Neurite consolidation is an active process requiring constant repression of protrusive activity

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During development, neurons extend projections that pathfind to reach their appropriate targets. These projections are composed of two distinct domains: a highly dynamic growth cone and a stable neurite shaft, which is considered to be consolidated. Although the regulation of these domains is critical to the appropriate formation of neural networks, the molecular mechanisms that regulate neurite shape remain poorly understood. Here, we show that calpain protease activity localizes to the neurite shaft, where it is essential for the repression of protrusive activity by limiting cortactin levels and inhibiting actin polymerization. Correspondingly, inhibition of calpain by branching factors induces the formation of new growth cones along the neurite shaft through cAMP elevation. These findings demonstrate that neurite consolidation is an active process requiring constant repression of protrusive activity. We also show that sprouting is, at least in part, accomplished by turning off the mechanism of consolidation.

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Introduction

One of the main characteristics of neurons is their shape, with every neuron extending long neurites to create a complex wiring network. Neurites in this network are branched, so that each neuron has the ability to establish connections with multiple targets. Not only is the process of neurite branching, also called sprouting, essential for the assembly of the neuronal network but it also has a central function in the injured central nervous system (CNS) (Raineteau and Schwab, 2001; Weidner *et al*, 2001; Hagg, 2006). Thus, understanding how neurons maintain and modify the morphology of their processes will be essential to understand the mechanisms of neuronal plasticity and repair. However, unlike axon

guidance or synaptogenesis, the question of how neurite shape is regulated has remained largely unexplored.

During nervous system development, neurites are guided by a growth cone, a very motile terminal structure that explores the surrounding environment and translates guidance information into movement. The neurite shaft is generated by a process called consolidation, which results from the collapse of the proximal part of the growth cone and the suppression of protrusive activity along the neurite (Luo, 2002; Dent and Gertler, 2003). It is interesting to note that consolidation is thought to be a punctual process after which the shaft is left in a non-stimulated state (Figure 1A). This model contrasts with what is known about other cell systems, where cell asymmetry requires active maintenance of both domains (Fais *et al*, 2000; Ridley *et al*, 2003). For example, during directional migration, the trailing edge is maintained by localized cell contraction and adhesion disassembly (Ridley *et al*, 2003). However, the possibility that the neurite shaft also has such a cell-autonomous mechanism of maintenance, that is, that consolidation keeps the shaft in a sustained non-protrusive state (Figure 1B), has not been considered.

Neurite morphological changes, such as growth cone guidance or neurite outgrowth, are largely governed by rearrangements of the cytoskeleton (Letourneau, 1996; Song and Poo, 2001; Dickson, 2002; Luo, 2002). Similarly, neurite sprouting is preceded by a reorganization of the cytoskeleton in the neurite shaft (Kalil *et al*, 2000; Dent and Kalil, 2001; Kornack and Giger, 2005). This includes microtubule fragmentation and local accumulation of F-actin, which is a required step for the formation of actin-driven protrusions, such as filopodia and lamellipodia, and subsequent branch generation (Loudon *et al*, 2006). As these protrusions are infrequently observed along the neurite shaft, it is most likely that repression of actin polymerization or nucleation is a key step in the process of consolidation. On the basis of this assumption, we adopted a candidate protein approach to examine whether there is a repressor controlling neurite consolidation and branching. We envisioned that (1) such repressors would be preferentially located, or active, at the neurite shaft, (2) such repressors would limit actin polymerization or nucleation in neurons and (3) their inhibition would induce sprouting along the consolidated shaft.

Here, we demonstrate that the protease calpain meets these criteria. Our results show that calpain is a repressor controlling neurite consolidation, and provide evidence that calpain's role in this process is to repress the formation of actin patches and therefore disable the formation of sprouts. We also show that induction of neurite sprouting by physiological branching factors is accomplished by turning off the pathway that maintains consolidation.

Results

Calpain is active along the neurite shaft

Calpain comprises a family of ubiquitous calcium-activated proteases recognized for their central role in cell death and

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Figure 1 Two mechanisms that could account for neurite consolidation and branching. Neurites have two morphologically distinct domains: the shaft and the growth cone. On contact with branching factors, a growth cone can be generated from the neurite shaft. Two mechanisms can account for these observations. (A) Model 1: the neurite shaft is in a default state resulting from insufficient stimulation, and sprouting is caused by stimulation of the shaft by branching factors. (B) Model 2: the neurite shaft is under constant repression, and local derepression by branching factors allows branches to sprout.

their deregulation in multiple diseases (Carragher, 2006; Saez *et al.*, 2006). More recently, calpain signalling has been implicated in regulating cell adhesion and actin remodelling during non-neuronal cell migration and spreading (Franco and Huttenlocher, 2005; Flevaris *et al.*, 2007), and also in regulating growth cone guidance and synapse physiology in neurons (Robles *et al.*, 2003; Wu and Lynch, 2006). These findings motivated us to explore whether calpain signalling also participates in neurite consolidation.

