

The Full-Length Form of the *Drosophila* Amyloid Precursor Protein Is Involved in Memory Formation

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The APP plays a central role in AD, a pathology that first manifests as a memory decline. Understanding the role of APP in normal cognition is fundamental in understanding the progression of AD, and mammalian studies have pointed to a role of secreted APP α in memory. In *Drosophila*, we recently showed that APPL, the fly APP ortholog, is required for associative memory. In the present study, we aimed to characterize which form of APPL is involved in this process. We show that expression of a secreted-APPL form in the mushroom bodies, the center for olfactory memory, is able to rescue the memory deficit caused by APPL partial loss of function. We next assessed the impact on memory of the *Drosophila* α -secretase *kuzbanian* (KUZ), the enzyme initiating the nonamyloidogenic pathway that produces secreted APPL α . Strikingly, KUZ overexpression not only failed to rescue the memory deficit caused by APPL loss of function, it exacerbated this deficit. We further show that in addition to an increase in secreted-APPL forms, KUZ overexpression caused a decrease of membrane-bound full-length species that could explain the memory deficit. Indeed, we observed that transient expression of a constitutive membrane-bound mutant APPL form is sufficient to rescue the memory deficit caused by APPL reduction, revealing for the first time a role of full-length APPL in memory formation. Our data demonstrate that, in addition to secreted APPL, the noncleaved form is involved in memory, raising the possibility that secreted and full-length APPL act together in memory processes.

Key words: α -secretase; APPL; *Drosophila*; *kuzbanian*; mushroom bodies; short-term memory

Introduction

APP is a major actor of AD (Zheng and Koo, 2011). APP is a transmembrane protein that undergoes two exclusive processing pathways: the nonamyloidogenic pathway initiated by the α -secretase produces a secreted APP form (sAPP α), while the amyloidogenic pathway leads to the production of amyloid- β peptides (A β), which can form toxic extracellular deposits in the brain of AD patients (Turner et al., 2003). For years, the amyloid hypothesis has placed A β at the center of the pathology etiology (Hardy and Selkoe, 2002).

To understand mechanisms underlying the pathology, it is fundamental to understand the role of APP in normal cognition, especially as disruption of APP normal function may contribute to AD (Neve et al., 2000). AD's first symptom is a decline of episodic memory (Förstl and Kurz, 1999), and several studies in mice reported that sAPP α enhances memory (Meziane et al., 1998; Ring et al., 2007). The physiological role of APP is particu-

larly difficult to study because of its numerous proteolytic metabolites. Furthermore, functional studies of the APP pathway in rodents are limited because of redundancy due to three APP-related genes and the lethality of the triple knock-out (Heber et al., 2000; Herms et al., 2004). In contrast, *Drosophila melanogaster* expresses a single nonessential APP ortholog, APP-Like (APPL). Importantly, APPL undergoes proteolytic pathways similar to that of APP, and the mammalian homologs of all secretases have been characterized in the fly (Poeck et al., 2012). Despite its relative simplicity, the fly brain is able to drive sophisticated behaviors. Particularly relevant for AD studies, *Drosophila* is extensively used as a model to study memory, and molecular processes at play are conserved from flies to mammals (McGuire et al., 2005). Neuronal structures underlying olfactory memory are well described. The mushroom bodies (MB), prominent bilateral structures of the fly brain comprising 4000 neurons, have been identified as the center of olfactory learning and memory (Heisenberg, 1998). We have recently shown that APPL is required for memory in the adult MB (Goguel et al., 2011), supporting the hypothesis that APP loss of function might contribute to the appearance of memory deficits in AD.

In the present study, we aimed to characterize which APPL forms are involved in memory. We analyzed short-term memory (STM) after overexpression in the adult MB of either a constitutively secreted APPL form (APPL^s; Torroja et al., 1996) or *kuzbanian* (KUZ), the *Drosophila* homolog of the mammalian α -secretase ADAM10 (Rooke et al., 1996; Carmine-Simmen et al., 2009). While APPL^s overexpression was fully able to rescue the STM deficit caused by an APPL partial loss of function, KUZ overexpression strikingly turned out to exacerbate this deficit.

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We show that in this context, KUZ overexpression generated a decrease of membrane-bound full-length APPL, and further demonstrate that expression of a membrane-bound mutant form of APPL (Torroja et al., 1996) was sufficient to rescue the STM deficit caused by *Appl* inhibition. Our data point to a requirement for memory processes of both secreted and nonproteolyzed forms of APPL.

Materials and Methods

Drosophila stocks. All fly strains were outcrossed to a Canton Special genetic background. The *UAS-kuzbanian* (*kuz*) line was kindly provided by D. Kretschmar (Carmine-Simmen et al., 2009). The *Appl^{td};MBSw* line was constructed using *Appl^{td}*-null mutant flies (Luo et al., 1992) and the Gal4-inducible MB247-Switch driver (Mao et al., 2004). The deficient *Appl^{td} w** line used for rescue experiments was a gift from J. M. Dura (Soldano et al., 2013). The *UAS-APPL*, *UAS-APPL^s*, and *UAS-APPL^{sd}* lines were described previously (Torroja et al., 1996, 1999). The specific *Appl-RNAi* used in this study is the transformant #108312 from the Vienna Drosophila RNAi Center. For behavioral experiments, flies were raised on standard medium at 18°C with 60% humidity under a 12 h light/dark cycle. As the *Appl* gene is located on the X chromosome, only females were analyzed in experiments that involved *Appl^{td}* genotypes. To induce transgene expression, the GeneSwitch system was used as described previously (Mao et al., 2004). A stock solution of RU486 (SPI-Bio; 10 mM in 80% ethanol) was mixed into molten food at 65°C to a final concentration of 200 μ M.

Behavior experiments. Flies were trained with classical olfactory-aversive conditioning protocols as described previously (Pascual and Pr  at, 2001). One to 2-d-old flies were kept on RU486-containing medium (RU) for 48 h before conditioning, and until testing when performed 24 h later. Training and testing were performed at 25°C with 80% humidity. Conditioning was performed on samples of 30–40 flies with 3-octanol (>95% purity; Fluka 74878, Sigma-Aldrich) and 4-methylcyclohexanol (99% purity; Fluka 66360, Sigma-Aldrich) at 0.360 and 0.325 mM, respectively. Odors were diluted in paraffin oil (VWR International, Sigma-Aldrich). Memory tests were performed with a T-maze apparatus (Tully and Quinn, 1985). Flies could choose for 1 min between two arms, each delivering a distinct odor. An index was calculated as the difference between the numbers of flies in each arm divided by the sum of flies in both arms. A performance index was then reported from the average of two reciprocal experiments with either octanol or methylcyclohexanol as conditioned stimulus. For odor-avoidance tests after electric shock exposure, and response to electric shock, flies were treated as described previously (Pascual and Pr  at, 2001).

