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Cell adhesion and actin dynamics factors promote axonal extension and synapse formation in transplanted *Drosophila* photoreceptor cells

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Abstract

Vision is formed by the transmission of light stimuli to the brain through axons extending from photoreceptor cells. Damage to these axons leads to loss of vision. Despite research on neural circuit regeneration through transplantation, achieving precise axon projection remains challenging. To achieve optic nerve regeneration by transplantation, we employed the *Drosophila* visual system. We previously established a transplantation method for *Drosophila* utilizing photoreceptor precursor cells extracted from the eye disc. However, little axonal elongation of transplanted cells into the brain, the lamina, was observed. We verified axonal elongation to the lamina by modifying the selection process for transplanted cells. Moreover, we focused on N-cadherin (Ncad), a cell adhesion factor, and Twinstar (Tsr), which has been shown to promote actin reorganization and induce axon elongation in damaged nerves. Overexpression of *Ncad* and *tsr* promoted axon elongation to the lamina, along with presynaptic structure formation in the elongating axons. Furthermore, overexpression of *Neurexin-1* (*Nrx-1*), encoding a protein identified as a synaptic organizer, was found to not only promote presynapse formation but also enhance axon elongation. By introducing *Ncad*, *tsr*, and *Nrx-1*, we not only successfully achieved axonal projection of transplanted cells to the brain beyond the retina, but also confirmed the projection of transplanted cells into a deeper ganglion, the medulla. The present study offers valuable insights to realize regeneration through transplantation in a more complex nervous system.

KEYWORDS
actin dynamics, axon growth, *Drosophila*, transplantation, visual system

1 | INTRODUCTION

Vision plays a pivotal role in the acquisition of external information. Vision is initiated upon the perception of light stimuli by the photoreceptor cells within the retina. These light stimuli are converted into electrical signals within the photoreceptor cells, which are subsequently conveyed to the brain through extensive axonal extensions. Therefore, impairments to the axons of the nerve connecting the retina to the brain result in vision loss. To reacquire vision, photoreceptor cells need to reestablish the neural circuitry connecting the retina to

the brain by extending novel elongated axons and forming synapses in the brain.

However, when axons are damaged, various impediments to axonal regeneration come into play, including the secretion of inhibitory molecules for axon elongation by glial cells and the formation of physical barriers due to scar tissue resulting from the injury, which collectively pose problems to nerve regeneration (Laha et al., 2017). The emergence of induced pluripotent stem (iPS) cells has provided a promising avenue for in vitro production of specific cells and tissues, attracting attention for potential regenerative transplantation approaches. In mammals, retinal ganglion cells (RGCs) play a vital role in transmitting information from the retina to the brain by extending axons. Successful transplantation of RGCs has been reported, and within these transplants, the integration of transplanted cells within the host organism and their responsiveness to light (MacLaren et al., 2006; Venugopalan et al., 2016). However, achieving precise control over the projection of transplanted cells into the brain remains challenging (Venugopalan et al., 2016). Notably, transplantation of iPS cell-derived RGCs revealed that while these cells displayed electrophysiological functionality, their axonal projections were limited to the optic nerve head and did not reach the brain (Oswald et al., 2021; Vrathasha et al., 2022). Regenerating neural circuits through transplantation necessitates specific elements, including cell adhesion factors facilitating attachment to neighboring tissues during axon elongation, axon guidance molecules responsible for directing the course of axonal growth, cytoskeletal molecules such as actin and tubulin, and molecules crucial for inducing the formation of synapses, which are essential for transmitting information within the nerve.

Previous research has shown that the reorganization of actin filaments plays a crucial role in promoting neural regeneration. Actin filaments, major constituents of the cytoskeleton that are formed through the process of actin polymerization and depolymerization, play a key role in maintaining cell shape and enabling cell motility. The actin-depolymerizing factor (ADF)/Cofilin is a known factor that promotes actin depolymerization. ADF/Cofilin regulates neurite outgrowth during development and is localized in the filopodia of growth cones (Flynn et al., 2012; Hylton et al., 2022). Additionally, studies have shown that overexpression of *Cofilin1* in injured sciatic nerves in mice induces axon elongation, thereby facilitating nerve regeneration (Tedeschi et al., 2019).

A number of factors have been recognized for their role in inducing synapse formation, which is critical for the transmission of information within neural networks (Connor & Siddiqui, 2023). Neurexins, serving as synapse organizers and cell adhesion factors, are single transmembrane proteins predominantly localized to the presynaptic region. Their function involves inducing synapse formation through interactions with neuroligins, expressed at postsynaptic sites (Gomez et al., 2021). In mammals, neurexins are encoded by three genes, with several promoters, encoding α-neurexin 1-3 and β-neurexin 1-3.

To achieve optic nerve regeneration through transplantation, we utilized the model organism *Drosophila*. In the *Drosophila* visual system, photoreceptor cells extend axons that project directly to the optic lobe. While the structure is notably less complex than that of mammalian systems, this model presents fewer barriers to axon outgrowth and displays a precise layered organization. Studies of the *Drosophila* visual system have extensively analyzed axon guidance and elongation, identifying a range of associated molecules (Hakeda-Suzuki et al., 2017; Takechi et al., 2021; Zang et al., 2021). Additionally, owing to the genetic manipulation tools available in *Drosophila*, it serves as an advantageous model organism for the comprehensive exploration of various genes, particularly in the context of transplantation studies. Previously, we established a method for transplanting photoreceptor precursor cells obtained from eye discs (Suzuki et al., 2018), enabling a detailed analysis of the factors pivotal in promoting optic nerve axon growth following transplantation. While cells overexpressing *N-cadherin* (*Ncad*), encoding a cell adhesion molecule, have been transplanted, their impact has been restricted to promoting axon elongation primarily within the retina, with minimal extension beyond the retina (Suzuki et al., 2018). Therefore, we further focused on cytoskeleton-related factors and synapse organizers.

Twinstar (*Tsr*), a cytoskeleton-related gene and the *Drosophila* homolog of ADF/Cofilin (Gunsalus et al., 1995), has been reported to be involved in neuroblast growth, axon elongation, and retina morphogenesis (Ng & Luo, 2004; Pham et al., 2008). In *Drosophila*, it has been shown that actin depolymerization and cleavage lead to cellular morphological alterations. Moreover, the synapse organizer Neurexin-1 (Nrx-1), the *Drosophila* homolog of α-neurexin, has been found to regulate synapse formation as in mammals (Li et al., 2007; Zeng et al., 2007). Nrx-1 has also been implicated in the maturation of rhodopsin, a vital component in phototransduction, as well as in microtubule stabilization (Banerjee & Riordan, 2018; Tian et al., 2013).

We confirmed the extension of axons from the retina to the lamina by modifying the selection process of transplanted cells. Our findings demonstrated that overexpression of *Ncad*, encoding a cell adhesion molecule, and *tsr*, encoding a factor which induces actin reorganization, significantly promotes axon elongation. Furthermore, in the lamina, we observed the formation of presynaptic structures in the elongated axons 6 days posttransplantation. Additionally, our results indicated that the introduction of *Nrx-1*, encoding a synapse organizer, accelerated the formation of presynaptic structures, suggesting the involvement of Nrx-1 in axon growth. Notably, we succeeded in replicating the precise projection of *Drosophila* photoreceptor cells to their target tissue, the medulla. Given the rarity of stable axonal projection to the brain in previous observations, the outcomes of this study are anticipated to offer valuable insights in order to achieve regeneration through transplantation.

