CHMP2B mutants linked to frontotemporal dementia impair maturation of dendritic spines

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Summary

The highly conserved ESCRT-III complex is responsible for deformation and cleavage of membranes during endosomal trafficking and other cellular activities. In humans, dominant mutations in the ESCRT-III subunit CHMP2B cause frontotemporal dementia (FTD). The decade-long process leading to this cortical degeneration is not well understood. One possibility is that, akin to other neurodegenerative diseases, the pathogenic protein affects the integrity of dendritic spines and synapses before any neuronal death. Using confocal microscopy and 3D reconstruction, we examined whether expressing the FTD-linked mutants CHMP2B\textsuperscript{introns5} and CHMP2B\textsuperscript{410} in cultured hippocampal neurons modified the number or structure of spines. Both mutants induced a significant decrease in the proportion of large spines with mushroom morphology, without overt degeneration. Furthermore, CHMP2B\textsuperscript{410} induced a drop in frequency and amplitude of spontaneous excitatory postsynaptic currents, suggesting that the more potent synapses were lost. These effects seemed unrelated to changes in autophagy. Depletion of endogenous CHMP2B by RNAi resulted in morphological changes similar to those induced by mutant CHMP2B, consistent with dominant-negative activity of pathogenic mutants. Thus, CHMP2B is required for spine growth. Taken together, these results demonstrate that a mutant ESCRT-III subunit linked to a human neurodegenerative disease can disrupt the normal pattern of spine development.

Key words: ESCRT, FTLD, Neurodegeneration, Synapse, Spine head, NeuronStudio

Introduction

The endosomal sorting complexes required for transport (ESCRT) are a set of four cytoplasmic protein complexes (ESCRT-0 to ESCRT-III), conserved from yeast to human. ESCRTs are central to a growing range of cellular activities, including the delivery of downregulated surface proteins to the lumen of late endosomes (Hurley and Emr, 2006; Slagsvold et al., 2006), the release of viruses at the plasma membrane (von Schwedler et al., 2003), cytokinesis of dividing cells (Carlton and Martin-Serrano, 2007) and autophagy of protein aggregates (Filimonenko et al., 2007). All of these diverse processes involve the budding and fission of cellular membranes, the latter of which seems to be directly catalysed by ESCRTs. Recent results show that ESCRTs are sequentially recruited to the cytosolic side of endosomal or plasma membrane microdomains, until ESCRT-III subunits locally polymerise into filaments. Transient assembly of ESCRT-III filaments on the membrane is a physical agent of membrane deformation and vesiculation (Fabrikant et al., 2009; Hanson et al., 2008; Lata et al., 2008; Muziol et al., 2006; Soksa et al., 2009; Wollert et al., 2009; Wollert and Hurley, 2010). Hence, ESCRT-III polymers are pivotal effectors of cellular membrane restructuring.

In keeping with its potent effects, the polymerisation of ESCRT-III is normally under tight regulation. The core subunits of ESCRT-III form a family of highly related proteins, the charged multivesicular body proteins (CHMP) 1–7, all of which contain a conserved oligomerisation and lipid-binding interface (the ESCRT-III or SNF-7 domain), linked to a C-terminal regulatory region (see Fig. 1). The latter mediates both intramolecular inhibition of the ESCRT-III domain and intermolecular interaction with subunit-specific factors that regulate filament dynamics (Bajorek et al., 2009; Muziol et al., 2006; Stuchell-Brereton et al., 2007; Zamborlini et al., 2006). Deletion of the regulatory region converts ESCRT-III subunits into dominant-negative mutants that are able to block ESCRT-dependent processes such as the release of HIV-1 virions from infected cells (Zamborlini et al., 2006).

