Development/Plasticity/Repair

X11/Mint Genes Control Polarized Localization of Axonal Membrane Proteins *in Vivo*

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Mislocalization of axonal proteins can result in misassembly and/or miswiring of neural circuits, causing disease. To date, only a handful of genes that control polarized localization of axonal membrane proteins have been identified. Here we report that *Drosophila* X11/Mint proteins are required for targeting several proteins, including human amyloid precursor protein (APP) and *Drosophila* APP-like protein (APPL), to axonal membranes and for their exclusion from dendrites of the mushroom body in *Drosophila*, a brain structure involved in learning and memory. Axonal localization of APP is mediated by an endocytic motif, and loss of *X11/Mint* results in a dramatic increase in cell-surface levels of APPL, especially on dendrites. Mutations in genes required for endocytosis show similar mislocalization of these proteins to dendrites, and strongly enhance defects seen in *X11/Mint* mutants. These results suggest that X11/Mint-dependent endocytosis in dendrites may serve to promote the axonal localization of membrane proteins. Since X11/Mint binds to APP, and abnormal trafficking of APP contributes to Alzheimer's disease, deregulation of X11/Mint may be important for Alzheimer's disease pathogenesis.

Introduction

Neurons are highly specialized, polarized cells that contain somatodendritic and axonal compartments, which are morphologically and functionally distinct (Higgins et al., 1997). Membrane and membrane-associated proteins restricted to dendrites or axons are important for diverse functions that include regulation of neurite growth, branching and guidance, and synaptic connectivity (Horton and Ehlers, 2003). Dysfunction and/or mislocalization of membrane proteins can produce misassembly and/or miswiring of neural circuits, causing neuropsychological disorders, such as Alzheimer's disease (AD), schizophrenia, autism, and epilepsy (Aridor and Hannan, 2000, 2002; Sutula, 2004; Stokin et al., 2005; Walsh and Engle, 2010). Accordingly, it is important to identify mechanisms by which membrane proteins are targeted to specific neuronal compartments.

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To date, only a handful of genes have been identified as being required for polarized localization of axonal membrane proteins. For example, silencing of *NEEP21* (*neuron-enriched endosomal protein of 21 kDa*) or *EHD1* (*Eps15 homology-domain containing protein 1*) causes somatodendritic accumulation of the cell adhesion molecule L1/NgCAM in dissociated hippocampal neurons (Yap et al., 2008; 2010; Lasiecka et al., 2010;). Similarly, silencing or expression of a dominant-negative against *myosin VI* disrupts polarized localization of L1/NgCAM in dissociated cortical neurons (Lewis et al., 2011). Here, we identify *X11/Mint* as a new regulator of polarized localization of multiple axonal membrane proteins *in vivo*. Mutations in the amyloid precursor protein (APP) cause familial forms of AD (Tanzi, 2012), the most common neurodegenerative disorder. Sequential cleavage of APP by proteases results in production of cytotoxic β-amyloid (Aβ), which leads to synaptic dysfunction and subsequent amyloid plaque formation in AD brains (Tanzi, 2012). X11/Mint proteins bind to the APP intracellular domain (AICD) (Borg et al., 1996), and are a component of Aβ aggregates (McLoughlin et al., 1999). Overexpression of *X11* in *APP* transgenic mice can ameliorate many AD-like deficits, including Aβ plaque formation, defects in long-term potentiation, and spatial memory impairments (Lee et al., 2003, 2004; Mitchell et al., 2009). In contrast, loss of *X11* can enhance AD-like pathology, resulting in increased Aβ levels and more pronounced electrophysiological deficits (Ho et al., 2006, 2008; Sano et al., 2006; Saito et al., 2008; Saluja et al., 2009; Kondo et al., 2010). How X11/Mint proteins act on APP is poorly understood. Several studies have suggested loss of *X11/Mint* affects trafficking and sorting of APP *in vitro* (Hill et al.,

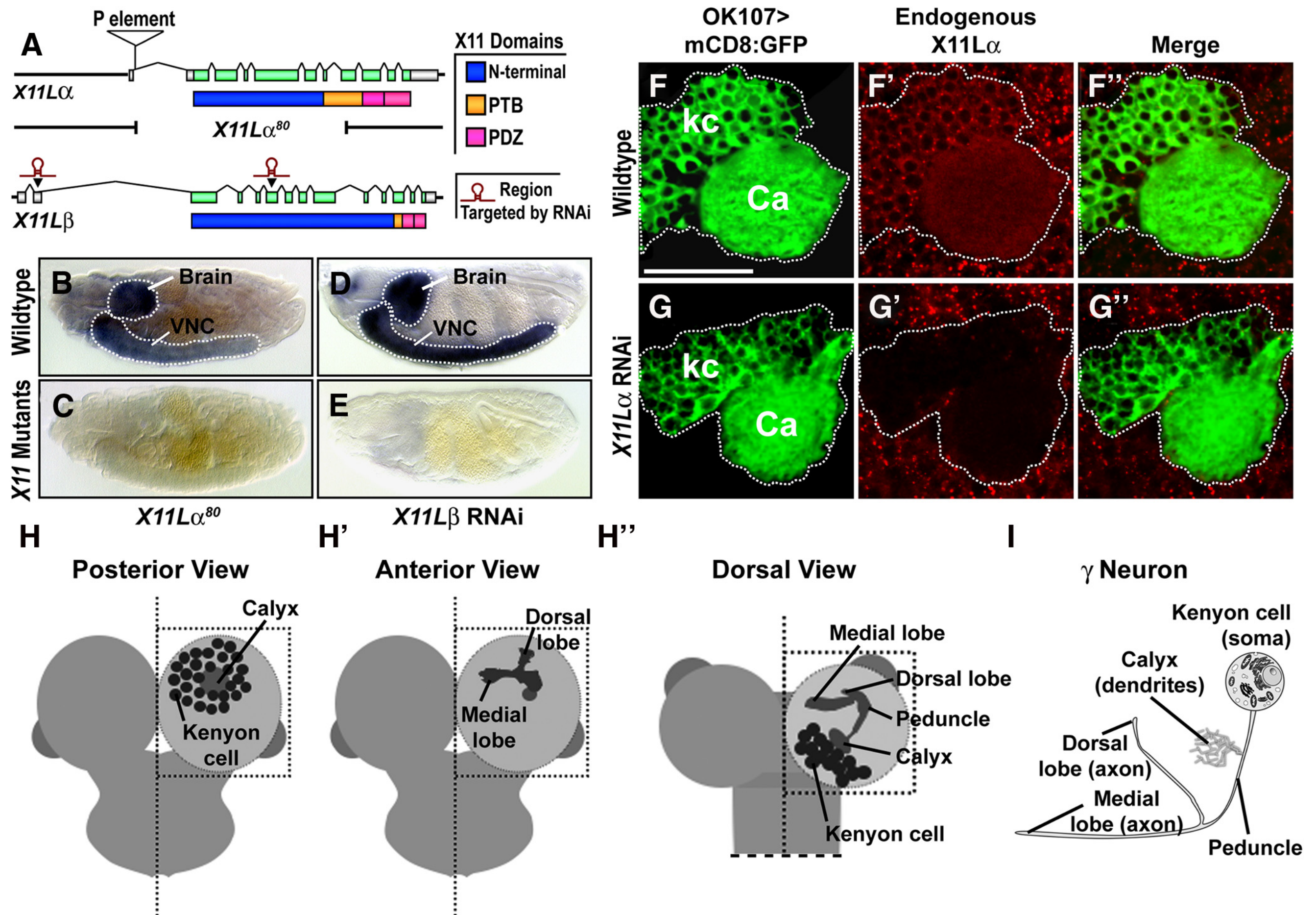

Figure 1. *X11* is expressed in the *Drosophila* CNS. ***A***, Schematic depicting *Drosophila X11* paralogs *X11Lα* and *X11Lβ*. X11 proteins consist of a N-terminal domain, PTB domain, and two PDZ domains. Loss-of-function mutant $X11L\alpha^{80}$ was generated by imprecise excision of a P element inserted in the 5′ UTR, which also excised a translational start codon, the N-terminal domain, and part of the PTB domain. *X11Lβ* was targeted by RNAi technology at two independent regions (black arrows). ***B–E***, *In situ* hybridizations against *X11Lα* (***B***, ***C***) or *X11Lβ* (***D***, ***E***) in stage 16/17 embryos indicate *X11/Mint* expression is highest in the CNS, which is outlined by a dotted line. ***C***, Embryos homozygous for $X11L\alpha^{80}$ show no detectable *X11Lα* transcript in the brain or ventral nerve cord (VNC) of the CNS. ***E***, Expression of an RNAi transgene against *X11Lβ* by pan-neuronal driver elav-GAL4 at 29°C dramatically reduces *X11Lβ* transcript levels. ***F–F″***, Larval brains stained with anti-*X11Lα* antibody indicate *X11Lα* (red) is expressed in the MB, labeled by mCD8-GFP (green) driven by MB-specific driver OK107-GAL4. Kenyon cells (kc) and the calyx (Ca) are visible, which are outlined by a dotted line. The calyx consists of densely packed dendrites, to the extent that cytoplasmic proteins exhibit a diffuse staining pattern when imaged by confocal microscopy. ***G–G″***, An RNAi transgene against *X11Lα* expressed by OK107-GAL4 at 29°C dramatically reduces X11Lα staining in the MB. Scale bar: (in ***F***) ***F–G″***, 50 μm. ***H–H″***, ***I***, Illustrations of the larval MB, which comprises exclusively γ neurons (***I***), depict three easy-to-identify neuronal compartments: Kenyon cells (cell bodies), the calyx (dendrites), and lobes (axons). Neurites from Kenyon cells project into the larval brain's posterior neuropile to give rise to dendrites in the calyx (***H***). Axons project further anteriorly as a fasciculated bundle (the peduncle), which eventually bifurcates in the brain's anterior neuropile to give rise to the medial or dorsal lobe, respectively (***H′***).

