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Mobility and cycling of synaptic protein–containing vesicles in axonal growth cone filopodia

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The spatial distribution and coordination of vesicular dynamics within growth cones are poorly understood. It has long been thought that membranous organelles are concentrated in the central regions of growth cones and excluded from filopodia; this view has dramatically shaped conceptual models of the cellular mechanisms of axonal growth and presynaptic terminal formation. To begin to test these models, we studied membrane dynamics within axonal growth cones of living rat cortical neurons. We demonstrate that growth cone filopodia contain vesicles that transport synaptic vesicle proteins bidirectionally along filopodia and fuse with the filopodial surface in response to focal stimulation, allowing for both local secretion of vesicular contents and rapid changes in the plasma membrane composition of individual filopodia. Our results suggest a new model in which growth cone filopodia are actively involved in both emitting and responding to local signals related to axon growth and early synapse formation.

Filopodia are functionally distinct from the palm of the growth cone, serving unique roles in axon development. Remarkably, changes in adhesion or signaling localized to a single filopodium can alter the behavior of the rest of the growth cone[1,2]. In addition, stable adhesion between two filopodia can initiate cell-cell contact[3] that is likely to contribute to mechanisms of axon guidance and synapse formation. Although filopodial dynamics are clearly important for neuronal development, the mechanisms of filopodial involvement in these processes remain poorly understood.

Since publication of the seminal electron micrographs of Tennyson[4], it has been thought that neuronal growth cone filopodia are mostly devoid of vesicles. Membranous organelles are said to be found in the central, microtubule-rich region of the growth cone, "as if somehow excluded from the peripheral regions of the growth cone, particularly the filopodia and lamellipodia"[5]. This consensus has remained strong despite several conflicting reports that membranous organelles occasionally appear in the periphery of fixed growth cones, as revealed by electron microscopy[6–8]. These reports were called into question when it was shown that growth-cone membranes are prone to artifacts of chemical fixation, yielding the appearance of intracellular membrane structures that are absent from rapidly frozen tissue[9–12]. Using differential interference contrast (DIC) video microscopy or FM dye uptake, vesicles have not been reported in the periphery of the growth cone, but appear abundant in the central region[13–15]. Thus, even in recent papers, it has been asserted that growth cone filopodia are free of vesicles[16,17]. This general consensus has limited the active roles proposed for growth cone filopodia in signaling and synaptogenesis.

Recently, however, the assumption that axonal growth cone filopodia are devoid of vesicles has been more seriously questioned by the discovery of the protein Sec6, a component of the exocyst complex, in growth cone filopodia[18]. Because the exocyst complex is important for targeting secretory vesicles to fusion domains[18], the presence of Sec6 in growth cone filopodia suggests that growth cone filopodia may contain vesicles. Using time-lapse confocal imaging, we show here that living axonal growth cone filopodia do indeed contain vesicles. These vesicles move rapidly and cycle within filopodia. On the basis of this evidence, we propose that regulated fusion of vesicles with the filopodial surface of the growth cone can lead to either rapid alteration of the local surface receptor composition or secretion of molecules, such as glutamate or the neurotrophins, that regulate filopodial dynamics.

RESULTS

To determine whether living axonal growth cone filopodia contain vesicles, we transfected 3–5 day *in vitro* (d.i.v.) primary cultures of postnatal rat visual cortical neurons with the synaptic vesicle marker VAMP2 tagged with enhanced green fluorescent protein (VAMP2-EGFP). We then carried out time-lapse confocal imaging. Filopodia were generally free of contact with other neurons. Clusters of VAMP2 appeared along a subset of axonal growth cone filopodia (65 ± 5.6% of filopodia, n = 105 filopodia in 21 growth cones; **Fig. 1a**). We next tested whether VAMP2 clusters associate with internal or surface membranes. Selective extraction of VAMP2 from the plasma membrane of fixed neurons revealed VAMP2-EGFP (**Supplementary Fig. 2** online) and endogenous VAMP2 (**Fig. 1e**) puncta within filopodia that were indistinguishable from the VAMP2-EGFP puncta seen in living neurons. We found that 78.3 ± 3.7% of phalloidin-labeled filopodia contained endogenous intracellular VAMP2 (n = 40 growth cones; **Fig. 1e**). Although growth