First, we analysed calpain expression and activity along consolidated neurites in cultured hippocampal neurons. Cultured hippocampal neurons are a well-characterized system for the study of neurite outgrowth, branching and neuronal polarity (Gallo and Letourneau, 1998; Dent *et al.*, 2004). As shown in Figure 2A and B, both major calpain isoforms, calpain-1 and -2, are expressed in young cultured neurons along all neurites, with higher expression in the neurite shafts (arrowheads) than at growth cones (open arrowheads). The distribution of both calpains was uniform in more mature neurons (Figure 2C and D; 6 days *in vitro*), and could be detected in axons and dendrites (Figure 2C–E). *In vivo*, calpain-2 was enriched in growing axons that extend from cortical neurons into the intermediate zone forming axon tracts (Supplementary Figure 1), and in neuronal cell bodies and processes in the adult brain, including both dendrites (Figure 2F) and axons (Supplementary Figure 4E). Therefore calpains are expressed along neurites, where they might proteolytically regulate cellular processes.

To assess whether neuronal calpain is active under basal conditions, we loaded neurons with the fluorescent reporter t-Boc-Leu-Met-CMAC (t-Boc) (Glading *et al.*, 2004), in the presence or absence of the reversible calpain-1 and -2 inhibitor ALLM. Using this reporter, we observed a strong and

dose-dependent drop in t-Boc fluorescence intensity with increasing ALLM (Franco and Huttenlocher, 2005) (Figure 2G), indicating that there is a basal level of active calpain in neurons. Similar results were obtained using the immunostaining of proteolysed fodrin (brain spectrin) (Roberts-Lewis *et al.*, 1994), an endogenous substrate of calpain, as an additional reporter of calpain activity (Figure 2G). Remarkably, the staining of calpain-2 and proteolysed fodrin in neurites that are only partially consolidated is highly associated with the morphological signs of consolidation (Figure 2H and I), and calpain-2 levels increase in parallel to culture maturation (Figure 2J). Similarly, although the location of total fodrin is widespread throughout the neuron (Figure 2K), extending into growth cones (Figure 2K), proteolysed fodrin immunostaining in neurites is limited to consolidated regions, which creates a sharp boundary between the neurite shaft (potentially rich in calpain activity) and the growth cone (rich in F-actin; Figure 2L). These observations indicate that under basal conditions calpain is active in neurons, in a location that is consistent with a possible role in neurite consolidation.

Calpain inhibition promotes neurite sprouting

To test whether calpain activity is required for the maintenance of neurite consolidation, we analysed the signs of deconsolidation, such as actin patch formation and growth of filopodia, in neurites treated with calpain inhibitors (Figure 3A). In young cultured hippocampal neurons (1 day *in vitro*, div), neurites exhibit actin patches that are clearly stained by phalloidin and range from small puncta to large patches, which often include associated filopodia (approximately 30% of total patches are protrusive). Consistent with a potential role of calpain in maintaining consolidation, treatment of cultured neurons for 30 min with the calpain inhibitor ALLM increased both the number of actin patches along neurites and the total amount of actin that accumulates in patches in each axon, which can be estimated using fluorescence intensity levels (Figure 3B–D). Another apparent weakening in consolidation observed on calpain inhibition was a change in the number and profile of protrusions along the neurites. In our experiments, most hippocampal neurons grown *in vitro* for 18–24 h had a major neurite that displayed fine filopodial protrusions and occasional thicker protrusions, which we called branches (Dotti *et al.*, 1988) (Supplementary Figure 2; Figure 3E; see also Materials and methods). We found that treatment of neurons with ALLM for as little as 5 min resulted in an increase in the number of filopodia extending along the neurite shaft (Figure 3E, F and H) without affecting neurite length (not shown). However, longer times resulted in a return to baseline number of filopodia while promoting an increase in the number of branches (Figure 3I for ALLM and Supplementary Figure 3 for a second calpain inhibitor, calpeptin), which indicates that the initially formed filopodia are transient and either disappear or become branches. Interestingly, a 30-min treatment where the calpain inhibitor was washed out after the first 5 min of incubation was more efficient than the sustained 30-min treatment in promoting collateral branches (5 + 25wo in Figure 3I), suggesting that re-consolidation after a limited period of deconsolidation accelerates the maturation of the newly formed protrusions. The initial sprouting of transient filopodia followed by more mature protrusions (longer and more stable)

Figure 2 Calpain spatial distribution and activity correlate with neurite maturation. (A, B) Calpain-1 and -2 are expressed in cultured neurons, as shown by immunolabelling of 1-day-old hippocampal neurons. Both calpain isoforms display lower levels in the growth cone (open arrowhead) than in the neurite shaft (arrowhead). (C–E) In polarized neurons (6 days *in vitro*), both isoforms are distributed throughout the entire cell, as shown by immunocytochemistry of calpain-1 and -2 together with dendritic (MAP2) and neuronal (TUJ-1) markers. (F) Immunohistochemistry of calpain-2 in the hippocampus CA1 field shows expression in neurons and their processes (arrowheads in F). (G) Calpain is active in neurons as demonstrated by a decrease in t-Boc reporter or proteolysed fodrin intensity in cells treated with different doses of the calpain inhibitor ALLM (w/o = wash out). Results are mean $\pm$ s.e.m. * P < 0.05. (H, I) Calpain-2 and proteolysed fodrin labelling is observed in neurites that are partially consolidated (cons.), whereas portions that remain non-consolidated (non-cons.) have very low levels of both proteins. (J) Western blot analysis of calpain-2 in 1-, 4- and 9-day *in vitro* cultures. (K, L) Immunolabelling of total (K) and proteolysed fodrin (L) showing that calpain activity (proteolysis of fodrin) is restricted to the soma and neurite shaft and stops at the shaft–growth cone boundary (arrowheads). Abbreviations: SLM: stratum lacunosum moleculare; SP: stratum pyramidale; SR: stratum radiatum; w/o: wash out. Scale bars: (A–D) = 10 μ m, (E, F) = 25 μ m, (H–L) = 10 μ m.