Among the ESCRT-III subunits, CHMP2B has received particular attention following the discovery that mutations in the CHMP2B gene underlie a dominantly heritable form of frontotemporal dementia (called FTD-3), a presenile brain disease caused by selective degeneration of cortical neurons (Skibinski et al., 2005). In a thoroughly studied Danish kindred, the FTD phenotype was tightly linked to a single point mutation at the splice acceptor site of CHMP2B exon 6. The mutation generates two distinct aberrant transcripts, CHMP2B\textsuperscript{introns5} and CHMP2B\textsuperscript{410}, both of which encode proteins with a defective C terminus (Skibinski et al., 2005) (see Fig. 1). Neurodegeneration has been hypothesised to result from perturbation of neuronal autophagy by these mutant subunits (Rusten and Simonsen, 2008). Consistent with the loss of the regulatory domain, in heterologous cells, both CHMP2B\textsuperscript{introns5} and CHMP2B\textsuperscript{410} form insoluble polymers, often coating endosomes, and cause dominant-negative inhibition of ESCRT-dependent processes, including autophagosome maturation (Filimonenko et al., 2007; Lee et al., 2007; Skibinski et al., 2005). In cultured cortical neurons, overexpression of CHMP2B\textsuperscript{introns5} was shown to induce retraction of dendritic arbors and subsequent cell death, partly through toxic build-up of autophagosomes (Lee et al.,
Middle: CHMP2Bintron5 forms multiple small clusters in the soma and dendrites, throughout the entire neuron, including the heads of dendritic spines (arrows).

Subcellular distribution of tagged CHMP2B proteins. Neurons were transfected with the indicated plasmids, processed for immunofluorescence staining with anti-tag antibody and imaged by confocal microscopy. Two representative cells are shown for each plasmid. Top: CHMP2Bwt diffuses throughout the entire neuron, including the heads of dendritic spines (arrows). Middle: CHMP2BΔ10 forms multiple small clusters in the soma and dendrites, and occasional larger aggregates. Bottom: CHMP2BΔ10 has a clustered distribution similar to that of CHMP2Bintrom5.

Results

Expression of CHMP2B variants in hippocampal neurons

To study the neuronal effects of CHMP2B proteins, hippocampal neurons were transfected at 10 days in vitro (DIV) with plasmids encoding N-terminally tagged CHMP2Bwt (wild type), CHMP2Bintrom5 or CHMP2BΔ10 (Fig. 1). Using immunofluorescent staining with anti-tag antibody, expression of these plasmids could be reliably detected up to 5 days after transfection. The subcellular distribution of the mutant proteins clearly differed from that of wild type (Fig. 1B; Fig. 2, left; Fig. 3, left). Wild-type CHMP2B was homogeneously distributed throughout the entire neuronal cytoplasm, including in axons, dendrites and dendritic spines (Fig. 1B, arrows). By contrast, both CHMP2Bintrom5 and CHMP2BΔ10 formed varying numbers of small bright puncta, which were found in the soma and proximal dendrites. Larger aggregates were occasionally observed in the soma. To determine whether expression of CHMP2B mutants perturbed autophagy in our system, we performed co-transfection experiments with LC3–GFP as an autophagosome marker. In a vast majority of neurons, expression of either CHMP2BΔ10 or CHMP2Bintrom5 failed to induce clustering of LC3–GFP (Fig. 2, b2, c2). In support of this, despite evaluation by two independent investigators, we could not detect any autophagy-associated drop in the diffuse pool of LC3–GFP. Thus, in the present setting, pathogenic CHMP2B mutants did not substantially enhance autophagosome accumulation. Furthermore, to determine whether the mutant CHMP2B puncta observed in transfected neurons were related to endosomes, transfected neurons were doubly stained with anti-tag antibody and antibodies raised against either the early endosomal marker EEA-1 (Fig. 3, a2–c2) or the late-endosomal–lysosomal protein LAMP-1 (Fig. 3, d2–f2). No colocalisation was observed with these markers. These data suggest that the CHMP2B aggregates that form under our transfection conditions are not associated with endosomes.