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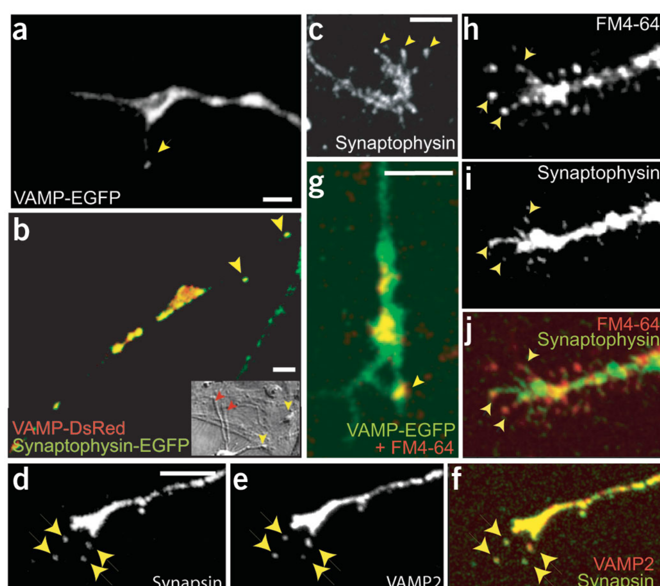

Figure 1 Vesicles containing synaptic-vesicle proteins are found in axonal growth cone filopodia. (**a**) Growth cones contain clusters of VAMP2-EGFP within their filopodia (arrow). (**b**) Filopodial VAMP2-DsRed clusters (red) colocalize with synaptophysin-EGFP (green); colocalization is indicated by yellow. Yellow arrows, filopodial clusters of colocalized synaptic proteins. Inset, transmitted light image showing all filopodia of growth cone. Red arrows, filopodia with no synaptic vesicle protein clusters. (**c**) Endogenous synaptophysin clusters in growth cone filopodia (arrows). (**d**–**f**) Endogenous synaptic vesicle proteins, synapsin (**d**) and VAMP2 (**e**), colocalize in axonal growth cone filopodia. (**f**) Colocalization of VAMP2 (red) and synapsin (green) is indicated by yellow in the overlay (arrows). (**g**) Neurons transfected with VAMP2-EGFP (green) were depolarized in the presence of FM4-64 (red). (**h**–**j**) Filopodial vesicles contain endogenous synaptophysin. (**h**) Neurons were loaded with FM4-64 by depolarization in the presence of the dye, washed and imaged. (**i**) Immediately after imaging, the neurons were fixed, labeled with anti-synaptophysin antibodies, and imaged again. (**j**) Overlay of the two images in **h** and **i**. Many FM4-64 vesicles contain synaptophysin (arrows). Since vesicles are dynamic within filopodia, it is expected that some of the vesicles moved after imaging of the FM4-64 signal but before fixation was complete. As a result, the two signals are not expected to show complete colocalization. Scale bars, 5 μm.

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cone morphologies vary, there were no clear morphological distinctions between filopodia or growth cones that contained filopodial VAMP2 clusters and those that did not.

Axonal growth cone filopodia also contained clusters of other synaptic vesicle proteins that colocalized with VAMP2. Endogenous synaptophysin and synapsin localized to puncta within filopodia (**Fig. 1c**,**d**). About 79.7 ± 11% (n = 6 growth cones) and 68.5 ± 10.2% (n = 11 growth cones) of filopodia contained synaptophysin and synapsin, respectively. Moreover, these synaptic vesicle–associated proteins colocalized with VAMP2. In co-transfected cells, synaptophysin-EGFP colocalized with VAMP2-DsRed (**Fig. 1b** and **Supplementary Fig. 3**). Endogenous synaptic vesicle proteins also colocalize within growth cone filopodia: 83.4 ± 6.7% (n = 10 growth cones) of clusters that contained either synapsin or VAMP2 contained both proteins (**Fig. 1d–f**). The presence of synaptic vesicle protein clusters within many of the filopodia suggests that axonal growth cone filopodia of cortical neurons contain either synaptic vesicles or synaptic vesicle precursors.

