1996). This process involves neuronal outgrowth, navigation and pathfinding that relies on a highly dynamic, fan-shaped, actin-rich structure located at the tip of axons and dendrites called a growth cone (Cajal, 1890). For over 100 years, neuroscientists have researched the nature of the guidance cues underlying these processes and developed an understanding of how these cues signal spatial information to growth cones to direct neural circuit formation (Kolodkin and Tessier-Lavigne, 2011). Many families of guidance cues have been characterized including Semaphorins, Netrins, Slits, Ephrins, morphogens, cell adhesion molecules (CAMs) and cytoskeletal-associated proteins (Yaron and Zheng, 2007).

Numerous studies have supported the idea that CAMs can guide axons by promoting adhesion, stimulating neuronal outgrowth, and functioning as signaling molecules in heterophilic or homophilic combinations (Moreland and Poulain, 2022; Pollerberg et al., 2013; Rutishauser, 1993). The immunoglobulin-like cell adhesion molecules (Ig-CAMs) that belong to the immunoglobulin superfamily and cadherins are the two major classes of CAMs. A role for CAMs in regulating axonal fasciculation and motor neuron guidance was first documented for the Ig-CAM Fasciclin 2 (Fas2) (Harrelson and Goodman, 1988; Lin and Goodman, 1994; Lin et al., 1994). Fas2 is homologous to the neural cell adhesion molecule (N-CAM) in vertebrates and is required for selective axon fasciculation in Drosophila. Ig-CAMs are also known to interact with signal transduction pathways during axonal guidance. For example, Fasciclin 1 (Fas1) genetically interacts with Abelson tyrosine kinase (Abl) with Fas1/Abl double mutants exhibiting commissural axon pathfinding defects (Elkins et al., 1990). Fasciclin 3 (Fas3), another Ig-CAM in Drosophila, is expressed by RP3 motor neurons and their synaptic targets (muscles 6 and 7) during embryonic development (Patel et al., 1987; Snow et al., 1989). Target recognition between the motor neuron growth cones and muscles is mediated through Fas3-dependent homophilic interactions. In addition, RP3 motor neurons form synapses on muscles that ectopically express Fas3, confirming that Fas3 acts as a synaptic recognition cue for these growth cones (Chiba et al., 1995). Additionally, when the Fas3-negative aCC and SNa motor neuron growth cones ectopically express Fas3, they innervate the Fas3-expressing muscles as alternative targets (Kose et al., 1997). However, motor neurons can correctly innervate the target muscles 6 and 7 in *Fas3* null mutants, suggesting redundancy in targeting mechanisms. These studies provide evidence for Fas3-dependent homophilic synaptic target recognition in the precise formation of neural circuits.

Once neural circuits are assembled during development, it is presumed that a distinct set of molecular programs is likely required to maintain their connectivity throughout the lifetime of the organism. Nonetheless, many mature neurons continue to express axon guidance proteins long after they have reached their targets and made functional connections. Indeed, the modENCODE high-throughput RNA-seq data confirms that axon guidance cues, including Semaphorins, Plexins, and CAMs (including *Fas3*) continue to be expressed in the adult *Drosophila* nervous system (Graveley et al., 2011). We have recently shown that members of the Semaphorin family of guidance cues have critical functions in the mature nervous system and are essential for maintaining neuronal survival, adult motility and longevity (Vaikakkara Chithran et al., 2023). Although this previous work showed that the expression of *Fas3* in adults is also critical for survival, the role of *Fas3* in the maintenance of adult neural circuits has not been examined. In this study, we utilize the well-characterized *Drosophila* olfactory circuit to explore the importance of stable expression of *Fas3* for neural circuit maintenance in adults.

In Drosophila, the olfactory lobe (OL) is composed of 56 neuropil regions, known as glomeruli (Laissue et al., 1999, Tanaka et al., 2012), each of which receives input from a specific population of olfactory sensory neurons (OSNs) that typically express a single type of odorant receptor (Couto et al., 2005; Fishilevich and Vosshall, 2005). Within each glomerulus, OSNs establish synapses with projection neurons (PNs), which serve as output neurons of the OL and can be compared to the mitral/tufted cells in mammals (Imai et al., 2010; Lledo et al., 2005). OSNs also make connections with local interneurons (LNs) that have arborizations confined within the OL, similar to interneurons found in the olfactory bulb of mammals, such as granule and periglomerular cells (Lledo et al., 2005; Stocker et al., 1990; Tanaka et al., 2012). Furthermore, PNs establish feedback connections with LNs (Liu and Wilson, 2013; Sudhakaran et al., 2012; Tanaka et al., 2009). LNs play multiple roles in shaping the output of the OL. One important function of LNs is their involvement in olfactory habituation, a phenomenon characterized by a reduced behavioral response to repeated or continuous exposure to an odorant (Twick et al., 2014). This reduced response is attributed to increased inhibitory input from LNs to odor-selective PNs (Das et al., 2011; Larkin et al., 2010; Sadanandappa et al., 2013; Sudhakaran et al., 2012). Interestingly, a genetic screen identified mutations in several CAMs, including Fas3, which disrupted olfactory habituation (Eddison et al., 2012), suggesting an important role in the proper development of the olfactory circuit. However, the role of Fas3 in the normal functioning of olfactory neurons in adults has not been examined. In this study, we present evidence demonstrating the essential role of continuous Fas3 expression in maintaining the olfactory circuitry in adult flies. Specifically, we reveal that Fas3 promotes cell survival in LNs, suggesting a critical function for CAMs in the maintenance of adult neurons.