2003; He et al., 2007; Shrivastava-Ranjan et al., 2008; Chaufty et al., 2012). However, how this is accomplished is still unclear.

Previously, we identified *Drosophila X11-like* (*X11Lα*) in an *in vivo* reporter-based genetic modifier screen as a regulator APP. *X11Lα* and human *X11α* function in a conserved manner, independent of APP proteolysis, to modulate activity of the AICD (Gross et al., 2008). *X11Lα* has also been reported to bind the APP ortholog, APP-Like (APPL) (Ashley et al., 2005). Here, we report a new requirement of X11/Mint in excluding APP/APPL and other axonally restricted membrane proteins from dendrites of neurons in the *Drosophila* mushroom body (MB) (Margulies et al., 2005). Furthermore, we present *in vivo* evidence that dendritic exclusion of APP/APPL and other axonal membrane proteins is dependent on endocytosis, and that X11/Mint proteins function in endocytosis and/or at Golgi to control polarized localization of axonal membrane proteins.

Materials and Methods

Molecular biology. A synthetic microRNA technology (miRNA) was used for RNAi silencing (Chen et al., 2007; Deng et al., 2008; Ganguly et al., 2008; Yun et al., 2008). To silence *X11Lβ*, the coding region and a 5′ UTR were independently targeted. To silence *X11Lα*, the coding region was targeted. PCR products of these miRNA precursors were cloned into pUASt. To generate UAS-*X11Lα*, the *X11Lα* cDNA [expression sequence tag (EST) clone from *Drosophila* Genome Research Center, LD20981], was subcloned into pUASt. All cloned PCR products were confirmed by DNA sequencing. Embryo *in situ* hybridization of either sex using digoxigenin-labeled probes against X11Lα or X11Lβ transcript was performed as described by Hemavathy et al. (1997).

Drosophila *genetics and strains.* The deletion mutant $X11L\alpha^{80}$ was generated by imprecise excision of a previously isolated P element (Gross et al., 2008) in the 5′ UTR of *X11Lα*. Breakpoints were mapped by genomic PCR followed by DNA sequencing. *UAS-hX11β* was obtained from Kenji Matsuno and Toshiharu Suzuki (Hase et al., 2002); *UAS-APPL* and *UAS-APPLΔci* from Kalpana White (Torroja et al., 1999); *UAS-Nod-LacZ* from Yuh Nung Jan (Clark et al., 1997); *UAS-Bazooka-*

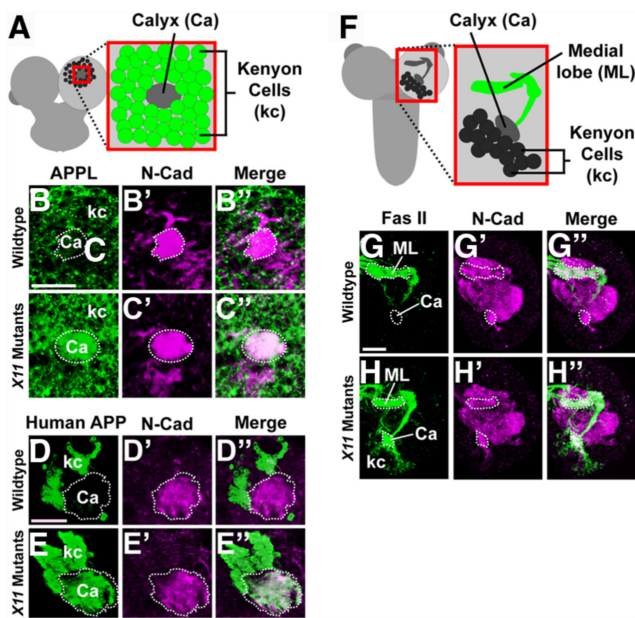

Figure 2. *X11* is required in the MB for polarized localization of human APP, APPL, and fasciclin II. ***B–E″***, ***G–H″***, Staining of brains against *Drosophila* N-cadherin (N-Cad, magenta) strongly labels the calyx of the MB, which is outlined by a dotted line, allowing for its identification. ***A***, An illustration depicts polarized localization of endogenous APPL (green) in Kenyon cells (kc) and the calyx (Ca) of a wild-type MB, viewed posteriorly. ***B–B″***, Wild-type first instar brains stained with anti-APPL antibody indicate APPL (green) is excluded from the MB's calyx. ***C–C″***, In contrast, brains of *X11* mutants stained against APPL show mislocalization of APPL to the calyx and increased levels in Kenyon cells. Scale bar: (in ***B***) ***B–C″***, 10 μm. ***D–D″***, Similarly, third instar brains exogenously expressing myc-tagged human APP_{695} by MB-specific driver OK107-GAL4 and stained with anti-Myc antibody indicate ectopic human APP (green) is mostly somatoaxonally localized, with very low levels detectable in the calyx. ***E–E″***, However, expression of RNAi transgenes against *X11* causes human APP to be dramatically increased in Kenyon cells and the calyx. Scale bar: (in ***D***) ***D–E″***, 50 μm. ***B–E″***, All brains stained against human APP or APPL were imaged from the posterior side. ***F***, Illustration depicts polarized localization of endogenous fasciclin II (green) in a wild-type MB, viewed dorsally. ***G–G″***, Wild-type first instar brains stained with anti-fasciclin II (Fas II) antibody indicate Fas II (green) is excluded from the calyx and Kenyon cells of the MB. ***H–H″***, Brains of *X11* mutants stained against Fas II show mislocalization of Fas II to the calyx and Kenyon cells, indicating loss of polarized localization. ***G–H″***, Brains stained against Fas II were imaged from the dorsal side. Mislocalization of Fas II is also visible in brains of *X11* mutants imaged from the posterior side. Scale bar, 10 μm.

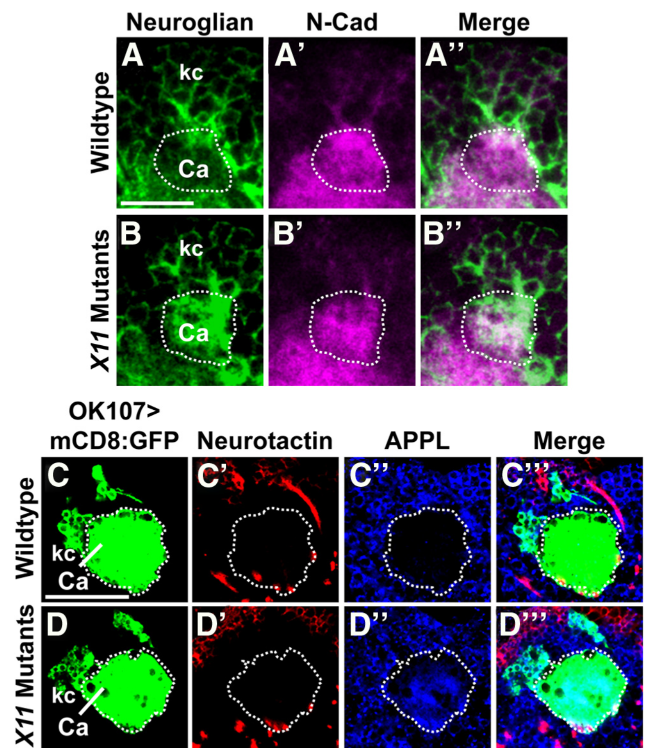

Figure 3. *X11* is not required in the MB for polarized localization of all axonal membrane proteins. ***A–B″***, Staining against *Drosophila* N-cadherin (N-Cad, magenta) strongly labels the calyx of the MB, which is outlined by a dotted line, allowing for its identification. ***A–A″***, Wild-type first instar brains stained with anti-neuroglian antibody indicate endogenous neuroglian (green) is mostly somatoaxonally localized in the MB, with very low levels detectable in the calyx (Ca). ***B–B″***, In contrast, brains of *X11* mutants stained against neuroglian show mislocalization of neuroglian to the calyx and increased levels in Kenyon cells (kc). Scale bar, 10 μm. ***C–D‴***, mCD8-GFP (green) expressed by MB-specific driver OK107-GAL4 labels Kenyon cells and the calyx, which is outlined by a dotted line, allowing for their identification. ***C–C‴***, Wild-type third instar brains stained with anti-neurotactin antibody indicate endogenous neurotactin (red) is similarly excluded from the MB's calyx. ***D–D‴***, However, brains of *X11* mutants stained against neurotactin suggest localization of neurotactin, unlike neuroglian or APPL (blue), is not affected by loss of *X11* in the MB. Scale bar, 50 μm. ***A–D‴***, All brains were imaged from the posterior side.