To determine whether filopodial synaptic vesicle protein clusters correspond to vesicles, neurons were stained with FM4-64 during depolarization with artificial cerebrospinal fluid (ACSF) containing high K^+ (80 mM), a protocol used extensively to label cycling synaptic vesicles at synapses[19]. Within labeled growth cones, 47.2 ± 4.9% of filopodia had detectable FM4-64 puncta (n = 33 growth cones). The growth cones did not stain with FM dyes in the absence of depolarization, indicating that there is not substantial constitutive endocytosis. In transfected neurons, clusters of VAMP2-EGFP were labeled with FM dye, whereas the rest of the filopodial membrane was not (**Fig. 1g**). In 61.5% of VAMP2-containing filopodia, VAMP2 clusters were stained with FM4-64 (n = 13 filopodia). To determine whether endogenous synaptic vesicle proteins also associate with vesicles, neurons were loaded with FM4-64, imaged, then fixed and immunolabeled for synaptophysin. FM4-64 and endogenous synaptic vesicle proteins were often found in the same vesicle (**Fig. 1h–j**). Since FM4-64 is trapped in endocytosed vesicles and cannot enter cells by crossing membranes, these results indicate that synaptic vesicle proteins are associated with vesicles in growth cone filopodia.

Time-lapse imaging of growth cones loaded with FM4-64 (**Fig. 2a–f**) or expressing VAMP2-EGFP (**Fig. 2g–j** and **Supplementary Video** online) revealed that vesicles move rapidly and bidirectionally along filopodia. Most active growth cones (69.4%; n = 36 growth cones) contained vesicles that moved within their filopodia. Vesicles moved at rates as high as 0.2 μm/s, consistent with the rates of some microtubule motors[20]. Interestingly, filopodial vesicles moved more slowly than expected for transport via KIF1a (1.5 μm/s), which is

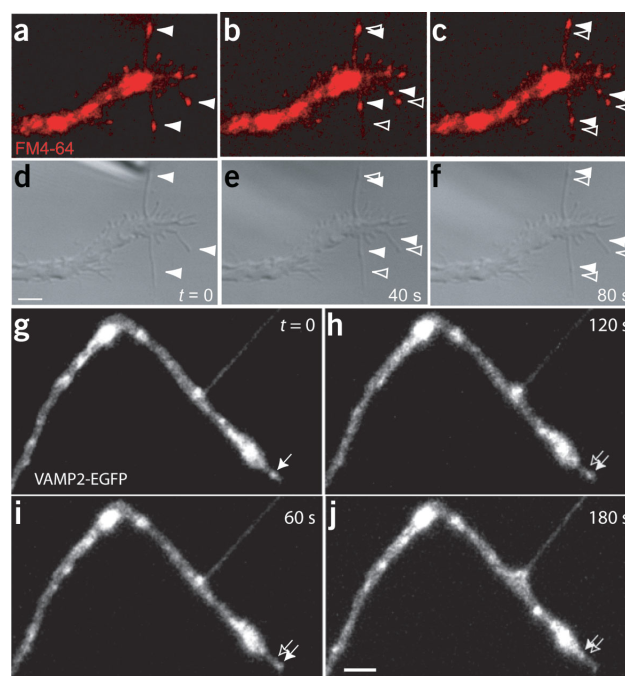

Figure 2 Vesicles move rapidly and bidirectionally along axonal growth cone filopodia. Time-lapse images were collected of neurons either loaded with FM4-64 (**a**–**c**) or transfected with VAMP2-EGFP (**g**–**j**). Filled arrows and arrowheads show the current positions of three vesicles in each frame; open arrows and arrowheads show the starting positions of each vesicle. (**d**–**f**) Phase contrast images show all filopodia for the images in **a**–**c**. Scale bars, 5 μm.