Accumulating evidence indicates that, in various neurodegenerative diseases, neuronal death is a late event, preceded by early disruption of dendritic spines and synapses, which itself is a pivotal pathogenic step (Morfini et al., 2009; Wei et al., 2009; Wishart et al., 2006). In FTD, extensive synaptic loss and reduction in the number of spines have been documented in diseased cortex (Ferrer, 1999; Lipton et al., 2001). Given its role in endosomal trafficking and plasma membrane deformation, ESCRT-III might plausibly control aspects of synaptic biology. Here, we examine the possible impact of CHMP2B mutants and CHMP2B depletion on the development of dendritic spines. CHMP2Bintrom5 and CHMP2BΔ10 was expressed in hippocampal neurons without causing apparent anomalies in endosomal morphology, autophagosome amount or cell viability. The mutants potently suppressed the growth of spine heads, strongly lowering the proportion of mushroom spines. Very similar effects were obtained with an siRNA targeting endogenous CHMP2B, consistent with dominant-negative action of the mutants. Physiologically, CHMP2BΔ10 caused a drop in the frequency and amplitude of spontaneous excitatory synaptic currents, with selective disappearance of large currents. We propose that CHMP2B and potentially ESCRT-III are required for the maturation of dendritic spines, and that pathogenic CHMP2B mutants perturb this process. To the extent that maturation correlates with functional potentiation, these results implicate ESCRT-III in synaptic plasticity. In the protracted course of FTD, CHMP2B mutations might affect synaptic homeostasis and plasticity long before inducing massive neuronal death.

2007; Lee and Gao, 2009). Surprisingly, however, CHMP2BΔ10 failed to increase neuronal death, even though its effects in heterologous cells on autophagy are similar to those of CHMP2Bintrom5 (Filimonenko et al., 2007). Furthermore, knockdown of CHMP2B by RNAi did not modify neuronal viability. These observations suggest that disease-linked CHMP2B mutations might affect neuronal physiology in ways other than by lethal malfunction of autophagic mechanisms.
To visualise the morphology of neurons that had been expressing mutant CHMP2B for several days, the cells were co-transfected with CHMP2B constructs and a plasmid encoding the fluorescent protein mCherry, allowing staining of the whole cytosol. At 5 days after transfection, 75–80% of neurons co-transfected with empty vector, CHMP2Bwt or CHMP2BΔ10, and 55% of neurons co-transfected with CHMP2Bintron5 were healthy, as judged from the overall integrity of their neurites and the absence of neuritic swellings (supplementary material Fig. S1). These percentages did not change much after 10 days, and were largely sufficient to allow meaningful analysis of dendritic arbors and spines. Only healthy neurons were retained for further analysis.

The effect of CHMP2B constructs on dendritic arborisation was determined by Sholl analysis of neuronal morphology. For this, we measured the rate of increase in total neuritic length as a function of radial distance from the cell body (supplementary material Fig. S2). CHMP2Bwt and CHMP2BΔ10 had no significant effect on arborisation. CHMP2Bintron5 induced a moderate (25%) decrease in average branching rate, but did not induce the large-scale retraction of dendrites that accompanies lethal perturbations of ESCRT-III (Lee et al., 2007).

Fig. 2. Lack of LC3–GFP clustering in neurons expressing mutant CHMP2B. Neurons were co-transfected at 10 DIV with the indicated CHMP2B plasmids (left), together with the autophagosomal marker LC3–GFP. The cells were fixed 5 days later and processed for immunofluorescence staining with anti-tag antibody and Alexa-Fluor-594-coupled secondary antibody. Confocal images were acquired in both the immunofluorescence and GFP channels. Diffuse LC3–GFP localisation predominated upon co-transfection with CHMP2Bwt (a1,a2), CHMP2BΔ10 (c1,c2) and in a large majority of cases with CHMP2Bintron5 (b1,b2). Autophagosome accumulation was seen in rare cells transfected with CHMP2Bintron5 (b3, b4, arrow). Arrowhead: no autophagosomes were detected in the dendrites. Scale bars: 10 μm.

