

# Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone

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## Summary

The amyloid precursor protein (APP) is a type I transmembrane protein of unknown physiological function. Its soluble secreted form (sAPP) shows similarities with growth factors and increases the *in vitro* proliferation of embryonic neural stem cells. As neurogenesis is an ongoing process in the adult mammalian brain, we have investigated a role for sAPP in adult neurogenesis. We show that the subventricular zone (SVZ) of the lateral ventricle, the largest neurogenic area of the adult brain, is a major sAPP binding site and that binding occurs on progenitor cells expressing the EGF receptor. These EGF-responsive cells can be cultured as neurospheres (NS). *In vitro*, EGF provokes soluble APP

(sAPP) secretion by NS and anti-APP antibodies antagonize the EGF-induced NS proliferation. *In vivo*, sAPP infusions increase the number of EGF-responsive progenitors through their increased proliferation. Conversely, blocking sAPP secretion or downregulating APP synthesis decreases the proliferation of EGF-responsive cells, which leads to a reduction of the pool of progenitors. These results reveal a new function for sAPP as a regulator of SVZ progenitor proliferation in the adult central nervous system.

Key words: APP, Subventricular zone, Neurogenesis, Proliferation

## Introduction

A $\beta$  peptides found in senile plaques accompanying Alzheimer's disease (AD) are formed by the amyloidogenic processing of a type-one integral membrane protein, the amyloid precursor protein (APP) (De Strooper and Annaert, 2000; Small et al., 2001). This processing requires cleavage at two distinct sites by the  $\beta$  and  $\gamma$  secretases. Considerable effort has been devoted to understanding the amyloidogenic processing of APP in relation to neurodegeneration and dementia, but the physiological functions of APP remain unclear. The ubiquitous expression of APP during development and in several adult tissues, along with its high degree of conservation across phyla, suggests a fundamental role in cellular physiology. In the central nervous system, the increase of APP expression during development coincides with the peak of neuronal differentiation and neurite outgrowth (Hung et al., 1992; Salbaum and Ruddle, 1994). APP-null mutant mice show diverse non-lethal defects, including reduced brain weight and reduced size of forebrain commissures (Zheng et al., 1995; Li et al., 1996; Magara et al., 1999). However, a clear interpretation of these mutant phenotypes has been hampered by a possible functional compensation by other APP family members, the amyloid precursor like proteins, APLP1 and APLP2 (Slunt et al., 1994; Wasco et al., 1992). Accordingly, combined APP/APLP2 inactivation leads to perinatal death (Heber et al., 2000; von Koch et al., 1997).

In addition to its still elusive role as a membrane-bound protein, APP can be cleaved to produce soluble derivatives

with biological activity. In non-pathological situations, APP is predominantly cleaved by the  $\alpha$ -secretase within the  $\beta$ -amyloid sequence, to release a soluble form of APP, sAPP (see Fig. 3A). sAPP is normally present in brain tissues and circulates in the cerebrospinal fluid (CSF) (Palmert et al., 1989). A number of *in vitro* studies have attempted to uncover sAPP neuronal functions, showing that sAPP enhances synaptogenesis, neurite outgrowth, cell survival and cell adhesion (Mattson, 1997). These growth-promoting properties, together with its structural similarities with cysteine-rich growth factors (Rossjohn et al., 1999), suggest that sAPP may function as a growth factor *in vivo*. Interestingly, infusion of sAPP into the brain increases synaptic density and improves memory retention (Meziane et al., 1998; Roch et al., 1994) and levels of sAPP are decreased in the CSF of individuals with AD (Lannfelt et al., 1995; Van Nostrand et al., 1992). Moreover, sAPP stimulates proliferation of neural stem cells isolated from the embryonic brain (Hayashi et al., 1994; Ohsawa et al., 1999).

The existence of adult neural stem cells and progenitors raises the possibility that sAPP may also have an effect on neurogenesis in the adult. The SVZ extends along the length of the lateral ventricle and, in this region, neural stem cells retain the capacity to produce new neurons throughout adulthood (Alvarez-Buylla and Garcia-Verdugo, 2002). In rodents, newly produced neuroblasts migrate rostrally toward the olfactory bulb where they differentiate into granular and periglomerular interneurons. Studies of SVZ cells and of their

lineage relationships have shown that SVZ astrocytes (type B cells) give rise to rapidly dividing transit amplifying precursors (type C cells) which in turn give rise to migrating neuroblasts (type A cells) (Doetsch et al., 1999a). Members of the epidermal growth factor (EGF) family stimulate the proliferation of a subpopulation of cells in the SVZ in vivo (Craig et al., 1996; Doetsch et al., 2002; Kuhn et al., 1997; Tropepe et al., 1997). These EGF-responsive cells have recently been identified as the transit amplifying C cells (Doetsch et al., 2002). They can be grown in culture as neurospheres (NS) (Doetsch et al., 2002), which exhibit stem cell properties: they self-renew in the presence of EGF and differentiate into neurons and glia upon EGF removal (Morshead et al., 1994; Reynolds and Weiss, 1992).

We show that the SVZ is a major binding site for sAPP in the adult brain and that sAPP binds EGF-responsive cells. In vitro, EGF stimulates sAPP secretion by NS cells and sAPP is required for full EGF mitogenic activity. In vivo, modulating levels of sAPP results in modifications of the number of EGF-responsive cells composing the SVZ transit amplifying compartment through regulation of their proliferation. This work therefore provides strong evidence for a function of sAPP in adult neurogenesis.

## Materials and methods

### sAPP-Fc production

Plasmids encoding human sAPP (695 amino acid form), sAPLP1 (up to amino acid 584) or sAPLP2 (up to amino acid 680 of APLP2 571) fused to the Fc fragment of human IgG1 were transfected into Cos cells. Purification of sAPP-Fc (or sAPLP1/2-Fc) from the medium was performed on a protein A-sepharose column by standard procedures.