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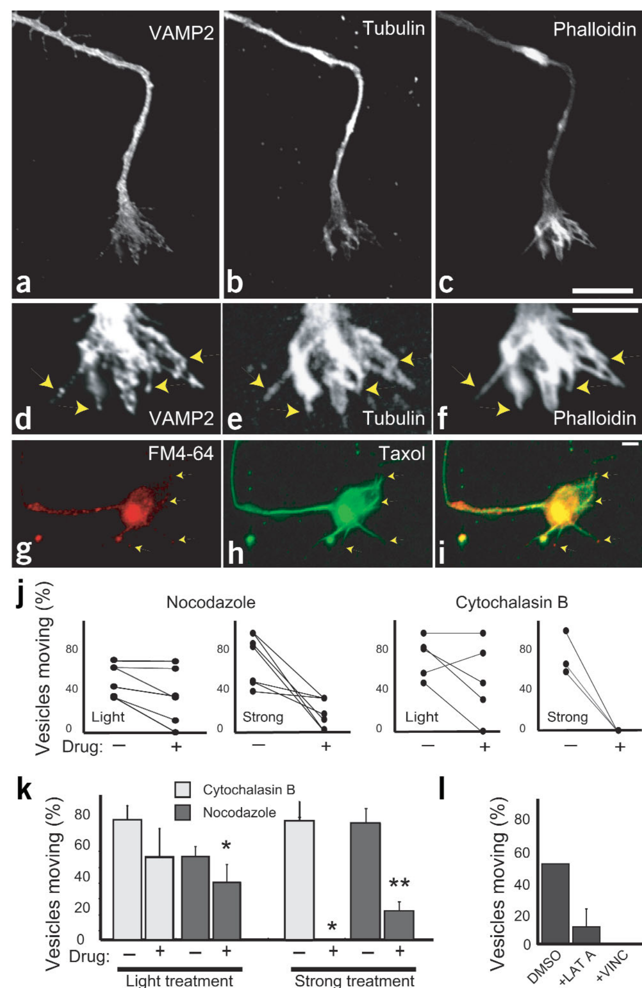

Figure 3 Movement of vesicles within axonal growth cone filopodia depends on microtubules. Triple-labeling with anti-VAMP2 antibodies (**a**,**d**), anti-tubulin antibodies (**b**,**e**) and phalloidin-543 (**c**,**f**), to label vesicles, microtubules and actin, respectively. Filopodia that contain vesicles also contain microtubules (arrows). (**a**–**c**) Low-magnification images of the triple labeling shown in **d**–**f**. Scale bars, 10 μm (**a**–**c**) or 5 μm (**d**–**f**). (**g**–**i**) Living neurons were loaded with FM4-64 (**g**) and paclitaxel-488 (Taxol in **h**) to label vesicles and microtubules, respectively. Microtubules appeared in filopodia that contained vesicles (arrows). Scale bar, 5 μm. (**j**–**l**) Treatment with microtubule and actin depolymerizing agents suggests that vesicular transport within filopodia depends on microtubules. Growth cones were imaged before (–) and after (+) treatment with nocodazole or cytochalasin B to depolymerize microtubules or actin, respectively. Pairs are shown before and after treatment in **j**. Light treatment, 20–30 min with 3 μM nocodazole ($n = 6$) or 4 μM cytochalasin B ($n = 5$). Strong treatment, >30 min with 10 μM nocodazole ($n = 7$) or cytochalasin B ($n = 4$). (**l**) In parallel experiments, growth cones were imaged with and without treatment for 4 h with 5 μM latrunculin A ($n = 12$) or vincristine ($n = 16$), to depolymerize actin and microtubules, respectively. The data represent the mean (± s.e.m.) percentage of vesicles moving during imaging. *$P < 0.05$, **$P < 0.01$. Statistical tests were repeated measures ANOVA for paired analysis (**g**) and ANOVA factorial for population analysis (**h**) with the Fisher's PLSD post-hoc test.