Fig. 3. Non-endosomal localisation of exogenously expressed CHMP2B proteins in hippocampal neurons. Neurons were doubly stained with monoclonal anti-tag antibody (a1–f1) together with polyclonal antibody against the respective marker (a2–f2), as indicated. Immunofluorescence was recorded by dual-channel confocal microscopy. Arrows indicate CHMP2B puncta. Note that they do not coincide with EEA1 (a2–c2) or LAMP1 (d2–f2) puncta. Scale bars: 10 μm.
CHMP2B mutants affect dendritic spine density and morphology

The dendritic morphology of neurons that were co-transfected with mCherry and CHMP2B plasmids was examined in detail by three-dimensional (3D) confocal imaging of mCherry fluorescence. Fig. 4A (a–d) shows representative views of neurons transfected with the different CHMP2B constructs. Stacks of serial optical sections were produced for each neuron and the NeuronStudio software package (Rodriguez et al., 2008; Rodriguez et al., 2006) was then used to generate 3D reconstructions of dendritic arbors and spines, and to count, measure and classify spines (see Materials and Methods). Fig. 4B shows a reconstructed dendritic segment obtained from a typical control neuron, and the results of automated identification of spine types. The volumes of the reconstructed spines followed a statistical distribution that closely resembled that described in a recent study of hippocampal spine dynamics (Yasumatsu et al., 2008) (supplementary material Fig. S3). Furthermore, the peak of our distribution (between 0.050 and 0.075 μm³) was in the same range as published values (0.058±0.034 μm³) previously obtained by electron microscopy for the spines of hippocampal neurons in dissociated culture at 14 DIV (Boyer et al., 1998). The distribution of spine lengths was also similar to previously published values (Fig. 4G). These data indicate that analysis by NeuronStudio correctly detected the spine population in our cultured neurons.

The density of spines (pooling all spine types) detected by this technique was determined for each neuron (Fig. 5A,B). Overexpression of tagged, wild-type CHMP2B did not change the average density compared to control (co-transfection with empty vector). By contrast, expression of CHMP2Bintron5 raised the average density of spines by 64% compared with control (Fig. 5A, column 3). However, many of these additional spines were very small (see below). These small spines were often hard to detect in uniformly thresholded 2D projections of image stacks, but were revealed by the 3D reconstruction procedure (supplementary material Fig. S4). Neurons expressing the CHMP2BΔ10 mutant also had a tendency to increase the density of their protrusions (average density 24% higher), but this trend did not reach significance (Fig. 5A, column 4).

To investigate the possible effect of CHMP2B mutants on spine dimensions, we determined the mean length of spines and the mean diameter of spine heads for each neuron. None of the CHMP2B constructs modified spine length (Fig. 5C,D). Wild-type CHMP2B did not change spine head diameter either. By contrast, mutant proteins strongly affected spine diameter. Expression of CHMP2Bintron5 induced a 40% reduction in average spine diameter, whereas CHMP2BΔ10 reduced average diameter by 32% (Fig. 5E,F). These changes in average spine dimensions reflect the selective loss of large-headed spines. Fig. 5G,H shows the normalised distributions of spine lengths and spine head diameters in neurons transfected with each of the four plasmids. Both mutants had a clear, comparable effect on the distribution of spine diameters (Fig. 5H). In control neurons, thin spines (diameter <0.2 μm) predominated on top of a distinct population of larger spines.

Fig. 4. Detection and classification of dendritic spines in transfected hippocampal neurons. (A) Neurons were co-transfected with the indicated constructs together with mCherry, fixed 5 days later and images of mCherry fluorescence were acquired by confocal microscopy. The figure shows maximal intensity projections of confocal image stacks. Control: empty pcDNA3 vector. The boxed regions in a–d (scale bar: 20 μm) were scanned at higher magnification in a1–d1 (scale bar: 5 μm). (B) Stacks of optical slices acquired in A served to generate 3D models of the dendrite, using the surface-defining Ray Burst algorithm implemented in NeuronStudio (Rodriguez et al., 2006). The dimensions of the modelled structures were automatically measured as described previously (Rodriguez et al., 2006) and used to identify and classify spines. Left: surface rendering of a segment from the dendrite shown in b1. Middle: maximal intensity projection of optical sections from the same segment. Right: automated spine detection and classification. Appropriately coloured dots are superimposed onto the spines. Yellow (t): thin spines; red (m): mushroom spines; blue (s): stubby spines.

A.