GFP from Daniel St. Johnston (Benton and St. Johnston, 2003); *UAS-mCD8-GFP*, *OK107-GAL4* from Liquin Luo (Lee et al., 1999). Flies carrying *elav-GAL4*, *OK107-GAL4*, *UAS-APC2-GFP*, *UAS-shi^{K44A}*, *α-adaptin^{06694}* (*AP2^{06694}*), *UAS-Dscam.exon17.1-GFP*, *UAS-GRASP65-GFP*, *UAS-KDEL-GFP*, *UAS-Rab5-GFP*, *UAS-Rab7-GFP*, or *FRT19A, tubP-GAL80, hs-FLP* were obtained from the Bloomington *Drosophila* Stock Center. For *UAS-X11Lβ.RNAi*, *UAS-X11Lα.RNAi*, and *UAS-X11Lα* transgenic animals, multiple independent fly lines were generated (Rainbow Transgenic Flies) and tested for each transgene. The recombinant stock *FRT19A, X11Lα^{80}, UAS-X11Lβ.RNAi* was assembled and confirmed essentially as described by Lee and Luo (1999). The stock *FRT19A, tubP-GAL80, hs-FLP; UAS-mCD8-GFP, OK107-GAL4* was constructed using standard genetic techniques. Mitotic clones of MB neurons homozygous for *X11Lα^{80}* and expressing *X11Lβ.RNAi* and *mCD8-GFP* under *OK107-GAL4* were generated as described by Lee et al. for MARCM (mosaic analysis with a repressible cell marker) (Lee et al., 1999).

Immunofluoresence and confocal microscopy. First and/or third instar larval brains of either sex were dissected in 1× PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Standard staining protocols were used (Ashburner, 1989) for immunocytochemistry. Primary antibodies were used at the following concentrations: rabbit anti-APPL antibody (1:500; Torroja et al., 1996), or rabbit anti-APPL (1:100; D-300, Santa Cruz Biotechnology), mouse anti-fasciclin II (1:10; Grenningloh et al., 1991; 1D4; Hybridoma Bank), mouse anti-Myc (1:100; Millipore), mouse anti-neuroglian (1:10; Hortsch et al., 1990a; BP 104, Hybridoma Bank), rat anti-DN-cadherin (1:20; Iwai et al., 2002; Hybridoma Bank), mouse anti-neurotactin (1:10; Hortsch et al., 1990b; BP 106, Hybridoma Bank), mouse anti-β-galactosidase (1:50; Sigma-Aldrich), rabbit anti-X11Lα (1:25; Gross et al., 2008). Secondary antibodies were purchased from Invitrogen and Jackson Immunoresearch and used at the manufacturer's recommended concentrations. Stained brains were mounted in Vectashield (H-1000, Vector Laboratory).

Confocal images were taken on a Bio-Rad MRC1024ES microscope using LaserSharp version 3.2 software. Complete series of optical sections were taken at 1 or 2 μm intervals. Samples from each experiment were imaged at equal intensity and magnification settings for comparison. Images were analyzed and assembled using ImageJ (United States National Institutes of Health).

APPL cell-surface labeling experiments. For analysis of cell-surface APPL in larval brains, dissected, live brains of either sex were incubated in Schneider's *Drosophila* Media (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and a rabbit polyclonal anti-APPL antibody against the extracellular domain of APPL (1:10; Torroja et al., 1996) for 4 h at 4°C. At which time, brains were moved to 30°C for 6 h to overnight. This chase step was necessary to allow efficient penetration of the APPL antibody through glia that ensheath the brains. During incubation of brains with APPL antibody, some APPL bound by antibody was internalized into neurons. Live brains were subsequently washed several

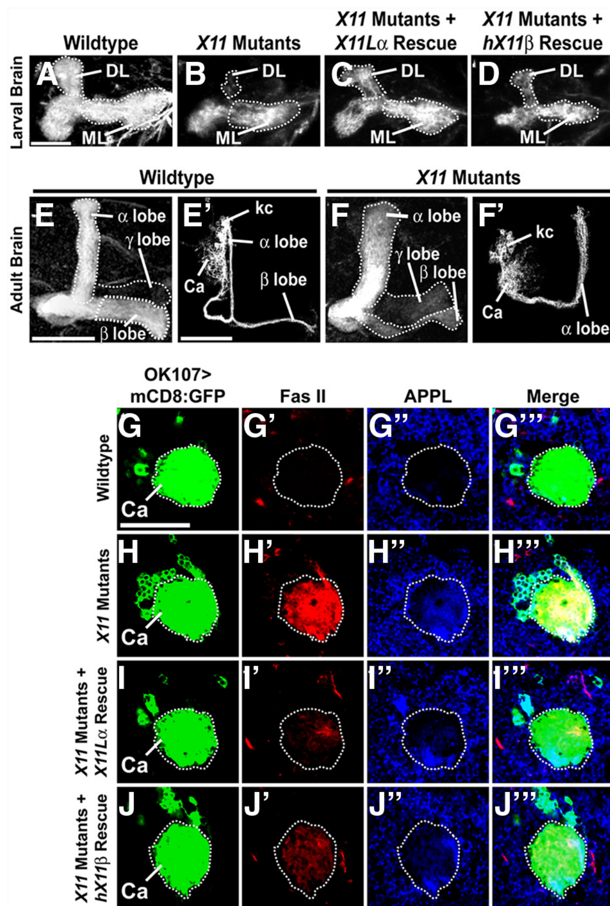

Figure 4. Ectopic expression of *X11Lα* or human *X11β* partially rescues defects in the MB of *X11* mutants. ***A–D***, First instar brains stained with anti-fasciclin II (Fas II) antibody. Scale bar, 10 μm. ***A***, In wild-type brains, Fas II staining labels the MB's dorsal lobe (DL) and medial lobe (ML), which are outlined by dotted lines. ***B***, In *X11* mutants the DL is drastically reduced and the ML misshapen, indicating aberrant axon development in γ neurons. These structural defects are partially rescuable by expression of *X11Lα* (***C***) or, to a lesser extent, human *X11β* (***D***) by pan-neuronal driver *elav*-GAL4. Expression of RNAi transgenes against *X11* by MB-specific driver OK107-Gal4 does not cause structural phenotypes in the larval MB, suggesting larval lobe defects in *X11* mutants may arise nonautonomously. ***E–F'***, However, MB-specific silencing of *X11* by RNAi does cause lobe defects in the adult brain. Adult brains stained against Fas II with MB-specific silencing of *X11* by RNAi show dramatic widening of the α lobe, shrinkage of the γ lobe, and severe thinning of the β lobe compared with wild type (***E*** vs ***F***). Mitotic clones from an MB neuroblast homozygous for $X11L\alpha^{80}$ and expressing RNAi *X11Lβ* and mCD8-GFP suggest that axonal tracts of α/β neurons project aberrantly to the α lobe (***E'*** vs ***F'***). Scale bar, 50 μm. ***A–F'***, Brains showing lobes of the MB were imaged from the anterior side. ***G–J'''***, mCD8-GFP (green) driven by OK107-GAL4 labels Kenyon cells (kc) and the calyx (Ca), which are outlined by dotted lines, allowing for their identification. In the third instar MB of *X11* mutants dendritically mislocalized APPL (blue) (***H''***) and fasciclin II (red) (***H'***) can be partially rescued to wild type (***G–G'''***) by expression of *X11Lα* (***I–I'''***) or, to a lesser extent, human X11β (***J–J'''***). ***G–J'''***, Brains showing the calyx were imaged from the posterior side. Scale bar, 50 μm.

times in ice-cold 1× PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. Fixed brains were then blocked for 1 h with 10% normal goat serum in 1× PBS at room temperature. Afterward, brains were incubated in secondary antibody in 1× PBS overnight at 4°C. The next day, brains were washed briefly in 1× PBS and fixed again for 5 min in 4% paraformaldehyde at room temperature to prevent loss of secondary antibody during later washes. Up until this point, brains were never exposed to permeabilizing conditions (i.e., detergent). Only after surface labeling of APPL was complete were standard staining protocols used (Ashburner, 1989). As a final step, brains were immunostained against DN-cadherin to label the neuropile, as described in the immunofluorescence and confocal microscopy section.

Monoclonal antibodies against neuroglian (BP 104), 1:5, or fasciclin II (1D4), 1:5, were used as negative staining controls for these experiments, to determine whether cell-surface labeling of APPL worked. The epitopes for these antibodies are reported to be strictly cytoplasmic (Hortsch et al., 1990a; Grenningloh et al., 1991). No immunostaining above background was ever observed in larval brains when using the described protein cell-surface labeling protocol with control antibodies. However, strong staining was observed when cell membranes were permeabilized using 0.1% Triton X-100.

Results

X11 is required for polarized localization of APP/APPL and multiple other axonal membrane proteins

Human and mammalian genomes contain three *X11/Mint* paralogs, *X11α/Mint1*, *X11β/Mint2*, and *X11γ/Mint3*, whereas the *Drosophila melanogaster* genome contains only two, *X11Lα* (CG5675) and *X11Lβ* (CG32677). All of these proteins contain a single phosphotyrosine binding (PTB) and two postsynaptic density protein, *Drosophila* disc large tumor suppressor, zonula occludens-1 protein (PDZ) domains (Rogelj et al., 2006; Vishnu et al., 2006). The two *Drosophila* X11L proteins share 85% amino acid identity in their PTB and PDZ domains, and the expression of both is restricted to differentiated neurons in the CNS (Hase et al., 2002; Vishnu et al., 2006). These findings raise the possibility that X11Lα and X11Lβ proteins are functionally redundant. While flies with partial loss of function of *X11Lα* are viable, and have only mild neuromuscular junction phenotypes (Ashley et al., 2005; Vishnu et al., 2006), phenotypes of flies lacking *X11Lβ* have not been reported. Utilizing a P element inserted in the 5′ UTR of *X11Lα*, we generated a large deletion, $X11L\alpha^{80}$ (Fig. 1*A*). $X11L\alpha^{80}$-mutant embryos contain no detectable *X11Lα* transcript (Fig. 1*B*,*C*), suggesting $X11L\alpha^{80}$ is a null allele. Since no loss-of-function mutants of *X11Lβ* are available, we generated two synthetic miRNA-expressing transgenic fly lines targeting two distinct regions of *X11Lβ* (Fig. 1*A*). Embryos from flies expressing either construct using the UAS-GAL4 system (Brand and Perrimon, 1993) show robust silencing of the *X11Lβ* transcript (Fig. 1*D*,*E*), and they produced highly similar if not identical phenotypes when coupled with $X11L\alpha^{80}$ or miRNAs targeting *X11Lα* we generated (Fig. 1*F–G''*). Flies lacking *X11Lα* ($X11L\alpha^{80}$), or flies with pan-neuronal silencing of *X11Lβ* alone, are viable and adults are wild type in appearance. In contrast, by pan-neuronal silencing of both *X11L* genes, or by combining the $X11L\alpha^{80}$-null allele with pan-neuronal expression of miRNAs targeted against *X11Lβ* (hereafter referred to as *X11* mutants in either case), we obtained early to mid-third instar larval lethality, suggesting that these two *X11L* genes carry out redundant functions in neurons.

