Specific Functions of *Drosophila* Amyloid Precursor-Like Protein in the Development of Nervous System and Nonneural Tissues

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ABSTRACT: ***Drosophila*** **amyloid precursor-like protein (APPL) is expressed extensively in the nervous system soon after neuronal differentiation. By utilizing different transgenic flies, we studied the physiological function of two APPL protein forms, membrane-bound form (mAPPL) and secreted form (sAPPL), in neural development. We found that neither deletion nor overexpression of APPL protein altered the gross structure of mushroom bodies in the adult brain. No changes were detected in cell types and their relative ration in embryo-derived cultures from all APPL mutants. However, the neurite length was significantly increased in mutants overexpressing mAPPL. In addition, mutants lacking sAPPL had numerous neurite branches with abnormal lamellate membrane structures (LMSs) and blebs, while no apoptosis was detected in these neurons. The abnormal neurite morphology was most likely due to the disorganization of the cytoskeleton, as shown by double staining of actin filaments and microtubules. Electrophysiologically, A-type K^+ current was significantly enhanced, and spontaneous excitatory postsynaptic potentials (sEPSPs) were greatly increased in APPL mutants lacking sAPPL. Moreover, panneural overexpression of different forms of APPL protein generated different defects of wings and cuticle in adult flies. Taken together, our results suggest that both mAPPL and sAPPL play essential roles in the development of the central nervous system and nonneural tissues.** © 2004 Wiley Periodicals, Inc. J Neurobiol 61: 343–358, 2004

***Keywords: Drosophila*; APPL; brain structure; cytoskeleton; K^+ currents**

INTRODUCTION

The β-amyloid precursor protein (APP) is the precursor of the Aβ peptide, the main component of senile plaque in Alzheimer's disease (AD). APP is well conserved across species, from APL-1 in *Caenorhabditis elegans* (Daigle and Li, 1993), APPL in *Drosophila* (Rosen et al., 1989), APP_{747} in *Xenopus* (Okado and Okamoto, 1992), to APP, APLP1, and APLP2 in mammals (Kang et al., 1987; Wasco et al., 1992, 1993, Sprecher et al., 1993), suggesting that it

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may have essential physiological functions. Alternatively spliced mRNAs encoded by the human *App* gene give rise to at least three different protein isoforms: APP_{695}, APP_{751}, and APP_{770} (Kitagchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). Multiple APP homologs and isoforms in mammals are functionally redundant, and deletion of all copies of *App* genes is lethal (Heber et al., 2000). In contrast, amyloid precursor-like protein (APPL) appears to be the single member of APP protein family in flies, and its deletion has no effect on viability and fertilization (Luo et al., 1992). Thus, *Drosophila* offers a unique opportunity to analyze the physiological functions of the APP protein.

Similar to human APP, APPL is synthesized as a 145-kDa membrane-bound form (mAPPL) consisting of extracellular, transmembrane, and cytoplasmic domains, and can be converted to a 130-kDa secreted form (sAPPL) (Luo et al., 1990). Among human APP isoforms, APPL is most similar to APP_{695}: both have three highly conserved regions, are expressed specifically in the nervous system, and lack the protease inhibitor domain (Rosen et al., 1989). These similarities suggest that through evolution a primordial neural-specific function has been selectively maintained for the APP protein family (Martin-Morris and White, 1990).

Accumulating evidence suggests that APP proteins are critically involved in neural development. Human APP proteins have been found to affect both cell survival and neurite extension of human neuroblastoma cells (LeBlance et al., 1992), probably through the adhesion function mediated by a common collagen-binding domain in the APP superfamily (Coulson et al., 2000). There is also evidence that the secreted form of APP may regulate cell survival of the fibroblast (Saiton et al., 1989). In addition, purified APP or the Aβ protein in the culture substrate can enhance neurite outgrowth capability of hippocampal neurons (Salinero et al., 2000) and peripheral sensory neurons (Koo et al., 1993).

The underlying mechanisms for the involvement of APP in neural development remain to be investigated. One possibility is that the protein may function through its association with cytoskeleton. APP proteins can physically bind to both microtubules and Tau protein with its C-terminal fragment *in vitro* (Smith et al., 1995; Islam and levy, 1997). They may also be associated with a cytoskeleton *in vivo*, although probably not through the Tau protein. Overexpression of the mutated APPL protein in flies leads to a vesicle transport defect in motoneurons, possibly through the competitive binding to kinesin (Kamal et al., 2000; Gunawardena and Goldstein, 2001). The inhibition of microtubule-associated transport, induced by overexpression of the Tau protein, can be further aggravated by cooverexpression of APPL (Torroja et al., 1999a), indicating the Tau-independent regulation of the cytoskeleton by the APPL protein.

APP may also affect neural development by regulating ion channels. APP proteins and their metabolic fragments have been shown to regulate K^+ and Ca^{2+} channels in several cell models (Fraser et al., 1997). Exogenous application of APP protein can activate K^+ channels and suppress neuronal activity in cultured embryonic hippocampal neurons (Furukawa et al., 1996). The Aβ peptide is able to induce morphological changes coinciding with increased K^+ and Cl^- channel activity in rat cortical astrocytes (Jalonen et al., 1997). Besides the channel activity, the expression level of K^+ channels is also influenced by the Aβ peptide in rat microglia (Chung et al., 2001).

Different forms of *Drosophila* APPL protein are enriched in different areas of the brain (Torrojia et al., 1996), and play different roles in synapse differentiation at the neuromuscular junction (Torrojia et al., 1999b). Deletion of APPL gene does not cause apparent morphological defect in both the appearance and paraffin section of adult heads (Luo et al., 1992). However, overexpression of the wild-type APPL protein exclusively in the nervous system can lead to defects of the wings and cuticle in adult flies, which is suggestive of neuroendocrine dysfunction (Torrojia et al., 1999a).

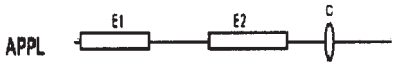
To further explore the normal function of APPL protein in the central nervous system (CNS), we examined the neuronal morphology, excitability, and brain structure with transgenic APPL flies overexpressing various forms of APPL. Our results show that overexpression of mutated APPL proteins in the nervous system does not disrupt the gross structure of the adult brain, but leads to a severe defect in non-neural tissues. Both deletion and overexpression of APPL protein can result in robust changes of the cytoskeleton and K^+ currents. Moreover, membrane-bound and secreted APPL proteins have different functions in neural development. Collectively, our results demonstrate that APPL protein plays an essential role in neural development.

MATERIALS AND METHODS

Drosophila Stocks

The *Drosophila* UAS-$Appl^+$, UAS-$Appl^{sd}$, UAS-$Appl^s$, and UAS-$Appl^{delCT}$ (UAS-$Appl^*$) lines were crossed with *Appl-*

Table 1 Different APPL Protein Forms in Wild Type and Mutants

Protein Forms	Fly Lines						
	CS	$APPL^{d}$	$APPL^{+}$	$APPL^{s}$	$APPL^{sd}$	$APPL^{delCT}$	$APPL^{s}$;$APPL^{sd}$
	+	−	+#	+	+	+	+
	—	—	—	#	—	—	#
	—	—	—	—	#	—	#
	—	—	—	—	—	#	—

The first row shows different forms of APPL proteins: "APPL" is the wild-type protein. "APPLS" is secreted form without C-terminal region. "APPLSD" has whole sequence with the cleavage site mutated. "APPLdelCT" has both cleavage site mutated and C-terminus deleted. The heterozygous flies were stated as APPL*. Fly lines include wild-type (CS), deletion mutant ($APPL^{d}$), and several overexpression mutants (APPL*). The intrinsic *Appl* gene (two copies) is indicated by +. # Stands for UAS-Gal4 promoted transcripts (one copy). The expression pattern dependents on Gal4 lines used.

Gal4 (Torroja et al., 1999a) to express different APPL proteins (see Table 1 for definitions) in a panneural manner. The heterozygous flies were stated as APPL*. These flies and the *Appl* null mutant ($APPL^{d}$) were generously provided by Dr. Kalpana White (Brandeis University) and Dr. Lawrence S.B. Goldstein (UCSD and Howard Hughes Medical Institute). Double mutant UAS-$Appl^{s}$;UAS-$Appl^{sd}$ was generated from UAS-$Appl^{s}$ (II) and UAS-$Appl^{sd}$ (III) with the balancer yw;Adv/*CyO*;Sb/TM6B. Two mushroom body (MB)-specific drivers, *OK107*-Gal4 and 247-Gal4 (Zars et al., 2000), were combined with UAS-EGFP respectively in the same method. Canton-S (CS) served as the wild-type control. All the flies were reared with the standard corn medium at room temperature (Guo et al., 1996).