B.
represented as a shoulder and tail in the distribution (interquartile range (IQR), 5.98 μm). This subset of larger spines was still present in neurons transfected with CHMP2Bwt (IQR, 6.00 μm), but strongly reduced following transfection with either CHMP2Bintron5 (IQR, 2.89 μm) or CHMP2BΔ10 (IQR, 3.12 μm). Because spines with similar head width might differ in the size of their neck, with important consequences for their physiology (Noguchi et al., 2005), we determined how CHMP2B mutants
affected the basic morphological categories of spines. Fig. 5I shows that neurons expressing either mutant had an abnormally low fraction of mushroom spines, identified by their head vs neck width ratio. The fraction of spines with mushroom morphology was decreased by ~50% with both CHMP2Bmuton5 and CHMP2BΔ10, because they were replaced by an enlarged proportion of thin spines. Stubby spines were also reduced in number, but to a smaller extent, by expression of CHMP2Bmuton5. The average diameter of spines in the mushroom spine subset (0.56 μm) did not significantly change in cells transfected with the mutants, confirming the consistency of morphotype identification.

Taken together, these data indicate that expression of a mutant ESCRT-III subunit linked to a human neurodegenerative disease can strongly affect the density and morphology of dendritic spines, and disrupt the normal pattern of spine development.

A CHMP2B mutant affects miniature excitatory synaptic currents
To determine whether the effects of mutant CHMP2B on spine morphology correlated with functional changes at synapses, we recorded excitatory synaptic currents from cultured hippocampal neurons that had been co-transfected with GFP together with empty vector, or together with vectors encoding wild-type CHMP2B or CHMP2BΔ10. Transfected cells were identified by GFP fluorescence. We monitored the frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) as an indicator of possible effects of plasmids on synaptic strength. In total, 16 out of 18 transfected neurons were synaptically active; the two inactive neurons were discarded from analysis. Sample traces that were representative of each transfection condition are shown in Fig. 6A. A sequence of 128 consecutive mEPSCs was randomly picked from each recording, and the sequences were pooled according to the transfected plasmid. Mean inter-event intervals were calculated for each pool. Intervals were significantly longer between mEPSCs observed in neurons expressing CHMP2BΔ10 than in controls (Fig. 6B). Thus, the mean frequency of mEPSCs was lower in CHMP2BΔ10-expressing cells than in control cells (1.47 vs 1.94 Hz), indicating a decrease in synaptic activity (Fig. 6B). The mean amplitude was slightly lower in mEPSCs of cells transfected with CHMP2BΔ10 than in those of control- or CHMP2Bwt-transfected neurons (12.6 vs 13.6 pA) (Fig. 6C). However, cumulative amplitude histograms indicated that CHMP2BΔ10 caused a
significant reduction in the frequency of large amplitude events, i.e. those belonging to the top quintile (Fig. 6D). mEPSCs with an amplitude larger than 20 pA (approximately twice the median amplitude), which represented up to 19% of mEPSCs in control neurons, were reduced by a third in neurons expressing CHMP2BΔ10. Conversely, the proportion of small amplitude events (<5 pA) rose from 6.8% in controls to 10% in neurons expressing CHMP2BΔ10 (Fig. 6E). The drop in the frequency of large currents was not observed in neurons that overexpressed CHMP2Bwt. In view of the known correlation between spine head size and amplitude of AMPA-receptor-mediated synaptic currents (Matsuzaki et al., 2001), these data are consistent with the morphologically observed reduction in mushroom spines. Mutant CHMP2B might cause a reduction in the proportion of the more potent synapses and an increase in the proportion of weak synapses. Endogenous CHMP2B is required for maturation of dendritic spines Because dysregulated CHMP2B mutants affect spine morphogenesis, the issue arises of whether the endogenous normal protein plays some role in this process. Endogenous CHMP2B was clearly detectable in extracts from cultured neurons at 8 DIV and increased in older cultures, in which synaptogenesis and spine maturation are known to take place (Fig. 7). Immunofluorescent staining of the neurons with the anti-CHMP2B antibody showed the protein to be widely distributed throughout soma, axons and dendrites, similar to data obtained with the tagged wild-type protein (Fig. 8). To disrupt endogenous CHMP2B expression, we transfected cultured hippocampal neurons at 10 DIV with a pSuper-based plasmid vector encoding a previously described CHMP2B-specific shRNA (Lee et al., 2007). The efficiency of RNAi was ascertained by western blotting of transfected baby hamster kidney (BHK) cells (Fig. 8A). Transfected neurons could be tracked and outlined by mCherry, which was coexpressed from the same plasmid. Immunofluorescent staining with anti-CHMP2B antibody confirmed the efficient downregulation of endogenous CHMP2B and dendritic spines