## 2 | MATERIALS AND METHODS

2.1 | Fly strains

Flies were kept at 25°C. The following stocks were used: UAS-*tsr* (BDSC 9235), UAS-*tsr.S3A* (BDSC 9237), UAS-*tsr.S3E* (BDSC 9239) (Ng & Luo, 2004), UAS-*Nrx-1*-SAM (BDSC 82741), *GMR*-myr-RFP (BDSC 7120), UAS-myr-RFP (II) (BDSC 7118), *elav*-Gal4 (Luo

FIGURE 1 Transplantation method and its modification in *Drosophila*. (a) Schematic diagram of steps for transplantation. Take out the eye discs from third-instar larvae, add dispase and collagenase, and homogenize by hand. After incubation at 25°C for 4 h, aspirate cells with a glass capillary and transplant to the adult fly. (b) Schematic diagram of the *Drosophila* visual system. The *Drosophila* visual system consists of the retina and the brain ganglia, including the lamina and the medulla. Cells are transplanted into the retina (red). (c) Third-instar larval eye discs labeled with GMR-myr-RFP. Cells expressing RFP (magenta) represent developing photoreceptor cells. (d) Third-instar larval eye discs labeled with *elav*-Gal4, UAS-FLP, GMR-FRT-stop-FRT-Gal4, and UAS-myr-RFP (abbreviated as *elav*-FLP GMR-FsF-Gal4). Cells expressing RFP (magenta) represent developing neurons and photoreceptor cells. (e, f) Photoreceptor cells were stained with mAb24B10 (green). The yellow dotted line indicates the lamina region. (e) Section of an untransplanted adult head. (f) Transplantation of cells labeled with *elav*-FLP GMR-FsF-Gal4. Observation of transplanted cells (magenta) in the *Drosophila* retina. Axon elongation to the lamina was observed in 18.9% of flies (*n* = 37). Scale bars in (c–f): 50 μm.
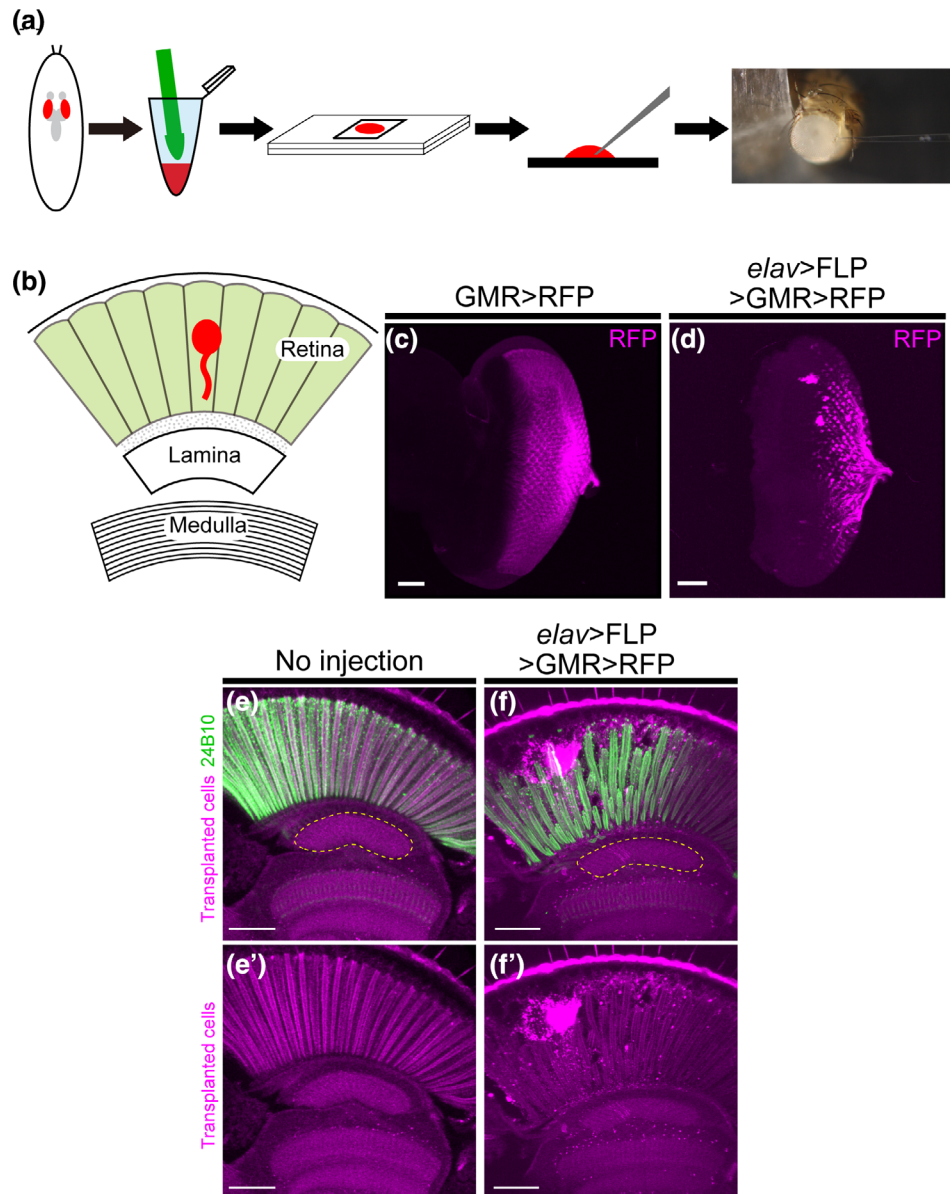
et al., 1994), UAS-FLP (BDSC 8208), GMR-FRT-stop-FRT-Gal4, and Brp-FRT-stop-FRT-GFP (Chen et al., 2014). Brp-FRT-stop-FRT-GFP (II) was generated by knock-in of the flip-out cassette with GFP (FRT-stop-FRT-GFP) into the *Brp* C-terminus.

2.2 | Transplantation

The experimental procedures for transplantation were described previously (Suzuki et al., 2018). The removed eye discs were dissociated and centrifuged at rcf 775g for 5 min. The precipitated cells were resuspended by pipetting and the cells were transferred to the chamber (Figure 1a). Coverslips coated with Concanavalin A (Wako) were placed over the chamber. Cells were incubated for 1.5 h with the coverslips down and for 2.5 h with the coverslips up in a humidity-controlled, light-shielded case (25°C). Glass capillaries were used for transplantation (0.75 mm inner diameter, 1 mm outer

diameter, NARISHIGE). Under an upright fluorescence microscope (Axio Plan2, ZEISS), cell clusters adhering to coverslips and expressing RFP were aspirated with a glass micropipette using a microinjector (NARISHIGE) filled with Mineral oil (Sigma). Parafilm was used to fix the heads of adult flies within 1 day after birth, and cell clusters were transplanted into the retina. Vaseline was also applied to the puncture site after transplantation to prevent the retina from drying out.