Cell Culture

The "giant" neuron cultures were derived from cytokinesis-arrested embryonic neuroblasts as described previously (Wu et al., 1990). Briefly, embryos were collected and incubated for 3–4 h at 25°C. After homogenization and centrifugation in the Schneider's insect medium (SIGMA) containing 20% fetal bovine serum (FBS, Gibco), 50 U/mL penicillin, 50 μg/mL streptomycin sulfate, and 200 ng/mL insulin (Sigma), cells were resuspended and plated on the coverslips with 1 μg/mL Cytochalasin B (CCB, Sigma) in the culture medium. Eight to 12 h after plating, CCB was removed by replacing with fresh culture medium. For the coculture method, neurons of the $APPL^{s}$ mutant were added to the $APPL^{sd}$ neuronal culture after CCB was removed. Without the inhibition of cytokinesis, the neuroblast cells of $APPL^{s}$ would divide and grow in clusters, with a much smaller soma size (about 3–5 μm) than the $APPL^{sd}$ "giant" neurons (about 20 μm). All cultures were maintained in humidified chambers at 21–23°C.

Morphology Study

Cultured cells were moved into normal saline (in m*M*: 128 NaCl, 2 KCl, 4 $MgCl_2$, 1.8 $CaCl_2$, and 35.5 sucrose, buffered at pH 7.1–7.2 with 5 HEPES), and cell images of random fields were collected under the Olympus microscope using 40× objectives. For each type of flies, more than 600 cells from three independent cultures were sampled 2, 4, or 6 days after plating. With calibrated software, the longest neurite of each cell was measured for the analysis of neurite length. The morphology of neurons was monitored throughout the culture period, and the percentage of cells with abnormal LMSs and blebs was counted at the 10th day.

To examine the *in vivo* morphology, UAS construction of different APPL mutants (UAS-*Appl**) was crossed with 247-Gal4;UAS-EGFP or UAS-EGFP;*OK107*-Gal4 to obtain the flies with both EGFP and various APPL proteins expressed specifically in the MBs. About 20 adult brains were dissected 2 or 10 days after eclosion. Three-dimensional images of MBs were obtained using two-photon confocal microscope under 40× water-immersion objectives, and were reconstructed with a Zeiss Lsmib Data Server program.

Immunocytochemistry

The methods for cell fixation and staining of actin filaments (AFs) and microtubules (MTs) were similar to those in mammalian cells (Willamsom et al., 1996; Challacombe et al., 1997). Briefly, cells (about 2 weeks in culture) were rinsed briefly and fixed for 10 min with 2% glutaraldehyde and 0.1% Triton in PHEM buffer. After rinsing with Ca^{2+}-Mg^{2+}-free phosphate-buffered saline (CMF-PBS), cells were blocked with soaking solution (2% BSA and 0.2% Triton in CMF-PBS) for 20 min. MTs were labeled with rat monoclonal antibody and secondary rabbit antirat antibody

with FITC fluorescence. AFs were labeled with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR). Primary antibodies of MTs and phalloidin were diluted into soaking solution at 1:600 and 1:50, respectively, and applied overnight at 4°C. Cells were rinsed with CMF-PBS followed by incubation in the secondary antibodies at 1:100 dilution for 30 min. After rinsing again, images of cells were collected under confocal laser scanning microscope.

Patch-Clamp Recording

Whole-cell patch-clamp recording was performed on neurons of wild-type or APPL mutants 3–5 days in culture. Patch electrodes were pulled from glass capillaries using a micropipette puller (model P-97, Sutter), and fire polished with a microforge (model MF-83, Narishige). Pipettes were filled with a solution containing (in m*M*): 144 KCl, 1.0 $MgCl_2$, 0.5 $CaCl_2$, and 5.0 EGTA, buffered at pH 7.1–7.2 with 10 HEPES. The pipette resistance was 4–6 MΩ when measured in the bath solution (the normal saline mentioned above, Yu et al., 1999). For K^+ currents study, 0.1 μM TTX and 100 μM $CdCl_2$ were added in the bath solution to block Na^+ and Ca^{2+} channels (Saito and Wu, 1991). An Axo-Patch 200B amplifier and pClamp software were used for voltage/current pulse generation and data acquisition. All figures were analyzed using software Clampfit and Origin.

RESULTS

Subtle Morphological Defects of Adult Brain in APPL Mutants

To address whether APPL is involved in the development of the adult brain, we examined the *in vivo* morphology of mushroom bodies (MBs), an important neuropile with highly stereotypic structure (Yang et al., 1995; Crittenden et al., 1998) and complexed reorganization process (Technau and Heisenberg, 1982). With EGFP labeling, two-photon microscopic images were collected and reconstructed. We found that the structure of MBs in various APPL mutants (UAS-*Appl**/+;UAS-EGFP/+;*OK107*-Gal4/+, see Table 1 for definition) was grossly normal. The localization of Kenyon cells, the orientation of their axons, and the calyx composed of cell bodies and dendrites were basically the same as those in wild type [+/+; UAS-EGFP/+;*OK107*-Gal4/+, Fig. 1(A)].

However, there was some subtle abnormal morphology of the lobes in some APPL mutants. The lobes were less uniform and the boundary was fuzzy in $APPL^d$, $APPL^{sd}$, and $APPL^{delCT}$ mutants [Fig. 1(A)]. Furthermore, the β/β' lobes from two hemispheres were fused together in 4 out of 40 brains of $APPL^{sd}$ flies [Fig. 1(A)], whereas this phenomenon has never been observed or reported in wild-type flies. To exclude the possibility of leaky expression of *OK107*-Gal4, we chose 247-Gal4, which shows the clearest expression pattern among MB-Gal4 lines, to repeat this experiment. There were still about 10% flies with fused lobes in 247-Gal4 promoted $APPL^{sd}$ mutant [Fig. 1(B)]. In a partially magnified scanning image, it is clear that two β' lobes, which are lower than β lobes in depth, came into fusion [Fig. 1(Bc)]. All these results indicate that APPL protein is not required for the gross organization of brain tissues, but is probably involved in the regulation of subtle morphology at the cellular level.

Abnormal Neurite Growth of APPL Mutant Neurons

To explore the cellular role of APPL protein in neural development, we examined the neuronal morphology of APPL mutants in the embryo-derived culture system. In this culture system, neuroblasts without neurites can develop into cells that have similar morphology, excitability, and molecular expression profile as mature neurons in adults (Wu et al., 1990; Saito and Wu, 1991), allowing a systemic examination of the role of APPL protein in the process of cell differentiation, neurite outgrowth, and extension.

According to the number of neurites, neurons were categorized into monopolar, bipolar, tripolar, and multipolar neurons. At the early culture stage (2 days after cell plating), neurite length of each type of neurons in all APPL mutants, including *Appl* deletion mutant ($APPL^d$) and various overexpression mutants, was similar to that in wild type, CS [Fig. 2(A)]. Furthermore, the proportion of different cell types has no obvious difference between CS and APPL mutants [Fig. 2(B)]. These results suggest that in flies, neither deletion nor overexpression of the APPL protein could interfere with the process of neurite outgrowth and cell type determination.

Although APPL mutants had a similar neurite length to wild type at the second day in culture, the difference became obvious a few days later. In 6-day cultures, all the mutants had significantly longer or shorter neurites than those of CS neurons (61.0 ± 1.0 μm). According to the neurite length, the mutants could be divided into two groups. One group with shorter neurites includes $APPL^d$ (55.8 ± 0.7 μm), $APPL^+$ (56.1 ± 0.7 μm), and $APPL^s$ (54.3 ± 0.7 μm), while the other group with longer neurites includes $APPL^{sd}$ (65.9 ± 0.9 μm) and $APPL^{delCT}$ (67.9 ± 1.2 μm). There is no significant difference among the members in each group [Fig. 2(C)]. Because both $APPL^{sd}$ and $APPL^{delCT}$ have abundant membrane-bound APPL (mAPPL), which is absent in $APPL^d$ and

