Kinesin-1 sorting in axons controls differential retraction of arbor terminals

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SUMMARY STATEMENT

Axonal transport and microtubule dynamics are differentially regulated between terminals in a single axon, and contribute to shaping the axonal arbor by differentially regulating retraction of terminals.

ABSTRACT

The ability of neurons to generate multiple arbor terminals from a single axon is crucial for establishing proper neuronal wiring. While growth and retraction of arbor terminals are differentially regulated within the axon, the mechanisms by which neurons locally control their structure remain largely unknown. In the present study, we found that the head domain of kinesin-1 (K5H) preferentially marks a subset of arbor terminals. Time-lapse imaging clarified that these arbor terminals are more stable than others, because of low retraction rate. Local inhibition of kinesin-1 in the arbor terminal by chromophore-assisted light inactivation (CALI) enhanced retraction rate. The microtubule turnover is locally regulated depending on the length from the branching point to the terminal end, but does not directly correlate with K5H. Conversely, F-actin signal values in arbor terminals correlate spatiotemporally with K5H, and inhibition of actin turnover prevented retraction. Results from the present study revealed a novel system mediated by kinesin-1 sorting in axons, which differentially controls stability of arbor terminals.
INTRODUCTION

To connect with multiple target cells, neurons elaborate the axonal arbor by controlling growth and retraction during development (Gibson and Ma, 2011; Kalil and Dent, 2014). Previous *in vivo* (Portera-Cailliau et al., 2005; Hua et al., 2005; Meyer and Smith, 2006; Stettler et al., 2006; Nishiyama et al., 2007) and *in vitro* (Bastmeyer and O’Leary, 1996; Ruthel and Hollenbeck, 2000) studies have revealed the diverse plasticity of arbor terminals in a single axon. The competition between different terminals of a single axon in cultured neurons (Hutchins and Kalil, 2008) suggests that neurons possess an intracellular system that coordinates the branched axonal shape by regulating growth and retraction of arbor terminals in a region-specific manner. Despite intensive studies on the axonal arborization, little is known about the intracellular mechanisms mediating terminal-dependent control of growth and retraction in the axonal arbor.

In collateral branch formation, filopodial and lamellipodial protrusions, which contain F-actin bundles, emerge from actin patches found along the axon shaft. Newly formed branches are then invaded by microtubules to become mature axonal branches (Gallo and Letourneau, 1999; Gallo, 2011; Lewis et al., 2013). It is thought that the balance between stabilization and destabilization of F-actin and microtubules determines the formation and growth of axonal branches. For example, activation of the actin nucleation factors, Arp2/3 and Cordon-bleu is required for filopodia formation from the actin patch, and inhibition of this pathway results in decreased branch number (Strasser et al., 2004; Ahuja et al., 2007; Spillane et al., 2011, 2012). The microtubule-severing enzymes, spastin and katanin, which provide a branching point at the axonal shaft, also control the number of axonal branches (Yu et al., 2008). In contrast, neurons that lack Kif2a, a kinesin family molecule that depolymerizes microtubules, exhibit increased axonal branch length (Homma et al., 2003). Thus, local control of F-actin and microtubule dynamics is crucial for shaping the axonal arbor. Nevertheless, intracellular systems that regulate individual arbor terminals in the axon have not been fully demonstrated.

Calcium is a major intracellular signaling molecule that regulates axonal growth (Kater and Mills 1991; Gomez and Spitzer, 1999; Tang and Kalil, 2005; Ageta-Ishihara et al., 2009). Previous studies suggest that localized Ca$^{2+}$ transients in restricted areas in
the arbor enhance axonal growth, accompanied by retraction of other terminal (Hutchins and Kalil, 2008). Cyclic nucleotides are thought to be candidates that send long-range signals mediating the competition (Hutchins, 2010), given that in axonal specification, the activation of cAMP in a single neurite mutually inhibits cAMP in other neurites to form a single axon (Shelly et al., 2010). The molecular mechanisms whereby these signals coordinately regulate extension or retraction of different arbor terminals have not been elucidated.

Kinesin-driven anterograde transport along microtubules has been shown to play critical roles in maintaining axonal morphology and function (Goldstein, 2001; Hirokawa and Takemura 2005; Salinas et al., 2008; Maday et al., 2014). Conventional kinesin (kinesin-1) consists of heavy chain (Kif5) dimmer and light chains. Kif5 contains a motor domain (also called the head domain) that hydrolyses ATP and moves along microtubules, whereas the carboxyl-terminal tail domain is required for cargo interactions. Because the tail domain inhibits motor function when not bound to cargos, deleting the tail domain results in constitutive activation of Kif5 (Coy et al., 1999). Intriguingly, the cleaved motor (head) domain of Kif5 (K5H) is selectively delivered to axons, but not dendrites (Nakata and Hirokawa, 2003; Jacobson et al., 2006), indicating the ability of K5H to discriminate between axons and dendrites. The control of microtubule dynamics has been shown to depend on the region within the single cell (Witte et al., 2008), and microtubule dynamics affect tubulin states, such as GDP/GTP binding, acetylation and tyrosination/detyrosination, and decoration by MAPs. Recent results from several reports, including from our group, suggest that K5H recognizes differences between microtubules on axon and dendrites (Reed et al., 2006; Konishi and Setou, 2009; Hammond et al., 2010; Nakata et al., 2011). Nevertheless, very little is known about the regulation of axonal transport within the arbor. Therefore, in the current study, we asked if there is any difference in efficiency of kinein-1-dependent axonal transport between terminals of a single axonal arbor. Results showed a selective K5H accumulation in a subset of axonal terminals, which contributed to the shape of the axonal arbor by differentially regulating retraction.
RESULTS

Kinesin-1 preferentially accumulates in a subset of axonal terminals

To study terminal dependency of kinesin-1-mediated axonal transport, we utilized cerebellar granule neurons (CGNs) that possess relatively simple axonal arbors (Bilimoria et al., 2010; Ito-Ishida et al., 2012). In culture, CGNs extend one or two axons that possess several arbor terminals (Bilimoria et al., 2010; Kubota et al., 2013) (Fig. 1A), thus are thought to be suitable for studying the diversity of arbor terminals. During the axonal arborization of CGNs in vitro, elimination of main axon terminal as well as massive extension of collateral branch can be observed (Fig. S1). In the current study, the analyses were carried out without distinguishing between main axonal process and branch formed through collateralization or bifurcation.