thought to carry synaptic vesicle precursors along axons[20], suggesting that alternate motors transport synaptic vesicle protein–containing vesicles within growth cone filopodia. Vesicle movements were not a result of filopodial extension or retraction, as movements could be seen within stable filopodia. Furthermore, when filopodia were mobile, vesicle and filopodial movements were not correlated. Because filopodia are highly dynamic and vesicles move rapidly into and out of filopodia, only a fraction of filopodia contain vesicles at any point in time. This may have contributed to past difficulties encountered in detecting these vesicles.

To determine the mechanisms of transport of filopodial vesicles, filopodia were characterized structurally using antibodies against tubulin, the main constituent of microtubules. We found that 89.6 ± 4.8% of filopodia, identified as actin-rich protrusions, that contained VAMP- or synaptophysin-labeled vesicles also contained tubulin. Conversely, 68.1 ± 8.1% of microtubule-containing filopodia contained vesicles ($n = 12$; **Fig. 3a–f**). Vesicles and microtubules were also labeled in living neurons using FM4-64 and fluorescently tagged taxol, respectively (**Fig. 3g–i**). In both living and fixed neurons, filopodia that contained vesicles tended to contain microtubules, suggesting that filopodial vesicles may be transported along microtubules.

To test whether filopodial vesicles move along microtubules or actin filaments, vesicle movements were examined after depolymerization of actin or microtubules. Filopodia containing FM4-64 or VAMP2-EGFP were imaged then treated with the pharmacological agents before imaging again. Short treatments with nocodazole, to depolymerize microtubules, decreased the percentage of moving filopodial vesicles, whereas brief depolymerization of actin with cytochalasin B did not (**Fig. 3j,k**). Longer treatments with higher doses of either cytochalasin B or nocodazole resulted in nearly complete disruption of transport (**Fig. 3j,k**). Finally, prolonged exposure to the alternative depolymerizing agents latrunculin A (depolymerizes actin) or vincristine (depolymerizes microtubules) almost completely inhibited vesicle movement within filopodia (**Fig. 3l**). Thus, both actin and microtubules are required for movement of vesicles in growth cone filopodia. The role of actin may be indirect, however, since the microtubule cytoskeleton is dependent on the actin network[13,21]. Therefore, the most conservative conclusion is that movement of vesicles within axonal growth cone filopodia depends on microtubules and may also depend on actin.

The presence of synaptic vesicle proteins in filopodial vesicles raised the question of whether synaptic vesicle–like cycling occurs within filopodia. To test this, after loading with FM4-64 as described above, filopodia were focally stimulated while imaging FM4-64 fluorescence (**Fig. 4a–c**). Decreases in FM4-64 fluorescence correspond to fusion of vesicles with the plasma membrane[19]. Nonspecific decreases in intensity were controlled for by determining the fluorescence of a neighboring axon during the same imaging period. After focal stimulation (30–90 s), 73.3% of filopodial vesicles ($n = 15$) lost more than 30% of their staining intensity. The mean percentage of puncta that were destained per filopodium (63.0%, $n = 9$) was significantly different than for unstimulated axons (8.3%, $n = 4$) during the same imaging periods (**Fig. 4c**; $P < 0.05$, ANOVA with Fisher's PLSD). DIC images were used to exclude from the analysis vesicles and filopodia that moved enough to confound the interpretation. The stimulation-dependent fusion of vesicles with the filopodial plasma membrane implies that these vesicles serve an important role within filopodia, possibly contributing to filopodial signaling and activity.