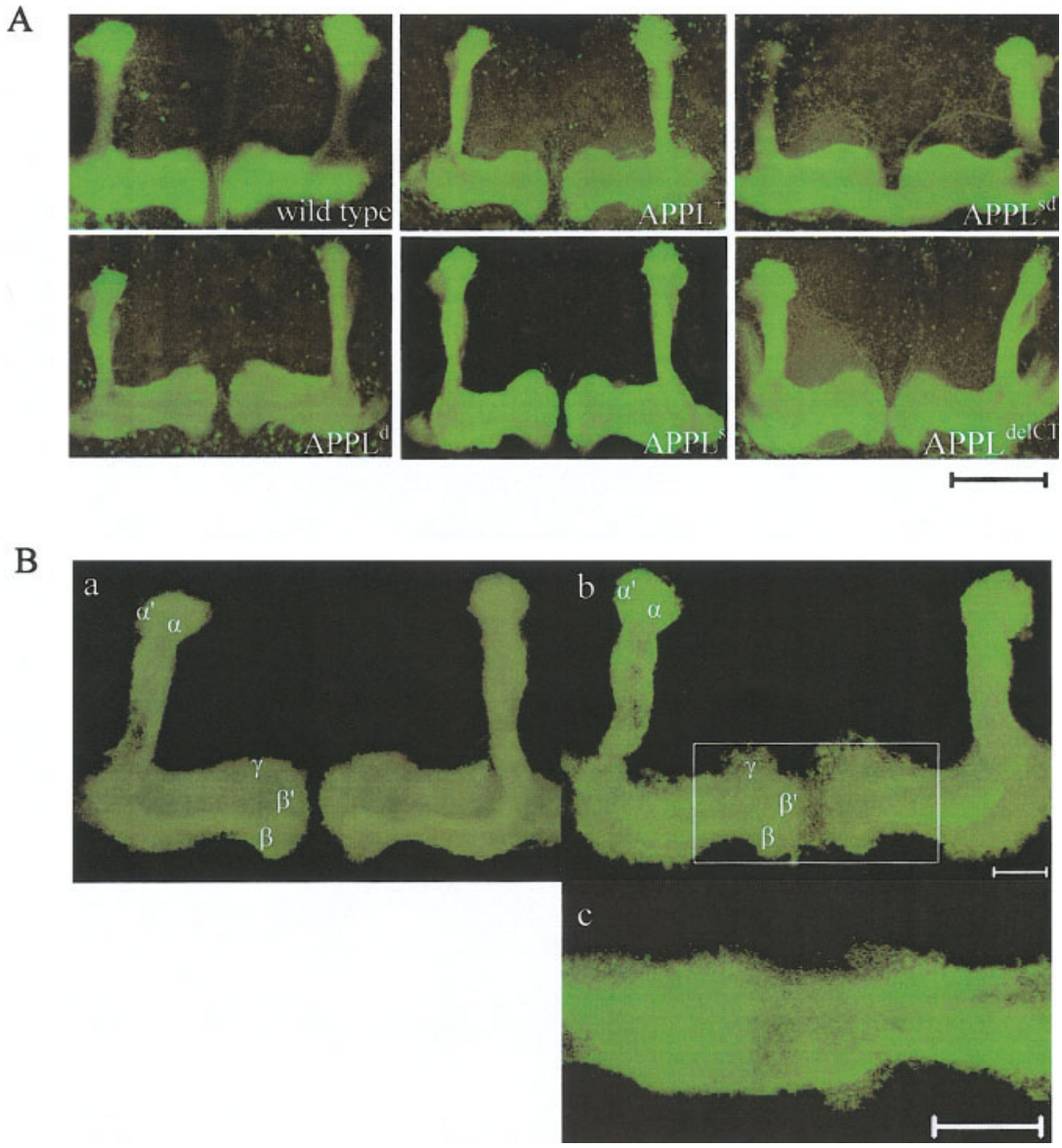


Figure 1 *In vivo* structure of mushroom bodies. (A) The two-photon confocal images of MBs lobes in adult flies of wild-type (+/+; UAS-EGFP/+; *OK017*-Gal4/+) and APPL mutants (APPL/^{d}Y or UAS-APPL*/+;UAS-EGFP/+; *OK107*-Gal4/+). The structure is grossly normal in all mutants. Note the fused β/β' lobes of APPL^{sd} MBs in about 10% flies. Scale bar: 50 μm. (B) The MBs structure showed by 247-Gal4. (a) Wild type (247-Gal4/+; UAS-EGFP/+). (b) APPL^{sd} (247-Gal4/UAS-*Appl*^{sd}; UAS-EGFP/+). MBs lobes are grossly normal as in wild type except the fuzzy boundary, and β' lobes of two hemispheres are fused together. (c) The magnified scanning image of the fusion part framed in (b). Scare bar: 20 μm.

APPL^{s}, these results thus support the idea that mAPPL plays an important role in neurite growth. Notably, neurite growth in APPL^{sd} and APPL^{delCT} is similar, suggesting that overexpression of mAPPL accelerates neurite extension in a C-terminus–independent manner.

However, the above notion cannot explain why APPL^{+} neurons with sufficient mAPPL had shorter neurites than CS. To test whether the secreted APPL protein (sAPPL) in medium could affect neurite extension, we cocultured APPL^{sd} neurons with APPL^{s} cells to examine the neurite length (see Materials and Methods for details). In the coculture system, sAPPL secreted from APPL^{s} neurons could inhibit the generation of abnormal neurite morphology in APPL^{sd} neurons (shown later in this article). However, the

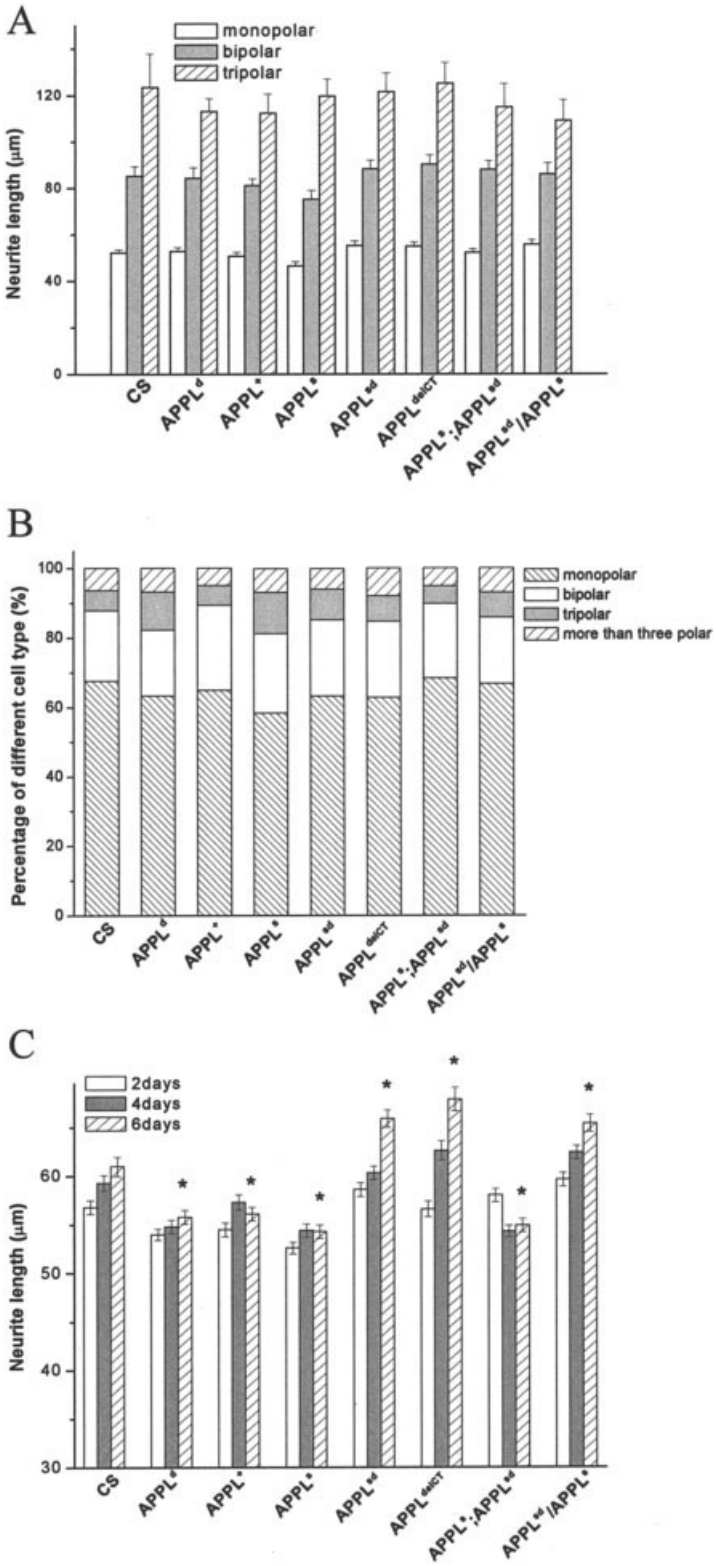


Figure 2 Neurite growth of different APPL mutants. (A) Three types of neurons in all APPL mutants have a similar neurite length to that of CS in 2-day cultures. (B) Similar proportion of cell types in terms of neurite number in different APPL mutants and wild type in 2-day cultures. (C) Quantitative analysis of neurite length through the culture. Compared with CS, neurites of 6-day cultures are significantly ($p < 0.001$, indicated by *) longer in $APPL^{sd}$, $APPL^{delCT}$, and $APPL^{sd}$/$APPL^{s}$ ($APPL^{sd}$ neurons cocultured with $APPL^{s}$ cells), or shorter in $APPL^{d}$, $APPL^{+}$, $APPL^{s}$, and $APPL^{s}$;$APPL^{sd}$ (double mutant).