CGNs introduced with an expression vector for K5H-EGFP (Kif5 head was fused to EGFP) were fixed at 7 DIV. As previously described (Nakata and Hirokawa, 2003; Jacobson et al., 2006), K5H selectively accumulates in the axon terminals (Fig. 1A). The signal intensity of K5H in each terminal varied largely, even in the same axonal arbor (Fig. 1A, arrowheads). To objectively evaluate K5H signal variation, signals at the distal terminal pairs were quantified and relative signal levels were calculated. Simultaneously, the relative length of terminal segment (from the branching point to the terminal end) was calculated as shown in Fig. 1B, and terminal segments were categorized merely based on the length. If K5H molecules are randomly delivered into two neighbouring terminals, the distribution of relative signal values should follow normal distribution. The quantile-quantile (q-q) plot of sample quantiles of relative K5H values vs. theoretical quantiles did not lie on a straight diagonal line, because some fractions revealed low (close to 0) or high (close to 1) relative K5H values (Fig. 1C), indicating that data were not normally distributed. The scatter plot of relative K5H values vs. the relative length of the same terminal pair revealed a mild, but significant, positive correlation (Fig. 1D, $r = 0.62$, $p < 0.001$, $n = 64$). These results indicated that K5H is preferentially delivered into a subset of arbor terminals that tend to be longer than neighbouring terminals in the CGNs.

We also cultured CGN at a low density (Kubota et al., 2013), and subjected the cells to immunocytochemistry (at 5 DIV) using antibodies specific to kinesin-1 (Fig. 1E) as previously described (Konishi and Setou, 2009). Results showed that
endogenous kinesin-1 signals in longer terminal segments are greater than in shorter terminal segments (Fig. 1E, $p < 0.01$, $n = 12$), suggesting that results observed by using K5H represent, at least in part, the nature of endogenous kinesin-1-mediated axonal transport. In addition, when we disrupted kinesin-1-mediated axonal transport by expressing the cargo binding (tail) domain (K5T), we found that length from branching point to axonal terminal became shorter compared with control neurons introduced with empty vector or vector for stalk region (K5S) (Fig. 1F, $p < 0.05$, $n = 37$). These results support our notion that selective delivery of kinesin-1 in a subset of arbor terminals might coordinate the shape of axonal arbors by controlling length of terminal segments.

**Retraction is inhibited in axonal terminals with accumulated K5H**

To determine whether kinesin-1-mediated transport efficiency in arbor terminals correlates with elongation or stability, we simultaneously observed changes in axonal morphology and K5H distribution within the axonal arbor by fluorescent time-lapse imaging. Although a majority of arbor terminals were stable, both growth and retraction occurred in a terminal-dependent manner at 5 DIV (Fig. 2A, B). K5H signals stably accumulated in a subset of arbor terminals in the most cases, but signals occasionally changed location to the other terminals. The rate of terminal growth was determined by differentiating the length of terminal segment at each time period. The values for elongation (i.e., positive growth) and retraction (i.e., negative growth) for each arbor terminal were then extracted. The scatter plot revealed that the average rate of elongation and retraction largely varied depending on the terminal (Fig. 2C, D). Comparisons of the average elongation rate between lower K5H terminals (i.e., relative K5H value $< 0.1$, $n = 77$) and K5H-enriched terminals (i.e., relative K5H value $> 0.1$, $n = 20$) revealed a significant median shift (0.17 μm/hr in lower K5H vs. 0.75 μm/hr in higher K5H, $p < 0.001$, Mann–Whitney U-test). Nevertheless, the relationship between K5H signal values and elongation rates remains unclear, because the high rate of elongation frequently occurred even in K5H-deprived arbor terminals, and also was not remarkable in terminals that exhibited very high K5H signals (Fig. 2C). Additionally, some arbor terminals deprived with K5H exhibited no growth rate, because they were removed by retraction within a short period of time. Conversely, the average retraction rate was consistently low in K5H-enriched arbor terminals (Fig. 2D), with statistical significance differences in median (0.90 μm/hr in lower K5H vs. 0.57 μm/hr in higher
K5H, $p < 0.05$, Mann-Whitney U-test). Consequently, while K5H-deprived terminal segments tended to become shorter, K5H-enriched terminal segments became longer or kept their length (Fig. 2E). Following these observations, we decided to focus our analysis on the relationship between differential terminal retraction and efficiency of kinein-1-mediated axonal transport in the arbor.

**Local inhibition of kinesin-1 enhanced retraction rate of arbor terminal**

We then applied chromophore-assisted light inactivation (CALI) to inhibit kinesin-1 activity in a terminal-dependent manner. KillerRed (KR) has been used as a genetically encoded photosensitizer for CALI (Destaing et al., 2010; Baumgart et al., 2012; Sano et al., 2014). To inhibit endogenous kinesin-1, CGNs were expressed with fusion protein consisting of KR and kinesin light chain (KR-KLC). KR-KLC fluorescence within a small circular area can be selectively bleached by illumination with a mercury lamp through the iris. About 60% and 80% loss of KR-KLC signal was observed within 30 s and 60 s of exposure, respectively (Fig. 3A, B). At axonal terminal segment, KR and KR-KLC were distributed throughout the process (Fig. 3C, D). Unlike K5H, it is expected that KR-KLC proteins exist as various state (e.g. as free forms, and as kinesin complex binding to various type of cargos). We illuminated axonal terminal and monitored fluorescence recovery. Recovery of KR signal reached a maximum in the first frame (15 min) after bleaching, at which half of signal relative to the pre-illumination was detected, and subsequently decayed (Fig. 3C, E). In addition, reduction of KR signal was observed even outside of the illuminated area. We concluded that a part of signal was lost during the photobleaching and subsequent imaging, and fast recovery of KR represents fast diffusion of free KR proteins. On the other hand, recovery of KR-KLC at 15 min was smaller compared with KR, then slowly increased and reached a similar level at 3 hr (Fig. 3D, E).