was confirmed by time-lapse imaging of 4div neurons transfected with DsRed (Supplementary Movie 1). Treatment of an axon with the calpain inhibitor calpeptin elicited an early response in the form of numerous transient filopodia and a later appearance of more stable protrusions (Supplementary Movie 1), as described in Figure 3E–I. As seen in Figure 3J, the protrusive response can start as rapidly as 30 seconds after calpain inhibition and it occurs both in axons (Figure 3J) and dendrites (see Figure). These results implicate calpain in the regulation of neurite sprouting and in controlling both axon and dendrite consolidation.

To determine the extent to which calpain inhibition also promotes neurite sprouting *in vivo*, we investigated the changes in plasticity induced by calpain inhibition in the hippocampus of adult mice, a well-characterized model of neuronal plasticity (Reprea and Ben-Ari, 1992).

Intraperitoneal injection of a calpain inhibitor successfully decreased the activity of calpain in the hippocampus as assessed by a decrease in proteolysed fodrin and an increase in full-length cortactin levels 90 min after injection (Supplementary Figure 4A and B). Interestingly, the levels of PSA-NCAM, a known reporter of structural plasticity (Durbec and Cremer, 2001; Bonfanti, 2006), were also increased, indicating that plastic reorganization of the hippocampus already started at this early time point (Supplementary Figure 4C). To assess neuronal morphological changes, we treated mice with calpain inhibitors for 2 days followed by 2 days without treatment to allow for new branches to develop and be detectable, or for new connections to form (Supplementary Figure 4A). We first analysed the effect of this treatment in young developing neurons by focusing on a neuronal population that is developing in the

Figure 3 Pharmacological manipulation of calpain alters sprouting *in vitro* and *in vivo*. (A) Schematic diagram of the experimental protocol followed in (B–G). Representative images (B, C) and quantification (D) of neurites showing the appearance of actin patches, stained with phalloidin and pseudocoloured to show intensity levels, in neurites treated with the calpain inhibitor ALLM. Representative images of neurites (E–G) showing induction of filopodia and branches by 10 μ M ALLM (quantified in H, I, wo = wash out). (J) Time-lapse images of a neurite before and after treatment with ALLM showing the generation of new sprouts (arrows). (K, L) Camera lucida-like reconstructions of PSA-NCAM-positive neurons from the subgranular layer of the adult dentate gyrus (control and calpain inhibitor-treated mice) are shown. PSA-NCAM-positive neurons exposed to calpain inhibitors have increased branching compared with control mice (quantification in M, N). Scale bars: (A–J) = 10 μ m, (K–L) = 25 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001.

adult brain: the immature neurons constantly generated at the subgranular zone (Figure 3K–N) (Zhao *et al*, 2006). PSA-NCAM can be used as a marker of these young neurons that are likely to be 1–2 weeks old (Supplementary Figure 5). Using this marker, we traced their soma and dendritic tree morphology in control and calpain inhibitor-treated mice and then quantified the number of branches and measured their length (Figure 3K and L). Remarkably, immature granule neurons (PSA-NCAM positive) from all four treated mice had longer and more branched dendritic trees than those from control (vehicle treated) mice (Figure 3M and N), confirming that calpain actively represses neurite consolidation and sprouting *in situ*. We then used sprouting and synaptic markers to explore the consequences of this modulation in plasticity induced by calpain inhibition in the adult hippocampus. GAP-43 is a membrane-bound protein found in the axonal growth cones of sprouting CNS axons (Goslin *et al*, 1988), and a well-known marker and key player of neurite sprouting and plasticity (Benowitz and Routtenberg, 1997; Oestreicher *et al*, 1997). We found that in animals treated with calpain inhibitor, the number of GAP-43 positive fibres populating the stratum lucidum was significantly increased (Supplementary Figure 4F and G). Sprouting was further assessed by monitoring the dendritic synaptic marker PSD-95 in the same hippocampal layer, which was visibly elevated in the CA3 dendrites of treated animals (Supplementary Figure 4H and I), and confirms plastic reorganization of both axons and dendrites in response to calpain inhibition. In summary, these results show that calpain maintains neurite consolidation and represses neurite sprouting in both immature and mature neurons not only *in vitro* but also *in vivo*.