### Immunostaining

For immunostaining on sections, the mice (Swiss, 2-month-old) were perfused (4% paraformaldehyde), their brains were removed, postfixed overnight and sectioned with a vibratome (frontal sections, 50  $\mu$ m). Sections were blocked in PBS, 10% fetal calf serum, 0.3% triton and incubated in primary antibodies or sAPP-Fc, sAPLP1-Fc, sAPLP2-Fc diluted in the same buffer for 36 hours at 4°C under agitation. The Fc-tagged proteins were used at a concentration of 10  $\mu$ g/ml. Antibodies were rabbit anti-APP C-terminal extremity, rabbit anti-EGFR (1:500, Santa-Cruz Biotechnology), mouse IgM anti-PSA-NCAM (1:1000, gift from G. Rougon), rabbit anti-S100 (1:1000, DAKO), rabbit anti-APLP1 (1:2000, anti-CT11) and rabbit anti-APLP2 (1:2000, DII2). Fluorescent secondary antibodies were FITC or Cy3 donkey anti-species (1:1000, Jackson Immunoresearch).

For whole-mount staining, the mice were perfused with 2% paraformaldehyde, the lateral wall of the lateral ventricle (injected side) was dissected and immunohistochemistry was performed as described (Doetsch et al., 1999b).

### Infusions in vivo

sAPP-Fc (200  $\mu$ g/ml), sAPLP2-Fc (200  $\mu$ g/ml), human IgG (200  $\mu$ g/ml, Sigma), batimastat (100  $\mu$ M, Glaxo Smith Kline), APP oligonucleotides [20  $\mu$ M, designed -10 to +10 around the mRNA initiation site and coupled to penetratin (Derossi et al., 1998)] or APLP2 oligonucleotides (20  $\mu$ M, designed -3 to +15 around the mRNA initiation site and coupled to penetratin) were diluted in 0.09% NaCl. At least six adult mice for each condition were infused continuously for 3 days into the lateral ventricle (coordinates: anterior/posterior, 0; lateral, 0.8 mm; dorsal, 2 mm, relative to bregma) using micro-osmotic pumps (1  $\mu$ l/hour, model 1003, Alzet). It is

assumed that the solution in the pump is diluted at least ten times upon infusion in the CSF.

Ara-C (2%, Sigma) in vehicle (0.9% saline) or vehicle alone was infused onto the surface of the brain of adult mice (coordinates: anterior/posterior, 0; lateral, 1.1 mm relative to bregma) for 6 days with micro-osmotic pumps (Alzet, model 1007) as described (Doetsch et al., 1999b).

### Quantification of proliferation

For quantification of EGF-responsive cell proliferation, infused animals ( $n=3$  for each condition) were injected intraperitoneally with BrdU (1 mg in 100  $\mu$ l) 1 hour before perfusion with 4% paraformaldehyde. Brains were removed, postfixed for 2 hours and cryoprotected in 20% saccharose overnight. They were frozen and 10  $\mu$ m thick sections were cut using a cryostat. Frontal sections (20 per brain) at different levels of the SVZ (between +1 mm and 0mm anterior to bregma) were double-labeled for EGFR (sheep anti-human EGFR, 1:100, Upstate Biotechnology) and BrdU (rat anti-BrdU, Accurate chemical, 1:200), and mounted with DAPI-containing mounting medium. Clearly identifiable EGFR immunopositive cells were scored individually for their BrdU immunoreactivity under the 100 $\times$  objective of a Leica fluorescence microscope.

For quantification of BrdU-positive cells in whole-mount preparations, cells were counted on a predefined area corresponding to the anterior subventricular zone using a computerized Biocom analysis system.

### Neurosphere culture and assays

NS were prepared, cultured in serum-free medium with EGF (20 ng/ml), passaged and differentiated to assess their multipotentiality as described (Doetsch et al., 1999a). All experiments were performed on NS that had been passaged several times.

To stain sAPP-binding sites on NS cells, 8-day-old in vitro (DIV) NS were plated onto poly-L-ornithin (15  $\mu$ g/ml) and laminin (10  $\mu$ g/ml) coated chamber-slides (16 well Lab-Tek, Nunc) in medium without EGF for 4 hours. Cells were fixed in 4% paraformaldehyde for 15 minutes, rinsed in PBS and blocked in PBS with 10% fetal calf serum for 1 hour. They were incubated overnight at 4°C in sAPP-Fc diluted to 10  $\mu$ g/ml in PBS with 10% fetal calf serum. The signal was developed as for sections. Cells were mounted with DAPI-containing vectashield mounting medium (Vector).

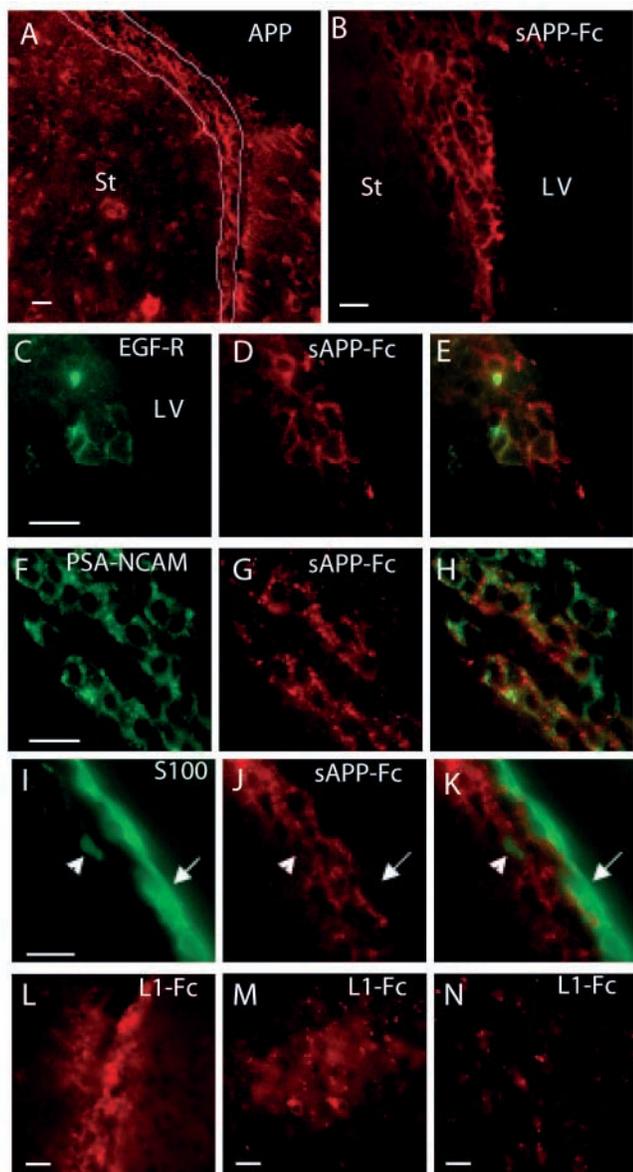
To measure sAPP secretion by NS, 8 DIV NS were centrifuged, resuspended in fresh medium and plated into 96-well plates (approximately 1000 NS per well in 50  $\mu$ l medium). After 15 hours, NS were centrifuged and the medium and cellular pellet from each separate well were processed independently for immunoblotting using the 22C11 antibody (Chemicon). The signal was developed using the application Image Gauge.