neurite length of cocultured $APPL^{sd}$ neurons (65.4 ± 0.9 μm) exhibited no significant difference to those cultured alone (65.9 ± 0.9 μm) [Fig. 2(C)]. We then hypothesized that intracellular accumulation of APPL protein might influence the neurite extension. To test this possibility, we generated the double mutant $APPL^{s}$;$APPL^{sd}$ (*Appl*-Gal4/+;UAS-$Appl^{s}$/+;UAS-$Appl^{sd}$/+), in which both sAPPL and mAPPL proteins were overexpressed. Interestingly, the neurite extension in $APPL^{s}$;$APPL^{sd}$ was completely inhibited [Fig. 2(C)]. This result provides direct evidence that excessive APPL protein inside the cells counteracts the neurite growth-promoting effect of mAPPL.

Abnormal Neuronal Morphology of APPL Mutants

Besides the change of neurite length, other abnormal morphology were observed in APPL mutant neurons. The typical morphology of cultured wild-type embryo-derived neurons is characterized by one to three main neurites and some simple branches without prominent growth cone (Wu et al., 1990). For most $APPL^{s}$ neurons, neurites seemed to be thick with few branches. In contrast, neurites branched numerously and interlaced with one another in most $APPL^{d}$, $APPL^{sd}$, and $APPL^{delCT}$ neurons [Fig. 3(A)]. Furthermore, in the 1-week culture of these mutant neurons, there were many lamellate membrane structures (LMSs) around neurite shaft or at the neurite terminal. Intriguingly, there were some protuberant blebs in the flat LMSs [Fig. 3(B)]. To quantify these phenomena, we counted the percentage of cells with either LMSs or blebs at the 10th day. The results showed that three mutants, $APPL^{d}$ (42.3 ± 2.1%), $APPL^{sd}$ (45.8 ± 2.9%), and $APPL^{delCT}$ (37.9 ± 5.1%), had a much higher rate of LMSs-positive cells than $APPL^{+}$ (1.0 ± 0.4%), $APPL^{s}$ (4.1 ± 0.9%), and CS (2.4 ± 0.8%), while only $APPL^{sd}$ and $APPL^{delCT}$ had blebs [Fig. 3(C)].

Because all the mutants with abnormal morphology lacked the sAPPL protein, we then examined whether sAPPL had an effect on $APPL^{sd}$ neurons using the coculture method. We found that the proportion of $APPL^{sd}$ neurons with LMSs was significantly decreased in cocultures (27.1 ± 2.7%), compared with those cultured alone (45.8 ± 2.9%) in the 10-day culture [Fig. 3(C)], suggesting that sAPPL secreted from $APPL^{s}$ cells could affect $APPL^{sd}$ neurons, and partly inhibit LMSs generation. With sAPPL expressed and secreted, few neurons had LMSs in double mutant $APPL^{s}$;$APPL^{sd}$ cultures (1.2 ± 0.3%) [Fig. 3(C)]. These results indicate that a certain amount of sAPPL expression is required for the normal morphogenesis of neurons. On the other hand, excessive sAPPL could also result in morphological defect, as there were fewer branches and many twigs at the distal end of neurites in $APPL^{+}$, $APPL^{s}$, and $APPL^{s}$;$APPL^{sd}$ neurons [Fig. 3(A)].

As it is generally recognized that APP proteins are closely related to neurodegeneration, the question is whether the formation of abnormal LMSs and blebs is due to apoptosis. The conventional method to detect apoptosis is TUNEL staining based on the labeling of DNA strand breaks. $APPL^{sd}$ neurons were chosen for TUNEL staining because this mutant exhibited most severe morphological defect both *in vivo* and *in vitro*. The serum deprivation-induced apoptosis of cultured neurons could be detected by the same deep staining as the positive control, in which DNase I was applied to degrade DNA chains artificially [Fig. 3(D a–b)]. However, none of $APPL^{sd}$ neurons, even those with large LMSs, had more staining than the negative control, which showed the background staining of neurons without DNase I digestion [Fig. 3(D c–d)]. This result is consistent with our observation that $APPL^{sd}$ cells could survive as well as the wild-type neurons for more than 2 weeks in culture, agreeing well with the notion that APPL mutation would not induce apoptosis (Gunawardena and Goldstein, 2001).

Because the abnormal neuronal morphology is not due to neurodegeneration, we therefore considered other possibilities. Based on the putative interaction between APP proteins and cytoskeleton proteins as suggested by previous studies (Smith et al., 1995; Islam and Levy, 1997), we hypothesized that mutation of APPL protein might influence the organization of cytoskeleton. With double labeling of actin filaments (AFs) and microtubules (MTs), we found that in $APPL^{sd}$ neurons, both AFs and MTs increased dramatically [Fig. 4(E)]. Unlike wild-type neurons, in which AFs were mainly concentrated in the growth cone at the distal end of neurites [Fig. 4(A)], AFs in $APPL^{sd}$ neurons were greatly increased and extended along neurite shaft [Fig. 4(C)] and around neurite terminal [Fig. 4(D)]. MTs in $APPL^{sd}$ neurons were tangled into knots in the middle of neurites [Fig. 4(C)], deviated from the shaft, and formed some small loops [Fig. 4(D–F)], in contrast to the regular parallel arrangement in wild-type neurons [Fig. 4(A,B)]. These results indicate that APPL protein mutation could disrupt the normal organization of cytoskeleton, and it is most likely that the abnormal organization of AFs and MTs results in the LMSs and blebs in $APPL^{sd}$ neurons.