Quantitative PCR. Flies were raised at 25°C before total RNA extraction from 50 female heads with the RNeasy Plant Mini Kit (Qiagen). Samples were submitted to DNase I treatment (BioLabs), and further reverse transcribed with oligo(dT)20 primers using the SuperScript III First-Strand kit (Life Technologies) according to the manufacturer's instructions. To specifically amplify the *Appl^s* cDNA, we used a primer (5'-CGCAGCGAGAAGCTCGATTA-3') that hybridizes at the site of the stop codon, which was introduced in the *Appl* sequence to generate the *APPL^s construct* (Torroja et al., 1996). We compared the level of the target cDNA to that of the α -*Tub4B* (CG1913) cDNA, which was used as a reference. Amplification was performed using a LightCycler 480 (Roche) in conjunction with the SYBR Green I Master (Roche). For each experiment, reactions were performed in triplicate for two dilutions of each cDNA. Each datum represents two to four independent experiments. Specificity and size of amplification products were assessed by melting curve analyses and agarose gel electrophoresis, respectively. Expression relative to the reference is expressed as a ratio ($2^{-\Delta C_p}$, where C_p is the crossing point).

Protein analyses. Proteins were extracted from 140 female fly heads after 48 h of RU induction at 18°C. Protein extracts were fractionated in RIPA buffer containing protease and phosphatase inhibitors (Roche) using sequential centrifugation as described previously (Lallemant et al., 2003). Samples were separated using SDS-PAGE gels containing a gradi-

ent of 3–8% acrylamide (Life Technologies) and transferred to nitrocellulose membrane (GE Healthcare). Membranes were cut into two parts and the higher one was probed with anti-APPL that binds to the APPL ectodomain (Ab952M; Torroja et al., 1996) at a dilution of 1:10,000. The lower part of the membrane was probed with either anti- γ -Tub (QG-17; Sigma-Aldrich, dilution 1:20,000) to analyze membrane fractions or anti- α -Tub (T6199; Sigma-Aldrich, 1:40,000 dilution) to analyze soluble fractions. Western blots were routinely developed using the ECL system (Life Technologies). Protein levels were quantified using ImageQuant TL software (GE Healthcare). Following normalization to loading controls, the levels of each APPL-reactive band were expressed as relative to the total amount of APPL detected in heterozygous *Appl^{td}/+*; *MBSw/+* flies from the same Western blot experiment. Three independent protein extractions were quantified 10 (membrane fractions) and 11 (soluble fractions) times on four independent Western blots.

Statistical analyses. Memory scores are displayed as mean \pm SEM. To compare performance indexes from more than two groups, statistical analyses were performed through a one-way ANOVA, followed by Newman–Keuls pairwise comparisons. Overall ANOVA *p* value is given in the legends along with the value of the corresponding Fisher distribution $F_{(x,y)}$, where *x* is the number of degrees of freedom for groups and *y* the total number of degrees of freedom for the distribution. Asterisks on the figure represent the least significant of the pairwise *post hoc* comparisons between the genotype of interest and its controls, following the usual nomenclature. To compare memory scores of two groups, Student's *t* tests were used with *p* < 0.05 as a significance threshold. mRNA quantification measurements were analyzed from $2^{-\Delta C_p}$ ratios in the same way.

Results

Several reports have shown that APP is involved in memory in mammals. In *Drosophila*, we recently showed that APPL expression is required in the MB of young adults for long-term memory (LTM) formation (Goguel et al., 2011). Because there are numerous APPL metabolites, we aimed to identify which APPL species are involved in memory processes. To this end, we addressed whether the expression of distinct mutant forms of APPL could rescue the memory deficit observed in flies containing a reduced level of APPL protein.

To analyze memory, we used a classical conditioning of an odor-avoidance response. In this paradigm, groups of flies are successively exposed to two distinct odors, only one of which is associated with electric pulses. Learning and STM are assessed immediately and 2 h, respectively, after one training cycle. Unlike STM, LTM is a consolidated memory whose formation relies on *de novo* protein synthesis (Tully et al., 1994), and it is assessed 24 h after five-spaced training sessions.

In our previous work (Goguel et al., 2011), we knocked down APPL expression in adult MB by RNAi using the conditional MB247-Switch driver (*MBSw*; Mao et al., 2004) and *UAS-RNAi* transgenes. The *MBSw* driver encodes a Gal4-Switch fusion protein that becomes active when flies are fed with the RU ligand (Roman et al., 2001). However, this knockdown strategy could not be used in the present study, as an *Appl* RNAi would target both endogenous *Appl* RNA and that resulting from the construct used to conduct rescue experiments. To circumvent this issue, we took advantage of *Appl^{td}*-null mutant flies (Luo et al., 1992), and generated *Appl^{td}* flies carrying the *MBSw* driver (*Appl^{td}; MBSw*). As previously described (Luo et al., 1992), homozygous *Appl^{td};MBSw* flies exhibited an impairment in electric shock avoidance (Fig. 1A), as well as an apparent defect in learning that might result from the impairment in shock reactivity (Fig. 1B). These data are consistent with the fact that *Appl* deletion leads to subtle abnormal morphology of the MB lobes (Li et al., 2004), and a more recent study showing the role of APPL in brain wiring during development (Soldano et al., 2013). Interestingly, Sol-