2.3 | Observation of transplanted flies

Transplanted flies were kept at 25°C for 3 or 6 days. The experimental procedures for agarose section, fixation, and immunostaining were described previously (Hakeda-Suzuki et al., 2011). Flies were sliced into 80 μm thick sections using a Linear slicer (Dosaka EM (D.S.K)). Samples were incubated with primary antibodies overnight at 4°C and with secondary antibodies at 25°C for 1 h. The following antibodies
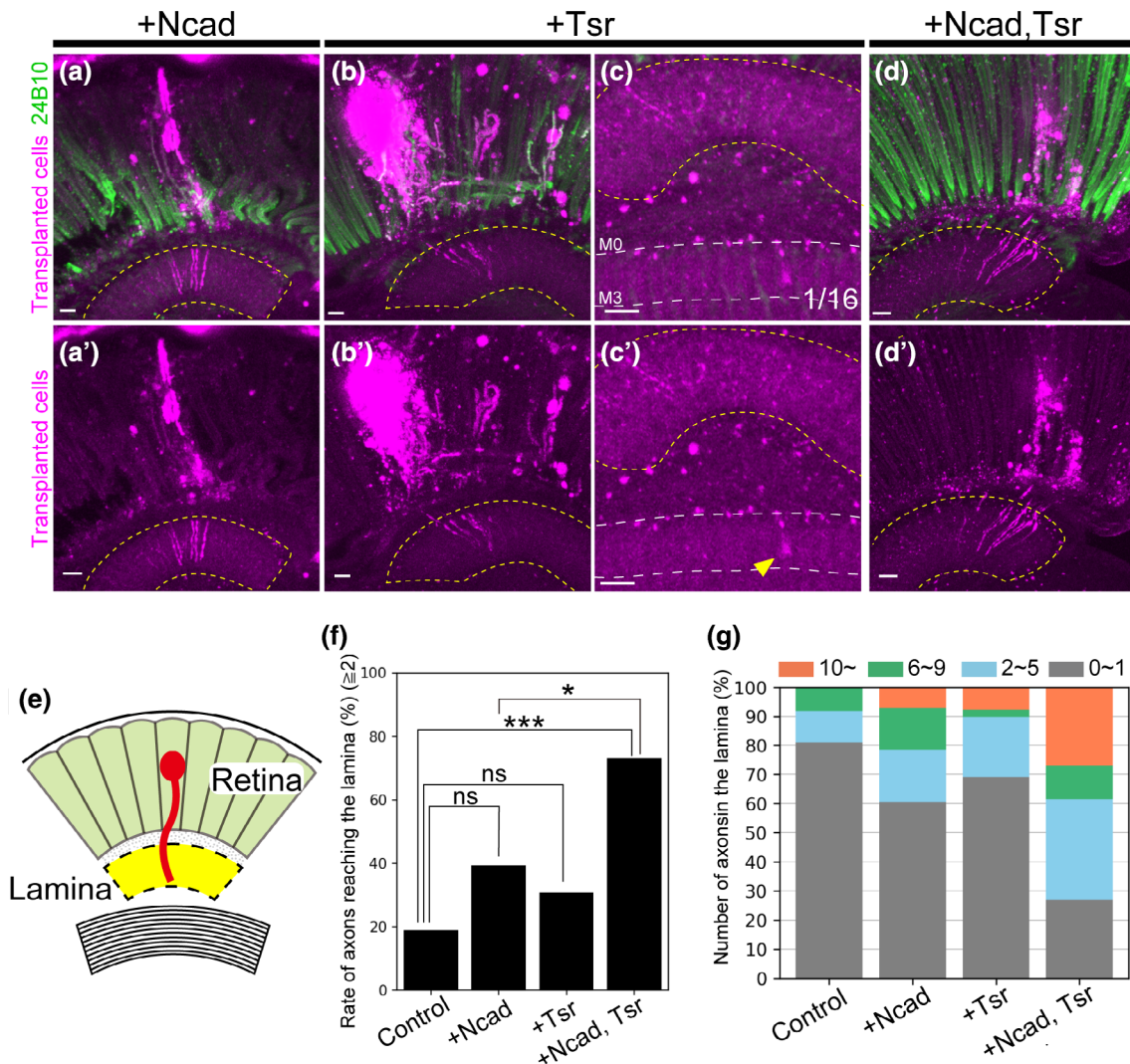
FIGURE 2 Transplantation of cells overexpressing N-cadherin and/or Twinstar. (a–d) Photoreceptor cells were stained with mAb24B10 (green). The yellow dotted line indicates the lamina region. (a, a') Cells overexpressing N-cadherin (*Ncad*) (magenta) showed axon elongation to the lamina in 39.3% of transplanted flies (*n* = 28). (b, b') Cells overexpressing Twinstar (*tsr*) (magenta) showed axon elongation to the lamina in 30.8% of transplanted flies (*n* = 39). (c, c') An axon elongating to the medulla was observed in one case of *tsr* overexpression. Yellow arrowheads indicate elongated axons, and white dotted lines indicate layers of the medulla. (d, d') Cells overexpressing *Ncad* and *tsr* showed axon elongating to the lamina in 73.1% of transplanted flies (*n* = 26). (e) Schematic of axon elongation to the lamina of transplanted cells. Axons reached the lamina (yellow) beyond the retina. (f) Percentage of flies with axon elongation to the lamina. Significant differences were observed between cells overexpressing *Ncad* and *tsr* and control cells and cells overexpressing *Ncad* alone. (g) Classification of the number of axons extending into the lamina. The number of axons was classified into four categories: ≤1 axon (gray), 2–5 axons (blue), 6–9 axons (green), and ≥10 axons (orange). In the control, no fly has more than 10 axons elongated to the lamina, while overexpression of *Ncad* and *tsr* resulted in more than five axons elongating to the lamina in about 40% of the transplanted flies. Scale bars in (a–d): 10 μm. n.s., $p \geq .05$, *$p < .05$, ***$p < .001$.

were used for immunohistochemistry: mAb24B10 (1:50, Developmental Studies Hybridoma Bank (DSHB)). The secondary antibodies were Alexa488- or Alexa633-conjugated (1:400, Life technologies).

2.4 | Observation of adult flies

The experimental procedures for adult brain dissection, fixation, and immunostaining were described previously (Hakeda-Suzuki et al., 2017). The following antibodies were used for immunohistochemistry:

mAb24B10 (1:50, DSHB). The secondary antibodies were Alexa633-conjugated (1:400, Life technologies).

2.5 | Imaging, quantification, and statistical analyses

All confocal images were obtained by a Nikon C2+ or Nikon A1 confocal microscope. The images were analyzed using NIS elements Imaris or Adobe Photoshop. Data in Figures 2, 3, and 5 were analyzed
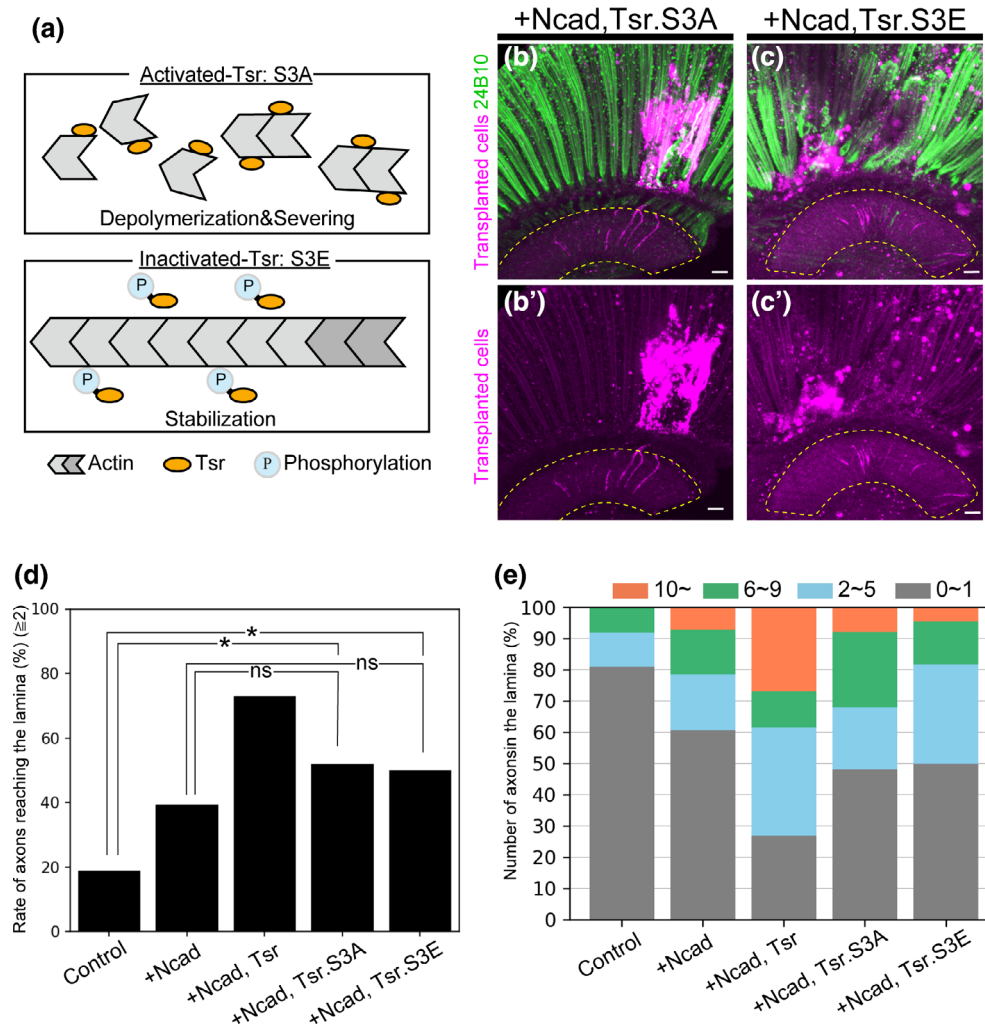
FIGURE 3 Transplantation of cells overexpressing Ncad and activated/inactivated Tsr. (a) Schematic diagram of activated/inactivated Tsr. Tsr is activated by dephosphorylation and inactivated by phosphorylation. (b, c) Photoreceptors were stained with mAb24B10 (green). The yellow dotted line indicates the lamina region. (b, b') Cells overexpressing *Ncad* and *tsr*.S3A (magenta) elongated axons to the lamina in 52% of transplanted flies ($n = 25$). (c, c') Cells overexpressing *Ncad* and *tsr*.S3E (magenta) elongated axons to the lamina in 50% of transplanted flies ($n = 22$). (d) Percentage of flies with axon elongation to the lamina. Significant differences were observed between cells overexpressing *Ncad* and *tsr*.S3A or *tsr*.S3E and control cells. No significant differences were observed compared to cells overexpressing *Ncad* alone. (e) Classification of the number of axons extending into the lamina. The number of axons was classified into four categories: ≤1 axon (gray), 2–5 axons (blue), 6–9 axons (green), and ≥10 axons (orange). When the activated/inactivated form of *tsr* was expressed, a decreasing trend in the number of axon elongations per fly was observed. Scale bar (b, c): 10 μm. n.s., $p \geqq .05$, *$p < .05$.

by Fisher's exact test using R Core Team (2023) (R Foundation for Statistical Computing, Vienna, Austria).