To evaluate NS cell proliferation, cells from dissociated NS were plated onto poly-L-ornithin, laminin-coated chamber-slides (16-well Lab-Tek, 2000 cells per well) and grown in EGF-containing medium (2 ng/ml) for 4 days. Cells were rinsed three times in fresh medium and cultured for 15 hours in medium with EGF (2 ng/ml), BrdU (1  $\mu$ M) and antibodies against sAPP (22C11, Chemicon; 6E10, Senetek) diluted to a concentration of 1.5  $\mu$ g/ml. Cells were fixed, processed for BrdU immunocytochemistry (mouse anti-BrdU, DAKO, 1:50), mounted with DAPI-containing mounting medium and the percentage of BrdU-positive nuclei was calculated.

## Results

### sAPP binding sites are present in the SVZ

APP is ubiquitously expressed in the brain and immunohistochemical staining of APP on brain sections shows that SVZ cells also express APP (Fig. 1A). Detection of sAPP signal transduction in different cell types (Greenberg et al.,



**Fig. 1.** sAPP-binding sites are present on SVZ cells. Single (A-D,F,G,I,J,L-N) or double (E,H,K) immunofluorescent stainings of frontal sections through the SVZ. (A) SVZ cells express APP. The SVZ is outlined by two white lines. (B) SVZ cells express sAPP-binding sites. (C-E) sAPP-binding sites (D) are present on EGFR immunopositive SVZ cells (C). (F-H) sAPP-binding sites (G) are present on PSA-NCAM immunoreactive neuroblasts (F). (I-K) S100 immunoreactive astrocytes (arrowheads) and ependymal cells (arrow) (I) do not express sAPP binding sites (J). (L-N) L1-binding sites are detected by the same technique in the SVZ (L), hippocampus (M) and cortex (N). St, striatum; LV, lateral ventricle. Scale bars: 10  $\mu$ m.

1994; Pietrzik et al., 1998) supposes the existence of a membrane receptor for sAPP. Saturable sAPP-binding sites have been detected in several cell lines (Hoffmann et al., 1999) but no receptor has yet been identified. We used a fusion protein consisting of the Fc domain of human immunoglobulin G (IgG) linked to sAPP (sAPP-Fc) to detect sAPP-binding sites on brain tissue sections. Interestingly, sAPP-Fc, but not control

human IgGs, prominently stained the surface of SVZ cells (Fig. 1B). Double-labeling showed that SVZ EGFR immunoreactive cells (type C cells) express sAPP-binding sites (Fig. 1C-E). sAPP-binding sites are also present on SVZ neuroblasts (type A cells), identified by PSA-NCAM staining (Fig. 1F-H). S100 immunopositive astrocytes (type B cells) and ependymal cells do not harbor sAPP binding sites (Fig. 1I-K). Under the staining conditions used, we did not detect sAPP-binding sites in other regions of the brain. Notably, we were unable to detect sAPP-binding in the hippocampus, the second major area where adult neurogenesis occurs (Kempermann, 2002).

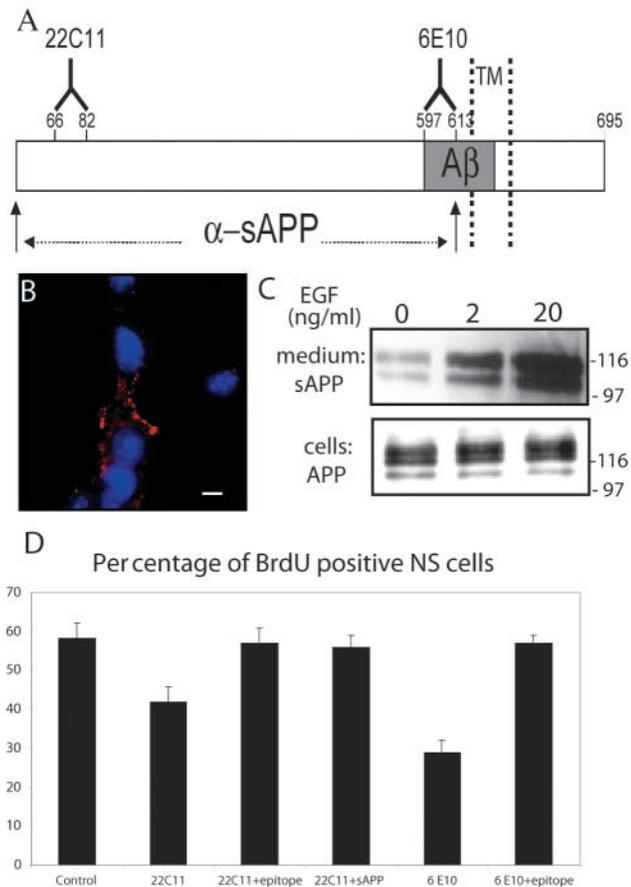
To verify if the prominent SVZ staining by sAPP-Fc and the absence of staining in other brain regions, in particular the hippocampus, reflects a heterogeneous penetration of sAPP-Fc within brain tissues, we used another Fc-tagged protein, L1-Fc (Doherty et al., 1995). In the same binding assay, L1-Fc binds sites in diverse regions of the brain, including the SVZ, the hippocampus and the cortex (Fig. 1L-N). This confirms that the detection of sAPP-binding sites in the SVZ is specific and that SVZ cells express high amounts of sAPP-binding sites. The possibility cannot be excluded, however, that low levels of binding sites or binding sites with lower affinities and below the limit of detection of our method are expressed in other brain regions.

