Amyloid precursor protein promotes post-developmental neurite arborization in the *Drosophila* brain

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The mechanisms regulating the outgrowth of neurites during development, as well as after injury, are key to the understanding of the wiring and functioning of the brain under normal and pathological conditions. The amyloid precursor protein (APP) is involved in the pathogenesis of Alzheimer's disease (AD). However, its physiological role in the central nervous system is not known. Many physical interactions between APP and intracellular signalling molecules have been described, but their functional relevance remains unclear. We show here that human APP and *Drosophila* APP-Like (APPL) can induce postdevelopmental axonal arborization, which depends critically on a conserved motif in the C-terminus and requires interaction with the Abelson (Abl) tyrosine kinase. Brain injury induces APPL upregulation in *Drosophila* neurons, correlating with increased post-traumatic mortality in *appl*^Δ mutant flies. Finally, we also found interactions between APP and the JNK stress kinase cascade. Our findings suggest a role for APP in axonal outgrowth after traumatic brain injury.

The EMBO Journal (2005) 24, 2944–2955. doi:10.1038/sj.emboj.7600757; Published online 28 July 2005

Subject Categories: development; neuroscience

Keywords: Alzheimer's disease; amyloid precursor protein; axonal outgrowth; brain injury; *Drosophila*

Introduction

During nervous system development, the outgrowth of neurites is tightly regulated to generate intricate patterns of neuronal connectivity required for a correct functioning of the brain. Under several conditions, like brain injury and neurodegenerative diseases, neuronal connections are lost, leading to functional impairment of the brain.

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Received: 4 April 2005; accepted: 6 July 2005; published online: 28 July 2005

The most prevalent neurodegenerative disorder in the western world is Alzheimer's disease (AD). AD is pathologically characterized by a reduction in synaptic contacts, correlating with impairments of higher cognitive function (DeKosky and Scheff, 1990) in addition to the accumulation of neurofibrillary tangles, and extracellular senile plaques containing the amyloid β peptide (A β) (Glennner and Wong, 1984). Head injury, another common cause of disruption of axonal contacts, can lead to AD-like pathology in the brain (Ikonomovic *et al*, 2004), and is a major environmental risk factor for this disease (Jellinger, 2004).

In this study, we focus on the amyloid precursor protein (APP), which is the precursor of A β in mammals (Kang *et al*, 1987). APP is a type-I membrane-spanning protein which is conserved in invertebrates and its *Drosophila* homologue, called APP-Like (APPL), is expressed in neurons (Martin-Morris and White, 1990). However, it does not contain an A β -peptide sequence, suggesting that the conserved physiological functions of APP do not involve A β .

Several genetic knockout models for APP and its homologues have been generated to gain insights into its physiology. Triple knockout mice for APP and its homologues are lethal and show focal cortical dysplasia (Herms *et al*, 2004). However, the mechanisms causing these phenotypes remain unresolved. Flies mutant for *appl* are viable, fertile and show some neurological defects, mainly in the peripheral nervous system. Disturbed locomotor behaviour (Luo *et al*, 1992), synapse formation at the neuromuscular junction (Torroja *et al*, 1999) and axonal transport (Gunawardena and Goldstein, 2001) in motor axons have been reported. Recently, also developmental defects in peripheral sensory organ formation were described (Merdes *et al*, 2004). Despite these advances, a mechanistic insight into the role of APP in the brain *in vivo* is still lacking.

Another approach to understand the mechanisms of APP signalling has been to perform *in vitro* studies to identify APP-binding partners and functional effects of APP on cultured cells. Some of these studies have attributed a role to the APP extracellular domain in neurite outgrowth, neuronal survival and proliferation (Jin *et al*, 1994; Caille *et al*, 2004). On the other hand, interaction studies using the APP intracellular domain (AICD) have identified a plethora of putative cytosolic binding partners for APP (Van Gassen *et al*, 2000; Turner *et al*, 2003). They include the adaptor proteins X11/Mint, Fe65 and Disabled (Dab), the JNK scaffolding protein JIP1-b and the Kinesin motor protein (Kamal *et al*, 2000; Matsuda *et al*, 2001). The *in vivo* relevance of these interactions, however, remains unclear.

In this study, we show that human APP and *Drosophila* APPL can induce postdevelopmental axonal arborization in the *Drosophila* CNS. This function of APP depends critically on the conserved YENPTY motif in the APP C-terminus, and requires interaction with the Abelson (Abl) tyrosine kinase

and the JNK signalling cascade, crucial in repair after axotomy in mammals. APPL, like mammalian APP, is strongly upregulated in regenerating neurons after traumatic brain injury, possibly to allow axonal arborization. Lack of this post-traumatic response in *appl* mutant flies increases mortality. We therefore propose a role for APP in axonal outgrowth after traumatic brain injury. These findings may have important implications in our understanding of some aspects of the pathophysiology of AD.