RESULTS

Fas3 is widely expressed in the adult Drosophila central nervous system

Using a *Fas3::GFP* protein trap allele combined with Bruchpilot (Brp) immunostaining, we examined the expression and localization of *Fas3* in the adult *Drosophila* central nervous system (CNS). *Fas3* is prominently localized in the olfactory lobes (yellow box in Fig. 1A), with lower levels observed in the optic lobes (white box in Fig. 1A), suboesophageal ganglion (yellow arrow in Fig. 1A), and a subset of neuronal populations in the ventral nerve cord (white arrows in Fig. 1A). This expression and localization were also confirmed by Fas3 immunoreactivity (Fig. 1B) and *Fas3-Gal4* driven expression of nuclear GFP (Fig. 1C). Within the olfactory circuit, it is

likely that Fas3 is expressed in a subset of neurons whose cell bodies lie adjacent the olfactory lobe (as observed by the *UAS-nuclear GFP* expression driven by *Fas3-Gal4* in Fig. 1C) and project their dendrites into the olfactory lobe (as seen by the strong expression of *Fas3-GFP* and anti-Fas3 labelling in the glomeruli of the olfactory lobe in Fig. 1A, B).

Fas3-Gal4 mediated Fas3 knockdown reduces adult Drosophila survival

To examine if *Fas3* expression in adults is essential for survival, we used the TARGET system (*Fas3-Gal4* with *tub-Gal80*^{ts}) to knockdown the expression of *Fas3* only in adult *Drosophila* in all *Fas3*-expressing cells (Fig. 2A) and monitored the survival of adult flies daily until day 22 post eclosion. We picked male flies for all the experiments. In the survival assay, we employed two controls; an isogenic host strain as a genetic background control for the GD RNAi library and a *UAS-dsRNA-GFP* control, both of which yielded similar results. On day 17, there was a significant reduction in survival in the *Fas3* knockdown flies, with only 87% of the knockdown flies surviving compared to 97% of the control flies. This impact on survival increased with age; by day 22, the survival rate of *Fas3* knockdown flies dropped to 47%, while 97% of the control flies survived (Fig. 2B), confirming that continued expression of *Fas3* in the adult is essential for longevity.

Fas3 is expressed in a subset of olfactory local interneurons

Due to high *Fas3* levels in the adult olfactory lobes (as shown in the yellow box in Fig. 1A) and the well-characterized circuitry of the adult *Drosophila* olfactory system (Fig. 3A), we examined the impact of *Fas3* knockdown on the maintenance of this circuit. To visualize the precise location of *Fas3* expressing neurons and their processes, we used *Fas3 Gal4* to drive *UAS*-*CD8::GFP*. We observed high expression in neurons around the adult olfactory lobe whose cell bodies lie adjacent to the OL (Fig. 3C). Among the three neuronal populations of the olfactory circuit (OSNs, PNs, LNs), the OSNs have their cell bodies located in the periphery and the PNs send their axonal projections into higher brain regions. However, we observed *Fas3* positive cell bodies right outside the olfactory lobe (Fig 1C) and after extensive examination we could not identify any projections directed to any higher brain regions (Fig 1A, B) suggesting that the *Fas3*-expressing neurons only send local projections and are likely to be LNs.

To confirm Fas3 expression in LNs, we examined co-labelling of *Fas3-nlacZ* reporter (Fig. 3D) with UAS-nGFP driven by one of three *LN-Gal4* lines, *GH298-Gal4* (Fig. 3E), *NP3056-Gal4* (Fig. 3F) and *189Y-Gal4* (Fig. 3G) (Chou et al., 2010; Liou et al., 2018). Almost all *GH298-Gal4* neurons were co-labelled by *Fas3-lacZ* (Fig. 3E'). Also, 85% of *NP3056-Gal4* neurons (Fig. 3F') and 50% of *189Y-Gal4* neurons (Fig. 3G') were co-labelled with *Fas3-lacZ*. This confirmed that *Fas3* is expressed in subsets of olfactory LNs.