We focused our studies on the *Drosophila* MB, a brain structure that shares functional similarities with the mammalian hippocampus in mediating learning and memory. AD patients show pronounced neuronal dysfunction in the hippocampus (Blennow, 2006). Immunostaining of wild-type larval brains shows that X11/Mint protein is present in the MB (Fig. 1*F–F''*; compare with Fig. 1*G–G''*, in which *X11* has been silenced) (Margulies et al., 2005). The MB of the early larval brain comprises exclusively a single class of neuron, the γ neuron. At later stages of the *Drosophila* lifecycle, the composition and circuitry of the MB becomes considerably more complex as additional classes of neurons (α, β, α', and β') are born and incorporated during extensive developmental remodeling (Lee et al., 1999). Accordingly, we limited our investigation mainly to early larval brains. Briefly, all γ neuron cell bodies (Kenyon cells) project their dendrites into the larval brain's posterior neuropile,

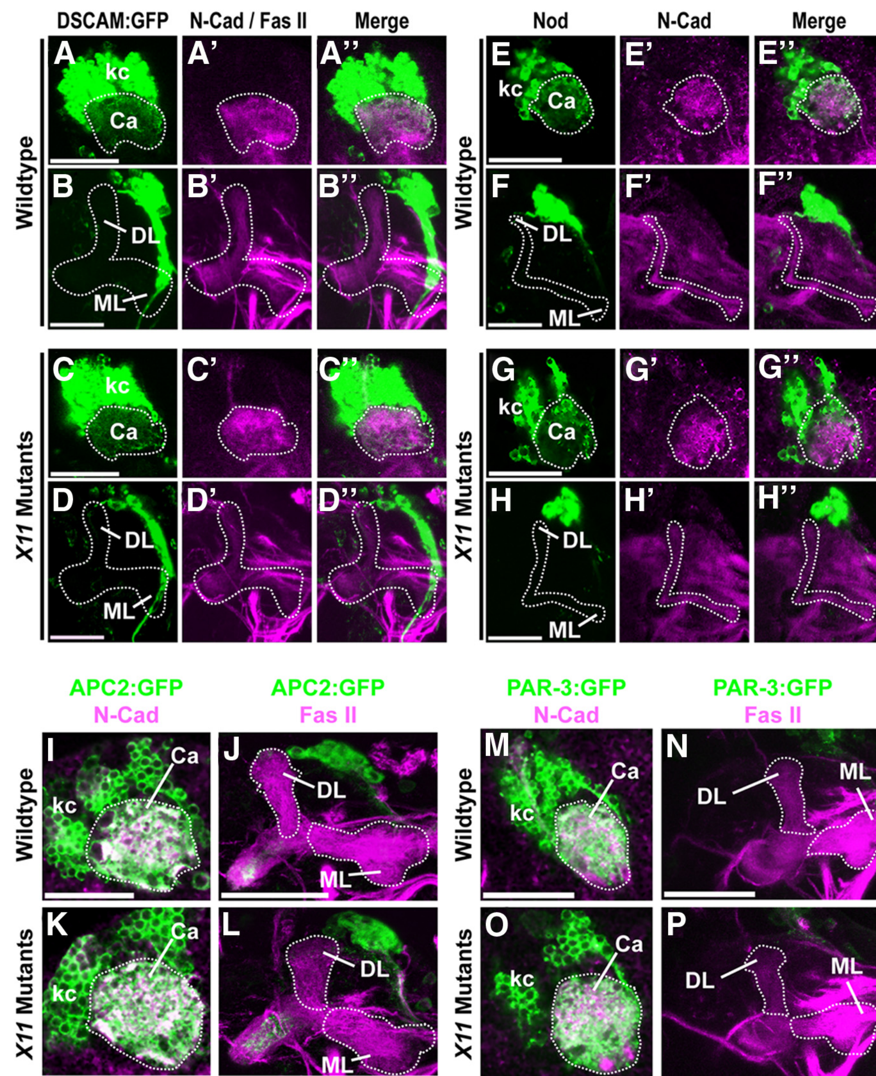

Figure 5. Neuronal polarity is unaffected in MB neurons of *X11* mutants. ***A–P***, Third instar brains expressing tagged dendritic proteins in the MB. Scale bar, 50 μm. Staining of brains against N-cadherin (N-Cad; magenta) identifies the calyx of the MB, while staining against either N-Cad (magenta) or Fas II (magenta) is used to identify the lobes. These structures are outlined by dotted lines. ***A–B″***, Wild-type brains expressing a dendritically restricted splice form of DSCAM-GFP (green), containing exon 17.1, driven by OK107-GAL4, show DSCAM[exon 17.1] is somatodendritically restricted. DSCAM[exon 17.1] is detectable in Kenyon cells (kc) and the calyx, but not in the medial lobe (ML) or dorsal lobe (DL) of the MB. ***C–D″***, Brains of *X11* mutants with MB-specific expression of DSCAM-GFP indicate DSCAM[exon 17.1] localization is not affected by loss of *X11*. ***E–F″***, Wild-type brains expressing Nod-LacZ (green) by OK107-GAL4, stained with anti-β-gal antibody, show Nod is also somatodendritically restricted. ***G–H″***, MB-specific expression of Nod-LacZ in brains of *X11* mutants, stained against β-gal, suggests that loss of *X11/Mint* does not change compartmental localization of Nod. ***I–P***, Similarly, MB-specific expression of APC2-GFP (green) or PAR-3/Bazooka-GFP (green) in wild-type brains (***I***, ***J***, ***M***, ***N***) versus in *X11* mutants (***K***, ***L***, ***O***, ***P***) indicates somatodendritic localization of APC2 and PAR-3/Bazooka is not affected by loss of *X11*.

which comprises the calyx (Fig. 1*H*). Axons of γ neurons in the larval MB fasciculate in the peduncle, which extends anteriorly, until each axon bifurcates to project one branch toward the brain's midline and another branch dorsally (Lee et al., 1999; Heisenberg, 2003). The fasciculated axons of these branches form the MB's medial lobe and dorsal lobe, respectively (Fig. 1*H*'). In summary, the structure of the larval MB is subdivided into three distinct, easily identifiable compartments that contain the cell bodies (Kenyon cells), axons (lobes), or dendrites (calyx) of γ neurons (Fig. 1*H*'',*I*).

APPL protein is also strongly expressed in the MB (Torroja et al., 1996; Li et al., 2004). We note that in wild-type γ neurons, APPL is exclusively somatoaxonally localized, being excluded from dendrites (Fig. 2*A–B*''). In contrast, polarized localization of APPL is severely disrupted in *X11* mutants. Instead of APPL being restricted to the Kenyon cells and lobes of the MB, in *X11* mutants APPL mislocalizes to the calyx (Fig. 2*C–C*''). Because of this finding, we also investigated localization of human APP ectopically expressed in *X11* mutants. Like endogenous APPL, ectopic human APP is mostly somatoaxonally restricted in wild-type MB neurons (Fig. 2*D–D*''), while in *X11* mutants, human APP is found in the calyx as well (Fig. 2*E–E*''). These results suggest that human APP contains a signal sufficient to specify its compartmental localization in γ neurons and that X11/Mint may use a conserved mechanism to exclude APP and APPL from dendrites of MB neurons.

Since APPL has previously been reported to form a tripartite complex with X11Lα and fasciclin II (Fas II) (Ashley et al., 2005), the *Drosophila* ortholog of neural cell adhesion molecule (NCAM), we also examined Fas II localization in the MBs of *X11* mutants. As with APP/APPL, Fas II is axonally restricted in wild-type γ neurons (Fig. 2*F–G*''). However, in *X11* mutants, we found that Fas II is similarly mislocalized to the calyx (Fig. 2*H–H*''). This mislocalization of Fas II is visible in brains imaged from their dorsal or posterior sides. To determine whether disruption of polarized localization in *X11* mutants is limited to APP/APPL and Fas II, or is more extensive, we examined larval MBs using antibodies against neuroglian (Nrg), an ortholog of L1/NgCAM, or the cell adhesion molecule neurotactin (Nrt). Neither Nrg nor Nrt share sequence homology with APPL or Fas II, or are known to bind X11/Mint. Nevertheless, like APPL and Fas II, both Nrg and Nrt are axonally restricted in wild-type γ neurons of the MB (Fig. 3*A–A*''). Interestingly, although we observed dendritic mislocalization of Nrg to the MB's calyx in *X11* mutants (Fig. 3*B–B*''), we did not detect similar mislocalization of Nrt (Fig. 3*C–D*'''). Together, these findings indicate that X11/Mint proteins regulate the polarized localization of a subset of axonal membrane proteins, including APP/APPL, Fas II, and Nrg, but not Nrt.