Results

APP promotes axonal arborization of the small lateral neurons ventral (sLNv) in the *Drosophila* brain

To investigate the effects and mechanisms of action of APP on postmitotic brain neuron morphology *in vivo*, we expressed APP in a well-characterized subset of *Drosophila* brain neurons called the sLNv. This group of four to five neurons is important in the regulation of circadian rhythm, and expresses a neuropeptide called pigment-dispersing hormone (PDH) (Kaneko and Hall, 2000). Their simple and stereotypical axonal pattern (Figure 1A–C) allows high-resolution studies of axonal arborization phenotypes. During larval and pupal development (Figure 1A), their axonal bundle grows dorsally, forming the axonal stem (Figure 1B, arrowhead), bends to the centre of the brain and sprouts into a small, flat, two-dimensional arbour (Figure 1B, arrow,

Figure 1C and D and Supplementary Figure S1A). Their morphology can therefore be visualized by confocal microscopy, using specific expression of an axonally transported reporter gene (e.g. membrane-bound CD8-GFP: Figure 1A–C) controlled by the PDH-Gal4 driver (Kaneko and Hall, 2000), or by immunostaining for the PDH neuropeptide, which is distributed throughout sLNv axons (Supplementary Figure S1C).

Expressing the neuronal isoform of human APP (APP695), or *Drosophila* APPL, had no effect on the early development of the axonal pattern of these cells (Figure 1A' and A'' and Supplementary Figure S2A), consistent with a lack of a sLNv axonal phenotype in *appl^d* mutant flies (data not shown). In contrast, APP or APPL expression resulted in a large increase in the area of the axonal arbour in adult flies (Figure 1B' and B'' and Figure 1C' and C''). Most axons grew along their right paths, but extended further and arborized over a larger two-dimensional area than in control flies (Figure 1C' and C'', arrows, and Figure 1D' and Supplementary Figure S1B). Thus, APP expression induces increased axonal extension and more extensive arborization in postmitotic brain neurons.

APP induces axonal arborization postdevelopmentally in a quantifiable, robust and dose-dependent way

To test whether APP-induced axonal arborization can be used to investigate APP signal transduction in neurons, we asked if

Figure 1 APP induces increased axonal arborization in the sLNv. (A) Confocal images of the sLNv in third instar larva show normal morphology when expressing human APP (A') or *Drosophila* APPL (A''). (B) Overview images of the adult sLNv, showing the dorsally projecting axons (arrowheads) and terminal arbours (arrows). (C) Detail of the sLNv terminal axonal arbour, which extends further and is more extensively arborized (arrows) when human APP (C') or *Drosophila* APPL (C'') is overexpressed. (D, D') Three-dimensional reconstructions rotated at 40° or 90° angles of the dorsal projections of the sLNv without and with overexpression of APP.

the effect of APP is (1) quantifiable, (2) independent of genetic background and (3) dose dependent.

To quantify the area of the axonal arbour, a confocal image representing the entire terminal arbour of the sLNv was imported into Scion Imaging software. The area was measured as detailed in Figure 2A (see also Materials and methods).

To determine the robustness of the observed axonal phenotype and the reliability of the area measurement, we studied the effects of APP expression in the sLNv of flies from different genetic backgrounds. Flies expressing APP were crossed to Canton S and *w¹¹¹⁸* fly lines, which are generally used as reference backgrounds. The qualitative phenotypes and quantitative area measurements were

Figure 2 The APP axonal arborization phenotype is quantifiable, dosage dependent and can be induced postdevelopmentally. (A) For quantification of the two-dimensional area of the terminal axonal arbour, the axonal stem was marked with a straight line (red vertical line). Between the points where the two terminal arbours deviate from this line, a horizontal line was drawn (horizontal red line). The outline of the axonal processes dorsal to this line was traced (blue line), and the area inside was measured. The distance between the intersection points of the vertical lines with the commissure served as a measure for brain size (green line). (B) Quantification of the axonal arbour area shows a robust increase, independent of genetic background, when APP is expressed ($***P < 0.001$ compared to control = yellow bars; error bars represent the 95% confidence interval). (C, D) Modifying the dose of APP expression by varying the temperature or the number of drivers (2X = 2 copies of the driver) modifies the effect on the axonal arbour area ($*P < 0.05$; $***P < 0.001$ compared to control = yellow bar; error bars represent the 95% confidence interval). (E) Flies raised at 18°C and switched to 28°C for 10 days as adults show outgrowth and arborization of axons (arrows) in different directions.

comparable in the different backgrounds with and without GFP expression (Figure 2B). This phenotype can therefore be used as a reliable and quantifiable readout for the effects of APP on axonal arborization.

Next, we investigated the dosage sensitivity of the observed phenotype. Since APP expression is controlled by the Gal4/UAS system (Brand and Perrimon, 1993), levels of expression are temperature sensitive. At 18°C a very low level, at 25°C an intermediate and at 28°C a high level of target gene expression are induced. Furthermore, we varied the number of copies of the Gal4 driver. In all cases the qualitative (Figure 2C) and quantitative (Figure 2D) phenotypes correlated with APP expression conditions, showing that this system is sensitive enough to be used for genetic interaction studies.

The observation that APP does not affect the early development of the sLNv axons suggests that APP's effects are largely postdevelopmental. To determine whether APP can also induce *de novo* outgrowth of mature axons in adult flies, we allowed APP-expressing flies to develop at 18°C, inducing only very low expression of APP. After eclosion, adult flies were shifted to 28°C for 10 days, thereby inducing high APP expression. This caused increased axon outgrowth and arborization (Figure 2E, arrows). Flies which were retained at 18°C for 10 days as adults, keeping APP levels low, did not show these phenotypes (data not shown).