Fas3 knockdown in the adult results in the death of olfactory circuit neurons

In order to examine the importance of Fas3 expression in neural circuit maintenance, understand why *Fas3* may be essential for adult survival, and due to its strong expression in the olfactory lobes, we decided to examine the impact of *Fas3* knockdown specifically on this circuit. As we observed a significant reduction in the survival of adult flies from day 17 post eclosion (Fig. 2B), we focused our analysis on circuit structure asked whether there was an impact on neuronal integrity before this time point. Specifically, we examined LNs and their projections at day 14, two weeks following initiation of Fas3 knockdown. Using Fas3-Gal4, we drove UAS-dsRNA-Fas3 with UAS-CD8::GFP or as a control we drove UAS-CD8::GFP alone. In the controls, we observed the normal distribution of CD8::GFP neuronal cell bodies located immediately adjacent to the olfactory lobes (indicated by white arrows in Fig. 4A). In contrast, in Fas3 knockdowns, we observed a striking, significant loss of these neurons (indicated by white arrows in Fig. 4B). This neuronal loss phenotype was observed with multiple dsRNA-Fas3 lines (Fig. S1). Fas3 antibody staining confirmed that Fas3 was severely reduced by day 14 (compare Fig. 4A', B'). Next, we observed an increase in anti-Caspase 3 immunolabelling as early as day 7, especially in the region surrounding the olfactory lobes (indicated by white arrows in Fig. 4A", B"). This suggested that loss of olfactory circuit neurons observed in Fas3 knockdowns is due to apoptotic cell death. It is interesting to note that the region of increased caspase3 appears to extend beyond Fas3 knockdown, suggesting possible apoptosis in other cells immediately surrounding the Fas3 expressing neurons. Further experiments will be required to examine the possible mechanisms underlying this cell death.

Reduced *Fas3* expression results in cell-autonomous cell death

To further confirm that *Fas3* knockdown-mediated cell death occurs in subsets of LNs, we used *LN-Gal4* drivers to restrict Fas3 knockdown to small groups of olfactory interneurons. We conducted this experiment using multiple *UAS-dsRNA-Fas3* lines. Due to the differences in the genetic backgrounds of the *UAS-dsRNA-Fas3* lines, we used a panel of relevant controls from each respective RNAi library. These are detailed in Supplementary Tables S2-S3. The strongest *NP3056-Gal4* mediated knockdown of *Fas3* resulted in a ~50% loss of *NP3056-Gal4* expressing neurons by day 14 (marked by white arrows in Fig. 5A-G); from 27.15 \pm 0.6 neurons in controls to 13.35 \pm 0.8 in *Fas3* knockdowns (P \leq 0.0001) (Fig. 5H, I). A similar cell-autonomous cell death phenotype was also observed when Fas3 was knocked down using other *LN-GAL4* drivers; *GH298-Gal4* (Fig. S2) and *189Y-Gal4* (Fig. S3). Taken together, our data confirm that *Fas3* knockdown results in cell-autonomous death of LNs.

Fas3 knockdown-mediated neuronal death is rescued by overexpressing *p35*

To better understand the time course of neuronal loss after *Fas3* knockdown, we used the panneuronal driver, *elav-Gal4*, to knockdown *Fas3*. The knockdown was again restricted to the adults using the TARGET system. Here, we examined neuronal numbers at days 3, 5, 7, 10 and 14 after the initiation of Fas3 knockdown (Fig. 6A1-A5, B1-B5). Pan-neuronal knockdown of *Fas3* recapitulated the neuronal death we observed when knocking down *Fas3* in subsets of olfactory neurons. After pan-neuronal Fas3 knockdown, we started to observe the loss of *Fas3* immunoreactivity by day 7, with significant death of olfactory circuit neurons evident by day 10 (Fig. 6A3-A4, B3-B4), which continued through day 14 (Fig. 6A5, B5). These results corroborate our observations using the *Fas3-Gal4* and *LN-Gal4* drivers and add a time course to the observed cell death.

Since we observed significantly increased cleaved Caspase-3 immunostaining in *Fas3* knockdowns (Fig. 4A", B") which is an indicator of apoptosis, we tested if neuronal death can be rescued by blocking the apoptotic pathway. Using *elav-Gal4*, we knocked down Fas3 in the presence of overexpressed p35, an anti-apoptotic protein. We also used *UAS-nGFP* to count neuronal numbers on day 14. We chose the area of LN neurons around each olfactory lobe as the region of interest (ROI) for quantification (indicated by the yellow box in Fig. 6C). Notably, co-expressing *UAS-p35* with *UAS-dsRNA-Fas3* completely rescued the neuronal death phenotype (Fig. 6C-D). The number of neurons (represented by nuclear GFP) in *Fas3* knockdowns was reduced by almost 50% compared to the control and p35 rescue (Fig. 6D). These results confirm that *Fas3* knockdown-mediated neuronal death can be rescued by blocking the apoptotic pathway.