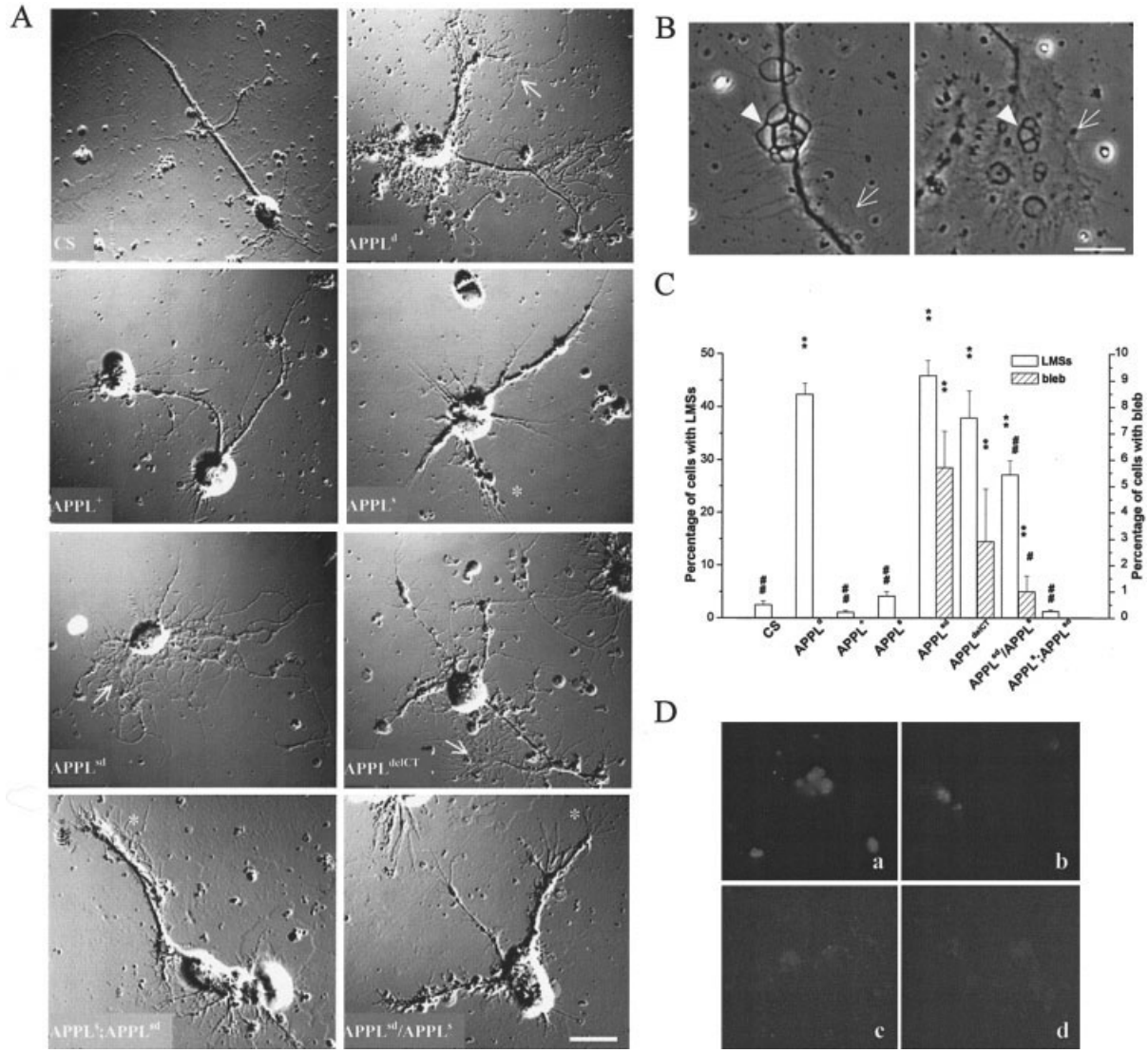


Figure 3 Abnormal neuronal morphology of APPL mutants. (A) The DIC photos of CS and APPL mutant neurons. There are numerous neurite branches in $APPL^{d}$, $APPL^{sd}$, and $APPL^{delCT}$ with LMSs marked by arrows. *Indicated the abnormal twigs in $APPL^{s}$, $APPL^{s}$;$APPL^{sd}$, and $APPL^{sd}$/$APPL^{s}$. (B) Two typical examples of LMSs (arrow) and blebs (arrowhead) around the neurite (left) or at distal end (right) of $APPL^{sd}$ neurons in a phase-contract microscope photo. Scale bar in (A) and (B): 20 μm. (C) Quantitative analysis of neurons with abnormal LMSs or blebs. The significant difference compared with CS and $APPL^{sd}$ is indicated as * and # ($p < 0.01$), or ** and ## ($p < 0.001$), respectively. Note the proportion of $APPL^{sd}$ neurons with LMSs or blebs is significantly decreased when cocultured with $APPL^{s}$ cells ($APPL^{sd}$/$APPL^{s}$). (D) No apoptosis is detected in $APPL^{sd}$ neurons with TUNEL staining. (a) Positive control with DNase I digestion. (b) Serum deprivation-induced apoptosis. (c) Staining of $APPL^{sd}$ neurons. (d) Negative control without enzyme solution. The staining of $APPL^{sd}$ neurons is comparable with negative control.

Changed Neural Excitability in APPL Mutant Neurons

Besides the morphological changes, is APPL protein involved in the regulation of neural function? We further studied whether APPL mutation could affect neural excitability by examining K^+ currents in the neuronal culture. Previous studies have shown that transient application of APP protein could influence K^+ currents in cultured embryonic hippocampal neurons (Furukawa et al., 1996). However, there is little evidence about the long-term effect of APPL protein on K^+ currents in neural development. The various

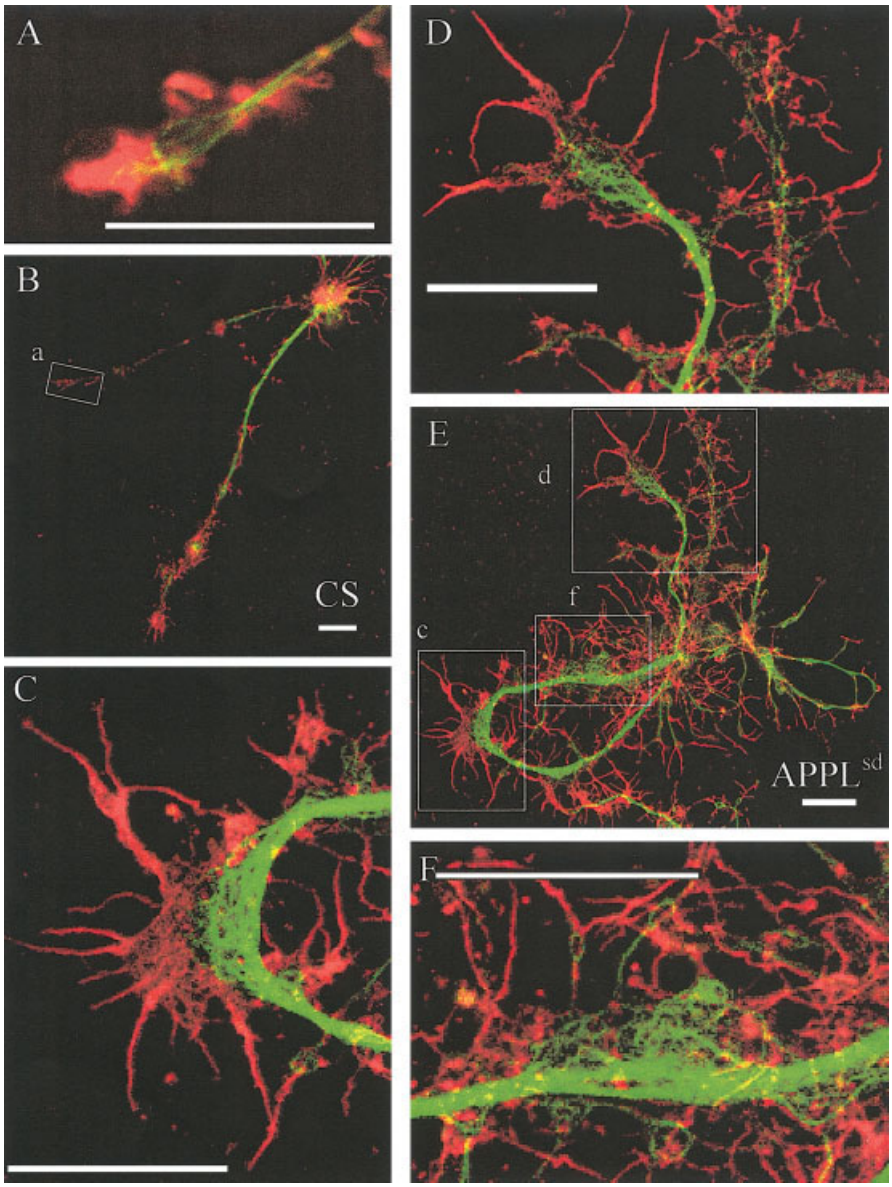


Figure 4 Double staining of AFs and MTs in CS and APPL^{sd} neurons. Two whole-cell photos of CS (B) and APPL^{sd} (E) are shown by double labeling of actin filaments (AFs, in red) and microtubules (MTs, in green). (A) The neurite terminal of CS neurons. (C, D, and F) Magnified images show the detail of expanded AFs and tangled MTs in APPL^{sd} neurons. Note some MTs deviate from the shaft and from some small loops (F). Scale bar: 15 μm.

APPL mutants provided the opportunity to address this issue in more physiological conditions.

We first studied the K^+ channel properties in cultured APPL^d neurons by whole-cell recording. As shown in Figure 5(A), when step-up depolarization pulses (from -70 mV, in 10-mV steps) were applied to voltage-clamped neurons, K^+ channels were activated at -50 mV in some APPL^d neurons (about 1/3), whereas no K^+ current was detected at this stimulus level in CS neurons. Moreover, the current amplitude at the same depolarization level (-10 mV) was greatly increased in APPL^d neurons (1.25 ± 0.19 nA), compared with CS (0.37 ± 0.03 nA, $p < 0.01$, t test), suggesting enhanced activation property or increased expression level of K^+ channels in APPL^d neurons. Furthermore, the normalized I/V curves of K^+ currents in APPL^d neurons shifted leftwards compared to that of CS neurons [Fig. 5(B)], which indicated that the activation dynamic of K^+ channels was changed by APPL protein deletion.