![Fig. 7. Levels of endogenous CHMP2B increase during neuronal maturation in culture.](image_url)

Sister cultures of hippocampal neurons maintained in plastic dishes were lysed at the indicated time points (DIV). The lysates were analysed by SDS-polyacrylamide gel electrophoresis and western immunoblotting with anti-CHMP2B antibody. The blots were reprobed with anti-actin antibody to normalise for loading error. (A) Representative blot. Leftmost lane: rat brain lysate, showing the pattern of CHMP2B in vivo. (B) Films were scanned and the optical density of the CHMP2B band was measured in grey levels for each lane. Band intensities were normalised by dividing each value by the intensity of the actin band in the same lane. Care was taken to obtain immunodetection in the linear range. The graph shows mean normalised intensities at three time points (n=3 cultures). Error bars: s.e.m.

![Fig. 8. Knockdown of endogenous CHMP2B in hippocampal neurons.](image_url)

(A) To verify the efficacy of the shRNA-encoding plasmid, the indicated plasmids were transfected in rodent (BHK) cells and equal protein amounts of transfected cell lysates were analysed by western immunoblotting with anti-CHMP2B antibody, or with anti-actin as a control. Lane 1: empty pSuper-mCherry plasmid; lane 2: shRNA-expressing plasmid; lane 3: shRNA-expressing plasmid co-transfected with vector encoding CHMP2B* (native CHMP2B cDNA with silent mutations at the siRNA target site). Note that the CHMP2B protein remaining after transfection of the shRNA plasmid largely originates from non-transfected cells in the culture. (B) Neurons were transfected at 10 DIV with the plasmids indicated on the left, fixed at 15 DIV and stained with anti-CHMP2B antibody and Alexa-Fluor-488-labelled secondary antibody. Confocal images were acquired in both the Alexa-Fluor-488 and mCherry channels. Representative images of transfected neurons are displayed. Control: empty pSuper-mCherry vector. Note the drop in immunofluorescence in the shRNA-expressing neuron (arrow) compared with surrounding cells, and the decreased green vs red ratio (overlay) compared with control or rescued cells. (C) Maximal intensity projections of image stacks, showing representative dendritic segments visualised by mCherry fluorescence in neurons transfected with the indicated plasmids.
expression in transfected neurons (Fig. 8B, middle panel). In agreement with published results (Lee et al., 2007), the CHMP2B shRNA did not affect neuronal viability. To verify the target specificity of RNAi, control experiments were performed in which CHMP2B shRNA was coexpressed with an RNAi-resistant CHMP2B mRNA (called CHMP2B*) (Fig. 8B, lower panel).

Confocal imaging of mCherry and spine analysis were performed as in the case of mutant CHMP2B (Fig. 8C, Fig. 9).

Depletion of endogenous CHMP2B increased the average density of spines, and the increase was fully reversed upon rescue by CHMP2B* (Fig. 9A,B). CHMP2B depletion did not significantly affect the length of spines (Fig. 9C,D). By contrast, a highly significant reduction in mean spine diameter per neuron was observed in shRNA-transfected cells compared with controls; this was rescued to a near-normal value upon coexpression of CHMP2B* (Fig. 9E,F). Similar to the case of neurons transfected with mutant CHMP2B, the size distribution of spine heads showed a loss of the large spine subset; this loss was largely reversed by rescue with RNAi-resistant CHMP2B (Fig. 9G). Spine classification revealed a 50% drop in the average proportion of mushroom spines, from 17% in control neurons to 9% in shRNA-transfected cells (Fig. 9H). This effect is equivalent to that observed upon expression of mutant CHMP2B. Upon coexpression of CHMP2B*, the average proportion of mushroom spines could be rescued to 13%, at which level the difference with control neurons was no longer significant (Fig. 9H). This demonstrates the specificity of the effects of CHMP2B shRNA. Taken together, these results indicate that CHMP2B is required for spine head expansion, a
Discussion

Here we found that, in cultured hippocampal neurons, two FTD-linked CHMP2B mutants strongly perturbed the normal pattern of dendritic spine development. Both mutants caused a striking decrease in the proportion of large spines with mushroom dendritic spine development. Both mutants caused a striking decrease in the proportion of large spines with mushroom dendritic spine development.