#### sAPP participates in the EGF-induced proliferation of adult SVZ progenitors in vitro

The two likely cellular targets of infused sAPP are neuroblasts (Type A cells) and EGF-responsive cells (Type C cells), which both express sAPP-binding sites. We have focused the present study on the effect of sAPP on EGF-responsive cells. These cells can be cultured and, in the presence of EGF, they proliferate and form small aggregates called neurospheres (NS). We first verified if NS cells can bind sAPP. Detection of sAPP-Fc on plated NS cells showed that they do bind sAPP, with a punctuate staining pattern at the cell surface (Fig. 2B). To determine whether NS cells also secrete sAPP, we immunoblotted media conditioned by NS cultured with 0, 2 or 20 ng/ml of EGF. EGF causes a concentration-dependent increase in the release of sAPP (Fig. 2C, upper panel). By contrast, cellular APP remains approximately constant at the three EGF concentrations (Fig. 2C, lower panel). It is noteworthy that a similar growth factor-induced sAPP release has been shown in different cell lines (Schubert et al., 1989; Slack et al., 1997).

This observation suggested that sAPP might participate in the EGF-induced proliferation of NS cells. To verify this point, NS were cultured for 15 hours with bromodeoxyuridine (BrdU), a marker of DNA synthesis (phase S), in the presence of the 22C11 antibody that binds a small domain between amino acids 66-82 in the APP N terminus (Fig. 2A). The addition of 22C11 antibody leads to a significant decrease in the percentage of BrdU-positive cells ( $42 \pm 4\%$ ) when compared with control conditions without antibody ( $58 \pm 4\%$ ) or to conditions in which the antibody was pre-adsorbed onto its epitope ( $57 \pm 4\%$ ). This decrease in proliferation was also reversed when sAPP (10  $\mu$ g/ml) was added to the medium with the antibody ( $56 \pm 3\%$ ) (Fig. 3D, *t*-test  $P < 0.001$ ).

The 22C11 antibody has been shown to induce intracellular signaling through binding to membrane APP, thus mimicking the action of an unknown endogenous ligand (Brouillet et al.,



**Fig. 2.** sAPP participates in the EGF-induced proliferation of neurospheres. (A) Schematic structure of human APP (695 amino acid form) indicating the positions of the epitopes of 22C11 and 6E10 antibodies.  $\alpha$ -sAPP, soluble APP; A $\beta$ , amyloid  $\beta$ -peptide; TM, transmembrane region. (B) sAPP-binding sites (red) are detected on plated undifferentiated NS as a punctuate membrane staining. Nuclei are counterstained with DAPI. Scale bar: 5  $\mu$ m. (C) A constant number of NS were seeded in media with different concentrations of EGF (0, 2 or 20 ng/ml). Secreted (upper panel) and cellular (lower panel) APP were detected by immunoblotting. Secretion of sAPP is augmented by increasing concentrations of EGF (2 ng/ml, 3.5 fold; 20 ng/ml, 5.9 fold increase relative to EGF-free medium) while cellular APP remains largely constant. (D) Percentage of BrdU-positive NS cells after 15 hours of culture in medium containing EGF (2 ng/ml) and BrdU without (control) or with antibodies directed against sAPP (22C11, 6E10). Both antibodies decrease proliferation of NS cells. This decrease is abolished by pre-adsorption of the antibodies with their epitopes or by co-incubating 22C11 and sAPP. Results are shown as mean $\pm$ s.e.m. of three independent experiments.

1999; Okamoto et al., 1995). We therefore followed the proliferation of NS cells in the presence of a different antibody (6E10) that binds another region comprised between amino acids 597 and 613 of the APP N terminus (Fig. 2A). Addition of this antibody also leads to a decrease in the percentage of BrdU-positive cells (29 $\pm$ 3%) and this decrease is also prevented by a pre-incubation of the antibody with its epitope (57 $\pm$ 2%) (Fig. 2D, *t*-test  $P$ <0.001). The similar decrease of proliferation provoked by two antibodies recognizing two distinct epitopes suggests that they act by sequestering the sAPP released into the medium upon EGF addition and not

through a ligand-like signaling activity. We also verified that the decrease in BrdU incorporation does not reflect a cell survival effect by counting the number of apoptotic TUNEL-positive cells. We found that the percentage of apoptotic cells remained unchanged after the addition of either anti-APP antibody (control, 7.2 $\pm$ 1.1%; 22C11, 7.4 $\pm$ 1.8%; 6E10, 7.8 $\pm$ 1.5%).

The effect of sAPP on NS cell differentiation was tested by adding sAPP-Fc or anti-APP antibodies in conditions that induce the differentiation of NS cells into neurons, astrocytes and oligodendrocytes. Compared with control conditions, the relative percentages of each differentiated cell types were found unaltered by these treatments (not shown), suggesting that sAPP does not play a role in NS differentiation in vitro.

Taken together, these results suggest that the EGF-induced proliferation of NS cells is partly dependent on the EGF-induced release of sAPP into the medium. However, sAPP is necessary but not sufficient as it does not induce cell division in EGF-free medium. Finally, the addition of exogenous sAPP-Fc (0.1 to 20  $\mu$ g/ml) to EGF-containing medium did not lead to a further increase in NS cells proliferation (not shown), a finding that may be explained by a saturating effect of the EGF-induced secretion of endogenous APP. We thus decided to turn to the in vivo situation to test the effects of sAPP gain and loss of function on the proliferation of adult progenitors.