We applied CALI to one side (longer terminal segment) of the terminal pairs of neurons, expressing KR or KR-KLC together with EGFP. Subsequently, effect of CALI application on the arbor terminals was examined by monitoring EGFP signals for 8 hr (Fig. 3F, S2). Since in a preliminary experiment, 60 s of illumination caused immortalization or disintegration of arbor terminal, we applied 30 s of exposure in this analysis to reduce photo toxicity. We measured difference in length of both target terminal segment and neighboring un-illuminated terminal segment at each time frame.
Most arbor terminals exhibited a limited retraction after CALI exposure in KR-expressing CGNs, whereas in KR-KLC-expressing CGNs, intermittent retraction was observed at arbor terminal exposed to CALI (target terminal) (Fig. 3F, S2). Although there was no clear time window that the boost of retraction occurred, retraction was not remarkable in a last few hours (Fig. S2). Given that KR-KLC signal was recovered to similar level as KR in 3 hr after photobleaching, we calculated the value of retraction in 3 hr (Fig 3G). Quantification of terminal retraction values revealed greater retraction in KR-KLC expressing CGNs (n = 18) compared with KR-expressing control neurons (n = 19) at target terminals (Fig. 3G, p < 0.05 Mann-Whitney U-test). In contrast, retraction of neighbouring shorter terminal segment that did not receive photo illumination (neighbouring terminal) was not greater in KR-KLC-expressing CGNs compared with control neurons (p = 0.11).

**Microtubule turnover is differentially regulated between neighbouring terminal segments**

To determine whether K5H accumulation in a subset of axonal terminals reflects a variation in microtubule content between terminal segments, we expressed mCherry-tubulin and K5H-EGFP in CGNs. Introduction of K5H-EGFP plasmid require high density culture, that made it difficult to detect endogenous tubulin in a single axonal arbor by immunocytochemistry. In addition, to estimate the amount of microtubules, signals of mCherry-tubulins that were incorporated in microtubules have to be separated from that of free mCherry-tubulins. To remove unpolymerized free tubulins, neurons were subjected to simultaneous extraction and fixation at 7 DIV (Fig. 4A). Signals at the terminal pairs were quantified, and relative signal levels were calculated (Fig. 4B). In the parts of terminal pairs, longer terminal segment enriched with K5H exhibited greater mCherry-tubulin signals (Fig. 4A). However, in other parts of the terminal pairs, greater K5H accumulation was observed in terminal segments with less mCherry-tubulin (Fig. 4A). Consequently, the overall correlation between K5H-EGFP values and mCherry-tubulin signals of the same terminal pair was not significant (Fig. 4B, r = 0.30, p = 0.33, n = 44). These results indicated that factors other than microtubule content may contribute to the preferential accumulation of K5H in longer terminal segments.
Previous studies focused on neuronal polarity suggest that kinesin-1 is preferentially transported in processes that contain acetylated and detyrosinated microtubules (Reed et al., 2006; Konishi and Setou, 2009; Hammond et al., 2010). Stable microtubules contain more detyrosinated and acetylated tubulins than dynamic microtubules (Janke and Bulinski, 2011). Therefore, we analysed microtubule turnover in axonal arbors. We applied fluorescence recovery after photobleaching (FRAP) by expressing an alpha-tubulin tagged with EGFP (EGFP-tubulin). In CGNs, EGFP-tubulin was detected throughout axonal arbors (Fig. 4C). We focused on pairs of arbor terminals and applied photobleaching in a rectangular area proximal (approximately 10 μm) to the branching point (Fig. 4C). Recovery of fluorescence in each terminal segment was measured at 40-s intervals. Because free tubulins are rapidly recovered in a matter of seconds, fluorescence recovery over time reflects mostly the turnover of EGFP-tubulins that exist as microtubules. Notably, when FRAP analysis was applied on terminal segments that possessed varying segment lengths, we found that recovery of EGFP-tubulin in longer axonal terminal segments was slower than in shorter ones (Fig. 4D). The dissociation rate of EGFP-tubulin, which was obtained by a mathematical fitting to the model (Edson et al., 1993; Hush et al., 1994) (see Materials and Methods), revealed that turnover of EGFP-tubulin was slower in longer terminal segments compared with shorter terminal segments (Fig. S3A, shorter terminal segment; \( k_{off} = 0.055 \pm 0.019/\text{min} \) vs. longer terminal segment; \( k_{off} = 0.017 \pm 0.006/\text{min}, n = 4 \)).

We also assessed the possibility that selective kinesin-1 transport directly correlates with microtubule stability in the terminal segments. In this experiment, FRAP was applied to CGN axons that simultaneously expressed EGFP-tubulin and K5H-mCherry. As described above, the length of the K5H-enriched terminals tended to be longer than neighbouring terminal segments (Fig. 1D). To investigate the direct correlation between K5H signal and turnover of EGFP-tubulin, we selected terminal pairs that were comparable in segment length (< 1.5-fold difference). When we analysed terminal pairs that possessed different amounts of K5H (> 2-fold), we found no remarkable difference in EGFP-tubulin recovery depending on K5H signal value (Fig. 4E, F). In addition, no significant correlation was observed between the K5H-signal value and dissociation rate of EGFP-tubulin (Fig. S3B).
**Correlation between length of terminal segment and reporters of microtubule stability**

We next performed immunocytochemistry by using antibodies that specifically recognize post-transcriptionally modified tubulins (Fig. 5A). The ratios of tyrosinated/detyrosinated tubulins and acetylated tubulins vary between neighbouring terminal segments with different lengths. Consistent with a previous report (Robson and Burgoyne, 1989; Ahmad et al., 1993; Brown et al, 1993), the ratio of modification gradually changed depending on the distance from the branching point (Fig. 5A). To account for region-dependent differences in tubulin modification within the terminal segment, we quantified staining profiles for distances from axonal branching points and distances from terminal ends (Fig. 5B, C). Notably, the ratio of tyrosinated/detyrosinated tubulin was greater in shorter terminal segments compared with longer terminal segments at the same distance from a branching point. The ratio difference increased in a distance-dependent manner, but was significant even at 10 μm from the branching point (Fig. 5C, \( p < 0.05, n = 11 \)). These results were consistent with the FRAP analysis. Similarly, the ratio of acetylated tubulin in shorter terminal segments was significantly less than the ratio at the same position in longer terminal segments relative to the branching point (Fig. 5C, \( p < 0.05 \) at 10 μm, \( n = 8 \)). Conversely, in both tyrosination/detyrosination and acetylation, staining profiles vs. distance from terminal ends were similar between shorter and longer terminal segments (Fig. 5C).