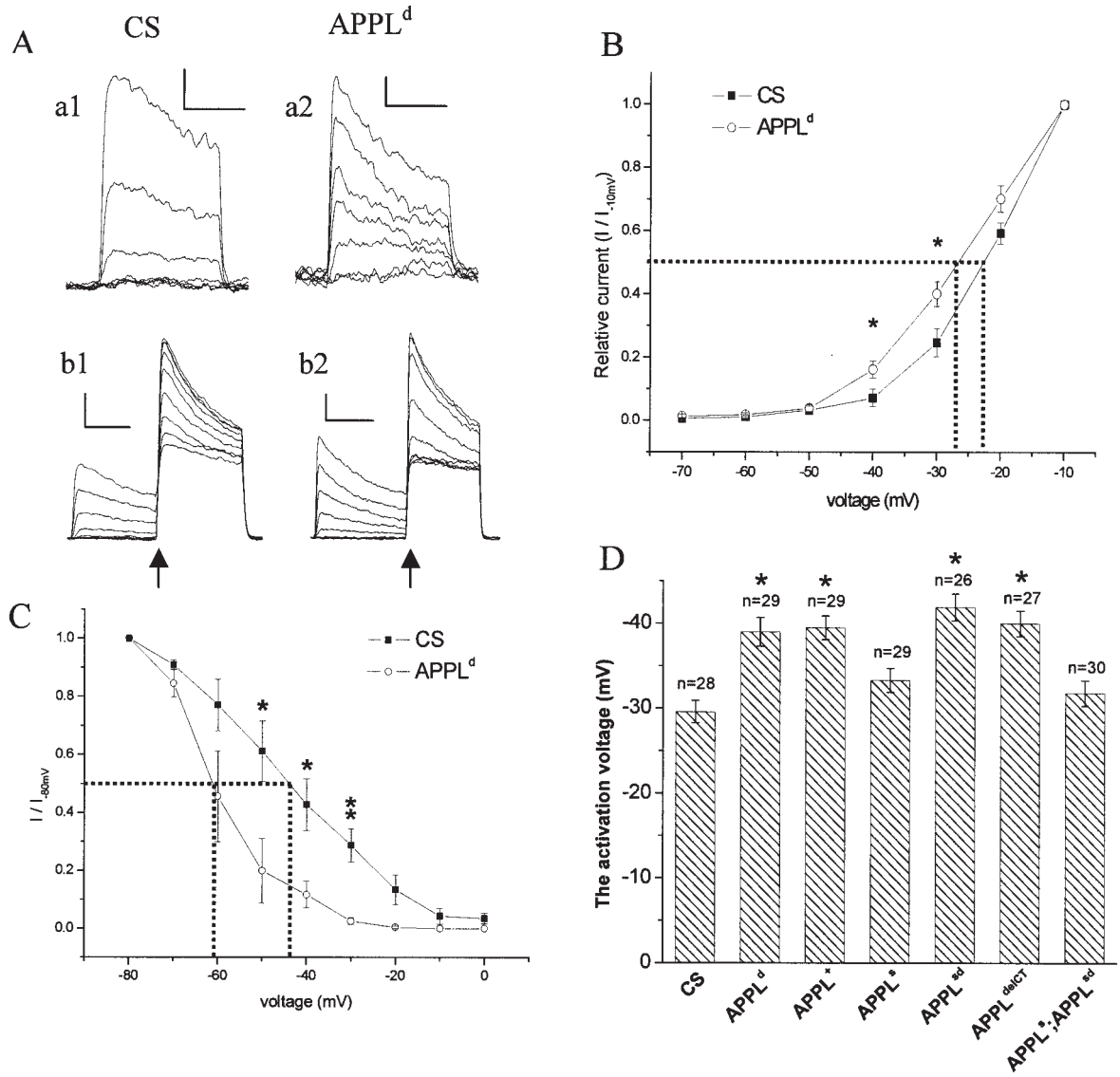


Figure 5 Abnormal K^+ currents in APPL mutants. (A) Changed K^+ currents in $APPL^d$ neurons (right) compared with wild-type (left) neurons. (a1–a2) Activation of K^+ currents induced by 100-ms step-up depolarization pulses from −70 mV in 10-mV steps, seven traces. Scale bar: 100 pA, 50 ms. (b1–b2) Inactivation of K^+ currents with double-stimulus by prepulse at −80 to 0 mV with 10-mV steps and test-pulse at 40 mV. Scale bar: 500 pA, 50 ms. (B) The normalized I/V curve of K^+ current in CS and $APPL^d$ neurons ($n = 8$). *Represents the signifcant difference at −40 and −30 mV ($p < 0.05$). The half-activated voltage is −23 and −27 mV in CS and $APPL^d$, repsectively. I_{-10mV} (current at −10 mV) equals 0.37 ± 0.03 nA in CS and 1.25 ± 0.18 nA in $APPL^d$ with significant difference ($p < 0.01$). (C) The inactivation of A-type current of CS and $APPL^d$ neurons ($n = 5$) measured at the point marked by the arrow in (A, b1–b1). I_{-80mV} is the current induced by test-pulse at 40 mV with prepulse at −80 mV. The half-inactivated voltage is about −28 and −52 mV, respectively. The significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$). (D) The average activation voltage of K^+ channel in all the mutants. With 5-mV step-up depolarizaiton, the threshold voltage to activate K^+ channgels is measured, and defined as activation voltage (V_a). *Indicates significant difference compared with CS ($p < 0.001$).

There are four kinds of voltage-dependent outward K^+ currents in cultured *Drosophila* embryo-derived neurons, including transient A-type current (I_A), delayed noninactivation current (I_K), calcium-dependent fast current (I_{CF}), and calcium-dependent slow current (I_{CS}) (Wu et al., 1990). Because Ca^{2+} currents were

blocked in our experiment, I_{CF} and I_{CS} were eliminated from the total K^+ currents at the same time. A-type channel can be activated at a more negative voltage and inactivated sooner, while delayed channel can only be activated at a higher voltage and will not be inactivated (Saito and Wu, 1991). Thus, I_A and I_K currents could be separated with the prepulse protocol (prepulse at −80 to 0 mV in 10-mV steps and test-pulse at 40 mV), and the currents at the beginning of the second stimulus stage exhibited the inactivation of A-type current [indicated by a arrow in Fig. 5(A b1–b2)]. We found that K^+ current of $APPL^d$ neurons was inactivated faster than that in CS neurons [Fig. 5(C)]. Because only I_A could be inactivated, the K^+ current detected at low voltage in $APPL^d$ neurons is the A-type K^+ current. This result indicates that APPL deletion leads to dynamic change of A-type K^+ current both on activation and inactivation.

With 5-mV step-up depolarization, the threshold voltage to activate K^+ channels was measured, and defined as activation voltage (V_a). The V_a was −39.0 ± 1.7 mV in $APPL^d$ neurons and −29.6 ± 1.3 mV in CS neurons with a significant difference [Fig. 5(D)]. Comparing the activation voltage of K^+ currents in different APPL mutants, we found that neurons expressing sufficient sAPPL protein, such as $APPL^s$ and double mutant $APPL^s$;$APPL^{sd}$, had a similar V_a as CS, while neurons without sAPPL protein, such as $APPL^{sd}$ and $APPL^{delCT}$, exhibited significantly decreased V_a comparable to that of $APPL^d$ neurons [Fig. 5(D)]. Thus, K^+ channels are likely regulated by the secreted APPL protein (sAPPL). It is unexpected that overexpression of wild-type APPL protein led to abnormal K^+ currents, similar to those APPL mutants lacking sAPPL [Fig. 5(D)]. This might be due to abnormal processing and secretion of APPL protein resulting from the overexpression in $APPL^+$ neurons.

Abnormal Spontaneous Postsynaptic Activity in APPL Mutant Neurons

The typical spontaneous postsynaptic currents (sPSCs) recorded in cultured *Drosophila* neurons are mediated by nicotinic acetylcholine receptors or GABA-gated chloride channels (Lee and O'Dowd, 1999; Yao et al., 2000). These two kinds of sPSCs were also identified in our cultured neurons, with characteristic decay time (τ) of about 2 and 11 ms, respectively [Fig. 6(A)]. In $APPL^s$ neurons, we found another kind of sPSC events with the decay value of more than 1000 ms. Superimposed on this slow depolarization current, there were many fast sPSCs, suggesting increased vesicle release [Fig. 6(B)]. Because these events were observed only in clustered

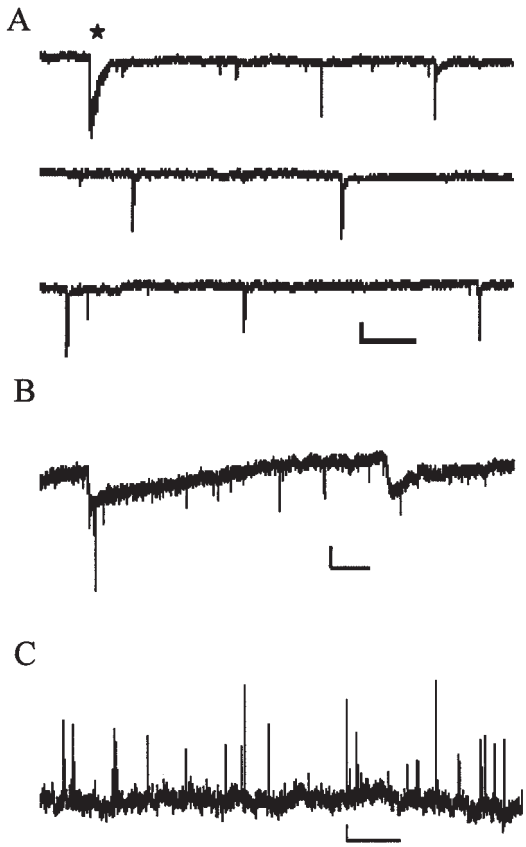


Figure 6 Abnormal spontaneous postsynaptic currents and potentials in APPL mutants. (A) Two kinds of sPSC in wild-type neurons. Star indicates a PSC with decay value(τ) about 11 ms. Others have a τ value of about 2 ms. Scale bar: 20 pA, 20 ms. (B) Spontaneous slow dpolarization in clustered $APPL^s$ neurons. Some fast sPSCs are superposed on the slow current. Scale bar: 20 pA, 10 s. (C) Spontaneous mEPSP in a single $APPL^{sd}$ neuron. Scale bar: 2 mV, 10 s.