To verify our pharmacological results, we directly altered the levels of calpain activity in developing neurons. As calpain-2 was the isoform, the protein levels and location of which during neuritogenesis best correlated with the observable calpain activity, we used a previously described protease-dead mutant of calpain-2, calpain-2-PD (Nuzzi *et al*, 2007), to inhibit its activity, and overexpressed the wild-type gene to increase calpain-2 activity (Figure 4). We transfected neurons with wild-type calpain-2-GFP, calpain-2-PD-GFP or with a control vector (YFP) at 2div, and then quantified the number of branches (not filopodia) 24 h after transfection (Figure 4A). Similar to ALLM treatment, molecular inhibition of calpain with calpain-2-PD induced an increase in the number of branches (Figure 4D, G and H). In contrast, calpain-2 overexpression reduced the number of branches generated during the 24-h period compared with control neurons (Figure 4B, C, E, F and H). This phenotype was not due to a compromise in the integrity of calpain-2-overexpressing neurons as it could be overcome by treating the transfected neurons with the calpain inhibitor calpeptin (Figure 4I and J) or ALLM (not shown). Similarly, neurons transfected with the protease-dead calpain-2 mutant, which already displayed more branches than control cells, failed to sprout in response to calpain inhibitors (Figure 4J). Therefore, inhibition of calpain by using specific pharmacological blockers or by expressing dominant-negative constructs mimics the effect of branching factors on actin polymerization and protrusion formation along the shaft. These findings suggest that calpain is a negative regulator of neurite sprouting and thus most likely to help maintain neurite consolidation.

Figure 4 Molecular manipulation of calpain-2 interferes with branching. (A) Schematic diagram of the experimental protocol followed in (B–H). (B–D) Images and (E–G) camera lucida-like drawings of 10 representative neurons transfected with YFP, calpain-2 or calpain-2-PD. An increase in calpain activity by overexpressing calpain-2 in cultured neurons led to a reduction of filopodia (C) and major branches (F) as compared with controls (B, E), whereas reduction of calpain activity by a protease-dead mutant calpain (calpain-2-PD) induced the opposite effect (D, G). (H) Quantification of the number of branches (not filopodia) in (E–G). Scheme (I) and quantification (J) of the branching of control and transfected neurons in response to the calpain inhibitor calpeptin. Results displayed as mean $\pm$ s.e.m. Asterisks denote statistical significance between groups (Kruskal–Wallis test, * P < 0.05, ** P < 0.01, *** P < 0.001). Scale bar = 10 μ m.

Cortactin is proteolysed by calpain to prevent sprouting

In non-neuronal cells, calpain-2 is known to regulate migration through the proteolysis of cortactin, an actin-binding

protein and Arp2/3 complex activator (Cosen-Binker and Kapus, 2006; Perrin *et al*, 2006). The result of this proteolysis is the repression of actin polymerization, which is likely to be the ultimate target of calpain for regulating consolidation. To test the possibility that the same pathway is involved in consolidation, we first analysed the expression of cortactin in neurons and its possible regulation by calpain. As in non-neuronal cells (Weed *et al*, 2000; Cao *et al*, 2003), cultured neurons showed widespread punctate staining of cortactin and high enrichment at the periphery of lamellipodia before neuritogenesis (Figure 5A). Once neurites emerged, cortactin accumulation was restricted to the growth cone and occasional patches, reflecting the distribution of F-actin (Figure 5B). In differentiated neurons, cortactin localized to both axon and dendrites, again reflecting the distribution of F-actin (not shown). Labelling of Arp3, the binding partner of cortactin in the Arp2/3 complex, was also restricted to the areas of F-actin accumulation where it colocalized with cortactin (Supplementary Figure 6), supporting a potential role of cortactin in regulating actin dynamics in neurons.

The minimal expression of cortactin within the neurite shaft during neuritogenesis suggests that calpain may also regulate cortactin in neurons. Consistent with this, pharmacological inhibition of calpain in cultured neurons induced a marked increase in the levels of full-length cortactin in a dose-dependent manner (Figure 5C; Supplementary Figure 7) that could also be detected at the neurite shaft (Figure 5D). Calpain inhibition also increased the percentage of F-actin patches that displayed cortactin immunoreactivity, which generally corresponded to protrusive patches (Figure 5E and E'). Not only did treated cells show three times more actin patches than the control neurons (not shown), but the percentage of those patches containing cortactin nearly doubled, which triggered an increase in the number of protrusive patches as well (from 30.0 to 65.1%; Figure 5E'). Thus, calpain regulates cortactin levels in neurons, probably by proteolysis of cortactin along the neurite shaft. This indicates that calpain might repress actin polymerization through regulation of cortactin levels in neurons. Alternatively, the increased accumulation of cortactin in F-actin patches could merely be a consequence of an indirectly induced protrusive activity. To discriminate between these two models, we selectively manipulated cortactin levels by transfecting neurons with cortactin-RFP, or YFP or pDsRed as a control (Figure 5F–K). Cells with elevated cortactin levels showed an increase in the number and complexity of branches (Figure 5F–I) and a noticeable spiny appearance of neurites at high magnification (Figure 5J and K). Indeed, fluorescent time-lapse imaging showed that neurons that overexpressed cortactin sprouted a large number of very motile, non-transient protrusions, in striking contrast to equivalent fields of control neurites, which had few filopodia that were mostly transient (Supplementary Movies 2 and 3). Cortactin-overexpressing neurons were also characterized by the presence of enlarged growth cones compared with controls. In the absence of antibodies to proteolysed cortactin, we cannot show that endogenous cortactin proteolysis is restricted to the shaft. Nonetheless, this assumption is supported by the findings that proteolysis of fodrin is limited to this region, that cortactin is visibly associated with actin at the growth cone and that sprouting can be elicited by an elevation of cortactin levels. Taken together, these results

suggest that cortactin is maintained at low levels by calpain, and increased cortactin levels is sufficient to activate the machinery required for sprouting along the shaft.