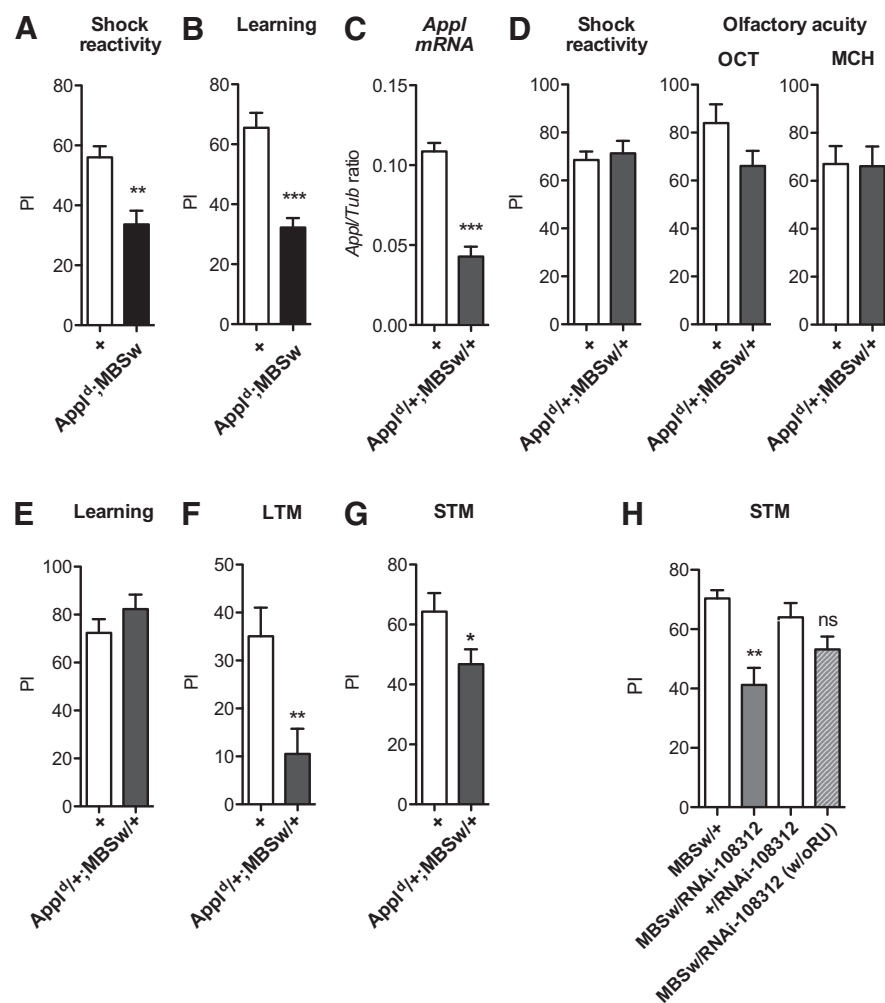


Figure 1. APPL LOF induces an STM deficit. **A, B.** Analysis of *Appl^Δ/MBSw* homozygous flies. **A**, *Appl^Δ/MBSw* flies exhibit a defect in shock reactivity (*t* test, ***p* < 0.005, *n* = 6). **B**, *Appl^Δ/MBSw* flies display a learning impairment. To assess learning ability, flies were submitted to one-cycle training and tested immediately after (*t* test, ****p* < 0.0001, *n* = 8). **C–G.** Analysis of *Appl^Δ/+; MBSw/+* heterozygous flies. **C**, qPCR analysis of *Appl* expression. Total RNA was extracted from wild-type (+) and *Appl^Δ/+; MBSw/+* fly heads, and further reverse transcribed with oligo(dT) primers. Resulting cDNA was quantified using tubulin (*Tub*) expression as a reference. Results shown are ratios to the reference (*t* test, ****p* < 0.0001, *n* = 3). **D**, Heterozygous *Appl^Δ/+; MBSw/+* flies display wild-type shock reactivity (*t* test, *p* = 0.6622, *n* = 6) and wild-type olfactory acuity (octanol, *t* test, *p* = 0.0954, *n* = 8; methylcyclohexanol, *t* test, *p* = 0.9390, *n* ≥ 7). **E**, Learning is not affected. Learning was assessed by testing flies immediately after one-cycle training (*t* test, *p* = 0.2717, *n* ≥ 7). **F**, *Appl^Δ/+; MBSw/+* flies exhibit an LTM deficit. To assess LTM, flies were submitted to five-spaced cycles, and memory was tested 24 h later (*t* test, ****p* < 0.005, *n* ≥ 15). **G**, *Appl^Δ/+; MBSw/+* flies exhibit an STM deficit. Flies were submitted to one-cycle training and tested 2 h later (*t* test, **p* < 0.05, *n* ≥ 11). **H**, Analysis of *Appl*-RNAi-expressing flies (*MBSw/RNAi-108312*). Unless indicated (w/o RU), flies were fed with RU for 48 h before conditioning. To assess STM, flies were submitted to one-cycle training and tested 2 h later. *MBSw/RNAi-108312* flies exhibit an STM impairment ($F_{(3,47)} = 7.507$, *p* = 0.0004, *n* ≥ 8; Newman–Keuls *post hoc*, *MBSw/RNAi-108312* vs *MBSw/+* ****p* < 0.001, *MBSw/RNAi-108312* vs *+ /RNAi-108312* ***p* < 0.005). *MBSw/RNAi-108312* flies not fed with RU display performances that were not different from the *+ /RNAi-108312* control (Newman–Keuls *post hoc* *p* > 0.05). Error bars indicate mean ± SEM. PI, Performance Index; OCT, octanol; MCH, methylcyclohexanol.

dano et al. (2013) showed that *Appl^Δ/+* heterozygous flies retaining one copy of the *Appl* wild-type gene do not undergo any MB developmental alteration. We thus hypothesized that *Appl^Δ/+; MBSw/+* heterozygous flies might constitute a tool to analyze the role of the APPL pathway in memory. As expected, measurement of *Appl* expression in *Appl^Δ/+; MBSw/+* fly heads by real-time PCR showed a strong reduction in *Appl* mRNA levels compared with wild-type (Fig. 1C). Interestingly, and in contrast to the *Appl^Δ/MBSw*-null mutant, *Appl^Δ/+; MBSw/+* heterozygous flies showed wild-type shock reactivity (Fig. 1D), and also wild-type olfactory acuity (Fig. 1D). Furthermore, *Appl^Δ/+; MBSw/+* flies displayed a normal learning (Fig. 1E). These data are consistent with

the fact that unlike *Appl^Δ*-null mutant, flies expressing 40% of the wild-type level of *Appl* mRNA possess brain structures involved in olfactory learning and memory that are not affected.

To better characterize the memory deficit of *Appl^Δ/+; MBSw/+* heterozygous flies, we analyzed their LTM performances. Consistent with the results previously observed with flies expressing specific *Appl* RNAi in adult MB (Goguel et al., 2011), *Appl^Δ/+; MBSw/+* heterozygous flies exhibited an LTM impairment (Fig. 1F). In addition, *Appl^Δ/+; MBSw/+* heterozygous flies exhibited a slight but significant STM defect compared with wild-type (Fig. 1G), whereas we previously observed that STM was not affected in *Appl* RNAi-G3- and *Appl* RNAi-42673-expressing flies (Goguel et al., 2011). In our previous study, APPL protein level was decreased by 39% in the MB as estimated by immunohistochemistry experiments (Goguel et al., 2011), whereas in the present work, *Appl* mRNA reduction in the fly head estimated by qPCR was 60%, suggesting that APPL protein level in the MB is lower in *Appl^Δ/+; MBSw/+* heterozygous flies than that previously achieved by RNAi induction. Thus, we hypothesized that the more APPL expression is reduced, the stronger the memory phenotypes observed. Indeed LTM is dependent on more advanced mechanisms than STM and is thus likely more sensitive to disturbance (Dubnau and Tully, 1998). To further address this question, we used a more efficient *Appl*-RNAi construct (RNAi-108312) to achieve a higher extent of APPL loss of function. Efficiency of *Appl* RNAi-108312 was assessed by driving its transcription with the pan-neuronal *elav*-Gal4 driver. qPCR analyses revealed that *elav/+; +/RNAi-108312* flies express an *Appl/Tub* ratio of 0.055 ± 0.003 relative to the *elav/+* control, whereas previously published results indicated relative ratios of 0.13 for *elav/+; 42673/+* and 0.25 for *elav/+; G3/+* flies (Goguel et al., 2011), showing that the RNAi-108312 construct leads to a higher *Appl* knockdown than

the RNAi constructs previously used. After 48 h of RU feeding, *MBSw/RNAi-108312* flies exhibited an STM deficit (Fig. 1H). This deficit was only observed when flies were fed with RU to induce RNAi expression (Fig. 1H), showing that it is specifically caused by RNAi induction in the adult MB. Therefore, decreasing APPL expression in the MB leads to a slight STM impairment that may not be observed when the reduction of *Appl* level is less substantial. The memory deficit is thus correlated to the extent of *Appl* level decrease in the MB. In conclusion, *Appl^Δ/+; MBSw/+* heterozygous flies should constitute an effective tool to conduct STM rescue experiments in APPL partial loss of function (LOF).