DISCUSSION

Previous studies have provided insight into the emergence and integration of neurons into the Drosophila olfactory circuit; however, very little is understood regarding the maintenance of these neurons in the adult. This study begins to reveal the importance of the stable expression of Fas3, an axon guidance cell adhesion protein, in the maintenance of the adult olfactory circuit. We demonstrated that adult-specific knockdown of Fas3 leads to the death of local olfactory interneurons which can be rescued by expressing the anti-apoptotic protein p35. Although the dynamic incorporation and function of local interneurons into the rodent olfactory circuit have been examined before (Adam and Mizrahi, 2010; Belluzzi et al., 2003), this is the first study to reveal a critical role for a guidance protein in the maintenance of Drosophila interneuron survival. Since we used the TARGET system to restrict the knockdown to the adult, the observed phenotypes are not a result of developmental defects but are due to defects in the maintenance of the adult circuit. To address the potential issue of off-target effects while using RNAi (i.e., knockdown of the expression of multiple genes by the same dsRNA/ shRNA), we employed several approaches. First, we picked UAS-dsRNA / -shRNA lines targeting Fas3 that were used in previous studies where no off-target effects were reported (Hu et al., 2013). In addition, the observed phenotypes were confirmed using five different Gal4 drivers (Fas3-Gal4, elav-Gal4, NP3056-Gal4, GH298-Gal4, 189Y-Gal4) and multiple different UAS-dsRNA/ -shRNA lines and controls (Supplementary Tables S2-S3). This includes UAS-dsRNA lines that are integrated into specific attP sites, ensuring that experimental and control UAS-transgenes are controlled for position effect (Heigwer et al., 2018).

Though there is evidence for intraglomerular plasticity in the adult *Drosophila* olfactory circuit (Berdnik et al., 2006), this study is the first to show cellular plasticity examine maintenance of the olfactory circuit at the level of a neuronal survival. population., resulting from a cell death pathway that is suppressed by cell adhesion proteins. Cellular plasticity in the adult olfactory system of several species is attributed to their sensory afferents and to subsets of

interneurons that are continuously replaced throughout adulthood (Bayramli et al., 2017; Durante et al., 2020; Fernandez-Hernandez et al., 2020 preprint; Graziadei and Okano, 1979; Monti Graziadei and Graziadei, 1979; Weiler and Farbman, 1997). Sustained neurogenesis and apoptosis of sensory neurons has been detected in the antennae of adult *Drosophila*, supporting an ongoing turnover in the olfactory system of adult flies (Fernandez-Hernandez et al., 2020 preprint). In vertebrates, olfactory sensory neuron replacement not only compensates for wearout processes in the periphery but also contributes to additional glomerular innervations in response to the organism's experience in adulthood (Jones et al., 2008). In the olfactory epithelium, where mature neurons die and are replaced throughout adult life, tight control over the mechanism of neuronal death is required to avoid tumorigenesis or premature depletion of olfactory sensory neurons. Both *in vivo* and *in vitro* studies have shown that olfactory sensory neurons die through an intrinsically programmed process, involving caspase activity and mediated by jun N-terminal kinase (JNK) signaling (Gangadhar et al., 2008).

In adult rodents, the local circuitry of olfactory interneurons is highly dynamic both at the population level and within individual cells. At the population level, a fraction of rodent interneurons undergo continuous replacement, while at the single-cell level, their dendritic morphology continues to change even after they reach maturity (Adam and Mizrahi, 2010; Belluzzi et al., 2003). The specific subpopulations of interneurons that either remain stable or undergo changes, as well as the extent of these changes, are still not fully understood. However, multiple studies provide compelling evidence for adult neurogenesis of olfactory interneurons in rodents (Kornack and Rakic, 2001; Ming and Song, 2011; Rosselli-Austin and Altman, 1979). Their survival has been shown to be dependent on sensory activity, with odor exposure or olfactory learning promoting neuronal survival (Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). In contrast, there is no evidence for adult neurogenesis of local olfactory interneurons in Drosophila. This rules out the possibility that the loss of Fas3 may be inhibiting the proliferation and differentiation of neural stem cells into local interneurons, thereby resulting in a reduced neuronal number. Moreover, the increased cleaved Caspase 3 labeling in the Fas3 knockdown brains and the p35 rescue of neuronal death imply that a caspase-dependent cell death pathway is activated in the LNs upon Fas3 knockdown. Although this may be a newly discovered role for Fas3, there is previous evidence associating cell death with the loss of expression of other cell adhesion molecules, such as integrins (Stupack, 2005), and axon guidance genes, such as Semaphorins and Plexins (Vaikakkara Chithran et al., 2023).