We further investigated the region-dependent frequency of microtubule growth in the arbor by introducing an EB3 plasmid that was fused to fluorescent proteins to label plus-ends of growing microtubules (Stepanova et al., 2003). EB3 particles were detected more frequently in distal regions compared with proximal regions, and shorter terminal segments contained more particles compared with longer terminal segments at the same distance from a branching point (Fig. 5D, \( p < 0.05, n = 9 \)). Additionally, profiles of EB3 particle numbers vs. distance from a terminal end were similar between shorter and longer terminal segments (Fig. 5D), which was consistent with analysis of tyrosination/detyrosination and acetylation of microtubules. Thus, differences in microtubule turnover between short and long terminal segments could be explained by a regional difference that is dependent on the distance from a terminal end. In a previous study, Seetapun and Odde (2010) provided a simple length-dependent model for selective accumulation of stable microtubules in axons.
during polarization, which does not require neurite-dependent control of a microtubule assembly. In this model, the growth rate of microtubules in minor and longer processes remained unaltered. However, because the traveling time depends on process length, the net turnover of microtubules is slower in longer process (i.e., axon) compared with shorter minor processes. We measured microtubule growth in axonal arbors by simultaneously monitoring EB3 particles in shorter and longer terminal segments (Fig. 5E). We found no length-dependent difference in the velocity of EB3 particles (Fig. 5E). These results support the notion that microtubule turnover in terminal segments is differentially regulated and dependent on distance from the terminal end, rather than by segment-specific microtubule regulation.

**F-actin distribution in the axonal arbor spatiotemporally correlates with K5H**

We next investigated the relationship between F-actin and K5H at arbor terminals. F-actin accumulates in the growth cone and is required to form filopodia and lamellipodia (Lewis et al., 2013). In axons of young CGNs (at 2 DIV), strong F-actin signals were observed in the growth cone by staining with phalloidin. However, at 5 DIV, a time point in which axons become thinner and form multiple terminals, F-actin signals at the axonal tip were highly restricted (Fig. S4A). To analyse detailed F-actin distribution in CGN axons, we expressed Lifeact, a marker for the visualization of F-actin (Riedl et al., 2008). In the axonal arbor, Lifeact signals co-localized with phalloidin signals at arbor terminals and protrusions (Fig. S4B). When we simultaneously expressed K5H and Lifeact in neurons, they co-localized in the axonal terminal (Fig. 6A). Plotting of relative Lifeact signal values vs. relative K5H signals in terminal pairs clearly revealed a positive correlation (Fig. 6B, $r = 0.83$, $n = 20$). Consequently, arbor terminals with greater K5H local levels also had significantly greater Lifeact signals (Fig. 6B, $p < 0.001$, $n = 15$). To analyse the temporal correlation, axonal arbors expressing K5H and Lifeact were subjected to time-lapse imaging analysis at 10-min intervals. In some arbor terminals, the K5H signal dramatically decreased or increased during the time-lapse period (Fig. 6C). In these arbor terminals, K5H signal changes were followed by changes in Lifeact signal values (Fig. 6C, D). We collected data from arbor terminals in which the K5H signal changed > 2-fold within 100 min. When the Lifeact signals between the two different time points (i.e., maximum K5H vs. minimum K5H) were compared, we found a significant difference (Fig. 6D, $p$
< 0.001, n = 5). These results suggested a spatiotemporal correlation between the K5H and Lifeact signals.

To determine whether F-actin regulation plays a role in the retraction that frequently occurs in K5H-deprived arbor terminals, we utilized Latrunculin A, which is shown to inhibit axonal retraction in sensory neurons by disrupting actin polymerization (Ahmad et al., 2000; Gallo et al., 2002). Neurons that express K5H-EGFP and mCherry were treated with Latrunculin A prior to time-lapse imaging. Changes in axonal morphology and distribution of K5H-EGFP were simultaneously analysed for 5 hr to determine the primary effect of inhibiting actin turnover. We observed that Latrunculin A-treated arbor terminals (n = 21) exhibited a lower average retraction rate compared with vehicle-treated control arbor terminals (n = 26) (Fig. 6E, median; 0.80 μm/hr in DMSO vs. 0.49 μm/hr Latrunculin A, p < 0.05, Mann-Whitney U-test). Together, these results indicate the possibility that actin-dependent mechanisms are involved in the axonal retraction that occurs in kinesin-1-deprived arbor terminals.
DISCUSSION

The intracellular systems involved in how neurons differentially regulate growth and stability of arbor terminals within the same axon remain largely unknown. Previous results indicate that intracellular Ca\(^{2+}\) mediates the competitive signal between different terminals of the same axon (Hutchins and Kalil, 2008). However, the mechanisms by which localized Ca\(^{2+}\) signals regulate the axonal structure region-specific manner remain to be elucidated. Other molecules that are differentially controlled between axonal terminals need to be identified. In this study, we found that K5H tagged by fluorescent protein, as well as endogenous kinesin-1, accumulate in a subset of axonal arbor terminals (Fig. 1). The q-q plot analysis of K5H signals in arbor terminals revealed that the differences in K5H signals between terminals are not due to random variation. We analysed terminal segment length > 20 \(\mu\)m to avoid newly formed protrusions that have not yet received microtubule invasion (Gallo, 2011). Furthermore, time-lapse imaging revealed that K5H signals occasionally and rapidly change their distribution, but local levels of K5H did not increase in a subset of arbor terminals that exhibit a long life span. These findings revealed selective sorting of kinesin-1-dependent transport in the axonal arbor. Time-lapse imaging analysis revealed that arbor terminals with high local levels of K5H exhibit a small retraction (Fig. 2). Consistently, local inhibition of kinesin-1 by CALI enhanced retraction specifically at illuminated arbor terminal (Fig. 3). Considering the function of kinesin-1-mediated axonal transport, the accumulation of cargo molecules in particular arbor terminals results in the deprivation of molecules in neighbouring terminals. Results from the present study suggest a potential intracellular system for competitive stabilization of arbor terminals via kinesin-1 sorting in the axonal arbor. Additionally, changes in length of terminal segment could provide feedback to kinesin-1-dependent transport as described below.