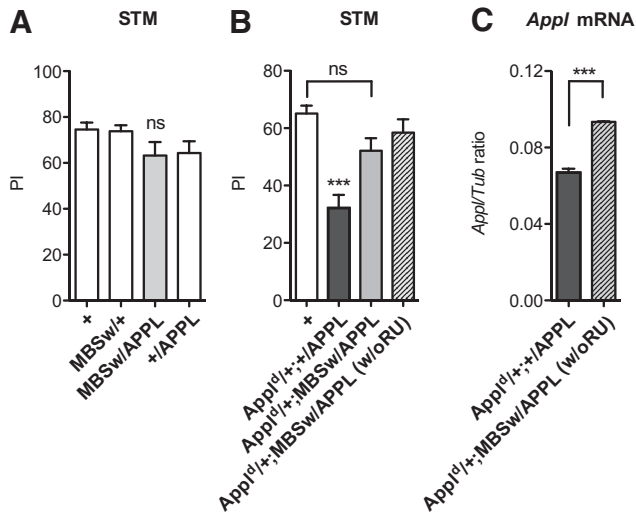


Figure 2. Expression in the MB of wild-type APPL rescues the memory deficit caused by APPL partial LOF. Unless indicated (**B**, **C**, w/o RU), flies were fed with RU for 48 h. **A**, **B**, Flies were submitted to one-cycle training, and memory was tested 2 h later. **A**, *MBSw/APPL* flies exhibit STM performances similar to control groups ($F_{(3,55)} = 2.143$, $p = 0.1060$, $n \geq 11$). **B**, Rescue experiments. *Appd1/+;MBSw/APPL* flies display a score significantly higher than that of *Appd1/+;+/APPL* flies ($F_{(3,84)} = 12.88$, $p = 0.0001$, $n \geq 19$; Newman–Keuls *post hoc*, $***p < 0.001$), and similar to wild-type (+) control flies (Newman–Keuls *post hoc*, $p > 0.05$). *Appd1/+;MBSw/APPL* flies not fed with RU exhibit scores similar to flies of the same genotype fed with RU (Newman–Keuls *post hoc*, $p > 0.05$). **C**, The *MBSw* driver leads to an increase in *Appl* levels in the absence of RU induction. Total RNA was extracted from *Appd1/+;+/APPL* and *Appd1/+;MBSw/APPL* fly heads, respectively, and further reverse transcribed. Resulting cDNA was quantified by qPCR using tubulin (Tub) expression as a reference (*t* test, $***p < 0.0001$, $n = 4$). Error bars indicate mean \pm SEM. PI, Performance Index.

To validate this tool we first expressed the wild-type APPL form and analyzed its capacity to restore the STM deficit of *Appd1/+;MBSw/+* heterozygous flies. When APPL overexpression was driven in the MB of wild-type flies (*MBSw/APPL*), it did not alter 2 h memory (Fig. 2A). APPL overexpression in the MB of *Appd1/+* heterozygous flies (*Appd1/+;MBSw/APPL*) led to a memory score similar to wild-type (Fig. 2B), showing that APPL overexpression in the MB leads to a rescue of the STM deficit caused by the deletion of one of the two copies of the *Appl* gene. When *Appd1/+;MBSw/APPL* flies were not fed with RU, STM scores were similar to wild-type and to flies of the same genotype fed with RU (Fig. 2B). Thus, we also observe a rescue of the memory deficit in the absence of RU induction. We hypothesized that this phenotype resulted from the expression in the MB of the *UAS-APPL* transgene, as Gal4-Switch may be partially active in the absence of RU feeding. To assess *Appl* expression in the absence of RU induction, we performed qPCR analyses. We observed that without induction, *Appd1/+;MBSw/APPL* flies expressed a significant increase in *Appl* mRNA levels compared with that of *Appd1/+;+/APPL* flies (Fig. 2C). Considering that RNA was extracted from the whole brain, whereas APPL expression due to the activity of the Gal4-Switch was restricted to the MB, APPL level of expression in the MB should be substantial. The memory impairment of *Appd1/+* flies is very likely caused by a functional deficit in the adult, so we conclude that APPL overexpression rescues the memory deficit caused by a partial reduction of endogenous APPL levels.

As the mammalian secreted fragment produced from the α -secretase cleavage pathway, sAPP α , is the prime candidate for a role in memory (Meziane et al., 1998; Bour et al., 2004; Ring et al., 2007; Taylor et al., 2008), we next aimed to analyze the secreted-

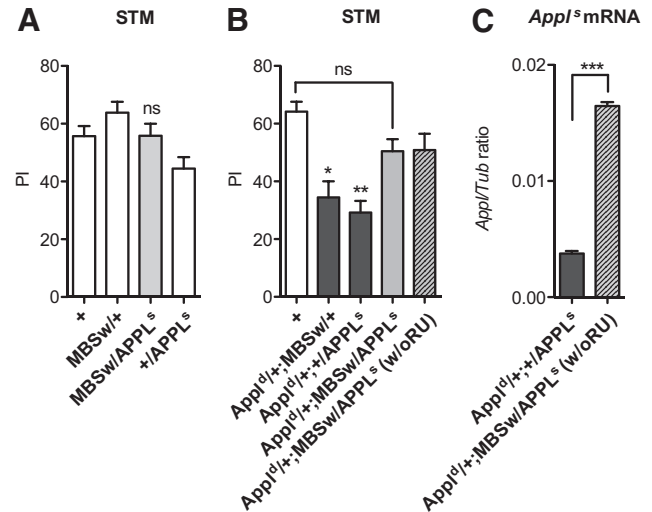


Figure 3. Expression of secreted APPL rescues the memory deficit caused by APPL partial LOF. Unless indicated (**B**, **C**, w/o RU), flies were fed with RU for 48 h. **A**, **B**, Flies were submitted to one-cycle training and memory was tested 2 h later. **A**, *APPL^s* overexpression in the MB does not impact STM. *MBSw/APPL^s* flies exhibit STM performances similar to control groups ($F_{(3,87)} = 4.625$, $p = 0.0048$, $n \geq 18$; Newman–Keuls *post hoc*, $p > 0.05$). *+/APPL^s* flies exhibit an STM score similar to wild-type (+) flies (Newman–Keuls *post hoc*, $p > 0.05$). **B**, Rescue experiments. *Appd1/+;MBSw/APPL^s* flies display a score significantly higher than that of *Appd1/+;MBSw/+* and *Appd1/+;+/APPL^s* flies, respectively ($F_{(4,84)} = 9.197$, $p < 0.0001$, $n \geq 15$; Newman–Keuls *post hoc*, *Appd1/+;MBSw/APPL^s* vs *Appd1/+;MBSw/+* $*p < 0.05$, *Appd1/+;MBSw/APPL^s* vs *Appd1/+;+/APPL^s* $***p < 0.005$), and similar to wild-type (+) control flies (Newman–Keuls *post hoc*, $p > 0.05$). *Appd1/+;MBSw/APPL^s* flies not fed with RU exhibit scores similar to flies of the same genotype fed with RU (Newman–Keuls *post hoc*, $p > 0.05$). **C**, *APPL^s* is expressed in the absence of RU feeding. Total RNA was extracted from *Appd1/+;+/APPL^s* and *Appd1/+;MBSw/APPL^s* fly heads and further reverse transcribed. cDNA resulting specifically from the *APPL^s* construct was quantified by qPCR using tubulin (Tub) expression as a reference (*t* test, $***p < 0.001$, $n = 2$). Error bars indicate mean \pm SEM. PI, Performance Index.