To assess whether an increase in cortactin levels is necessary to induce branching in response to calpain inhibitors, we used a dominant-negative form of cortactin and expressed it in 2div hippocampal neurons for 24 h. This mutant has been previously described to function as a dominant negative of cortactin but not of N-WASP (Weed *et al*, 2000; Weaver *et al*, 2002), and consists of the Arp3 interacting domain of cortactin (NTA) fused to an HA tag (cloned into the pCIG (GFP IRES) vector), but lacks the actin-binding domains and C terminus (Weed *et al*, 2000). We found that this construct prevented the robust increase in branching induced by cortactin overexpression (Figure 5L–Q; see also Supplementary Figure 8), which demonstrates that cortactin induces membrane protrusion through interaction with Arp3 in neurons. Expression of NTA also prevented the increase in branching induced by bath application of the calpain inhibitor calpeptin (Figure 5R–U). These results indicate that proteolysis of cortactin by calpain is a key step in calpain regulation of neurite consolidation.

PKA regulates calpain activity in neurons

The restricted distribution of calpain activity to the soma and neurite shaft does not reflect the localization of calpain. This suggests that an upstream regulator must be important in regulating domain-specific activity of calpain. Previous studies in neuronal and non-neuronal cells have shown that activation of calpain can be stimulated by high calcium levels or direct phosphorylation by Erk (Huttenlocher *et al*, 1997; Glading *et al*, 2004). In addition, phosphorylation by PKA represses calpain in response to high levels of cAMP in migrating cells (Shiraha *et al*, 2002) (Figure 6A). To determine the role of these upstream regulators in maintaining calpain activation along the neurite shaft, we first asked whether their pharmacological manipulation could modulate basal calpain levels in neurons using the t-Boc assay (Figure 6A–C). Both inhibition of calcium binding to calpain, using a calcium chelator and a specific calpain inhibitor that prevents calcium binding, as well as inhibition of Erk, failed to decrease calpain basal levels (Figure 6B). In contrast, manipulation of PKA activation resulted in changes in calpain activity: elevation of PKA activity using a cAMP analogue resulted in direct phosphorylation of calpain by PKA (Figure 6D) and inhibition of its activity (Figure 6C), whereas pharmacological inhibition of PKA induced calpain overactivation (Figure 6C). These results indicate that calpain activity in neurons is regulated by repression, and that the repressor is PKA. Because the t-Boc reporter assay lacks subcellular resolution, we analysed proteolysed fodrin immunolabelling to evaluate the significance of cAMP regulation of calpain activity along the shaft. Elevation of cAMP levels using the cAMP analogue induced a noticeable decrease in calpain activity over the entire neurite length, as measured by the intensity and distribution of proteolysed fodrin along the neurite (Figure 6E and F). Conversely, inhibition of PKA increased the extent of proteolysed fodrin into growth cones and filopodia (Figure 6G open arrowheads). This confirms that calpain activity in neurons is regulated by cAMP levels, and suggests that differential

activation of PKA at the growth cone versus the neurite shaft is responsible for the shaft-specific activation of calpain.

Regulation of calpain activity in response to branching factors

The same cues that induce the development of collateral branches *in vivo*, such as neurotrophins and guidance cues, can be used to elicit neurite branching *in vitro* (Gallo and Letourneau, 1998; Dent *et al.*, 2004). If calpain is indeed

responsible for neurite consolidation, it must be inactivated to allow the formation of new branches. To evaluate the regulation of calpain activity during sprouting, we treated cultured hippocampal neurons with known branching factors and monitored calpain activity using the t-Boc reporter. NT3 stimulation of neurite branching was accompanied by a transient decrease in calpain activity that was apparent between 20 and 30 min after bath application (Figure 7A and B). A similar regulation of calpain was also

seen with an additional branching factor, BDNF (Figure 7C and D). This coincided with an increase in the levels of full-length cortactin and its association with cytoskeleton (Figure 7E and F), which was also observed in response to netrin-1, an additional branching factor (Supplementary Figure 9). Furthermore, pre-incubation of neurons with inhibitors for 30 min prior to NT3 addition confirmed that the decrease in calpain activity was dependent on PKA and not on calcium or Erk activation (Figure 7G). Thus, calpain is inhibited by branching factors, consistent with a possible transient deconsolidation of the shaft during neurite branching.

Finally, we investigated whether inhibition of calpain is required for neurite sprouting in response to branching factors. We first tested the responses of neurons transfected with control vectors to bath application of NT3, BDNF and netrin-1 for 1 h (Figure 7H). In all experiments, 1 h of treatment was enough to induce a clear increase in the number of branches (not filopodia) extending from the major neurite (Figure 7I and J; Supplementary Figure 10). Using this assay, we tested whether deregulation of calpain or inhibition of cortactin can affect the sprouting elicited by branching factors (Figure 7I and J). Overexpression of calpain-2 abolished neurite branching in response to NT3, BDNF and netrin-1

Figure 6 PKA regulates basal calpain activity in neurons. (A) Schematic diagram of the experiment shown in (B, C) indicating possible calpain regulators, the agents used to modulate them and the detection system (t-Boc). Total calpain activity in cultured neurons treated with calcium or Erk inhibitors is not affected (B), whereas PKA activity inversely regulates calpain activity, therefore acting as a repressor (C) (mean $\pm$ s.e.m). (D) Immunoprecipitation of calpain-2 followed by western blot detection of proteins phosphorylated by PKA showing that calpain-2 is phosphorylated by PKA in neurons. (E–G) Subcellular localization of proteolysed fodrin in neurons depends on PKA. Although calpain activity (proteolysed fodrin) localizes to the soma and shaft, the activity along the shaft is reduced in neurons treated with 8Br-cAMP (F) and increased—to the extent of invading the growth cone—in neurons treated with PKA inhibitors (G). The shaft-growth cone boundary is indicated with white arrows, whereas the new limits of proteolysed fodrin staining are shown with open arrows. Scale bars = 10 μ m. ** P < 0.01, *** P < 0.001.