3 | RESULTS

3.1 | The modified transplantation method enhances axonal extension

To select cells for transplantation, cells were previously labeled using GMR-myr-RFP (Suzuki et al., 2018). GMR (Glass Multimer Reporter) originates from the promoter sequence of *Drosophila* Rh1, serving as the binding site for the transcription factor Glass (Ellis et al., 1993;

Hay et al., 1994). Glass exhibits expression not only in photoreceptors, which are neuronal cells, but also in cone cells and pigment cells of the eye, which are non-neuronal cells, suggesting that Glass plays a role in promoting the differentiation of non-neuronal cells (Ellis et al., 1993; Morrison et al., 2018; Moses & Rubin, 1991). These findings suggest that cells labeled with GMR-myr-RFP included non-neuronal cells. We hypothesized that the low percentage of photoreceptor cells in the transplanted cells prevented bundling of axons by non-neuronal cells, which would have limited axon growth of the transplanted cells to the retina. Therefore, we attempted to reduce non-neuronal cells by using *elav*-Gal4, a neuron-specific Gal4 driver.

In *Drosophila* transplantation, eye discs are initially extracted from third instar larvae, dissociated, and cultured. The cultured cells are

then transplanted into adult flies (Figure 1a). In the *Drosophila* visual system, photoreceptor cells extend axons from the retina, a component of the compound eye, projecting these axons to the lamina and medulla—ganglia in the brain. Having transplanted cells into the adult retina, we anticipated that the transplanted cells would project axons towards the lamina or medulla (Figure 1b). The transplanted cells were labeled by combining *elav*-Gal4 with UAS-FLP, GMR-FRT-stop-FRT-Gal4, and UAS-myr-RFP to selectively label only the neuronal photoreceptors among the cellular components of the compound eye (abbreviated as *elav*-FLP GMR-FsF-Gal4 hereinafter). This combined approach, involving *elav*-Gal4 and GMR-FRT-stop-FRT-Gal4, not only targeted neurons exclusively but also amplified the expression of RFP in the transplanted cells, thereby aiding in the visualization of these cells.

In the eye disc, cells labeled with *elav*-FLP GMR-FsF-Gal4 exhibited a reduction in labeled photoreceptor cells compared to those labeled with GMR-myr-RFP (Figure 1c,d). Previously, transplantation of cells labeled with GMR-myr-RFP resulted in axon elongation limited to the retina, without any observed axonal projection to the lamina (Suzuki et al., 2018). In contrast, upon transplantation of cells labeled with *elav*-FLP GMR-FsF-Gal4, transplanted cells were observed in the retina, and axon elongation towards the lamina was observed in 18.9% of the transplanted flies (Figure 1e,f). This occurrence might be attributed to the increased presence of photoreceptor cells among the transplanted cell population.

3.2 | Co-overexpression of *Ncad* and *tsr* promotes axonal extension in transplants

To enhance the axonal elongation of cells labeled with *elav*-FLP GMR-FsF-Gal4 towards the lamina, we transplanted cells overexpressing *Ncad* using the Gal4-UAS system. Previous investigations have demonstrated that the transplantation of cells overexpressing *Ncad*, encoding a cell adhesion factor, promotes axon elongation in the retina (Suzuki et al., 2018). Therefore, we attempted to enhance the reachability of the lamina by overexpressing *Ncad* in cells labeled with *elav*-FLP GMR-FsF-Gal4. Despite the transplantation of *Ncad*-overexpressing cells resulting in axon elongation to the lamina in 39.3% of the flies, there was no significant difference compared to controls (Figure 2a,e,f).

To further promote axon elongation to the lamina, we focused on Tsr, a *Drosophila* homolog of ADF/Cofilin. Tsr is implicated in actin dynamics, contributing to actin depolymerization and cleavage. Prior research has demonstrated that overexpression of *Cofilin1*, the mouse homolog of *Tsr*, in damaged mouse neurons promotes nerve regeneration (Tedeschi et al., 2019). Consequently, we attempted to transplant cells overexpressing *tsr*, leading to axonal elongation to the lamina in 30.8% of flies. However, no significant difference was observed compared to controls (Figure 2b,e,f). In one case, overexpression of *tsr* resulted in axon elongation beyond the lamina, extending into the M2 and M3 layers of the medulla (Figure 2c).

We presumed that overexpression of either *Ncad* or *tsr* would promote axon elongation to the lamina. However, no significant difference was observed compared to controls (Figure 2f). We considered that a combination of Ncad, promoting adhesion to surrounding tissues, and Tsr, generating a driving force through actin depolymerization and cleavage, could potentially promote axon elongation. Subsequently, we attempted to transplant cells overexpressing both *Ncad* and *tsr*. The transplantation of cells overexpressing both *Ncad* and *tsr* led to axon elongation to the lamina in 73.1% of flies. In comparison to controls and *Ncad* alone, overexpression of *Ncad* and *tsr* significantly promoted axon elongation (Figure 2d–f) (control: 18.9%).

We also analyzed the number of axons extending into the lamina per fly. In controls, no more than 10 axons extended into the lamina; however, when *Ncad* or *tsr* was overexpressed, more than 10 axons extended into the lamina in some flies. Furthermore, overexpression of *Ncad* and *tsr* led to more than five axons elongating to the lamina in about 40% of the transplanted flies, indicating an increase in the number of axons elongating to the lamina (Figure 2g).

These results suggest that the combination of Ncad, a cell adhesion factor, and Tsr, which facilitates actin reorganization, promotes axon elongation beyond the retina into the lamina.

3.3 | Both actin rearrangement and stabilization are crucial for axonal extension

Cofilin undergoes activation through dephosphorylation of its Ser3 residue and inactivation through phosphorylation of the same residue (Agnew et al., 1995; Moriyama et al., 1996). Activated Cofilin binds to actin, facilitating depolymerization, whereas inactivated Cofilin is unable to bind to actin, leading to the stabilization of the actin fiber (Figure 3a). Similarly, in Tsr, the *Drosophila* homolog of ADF/Cofilin, the unphosphorylated form of Tsr is active, while the phosphorylated form is inactive. To examine whether activated Tsr facilitates axon elongation of transplanted cells into the lamina or, conversely, whether inactivated Tsr inhibits axon elongation, we transplanted cells overexpressing activated/inactivated Tsr.

In the mushroom body, the other nervous system of the fly, the activated form of Tsr (Tsr.S3A) demonstrates a rescue of Tsr defects comparable to the wild type (Ng & Luo, 2004; Sudarsanam et al., 2020). Similarly, in mice, activated Cofilin1 has been observed to promote axon elongation to a degree equivalent to the wild type (Tedeschi et al., 2019). When we transplanted cells overexpressing *tsr.S3A*, mimicking the activated form, and *Ncad*, axon elongation to the lamina was observed in 52% of flies (Figure 3b,d). In contrast to activated Tsr, inactivated Tsr (Tsr.S3E) is known to be unable to sufficiently rescue Tsr defects in flies (Ng & Luo, 2004; Sudarsanam et al., 2020). Nonetheless, when *tsr.S3E* and *Ncad* were overexpressed, axon elongation to the lamina was observed in 50% of flies (Figure 3c,d).

The axonal elongation observed in both Ncad + activated Tsr (52%) and Ncad + inactivated Tsr (50%) does not show a significant difference compared to the overexpression of *Ncad* alone (Figure 3d).

However, a significant difference is noted when compared to controls. This suggests that the overexpression of *Ncad* and *tsr*, whether in the activated or inactivated form, tends to promote axonal elongation. However, the most notable enhancement of axon elongation is observed when *Ncad* is co-overexpressed with wild-type *tsr*. Comparing the number of elongated axons, the percentage of flies with 10 or more axons reaching the lamina was higher in wild-type *tsr* overexpression than in the activated/inactivated *tsr* overexpression (Figure 3d,e).