APPL form. For this purpose, we took advantage of a constitutively secreted-APPL mutant protein. A stop codon introduced in the *Appl* sequence generates a 788 aa soluble N-terminal fragment of APPL (APPL^s; Torroja et al., 1996), which has been put under control of the *UAS* expression system to allow for controlled expression patterns (*UAS-APPL^s*; Torroja et al., 1999). To assess APPL^s function in memory, we induced its expression in the MB for 48 h before conditioning and analyzed memory performances 2 h after. In flies expressing a wild-type level of endogenous APPL, overexpression of APPL^s in the MB (*MBSw/APPL^s*) led to a memory score that was not significantly different from that of control flies (Fig. 3A), showing that expression of a secreted-APPL form in the MB of young adult flies does not increase their STM performances. We next analyzed whether APPL^s expression in the MB of APPL partial LOF flies could rescue their STM deficit. Heterozygous *Appd1/+;MBSw/+* and *Appd1/+;+/APPL^s* flies showed an STM deficit (Fig. 3B), while APPL^s expression (*Appd1/+;MBSw/APPL^s*) led to the restoration of a wild-type STM score (Fig. 3B). This result shows that a secreted form of APPL is involved in memory processes. We observed that *Appd1/+;MBSw/APPL^s* flies that were not fed with RU also exhibited a wild-type STM score (Fig. 3B). To assess APPL^s expression in the absence of RU, we performed qPCR analyses. We observed an intrinsic level of APPL^s expression in the absence of Gal4 driver (Fig. 3C, *Appd1/+;+/APPL^s*). In the presence of the *MBSw* driver, *Appd1/+;MBSw/APPL^s* flies not fed with RU expressed a fivefold increase of APPL^s mRNA compared with the

control (Fig. 3C). This result indicates that in the absence of RU, the Gal4-Switch protein drives expression of the *UAS-APPL^s* transgene. In conclusion, expression of secreted APPL is able to rescue the memory deficit caused by the reduction of endogenous APPL.

To get further insight into secreted-APPL function in memory, we next analyzed the influence on memory of the overexpression of the *Drosophila* α -secretase, KUZ (Rooke et al., 1996), the enzyme that initiates the nonamyloidogenic pathway (Carmine-Simmen et al., 2009). Wild-type KUZ-expressing flies (*MBSw/kuz*) exhibited a memory score that was not significantly different from that of *+/+kuz* control flies (Fig. 4A), showing that, similarly to APPL^s, KUZ overexpression in the MB of young adult flies has no impact on STM. We next studied whether overexpression of the α -secretase could rescue the memory deficit caused by APPL partial LOF. Unexpectedly, KUZ-expressing flies (*Appl^{d/+};MBSw/kuz*) showed an STM score significantly lower than their genetic controls (Fig. 4B). Thus, not only did KUZ overexpression fail to rescue the memory deficit caused by APPL partial LOF, but even more, it exacerbated this deficit. *Appl^{d/+};MBSw/kuz* flies exhibited similar scores whether or not they were fed with RU for 48 h before conditioning (Fig. 4B). *kuz* mRNA quantification indicated that KUZ is overexpressed in the absence of RU feeding (data not shown), showing again that the Gal4-Switch driver is partially active under these conditions. *Appl^{d/+};MBSw/kuz* flies fed with RU and tested immediately after conditioning exhibited wild-type learning (Fig. 4C). Their ability to avoid electric shocks as well as their olfactory acuity to each odor after electric shock exposure were unaffected (Fig. 4D), showing that they perceived the stimuli used for conditioning normally. These data suggest that brain structures required for olfactory memory are not affected in *Appl^{d/+};MBSw/kuz* flies.

We next verified the level of *kuz* overexpression in wild-type and APPL LOF genetic contexts. qPCR analyses revealed that the level of *kuz* mRNA was similar in *MBSw/kuz* and *Appl^{d/+};MBSw/kuz* fly heads (Fig. 4E), indicating that the augmentation of the memory deficit observed in *Appl^{d/+};MBSw/kuz* flies is not due to an increase in KUZ expression level compared with that of *MBSw/kuz* flies. Altogether, the data show that in an APPL partial LOF background, overexpression of the α -secretase, KUZ, is deleterious for memory. This result in apparent contradiction to that observed with APPL^s overexpression suggests that increasing secreted APPL is not the only consequence of KUZ overexpression.

KUZ has substrates additional to APPL (Pan and Rubin, 1997; Lieber et al., 2002). However, because KUZ overexpression affects STM only when APPL levels are reduced, the results indicate that the memory deficit is caused by an interaction between KUZ and the APPL pathway. To get an insight into this interaction, we first analyzed whether the memory deficit induced by KUZ overexpression in *Appl^{d/+};MBSw/kuz* flies was linked to a modulation of endogenous *Appl* expression. *Appl* mRNA level was similar in *Appl^{d/+};MBSw/+* and *Appl^{d/+};MBSw/kuz* flies (Fig. 5A), showing that the mechanism by which KUZ exacerbates the memory deficit is not through modulation of *Appl* transcription, but rather through APPL processing. We next analyzed the effect of KUZ overexpression on APPL protein steady-state levels. Western blots were performed after fractionation of fly head lysates. As expected, the data show that APPL-secreted forms revealed in the soluble fraction were enriched upon KUZ overexpression (Fig. 5B). In addition, we observed that full-length APPL (fl-APPL) levels present in the membrane fraction were decreased by 30% in *Appl^{d/+};MBSw/kuz* compared with