$APPL^s$ neurons, we hypothesize that it is attributed to the spontaneous secretion of APPL protein. Previous studies showed that neurons exposed to APP protein were depolarized slowly, and the frequency of fast sPSCs was increased (Furukawa et al., 1996). Because APPL protein secreted from $APPL^s$ neurons could affect the neurite morphogenesis of other cells, it was conceivable that the sAPPL protein in the medium could also stimulate and slightly depolarize the neurons, similar to the effect of purified APP protein.

In low-density cultured $APPL^{sd}$ neurons, untouched with each other, a number of spontaneous excitatory postsynaptic potentials (sEPSPs) were detected [Fig. 6(C)]. Similar spontaneous activity was reported only in clustered wild-type neurons (see also Lee and O'Dowd, 1999), and was rarely observed in single CS neurons in our experiment. Using FM1-43

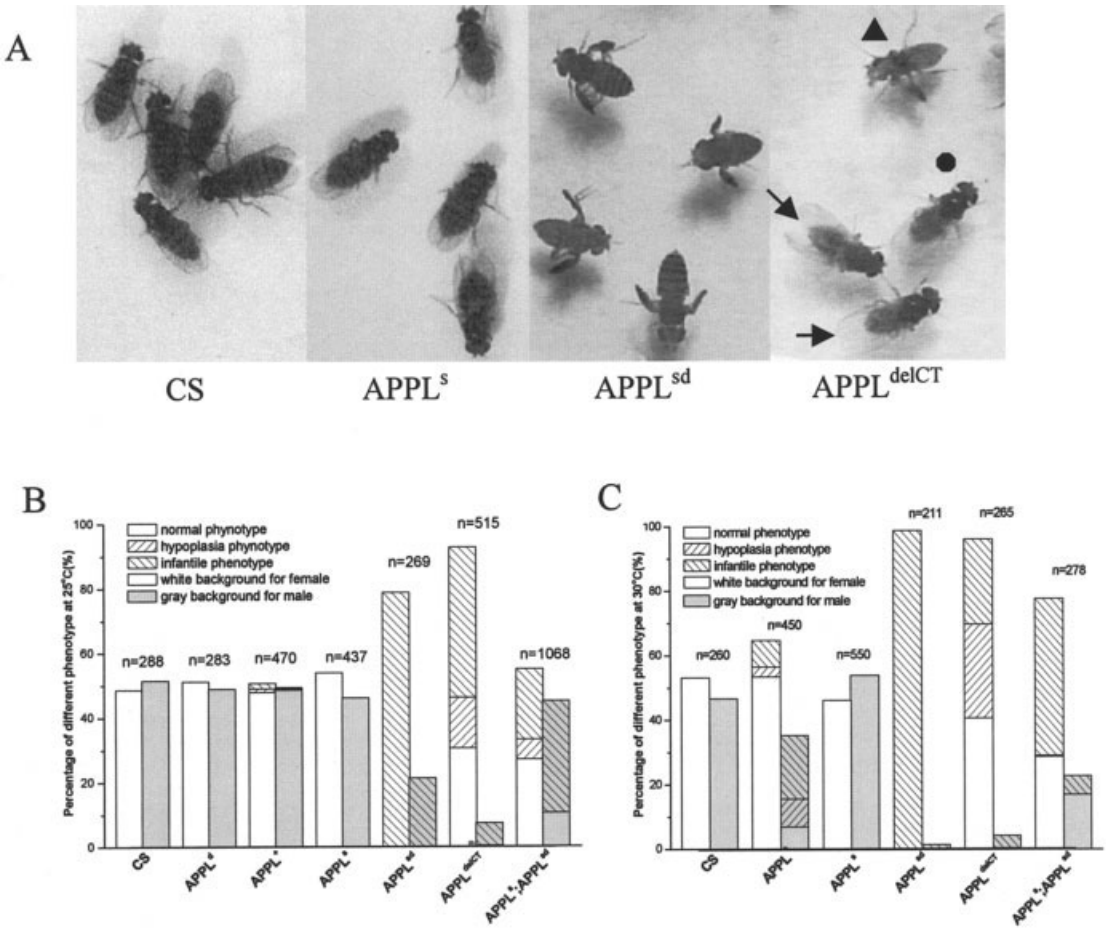


Figure 7 Different developmental defects of nonneural tissues in APPL mutants. (A) Different phenotypes in wild-type and mutants. APPL^{s} adult flies have normal phenotype as CS. All the APPL^{sd} flies fail to expend wings and harden cuticle, called infantile phenotype. APPL^{delCT} flies exhibit normal (round), infantile (arrowhead), or hypoplasia (abnormal wings, arrow) phenotypes, which can also be found in APPL^{+} and APPL^{s};APPL^{sd} mutants. (B and C) Quantitative analysis of the proportion of different phenotypes in terms of females and males at 25°C (B) and 30°C (C), respectively. The disproportion of female over male flies in some APPL mutants demonstrated the harmful effect of mutated APPL proteins on fly viability.

staining, we examined the functional synapses in single APPL^{sd} neurons. Punctate fluorescent staining along the neurites was detected (data not shown), indicating the formation of autosynapses between interlaced branches of neurites in single APPL^{sd} neurons. The increased spontaneous activities of neurons are likely due to the formation of functional synapses, which could greatly influence the neural excitability.

Panneural APPL Mutants Exhibit Severe Defects in Nonneural Tissues

Although APPL protein is expressed exclusively in the nervous system (Torroja et al., 1996), previous study showed that overexpression of wild-type APPL protein resulted in the failure of wing expansion and cuticle hardening, a phenotype termed "infantile," in less than 10% progeny (Torroja et al., 1999a). In our study, adult flies of various panneural APPL mutants showed different degrees of defects in wings and cuticle. Strikingly, all the APPL^{sd} flies exhibited infantile phenotype [Fig. 7(A,B)], which were much more severe than APPL^{+} flies (2.1%). APPL^{d} flies were normal in appearance, indicating that the absence of APPL protein would not lead to infantile phenotype. Thus, the severe defects of APPL^{sd} flies should mainly be attributed to the excessive mAPPL, but not the deficiency of sAPPL. About 53.6% of APPL^{delCT} flies had infantile phenotype, and less than 15.7% could harden the cuticle but expand their wings

Table 2. The Phenotype of Different APPL Mutants

Phenotype	Fly Lines						
	CS	$APPL^{d}$	$APPL^{+}$	$APPL^{s}$	$APPL^{sd}$	$APPL^{delCT}$	$APPL^{s}$,$APPL^{sd}$
MBs structure	normal	normal	normal	normal	mild	normal	ND
Neurite extension	normal	increase	decrease	decrease	increase	increase	decrease
Neuronal morphology	normal	severe	normal	normal	severe	severe	normal
K^{+} currents	normal	severe	severe	normal	severe	severe	normal
Wings and cuticle	normal	normal	mild	normal	severe	mild	mild
Viability	normal	severe	mild	normal	severe	mild	mild

"Normal," "mild," and "severe" indicate different phenotype. Neurite extension is distinguished as "increase" and "decrease." "ND" represents not done.

abnormally [Fig. 7(A,B)]. This less severe phenotype is named hypoplasia by us, which was also found in a subpopulation (6.2%) of $APPL^{s}$;$APPL^{sd}$ flies [Fig. 7(B)]. The lower proportion of infantile phenotype in $APPL^{delCT}$ compared to $APPL^{sd}$ flies suggests that the harmful effect of excessive mAPPL in development was partly C-terminal dependent.