Two lines of evidence indicate that the impairment in spine maturation described here occurs through a mechanism that is distinct from this toxic process. First, the changes in spine morphology were observed in healthy neurons. In particular, expression of CHMP2BΔ10 or knockdown of endogenous CHMP2B potently affected spines, even though we found, in agreement with published data, that these perturbations of CHMP2B activity did not diminish neuronal viability. Second, alterations of spines did not correlate with changes in dendritic complexity. CHMP2BΔ10 elicited no detectable loss of dendritic branching and, in our conditions, CHMP2BΔ10 only induced a 25% decrease in branching rate. This reduction remained modest compared with the 80% shortening of total dendritic length shown to occur in the early phase of CHMP2BΔ10-induced cell death (Lee et al., 2007).

The CHMP2B shRNA caused a reduction in dendritic branching, but this might have been an off-target effect because, unlike the reduction in mushroom spines, it was not suppressed by coexpression of an RNAi-insensitive CHMP2B cDNA (A.B. and Y.G., data not shown). Third, when expressed in hippocampal neurons, neither of the CHMP2B mutants caused a clear-cut increase in LC3–GFP condensation onto autophagosomes. By contrast, a lethal degree of CHMP2BΔ10 overexpression has been shown to induce strong clustering of LC3–GFP; furthermore, preventing autophagosome formation retarded the neuronal death caused by CHMP2BΔ10 (Lee et al., 2007; Lee and Gao, 2009). Beyond some threshold level of expression, the ability of CHMP2B mutants to block the ESCRT-dependent fusion of autophagosomes to lysosomes (Filimonenko et al., 2007; Urwin et al., 2010) might favour the proliferation of toxic autophagosomes. The difference with our results might arise in part from the relatively low level of mutant CHMP2B expression we used in hippocampal neurons. Indeed, with fourfold higher amounts of CHMP2BΔ10 plasmid, leading to higher expression, we observed toxicity similar to that described by Lee et al. (Lee et al., 2007) (although comparison is difficult, because Lee et al. did not mention the amounts of plasmid in their transfection assays). The non-endosomal location of mutant CHMP2B proteins in our experiments also seem at first sight to be at variance with previously published results from other cell types. However, HA-tagged CHMP2BΔ10 did accumulate around endosomes when overexpressed in BHK cells (A. Belly, unpublished data). It should also be noted that, even in heterologous cells, a fraction of CHMP2BΔ10 and CHMP2BΔ10 aggregates consistently fail to colocalise with endosomal markers (Skibinski et al., 2005) (A.B., unpublished). In transfected hippocampal neurons, presumably similar non-endosomal aggregates largely predominated. The occurrence of non-endosomal CHMP2B clusters is also consistent with the fact that, in vitro, activated ESCRT-III subunits can form large polymers in the absence of lipids (Lata et al., 2008). Our attempts to identify further components of the mutant CHMP2B clusters have so far been unsuccessful, except for the finding that endogenous CHMP2B is sometimes concentrated in CHMP2BΔ10 granules. This suggests that native CHMP2B can be trapped in polymeric structures together with the mutant. Because much of the endogenous CHMP2B seemed to remain outside of the mutant CHMP2B puncta, sequestering of the native protein in the aggregates is unlikely to explain the apparent
Materials and Methods

Plasmids and antibodies

The mCherry plasmid was a kind gift from Pascal Dournaud (INSERM, Paris, France). The LC3–GFP plasmid was a kind gift from Tamotsu Yoshimori (National Institute of Genetics, Mishima, Japan). The GFP plasmid was pEGFP-C1 (Clontech).
equivalent distances from the soma were selected, and each of those was scanned at 1.5× electronic zoom.

