Drosophila Neprilysin 1 Rescues Memory Deficits Caused by Amyloid- β Peptide

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Neprilysins are Type II metalloproteinases known to degrade and inactivate a number of small peptides, in particular the mammalian amyloid- β peptide (A β). In *Drosophila*, several neprilysins expressed in the brain are required for middle-term (MTM) and long-term memory (LTM) in the dorsal paired medial (DPM) neurons, a pair of large neurons that broadly innervate the mushroom bodies (MB), the center of olfactory memory. These data indicate that one or several peptides need to be degraded for MTM and LTM. We have previously shown that the fly amyloid precursor protein (APPL) is required for memory in the MB. We show here that APPL is also required in adult DPM neurons for MTM and LTM formation. This finding prompted us to search for an interaction between neprilysins and *Drosophila* A β (dA β), a cleavage product of APPL. To find out whether dA β was a neprilysin's target, we used inducible drivers to modulate neprilysin 1 (Nep1) and dA β expression in adult DPM neurons. Experiments were conducted either in both sexes or in females. We show that Nep1 inhibition makes dA β expression detrimental to both MTM and LTM. Conversely, memory deficits displayed by dA β -expressing flies are rescued by Nep1 overexpression. Consistent with behavioral data, biochemical analyses confirmed that Nep1 degrades dA β . Together, our findings establish that Nep1 and dA β expressed in DPM neurons are functionally linked for memory processes, suggesting that dA β is a physiological target for Nep1.

Key words: amyloid peptide; dorsal paired medial neurons; *Drosophila*; learning and memory; neprilysin; olfactory conditioning

Significance Statement

Neprilysins are endopeptidases known to degrade a number of small peptides and in particular the amyloid peptide. We previously showed that all four neprilysins expressed in the *Drosophila* brain are involved in specific phases of olfactory memory. Here we show that an increase in the level of the neprilysin 1 peptidase overcomes memory deficits induced by amyloid peptide in young flies. Together, the data reveal a functional interaction between neprilysin 1 and amyloid peptide, suggesting that neprilysin 1 degrades amyloid peptide. These findings raise the possibility that, under nonpathological conditions, mammalian neprilysins degrade amyloid peptide to ensure memory formation.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that first manifests as a memory decline. Its dominant histological hallmark is the accumulation of amyloid- β peptides (A β) produced by the processing of the amyloid precursor pro-

tein (APP) following the amyloidogenic pathway (Turner et al., 2003). This proteolytic pathway is initiated by the rate-limiting enzyme β -secretase (BACE) (Cole and Vassar, 2007). As for any protein, the A β steady-state level results from a balance between its synthesis and its degradation. Neprilysins (Nep), known as membrane metallo-endopeptidases, have been highlighted as the main enzyme for A β degradation in humans (Turner, 2003; Nalivaeva et al., 2012). It has been shown that Nep level and activity decrease during AD progression, suggesting that a reduction in A β degradation may contribute to the development of the disease (Yasojima et al., 2001; Caccamo et al., 2005; Wang et al., 2010; Zhou et al., 2013). In mouse models of AD, an increase in Nep activity ameliorates the memory deficits observed (Poirier et al., 2006; Spencer et al., 2008; Park et al., 2013), whereas Nep deficiency worsens them (Hüttenrauch et al., 2015).

Drosophila melanogaster is a model system well suited to analyzing the influence of the APP pathway on memory. Impor-

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tantly, the fly genome contains homologs of 75% of human disease-related genes (Reiter et al., 2001), and molecules and mechanisms underlying memory processes are conserved from flies to mammals. Although relatively simple, the fly brain is highly organized and sustains complex forms of learning and memory. The mushroom bodies (MB) constitute the central integrative brain structure for olfactory associative memory. They are composed of ~4000 neurons, the Kenyon cells (KC), classified into three subtypes whose axons form five lobes: two vertical (α and α') and three medial (β , β' , and γ) (Crittenden et al., 1998).