### sAPP increases proliferation of EGF-responsive adult progenitors in vivo

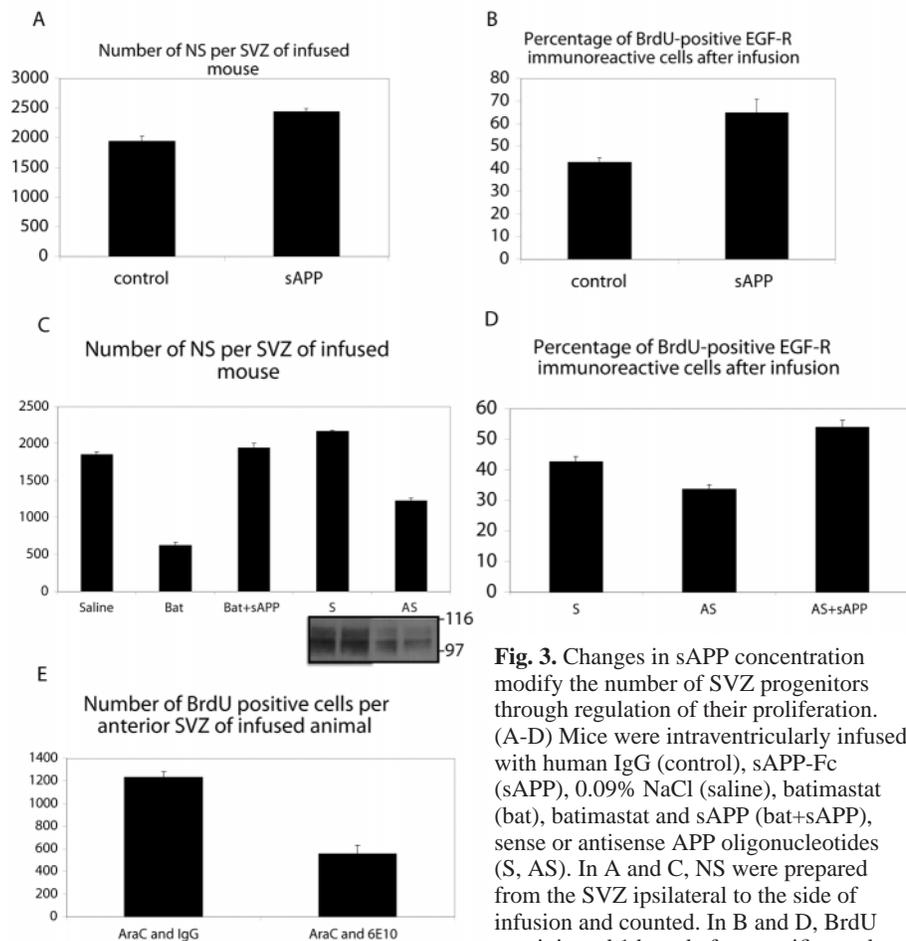
To determine whether sAPP also acts on EGF-responsive SVZ cells in vivo, we unilaterally infused sAPP-Fc (200  $\mu$ g/ml) or human IgG (same concentration) into the lateral ventricle for 3 days and prepared NS from the SVZ ipsilateral to the side of the infusion. The number of NS growing from each SVZ reflects the total number of NS precursors present in the SVZ at the time of dissection (Morshead et al., 1994). The average number of NS obtained from the SVZ of control mice infused with human IgG was 1940 $\pm$ 89. This number, and therefore the number of progenitors present in vivo, increased up to 2444 $\pm$ 49 after infusion of sAPP-Fc (Fig. 3A, *t*-test  $P$ <0.01).

These NS were tested for their ability of self-renewal and their differentiation properties and showed no difference as compared to control NS (not shown). Because NS precursors are mainly EGF-responsive Type C cells (Doetsch et al., 2002), this suggested an increase in the number of these progenitors. To assess whether this increase reflected their enhanced proliferation, BrdU was injected, 1 hour before sacrifice, into animals that had been infused for 3 days and the cells double-stained for BrdU and EGFR were counted. A larger fraction of EGF-responsive cells were BrdU-positive in sAPP-Fc infused mice (64.9 $\pm$ 6%) compared with control mice infused with human IgG (43 $\pm$ 2%) (Fig. 3B, *t*-test  $P$ <0.01). This suggests that sAPP positively regulates the proliferation of EGF-responsive SVZ adult progenitors in vivo.

### Decreasing sAPP reduces the proliferation of EGF-responsive adult progenitors in vivo

The  $\alpha$ -secretase activity, which allows the production of sAPP from APP, can be inhibited by hydroxamic acid-based zinc metalloprotease inhibitors such as batimastat (Parvathy et al., 1998).

Therefore, to evaluate the effect of a reduction of sAPP



**Fig. 3.** Changes in sAPP concentration modify the number of SVZ progenitors through regulation of their proliferation. (A-D) Mice were intraventricularly infused with human IgG (control), sAPP-Fc (sAPP), 0.09% NaCl (saline), batimastat (bat), batimastat and sAPP (bat+sAPP), sense or antisense APP oligonucleotides (S, AS). In A and C, NS were prepared from the SVZ ipsilateral to the side of infusion and counted. In B and D, BrdU was injected 1 hour before sacrifice and

the percentage of BrdU-positive EGFR immunoreactive cells on the side of infusion was counted. Results are shown as mean $\pm$ s.e.m. of six infused mice. (A,B) Augmentation of sAPP concentration leads to an increased number of EGF-responsive NS progenitors in the SVZ (A) and to their increased proliferation (B). (C) Reduction of sAPP secretion by batimastat or of APP synthesis by antisense oligonucleotides decreases the number of EGF-responsive NS progenitors. (Bottom panel) APP immunoblotting of the SVZ from two sense (S) or two antisense (AS) oligonucleotide-infused mice reveals a 30% reduction in APP levels in AS. (D) Reduction of APP synthesis through antisense oligonucleotide infusion downregulates EGFR immunoreactive cell proliferation and sAPP compensates this downregulation. (E) Ara-C infusion was followed by a 3 day intraventricular infusion of control IgG (Ara-C and IgG) or 6 E10 antibody (Ara-C and 6 E10) and the total number of BrdU-positive cells was counted in whole-mount preparations of the anterior SVZ. During SVZ regeneration, reduction of circulating sAPP downregulates EGF-responsive cell proliferation.