The C-terminal domain of APP mediates its effects on axonal arborization

The data above show that APP can induce axonal outgrowth and arborization in adult brain neurons. Previous studies, using cultured neurons, have suggested that APP can affect neurite outgrowth by interacting with the extracellular matrix via its N-terminal domain (Jin *et al*, 1994). The APP C-terminus, on the other hand, can interact with several intracellular signalling molecules (Turner *et al*, 2003). To identify the domain of APP that induces axonal arborization in the sLNv, we expressed the full-length and two truncated Myc-tagged forms of APP (Fossgreen *et al*, 1998; Figure 3A). The truncated protein lacking the C-terminus (APPΔCT) failed to induce a significant increase in axonal arborization (Figure 3B and D), despite its localization to axons (Figure 3D) and detection levels not significantly different from those of APP (Supplementary Figure S3D). To test whether the APP N-terminus is also required to activate axonal arborization, we expressed a truncated form lacking most of the N-terminus (APPΔNT also known as SPA4CT; Fossgreen *et al*, 1998), which resulted in increased axonal arborization (Figure 3B and D). Surprisingly, its immunohistochemical signal was much weaker in the cell bodies (Figure 3C' and Supplementary Figure S3D) and not detectable in axons (Figure 3D and Supplementary Figure S3B and C), which may be due to a decreased stability of this truncated protein. These results combined suggest that the APP C-terminus is necessary and sufficient to activate the axonal arborization signal.

APP interacts with the Abl tyrosine kinase signalling pathway to induce axonal arborization

To better understand how APP would control axonal outgrowth signals via its C-terminus, we wanted to identify the C-terminal motif(s) required for this function. The APP

C-terminus contains two important conserved domains (Figure 4A): a Go-protein-binding and -activating domain (Nishimoto *et al*, 1993) and the YENPTY protein interaction domain, which is 100% conserved in *Drosophila* APPL (Luo *et al*, 1990; De Strooper and Annaert, 2000). To test the importance of these domains in APP-induced axonal outgrowth, we expressed APP forms in which either of these motifs was removed (Figure 4A; Merdes *et al*, 2004). Expression of all transgenes was confirmed by immunostaining with the 4G8 antibody (Figure 4C–G, insets), and quantification of the terminal axonal arbour areas in all conditions tested is provided in Supplementary Figure S2. Upon expression of APP lacking parts of the Go-protein-binding domain, axonal arborization phenotypes were still induced (Figure 4C and D). In contrast, removal of the YENPTY motif rendered APP incapable of increasing arborization, and in rare cases (4/24) reduced the normal axonal arbour (Figure 4E). The YENPTY motif of APP is required for the binding of adaptor proteins, including Fe65, X11/Mint and Dab1. Binding studies using point mutations in APP have furthermore shown that binding of Fe65 and X11/Mint to APP depends on the Y682 residue, but not Y687 of APP (Borg *et al*, 1996). In contrast, Dab1 and its *Drosophila* homologue Dab require both intact Y682 and Y687 for APP binding (Trommsdorff *et al*, 1998; Merdes *et al*, 2004). Therefore, we expressed two forms of APP carrying point mutations in Tyrosine residues 682 or 687. Both failed to induce significant axonal arborization (Figure 4F and G), suggesting that the APP effect is more likely mediated by Dab. Dab acts as an adaptor molecule for the Abl tyrosine kinase (Howell *et al*, 1997), which is essential for regulating correct axonal outgrowth in *Drosophila* (Lanier and Gertler, 2000). Furthermore, activated Abl has been proposed to phosphorylate the Tyr 682 residue of APP, creating a docking site for its own SH2 domain (Zambrano *et al*, 2001). Together, these data point to Abl as a strong candidate for the executor of the APP axonal arborization signal.

If the APP signal were transduced via interaction with Abl, reduction of its levels or activity should reduce the effect of APP on axonal arborization. Taking advantage of the quantifiability and robustness of the APP phenotype, we performed genetic interaction studies between APP and Abl. As the APP phenotype is only detectable in adult sLNv and *Abl* mutant flies are lethal before adulthood, we expressed APP in flies heterozygous for *Abl*^d or *Abl*^l, both strong hypomorphic alleles of Abl (Henkemeyer *et al*, 1987). Heterozygosity for either *Abl* allele suppressed the APP-induced increase in axonal arborization (Figure 4H–J and Supplementary Figure S2D), showing that the APP phenotype depends critically on the levels of Abl. To test whether APP signalling through Abl depends on its tyrosine kinase activity, we coexpressed a kinase-dead form of Abl (Wills *et al*, 1999a) together with APP in the sLNv. This also reduced APP-induced axonal arborization (Figure 4K and Supplementary Figure S2D), probably by competition for APP binding with the endogenous protein. We can thus conclude that both normal levels of Abl signalling components, as well as intact interaction domains with its scaffold Dab, are required for APP to induce axonal arborization, strongly suggesting that Abl is indeed the executor of the APP axonal arborization signal.