In transplantation, overexpression of either activated or inactivated *tsr* with *Ncad* did not promote axon elongation to the same degree as observed with overexpression of wild-type *tsr* and *Ncad*. Additionally, notable differences were observed in the number of axons reaching the lamina. These findings suggest that Tsr plays a crucial role in promoting axon elongation by reversibly cycling between phosphorylated and dephosphorylated states.

3.4 | Presynaptic structures can be formed in axons extended to the lamina

To regenerate neural circuits, synapses must be formed following axon elongation. To assess synapse formation in axons extending to the lamina from transplanted cells, we employed Brp as a presynaptic marker. Brp, a key component of the active zone in the presynapse, is visually observed as distinct dots in the lamina (Figure 4a,b). As the number of Brp dots labeled with GFP using the STaR method approximately corresponds to the number and distribution of T-bars observed through electron microscopy, Brp serves as a marker for both synapse construction and disassembly, making it a suitable indicator for presynapse formation (Araki et al., 2020; Chen et al., 2014; Osaka et al., 2022; Sugie et al., 2015). Consequently, we examined the formation of presynaptic structures in axons elongated to the lamina 3 days after transplantation by specifically labeling transplanted cells with Brp (Brp-GFP).

Transplantation of cells overexpressing *Ncad* and *tsr* with Brp-GFP revealed the presence of Brp-GFP signals throughout the axons extending to the lamina after 3 days. However, at this time point, the Brp-GFP signal pattern perfectly overlapped with the axon plasma membrane (RFP), and thus no obvious punctate signal indicative of presynapse formation was observed, suggesting that presynapse induction was not sufficiently triggered in the elongated axons (Figure 4c). Considering that the short duration between transplantation and fixation may have contributed to the absence of presynapse formation, we extended this period to 6 days. After 6 days, Brp-GFP signals perfectly overlapped with the axonal membrane in some flies as at 3 days. However, in two of the cases with axon elongation to the lamina, punctate signals stronger than the membranous RFP signal were observed at the lamina, confirming the formation of presynaptic structures (Figure 4d,e, *n* = 2/5).

In summary, overexpression of *Ncad* and *tsr* promotes axon elongation but necessitates a relatively extended period of 6 days for presynapse formation.

3.5 | Nrx-1 not only induces presynapse formation but also promotes axonal extension

To increase the efficiency of presynapse formation in axons extending into the lamina, we attempted to transplant cells overexpressing *Nrx-1* in addition to *Ncad* and *tsr*. Neurexins, as cell adhesion factors, are localized to the presynapse and are known to induce synapse formation (Gomez et al., 2021). *Drosophila* Nrx-1 has been demonstrated to be essential for synapse formation and exhibits widespread expression from embryonic to adult stages (Li et al., 2007; Zeng et al., 2007).

We transplanted cells overexpressing *Ncad*, *tsr*, and *Nrx-1* with Brp-GFP and observed axonal elongation after 3 days. Axonal elongation to the lamina was observed in 69.6% of the flies (Figure 5a,g,h). Notably, punctate Brp-GFP signals were observed in the lamina, confirming the formation of presynaptic structures, in 26.1% of the transplanted flies (Figure 5b,g,i, *n* = 6/23). Clearly, with overexpression of *Ncad*, *tsr*, and *Nrx-1* a trend towards enhanced presynaptic formation was observed in comparison to overexpression of *Ncad* and *tsr* (Figure 5i). Although this difference was not statistically significant, we proceeded to transplant cells overexpressing *Nrx-1* exclusively. This resulted in axon elongation to the lamina in 65% of the flies, with presynapse formation confirmed in 30% of the transplanted flies (Figure 5c,d,g,h,i, *n* = 6/20).

As 65% of axons reached the lamina, exclusive overexpression of *Nrx-1* represented a significant difference compared to controls. This suggests that Nrx-1 may not only enhance the efficiency of synapse formation but also play a role in axon elongation to the lamina. Therefore, we transplanted cells overexpressing *tsr* and *Nrx-1* to investigate whether the combination of Tsr, promoting cytoskeletal reorganization, and Nrx-1, a cell adhesion molecule, would enhance axonal elongation to the lamina. The transplantation of cells overexpressing *tsr* and *Nrx-1* resulted in axon elongation to the lamina in 76.2% of the transplanted flies, the highest percentage observed (Figure 5e–h). Punctate Brp-GFP signals were observed in 52.4% of the transplanted flies, confirming the formation of presynaptic structures (Figure 5f,i, *n* = 11/21). Notably, in terms of the number of elongated axons per fly, overexpression of *Nrx-1* alone yielded the highest percentage of flies with 10 or more axons (Figure 5j).

The introduction of *Nrx-1* shortened the time to presynapse formation. While the primary purpose of introducing *Nrx-1* was to induce presynapse formation, all combinations with additional Nrx-1 seemed to promote both axon elongation and presynapse formation. Notably, axon elongation to the lamina was observed even when only *Nrx-1* was overexpressed, suggesting the involvement of Nrx-1 in axon elongation.

3.6 | Transplanted cells elongate axons to the medulla beyond the lamina

We successfully verified the axon elongation of transplanted cells to the lamina beyond the retina, as well as the furthest target tissue of the photoreceptor, the medulla. When only *tsr* was overexpressed,
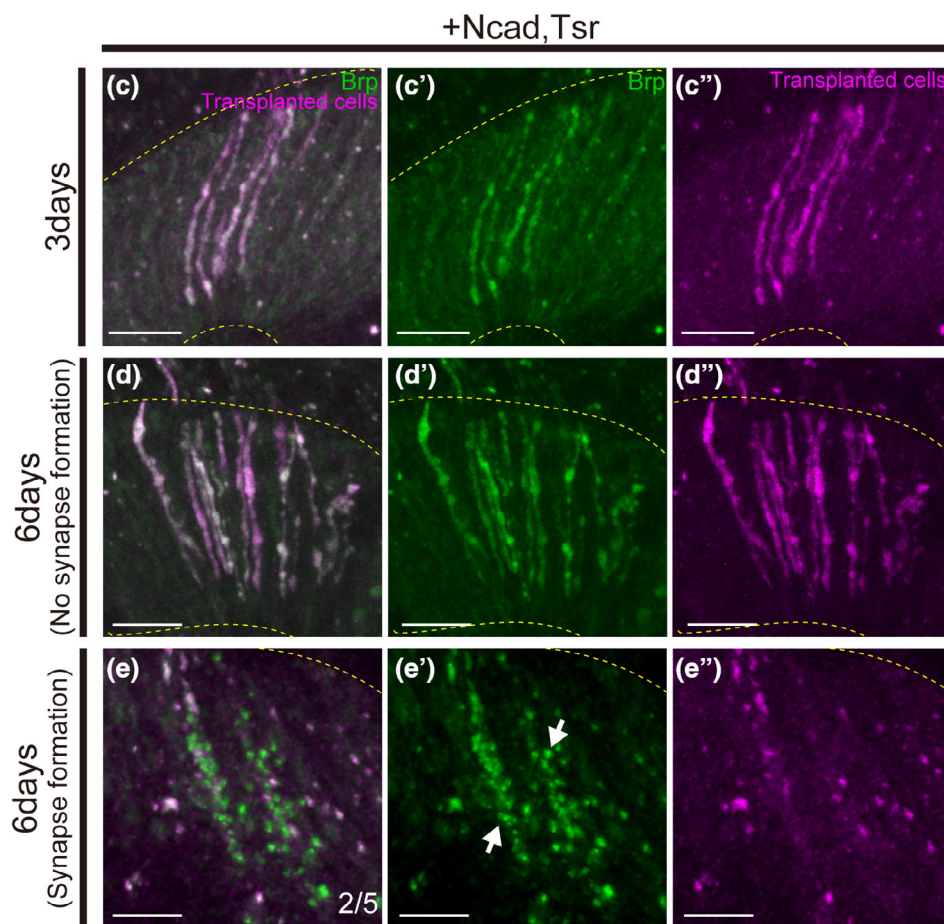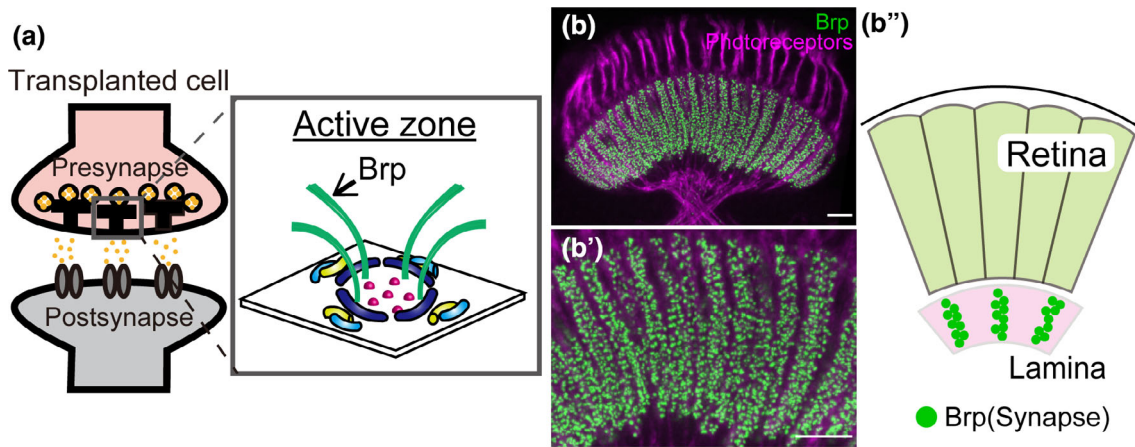
FIGURE 4 Presynapse formation in axons elongating into the lamina. (a) Schematic diagram of the synapse. The active zone is a structure present in the presynapse, and Brp is one of the active zone components. (b) Localization of endogenous Brp in the adult Lamina. Brp (green) is observed as dots in the lamina. Photoreceptor cells were stained with mAb24B10 (magenta). (c–e) Transplanted cells are labeled with RFP (magenta), Brp is labeled with GFP (green). The yellow dotted line indicates the lamina region. (c–c″) Cells overexpressing *Ncad* and *tsr* with Brp-GFP showed axon elongation to the lamina 3 days after transplantation and a Brp signal throughout elongated axons ($n = 10/16$). No punctate signal was observed. (d–d″) At 6 days after transplantation, axon elongation to the lamina was observed, and the Brp signal was detected throughout axons ($n = 5/7$). (e–e″) Punctate signals (green) were observed in the lamina in two of the cases elongating axons to the lamina ($n = 2/5$). In this fly 25 or 33 synapses were observed. The white arrow indicates the distinct synapse. Scale bar (b–e): 10 μm.