Along with mislocalization of multiple axonal membrane proteins, we observed structural defects in the MB lobes of *X11* mutants. Formation of the dorsal lobe during MB development is aberrant in most *X11* mutants, causing it to be severely truncated (Fig. 4*A*,*B*). In addition, the medial lobe appears frayed and misshapen, indicating abnormal axon development. When we specifically silence *X11/Mint* expression in MB, which is a structure dispensable for viability (Margulies et al., 2005), flies survived to adulthood, with gross structural

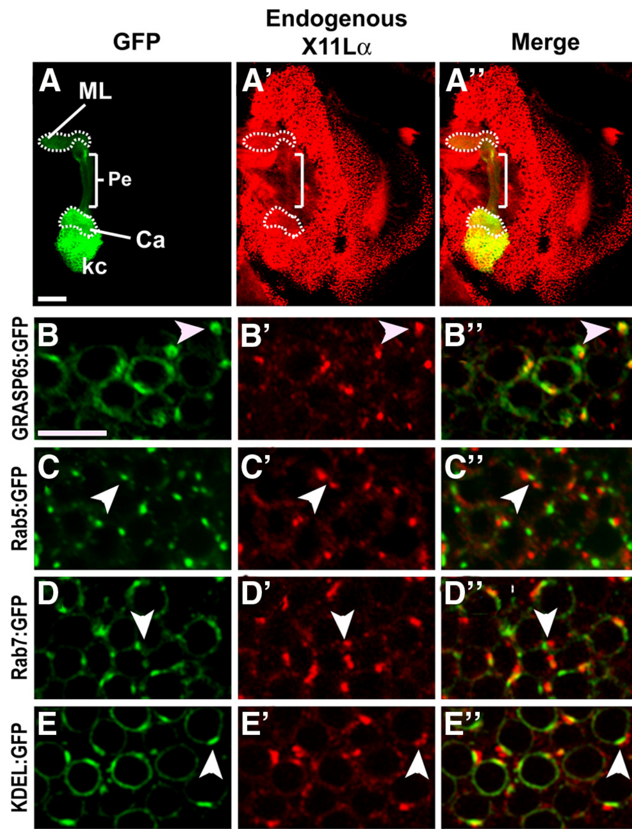

Figure 6. X11Lα is present at Golgi, dendrites, and axons in MB neurons. ***A–A″***, Third instar wild-type brains stained against X11Lα indicate moderate levels of X11Lα are present in the peduncle (Pe), calyx (Ca), medial lobe (ML), and dorsal lobe of the MB, which are labeled by mCD8-GFP (green) expressed by OK107-GAL4. Scale bar, 50 μm. ***B–E″***, Third instar brains stained against X11Lα expressing various GFP-tagged subcellular markers by MB-specific driver OK107-GAL4. Scale bar, 10 μm. ***B–B″***, Larval brains stained with anti-X11Lα antibody show strong X11Lα (red) colocalization with the Golgi marker GRASP65-GFP (green) in Kenyon cells (kc). ***C–C″***, In contrast, X11Lα staining poorly colocalizes with the early endosome marker Rab5-GFP (green) and only mildly colocalizes with the late endosome marker (***D–D″***) Rab7-GFP (green) or the endoplasmic reticulum marker (***E–E″***) KDEL-GFP (green).

defects in the lobes (Fig. 4*E–F*'). Importantly, we were able to partially rescue axonal membrane protein mislocalization and structural defects in the MB of *X11* mutants by ectopically expressing *X11Lα* or human *X11β* (Fig. 4*C*, *D*, *G–J*'''). These results indicate that functions of X11/Mint are partially conserved between flies and humans.

Neuronal polarity is unaffected in X11 mutants

Mislocalization of axonal proteins in *X11* mutants could be due to loss of dendritic compartment identity. To investigate this possibility, we compared the localization of several putative dendritic markers in the MB of *X11* mutants versus wild-type animals. A dendritically restricted splice form of the *Drosophila* membrane protein DSCAM (Down syndrome cell adhesion molecule), which contains exon 17.1 (Wang et al., 2004), is expressed exclusively in membrane of the somatodendritic compartment in wild-type MB neurons (Fig. 5*A–B*''). Importantly, in *X11* mutants, this localization was unchanged (Fig. 5*C–D*''). Nod, APC2, and PAR-3/Bazooka, well characterized cytoskeleton regulators, also exhibited restricted somatodendritic localization in the MB of wild-type animals (Rolls et al., 2007) and *X11* mutants (Fig. 5*E–P*). In summary, neither DSCAM[exon 17.1], Nod, APC2, nor PAR-3/Bazooka mislocalizes to axons of γ neurons lacking

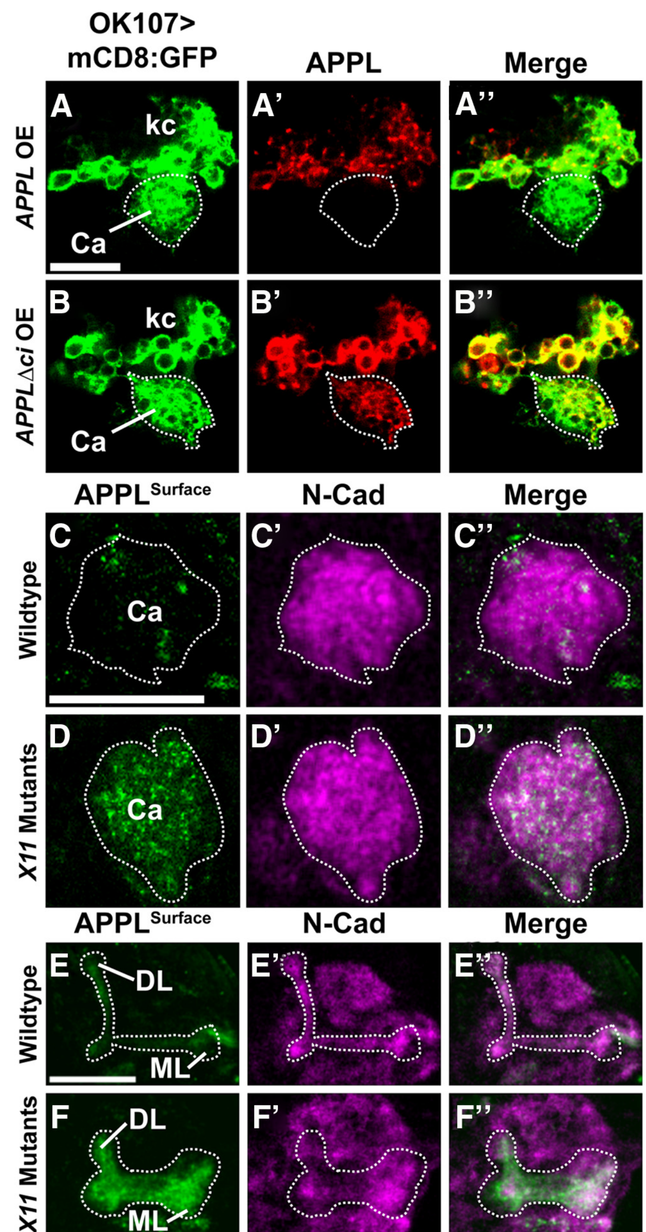

Figure 7. APPL requires an endocytic motif for dendritic exclusion, and is increased on the dendritic surface of MB neurons in *X11* mutants. ***A–B″***, mCD8-GFP (green) expressed in larval brains by MB-specific driver OK107-GAL4 labels Kenyon cells (kc) and the calyx (Ca; dotted line). Scale bar, 10 μm. ***A–A″***, First instar brains homozygous for *appl^d^*, a null allele, overexpressing wild-type *APPL* by OK107-GAL4, and stained with anti-APPL antibody indicate ectopic APPL (red) is excluded from the calyx. ***B–B″***, In contrast, similar MB-specific expression of *APPLΔci*, which removes the highly conserved motif GYENPTY, shows APPL staining mislocalized to the calyx. ***C–F″***, Since the GYENPTY motif is involved in endocytosis of APPL, live third instar brains of *X11* mutants were labeled with an anti-APPL antibody against APPL's extracellular domain to detect cell-surface levels of APPL. Staining of brains against *Drosophila* N-cadherin (N-Cad, magenta) labels the calyx and lobes of the MB, which are outlined by dotted lines, allowing for their identification. Brains of *X11* mutants show abnormal levels of APPL (green) on the surface of the calyx (***D–D″***) versus wild type (***C–C″***). Scale bar, 50 μm. In addition, levels of APPL appear increased on the surface of the MB's lobes in *X11* mutants (***F–F″***) versus wild type (***E–E″***). These results indicate APPL's endocytosis at the plasma membrane in dendrites and axons in MB neurons may be impaired.

X11/Mint. These findings, together with our previous observation that axonally restricted neurotactin (Nrt) does not mislocalize to dendrites in *X11* mutants, indicate that neuronal polarity is unaffected in MB neurons lacking *X11/Mint*. Thus, a disruption

of polarized localization of axonal proteins in *X11* mutants must have a different cause.