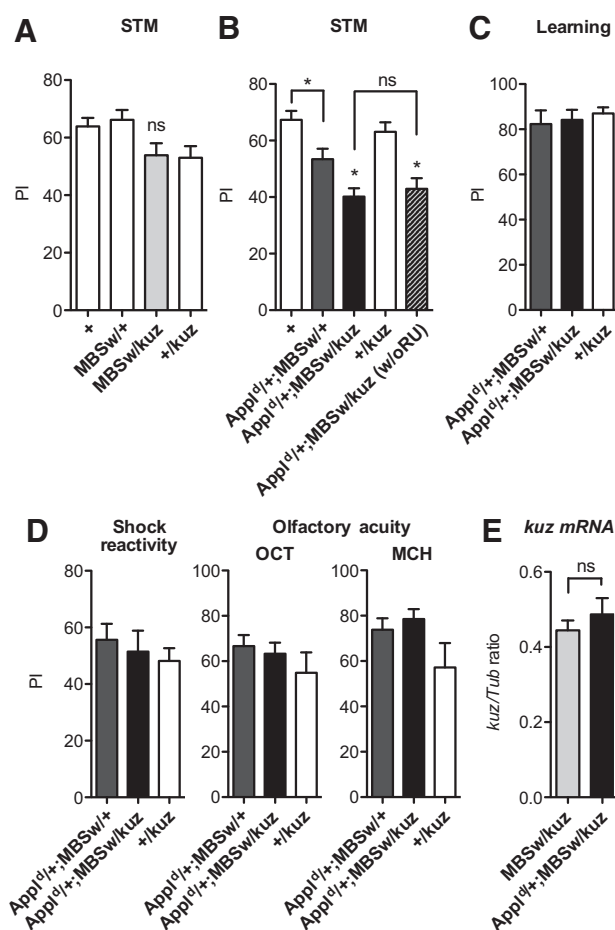


Figure 4. KUZ overexpression exacerbates the memory deficit caused by APPL partial LOF. Unless indicated (**B**, w/o RU), flies were fed with RU for 48 h before conditioning or total RNA extraction to induce the expression of the *UAS-kuz* transgene. **A**, **B**, Flies were submitted to one-cycle training and tested 2 h later. **A**, KUZ overexpression in wild-type flies does not impact STM. The score of *MBSw/kuz* flies is similar to that of the genetic control groups ($F_{(3,90)} = 3.342$, $p = 0.0229$, $n \geq 21$). **B**, KUZ overexpression in APPL partial LOF flies exacerbates their STM deficit. Heterozygous *Appl^{d/+};MBSw/+* flies exhibit an STM score significantly lower than the wild-type (+) control ($F_{(4,135)} = 11.83$, $p < 0.0001$, $n \geq 25$; Newman–Keuls *post hoc*, $*p < 0.05$). *Appl^{d/+};MBSw/kuz* flies perform significantly worse than both genetic controls (Newman–Keuls *post hoc*, $*p < 0.05$). *Appl^{d/+};MBSw/kuz* flies not fed with RU display scores significantly different from control groups (Newman–Keuls *post hoc*, $*p < 0.05$) and similar to *Appl^{d/+};MBSw/kuz* flies fed with RU (Newman–Keuls *post hoc*, $p > 0.05$). **C**, Learning is not affected. Learning was assessed by testing flies immediately after one-cycle training. *Appl^{d/+};MBSw/kuz* flies exhibit scores indistinguishable from controls ($F_{(2,21)} = 0.2516$, $p = 0.7801$, $n \geq 7$). **D**, Neither shock reactivity ($F_{(2,42)} = 0.3435$, $p = 0.7113$, $n \geq 13$) nor olfactory acuity (octanol, $F_{(2,40)} = 0.8994$, $p = 0.4153$, $n \geq 9$; methylcyclohexanol, $F_{(2,42)} = 2.694$, $p = 0.0798$, $n \geq 9$) is impaired in *Appl^{d/+};MBSw/kuz* flies. **E**, qPCR analysis of *kuz* expression. Total RNA was extracted from *MBSw/kuz* and *Appl^{d/+};MBSw/kuz* fly heads, respectively. Resulting cDNA was quantified using tubulin (Tub) expression as a reference. Results shown are ratios to the reference. *kuz* mRNA level is similar in *MBSw/kuz* and *Appl^{d/+};MBSw/kuz* flies (*t* test, $p = 0.4184$, $n = 3$). Error bars indicate mean \pm SEM. PI, Performance Index; OCT, octanol; MCH, methylcyclohexanol.

Appl^{d/+};MBSw/+ control flies (Fig. 5C). The results thus show that KUZ overexpression leads to a substantial increase of secreted APPL and a concomitant decrease of membrane-bound fl-APPL forms, suggesting that the deleterious impact on memory of the α -secretase overexpression is caused by a decrease of membrane fl-APPL protein.

To get an insight into the influence of fl-APPL on memory, we used the APPL^{sd} (APPL secretion-defective) mutant form of APPL (Torroja et al., 1996, 1999). APPL^{sd} carries a short deletion

encompassing the α - and β -cleavage sites that thus prevents its proteolytic cleavage. In consequence, APPL^{sd} is only expressed as a transmembrane protein. When overexpressed for 48 h in the MB of wild-type flies (*MBSw/APPL^{sd}*), APPL^{sd} mutant form did not affect STM (Fig. 6A). We next overexpressed APPL^{sd} in an APPL partial LOF genetic background. Interestingly, *Appl^l/+;MBSw/APPL^{sd}* flies fed with RU showed a STM performance significantly higher than that of *Appl^l/+;MBSw/+* and *Appl^l/+;+/APPL^{sd}* flies, and indistinguishable from wild-type flies (Fig. 6B). In contrast, in the absence of RU-feeding, *Appl^l/+;MBSw/APPL^{sd}* flies exhibited scores that were indistinguishable from that of *Appl^l/+;MBSw/+* and *Appl^l/+;+/APPL^{sd}* flies, and significantly lower than that of wild-type flies (Fig. 6B). The data show that the induction of APPL^{sd} transient expression in the adult MB rescues the STM defect caused by *Appl* reduction. This result also demonstrates that the STM deficit observed in *Appl^l/+;MBSw/+* flies (Fig. 1G) is indeed the result of a functional deficit in the adult. In conclusion, APPL^{sd} is able to fully rescue the memory deficit caused by APPL partial LOF, consistent with the fact that KUZ overexpression leads to decreased fl-membrane-bound APPL, and is associated with an exacerbation of memory deficits.

We next aimed to analyze whether the distinct APPL forms used in this study could rescue the LTM deficit caused by the reduction of endogenous APPL levels. As expected, we observed that overexpression of the wild-type APPL form could totally rescue the LTM impairment caused by APPL partial LOF (Fig. 7A). In contrast, neither APPL^s nor APPL^{sd} expression could rescue the LTM deficit (Fig. 7B,C). These data thus indicate that overexpression of either secreted APPL or fl-membrane-bound APPL alone is unable to restore wild-type LTM. Although it is difficult to conclude from these negative results, it is possible that both sAPPL and fl-APPL are required for LTM formation, but that either their ratio or their respective abundance is not able to sustain LTM.

Discussion

The majority of studies into APP biology have focused on pathogenic mechanisms. However, it remains crucial to understand the normal physiological function of APP, especially as it is possible that APP loss of function elicits early cognitive impairment in AD patients. Here, we show that overexpression of secreted APPL rescues the short-term memory deficit caused by a reduction of APPL level. In sharp contrast, overexpression of the α -secretase, KUZ, which produces sAPPL, exacerbates the memory impairment, a phenotype that is likely due to a deficit in full-length APPL protein level. Supporting this hypothesis, we further dem-