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one case was observed with axon elongation reaching the M2–M4 layers of the medulla beyond the lamina (Figures 2c, 6f). When only *Nrx-1* was overexpressed, we observed axon elongation to the lamina and the formation of presynaptic structures. Additionally, in one case, axon elongation to the medulla was noted, accompanied by the formation of presynaptic structures in the elongated axon (Figure 6a,b,e,f). When *tsr* and *Nrx-1* were overexpressed, axon elongation to the medulla was observed in four samples with axons extending to the lamina. The most extended axon reached the M6 layer, and the formation of presynaptic structures was confirmed in
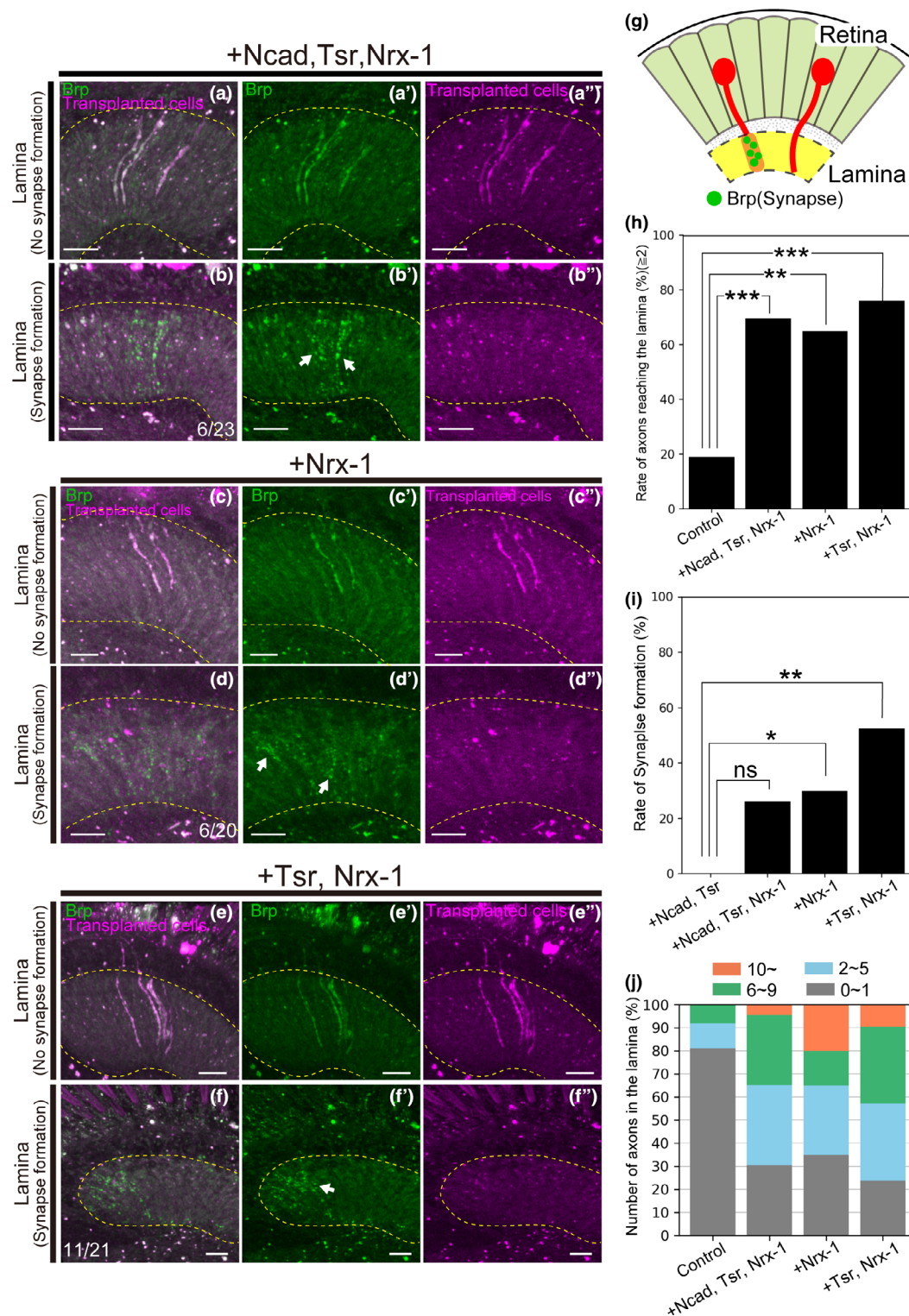

FIGURE 5 Legend on next page.

half of the flies with axon elongation to the medulla (Figure 6c–f). One case of axon elongation beyond the lamina to the medulla was also observed when activated/inactivated *tsr* and *Ncad* were overexpressed or when *Ncad*, *tsr*, and *Nrx-1* were overexpressed (Figure 6f). When *Ncad* and *tsr* were overexpressed, axon elongation to the lamina was not observed (Figure 6f). To examine the cell types of transplanted cells, the results of staining with an antibody against Rh1, an R1–R6 photoreceptor-specific rhodopsin, showed that few cells overexpressing *Ncad* and *tsr* were stained with Rh1 (data not shown). R7 and R8 photoreceptors project to the medulla, and based on the staining results, the transplanted cells with axons reaching the lamina or the medulla are likely to be R7 or R8 photoreceptors. However, rhodopsin is expressed during the late pupal stage (Earl & Britt, 2006). In the present study, cells taken from the eye discs of third instar larvae were transplanted and observed 3 days (72 h) later, potentially overlapping with the period of rhodopsin expression. Consequently, there is a possibility that the staining was conducted when rhodopsin levels were too low to be detected. Thus, we cannot exclude the possibility that the transplanted cells may have comprised R1–R6 photoreceptors.