To further confirm a role of Abl in axonal arborization, we tested whether activation of Abl on its own is sufficient to

Figure 3 The C-terminal domain of APP mediates its effects on axonal arborization. (A) Schematic representation of the truncated APP constructs. The red boxes represent the A β domain, which is recognized by the 4G8 antibody. (B) Quantification of axonal arbor areas (***) compared to control = yellow bar; error bars represent the 95% confidence interval). (C) Cell bodies of LNV in which different truncated constructs of APP are expressed show 4G8 immunoreactivity; (C') is taken at higher laser power in the red channel. (D) Terminal axonal arbours of sLNv expressing full-length APP and APP Δ CT show 4G8 immunoreactivity, while sLNv expressing APP Δ NT do not. Axonal arborization is increased by expression of APP and APP Δ NT, but not by APP Δ CT.

induce increased arborization. Overexpression of wildtype *Drosophila* Abl induced a small increase in axonal outgrowth (Figure 4L and Supplementary Figure S2C). Abl tyrosine kinase activity is tightly regulated by intramolecular inhibition (Barila and Superti-Furga, 1998), possibly explaining its limited effect. To overcome this self-inhibitory effect, we expressed constitutively active BCR–Abl fusion proteins. These proteins can substitute for loss of function of *Drosophila* Abl (Fogerty *et al*, 1999). Expression of activated Abl in the sLNv induced strong axonal extension and arborization phenotypes very similar to those induced by APP (Figure 4M and N). Finally, we expressed a kinase-dead form of Abl (Wills *et al*, 1999a), which had no effect (Figure 4O). If Abl is indeed the downstream signalling partner of APP, we would expect the Abl effect on axonal arborization to be independent of APP. To test this, we expressed an activated form of Abl in an *app^Δ* mutant background and found that the axonal arborization phenotype was still induced

(Figure 4P). These findings show that Abl tyrosine kinase activity is not only necessary but also sufficient to increase axonal arborization.

As Abl signalling has been shown to modify axonal outgrowth by affecting the cytoskeleton, we next tested the involvement of other proteins which are important in cytoskeleton reorganization and are functionally linked to Abl. Although Abl has been suggested to modify actin dynamics by directly binding to F-actin (Woodring *et al*, 2003), it also interacts genetically with the actin-binding protein Profilin, which is required for the normal dynamics of actin filaments (Wills *et al*, 1999b). To test the requirements for Profilin in APP signalling, we tested genetic interactions with *chickadee* (*chic*), the gene encoding the *Drosophila* homologue of Profilin. Regulation of Profilin has recently been shown to affect sLNv axonal arborization (Reeve *et al*, 2005). Heterozygosity for *chic²²¹* or *chic⁰¹³²⁰*, both null alleles of *chic* (Verheyen and Cooley, 1994), also suppressed the

Figure 4 The Abl signalling cascade is necessary and sufficient to induce axonal arborization. (A) Amino-acid composition of the APP C-terminus, showing the conserved domains and the amino acids changed or removed in the different constructs. (B–S) Confocal images showing the axonal arbor of sLNv visualized with anti-PDH immunostaining (H–K, P–S) or CD8-GFP coexpression (B–G, L–O). (C, D) Expression of APP forms lacking the PEER or Go-protein-binding domain induces increased axonal outgrowth. (E) Deletion of the YENPTY motif (E) and point mutations in Y682 (F) or Y687 (G) of the APP C-terminus abolish its effect on axonal arborization. (C–G, insets) Cell bodies stained with 4G8 antibody (red). (H–K) The APP phenotype is suppressed by genetic reduction of Abl (J), or by coexpression of a kinase-dead form of Abl (K). (L–O) Expression of wildtype Abl has a small effect on axonal outgrowth (L, arrow), while activated forms of human Abl induce marked increases in axonal arborization (M, N). Expression of kinase-dead Abl has no effect (O). (P) Activated Abl induces axonal arborization in *ap^{pl}^d* mutant flies. (Q–S) Genetic reduction of Chic (Q) suppresses the APP phenotype, while genetic reduction of Ena (R) or Tsr (S) does not have an effect. Quantification of all phenotypes is shown in Supplementary Figure S2D.

APP-induced increase in axonal arborization (Figure 4Q and Supplementary Figure S2D). This result suggests that the APP effect on axonal outgrowth requires modification of the actin cytoskeleton mediated by Abl and Profilin.

Another molecule putatively involved in Abl signalling to actin is Enabled (*Ena*), the homologue of mammalian Mena (Wills *et al*, 1999a), which has also been shown to bind an APP adaptor molecule: Fe65 (Ermeikova *et al*, 1997). However, heterozygosity for *Ena* did not interfere with the APP effect on the axonal arbor (Figure 4R), consistent with the lack of a Fe65 homologue in *Drosophila*. Finally, to exclude that general interference with actin-binding proteins suppresses APP phenotypes, we tested the effects of APP expression in flies heterozygous for *twinstar*, the homologue of Cofilin (Gunsalus *et al*, 1995). Also, in this case APP-induced axonal arborization was not affected (Figure 4S and Supplementary Figure S2D).