Besides the abnormal wings and cuticle, decreased motility and viability have also been observed in some APPL mutants. Only $APPL^{s}$ flies could eclosion with the same motility and viability as wild-type CS. $APPL^{d}$ flies were much weaker than CS at 25°C, and died at the pupae stage when the temperature was raised to 30°C. $APPL^{sd}$ flies were too sluggish and weak to climb on the standpipe, and could rarely survive after eclosion even at 25°C. Flies of $APPL^{+}$, $APPL^{delCT}$, and $APPL^{s}$;$APPL^{sd}$ exhibited different degrees of decreased motility and viability according to their defects in appearance. When the temperature was raised to 30°C, more pupae, especially males, failed to eclosion (as in $APPL^{sd}$), and more adult flies exhibited infantile phenotype [as in $APPL^{delCT}$ and $APPL^{s}$;$APPL^{sd}$, Fig. 7(C)]. Because *Appl*-Gal4 is located on the X-chromosome, all mutated APPL proteins were expressed at a higher level in males due to dosage compensation. Thus, the disproportion of female over male flies within the population of $APPL^{sd}$ and $APPL^{delCT}$ flies [Fig. 7(B,C)] indicates the harmful effect of mutated APPL proteins on fly viability. The direct correlation between fly viability and either the expression level or the type of mutated APPL protein demonstrates that normal function of APPL protein in the nervous system is required for the development of nonneural tissues.

DISCUSSION

Despite considerable studies of APP proteins on the pathogenesis of Alzheimer's disease (AD), there is little understanding about its physiological functions in the central nervous system (CNS). Using transgenic flies, we systematically studied the normal functions of *Drosophila* APPL protein in neural development. Our results indicate that APPL protein is involved in the regulation of cytoskeleton and K^{+} channels. Furthermore, both the precise expression level and normal processing of APPL protein are essential for the neural function, although not for the organization of gross brain structures. The specific roles of APPL protein imply the possible relationship between the normal function of APP proteins and the development of AD.

APPL Protein Plays Essential Roles in Neural Development

Although previous studies suggest that APPL protein deletion could not lead to obvious morphological defect in the adult heads of flies (Luo et al., 1992), our results show that APPL protein deletion indeed leads to robust changes in neuronal morphology and physiology [Figs. 2(C) and 5]. In addition, the motility and viability of $APPL^{d}$ flies are obviously decreased [Fig. 7(C)]. On the other hand, overexpression of wild-type APPL protein can also lead to developmental defects in neuronal morphology, excitability, and adult phenotype (Figs. 2, 3, 5, and 7). These results demonstrate that the precise expression level of APPL protein is required for both normal morphology and the function of neurons, suggesting the essential roles of APPL protein in neural development.

In flies, two forms of APPL protein, sAPPL and mAPPL, can be identified from embryonic extracts and primary cultures, and sAPPL is converted from mAPPL soon after it is transported to the cytoplasm (Luo et al., 1990). Similar to the previous studies on neuromuscular junction (NMJ) (Torroja et al., 1999b), our results show that overexpression of different mutated APPL proteins leads to different developmental

defects (Table 2). Moreover, it is interesting that two APPL protein forms have opposite effects on neural development, such as neurite extension and morphogenesis (Figs. 2–3). Thus, we hypothesize that besides the expression level, normal processing of APPL protein is also essential for its physiological function in neural development.

APPL Protein and Cytoskeleton

APP proteins have been implicated to be involved in the regulation of the cytoskeleton either by direct binding to MTs (Smith, 1995; Islam and levy, 1997), or through microtubule-associated protein, Tau (Greenberg et al., 1994; Busciglio et al., 1995). However, our findings raised other possibilities. We show that the severe disorganization of AFs and MTs in $APPL^{sd}$ neurons results from the lack of sAPPL, and can be rescued by the application of sAPPL protein using the coculture method [Fig. 3(C)], indicating that the secreted APPL protein in the culture medium is able to inhibit the abnormal increase of AFs and MTs. Thus, we hypothesize that a certain amount of sAPPL is necessary for neuronal morphogenesis, acting between cells and neurites in a paracrine-inhibition manner.

Previous study shows that in flies, overexpression of Tau protein can lead to axonal transport defect and abnormal infantile phenotype, which both putatively result from cytoskeleton-associated neural dysfunction. The infantile phenotype can be further aggravated by cooverexpressing with APPL protein (Torroja et al., 1999a), and is even much more severe in flies overexpressing APPLSD protein alone (Fig. 7). These findings suggest that the APPL protein is involved in the regulation of cytoskeleton independently on the Tau protein. Our study on Tau mutants shows that the morphological defects in $APPL^{sd}$ neurons, such as increased branches and LMSs, are not found in neurons overexpressing wild-type or mutated Tau proteins (data not shown). These results suggest that APPL protein can affect the regulation of cytoskeleton in a Tau-independent manner.

APPL Protein and Neural Excitability

Various effects of APP protein and its metabolic fragments on K^+ and Ca^{2+} channels have been studied in several systems (Fraser et al., 1997). However, there is no direct evidence for the actual roles of APP protein in the regulation of ion channels in neural development. Our results demonstrate that in APPL mutant neurons, the lack of sAPPL protein can result in a persistent change of K^+ channels on both dynamic property and current amplitude (Fig. 5). Previous studies show that bath application of sAPP protein is able to enhance the voltage-dependent K^+ currents and suppress neural excitability (Furukawa et al., 1996). We hypothesize that absence of sAPPL protein in development could lead to less activation of K^+ channels, and might consequently upregulate K^+ channels both on expression level and dynamic property. K^+ currents can directly affect neural excitability by regulating the frequency of action potentials (Zhao and Wu, 1997). Our finding provides direct evidence that APPL protein is involved in the regulation of neural excitability of the CNS, mainly through the activation of K^+ channels by secreted APPL form.

In addition, we find that besides the change in K^+ channels, APPL mutants lacking sAPPL all exhibit abnormal neurite morphology. Torroja et al. (1999b) showed that neuronal activity could drive APPL-mediated bouton differentiation in NMJ. Is there any relationship between the change of neuronal morphology and excitability in the CNS? The abnormal LMSs has never been observed or reported in other mutant neurons, including various ion channel mutants, suggesting that the generation of this particular morphology is not induced by the change of neural excitability. On the contrary, numerous neurite branches and functional synapses lead to a great increase of spontaneous EPSPs in $APPL^{sd}$ neurons [Fig. 6(C)]. These abnormal neural activities might induce the downregulation of neural excitability as a feedback, probably by upregulating K^+ currents. Thus, the abnormal neuronal morphology in APPL mutants may indirectly influence neural excitability in the CNS. Furthermore, the dynamic change of cytoskeleton in APPL mutants might influence the formation and plasticity of synapse (Luo, 2002). In conclusion, besides the enhancement on K^+ channels of sAPPL, regulation of neuronal morphology is another way that APPL protein affects neural excitability and plasticity.

APPL Protein Is Required for Normal Neural Function

Although our *in vitro* studies show that mutation of APPL protein can lead to many neuronal defects, the *in vivo* brain structure of APPL mutants is grossly normal (Fig. 1). Are the developmental effects of APPL protein limited to the *in vitro* system? The subtle defects, especially the lobe fusion, in MBs of $APPL^{sd}$ flies indicate that APPL protein is indeed involved in the development of the CNS. Furthermore, the severe defects of wings and cuticle in adult

flies of APPL mutants reveal that APPL protein is required for the development of nonneural tissues. Because APPL protein is expressed exclusively in the nervous system, the infantile phenotype putatively results from neuroendocrine dysfunction (Torroja et al., 1999a).

Besides the neuroendocrine, we demonstrate that APPL protein is involved in many other neural functions, such as neural excitability and cytoskeletal dynamics, which is closely related to synaptic formation and plasticity (Luo, 2002). Our behavior study shows that MB-localized APPL mutants exhibit defective short-term memory in olfactory learning task (data not shown). Incorporated with the results obtained from the neuronal cultures, this finding suggests that dysfunction of the APPL protein could lead to memory defect without inducing the generation of the Aβ peptide or apoptosis [Fig. 3(D)]. Based on the information derived from flies, we hypothesize that in humans, abnormal expression or processing of APP proteins might lead to dysfunction of the CNS, before the Aβ peptide deposition and cell death. The cognitive defects may result from abnormal function of APP proteins in the CNS, especially at the early stage of AD. Continued studies based on the findings reported here will help to understand the normal functions of the APP protein family and the pathogenesis of AD.

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