The mechanisms by which kinesin-1 gets sorted into a subset of arbor terminals in the axonal remain to be clarified. Our results showed that microtubules in longer terminal segments are more stable than neighbouring shorter terminal segments when compared in the area proximal to the branching point (Fig. 4). Consistently, posttranslational modifications of tubulins abundant in stable microtubules (i.e., detyrosination and acetylation) are greater in longer axonal terminal segments (Fig. 5).
Thus, kinesin-1-dependent terminal selection can be explained by preferential recruitment to stable microtubules as described in other paradigms (Reed et al., 2006; Hammond et al., 2010; Dunn et al., 2008). In the previous study, we have reported that inhibition of tubulin tyrosination in immature hippocampal neurons disrupts polarized K5H transport into axons (Konishi and Setou, 2009). In the current study, although inhibition of tubulin tyrosination by siRNA for tubulin tyrosination ligase (TTL) tended to inhibit the terminal-dependent accumulation of K5H in CGNs, no statistical significance was obtained (data not shown). Thus, function of tyrosination/detyrosination alone might be limited in this paradigm. It is possible that other differences (e.g., MAPs, GTP/GTP-binding state, microtubule numbers) could also contribute to this process, because they are possibly affected by microtubule dynamics. Studies focused on neural polarity have shown the contribution of multiple factors that can be differentially coordinated dependent on the situation and neuronal types (Barnes and Polleux 2009). Further studies are required to fully demonstrate the molecular mechanisms of kinesin-1 sorting in the axonal arbor.

Microtubules undergo repetitive growth and shortening until they reach the axonal terminal, where most microtubules cause shrinkage (catastrophe). According to the polarization model by Seetapun and Odde (2010), because of increased traveling time from the soma, microtubule turnover is slower in the axon due to process length. Under certain conditions, the region closer to the terminal contains a larger number of growing microtubule plus-end than regions further from the terminal. Consequently, the longer process contains older microtubules than shorter, minor processes. Although microtubules in mature axons are segmented (Yu and Baas, 1994), our observations suggest that this model can be adopted to explain microtubule difference in axonal arbors. First, there is a positive correlation between length of terminal segment and microtubule turnover. Second, distribution of reporters for microtubule turnover depends on distance from terminal end. Third, there is no segment-dependent difference in velocity of EB3 particle. Obviously, other factors could also regulate microtubules, either stochastically or in a region-specific manner via cleavage or stabilization (Yu et al., 2008; Homma et al., 2003; Peris et al., 2009). Indeed, we found only a moderate correlation between K5H signals and length of terminal segment, and K5H distribution can be suddenly altered prior to a change in length of terminal segment. These factors may play critical roles in switching between a stable and unstable state in arbor...
terminals.

As described above, longer terminal segments contain more stable microtubules and tend to contain greater K5H signals. Previous studies have reported that kinesin-1 transports molecules involved in the stabilization of microtubules (Kimura et al., 2005). However, microtubule turnover in terminal pairs with comparable segment lengths, but differences in K5H signals, was not remarkably different. This observation contradicts the notion that kinesin-1-dependent inhibition of retraction is mediated by selective stabilization of microtubules. Conversely, there was a spatiotemporal correlation between K5H and F-actin signals in arbor terminals, and disruption of axonal turnover with Latrunculin A significantly inhibited retraction (Fig. 6), even in arbor terminals with low K5H signals. Actin regulation has been shown to play an important role in axonal growth, as well as retraction (Giannone et al., 2009). Retraction cues induce growth cone collapse by destabilizing F-actin in the axonal terminal. Conversely, RhoA-myosin II driven contractility along intra-axonal F-actin mediates axonal retraction (Gallo et al., 2002; Gallo, 2006). Therefore, reorganization of F-actin is crucial for the retracting axon to change its structure. Although detailed molecular mechanisms remain to be elucidated, our current study raised a possible link between the differential control of terminal stability and actomyosin-mediated axonal retraction.
MATERIALS AND METHODS

Cell culture

Dissociated CGN culture was prepared according to the previous study (Konishi et al., 2004) with modifications. In brief, cerebella isolated from Slc:ICR mice (postnatal days 5-6) were digested with trypsin, and dissociated neurons were placed in Basal Medium Eagle (BME; Sigma-Aldrich, St Louis, MO) supplemented with 10% calf serum (Thermo Fisher Scientific, Waltham, MA), 1 mg/ml penicillin, 1 mg/ml streptomycin, 2 mM glutamine and 25 mM KCl. Neurons were spread on the plate that has been coated with poly-L-ornithine (Sigma-Aldrich). Low-density CGN cultures were prepared by co-cultivating with high-density CGN culture as described previously (Kubota et al., 2013). Animals were treated according to the institutional ethical guidelines of University of Fukui.

Transfection

Plasmid DNAs were introduced in CGNs by calcium-phosphate method. Prior to the transfection, CGN culture was washed with Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) for twice, then placed in DMEM and incubated at 37 °C in CO₂ chamber. DNAs solution containing 250 mM CaCl₂, were gradually mixed with same amount of 2×HBS solution (270 mM NaCl, 9.5 mM KCl, 1.4 mM Na₂HPO₄, 15 mM Glucose, 42 mM Hepes). After 15 min, DNA mixture was added to cells and incubated for 15 min in CO₂ incubator. Finally, the CGN culture was washed twice with DMEM and placed in original media. In the case of low-density culture, dissociated neurons were placed in DMEM that contain plasmids, and exposed to the square electric pulses (140 V, 5 ms for two times) before plating as described previously (Kubota et al., 2013). To construct a plasmid for KR-KLC, full length KLC1 cDNA was amplified by polymerase chain reaction, and inserted into pKillerRed-C vector (Evrogen, Moscow, Russia). Plasmids for EGFP-tubulin (#30487, gift from Tso-Pang Yao), mCherry-tubulin (#26768, gift from Torsten Wittmann), tdTomato-EB3 (#58090, gift from Michael Davidson) and pLifeAct-mTurquoise2 (#36201, gift from Dorus Gadella), were obtained from Addgene. Plasmids for EGFP-EB3, K5H-EGFP, and Dominant-negative Kif5s were described previously (Konishi and Setou, 2009; Okamoto et al., 2015). K5H-mCherry was constructed by inserting K5H sequence into pmCherry-N vector.
(BD Biosciences, Franklin Lakes, NJ).