Figure 5 Cortactin is proteolysed by calpain to prevent sprouting. (A, B) Cortactin accumulates at actin-rich regions of cultured hippocampal neurons before and after the emergence of neurites (arrowheads). Cortactin immunolabelling is shown. (C) Western blot of cortactin in ALLM-treated cultures showing an increase in cortactin protein levels upon calpain inhibition. Loading control corresponds to cortactin antibody nonspecific band (Supplementary Figure 6). (D) Quantification of cortactin fluorescence along the neurite shaft of neurons treated with calpain inhibitors (45 min after treatment). (E, E') Correlation between cortactin accumulation and the presence of protrusions in F-actin patches (arrowheads) of DMSO- or ALLM-treated neurites (two experiments, n = 50 patches per experiment). Images in (E) correspond to sample images of different patches and the bar graph in (E') shows that the main change after ALLM treatment is a reduction of the non-protrusive patches that have no cortactin accumulation in exchange for an increase in the cortactin-rich protrusive patches. (F, G) Neurons transfected with cortactin-RFP display increased branching compared with control (pDsRed). (H, I) Quantification of the branching complexity of neurons overexpressing cortactin. (J, K) High magnification of representative neurites from the same experiment highlights the spiny phenotype of cortactin-overexpressing cells (K) compared with controls (J). (L–O) Transfection of the cortactin dominant-negative HA-NTA (N–O) reverses the hyperbranched phenotype of cortactin-overexpressing neurons (as shown in (M)). Schematic diagram (P) and quantification (Q) of the reversal experiment. Schematic diagram (R) and quantification (S) of branches in neurons transfected with cortactin dominant-negative HA-NTA or control (pCIG, cloning vector) and treated with calpeptin for 60 min. Overexpression of HA-NTA abolishes the branching response induced by calpeptin. (T, U) Time-lapse imaging of control (T), pCIG and NTA-transfected neuron dendrites (U) before and after the addition of calpeptin. Three frames of a 5-min video are shown overlapped coded in RGB (red: initial frame; green: 2.5 min frame; blue: 5 min frame). Movement is seen as colours, whereas static structures overlap and generate white (note the appearance of colours in T, arrowhead). Colours along the shaft in (U) result from vibration of the image, not from protrusive activity. No new protrusions (colours) are formed in NTA neurons after the addition of calpeptin. Results displayed as mean $\pm$ s.e.m. Asterisks denote statistical significance between groups (Kruskal–Wallis test, ** P < 0.01, *** P < 0.001) Scale bar = 10 μ m.

Figure 7 Inhibition of calpain is required for neurite branching. (A) Calpain activity in neurites exposed to 50 ng/ml of NT3 for 20 min (B) is decreased as compared with controls (A), seen as intensity of fluorescence of t-Boc. (C, D) Schematic diagram and experimental data of a time course of calpain activity after BDNF or NT3 treatment using t-Boc. Branching factor or control (stars) was added at different times before t-Boc (C) was measured and compared with control (D). (E, F) Western blots of cortactin levels in neurons at different time points following NT3 addition (E), or associated with cytoskeleton at 45 min after NT3 addition. (G) NT3 reduction of calpain activity, measured as a change in the intensity of fluorescence of t-Boc reporter, can be blocked by PKA inhibitors. Schematic diagram of the experiment (H), and quantification of branches in neurons transfected with calpain-2 (I) or dominant-negative HA-NTA (J) and treated with NT3, netrin-1 or BDNF for 60 min. Inactivation of calpain and an increase in cortactin activity are required for branching. Results displayed as mean $\pm$ s.e.m. Asterisks denote statistical significance between groups (Kruskal–Wallis test, * P < 0.05, ** P < 0.01, *** P < 0.001). Scale bar = 10 μ m.

(Figure 7I), and neurites overexpressing the cortactin dominant-negative NTA also showed reduced branching response (Figure 7J). As overexpression of calpain does not alter the ability of neurons to generate branches, as shown with calpain pharmacological inhibitors (Figure 4J), these results show that all three branching factors need to induce a sufficient reduction in calpain activity to promote neurite branching. Taken together, our experiments indicate a role for calpain in keeping the neurite shaft consolidated and support a model in which branching factors induce deconsolidation of the neurite shaft through the inhibition of calpain and upregulation of cortactin.

Discussion

Unlike axon guidance or synaptogenesis, the question of how neurite shape is regulated has remained largely unexplored.

Our results show that neurite consolidation requires constant repression of protrusive activity, and that this repression is mediated by calpain. This constant repression model provides an explanation for how a neurite shaft is generated from a growth cone and how branching factors reverse this consolidation process (Figure 8). It also illustrates the conservation of cell-shape regulatory pathways between non-neuronal and neuronal cells (Supplementary Figure 11).