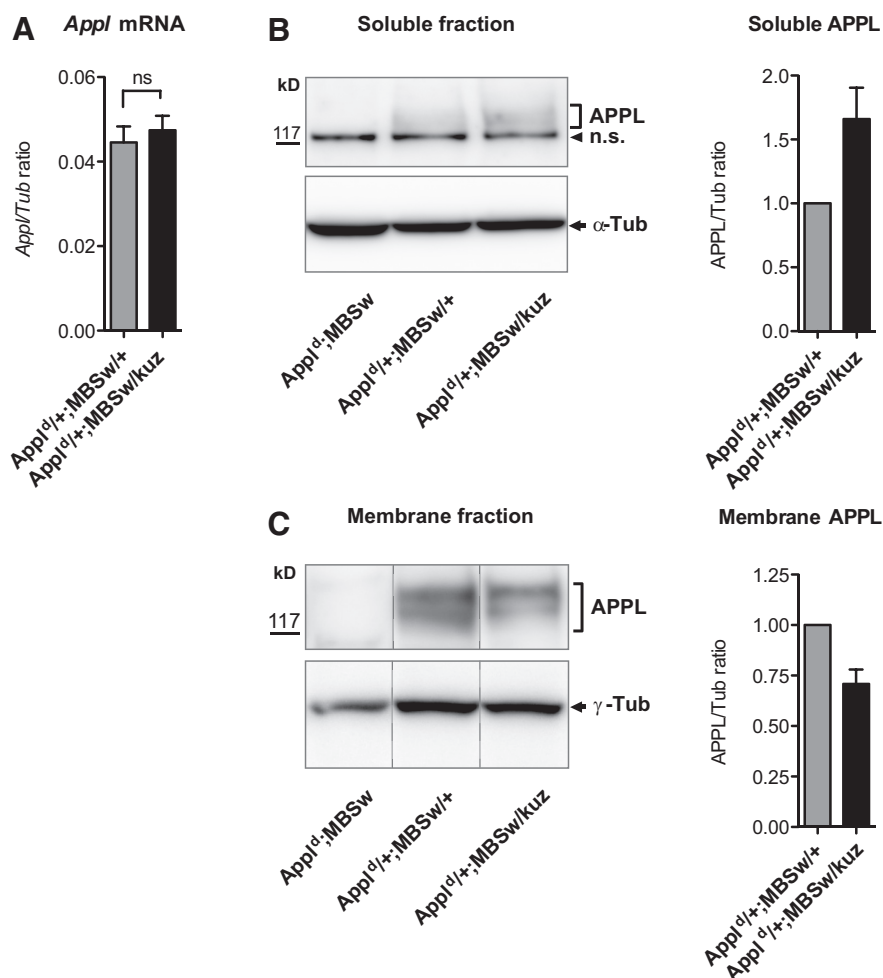


Figure 5. Impact of KUZ overexpression on *Appl* transcription and APPL processing. Flies were fed with RU for 48 h before RNA or protein extraction. **A**, qPCR analyses. Total RNA was extracted from *Appl^l/+;MBSw/+* and *Appl^l/+;MBSw/kuz* fly heads, and resulting cDNA was quantified using tubulin (Tub) expression as a reference. Results shown are ratios to the reference. *Appl* mRNA level is similar in *Appl^l/+;MBSw/+* and *Appl^l/+;MBSw/kuz* flies (*t* test, $p = 0.5814$, $n = 3$). **B, C**, Western blot analyses. Protein extracted from fly head lysates was fractionated ($n = 3$), and resulting blots were stained using an N-ter APPL-specific antibody. Left, Western blots. Right, Quantification. Soluble ($n = 11$) and membrane ($n = 10$) species are expressed as ratios to α -Tub and γ -Tub, respectively, relative to the *Appl^l/+;MBSw/+* control. **B**, Analyses of soluble fractions. Secreted-APPL level is increased in *Appl^l/+;MBSw/kuz* flies compared with *Appl^l/+;MBSw/+*, while it is not detected in *Appl^l;MBSw*-null flies. n.s., Nonspecific band resulting from a cross reaction. **C**, Analyses of membrane fractions. Left, Dotted lines separate lanes that were run on the same gel, but not side to side. fl-APPL level is decreased in *Appl^l/+;MBSw/kuz* flies compared with *Appl^l/+;MBSw/+* control flies. Error bars indicate mean \pm SEM.

onstrate that expression of a nonprocessed APPL mutant form is able to restore wild-type memory in an APPL partial loss of function background.

In the past, two main strategies have been considered as therapeutic approaches for AD. First, inhibition of the β - or γ -secretase has been used to achieve an inhibition of A β toxic production (Wolfe, 2008). However, reduction of A β production is not only an ineffective approach for AD (Callaway, 2012; Xia et al., 2012), it also can actually promote further pathology, as these enzymes have numerous substrates (Randall et al., 2010). A second proposed approach has been to inhibit the amyloidogenic pathway by activating the α -processing of APP (Fahrenholz, 2007). In addition to the potential beneficial inhibition of the amyloidogenic pathway, the advantage of this type of approach is to also increase the production of sAPP α (Vincent and Govitrapong, 2011). Indeed, decreased CSF sAPP α levels were found in familial and sporadic AD patients (Lannfelt et al., 1995; Sennvik

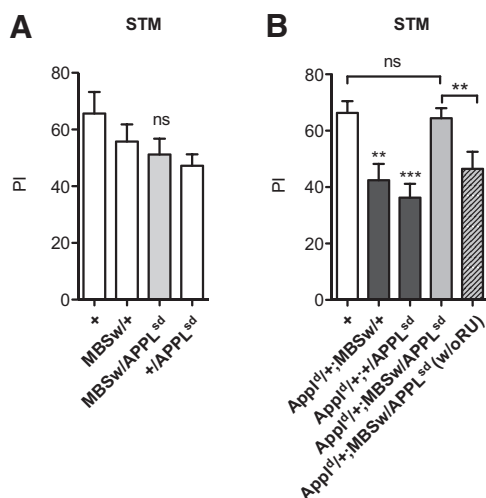


Figure 6. Transient expression of membrane-anchored APPL^{sd} rescues the memory deficit caused by APPL partial LOF. Unless indicated (**B**, w/o RU), flies were fed with RU for 48 h before conditioning. Flies were submitted to one-cycle training and tested 2 h later. **A**, MBSw/APPL^{sd} flies exhibit STM performances similar to control groups ($F_{(3,39)} = 1.693$, $p = 0.1858$, $n \geq 7$). **B**, APPL^{sd} flies display a memory score significantly higher than that of APPL^{sd} flies ($F_{(4,85)} = 8.023$, $p < 0.0001$, $n \geq 13$; Newman–Keuls post hoc, $App1^{+/+};MBSw/APPL^{sd}$ vs $App1^{+/+};MBSw/+$ *** $p < 0.005$, $App1^{+/+};MBSw/APPL^{sd}$ vs $App1^{+/+};+/APPL^{sd}$ *** $p < 0.001$), and similar to wild-type (+) flies (Newman–Keuls post hoc, $p > 0.05$). APPL^{sd} flies not fed with RU exhibit scores indistinguishable from that of APPL^{sd} flies and APPL^{sd} flies (Newman–Keuls post hoc, $p > 0.05$), and significantly lower than wild-type (+) (Newman–Keuls post hoc, * $p < 0.05$). APPL^{sd} flies not fed with RU exhibit an STM score significantly decreased compared with the flies of the same genotype fed with RU (Newman–Keuls post hoc, ** $p < 0.005$). Error bars indicate mean \pm SEM. PI, Performance Index.