**Immunocytochemistry**

For immunocytochemistry, neurons were fixed for 15 min at room temperature with paraformaldehyde solution (4 % paraformaldehyde in PBS) followed by permeabilization with 0.2-0.4 % Triton X-100 in PBS for 15 min, and incubated further (1 hr) with blocking solution (5 % goat serum, 3 % BSA and 0.02 % tween-20 in PBS). Cells were then incubated with primary antibodies in blocking solution at 4 °C for overnight, followed by incubation with secondary antibodies (2-3 hr, at room temperature). To stain nucleus and F-actin, Hoechst 33258 (Sigma-Aldrich) and Rhodamine-Phalloidin (Cytoskeleton Inc., Denver, CO) were respectively used. Monoclonal antibody against alpha-tubulin (1:1000; 12G10) was obtained from Developmental Studies Hybridoma Bank of University of Iowa. Monoclonal antibodies against β-tubulin (1:1000; Tub2.1, Cy3-conjugated), Tyr-tubulin (1:2000; TUB-1A2), Ac-tubulin (1:10000; 6-11B-1), and kinesin heavy chain (1:50; clone H2) were obtained from Sigma-Aldrich. An antibody against detyrosinated tubulin (1:1000; #AB3201) was purchased from Merck Millipore (Darmstadt, Germany). For secondary antibodies, goat anti-mouse or anti-rabbit IgG conjugated to Alexa Fluor dyes (1:1000; Thermo Fisher Scientific) were used.

**Cell imaging and data quantification**

Images of fixed neurons were obtained under an Axiovert 200M microscope equipped with Axiocam MRm digital camera (Carl-Zeiss, Oberkochen, Germany), and were analyzed using ImageJ software (National Institute of Health). In some experiments, confocal laser scan microscope (LSM 5 PASCAL; Carl-Zeiss) or ApoTome.2 (Carl-Zeiss) was used to obtain sectionized images (see below). In the analysis of arbor terminals, the processes that have more than 20 μm in length were selected to exclude short protrusions.

**Time-lapse imaging of axonal arbor**

For time-lapse imaging, neurons were cultured on glass bottom plates (Iwaki, Chiba, Japan) attached by flexiperm (Sarstedt, Nümbrecht, Germany). Minimal Essential Medium (MEM; Thermo Fisher Scientific) that does not contain Texas Red
was used instead of BME. Images were acquired at 10-20 min intervals by using Axiovert 200M equipped with MRm monochromatic digital camera (Carl-Zeiss). During the imaging, neuron culture was kept at 36.7 °C in stage top incubator (ZILCS; Tokaihit, Shizuoka, Japan) supplied with 5 % CO₂, which was set on the scanning stage. Stage position and camera were controlled by AxioVison software (Carl-Zeiss). Images were collected randomly from neurons whose axonal morphology was clearly observed, and degenerating neurons were excluded from the analysis. Axonal growth rate was calculated by differentiating the value of segment length in each frame. The positive and negative change was defined as “elongation” and “retraction”, respectively. In the inhibition of actin turnover, Latrunculin A (WAKO, Osaka, Japan) was added to the media prior to placing culture in the chamber.

**Chromophore-assisted light inactivation (CALI)**

CGNs were introduced with expression vectors for KR or KR-KLC1 together with EGFP plasmid at 2 DIV, and subjected to CALI at 4 or 5 DIV. For CALI experiments, cells were placed in the stage top incubator, and small circular area defined by the iris (about 100 μm diameter) was illuminated with a 100 W mercury arc lamp (HBO 100) through 20× objective lens (Plan-Apochromat, NA 0.8) and a band path filter (Ex BP/565/30) (Carl-Zeiss). To analyse stability of arbor terminal, more than half the area from terminal end of longer terminal segment was illuminated for 30 s, and monitored by at 15-min intervals for 8 hrs. Time-lapse images of all axonal arbors that did not undergo degeneration were quantified. To remove the position shift, time-series of axonal arbor images was aligned using StackReg plugin (Philippe Thévenaz, Swiss Federal Institute of Technology Lausanne) of ImageJ before measuring a frame-to-frame difference in the length of terminal segment.

**Analysis of microtubule content**

To analyze microtubule content, mCherry-tubulin was expressed in neurons. Neurons were then simultaneously fixed and permeabilized as described previously (Witte et al., 2008) in the solution containing 60 mM Pipes (pH6.9), 25 mM Hepes, 5 mM EGTA, 1 mM MgCl₂, 0.25 % glutaraldehyde, 3.7 % paraformaldehyde, 3.7 % sucrose, and 0.1 % Triton X-100, for 20 min at room temperature in order to remove unpolymerized tubulins. The mCherry-tubulin signals in each terminal segment (in 20
μm from the branching point) were quantified.

**Fluorescent recovery after photo-breaching (FRAP) analysis of tubulins**

For FRAP analysis of tubulins, a confocal microscope (LSM 5 PASCAL, Carl Zeiss) equipped with 488 nm argon laser was used. CGNs that have been introduced expression plasmids for EGFP-tubulin with or without K5H-mCherry was placed in stage top incubator as described above. The rectangular region in the pair of axonal terminal segments that is about 10 μm distal from the branching point was analyzed. After photobleaching the interested region by 63× objective lens, fluorescence recovery was monitored every 40 s. Data was quantified by using ImageJ. Fluorescence recovery rate was calculated from a single exponential function, as described previously (Hush et al., 1994) by following equation (1), where \( \text{frap}(t) \) is the recovered fluorescence at each time point and \( C_{eq} \) is the total amount of complex. The dissociation constant, \( k_{off} \) was calculated from plots by fitting and used to calculate the half time of recovery (2).

\[
\text{frap}(t) = C_{eq} e^{k_{off}t} \quad (1)
\]

\[
t_{1/2} = \frac{ln2}{k_{off}} \quad (2)
\]

**EB3 experiments**

CGNs (5 DIV) that have been introduced with EB3 plasmids by calcium phosphate method were analyzed. For the fixation, neurons were immediately placed in the cold methanol for 20 min and rehydrated with PBS. For the live cell imaging, neurons were placed in the stage top incubator, and fluorescence images of EB3 particles was obtained by ApoTome.2 and MRm camera at 3-s intervals under 63× objective lens. Velocity of EB3 spot was quantified by using Multiple Kymograph plugin (J. Rietdorf and A. Seitz, European Molecular Biology Laboratory) of ImageJ.

**Statistical analysis**

Compiled data in bar graphs are expressed as mean ± SEM. If not indicated, we used the two-tailed Student’s t-test for statistical analysis. The levels of significance are indicated as follows: ** ** ** \( P < 0.001 \), ** \( P < 0.01 \), * \( P < 0.05 \).
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COMPETING INTERESTS

No competing interests declared

AUTHOR CONTRIBUTIONS

T. S., K. M., N. S. and Y. K. analyzed axonal morphology and microtubules, and T. I., M. K., analyzed the F-actin. H. T. contributed to the data analysis, and M. S. and Y. K. designed the experiments and wrote the paper.