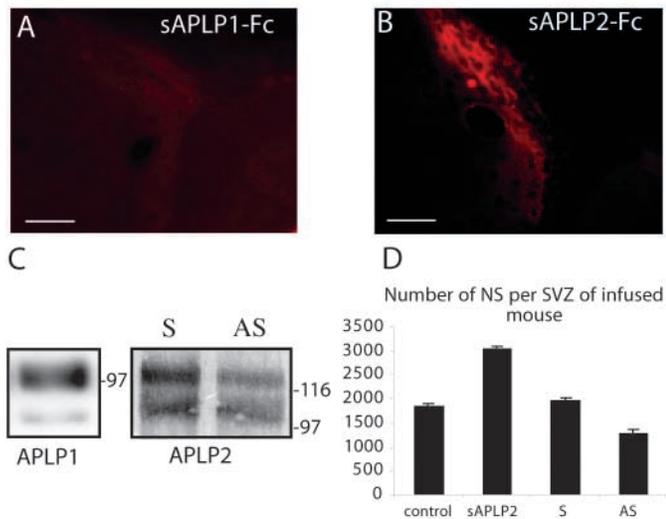
concentration on EGF-responsive SVZ cells, we unilaterally infused batimastat (100  $\mu$ M) into the lateral ventricle for 3 days and prepared NS from the SVZ, as above. The number of NS was reduced from 1856 $\pm$ 36 in control mice to 626 $\pm$ 34 after batimastat infusion (Fig. 3C, *t*-test  $P$ <0.001). Because batimastat probably inhibits the cleavage of several membrane proteins in addition to APP, we tested the specificity of the effect by co-infusing sAPP-Fc (200  $\mu$ g/ml) and batimastat before preparing NS. As shown in Fig. 3C, sAPP addition fully reversed the effect of batimastat, restoring the number of NS back to control levels (1948 $\pm$ 60). This strongly suggests that the reduction of SVZ progenitors upon batimastat infusion is a consequence of a decrease in the cleavage of APP, sAPP and not of another unknown factor.

As an independent means of assessing the effect of sAPP reduction, we infused an antisense oligonucleotide (20  $\mu$ M) directed against APP and linked to penetratin for internalization (Derossi et al., 1998). This linkage catalyses an extremely efficient addressing of the oligonucleotide into the cell cytoplasm and nucleus (Allinquant et al., 1995). It allows the use of low concentrations of oligonucleotides, thus preventing undesirable effects of the polyanionic nature of oligonucleotides. Quantification of APP in the SVZ by immunoblot shows a 30% reduction in antisense- as compared to sense-infused animals (Fig. 3C). The number of NS growing after infusion of sense oligonucleotides was 2167 $\pm$ 18, whereas infusion of antisense oligonucleotides led to a reduction to 1229 $\pm$ 34 (Fig. 3C, *t*-test  $P$ <0.01). This indicates that a reduced synthesis of APP leads to a reduction in the number of EGF-responsive progenitors.

To evaluate if, as anticipated from the in vitro studies, this reduction of the pool of progenitors was a consequence of their reduced proliferation, we injected BrdU into infused animals and calculated the percentage of EGF-responsive cells labeled with BrdU, as above. A lesser fraction of EGF-responsive cells were BrdU-positive in antisense-infused (33.8 $\pm$ 1.4%) compared with sense-infused mice (42.7 $\pm$ 1.8%) (Fig. 3D, *t*-test  $P$ <0.01). Because antisense treatment reduces APP synthesis and thus both the transmembrane form and its released extracellular domain (sAPP), we tested whether sAPP could rescue this reduction of proliferation. This was indeed the case as the percentage of BrdU-positive EGFR immunoreactive cells was above control levels in animals co-infused with antisense and sAPP (54 $\pm$ 2.2%) (Fig. 3D, *t*-test  $P$ <0.01). This suggests that the

reduction of proliferation of SVZ progenitors upon antisense infusion results from a decrease in sAPP and confirms that a reduction of sAPP level leads to reduced proliferation of adult SVZ progenitors.

Taken together, these data strongly suggest that sAPP regulates the proliferation of SVZ progenitors. However, as illustrated in Fig. 1, sAPP-binding sites are present in vivo both on Type C cells (EGFR-expressing progenitors) and Type A cells (PSA-NCAM-expressing neuroblasts). To verify that sAPP directly acts on type C cells and that sAPP-induced progenitor proliferation does not happen through an indirect action on type A cells, we took advantage of the well-defined system of SVZ regeneration after infusion of the antimetabolic drug cytosine- $\beta$ -D-arabinofuranoside (Ara-C) (Doetsch et al.,



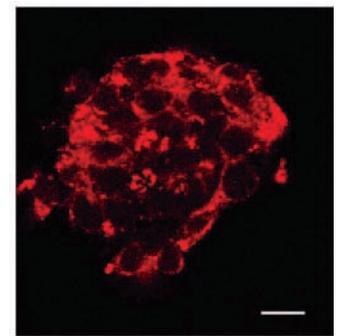
**Fig. 4.** sAPLP2 is functionally redundant with sAPP in the SVZ. (A) SVZ cells do not express sAPLP1 binding sites. (B) SVZ cells express sAPLP2 binding sites. (C, left panel) APLP1 immunoblotting showing APLP1 expression in the SVZ. (C, right panel) APLP2 immunoblotting of the SVZ from sense (S) or antisense (AS) APLP2 oligonucleotides infused mice reveals a 30% reduction in APLP2 levels in AS. (D) Augmentation of sAPLP2 concentration upon sAPLP2-Fc infusion increases the number of EGF-responsive NS progenitors in the SVZ, whereas reduction of APLP2 synthesis by antisense oligonucleotide infusion decreases their number. Results are shown as mean ± s.e.m. of six infused mice. Scale bars: 10  $\mu$ m

1999b). In this model, after elimination of all Type C and A cells by Ara-C, the SVZ regenerates rapidly with a sequential reappearance of type C (2 days after termination of Ara-C treatment) and A cells (4 days after treatment). This system allows one to follow the response of type C cells in the absence of neuroblasts. We thus infused antibodies directed against sAPP (6 E10, 200  $\mu$ g/ml) or control IgG (same concentrations) during the first 3 days of regeneration before the reappearance of type A cells. BrdU was injected 1 hour before sacrifice to count the total number of proliferating cells on whole-mount preparations of the anterior SVZ. The number of BrdU-positive cells was significantly reduced in antibody-infused animals (557 ± 78) when compared with control animals (1235 ± 45) (Fig. 3E, *t*-test  $P < 0.01$ ). This strongly suggests that a reduction of circulating sAPP downregulates type C cell proliferation and that this reduction is a direct effect of sAPP on type C cells.