Glutamatergic regulation of filopodial dynamics has recently been shown to be important for synapse formation[22]. Since filopodial vesicles contain synaptic vesicle proteins and undergo regulated fusion, we tested whether they might release glutamate. The best indicator of whether glutamate is packaged into vesicles is the presence of the vesicular glutamate transporter VGlut1 (ref. 23). Immunofluorescence labeling revealed that clusters of VGlut1 are found along

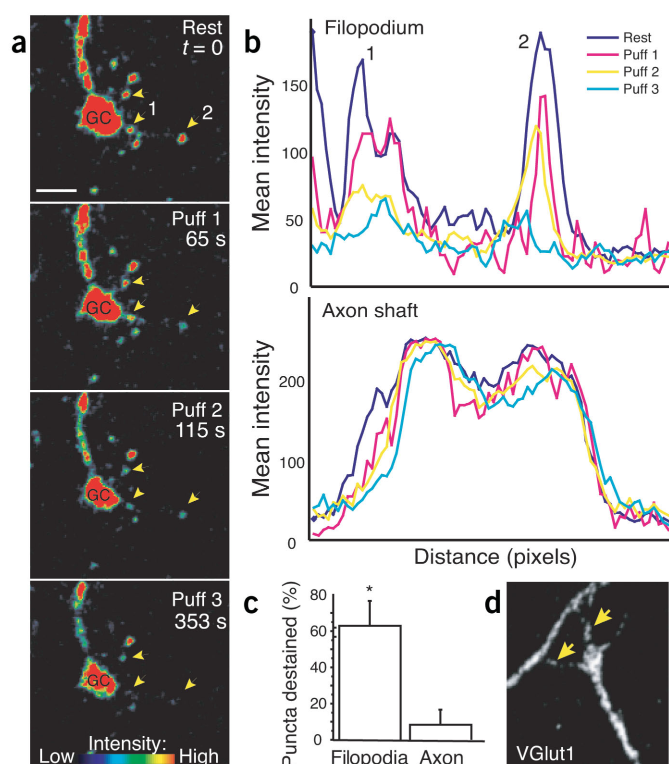

Figure 4 Filopodial vesicles fuse with the axonal growth cone plasma membrane. (**a**) A growth cone loaded with FM4-64 (Rest). Images are pseudocolored (see scale; GC, growth cone). Vesicles (arrows) are increasingly destained in response to local stimulation (Puffs 1–3) and subsequent membrane fusion. Scale bars, 5 µm. (**b**) Mean FM4-64 intensities along each filopodium before (Rest) and after (Puffs 1–3) successive rounds of stimulation. Peaks in the initial intensity profiles correspond to vesicles (numbered arrows in **a**). Whereas filopodial vesicles destained upon stimulation (upper panel), intensities within neighboring, unstimulated axons did not (lower panel). (**c**) The mean (± s.e.m.) percentage of puncta within each filopodium that were destained, losing >30% of their average initial fluorescence (n = 15 and 14 for filopodial and axon shaft puncta, respectively). *$P < 0.05$, ANOVA with Fisher's PLSD. (**d**) Immunofluorescence labeling of growth cone filopodia for the vesicular glutamate transporter VGlut1 (arrows) indicates that vesicle fusion is likely to result in glutamate release.

growth cone filopodia (**Fig. 4d**; n = 9 filopodia), suggesting that glutamate can be released from synaptic vesicle–like vesicles in filopodia. Dendrites should detect glutamate emitted from nearby filopodia since glutamate receptors are clustered in dendrites of cortical neurons at 3–5 d.i.v.[24]. Although the global effects of glutamate release remain unresolved, local release of glutamate is likely to stabilize otherwise-transient interactions between axonal growth cone filopodia and dendritic filopodia, which are thought to facilitate synaptogenesis[25]. In support of this hypothesis, neurotransmitter reduces the motility of both axonal and dendritic filopodia[16,26,27]. Previous reports have shown that motor neurons release acetylcholine before synapse formation[28,29], but these are the first data to show that transmitter release could occur from a single growth cone filopodium.