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Fig. 1. The distribution of kinesin-1 mediated transport in axonal arbors. (A) CGNs that were transfected with plasmids for K5H-EGFP and mCherry were analysed at 7 DIV. Magnified images at the axonal arbor (dashed square) are shown in bottom. K5H-EGFP is selectively transported in the axon (arrows) and accumulated in a part of arbor terminals (filled arrowhead). In other terminals (open arrowheads), K5H-EGFP signal was weak or undetectable. Scale bars on the top and bottom indicate 50 μm and 20 μm, respectively. (B) Schematic representation of the data quantification of K5H-EGFP signals and length of terminal segment. The terminal pairs (more than 20 μm the length) were selected for the analysis. Formulas for relative K5H-EGFP signal value as well as relative length of terminal segment were shown. (C) The q-q plot of relative K5H-EGFP signal values vs. theoretical quantiles. Note that the data plots do not align on a diagonal line. (D) The scattering plot of relative length of terminal segment vs. relative K5H-EGFP value. Moderate but significant positive correlation between two datasets was revealed (r = 0.62, p < 0.001, n = 64). (E) Low density
culture of CGNs was stained with antibody against Kif5. The Kif5 signal value at axonal terminal is higher in longer terminal segments compared with shorter terminal segments \((p < 0.01, n = 12)\). Scale bar indicates 50 μm. (F) Inhibition of axonal transport mediated by kinesin-1. CGNs were introduced with plasmid for tail region of Kif5 (K5T), which disrupts the interaction with cargos, or stalk region of Kif5 (K5S) as a control. Although the longest path from soma to distal terminal end of axon tends to be shorter in K5T expressing neurons, no significance was obtained in this analysis \((p = 0.06\) and 0.29 compared with vector and K5S, respectively). The length from branching point on the longest path to axonal terminals in K5T expressing neurons was significantly shorter than that of control neurons \((p < 0.05, n = 37)\).
Fig. 2. Time-lapse imaging of K5H distribution and axonal arborization. (A, B) CGNs that were transfected with plasmids for K5H-EGFP and mCherry were subjected to the time-lapse imaging analysis at 5 DIV. Images were taken by 15 min/frame for over night, and parts of time-lapse images at indicated time obtained from two different neurons were shown. Examples of arbor terminals that possess relatively high (a, a’) or low (b, b’) K5H signal were marked. Elongation and retraction of arbor terminals were indicated by filled and open arrowheads, respectively. (C, D) The scattering plots of average elongation rate of arbor terminal vs. relative K5H-EGFP value (C), and average retraction rate of arbor terminal vs. relative K5H-EGFP value (D). Relative K5H-EGFP
level in each terminal was determined by calculating the signal ratio to the total signal value in the axonal arbor, and averaged value within the time window subjected to the analysis was revealed. For the terminals that exist only for a certain period, averaged value within the existing period was calculated. Data were collected from 97 arbor terminals in multiple neurons. Each circle in the plots represents the individual arbor terminal. For statistical analysis, data sets were separated into two groups (i.e. relative K5H value < 0.1, \( n = 77 \), and relative K5H value > 0.1, \( n = 20 \)). Significant median shift was detected both in elongation and retraction (elongation; \( p < 0.001 \), retraction; \( p < 0.05 \), Mann–Whitney U-test). (E) The scattering plot of average difference in length (i.e. elongation rate – retraction rate) vs. relative K5H-EGFP value. Scale bars indicate 50 \( \mu \text{m} \).
Fig. 3. CALI-mediated local inhibition of kinesin-1 in the arbor terminal. (A) Region dependent photobleaching of KR-KLC. CGNs expressing KR-KLC (orange) and EGFP (green) were applied with photobleaching within the indicated circular area. Images taken before (0 s) and after photobleaching for 30 s and 60 s were revealed. Scale bar indicates 100 μm. (B) Signals for KR-KLC and EGFP in soma of CGNs during photobleaching (n = 6). Fluorescence of KR-KLC, but not EGFP is rapidly decreased by illumination. (C, D) Distribution and recovery after photobleaching of KR and KR-KLC signals at axonal terminal. CGNs expressing KR (C) or KR-KLC (D) were applied with photobleaching at axonal terminal. A region of about 50 μm in diameter from terminal (dashed line indicates approximate position of border) was illuminated for 60 s, and recovery of signals within 20 μm from terminal (bracket) was
monitored at indicated time. Scale bar indicates 20 μm. (E) Quantified results of fluorescence recovery of KR (green; $n = 6$) and KR-KLC (orange; $n = 5$) in axonal terminals. Signal value was normalized with EGFP signals. Fluorescence recovery of KR-KLC was slower than that of KR. At 3h after photobleaching, signals of KA and KR-KLC became similar level. (F, G) CALI analysis on arbor terminal pairs expressing KR (left) and KR-KLC (right). Representative arbor terminals before (Pre) and after CALI at indicated time as shown (F). Arbor morphology was visualized with EGFP signals. Longer terminal that received CALI (Target terminal; T) and shorter unilluminated terminal (Neighbouring terminal; N) were indicated (F). Filled and open arrowheads indicate the each arbor terminals. Values of retraction in 3 hr after CALI were quantified and presented in box plot with outliers (open circles) (G). $p$ values on the bottom indicate the results statistical analysis (Mann–Whitney U-test) between the axonal terminals expressing KR ($n = 19$) and KR-KLC ($n = 18$). Scale bar indicates 20 μm.
Fig. 4. Analysis of microtubule differences in neighboring terminal segments. (A) Neurons at 7DIV that have been introduced with the plasmid for mCherry-tubulin and K5H-EGFP were simultaneously fixed and permeabilized to remove unpolymerized mCherry-tubulins. Two representative terminal pairs possessing shorter (S) and longer (L) terminal segments were shown. Scale bar indicates 50 μm. MT; microtubules. (B) The scattering plot of relative mCherry-tubulin value vs. relative K5H-EGFP value (filled circles). Only a small correlation between two datasets was observed ($r = 0.30, p =0.33, n = 44$). The plot for relative length of terminal segment vs relative K5H-EGFP value was also shown (open circles) as in Fig. 1D. (C) Neurons were introduced with the plasmid for EGFP-tubulin, and microtubule turnover in the pairs of terminal segments that is different in length (> 2-fold) were analyzed by FRAP. The photobleached region (about 10 μm distal from the branching point) across the shorter (S) and longer (L) terminal segments was indicated by rectangles. The recovery was monitored every 40 s. (D) Quantified results of fluorescence recovery of EGFP-tubulin in axonal terminals. Fluorescence recovery of EGFP-tubulin is faster in shorter terminal segments (upper) compared with longer terminal segments (lower) (data obtained from...
4 experiments are shown). (E) Neurons were introduced with plasmids for EGFP-tubulin and K5H-mCherry, and pairs of terminal segments that have comparable length (< 1.5-fold difference) were subjected to FRAP as in (C). A pair of axonal terminals expressing low (L) and high (H) amount of K5H is revealed. (F) The fluorescence recovery of EGFP-tubulin in terminal pairs that have similar segment length but show difference in K5H signals (> 2-fold, n = 6). Graphs represent mean ± SEM. Scale bars indicate 20 μm.
Fig. 5. Regulation of microtubules depends on the length of terminal segment in axonal arbors. (A) Distribution of tyrosinated (Tyr) and detyrosinated (Δtyr) tubulins as well as acetylated (Ac) tubulins in CGN axonal arbors detected by immunocytochemistry. Shorter (S) and longer (L) axonal terminal segments were indicated (arrowheads). Scale bar indicates 20 μm. (B) Schematic representations of the data quantification for the analysis of region dependent tubulin modification. The signal profiles for the distance from branching point or distance from terminal ends were calculated. (C) Quantifications of the region dependent tyrosination and acetylation on the tubulins in shorter (S; orange) and longer (L; green) terminal segments. Tyrosinated tubulin (Tyr-tub) level was determined by taking the ratio of tyrosinated and detyrosinated tubulins, whereas acetylated tubulin (Ac-tub) level indicates the ratio to tubulin signal. Tyr-tub and Ac-tub level in each region was normalized by the signal at
the branching point (Tyr; \( n = 11 \), Ac; \( n = 8 \)). (D) The EB3-expressing neurons were fixed with methanol, and the number of EB3 particles in every 10 \( \mu \text{m} \) on the terminal segment was measured. The number of EB3 particles vs. the distance from branching point (upper) as well as the distance from terminal end (lower) was presented (\( n \geq 9 \)). Graphs represent mean ± SEM. (E) CGNs expressing EB3 that was fused to fluorescent protein were subjected to time-lapse imaging. Data were acquired from arbor terminal pairs that have difference in segment length. Scale bar indicates 20 \( \mu \text{m} \). The magnified images of EB3 particles (dashed squares) are shown in bottom. Representative kymographs for shorter (S) and longer (L) terminal segments were shown in top right. The graph (bottom right) represents velocity of EB3 particles calculated from kymographs (\( n \geq 31 \)).
Fig. 6. Distribution of F-actin in arbor terminals of CGNs. (A) Axonal arbor of CGN that express Lifeact and K5H. (B) Scatter plots of Lifeact signal values at arbor terminal versus K5H signals reveals positive correlation (left; \( r = 0.83, n = 20 \)). Lifeact signal is significantly higher in the arbor terminals that contain higher K5H signal compared with the arbor terminals that contain lower K5H signals (right; \( n = 15 \)). (C) Simultaneous alternation of K5H and Lifeact distribution in arbor terminals of living neurons. Representative images of an axonal arbor in which K5H signal was largely altered (arrowheads) during imaging were shown. (D) Quantified result of the time-lapse imaging shown in (C). Imaging data from five different axonal arbors were subjected to statistical analysis (right). Data were collected from arbor terminals in which > 2-fold change in K5H signal was occurred during the time-lapse imaging (100 min) \((n = 5)\). (E) Inhibition of actin turnover disrupts the retraction of arbor terminals. CGNs that had been transfected with vectors for mCherry and K5H-EGFP were treated with 1 μM Latrunculin A or DMSO, followed by time-lapse imaging. Average retraction rate of arbor terminals were measured as in Fig. 2, and shown in box plot. Latrunculin A treatment inhibited the retraction of arbor terminals \((p < 0.05, \text{DMSO}; n = 26, \text{Latrunculin A}; n = 21, \text{Mann–Whitney U-test})\). Scale bars indicate 50 μm.
Supplementary Information