Calpain signalling maintains neurite consolidation along the neurite shaft

Our finding that calpain activity, reported by fodrin proteolysis, closely corresponds to the consolidated domains of the neuron is striking, and prompted us to investigate the possible role of calpain in regulating this process. Whereas full-length fodrin is present throughout the cell, including growth cones (Figure 2K; Letourneau and Shattuck, 1989; Sobue,

Figure 8 A model for calpain maintenance of neurite consolidation. We propose that calpain functions as a repressor of the protrusive activity needed for branching or growth cone formation, therefore maintaining the shaft consolidated. Below a threshold level of active PKA, calpain is left unrepressed and it prevents actin patch formation though proteolysis of cortactin (soma and shaft). Levels of cAMP and active PKA are higher at the growth cone, so PKA inhibits calpain that allows de-repression of cortactin and actin polymerization. Acute elevation of cAMP at the consolidated areas (centre) reproduces the signalling of the growth cone and leads to deconsolidation and branch creation.

1990), proteolysed fodrin is restricted to the neurite shaft, beginning at the first signs of consolidation and persisting throughout development. Not only do these results implicate calpain in the consolidation process but they also raise the possibility that proteolysis of fodrin is used by calpain to induce a local disconnection of actin filaments from the plasma membrane (Hu and Bennett, 1991). Such a pattern of activation is consistent with the reported role of calpain-2 in non-neuronal migrating cells, where it preferentially localizes to the rear and represses the formation of protrusions (Franco *et al*, 2004; Flevaris *et al*, 2007). In addition to the localization of calpain, we provide pharmacological and molecular data (overexpression and dominant negative) that lead us to propose that calpain has a critical function in maintaining neurite consolidation. Although pharmacological inhibition of calpain and a dominant-negative calpain-2 promoted neurite branching, we do not know whether a specific knockdown of calpain-2 can also result in the formation of hyperbranched neurites, a task that has so far proven difficult. However, in support of our conclusions, the knockdown in hippocampal neurons of the calpain-2 target cortactin results in the marked reduction of dendritic protrusions (Hering and Sheng, 2003), demonstrating a new and important function for calpain in the prevention of neurite sprouting.

The growth, guidance and branching of neuronal processes in the nervous system is controlled by multiple extracellular cues (Chilton, 2006), but can also be modulated intracellularly (Zhou and Snider, 2006). In this report, we demonstrate that inhibition of calpain enhances the capacity of young granule neurons to branch *in vivo*, indicating that calpain is constitutively activated in growing neurites and regulates neuronal morphogenesis by controlling the capacity

of these neurites to sprout. We also found that pharmacological inhibition of calpain *in vivo* promotes axonal and dendritic plasticity, which could lead to enhanced synaptogenesis. Although this latter observation is to be interpreted carefully, given the known role of calpain in directly remodelling synapses, including the proteolysis of PSD-95 (Liu *et al*, 2008), it is reasonable to expect closely related processes such as synaptogenesis and morphological plasticity (protrusion formation or repression) to share signalling pathways, making it difficult to discriminate between them or to interfere with one and not with the other. It thus seems that calpain functions as a repressor that maintains the neurite shaft consolidated, therefore limiting neurite plasticity both during neuronal morphogenesis and in the maintenance of mature neuronal projections.

Our data suggest that a key downstream effector of calpain in regulating neurite consolidation is cortactin: it is regulated by calpain in neurons, its overexpression induces extensive branching and a dominant-negative form prevents branching. Indeed, overexpression of cortactin is sufficient to induce the sprouting of previously aspiny neurons (Hering and Sheng, 2003), and cortactin is required for the formation of dendritic spines in mature neurons (Hering and Sheng, 2003; Gray *et al*, 2005). Thus, cortactin seems to regulate the formation of new branches by controlling the initial sprouting of filopodia. Several aspects of our findings on cortactin deserve additional comment. First, we found that cortactin is constantly proteolysed by calpain in neurons, presumably along the soma and neurite shaft. This implies a regulation of cortactin activity by protein levels, a regulation known for other proteins such as β -catenin but so far unreported for cortactin. Indeed, cortactin overexpression in neurons results in a hyper-branched and hyper-motile phenotype and all three stimuli used to induce sprouting—calpain inhibitors, neurotrophins and netrin-1—produced an elevation of cortactin levels. Finally, when calpain is overexpressed, or a dominant-negative form of cortactin is expressed, the sprouting response of the neuron to branching factors is attenuated or even prevented. This result strongly implies that neurite branching is accomplished, at least in part, by inhibiting calpain and therefore by ‘relieving’ the downstream pathway.