Drosophila local interneurons (LNs) are diverse in their neurotransmitter profiles, glomerular innervation patterns, and odor response properties. Neuronal subpopulations labelled by *NP3056-*, *GH298-*, and *189-Gal4* drivers show a patchy glomerular innervation pattern in the olfactory lobe. Each of the *LN-Gal4* drivers label a subset of both larval and adult LNs with at least 189-Gal4 and NP3056-Gal4 showing no overlap of expression (Chou et al., 2010). No two neurons have identical innervation patterns, suggesting that these innervation patterns may be established through cell-cell interactions among LNs (Chou et al., 2010). It may be speculated that the expression of *Fas3*, a homophilic cell adhesion molecule, is required for maintaining the

LN-LN interactions in adults which in turn is required for maintaining their patchy innervation. A role for CAMs in maintaining axonal morphologies has been demonstrated previously. For example, it has been shown that JNK signaling is required cell autonomously for axon pruning in mushroom body neurons by negatively regulating the plasma membrane localization of Fas2, another cell adhesion molecule of the Ig superfamily (Bornstein et al., 2015). Overexpression of Fas2 or other CAMs such as Fas1, Fas3 or Neuroglian (Nrg) was sufficient to inhibit pruning and high levels of CAMs were observed in unpruned axons. Thus, it is possible that Fas3 knockdown disrupts LN-LN interactions, triggering changes in their axonal/ dendritic morphologies and glomerular innervation, eventually causing neuronal degeneration and death. The importance of maintaining cell-cell interactions is also evident from the aptly called "community effect" observed among precursor cells during development (Saka et al., 2011). Cells in the developing embryo are in constant communication with their neighbors and this interaction is necessary for them to maintain tissue-specific gene expression and differentiate in a coordinated manner. Loss of signaling from neighbors can cause cellular dedifferentiation and death. Similarly, in the adult brain, the loss of cell-cell communication following Fas3 knockdown may be resulting in neuronal degeneration and death. In addition, Fas3 is known to genetically interact with other cell adhesion genes including armadillo (Greaves et al., 1999), shotgun (Greaves et al., 1999), Toll (Rose and Chiba, 1999), and Nrg (Lu et al., 2014). Ongoing studies will examine if loss of Fas3 downregulates the expression of other CAMs eventually contributing to cell death.

To maintain survival over the course of their lifetimes, neurons have the ability to adapt certain biological processes to prevent cell death. Cell death pathways are often activated in developing neurons to fine-tune the number of neurons required for the precise formation of neural circuits (Davies, 2003; Kole et al., 2013; Oppenheim, 1991). However, mature neurons promote survival by employing multiple mechanisms to prevent cell death. This shift from permitting cell death to ensuring cellular survival is imperative in mature neurons, as in most cases they must maintain the neuronal circuitry for an organism's entire lifetime (Benn and Woolf, 2004; Kole et al., 2013). We speculate that maintaining cell-cell interactions by continued expression of guidance cues and cell adhesion proteins molecules such as *Fas3* is a survival mechanism in adult neurons and its loss releases the apoptotic breaks in mature neurons, resulting in cell death. However, the mechanisms downstream of Fas3 loss that result in death require further detailed analysis. Previous studies have discovered several intracellular signaling cascades that are activated upon the homophilic binding of CAMs (Kiryushko et al., 2004). For example, Leukocyte-antigen-related-like (Lar), a protein that resembles CAMs of the Ig superfamily, controls motor axon guidance in *Drosophila* through protein-tyrosine-phosphatase activity (Krueger et al., 1996). The intracellular region of Fas3 contains two Serine residues that are potential sites of phosphorylation by Protein Kinase C and a Tyrosine residue that may facilitate the attachment of adapter proteins responsible for internalization into coated pits (Snow et al., 1989). Phosphorylation of CAMs such as integrins, N-CAM and L1 has been extensively studied and recognized as a fundamental event in regulating interactions with cytoplasmic

proteins to induce alterations in cell adhesion and signaling (Gahmberg and Grönholm, 2022; Matthias and Horstkorte, 2006; Wong et al., 1996). Furthermore, previous research has suggested a role for coated pits in mediating interactions between neuronal growth cones and neighboring cells (Bastiani and Goodman, 1984). Thus, loss of *Fas3* may result in the impairment of cell signaling, endocytosis and protein trafficking, leading to the disruption of cell adhesion and possibly cell death. Further research on intracellular signaling cascades following Fas3 homophilic binding is required to understand how *Fas3* maintains cell survival in LNs.

The main goal of this study was to examine whether *Fas3* has an essential function in the maintenance of adult neural circuits. In addition to the maintenance of adult olfactory neurons, we also demonstrated that *Fas3* is necessary for the survival of adult *Drosophila* which corroborates our previous finding that pan-neuronal expression of axon guidance genes in adults is essential for longevity (Vaikakkara Chithran et al., 2023). The observed reduction in the survival rate upon *Fas3* knockdown could result from the dysfunction of any number of neuronal populations or non neuronal tissues that express Fas3 in the adult *Drosophila* nervous system. Though we have focused on the role of *Fas3* in the adult olfactory circuit in this study, it is important to note that *Fas3* is also expressed in the optic lobes, suboesophageal ganglion, ventral nerve cord and non neuronal tissues. Compromising Fas3 function in multiple neural circuits or none neuronal tissues could result in reduced organismal survival. Examining these additional neuronal populations will determine if *Fas3* is required to maintain their survival during adulthood, similar to its function in adult LNs.