Although the elongation to the medulla was limited, we successfully confirmed axonal elongation of transplanted cells to the medulla, the furthest target tissue of *Drosophila* photoreceptor cells, by introducing *Ncad*, *tsr*, and *Nrx-1*. While no clear correlation was observed between Ncad, Tsr, and Nrx-1, overexpression of *Nrx-1* tended to promote axon elongation to the medulla. In particular, axon elongation to the medulla was most frequently observed when cells overexpressing *Nrx-1* and *tsr* were transplanted. The exact mechanism underlying the induction of axon elongation to the medulla by these factors remains unclear and constitutes a subject for future investigation. These results suggest that Nrx-1 may play a role not only in the formation of presynaptic structures but also in axon growth to the medulla.

4 | DISCUSSION

In this study, we enhanced the axonal extension of transplanted cells from the retina to the lamina by modifying the selection process of transplanted cells. Furthermore, our results showed that overexpression of *Ncad* and *tsr* in cells selected through this process facilitates axon elongation. Presynapse formation in the elongated axons in the lamina was confirmed 6 days after transplantation. The introduction of *Nrx-1* led to a significant reduction in the time required for presynapse formation, shortening it to 3 days, suggesting that Nrx-1 not only promotes presynapse formation but is also involved in axon elongation. Overexpression of *Ncad*, *tsr*, or *Nrx-1* promoted axonal projection beyond the retina, successfully replicating the accurate projection of *Drosophila* photoreceptor cells to their furthest target tissue, the medulla. In conclusion, these results suggest that overexpression of *Ncad*, *tsr*, and *Nrx-1* is associated with the promotion of axon elongation in transplantation. These factors emerge as effective contributors to the regeneration of neural circuits through transplantation.

4.1 | Actin depolymerization and cell adhesion promote axon elongation

Axon elongation is thought to occur with the driving force of retrograde actin flow by actin turnover and Myosin II in the growth cone and adhesion to surrounding tissues, which pushes the cell membrane in the direction of elongation, thereby protruding the growth cone (Dent & Gertler, 2003; Lowery & Van Vactor, 2009; Suter & Forscher, 1998; Vitriol & Zheng, 2012). ADF/Cofilin plays a critical role in actin turnover and retrograde actin flow, contributing to neurite formation (Flynn et al., 2012; Tedeschi et al., 2019). We attempted to promote axon extension beyond the retina into the lamina by introducing *tsr*, which encodes a protein that induces actin reorganization. Previous research has demonstrated that Cofilin1, a protein that facilitates actin depolymerization and cleavage, promotes the elongation of injured axons in the central nervous system of mice (Tedeschi et al., 2019). Nevertheless, overexpression of *tsr* alone, a homolog of ADF/Cofilin, in transplanted cells did not markedly enhance axon elongation to the lamina beyond the retina, contrasting with findings from previous studies. In transplantation, cells conditioned in vitro must extend axons within the organism, having already completed their development. The expression of factors promoting actin

FIGURE 5 Nrx-1 shortens the latency and promotes the formation of presynaptic structures. (a–f) Transplanted cells were labeled with RFP (magenta). Photoreceptors were stained with mAb24B10 (green). The yellow dotted line indicates the lamina region. (a-a") Cells overexpressing *Ncad*, *tsr*, and *Nrx-1* with Brp-GFP elongated axons to the lamina in 69.6% of flies 3 days after transplantation ($n = 23$). Brp signals throughout the elongated axons were observed. (b–b") Punctate Brp signals were identified on the elongated axons in 26.1% of transplanted flies ($n = 6/23$). (c–c″) Cells overexpressing *Nrx-1* with Brp-GFP elongated axons to the lamina in 65% of flies 3 days after transplantation ($n = 20$). Brp signals throughout the elongated axons were observed. (d–d″) Punctate Brp signals were identified on the elongated axons in 30% of transplanted flies ($n = 6/20$). (e-e") Cells overexpressing *tsr* and *Nrx-1* with Brp-GFP elongated axons to the lamina in 76.2% of flies 3 days after transplantation ($n = 21$). Brp signals throughout the elongated axons were observed. (f–f″) Punctate Brp signals were identified on the elongated axons in 52.4% of transplanted flies ($n = 11/21$). (g) Schematic of axons that formed and did not form synapses in the lamina. (h) Percentage of flies with axon elongation to the lamina. Significant differences were observed between cells overexpressing *Ncad*, *tsr*, and *Nrx-1*, overexpressing *Nrx-1*, or overexpressing *tsr* and *Nrx-1* and controls. (i) Percentage of presynapse formation in extended axons. Significant differences were observed between cells overexpressing *Nrx-1* or overexpressing *tsr* and *Nrx-1* and cells overexpressing *Ncad* and *tsr*. (j) Classification of the number of axons extending into the lamina. The number of axons was classified into four categories: ≤1 axon (gray), 2–5 axons (blue), 6–9 axons (green), and ≥10 axons (orange). The percentage of flies with 10 or more elongating axons was the highest in *Nrx-1* overexpression. Scale bar (a–f):10 μm n.s., $p \geqq .05$, *$p < .05$, **$p < .01$, ***$p < .001$.
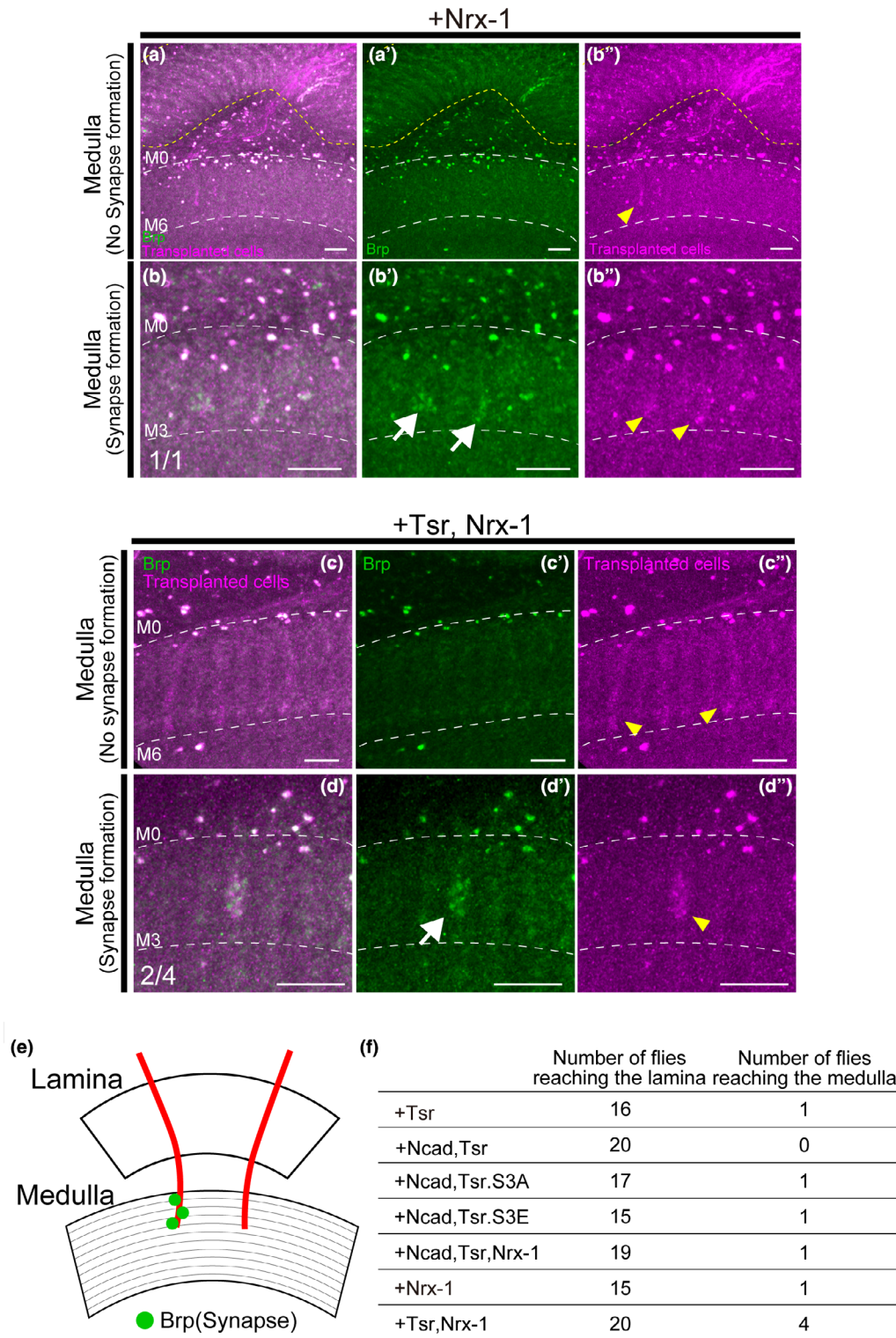
FIGURE 6 Axon elongation of transplanted cells beyond the lamina to the medulla. (a–d) Transplanted cells are labeled with RFP (magenta), Brp is labeled with GFP (green). The yellow dotted line indicates the lamina region. Yellow arrowheads indicate axons and white arrows indicate distinct synapses. (a–a") Cells overexpressing *Nrx-1* elongated axons to the M2–M5 layers of the medulla in one case among flies with axon elongation to the lamina ($n = 1/15$). (b–b") Punctate Brp signals were found on the axons elongating to the medulla of cells overexpressing *Nrx-1*. (c–c") Cells overexpressing *tsr* and *Nrx-1* elongated axons to the medulla in four cases among flies with axon elongation to the lamina ($n = 4/20$). The most elongated axons reached the M6 layer of the medulla. (d–d") Punctate Brp signals were found on the axons elongating to the medulla of cells overexpressing *tsr* and *Nrx-1*. (e) Schematic of axons that elongated to the medulla and formed synapses and axons that did not form synapses. (f) The number of flies with axon elongation to the medulla among flies with axon elongation to the lamina. The rate of reaching the medulla was higher in transplants of cells overexpressing *tsr* and *Nrx-1*. Scale bar (a–d): 10 μm.