DISCUSSION

Neuronal growth cones contain many vesicles of varying sizes, concentrated in the growth cone central domain. The density of membranous organelles in the central region has made it difficult to characterize the movements and cycling of vesicles within growth cones. In addition, until recently, studies of membrane dynamics in growth cones were limited by the available methodologies, such as DIC imaging or labeling with relatively nonspecific dyes[13–15,30], which has obscured analysis of individual classes of vesicles. For example, using these methods, it has been shown that large vacuoles appear throughout the growth cone, then move retrogradely toward the base of the growth cone within minutes after they first appear[30]. Orthograde movements have been observed much less frequently in growth cones[5].

By examining filopodia, which are not organelle-rich, we were able to characterize the transport and fusion of a particular class of vesicles, identified by their synaptic protein cargo. We have shown here not only that living axonal growth cone filopodia contain vesicles, but also that these filopodial vesicles exhibit important functional properties and molecular profiles that are characteristic of synaptic vesicles at mature synapses. These vesicles move bidirectionally, frequently switching direction even at sites other than the ends of the filopodia. Moreover, the movement depends on microtubules and occurs at rates comparable to those of multiple kinesin superfamily motors known to be expressed in axons and growth cones[20,31]. For example, KIF2, KIF3 and conventional kinesin move at rates ranging from 0.3–0.5 µm/s and are all found in axons[20]. Members of the N-type KIF family move at rates as slow as 0.05 µm/s, and KIF4, a member of this family, is found in growth cones[20,31].

It is commonly thought that growth cone vesicles mainly serve as membrane donors for growth; however, there is some indirect evidence that growth cones might secrete neurotransmitter via vesicles similar to synaptic vesicles. Norepinephrine is stored in small (40–60 nm) vesicles in cultured rat sympathetic neuron growth cones[8]; however, similar evidence has been lacking for other types of neurons, largely because antibodies to neurotransmitters usually label the entire cytoplasm of the growth cone[5]. Acetylcholine is secreted from motor neuron growth cones in culture[28,29], and isolated growth cones from rat forebrain release GABA[32]. Although neurotransmitter release from isolated axons is calcium-dependent, it is unclear whether the release is from vesicular stores or directly from the cytoplasm[28,29]. Here we show that vesicles that contain a number of synaptic vesicle proteins—including the vesicular glutamate transporter VGlut1—are present in axonal growth cone filopodia where they fuse with the surface and presumably release glutamate in response to local stimulation.

Growth cone filopodia are important structures that have behaviors and characteristics distinct from those of the palm of the growth cone. Filopodia are specifically interesting in part because their growth can be monitored to correlate whether or not dynamics of particular vesicles are related only to addition of membrane to the surface for growth. This correlation has not been possible in past studies of membrane dynamics in growth cones in general. Movements of the filopodial vesicles we have investigated are uncorrelated with elongation or retraction of filopodia and are therefore unlikely to function solely as membrane donors for growth.

We have demonstrated that synaptic-like vesicles fuse with the filopodial surface in response to focal stimulation. Fusion releases vesicular contents; therefore, in addition to the well-established ability of filopodia to detect signals from potential synaptic partners[33], neuronal growth cone filopodia can also emit signals. When released from a single filopodium, highly localized secreted signals could be involved in multiple processes during develop-

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ment, such as focal priming of potential postsynaptic sites for contact and eventual synaptogenesis.

More generally, the presence of fusion-competent vesicles within filopodia allows for rapid, localized regulation of the composition of the plasma membrane of a single growth cone filopodium. Proteins important for axonal pathfinding, target selection or growth factor signaling might be delivered quickly to the filopodial surface via these vesicles. Before this study, models of filopodial function required proteins either to be constantly on the filopodial surface or to be delivered selectively, via an unknown mechanism, to the filopodial membrane after insertion within the growth cone central domain or the axon shaft. The results presented here support a new model in which rapid vesicular transport to, and fusion within, growth cone filopodia allow the contents of an individual filopodium to be quickly altered in response to signals communicated by a potential target. The present data alter the way we must think about filopodial function, suggesting that growth cone filopodia and their vesicles have active roles in growth factor signaling, axon guidance and synapse formation.