While these results cannot exclude some role for Ena and Tsr in APP's effects, they clearly identify Abl and Profilin as essential downstream signalling molecules for APP. Importantly, reduction of Abl or Profilin when the APP transgene is not expressed does not influence the area of the terminal arbores (Supplementary Figure S2D), showing that these genetic modifications specifically affect the APP phenotype.

APPL expression is upregulated after traumatic brain injury in *Drosophila*

While APP upregulation and its interaction with the Abl tyrosine kinase have a profound impact on postdevelopmental axonal arborization, no obvious developmental axonal phenotypes have been found in the CNS of *ap^{pl}^d* mutant animals (Luo *et al*, 1992). This, combined with the fact that APP can induce axonal arborization in mature adult CNS

neurons, led us to speculate that APP upregulation could be relevant in specific circumstances later in life. APP expression has been shown to be upregulated after brain injury in humans and mammalian model organisms (Murakami *et al*, 1998; Van den Heuvel *et al*, 1999). To test if *Drosophila* APPL is also upregulated in response to neuronal injury, we developed a method for inducing injuries in the brains of adult flies. Flies were anaesthetized and immobilized, after which a small insect needle was inserted through the head as shown in Figure 5A (see also Materials and methods). The brains of surviving flies were dissected 48 h after injury and labelled with TRITC-Phalloidin, an actin dye, as well as with Elav, a nuclear pan-neuronal marker, and APPL antibodies. Neuronal injury causes a dramatic increase of APPL staining in the damaged optic lobe as compared to the undamaged side, which served as an internal control for the staining (Figure 5B).

To further analyse the spatial properties of injury-induced APPL upregulation, we injured adult *Drosophila* brains at different sites. In all cases we dissected a minimum of six fly brains and compared representative confocal sections at the level of the α - and β -lobes of the mushroom bodies of undamaged brains or brain halves (Figure 5C–E) with identical sections taken from injured brains. Upregulated APPL signals were specifically associated with the injured brain areas (Figure 5F and G), and were strongest in the cortical areas where the neuronal cell bodies reside. Three-dimensional reconstructions of the brains represented in Figure 5F and G confirmed our findings from single confocal sections (Supplementary Figure S4).

To determine the temporal characteristics of APPL upregulation, we damaged the brains of approximately 50 adult flies. Brains were dissected at 48 h, 4 or 7 days after trauma. We found that APPL signals were sustained up to 7 days after injury, at which point they became less pronounced, depending on the extent of the residual damage (Figure 5F–I).

To establish the association between the damaged area and APPL upregulation in neurons at the cellular level, we performed a higher resolution study of damaged brains 48 h after injury. Strong APPL signals were associated with neuronal cell bodies in the cortical areas adjacent to the damaged tissue (Figure 5J and K, arrowheads), as determined by costaining with Elav and TRITC-Phalloidin staining. High APPL levels could also be detected in neuronal processes extending towards the damaged areas (Figure 5J and K, arrows). Finally, to exclude that the APPL antibody would bind nonspecifically to the damaged brain areas, we immunostained damaged brains of *appl^Δ* mutant animals 48 h after injury. No APPL signal was detected (Figure 5L).

Therefore, a temporary neuronal APP/APPL upregulation specifically in damaged brain areas is a conserved response

to traumatic brain injury between *Drosophila* and mammals. If this conserved response is physiologically important, one would expect to see a selective disadvantage in animals lacking it. To test this, we compared the mortality rate of *appl^Δ* mutant flies versus Canton S wildtype controls. While *appl^Δ* flies do not show significantly impaired survival under standard conditions, we did observe a large increase in the mortality of mutant versus wildtype flies 1 and 2 weeks after brain trauma (Figure 5M). This suggests that the ability to upregulate APPL is important to cope with the consequences of brain injury in *Drosophila*.

JNK signalling is activated after traumatic brain injury and is required for APP to induce axonal arborization

Jun N-terminal kinase (JNK) signalling is important in several developmental contexts and has also been implicated in wound repair in *Drosophila* (Martin-Blanco, 1997; Galko and Krasnow, 2004). The JNK-signalling pathway in *Drosophila* consists of the JNK kinase, Hemipterous (*Hep*), which activates Basket (*Bsk*), the JNK homologue, which in turn phosphorylates and activates the transcription factors *Jun-Related Antigen* (*Jra*) and *Kayak* (*Kay*), the homologues of mammalian Jun and Fos, respectively. Upon activation of the JNK cascade, target genes are expressed, among which several are involved in the organization of the cytoskeleton, notably *Profilin* (Jasper *et al*, 2001). Furthermore, *Jun* has recently been shown to be essential for correct axonal regeneration after axotomy in mammals (Raivich *et al*, 2004).

To further investigate the conservation in the responses to traumatic brain injury in *Drosophila*, we examined the expression of the JNK target gene *puckered* (*puc*), using the *puc-lacZ* enhancer trap reporter (Martin-Blanco *et al*, 1998), in fly brains after injury. Control brains show widespread, low-level expression of this reporter (Figure 6A). However, 48 h after injury, a much stronger signal was detected in damaged brain regions (Figure 6B), which was further supported by immunostaining for Jun, another JNK target gene (data not shown). This signal colocalized significantly, but not completely, with APPL especially close to the injury site (Figure 6B', arrowheads). Trauma thus causes both APPL upregulation and JNK cascade activation. Since JNK signalling is known to upregulate Profilin expression (Jasper *et al*, 2001) as well as other cytoskeletal modifying proteins, it may be important to provide the effectors for APP-induced, Abl-dependent actin remodelling.