MATERIALS AND METHODS

Drosophila genetics

Reducing axon guidance gene expression by RNAi in the adult nervous system was carried out using the TARGET system. Gal4 driver line virgin females (see Supplementary Table S1) were crossed with *UAS-dsRNA/ -shRNA* males (see Supplementary Table S2) or the appropriate negative control line males (see Supplementary Table S3). The crosses were set up at 18°C and the progeny (F1) was switched to 29°C immediately after eclosion to repress Gal80^{ts} and activate the Gal4-UAS system. All analyses were performed on day 14 old adult male flies unless otherwise stated.

Drosophila melanogaster stocks used in this study were maintained on standard cornmeal food, at 18, 25 or 29°C in environment rooms set at 70% humidity. The stocks used in this study were received from Bloomington *Drosophila* Stock Center (BDSC), Vienna *Drosophila* Resource Center (VDRC) and Kyoto *Drosophila* Stock Center (DGRC) and are listed in Supplementary Tables S1-S4.

Survival assay

F1 progeny males were collected immediately after eclosion and switched to 29°C. They were maintained in vials of 10 and their survival was recorded every day for the next 20 days. Flies were flipped into fresh vials every week. Multiple UAS-dsRNA/-shRNA lines and controls were

tested and for each line, 3 vials containing 10 flies were assayed. All results were analyzed using two-way ANOVA and Tukey's multiple comparison tests using GraphPad Prism.

Immunohistochemistry and imaging

Adult male Drosophila brains and VNCs were dissected in ice-cold 1x PBS, over a period of 30 minutes. The tissue was fixed, on poly-Lysine coated slides, in 4% PFA at room temperature (RT) for 30 minutes followed by 3x 5 minutes washes with 0.1% PBT. The samples were blocked in 5% PBTN for 2 hours at RT, and then incubated in primary antibodies overnight at 4°C. The next day, samples were washed 3x 30 minutes in 0.1% PBT. They were then blocked in 5% PBTN for 1 hour at RT and incubated in secondary antibody for 3 hours at RT in the dark. For each experiment, controls and knockdowns were dissected and labelled in parallel. The samples were washed 3x 30 minutes in 0.1% PBT. They were mounted in Vectashield mounting media (Vector Laboratories Inc.), coverslipped and sealed with nail polish. Primary antibodies used in this study were as follows: mouse anti-Bruchpilot ne82 (DSHB, 1:20), mouse-anti-Fasciclin III 7G10 (DSHB, 1:400), mouse-anti-β-gal (DSHB, 1:50), chicken-anti-β-gal (Abcam, 1:1000), rabbit-anti-cleaved caspase 3 (Asp175) (Cell Signaling Technology, 1:300). Secondary antibodies used in this study were as follows: donkey-anti-mouse cy3 (Jackson, 1:250), donkeyanti-chicken cy3 (Jackson, 1:250), donkey-anti-rabbit cy3 (Jackson, 1:250). All images were acquired using the Zeiss LSM 880 Fast Airyscan inverted confocal microscope in a Z-stack. Images were processed using the 'Airyscan processing' feature using the Zen-black software. The images were acquired in multiple tiles and stitched post Airyscan processing. The images included in this study are maximum-intensity projections of representative images and all images were acquired with the same parameters.

Neuronal quantification and Statistical analysis

In *elav-Gal4* driven experiments, the area around the olfactory lobe was selected as the region of interest and neurons (represented by nlsGFP) were quantified using a MATLAB program kindly provided by Katie Goodwin (University of British Columbia). In *LN-Gal4* driven experiments, the olfactory circuits neurons were quantified manually in a 3D-model (using Zen-blue image processing software). The results were analysed using one-way ANOVA and Tukey's multiple comparison tests using GraphPad Prism.

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Competing interests

The authors declare no competing interests.

Author contributions

Conceptualization: A.V.C, D.W.A, T.OC; Methodology: A.V.C; Validation: A.V.C, D.W.A, T.OC; Formal analysis: A.V.C; Investigation: A.V.C; Writing- original draft: A.V.C; Writing- review & editing: A.V.C, D.W.A, T.OC; Visualization: A.V.C; Supervision: D.W.A, T.OC; Funding acquisition: A.V.C, T.OC

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Data availability

The datasets generated during and/ or analyzed during the current study are available from the corresponding author on request.

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FIGURE LEGENDS All changes have been highlighted.

Fig. 1. *Fas3* is widely expressed in the adult *Drosophila* CNS. *Fas3* is expressed in the adult *Drosophila* olfactory lobes (yellow box), optic lobes (white box), suboesophageal ganglion (yellow arrow) and ventral nerve cord (white arrows) as shown by (A) *Fas3-GFP* fusion protein expression, (B) immunostaining using a mouse-anti-Fas3 antibody and (C) *UAS-nuclear GFP* (*nGFP*) expression driven by *Fas3-Gal4*. The scale bars represent 50 μ m. Images shown are maximum intensity projections. Genotypes: (A) *w*; Fas3-GFP* (B) *Oregon R* (C) *w; Fas3-Gal4/+; UAS-nGFP/+*. See Supplementary Tables S1 and S4 for the additional details on the fly lines.