### sAPLP2 is functionally redundant with sAPP in the SVZ

This study points towards a role for sAPP in regulating the proliferation of cells composing the SVZ transit amplifying compartment. APP is a member of a larger gene family, including the two amyloid precursor-like proteins APLP1 and APLP2 (Slunt et al., 1994; Wasco et al., 1992). APLPs are highly homologous to APP and can be processed in a similar way, leading to the secretion of their ectodomains (sAPLP) (Paliga et al., 1997; Slunt et al., 1994). It is thus possible that sAPLPs and sAPP have redundant functions in the SVZ.

As illustrated in the western blot of Fig. 4C, both APLP1 and APLP2 are expressed in the SVZ. However, when Fc-



**Fig. 5.** sAPP-binding sites are present on NS from the embryonic brain (E14). Scale bar: 10  $\mu$ m.

tagged sAPLPs were used to detect if binding sites are present on brain tissue sections, it was found that sAPLP2-Fc stained SVZ cells similarly to sAPP-Fc (Fig. 4B), whereas sAPLP1-Fc showed no detectable binding (Fig. 4A). We therefore assessed whether sAPLP2 could stimulate the proliferation of EGF-responsive cells by infusing sAPLP2-Fc in the lateral ventricle and counting the number of NS growing from the infused SVZ. The average number of NS obtained from control mice infused with human IgG was 1862 ± 58 and increased up to 3050 ± 50 upon sAPLP2-Fc infusion (Fig. 4D, *t*-test  $P < 0.01$ ). Conversely, infusion of a penetratin-linked antisense oligonucleotide directed to APLP2, which leads to a 30% decrease in APLP2 synthesis (Fig. 4D), was paralleled by a significant decrease in the number of NS (1276 ± 104). This decrease was not observed with a control sense oligonucleotide (1972 ± 68) (Fig. 4D, *t*-test  $P < 0.01$ ).

Increasing or decreasing sAPLP2 concentrations thus leads, respectively, to an increase or a decrease in the number of SVZ EGF-responsive progenitors, a result very similar to that obtained with sAPP. This suggests that both molecules serve similar and possibly redundant physiological functions in the SVZ.

## Discussion

This study points towards a new site of action and physiological function of sAPP in the adult mammalian brain. We show that sAPP-binding sites are prominently present in the SVZ on Type C cells characterized by the expression of the EGFR and on type A cells stained with the anti-PSA-NCAM antibody. A role for sAPP in the control of type C cell proliferation is supported by *in vitro* and *in vivo* experiments. *In vitro*, NS proliferation is dependent on the presence of EGF in the culture medium but also on that of sAPP released from the cells upon EGF addition. This indicates that sAPP plays a part in adult EGF-responsive cell proliferation and is further supported by *in vivo* approaches. Indeed, *in vivo* gain and loss of function of sAPP increases and decreases, respectively, the number of EGF-responsive SVZ progenitors composing the transit amplifying compartment (C cells) through modulation of their proliferation. It can thus be proposed that sAPP acts as a paracrine or autocrine factor mediating the proliferative effect of EGF on EGF-responsive cells. As a consequence, changes in the levels of circulating sAPP are likely to have direct consequences on the SVZ neurogenic potential by modifying the number of cells composing its transit amplifying compartment.

### Specificity of sAPP binding and activity

The binding of sAPP-Fc on both Type A and Type C cells clearly indicates that the protein probably has multiple sites of action and may not only influence the rate of division of Type C cells but also the division and migration of Type A cells. Although we do not preclude an effect on Type A cells, the experiments on Ara-C-treated animals demonstrate that the effect on Type C cell proliferation is not an indirect consequence of an effect on type A cells. This justifies the focus put, in this study, on the role of sAPP in Type C cell growth regulation.

Another aspect of specificity is the lack of binding of sAPP-Fc in the dentate gyrus, a second site of neurogenesis in the adult CNS. The absence of sAPP-Fc binding is not due to a lack of penetration of sAPP-Fc because a similar reagent, L1-Fc, binds both SVZ and hippocampal cells. This means that, at least in the adult, the number of sites or their affinity is lower than in the SVZ. This correlates with an absence of biological activity of sAPP-Fc, as monitored by the number of BrdU-labeled cells in the dentate gyrus after infusion of sAPP-Fc [on 10 representative sections, this number was  $51 \pm 5$  in sAPP-Fc infused animals and  $54 \pm 3$  in control animals (mean  $\pm$  s.e.m.,  $n=3$ )]. This finding is apparently at odds with some *in vitro* studies on hippocampal cells showing that sAPP or smaller peptides derived from sAPP have a trophic action or influence neuronal excitability (Mattson, 1997). Some of these studies provide evidence for an underlying signal transduction pathway suggesting the existence of a receptor for sAPP. However, most of the above-mentioned studies have been performed on embryonic cells or on cell lines and not on adult brain or NS cells derived from adult brain.

The absence of sAPP-binding sites in the dentate gyrus of the hippocampus and its ineffectiveness at promoting proliferation in this region also suggests that, in the adult, its role in neurogenesis could be limited to the SVZ. This is consistent with the fact that dentate gyrus neurogenesis is regulated differently from SVZ neurogenesis. In particular, the dentate gyrus progenitors are more restricted in their potentiality than SVZ progenitors and cannot be cultured as NS (Seaberg and van der Kooy, 2002). In line with this, an EGF-responsive transit amplifying compartment similar to the SVZ type C cells, shown here to be regulated by sAPP, is missing in the dentate gyrus (Seri et al., 2001).