Fig. S1. Time-lapse imaging of CGN axons in culture. CGNs that were transfected with a plasmid for mCherry were subjected to the time-lapse imaging at 2 DIV (A) and 5 DIV (B). Images were taken by 20 min/frame, and representative images at indicated time were shown. Examples of arbor terminals that had been existed at the most distal position (open arrowheads), and newly formed collateral branches that revealed massive extension (filled arrowheads) were indicated. Scale bars indicate 50 µm.
Fig. S2. Analysis of the position of axonal terminal after CALI. (A-D) Difference in the position of terminals after CALI expressing KR (A, C; \( n = 19 \)) or KR-KLC (B, D; \( n = 18 \)). CALI was applied to the longer side of terminal segment pairs. Data for each terminal which received CALI (A, B) or their neighboring terminal (C, D) was shown. (E, F) The averaged results of A-D were revealed. In axonal arbors of KR expressing neurons, target terminals tend to be stable than shorter neighboring terminals (E). This trend was not observed in KR-KLC expressing neurons (F).
Fig. S3. Quantification of microtubule turnover in FRAP analysis. (A) Dissociation rate ($k_{off}$, left) and half time recovery ($t_{1/2}$, right) of EGFP-tubulin were calculated from FRAP data obtained from experiments shown in Fig. 4D (see Materials and Methods). Turnover of EGFP-tubulin is slower in longer terminal segments, compared with shorter terminal segments ($p < 0.05$, $n = 4$). (B) Dissociation rate of EGFP-tubulin in each terminal segment was calculated from FRAP data obtained from the experiments shown in Fig. 4F. There is no clear correlation between dissociation rate and K5H signal ratio.
Fig. S4. Distribution of F-actin in the CGN axonal arbor. (A) F-actin signals detected in CGN axons. CGNs fixes at 2 DIV or 5 DIV were stained with phalloidin together with antibody against tubulin. Scale bar indicates 20 µm. (B) Axonal arbor of low-density culture of CGN that express Lifeact was stained with phalloidin. Distribution of Lifeact and phalloidin were overlapped in axonal terminals and protrusions. Scale bar indicates 10 µm.