The finding that calpain expression cannot account for the domain-specific location of calpain activity implies that the upstream regulators of calpain determine the extent and location of consolidation. Interestingly, we found that cAMP, a well-known promoter of neurite plasticity and branching (Gallo and Letourneau, 1998; Kalil *et al*, 2000; Spencer and Filbin, 2004), is responsible for calpain domain-specific activation in neurons. Interestingly, it is not sustained activation of calpain at the neurite shaft, but sustained repression at the growth cone through PKA, that keeps calpain active along the neurite and inactive at the growth cone. These results imply that, in non-pathological conditions, neuronal calpain appears to be regulated by repression. This resembles the regulation of GSK-3 β in neurons, which is active throughout the cell with the exception of the growth cone, where local activation of PI3K results in an inactive pool of GSK-3 β (Eickholt *et al*, 2002). Indeed, axon branching is known to require interactions between actin and microtubules (Dent and Kalil, 2001), and inhibition of GSK-3 β , a regulator of microtubules, promotes neurite branching (Jiang *et al*, 2005). The similar pattern of subcellular regulation and

the fact that both calpain and GSK-3 β are 'repressors', controlling complementary elements of the cytoskeleton, supports the concept of a 'repression model' and an interaction between both pathways during branching seems plausible. It is then reasonable to propose that phosphodiesterases might localize, or be preferentially active, along the neurite shaft, playing an equivalent role to that of PTEN and thus helping create the border between shaft and growth cone.

Although there are multiple upstream regulators of calpain, including calcium and Erk (Shiraha *et al.*, 2002; Glading *et al.*, 2004), we found only cAMP/PKA to be involved in calpain regulation during neurite consolidation and sprouting. This contrasts with previous literature in which local calcium transients were shown to promote neurite branching (Tang *et al.*, 2003; Tang and Kalil, 2005; Hutchins and Kalil, 2008), and in particular in response to netrin-1 (Tang and Kalil, 2005). However, our results showing that calcium does not modulate calpain basal levels in neurons are not inconsistent with a role for calcium in neurite branching. As discussed above, additional pathways are likely to be involved in the regulation of consolidation, and branching factors, such as netrin-1, could target and even require several of these for adequate deconsolidation. How all these pathways fit into the picture remains to be seen.

Cell polarity and the two neurite domains

Our results suggest that shaft and growth cone 'phenotypes' may be switched by turning on or off certain signalling pathways in which calpain is a key player. This implies that neurite branching and neurite consolidation are intimately connected, as the result of two states of a pathway: one state represses protrusions, whereas the other promotes them. This observation challenges the common assumption that the neurite shaft is in a dormant state, assumed by the apparent stability of actin and microtubules along it (Dent and Gertler, 2003). Instead, it implies that neurite consolidation is not a punctual event occurring at the growth cone neck, but involves the maintenance of the shaft non-protrusive state by constant repression of actin dynamics. In addition, it implies that neurite plasticity is internally regulated by the neuron and that protrusive potential is not absent from the shaft but repressed.

The observation that neurite shaft morphological inactivity requires constant repression is reminiscent of non-neuronal cell polarization, where asymmetry of membrane protrusions is achieved by promotion of actin protrusions in one domain and repression in the other (Fais *et al.*, 2000; Ridley *et al.*, 2003) (Supplementary Figure 11). By analogy, growth cones and neurite shafts could also be regarded as two compartments of a polarized cell that are generated by differential consolidation, which occurs only along the shaft and soma (Supplementary Figure 11). Our results also focus attention on the remarkable conservation of the signalling mechanism involved in regulating these two domains (Perrin *et al.*, 2006). It appears that during evolution, establishment of cell asym-

metry by coordinated repression and promotion of protrusions provided a useful set of molecular tools that could be readily co-opted for different cell systems, including the division of neurites in shaft and growth cone and the maintenance of the shaft by consolidation. Given our much broader knowledge of the signalling involved in directional migration, and the anticipation that further signalling similarities exist, we could use this understanding to fill the current gap of knowledge about neurite consolidation and the regulation of plasticity.

Materials and methods

Cell culture and transfection

Neurons were isolated from the hippocampus of CD1 mouse embryos at 16–18 days postcoitum or postnatal day 0 and were cultured on poly-D-lysine-coated coverslips (Sigma). Neurons were maintained in neurobasal medium supplemented with glucose, glutamine and B27 (Invitrogen) (Mingorance-Le Meur *et al.*, 2007). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum. Mouse primary neurons were transfected at 2 days *in vitro* with lipofectamine 2000 (Invitrogen) and HEK293 cells with Fugene-HD (Roche).

In vivo experiments, tissue collection and immunohistochemistry

For calpain immunostaining, time pregnant and non-pregnant adult CD1 mice (Charles River) were used. Perfusion and processing of the tissue for free floating immunohistochemistry was performed as described (Mingorance *et al.*, 2004). For the *in vivo* sprouting experiments, calpain inhibitor III (CI-III, also MDL28170; Calbiochem) (Markgraf *et al.*, 1998) 12.5 mg/kg was administered i.p. in four adult CD1 mice. CI-III stock was prepared in DMSO and dissolved 1000 $\times$ and dissolved in saline (0.9% NaCl) for i.p. administration. This inhibitor was chosen because it was the calpain inhibitor better characterized in the *in vivo* literature and it is known to cross the blood-brain barrier following systemic administration and inhibit calpain in the CNS at the dose used (Markgraf *et al.*, 1998; Kunz *et al.*, 2004; Yu and Geddes, 2007). Four additional mice received a 0.1% solution of DMSO as a control. Animals received two injections, 72 and 48 h before perfusion. This time point was chosen as the most appropriate timing to detect sprouting of hippocampal tracts based on previous observations (Cantalupo and Routtenberg, 1996). Tissue processing and immunohistochemistry was performed as described for calpain immunostaining. Four additional mice (two per group) were killed 90 min after a single injection and their hippocampus were immediately removed and lysed for western blot analysis.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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