A function for sAPP in regulating the proliferation of different cell types has been proposed in previous studies performed on cell lines (Hayashi et al., 1994; Hoffmann et al., 2000; Ohsawa et al., 1999; Pietrzik et al., 1998; Saitoh et al., 1989). Interestingly, sAPP has been described as an autocrine regulator of epidermal basal cell proliferation (Hoffmann et al., 2000), which, similar to SVZ progenitor proliferation, is under the control of TGF $\alpha$ , a member of the EGF family that acts through the EGF receptor. Cultured keratinocytes also exhibit sAPP-binding sites similar to the ones we observed in the SVZ or on cultured NS. In line with the results presented here, sAPP was described to stimulate proliferation of embryonic neural stem cells (Hayashi et al., 1994; Ohsawa et al., 1999). Accordingly, we could detect sAPP-binding sites not only on adult NS cells and SVZ but also on NS cells cultured from the embryonic brain (Fig. 5). Consequently, whereas this study is limited to adult neurogenesis, it is possible that sAPP also triggers progenitor proliferation at early developmental stages.

This possibility is supported by the reduced brain volume of mice deleted for APP (Zheng et al., 1995) and by a study on transgenic mice overexpressing a mutant form of APP (Bondolfi et al., 2002), revealing that these mice have 10-15% more cortical neurons than wild-type control mice. This, in view of the present results, could be a consequence of an increased neurogenesis consecutive to an increase in sAPP concentration.

### sAPP and transmembrane APP

The *in vitro* experiments demonstrate that the proliferation of NS in response to EGF is blocked in the presence of two antibodies that recognize two distinct epitopes in the N-terminal domain of APP. The antibodies could either act as agonists for transmembrane APP, as described for 22C11 (Brouillet et al., 1999; Okamoto et al., 1995), or by sequestering sAPP and thus preventing its availability to receptors. The latter hypothesis is more likely because the two antibodies recognizing two different epitopes have similar effects and it has never been reported that 6E10 mimics an APP ligand.

Also in favor of a direct role for sAPP as opposed to transmembrane APP, is the effect of inhibition of sAPP formation through batimastat infusion, which is fully reversed by the infusion of sAPP. In addition, antisense infusions are extremely demonstrative as the decrease in transmembrane APP is compensated by sAPP in the absence of a restoration of the normal concentrations of the transmembrane form. These data, together with the specificity of action of sAPP on the SVZ, which presents the highest concentration of sAPP-binding sites, support the idea that it is sAPP that triggers Type C cell proliferation and that, as far as this activity is concerned, transmembrane APP acts as a precursor. This, indeed, does not preclude other functions associated with this transmembrane form.

### sAPP and sAPLP2 as EGF/TGF $\alpha$ co-factors

The signaling pathway triggered by sAPP was not investigated here. It is important to note that, in the model that we propose, the activity of EGF requires that sAPP be present in the medium. sAPP is necessary, but not sufficient for EGF activity (it cannot replace EGF) and thus, in this model, acts as an EGF co-factor for Type C cell proliferation. It would be of great interest, in the future, to identify which pathways are co-regulated by the two molecules. To this extent, it is interesting that two studies have reported a stimulation of MAP kinase activity by sAPP (Greenberg et al., 1994; Pietrzik et al., 1998). It is also striking that, *in vitro*, the addition of sAPP in the presence of EGF does not further enhance NS proliferation. This situation differs from that observed *in vivo*, where the infusion of sAPP has a growth-promoting effect. This difference between the *in vitro* and *in vivo* situations might be due to the low levels of sAPP at the vicinity of their target cells, possibly owing to its fast clearance in the CSF. If so, sAPP would be a limiting factor for Type C cell proliferation in the adult SVZ.

We show that sAPLP2 also binds SVZ cells and acts similarly to sAPP on EGF-responsive progenitors, demonstrating a physiological function for sAPLP2. These results confirm the functional redundancy between APP and APLP2, postulated on the basis on their high degree of

homology (up to 72% identical in the cysteine-rich regions of their ectodomains) (Wasco et al., 1992) and from the lethal phenotype of the double knockout mice (Heber et al., 2000; von Koch et al., 1997) as opposed to minor phenotypes of viable single knockouts (Li et al., 1996; Zheng et al., 1995). This redundancy associated with the structural similarities between the two proteins and the fact that sAPP is a limiting factor, as proposed above, provides a rational for the fact that the two proteins have the same effects on Type-C cell proliferation and suggests that they act on the same receptors. The absence of compensation between the two molecules in the adult loss-of-function experiments is also in agreement with their limiting concentrations. This is not contradictory with the compensation observed in the genetic deletions as gene deletion in the embryo sets up permanent compensation phenomena not observed in the acute adult treatments used in this study.

### sAPP and AD

The present findings might have implications for the understanding of AD. The extent and significance of adult neurogenesis in the human brain is a matter of controversy. However, human and rodent SVZ cells express similar markers, including the EGFR (Weickert et al., 2000). A subset of human SVZ cells have been shown to divide (Eriksson et al., 1998) and the human SVZ is neurogenic in vitro (Kirschenbaum et al., 1994). This supports the view that SVZ neurogenesis is similarly regulated in all mammals. In the light of the reduction in sAPP concentrations in brains from individuals with AD (Lannfelt et al., 1995; Van Nostrand et al., 1992), the present data that link sAPP levels and the number of SVZ progenitors, suggest that a deficit in neurogenesis could play a role in some aspects of AD pathogenesis. Interestingly, individuals with AD show very early odor discrimination defects (Nordin and Murphy, 1998) as do mutant mice with reduced SVZ neurogenesis (Gheusi et al., 2000). Even though impaired olfaction in AD could be a consequence of cortical neurodegeneration, reduced olfactory bulb neurogenesis could participate in this phenomenon. Similarly, it is interesting that the primate SVZ gives rise to neurons destined to the amygdala and pyriform cortex (Bernier et al., 2002), two regions important for emotions and memory and atrophied in AD (Callen et al., 2001). Added to the implication of adult neurogenesis in learning and memory (Scharff, 2000), this observation suggests that some cognitive impairments in AD might result from a defect in SVZ neurogenesis.

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