Fig. 2. *Fas3-Gal4* mediated *Fas3* knockdown reduces adult *Drosophila* survival from day 17. (A) A schematic of the TARGET system utilized to restrict the *Fas3* knockdown spatiotemporally to adult neurons. After eclosion, flies are moved to the permissive temperature (29°C) to initiate *dsRNA/ shRNA-Fas3* expression. (B) Knockdown of *Fas3* specifically in *Fas3*expressing cells reduces adult survival. '*' indicates the time points when the survival of *Fas3* knockdowns is significantly different from that of both the injection strain control and the GFP control. Data shown are mean \pm SEM. '*' denotes P \leq 0.05, '**' denotes P \leq 0.01, and '***' denotes P \leq 0.001 (two-way ANOVA and Tukey's multiple comparison tests; n=30). Genotypes: 'Injection strain' is *UAS-Dicer2; Fas3-Gal4/+; tubGal80^{ts}/*+. 'dsRNA-GFP' is *UAS-Dicer2; Fas3-Gal4/ UAS-dsRNA-GFP; tubGal80^{ts}/*+. 'dsRNA-Fas3' is *UAS-Dicer2; Fas3-Gal4/ UASdsRNA-Fas3 (#1); tubGal80^{ts}/*+. See Supplementary Tables S1-S3 for the additional details on the fly lines.

Fig. 3. *Fas3* is expressed in olfactory local interneurons. (A) A schematic of the adult *Drosophila* olfactory circuit, adapted from Seki et al., 2010. The axons of OSNs from the periphery project into distinct neuropil regions called glomeruli in the olfactory lobe. Within a given glomerulus, OSNs (yellow and green neurons) form synapses with PNs (blue) and LNs (red). The cell bodies of PNs and LNs lie outside the olfactory lobes. PNs extend their dendrites within specific glomeruli and send their axonal projections to higher centers (blue arrow) in the brain, whereas LNs send their projections locally, within the olfactory lobe. (B) Nuclear lacZ expression in *Fas3*-positive cells shows that the cell bodies are grouped around the adult olfactory lobes. (C- E) The Gal4 drivers *GH298-Gal4*, *NP3056-Gal4* and *189Y-Gal4* are expressed in a subset of LNs. (C'- E''') *Fas3* is co-expressed in a subset of the *LN-Gal4* neurons. The numbers shown in the bottom left corner of each merged panel indicate the number of cells labelled by *LN-Gal4*> *UAS-nGFP* (green), *Fas3-lacZ* (red) and both (yellow). (C'-C''') All the neurons labelled by *GH298-Gal4* were also labelled by *Fas3-lacZ* (n=10). (D'-D''') Approximately 85% of the neurons labelled by *NP3056-Gal4* also showed *Fas3-lacZ* (n=10). The

scale bars represent 20 μm in B-E and 10 μm in C'-E'''. Images shown are maximum intensity projections. Genotypes: (B) *Fas3-lacZ, CyO/ In(2LR)Gla, wg^{Gla-1}.* (C-C''') *w; Fas3-lacZ, CyO/+; UAS-nGFP/ GH298-Gal4.* (D-D''') *w; Fas3-lacZ, CyO/+; UAS-nGFP/ NP3056-Gal4.* (E-E''') *w; Fas3-lacZ, CyO/ 189Y-Gal4; UAS-nGFP/+.* See Supplementary Tables S1 and S4 for the additional details on the fly lines.

Fig. 4. *Fas3* **knockdown in all** *Fas3*-**expressing cells results in widespread death of olfactory neurons.** (A, B) Impact of *Fas3* knockdown on olfactory circuit neurons at day 14 post eclosion. The loss of neurons after *Fas3* knockdown is evident from comparing the *UAS-CD8 GFP* labelled neurons in the controls (white arrows in A) and the lack of these neurons in the knockdowns (white arrows in B) (n=10). (A', B') Immunostaining using a mouse-anti-Fas3 antibody in controls and knockdowns confirms *Fas3* knockdown by day 14 (n=10). (A", B") Cleaved caspase 3 immunolabelling at day 7 confirms that caspase activity is significantly increased in *Fas3* knocked down brains (white arrows in B") as compared to that in the controls (white arrows in A") (n=10). The white dotted circles outline the location of the adult olfactory lobes. The scale bars represent 20 µm. Images shown are maximum intensity projections. Genotypes: (A, A', A") *UAS-Dicer2; Fas3-Gal4/ UAS-dsRNA-lacZ; tubGal80^{ts}/ UAS-CD8 GFP* (B, B', B") *UAS-Dicer2; Fas3-Gal4/ UAS-dsRNA-fas3 (#1); tubGal80^{ts}/ UAS-CD8 GFP*. See Supplementary Tables S1-S3 for the additional details on the fly lines.