X11/Mint is localized to Golgi, dendrites, and the axon of the MB neurons

To gain insight into how X11/Mint controls polarized localization of membrane proteins at the cellular level, we examined the expression pattern of X11Lα using an anti-X11Lα antibody (Gross et al., 2008). In the brain, X11Lα is expressed in most, if not all, neurons (Fig. 4*A–A*''). In γ neurons of the MB, X11Lα exhibits a diffuse, but still highly specific, staining pattern in the lobes and calyx of the MB. The subcellular localization of *Drosophila* X11 proteins has not been reported. To explore this question, we double-labeled MB neurons using various markers for subcellular compartments. X11Lα has a punctate cytoplasmic pattern that strongly colocalizes with the Golgi marker GRASP65-GFP (Barr et al., 1997) in the soma of γ neurons (Fig. 6*B–B*''). X11Lα was also localized adjacent to structure labeled with an early endosomal marker Rab5-GFP (Fig. 6*C–C*''), a late endosomal marker, Rab7-GFP (Fig. 6*D–D*''), and an endoplasmic reticulum (ER) marker, KDEL-GFP (lysine–aspartic acid–glutamic acid–leucine-GFP) (Fig. 6*E–E*''). However, as the MB neurons are quite small, due to limitations on the resolution of images obtained by confocal microscopy, we cannot exclude the possibility that X11Lα may also partially colocalize with these compartments.

An endocytic motif (GYENPTY) in APP/APPL is required for its dendritic exclusion in MB neurons

Previously we reported that X11Lα regulates APP through its intracellular domain (AICD) (Gross et al., 2008), which contains a putative GYENPTY motif. This highly conserved motif is necessary for X11/Mint to directly bind to APP and APPL (Borg et al., 1996; Ashley et al., 2005). The GYENPTY motif has also been suggested to be important for recruitment of many membrane proteins, including APP, to clathrin-coated pits at the plasma membrane via binding to clathrin adaptor proteins to facilitate their rapid endocytosis (for review, see Bonifacino and Traub, 2003). To determine whether this motif might be important for dendritic exclusion of APP/APPL in neurons, we ectopically expressed a mutant form of APPL with the GYENPTY motif removed (APPLΔci) (Torroja et al., 1999; Ashley et al., 2005) in larval MB neurons null for endogenous *appl*. Interestingly, MB-specific expression of *applΔci*, but not wild-type *appl*, led to mislocalization of APPL to the calyx (Fig. 7*A–B*''). This suggests that the GYENPTY motif in APP/APPL's cytoplasmic domain is required for APPL's dendritic exclusion in MB neurons, and that endocytosis of APPL from dendrites may contribute to its polarized localization.

APPL accumulates on the surface of MB neurons in *X11* mutants

To explore the possibility that lack of *X11/Mint* leads to an endocytosis defect, we asked whether APPL in the *X11*-mutant brain accumulates on the surface of MB neurons. Live, larval brains were labeled with a polyclonal antibody against the extracellular domain of APPL (Torroja et al., 1996). Binding of anti-APPL antibody was done in the absence of membrane-permeabilizing detergent, so as to label only cell-surface APPL. Mislocalized APPL was observed on the dendritic membrane of the MB's calyx in *X11* mutants (Fig. 7*E–E*''), whereas little or no APPL was detectable on the dendritic membrane in the calyx of wild-type brains (Fig. 7*D–D*''). In addition, we observed increased levels of

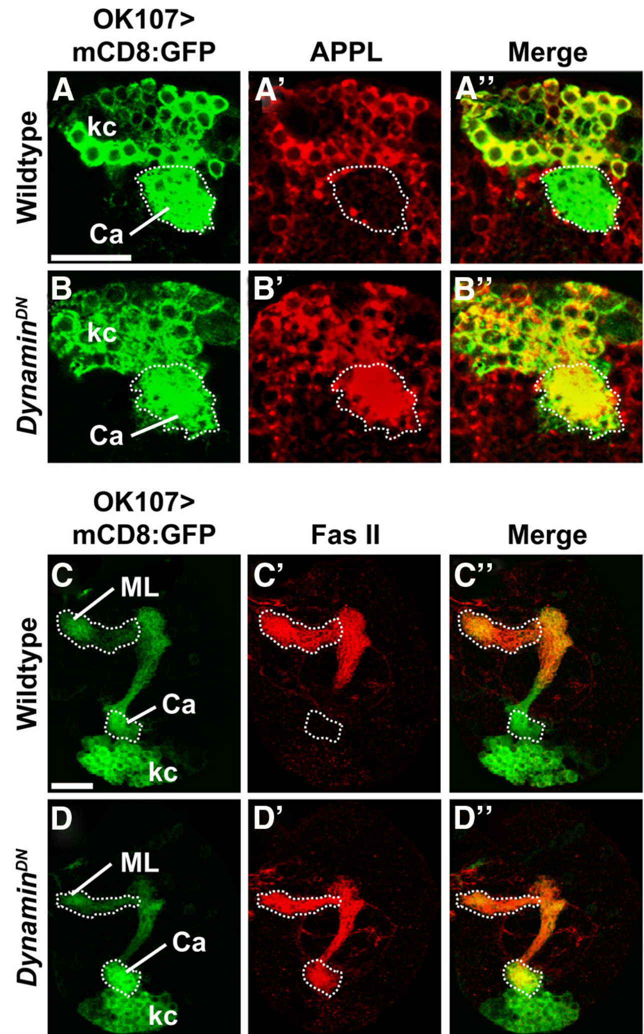

Figure 8. Dynamin-dependent endocytosis is required for dendritic exclusion of APPL and other axonal membrane proteins in MB neurons. ***A–D''***, mCD8-GFP (green) expressed in first instar brains by MB-specific driver OK107-GAL4 labels Kenyon cells (kc), the medial lobe (ML), and the calyx (Ca), which are outlined by dotted lines. Scale bar, 10 μm. Brains stained with anti-APPL antibody strongly overexpressing dominant-negative *dynamin/shibire* (Dynamin^{DN}) by OK107-GAL4 at 29°C show mislocalization of endogenous APPL (red) to the calyx of the MB (***A–A''*** vs ***B–B''***). Similarly, brains stained with anti-fasciclin II (Fas II) antibody strongly overexpressing *dynamin^{DN}* in the MB show mislocalization of endogenous Fas II (red) (***C–C''*** vs ***D–D''***) to the calyx. Brains stained against APPL were imaged from the posterior side; brains stained against Fas II were imaged from the dorsal side.

cell-surface APPL on the MB's lobes in *X11/Mint* mutants versus wild-type animals (Fig. 7*E–F*''). The accumulation of APPL on the surface of the MB's calyx and lobes in *X11* mutants is consistent with the idea that X11/Mint regulates the trafficking and/or endocytosis of APPL at the surface of neuronal compartments in γ neurons.

Inhibition of dynamin/shibire causes APPL and other axonal membrane proteins to mislocalize to dendrites in MB neurons

An accumulating body of evidence suggests that in mammalian neurons several axonal membrane proteins, including the cell adhesion molecule L1/NgCAM, are trafficked from the trans-Golgi network (TGN) to the somatodendritic surface, where they are rapidly removed by endocytosis for sorting into carrier vesicles targeted to the axon (Sampo et al., 2003; Bel et al., 2009;

Winckler and Yap, 2011). To investigate whether the polarized localization of APPL might depend on a similar mechanism in larval MB neurons, we sought to inhibit most types of endocytosis by expressing in the MB a dominant-negative form of *dynamin/shibire* (*shi*^{DN}) that is defective in hydrolysis of bound GTP, thus resulting in defective endocytosis (van der Bliek et al., 1993; Moline et al., 1999). In the presence of strong *shi*^{DN} expression, the lobes of the MB showed only mild structural phenotypes, but APPL was strongly mislocalized to the MB's calyx (Fig. 8*A*–*B*"). Dendritic mislocalization of Fas II (Fig. 8*C*–*D*") and neuroglian (data not shown) were also observed in MB neurons with strong *shi*^{DN} expression. These results suggest that interference with dynamin-dependent endocytosis *in vivo* disrupts polarized localization of APP/APPL and other axonal membrane proteins in γ neurons, and that dynamin/shibire may have an important role in establishing and/or maintaining polarized localization of multiple axonal membrane proteins in the *Drosophila* CNS.