turnover and increasing retrograde actin flow, such as Tsr, is expected to be insufficient for stabilizing axons due to their inability to adhere to the surrounding tissue. Consequently, we assumed that the adaptation to the transplanted environment is hindered, and Tsr alone does not adequately facilitate axon elongation into the lamina.

Ncad, which promotes axon elongation when co-expressed with Tsr, plays a role in stabilizing growth cones through filopodial dynamics (Mehmet Neset Özel et al., 2015). The cytoplasmic domain of cadherin binds to β-catenin, which associates with actin fibers via α-catenin, leading to enhanced cell adhesion (Noordstra et al., 2023). The driving force generated by the increased retrograde actin flow due to enhanced actin turnover by overexpression of *tsr*, combined with stable adhesion to the surrounding environment via catenin by Ncad, may have pushed the plasma membrane in the direction of elongation and protruded the growth cone. Consequently, axon elongation beyond the retina into the lamina could be induced.

Overexpression of *Ncad* and the activated/inactivated form of *tsr* did not exhibit a significant difference compared to *Ncad* overexpression alone, although a notable distinction was observed compared to controls, suggesting a tendency to promote axon elongation regardless of whether Tsr is active or inactive. Previous research has demonstrated that activated Cofilin promotes axon elongation to a similar extent as the wild type (Endo et al., 2003; Tedeschi et al., 2019), reinforcing the notion that activated Tsr plays a crucial role in axon elongation. On the contrary, the influence of inactivated Tsr on axon elongation has been reported to be both promoting and non-promoting (Ng & Luo, 2004; Sudarsanam et al., 2020). Our findings indicated that overexpression of *Ncad* and the inactivated form of *tsr* tended to facilitate axon elongation compared to controls. Two potential reasons for this promotion are considered. The first reason is actin stabilization. Since axon elongation was observed in control cells, it is presumed that the transplanted cells inherently possessed the ability to elongate axons. The introduction of the inactivated form of *tsr* is thought to have inhibited the depolymerization of actin fibers, leading to their stabilization. This may explain the observation of axons in the lamina without retraction. The second possibility is a shift in the ratio of inactivated to activated Tsr. Tsr (Cofilin) undergoes phosphorylation and dephosphorylation, regulated by LIM-kinases and Slingshot (Van Troys et al., 2008). Since our experiments were conducted in the presence of endogenous Tsr, overexpression of the inactivated form of *tsr* might have influenced axon elongation by modulating the ratio of activated to inactivated forms of endogenous Tsr. The role of the inactivated form of Tsr in axon elongation remains controversial. While overexpression of *Ncad* and activated/inactivated *tsr* promoted axon elongation compared to controls, it reduced the number of axons reaching the lamina, implying that either activation or inactivation alone is insufficient for axon elongation. Hence, a reversible change in the phosphorylated and dephosphorylated forms of Tsr is considered crucial for the process of axon elongation.

While the introduction of *Nrx-1* was intended to induce synapse formation, its potential involvement in axon elongation is also suggested. Previous studies have identified molecules that play dual roles in both synapse formation and axon stabilization (Astigarraga et al., 2010; Hakeda-Suzuki et al., 2017; Holbrook et al., 2012), suggesting that synapse formation causes axonal stabilization (Özel et al., 2019). One potential explanation for the dual role of Nrx-1, promoting both synapse formation and axon elongation, could be that the synapses induced by Nrx-1 contribute to axonal stabilization, thereby suppressing axonal retraction. Another potential mechanism contributing to the role of Nrx-1 in axon elongation is the stabilization of microtubules. Previous research showed that the stabilization of microtubules is pivotal for axon elongation (Blanquie & Bradke, 2018). It is known that Nrx-1 has genetic interactions with the microtubule-associated factor Futsch and that microtubule cleavage is triggered by *Nrx-1* expression (Banerjee & Riordan, 2018). Our observations of axon elongation to the lamina in controls suggest that the transplanted cells inherently possessed the ability to elongate axons. Axon elongation to the lamina was observed even in the transplantation of cells overexpressing *Nrx-1* alone, which is presumably attributed to the non-retraction of elongated axons resulting from microtubule stabilization induced by Nrx-1. Moreover, the greatest axon elongation was observed after overexpression of *tsr* and *Nrx-1*, which may be related to the actin ring. Actin is present not only in growth cones but also in axons, forming a ring structure called an actin ring (Xu et al., 2013). Previous research indicates that actin rings play a role in microtubule stabilization (Qu et al., 2017). The depolymerization and cleavage of actin induced by Tsr may have influenced the formation of actin rings, consequently stabilizing the microtubules within the axons and ultimately promoting axon elongation.

4.2 | Presynapse formation in extended axons is induced by Nrx-1

In transplantation of cells overexpressing *Ncad* and *tsr*, presynapse formation could not be confirmed 3 days after transplantation, but it could be confirmed 6 days later. While the RFP signal was weak in regions where the Brp-GFP signal was detected, we attribute this to the thickening of the elongated axons as they matured. The presence of debris is typically associated with neurodegeneration (Osaka et al., 2022). If the observed Brp-GFP signals were indicative of neurodegeneration, a corresponding RFP signal would be expected. However, no such RFP signal was observed. These findings suggest that the axons undergoing synaptogenesis became thicker as they matured, leading to a diminished RFP signal, with only the Brp-GFP signal being detectable. The delayed formation of presynaptic structures by cells overexpressing *Ncad* and *tsr* may be attributed to the role of Ncad in stabilizing synapse formation rather than initiating it. Previous studies have demonstrated that Ncad plays a role in stabilizing synapses but is not indispensable for the actual formation of synapses (Suzuki & Takeichi, 2008). Moreover, other synaptic organizers have been identified (Connor & Siddiqui, 2023). Overexpression of *Ncad* alone might not be fully capable of initiating synapse formation.

Overexpression of *Nrx-1* promoted the formation of presynaptic structures. Previous studies have demonstrated that Nrx-1 is a key

organizer of synapse formation (Gomez et al., 2021). Consistent with this, our results showed that Nrx-1 significantly curtails the time of presynapse formation, emphasizing its role in promoting formation of presynaptic structures. In contrast, synapse formation was observed in approximately half of the transplanted animals. To enhance the rate of synapse formation, molecules associated with synapses, such as Munc13-1 and RIM, known to reside in the active zone and play a role in priming synaptic vesicles (Südhof, 2012), have been shown to inhibit axon elongation (Hilton et al., 2022). Considering that synapse formation may contribute to axon stabilization, as discussed earlier, the expression of synapse-related molecules during the transplantation stage could impede axon elongation. Therefore, precise timing regulation, such as employing a light-dependent gene expression system (de Mena & Rincon-Limas, 2020; Qian et al., 2023), might be crucial to control the expression timing effectively.

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