METHODS

All studies were conducted with an approved protocol from the University of California Davis Animal Care and Use Committee, in compliance with NIH guidelines for the care and use of experimental animals.

Neuronal cultures. Cultures were prepared from postnatal rat visual cortex essentially as described previously[24]. Neurons were either grown directly on a monolayer of rat cortical astrocytes or on coverslips coated with poly-L-lysine and inverted over astrocyte monolayers[34].

Transfections. Neurons were transfected with Lipofectamine 2000 (Invitrogen) 24–48 h before imaging[24], using 4–7 ng DNA and about 4 μl Lipofectamine per mm^2. Transfection with small amounts of DNA and short expression times yielded expression that was not significantly higher than endogenous levels in neighboring axons. With double transfection, localization of each protein appeared similar to expression alone. The pattern of VAMP2-DsRed staining of axons appeared identical to that for VAMP2-EGFP. Neurons were healthy and their growth cones active: filopodia often explored the local environment, and VAMP2-containing clusters moved within axons.

Imaging. Growth cones were imaged with a Zeiss Pascal confocal system using a 40× objective lens. Imaging was conducted with constant perfusion with warm (30–35 °C) ACSF (120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose and 20 mM HEPES; pH 7.2 with 0.2% sorbitol). Laser power was low (6% or less for 488 nm Ar and 18 % or less for 543 nm HeNe) to avoid phototoxicity. Images were collected every 10 s, with 3 s or shorter scan times. For dual-color imaging, channels were collected sequentially to eliminate bleed-through. Axons were identified using morphological criteria (**Supplementary Fig. 1** and **Supplementary Methods** online). Filopodia were identified as thin (typically <1 μm diameter), elongated protrusions extending from the leading edges of growth cones. Active growth cones were defined as having vesicle movements somewhere within the growth cone or its axon.

Immunofluorescence. Neurons were fixed, permeabilized, labeled, and then mounted in Fluoromount (Fisher) containing DABCO (Sigma). For more detail and antibody information, see **Supplementary Methods** online. The average numbers of filopodia on living and fixed neurons were not significantly different.

FM4-64 loading and unloading. Vesicles were loaded with FM4-64 (Molecular Probes) in high-K^+ ACSF (38 mM NaCl, 85 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose and 20 mM Hepes, pH 7.2 with 0.2 % sorbitol) containing 10 or 15 μM FM4-64. Images were collected after washing at least 10 min in the absence of dye. This labeling protocol is expected to underestimate the total vesicle pool (see **Supplementary Methods** online).

Filopodia were focally stimulated and FM4-64 unloaded by picospritzing high K^+ ACSF containing 300 mM sucrose (to visualize the area of stimulation) while collecting images. The region of application was kept small (2–10 mm) by placing a suction pipette (10 mm) opposite the stimulation pipette (1 mm)[35]. The intensity was measured along a line drawn down the length of the filopodium and peak mean intensities compared before and after stimulation. Mean intensities were averages for all frames before and after stimulation.

Retrospective immunofluorescence. Neurons were loaded with FM4-64, imaged, then immediately fixed and immunolabeled for synaptophysin. The same cell was imaged again, and the FM4-64 and synaptophysin images were superimposed. Colocalization is expected to be underestimated by this method (see **Supplementary Methods** online for details).

Pharmacological manipulations. Neurons were imaged, incubated for 20–30 min with 4 μM cytochalasin B[13,36] or 3 mM nocodazole[36–38] and imaged again in the presence of the drugs. Alternatively, neurons were treated for >30 min with 10 μM nocodazole or cytochalasin B. In additional experiments, growth cones from sister cultures were imaged after incubation for 4 h with 5 μM latrunculin A, vincristine or vector (DMSO).

Analysis. Analyses were performed using Image Pro (Media Cybernetics), StatView (SAS), Excel (Microsoft), and S-Plus (Mathsoft). For explanation of why maximum rates are presented, see **Supplementary Methods** online. Statistical analyses were ANOVA factorial or repeated measures ANOVA with Fisher's PLSD.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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