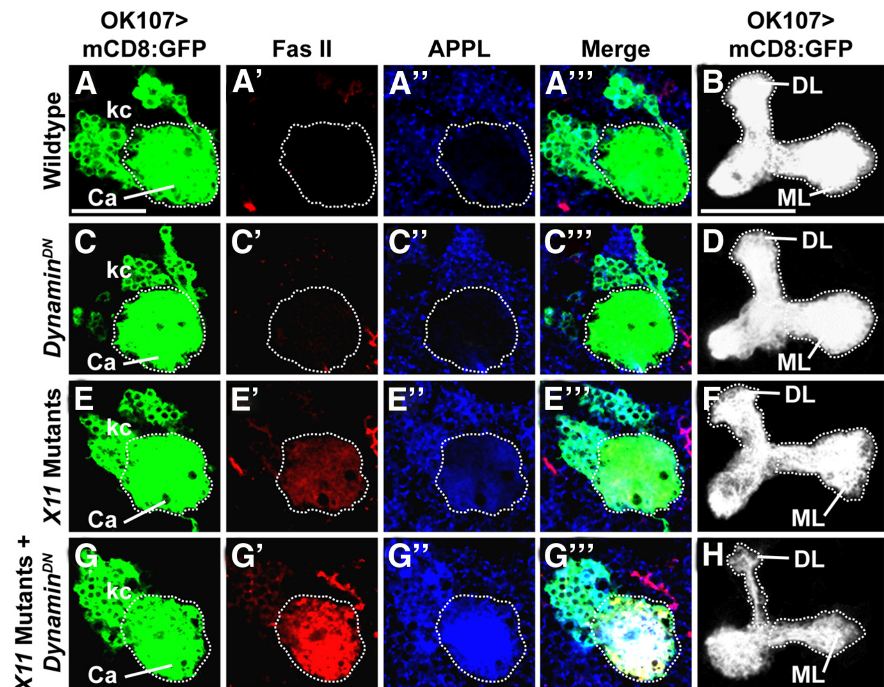

Figure 9. Attenuation of dynamin-dependent endocytosis enhances MB phenotypes in *X11* mutants. ***A–H***, mCD8-GFP (green) expressed in third instar brains by MB-specific driver OK107-GAL4 labels Kenyon cells (kc), the calyx (Ca), the medial lobe (ML), and the dorsal lobe (DL). In addition, the calyx and lobes are outlined by dotted lines. Scale bar, 50 μm. Larval brains weakly expressing RNAi transgenes against *X11* by MB-specific driver OK107-GAL4 at 25°C and stained with anti-APPL and anti-fasciclin II (Fas II) antibodies show moderate mislocalization of APPL (blue) and Fas II (red) to the calyx and Kenyon cells (***A–A'''*** vs ***E–E'''***) and exhibit mostly wild-type-appearing lobes (***B*** vs ***F***). Furthermore, brains weakly expressing *dominant-negative dynamin/shibire* (*Dynamin*^{DN}) by OK107-GAL4 at 25°C show almost no mislocalization of APPL or Fas II to the calyx (***A–A'''*** vs ***C–C'''***) and have wild-type-appearing lobes (***B*** vs ***D***). In dramatic contrast, simultaneous weak expression of *dynamin*^{DN} and RNAi *X11* in the MB strongly enhances mislocalization of APPL and Fas II to the calyx and Kenyon cells (***E–E'''*** vs ***G–G'''***) and causes severe malformation of the dorsal and medial lobes (***F*** vs ***H***).

Dynamin/shibire and AP-2 mutants synthetically interact with *X11* mutants

Based our findings that either lack of X11 or disruption of dynamin-dependent endocytosis led to mislocalization of axonal proteins, we further investigated whether there is a sensitive, synthetic interaction between loss of *X11* function and disruption of endocytosis. Adult flies with loss of *X11*/*Mint* or overexpression of *shi*^{DN} in the MB are viable and fertile. However, flies expressing RNAi to *X11* and *shi*^{DN} in the MB are embryonic lethal, indicating a robust genetic interaction exists between *X11*/*Mint* and *dynamin*/*shibire*. As the MB is dispensable for viability (i.e., flies lacking MBs are viable), this synthetic lethality is likely due to a basal level expression of the MB driver, suggesting a synthetic interaction between reduction of X11 function and decrease in endocytosis.

To further explore this genetic interaction at the level of APPL localization, we raised *X11-shibire* double mutants at a lower temperature than normal. Since GAL4-dependent expression is decreased at lower temperatures (Brand and Perrimon, 1993; Duffy, 2002), this approach results in more modest decreases in dynamin/shibire function and X11 expression, leading to the emergence of viable larvae. Weak expression of *shi*^{DN} in γ neurons causes almost no somatic or dendritic accumulation of APPL or Fas II, nor does it produce any noticeable defects in MB lobe morphology (Fig. 9*A*–*D*). Similarly, weak expression of RNAi *X11* in MB neurons causes only mild dendritic mislocalization of APPL and Fas II and produces no defects in MB lobe morphology. However, weak expression of *shi*^{DN} and RNAi to *X11* in the MB together results in dramatic enhancement of somatodendritic APPL and Fas II mislocalization and aberrant anatomical development of the medial and dorsal lobes (Fig. 9*E*–*H*). This result indicates that a modest reduction in dynamin-dependent endocytosis significantly exacerbates axonal membrane protein mislocalization and structural phenotypes seen in *X11* mutants.

We then investigated whether a hypomorphic allele of α-adaptin (*AP2*^{06694}) also enhances dendritic mislocalization of axonal membrane proteins and structural defects in the MB of *X11* mutants. *AP2*^{06694} disrupts function of the α subunit of the clathrin adaptor protein-2 (AP-2) complex that binds PIP_2 (phosphatidyl-4,5-bisphosphate) at the plasma membrane to facilitate endocytosis (González-Gaitán and Jäckle, 1997). Brains homozygous for the partial loss-of-function mutant *AP2*^{06694} contain no somatic or dendritic mislocalization of APPL or Fas II in γ neurons and the MB lobes appear largely wild type (Fig. 10*A*–*D*). As noted previously, brains strongly expressing RNAi *X11* in MB neurons show mislocalization of APPL and Fas II to the calyx, while the MB lobes appear largely wild type (Fig. 10*E*,*F*). Interestingly, larvae mutant for both *X11* and *AP2*^{06694} show a pronounced increase in dendritic mislocalization of APPL and Fas II in γ neurons. They also show MB lobe phenotypes strikingly similar to those of animals with pan-neuronal loss of *X11* (Fig. 10*G*,*H*; compared with Fig. 4*B*). This result suggests that a reduction in clathrin-mediated endocytosis at the plasma membrane aggravates dendritic mislocalization of axonal membrane proteins and accelerates the appearance of developmental defects in *X11*/*Mint* mutants. Together, these robust genetic interaction studies suggest the possibility that either X11 proteins directly regulate endocytosis, or they act in a highly cooperative fashion with endocytosis to control polarized localization of certain axonally restricted membrane proteins, including APP/APPL.

Discussion

Here, we report the identification of a new set of evolutionarily conserved proteins that control polarized axonal protein local-

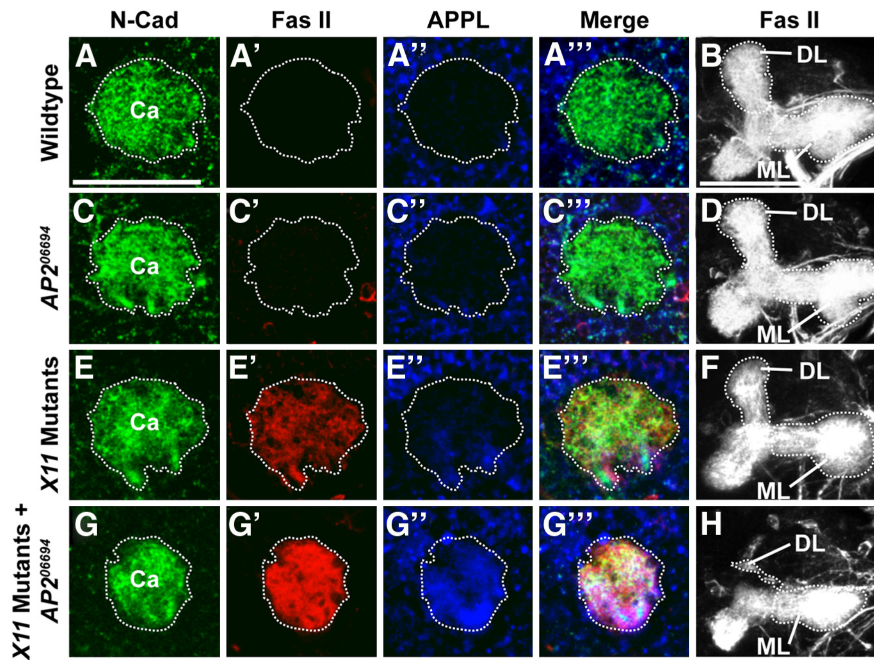

Figure 10. Attenuation of AP2-dependent endocytosis enhances MB phenotypes in *X11* mutants. ***A–H***, Staining of third instar brains against *Drosophila* N-cadherin (N-Cad, green) identifies the MB's calyx (Ca), which is outlined by a dotted line. In addition, staining against fasciclin II (Fas II) identifies the MB's dorsal lobe (DL) and medial lobe (ML), which are also outlined by dotted lines. Scale bar, 50 μm. Larval brains expressing RNAi transgenes against X11 by MB-specific driver OK107-GAL4 at 25°C and stained with anti-APPL and anti-Fas II antibodies show mislocalization of APPL (blue) and Fas II (red) to the calyx and Kenyon cells (***A–A‴*** vs ***E–E‴***) and exhibit mostly wild-type-appearing lobes (***B*** vs ***F***). Furthermore, brains homozygous for α-adaptin ^{06694} (AP2 ^{06694}), a hypomorphic allele of the α subunit of *clathrin adaptor protein-2* (*AP-2*), which binds PIP_2 (phosphatidyl-4,5-bisphosphate) at the plasma membrane, show no detectable staining of APPL or Fas II in the calyx (***A–A‴*** vs ***C–C‴***) and have wild-type-appearing lobes (***B*** vs ***D***). In contrast, MB-specific silencing of *X11* by RNAi in brains homozygous for *AP2* ^{06694} noticeably enhances mislocalization of APPL and Fas II to the calyx and Kenyon cells (***E–E‴*** vs ***G–G‴***) and causes severe malformation of the dorsal lobe and, to a lesser extent, the medial lobe (***F*** vs ***H***).

ization. First, we demonstrate that *X11*/*Mint* genes are required *in vivo* for dendritic exclusion and/or polarized localization of multiple, but not all, axonal membrane proteins. This list of proteins includes human APP, APPL, Fas II, and neuroglian, but not neurotactin. Mislocalization of these proteins in *X11* mutants is not due to alteration of identity of the dendritic compartment. Thus, X11/Mint controls polarized localization of axonal membrane proteins through a process distinct from those required to establish and maintain these compartments. Second, we find that X11 is localized to the Golgi and also in areas adjacent to ER and endosomes. Third, we present *in vivo* data indicating that dendritic exclusion of APPL depends on an endocytic motif in APPL, and that APPL accumulates on the dendritic surface in *X11* mutants. Fourth, we show direct disruption of endocytosis also leads to axonal protein mislocalization. Last, we establish that a modest reduction of X11 function and a modest disruption of endocytosis result in a dramatic synthetic genetic interaction. Our findings suggest the interesting possibility that X11/Mint proteins regulate endocytosis of certain axonal membrane proteins in dendrites (see below).