**Image analysis**

Image files were processed with MetaMorph (Molecular Imaging). All figures showed maximal intensity projections. For analysis of spines, serial image files corresponding to z-stacks of 20–30 optical sections per dendritic segment were directly processed with NeuronStudio, a software package specifically designed for spine detection and analysis (Rodriguez et al., 2008; Rodriguez et al., 2006) (http://www.msnm.edu/cnic/tools.html). Voxel size was 0.143×0.143×0.200 μm. The fluorescence threshold for inclusion in objects was dynamically set for each local sampling of the dendrite surface by the Isodata segmentation algorithm implemented in NeuronStudio. The seed location for automatic dendrite tracing was typically set near the base of a major proximal dendrite. At bifurcation points, the attach ratio was 1.5 and neurites shorter than 5 μm were not retained. After modelling of the dendrite surface, protrusions with a minimum volume of 5 voxels (0.020 μm³), length of between 0.2 μm and 3 μm and a maximal width of 3 μm were retained as spines. Following default settings of the program and the empirical classification rule defined by Rodriguez et al. (Rodriguez et al., 2008), spines with a minimum head diameter of 0.35 μm and minimum head vs neck ratio of 1.1 were classified as mushroom spines. Non-mushroom spines with minimum volume of 10 voxels (0.040 μm³) were classified as stubby spines. All other spines were considered thin. The typical percentages of the three spine types that we obtained were close to those found by Rodriguez et al. (Rodriguez et al., 2008). Measurements obtained by NeuronStudio were transferred to a spreadsheet (Excel or Gnuplot) for analysis. Sholl analysis was also performed with NeuronStudio, using single-plane lower magnification views centred on the cell soma. For each neuron, a series of 2.5-μm-spaced concentric circles centred on the soma were drawn as reference marks, starting with a circle just large enough to enclose the cell body. The total length of neurites within two consecutive circles was measured, and the measurement was repeated for increasingly large circle radii. Stepwise increases in neuritic length were plotted as a function of radial distance from the soma.

**Cell degeneration assay**

Neurons were transfected with 0.5 μg CHMP2B plasmid and 2.5 μg mCherry as above. After fixation and mounting onto slides, neurons were observed under an epifluorescence microscope (Zeiss Axiovert 200M) with a 63× objective lens and a Rhodamine filter, and scored as dead or alive according to morphological criteria (see Results). The actual presence of coexpressed CHMP2B was verified by immunofluorescent staining with anti-tag antibody, using the GFP filter.

**Electrophysiology**

Spontaneous tetrodotoxin-resistant mEPSCs were recorded by means of the whole-cell configuration of the patch clamp method (Hamill et al., 1981). The recording medium contained (in mM): 136 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, 0.0005 TTX, pH 7.4 (NaOH). Patch pipettes were made with borosilicate glass capillaries (1.5 mm outside diameter). The total length of neurites within two consecutive circles was measured, and the measurement was repeated for increasingly large circle radii. Stepwise increases in neuritic length were plotted as a function of radial distance from the soma.

**Statistical analysis**

The statistical significance of overall variations within sets of conditions was determined by using two-way ANOVA. For analysis of mEPSC frequency, the significance of the transfection effect was confirmed by two-way ANOVA on a balanced subset of mEPSCs, separating the effect of plasmid identity from intrinsic neuron-to-neuron variation owing to the variability in innervation in the culture. The significance of differences between multiple frequency distributions was assessed with the χ² test. A post-hoc test of pairwise differences was used the Marascuilo procedure (NIST Handbook of statistics, http://www.itl.nist.gov/div898/handbook/). For Sholl analysis, curves were compared by two-way ANOVA, separating the effect of plasmid identity from that of radial distance. Post-hoc comparisons between curves were performed by the Bonferroni method. All calculations were performed with the statistics-oriented spreadsheet Gnuplot (http://projects.gnome.org/gnuplot/) or with OpenStat (http://statpages.org/miller/opensat). All error bars show s.e.m.

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**Supplementary material available online at**

http://jcs.biologists.org/cgi/content/full/123/7/2943/DC1

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**CHMP2B and dendritic spines**

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