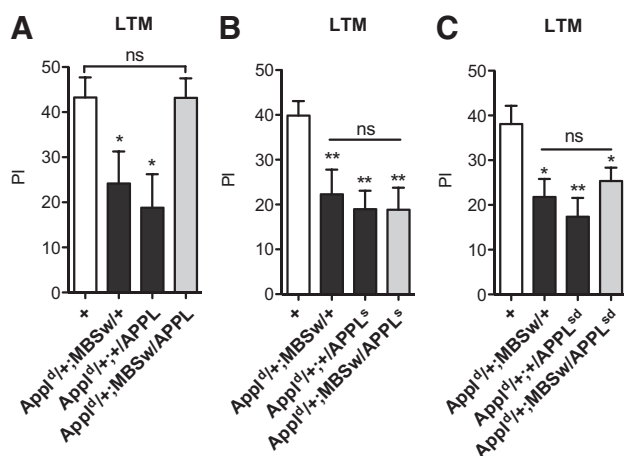


Figure 7. Analyses of LTM rescue. Flies were fed with RU for 48 h before five-spaced cycle conditioning, and also for 24 h until memory tests. **A**, Expression of wild-type APPL restores wild-type LTM in APPL^{sd} heterozygous flies. APPL^{sd} flies exhibit a score significantly higher than that of APPL^{sd} flies and APPL^{sd} flies ($F_{(3,51)} = 4.768$, $p = 0.0055$, $n \geq 11$; Newman–Keuls post hoc, * $p < 0.05$), and indistinguishable from wild-type (+) flies (Newman–Keuls post hoc, $p > 0.05$). **B**, APPL^{sd} overexpression does not rescue the LTM deficit caused by APPL partial LOF ($F_{(3,85)} = 5.672$, $p = 0.0014$, $n \geq 18$). APPL^{sd} flies display a memory deficit similar to that of APPL^{sd} flies and APPL^{sd} flies, respectively (Newman–Keuls post hoc, $p > 0.05$), and significantly lower than wild-type (+) flies (Newman–Keuls post hoc, ** $p < 0.005$). **C**, APPL^{sd} overexpression does not rescue the LTM deficit caused by APPL partial LOF ($F_{(3,95)} = 5.460$, $p = 0.0017$, $n \geq 22$). APPL^{sd} flies display a memory deficit similar to that of APPL^{sd} flies and APPL^{sd} flies (Newman–Keuls post hoc, $p > 0.05$), and significantly different from that of wild-type (+) flies (Newman–Keuls post hoc, * $p < 0.05$). Error bars indicate mean \pm SEM. PI, Performance Index.

et al., 2000), and correlated with poor memory performance in patients with AD (Almkvist et al., 1997; Fellgiebel et al., 2009). Thus, *in vitro* and *in vivo* studies indicate that sAPP α is down-regulated during AD. Numerous analyses have shown that sAPP α ectodomain has neurotrophic and neuroprotective effects in different models of neuronal stress (Mattson et al., 1993; Smith-Swintosky et al., 1994; Copanaki et al., 2010; Corrigan et al., 2011). In addition, sAPP α exhibits memory-enhancing properties. Intracerebroventricular infusion of anti-sAPP α serum was deleterious for memory, while that of sAPP α was beneficial (Meziane et al., 1998; Bour et al., 2004; Taylor et al., 2008). However, these studies relied on an exogenous excess of sAPP α and mechanisms of action and potential targets remained to be elucidated. With knock-in mice experiments, Ring et al. (2007) showed that sAPP α was sufficient to correct the impairments in spatial learning and long-term potentiation that are present in APP KO mice. Here we show in *Drosophila* that sAPPL is able to fully rescue the STM deficit caused by a reduction in endogenous APPL level, thus establishing that an APPL soluble form plays a role in memory, and giving further support for a role of secreted forms in memory in mammalian systems.

When the fly α -secretase, KUZ, was overexpressed in the adult MB, we did not observe any STM-enhancing effect and, unexpectedly, KUZ overexpression in the MB of flies with an APPL partial loss of function exacerbated their memory impairment. Thus, KUZ overexpression was actually deleterious for memory, rather than beneficial. These results contrast with a previous study showing that overexpression of the mammalian α -secretase ADAM10 in an AD mice model led to an increase in sAPP α , and was able to overcome APP-related learning deficits (Postina et al., 2004). However, these studies showed that α -secretase activation has a positive impact on memory exclusively under conditions where human APP is overexpressed. In wild-type mice, results were not clear because overexpression of either the wild-type or an inactive form of the bovine ADAM10 altered learning and memory (Schmitt et al., 2006). Furthermore, ADAM10 has many substrates (Vincent and Checler, 2012), and no evidence was brought to link the memory deficit to APP.

Interestingly, we observed that KUZ overexpression decreases membrane nonproteolyzed APPL level, suggesting that its negative impact on memory in APPL LOF flies is linked to a reduction of nonproteolyzed APPL level. Therefore, strategies aimed at increasing APP α -cleavage may not be appropriate as this could provoke a decrease of fl-APP levels that might be deleterious to APP function.

Transient expression of a constitutive membrane-bound mutant APPL has the capacity to fully rescue the STM deficit caused by APPL partial loss of function. Thus, both sAPPL and fl-APPL appear to be involved in memory processes. This is in apparent contradiction with the observation that mammalian sAPP α was sufficient to correct spatial learning deficit of APP KO mice (Ring et al., 2007). However, in this study APP-like proteins APLP1 and APLP2 were preserved, and as it is known from double KO analyses that the three APP homologs exert functional redundancy (Anliker and Müller, 2006), they may have compensated for the loss of essential fl-APP functions. In consequence, one cannot attribute the memory function exclusively to sAPP α .

If both fl-APPL and sAPPL carry the capacity to restore wild-type STM in APPL partial LOF flies, it is puzzling to observe that KUZ overexpression in this genetic context is deleterious for memory. Indeed, in addition to causing a decrease in fl-APPL, KUZ overexpression leads to a concomitant increase in sAPPL

that should be able to complement fl-APPL deficiency. We suggest that in this context, fl-APPL level is below threshold so that even high levels of sAPPL cannot restore a wild-type memory. This hypothesis is supported by protein quantification experiments showing a 30% decrease in fl-APPL level. Because APPL was extracted from the whole brain, whereas KUZ overexpression was only driven in a subset of neurons, the effective fl-APPL decrease in the MB must be much higher than 30%.

In mammalian cells under steady-state levels, ~10% of APP is located at the plasma membrane (Thinakaran and Koo, 2008). APP has long been suggested to act as a cell-surface receptor (Kang et al., 1987); however, such a function has not been unequivocally established. Several reports have shown that APP exists as homodimers (Scheuermann et al., 2001; Soba et al., 2005; Dahms et al., 2010). *Cis*-dimerization of APP would represent a potential mechanism for a negative regulation of APP functions and a concomitant impact on A β generation via an increase in β -processing (Scheuermann et al., 2001; Hashimoto et al., 2003; Lu et al., 2003; Kedikian et al., 2010; Lefort et al., 2012). Interestingly, it has been suggested that APP is a receptor for sAPP α as its binding could disrupt APP dimers (Gralle et al., 2009).

In *Drosophila*, Wentzell et al. (2012) reported that the secreted N-terminal ectodomain of APPL acts as a soluble ligand for neuroprotective functions. Furthermore, coimmunoprecipitation experiments from transfected *Drosophila* MB intrinsic cells revealed a physical interaction between fl-APPL and sAPPL, suggesting that sAPPL could be a ligand for fl-APPL (Wentzell et al., 2012). Our data showing the involvement of both membrane fl-APPL and sAPPL in memory are consistent with the hypothesis that sAPPL could be a ligand for its own fl-APPL precursor.

In conclusion, our data reveal for the first time a role for membrane fl-APPL in memory, opening new questions about APP nonpathological functions and relations between secreted and full-length forms in memory processes.

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