Fig. 5. *Fas3* **knockdown in a subset of LNs results in restricted neuronal death.** (A-D) *NP3056-Gal4* driven knockdown of *Fas3* using the GD RNAi library results in neuronal death by day 14 post eclosion (white arrows). (E-G) Knockdown of *Fas3* using the TRiP RNAi library shows the same impact on LNs (white arrows). The LN neuron subset is labeled with nGFP driven by *NP3056-Gal4* (A-G). The scale bars represent 20 µm. Images shown are maximum intensity projections. (H) Neuronal quantification confirming the reduction of neurons observed in the images A-D. (I) Neuronal quantification confirming the neuronal death observed in the images E-G. Data shown are mean \pm SEM. 'ns' denotes P > 0.05, '*' denotes P \leq 0.05, and '****' denotes P \leq 0.0001 (one-way ANOVA and Tukey's multiple comparison tests; n=20). Genotypes: (A) *w; tubGal80^{ls}, UAS-nGFP/+; NP3056-Gal4/+* (B) *w; tubGal80^{ls}, UAS-nGFP/UAS-dsRNA-lacZ; NP3056-Gal4/+* (C) *UAS-Dicer2; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#1); *NP3056-Gal4/+* (D) *w; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#2); *NP3056-Gal4/+* (E) *w; tubGal80^{ls}, UAS-nGFP/ attP40; NP3056-Gal4/+* (F) *w; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#3); *NP3056-Gal4/+* (G) *w; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#3); *NP3056-Gal4/+* (S) *w; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#3); *NP3056-Gal4/+* (F) *w; tubGal80^{ls}, UAS-nGFP/ UAS-dsRNA-Fas3* (#3);

Fig. 6. *Fas3* knockdown-mediated neuronal death can be rescued by overexpressing p35. (A1-A5, B1-B5) Impact of a pan-neuronal knockdown of *Fas3* on the olfactory circuit as shown from representative images on days 3, 5, 7, 10 and 14 post eclosion. A significant reduction in the neuronal number is evident in the knockdowns on day 10 (A4 vs B4) and day 14 (A5 vs B5).

Fas3 immunolabelling is also significantly lower in the knockdowns starting from day 10 (A4 vs B4, A5 vs B5). The knockdown brains from earlier time points do not seem to be any different from the controls (A-A3 vs B1-B3). (C-D) Rescue of *Fas3* knockdown-mediated neuronal death by overexpressing *p35*. (C) A representative image used for quantification. The nuclear GFP driven by *elav-Gal4* in the area surrounding the olfactory lobes (ROI bordered by a yellow box) was quantified. (C', C", C"') Representative images of the ROI used for neuronal quantification. *elav-Gal4* mediated knockdown of *Fas3* results in the loss of LN (C' vs C"), which is rescued by overexpressing *p35* (C"'). All scale bars represent 20 µm. Images shown are maximum intensity projections. (D) Quantification of neurons in the controls, knockdowns and p35 rescue, confirming a complete rescue of neuronal death. Data shown are mean \pm SEM. 'ns' denotes P > 0.05, and '**' denotes P ≤ 0.01 (one-way ANOVA and Tukey's multiple comparison tests; n=20). Genotypes:_(A1- A5) *elav-Gal4*, *UAS-Dicer2; tubGal80^{ts}*, *UAS-nGFP/*+ (B1- B5) *elav-Gal4*, *UAS-Dicer2; tubGal80^{ts}*, *UAS-nGFP/*+ (C') *elav-Gal4*, *UAS-Dicer2; tubGal80^{ts}*, *UAS-nGFP/*+ (C') *elav-Gal4*, *UAS-Dicer2; UAS-dsRNA-Fas3* (#1)/+; *tubGal80^{ts}*, *UAS-nGFP/*+ (C') *elav-Gal4*, *UAS-Dicer2; UAS-nGFP/*+ (C') *elav-Gal4*, *UAS-Dicer2; UAS-nGFP/*+

(C^{***}) *elav-Gal4*, *UAS-Dicer2*; *UAS-dsRNA-Fas3* (#1)/+; *tubGal80^{ts}*, *UAS-nGFP/ UAS-p35*. See Supplementary Tables S1-S3 for the additional details on the fly lines.







Adult age (days)



Fas3-lacZ (mouse-anti-β-Gal) NP3056-Gal4 > UAS-nGFP



GH298-Gal4 > UAS-nGFP











Fas3-Gal4 > UAS-CD8 GFP Day 14

mouse-anti-Fas3

Day 14

rabbit-anti-cleaved caspase3

Day 7

GD injection strain

attP40 injection strain

dsRNA-lacZ

	*		

\$			
*	***	ns	
lacZ	dsRNA-J	Fas3 a	lsRNA-Fa

(#1)

NP3056-Gal4 > *UAS-nGFP* Day 14

dsRNA-Fas3 (#1)

dsRNA-RFP

IS3

(#2)

dsRNA-Fas3 (#2)

dsRNA-Fas3 (#3)

*** **** dsRNA-RFP dsRNA-Fas3

(#3)

 $\left(\right)$

5

2

elav-Gal4 > UAS-nGFPmouse-anti-Fas3