To test whether the activation of the JNK cascade also affects axonal outgrowth and arborization, we ectopically activated this signalling pathway in the sLNv by expressing a constitutively active JNK kinase Hemipterous (*HepCA*; Adachi-Yamada *et al*, 1999). This resulted in drastic axonal

Figure 5 APPL expression is increased in neurons after traumatic brain injury in *Drosophila*. (A) Schematic of a *Drosophila* head showing the points of entry and exit of the insect pin used for inducing brain injury (adapted from *Drosophila* protocols, CSHL press). (B) Three-dimensional reconstruction of a *Drosophila* brain showing increased expression of APPL in the damaged optic lobe 48 h after injury. The deduced tract of the insect pin is represented by a white arrow. (C) Schematic of a confocal section of a *Drosophila* brain half stained with TRITC-Phalloidin (red), anti-Elav (a marker for neuronal nuclei; blue) and anti-APPL (green). The same stainings are used in panels D–L. The brain landmarks used to define the depth of confocal sections in panels D, F–I, L are highlighted. (D) Three-dimensional reconstruction of an undamaged adult brain rotated 90°. (E) Confocal section showing the normal staining pattern in an undamaged adult brain. (F, G) Confocal sections of brains 48 h after trauma, showing increased APPL immunoreactivity surrounding the damaged tissue (arrows). (H) At 4 days after trauma, an increased APPL signal is still observed. (I) At 7 days after trauma, only a weak APPL signal is detectable. (J, K) Higher magnification of (F), showing APPL upregulation in neuronal cell bodies (arrowheads) and axons (arrows) surrounding the damaged tissue (black gap in TRITC-Phalloidin staining). (L) No APPL signal is detected in damaged *appl^Δ* brains. (M) Graphic representation of the mortality of wildtype versus *appl^Δ* mutant flies with or without brain trauma (* $P < 0.05$; *** $P < 0.001$).

Figure 6 APPL and JNK signalling are coactivated after traumatic brain injury in *Drosophila*. (A–C) Confocal sections of *puc-LacZ* transgenic *Drosophila* brains triple labelled for APPL (green), TRITC-Phalloidin (red) and BGal (blue, representing levels of JNK signalling). (A) Low levels of APPL and BGal expression are found in an undamaged brain. (B) Increased APPL and BGal expression are found in traumatized areas 48 h after trauma. (B') Higher magnification of (B), showing partial colocalization (arrowheads) of APPL and BGal expression around the tissue damage (white line). (C) BGal expression is also increased in damaged *aptl*^Δ mutant brains. (D) Ectopic activation of JNK signalling by expression of HepCA induces axonal overextension without increased arborization. Inactivation of JNK signalling by expression of BskDN does not affect the normal development of the sLNv axonal arbour, but suppresses APP-induced axonal outgrowth, while it has no effect on Abl-induced axonal arborization. (E) Schematic representation of proposed events following brain injury in *Drosophila*.

overextension phenotypes (Figure 6D). However, we did not observe strong arborization, suggesting some differences in the downstream molecular events as compared to APP signalling via Abl. Blocking this cascade by expression of a dominant-negative form of Basket (BskDN; Adachi-Yamada *et al*, 1999), the *Drosophila* homologue of JNK, had no effect on the normal development of the terminal axonal arbour (Figure 6D and Supplementary Figure S2D).

Thus far, our data indicate that both APP expression and JNK signalling are activated in response to neuronal injury, and that both can induce axonal outgrowth. We therefore sought to determine the relationship between the two pathways. At least three possibilities exist: (1) JNK may act at some point downstream of APP. In this case, APP-induced outgrowth should require JNK, but not the reverse. (2) APPL

may act downstream of JNK, in which case APPL would be required for JNK-induced outgrowth, but not the reverse. (3) Both signals act independently and in parallel. If so, neither would be required by the other.

To distinguish between these possibilities, we first expressed HepCA in an *aptl*^Δ mutant background. HepCA still induced significant axonal extension under these conditions (data not shown), suggesting that APPL is not an essential downstream factor in JNK signalling. Conversely, expression of APP while blocking JNK signalling by expression of BskDN suppressed APP-dependent arborization (Figure 6D and Supplementary Figure S2D). To test whether this interaction was limited to the kinase itself or whether also other factors in the JNK cascade are important, we tested genetic interactions between APP and the downstream transcription factors

of JNK. Reduction of *Kay* (Figure 6D), the *Drosophila* homologue of Fos, suppressed the APP axonal arborization phenotype as did, to a lesser extent, *Jra*, the homologue of Jun (Supplementary Figure S2D). These results suggest that JNK signalling is required to allow the transcription of targets needed for the efficient transduction of the APP signal. Therefore, both Abl and JNK act downstream of APP to induce increased axonal arborization. To test whether the two pathways also act in parallel on axonal outgrowth, we coexpressed activated Abl together with Bsk-DN in sLNv. BskDN failed to suppress Abl activation (Figure 6D), suggesting that JNK signalling acts in parallel to Abl.