The neurons of *Drosophila melanogaster* provide an excellent system in which to study the polarized localization of membrane proteins *in vivo* (Rolls et al., 2007). Although *Drosophila* neurons are predominantly unipolar, they have been shown to compartmentally restrict membrane proteins, as observed in neurons of vertebrates (Rolls, 2011). Most of the work on polarized axonal protein localization has been performed in mammalian cultures of dissociated neurons. As effective as these culture systems are in identifying and investigating the role of these proteins, it remains possible that cultured neurons are separated from other important sources of neuronal protein localization cues. The *Drosophila* MB offers an important system in which to dissect the mechanisms of protein localization *in vivo*.

In mammalian systems, several mechanisms have been postulated for establishing and maintaining polarized localization of axonal membrane proteins. Some axonal membrane proteins are thought to be trafficked along a "direct transport pathway" through the TGN, from which they are sorted and packaged into distinct carrier vesicles bound for the axon (Horton and Ehlers, 2003; Winckler and Yap, 2011). In contrast, axonal membrane proteins trafficked along an "indirect transport pathway" are first localized to the somatodendritic surface, with endocytosis being required for their rapid removal and proper localization to the axon (Sampo et al., 2003; Wisco et al., 2003).

How do X11/Mint proteins regulate axonal membrane protein localization? Our data favors a model where X11/Mint proteins control polarized localization of axonal membrane proteins via an "indirect transport pathway." The strong synthetic genetic interactions we observed between *X11/Mint* and *dynamin*, and to a lesser extent between *X11/Mint* and *α-adaptin*, indicate that X11/Mint likely regulates, either directly or indirectly, endocytosis of certain axonal membrane proteins from the dendritic membrane (Fig. 11*A*). Our identification of X11Lα at the Golgi is also consistent with a model in which X11/Mint exerts control over polarized localization of axonal membrane proteins at the TGN. Thus, X11/Mint may also be required at the TGN for sorting and/or packaging of APP and other axonal membrane proteins into carrier vesicles bound for the axon via a "direct transport pathway" rather than dendrites (Fig. 11*B*). These two models are not necessarily mutually exclusive. X11/Mint has been described as an adaptor protein with many diverse functions (Rogelj et al., 2006; Sakamoto and Seiki, 2009; Hara et al., 2011). Thus, it is possible that X11/Mint acts both in endocytosis and at Golgi to control polarized localization of certain axonal membrane proteins, such as APP/APPL. For example, in *C. elegans*, *X11/LIN-10* is required for retrograde transport of endocytosed AMPA-type glutamate receptor GLR-1 from dendrites to Golgi outposts and the Golgi apparatus (Zhang et al., 2012). Similarly, X11/Mint proteins may also mediate sorting of dendritically localized protein from the TGN, and/or mediate return of dendritically mislocalized axonal membrane proteins to the Golgi in neurons of *Drosophila*.

Several observations in mammalian systems support a role of X11/Mint proteins in regulating endocytosis. First, X11/Mint physically binds both clathrin and PIP_2 (Okamoto and Südhof, 1997), which can independently bind AP-2 to initiate assembly of clathrin-coated pits and dynamin to control fission in endocytosis (Di Paolo and De Camilli, 2006). Second, expression of X11/Mint's PTB domain, as a dominant-negative, reduces endocytosis of APP with ApoER2 (apolipoprotein E receptor 2) in cultured neuroblas-

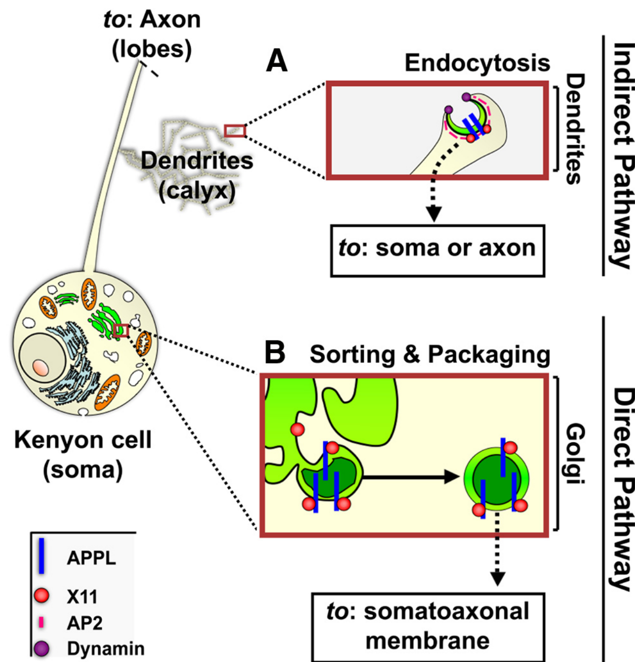

Figure 11. Cellular mechanisms of X11/Mint for controlling polarized localization of APP/APPL in MB neurons. Depicted is a γ neuron of the MB in a larval brain, containing Golgi (green) and other subcellular organelles in the soma. ***A***, Trafficking of the somatoaxonally restricted membrane protein APPL (blue) from Golgi leads to localization of APPL to dendrites. Once on the dendritic membrane, APPL is rapidly removed by dynamin (purple)-dependent endocytosis. This process is cooperatively facilitated by clathrin adaptor protein AP-2 (magenta) and binding of X11/Mint (red) to an endocytic motif (GYENPTY) in the intracellular domain of APPL. Those APPL proteins that reach the axon via endosomes have traveled there by an "indirect pathway." With loss of X11 function, endocytosis of APPL is impaired, which causes APPL proteins localized to the dendritic surface to accumulate there. ***B***, At Golgi, where X11/Mint is highly concentrated, APPL proteins may be preferentially sorted and packaged into axon-bound vesicles. APPL proteins delivered to the axon via Golgi-budded carrier vesicles have traveled there by a "direct pathway." If X11/Mint is required for sorting and/or packaging of APPL protein, then loss of X11/Mint function will cause APPL protein to be localized to both axon-bound and dendrite-bound vesicles. Since neither mechanism is mutually exclusive, X11/Mint may control polarized localization of APP/APPL both in dendrites and at Golgi.

toma cells (He et al., 2007). Finally, endocytosis of APP is significantly impaired in dissociated cortical neurons cultured from Cre-conditional *X11α-X11β-X11γ* knock-out mice (Chaufty et al., 2012). It is thus possible that *Drosophila* X11/Mint proteins can regulate endocytosis of APP/APPL at the plasma membrane. Work in mammalian cells also supports a role of X11/Mint proteins at the TGN. X11γ/Mint3 may control sorting and/or packaging of membrane proteins into secretory vesicles at the TGN (Teber et al., 2005; Zhang et al., 2009). Silencing of *X11γ/Mint3* expression in several mammalian cell lines causes APP and other membrane proteins to preferentially localize to endosomes and/or the plasma membrane rather than at the TGN (Hill et al., 2003; Han et al., 2008; Shrivastava-Ranjan et al., 2008).

X11/Mint proteins may constitute a new group of proteins that localize other membrane proteins. In *C. elegans*, X11/LIN10 is required for polarized localization of the epidermal growth factor receptor to the basolateral membrane of vulva precursor cells (Rongo et al., 1998; Whitfield et al., 1999). Indeed, X11/LIN10 may function similarly to the Par3/Bazooka/aPKC (Suzuki and Ohno, 2006) to localize proteins in various contexts. One reason that this function of X11/Mint has not been uncovered previously may be due to the fact that there are three X11/Mint proteins in mammals, and they serve partially redundant functions.

Mutations in APP cause AD (Tanzi, 2012). Interestingly, changes in the transport and/or trafficking of APP have been suggested to promote AD pathogenesis by increasing amyloidogenic proteolysis of APP, and therefore production of the cytotoxic peptide Aβ (Cataldo et al., 1997; Stokin et al., 2005). Notably, NEEP21, which is required for endosomal trafficking and polarized localization of the axonal membrane protein L1/NgCAM (Yap et al., 2008), was also identified as an interactor of APP and modulator of Aβ production (Norstrom et al., 2010). Thus, it will be interesting to determine whether loss of X11/Mint effects polarized localization and/or processing of APP in the adult brain, thereby contributing to the dysregulation and failure to maintain synaptic plasticity, and subsequently leading to AD.

Our findings suggest that mutations in *X11/Mint* could also lead to other neuropsychiatric disorders besides AD. Intriguingly, *X11α* and *X11β* knock-out mice suffer from epileptic seizures (Saito et al., 2012), due to a malfunction of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. It remains to be seen whether the HCN channel is mislocalized in these knock-out mice. In addition, several studies have suggested that duplications of *X11/Mint* in the human genome might play a role in schizophrenia (Kirov et al., 2008) or autism (Maddox et al., 1999; Sutcliffe et al., 2003). However, it is not clear whether these partial duplications cause gain or loss of function. Thus, it will be interesting to determine whether these mutations in *X11* can disrupt polarized localization of axonal membrane proteins and/or cause structural defects during neurodevelopment.

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