Next, we asked whether, APPL activates JNK in response to brain injury. The fact that the APPL and puc-lacZ signals do not completely overlap after trauma (Figure 6B and B') may mean that APP and JNK are activated independently and then interact. To further dissect the interdependence of APPL and JNK signalling, we induced injury in *appl*^d mutant brains. Significant activation of JNK signalling was still observed (Figure 6C), which means that activation of JNK after injury does not depend critically on the presence of APPL. Conversely, we found that ectopic activation of JNK signalling is not sufficient to induce APPL expression (data not shown). Therefore, whereas both signals are required to induce axonal arborization, the two pathways appear to be activated in parallel in response to brain injury. Interestingly, a molecular link between APP and the JNK pathway via a JNK scaffolding molecule has been previously suggested both in mammals (JIP1-B; Matsuda *et al*, 2001) and in *Drosophila* (APLIP1; Taru *et al*, 2002). We confirmed this physical interaction with GST-pulldown studies of APLIP1 to human APP (Supplementary Figure S5). We thus conclude that APP and JNK signalling are tightly linked in the context of axonal outgrowth, and their coactivation after traumatic brain injury strongly suggests that their interaction may be important in axonal outgrowth after trauma.

Discussion

In this work, we show that APP and its *Drosophila* homologue APPL promote *de novo* axonal arborization in the *Drosophila* brain. Interestingly, this axonal arborization phenotype can also be induced when high levels of APP are induced only in fully mature adult neurons, a situation similar to that after traumatic brain injury in adult organisms. In contrast to previous reports in cell culture (Jin *et al*, 1994), the effect of APP depends critically on its intracellular domain. To elucidate the mechanisms used by APP to induce axonal arborization, we investigated the importance of different conserved residues in the APP molecule and performed genetic interaction studies. We show that deletions and point mutations in APP domains known to mediate physical interactions between APP and components of the Abl signalling pathway (Trommsdorff *et al*, 1998), including Abl itself (Zambrano *et al*, 2001), abolish the APP effect on axonal outgrowth. Furthermore, APP signalling depends critically on the levels and activity of the Abl tyrosine kinase. Consistently, activation of Abl is sufficient to induce axonal arborization of the sLNv, downstream of APPL. We also found that the actin-binding protein Profilin, itself known to mediate Abl effects on axons, is required for APP-dependent axonal arborization, suggesting a functional link between

APP and the reorganization of the actin cytoskeleton. It is noteworthy that the Abl adaptor protein Dab is an important factor in neuronal migration (Sheldon *et al*, 1997). As such, it would be interesting to investigate whether the focal dysplasia seen in the triple APP knockout mice (Herms *et al*, 2004) is also linked to the Abl tyrosine kinase cascade.

Since we and others did not find axonal outgrowth defects in the CNS of *appl* mutant *Drosophila* and because APP was able to induce axonal outgrowth phenotypes in fully mature CNS neurons, we reasoned that APPL might have a role in specific postdevelopmental contexts like brain injury. We therefore developed a model for the induction of brain damage in adult *Drosophila*. Expression of APPL is increased for several days, specifically in neurons surrounding damaged brain areas after injury, and flies mutant for *appl* show increased post-traumatic mortality, suggesting that APPL has an important physiological role under these conditions.

Many neurons in the injured brain areas also show activation of the JNK-signalling cascade. JNK signalling is essential for correct regeneration of axons after injury in mammals (Raivich *et al*, 2004). We found that APP-induced axonal arborization depends on intact JNK signalling. This functional link can be explained by the presence of a molecular link between the pathways provided by the physical binding of JIP/APLIP1 to APP, as well as JNK-signalling components. On the other hand, downstream effectors like Profilin may be functionally controlled by APP/Abl, while being under the transcriptional control of JNK signalling.

Our data combined suggest a model (Figure 6E) which brings together several of the proposed APP-binding partners in the context of axonal arborization and trauma response *in vivo*. In this model, we propose that acute trauma results in increased APPL expression and independent, but simultaneous, JNK signalling activation. JNK activity provides, via transcriptional activation of Profilin and other cytoskeletal regulators, a permissive environment for remodelling of the actin cytoskeleton. Regulation of Abl signalling by APP ensures that these components are controlled functionally, resulting in an appropriate neuronal response to axonal damage.

Our findings thus provide the first *in vivo* evidence for a role of APP in axonal arborization in the central nervous system and propose an integrated explanation for a number of intriguing, but thus far separate, observations from biochemical and cell culture studies in various contexts. Interestingly, while expression of the membrane-bound C-terminal fragment of APP (APP-CTF) is sufficient to induce axonal arborization, expression of the AICD, which results from the γ -secretase-mediated cleavage of APP-CTF, does not (our unpublished observations). We therefore speculate that the cleavage of APP-CTF by γ -secretase may terminate APP activity and therefore regulate APP-Abl signalling.

Our findings may also have implications on the understanding of some aspects of the pathophysiology of AD. We hypothesize that during adult life stressful and/or traumatic events in the brain cause APP upregulation. As a side-effect of this, A β peptides are generated, which may cause further disruption of neuronal connections (Tsai *et al*, 2004). Whether A β peptides have any function under trauma conditions in mammals or are just a toxic side product of the cleavage remains to be seen. This hypothesis would, in part,

explain the strong epidemiological relationship between brain trauma and AD (Jellinger, 2004), as well as the reports of AD-like brain pathology after severe head trauma (Ikonomic *et al*, 2004). Finally, it would be interesting to investigate whether the downstream components of APP signalling identified in this work are AD susceptibility loci, leading to an inefficient neuronal trauma response and thus longer-lasting APP upregulation after brain injury.

Materials and methods

Drosophila stocks

Transgenic lines containing the different mutated APP constructs were a gift from G Merdes. *UAS-Abl*, *UAS-AblKD* and constitutively active forms of Abl: *UAS-P210Abl* and *UAS-P185Abl* lines, were obtained from J Fogerty. *appt^d* and *UAS-APPL* lines were obtained from L Goldstein, the *pdf-gal4* line from P Taghert, and *Hep1* and *puc-lacZ* lines from D Bohmann. The *UAS-APP*, *UAS-APPΔCT*, *UAS-APPANT*, *Ena²⁰²⁹*, *Abl¹*, *Abl⁴*, *UAS-BskDN*, *UAS-hep^{CA}*, *Kay¹*, *Jra^{IA109}*, *Chic²²¹*, *Chic⁰¹³²⁰*, *Tst^{k05633}*, *w¹¹¹⁸*, *Canton S* and *UAS-CD8GFP* lines were obtained from the Bloomington *Drosophila* stock centre.

Immunohistochemistry

Brains of wandering third instar larva or adult flies (within 4 days of eclosion) were dissected and processed as described in Hassan *et al* (2000). The APPL antibody (used in a dilution of 1/10 000) was obtained from K White, the PDF antibody (1/1000) from P Taghert, the Elav (1/500) antibody from the Developmental Studies Hybridoma Bank (University of Iowa). For Myc staining the polyclonal antibody (Upstate), for GFP staining rabbit anti-GFP (Molecular Probes) (1/1000), and for APP the 4G8 antibody (1/100) from Abcam or the B63.5 antibody (1/8000) were used. For actin labelling, brains were incubated with 1/1000 TRITC-Phalloidin (SIGMA) in PBT for 2 h at room temperature.

Quantification of axonal arbour area

Unless mentioned explicitly, all flies were raised at 28°C. For quantification of the sLNv terminal arbour area, whole-mount adult brain preparations were imaged with a Biorad 1024 confocal system in the plane of the terminal axonal arbour using PDH or GFP antibody staining. For quantitative analysis, these images were imported in Scion imaging software, converted to greyscale, and the area was measured as described in Figure 2A. Area measurements were normalized for brain size, using the square of the distance

between the two dorsal projections divided by the square of the average distance in control brains as a correction factor. The resulting corrected area is represented in the graphs. Statistica 6.0 software was used to calculate the means and error bars (95% confidence interval) of these area measurements. Student's *t*-tests were used to calculate the significance in difference between the mean arbour areas, as indicated by the *P*-values in the graphs. *P*-values >0.05 were considered nonsignificant. Graphing was done in Excel and figures were prepared in Adobe Photoshop 7.0.

Traumatic brain injury model in *Drosophila*

To induce brain injury, adult flies were collected within four days of eclosion, CO₂ anaesthetized and immobilized in a cutoff 100-μl pipette tip. The fly head was pierced by inserting a 0.1-mm insect pin (Fine Science Tools) below the left antenna, through the left optic lobe and with the dorsal side of the head as exit point (Figure 5A). Flies were left to recover on standard fly food and maintained at 25°C. Surviving flies were dissected at the times indicated. After immunostaining, Z-stacks at 2 μm distance were collected from whole-mount adult brains. Representative images in the focal plain of the mushroom bodies are used in Figure 5. For three-dimensional reconstructions, 0.2 μm stacks were collected and processed using Biorad software. For survival experiments, newly eclosed flies of different genotypes were collected, counted and mixed to blind the observer. Cohorts of flies were traumatized within 48 h after eclosion and maintained at 25°C on standard fly food, which was refreshed every 1 or 2 days. The genotype of dead flies was determined based on the missing of one or more scutellar bristles in the *appt^d* background (Merdes *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Drs G Merdes, J Fogerty, K White, L Goldstein, D Van Vactor, P Taghert, D Bohmann, B Dickson, E Levy, E Giniger, the Bloomington Stock Center and the Developmental Studies Hybridoma Bank for materials. We are grateful to Drs B Dickson, J Eggermont, P Marynen, J Schulz and members of the Hassan lab for critical discussions, S Vilain for graphic assistance and W Annaert for advice on 3 dimensional reconstructions. This work was supported by VIB, a Concerted Research Action (GOA) grant from the University of Leuven (to BDS and BAH), the EU-APOPIs, Alzheimer's Association Pioneer Award (BDS) and a Fund for Scientific Research (FWO) Aspirant doctoral fellowship (ML).

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