Four Neps (Nep1–4) are expressed in the *Drosophila* CNS (Meyer et al., 2011; Sitnik et al., 2014). We previously showed that they were involved in both middle-term (MTM) and long-term memory (LTM) in the dorsal paired medial (DPM) neurons, a pair of large neurons that broadly innervate all the MB lobes (Turrel et al., 2016). DPM neurons are thought to regulate the MB outcome with a feedback system, a process involved in MTM formation (Keene et al., 2004). Nep1 expression is also required in the MB for MTM and LTM, and functional redundancy is observed between Nep2, Nep3, and Nep4 for LTM formation in the MB (Turrel et al., 2016). The Nep targets involved in memory processes are still unknown.

The fly expresses a single nonessential APP ortholog called APP-Like (APPL) (Rosen et al., 1989; Luo et al., 1992). Importantly, APPL follows proteolytic pathways similar to APP (Poeck et al., 2012), and the homologs of all mammalian secretases have been characterized in the fly (Rooke et al., 1996; Boulianne et al., 1997; Hong and Koo, 1997; Carmine-Simmen et al., 2009). APPL silencing in the MB of adult flies was shown to specifically disrupt MTM and LTM (Goguel et al., 2011; Bourdet et al., 2015a).

It has been shown previously that Nep2 can degrade human A β 42 (Finelli et al., 2004; Cao et al., 2008). However, whether or not a *Drosophila* Nep degrades endogenous A β is still an open question. Although there is no sequence similarity at the level of A β sequences between APP and APPL, a *Drosophila* A β -like peptide (dA β) was identified in old flies overexpressing APPL (Carmine-Simmen et al., 2009).

In the work reported here, using conditional expression, we modulated Nep1 and dA β levels in young adult flies. Together, our findings reveal a functional interaction between Nep1 and amyloid peptide for memory processes.

Materials and Methods

Drosophila stocks. *D. melanogaster* wild-type strain Canton Special (CS) and mutant flies were raised on standard medium at 18°C and 60% humidity with a 12 h light/dark cycle. All the strains used for memory experiments were outcrossed to the CS background. *Nep1* and *Appl*-RNAi lines were obtained from the Vienna Drosophila Resource Center (*Nep1A*: 27537; *Nep1C*: 108860; *Appl*: 108312 and 42673). To overexpress Nep1, we used the P{EPgy2}Nep1^{EY21255} line (Nep1^{EY21255}) obtained from the Bloomington Drosophila Stock Center (Indiana University, #22465). UAS-APPL (APPL) was previously described by Torroja et al. (1999). UAS-dA β (dA β) and UAS-dBACE (dBACE) were kindly provided by Doris Kretschmar (Carmine-Simmen et al., 2009). To generate a Nep1 mutant, a 2.59 kb PCR fragment was amplified using the Nep1 cDNA as a template (Drosophila Genomics Resource Center, Bloomington, IN, #GH03315), and cloned into the BglII site of pUAST (Brand and Perrimon, 1993). The resulting plasmid was verified by sequencing, and the 1.24 kb BstEII-KpnI restriction fragment was replaced by the mutant fragment obtained from GenScript. The mutated Nep1 (Nep1^{mut}) displays a mutation in position 685 (E $\rightarrow$ V) that disrupts the catalytic activity of the enzyme (Devault et al., 1988). GAL4 drivers used were VT64246 (Vienna Drosophila Resource Center, #264246) for expression in DPM neurons and *c739* for expression in MB α/β neurons. We used the TARGET system to specifically induce RNAi expression in adults

(McGuire et al., 2003). To achieve GAL4 induction, flies were kept at 30°C for 3 d before conditioning, and until memory test for LTM analyses.

Behavioral experiments. Flies were trained with classical olfactory aversive conditioning protocols as described by Pascual and Preat (2001). Training and testing were performed at 25°C and 80% humidity. Conditioning was performed on samples of 25–35 flies between 4 and 5 d of age with 3-octanol (~95% purity, Sigma-Aldrich) and 4-methylcyclohexanol (99% purity, Sigma-Aldrich) at 0.360 mM and 0.325 mM, respectively. Odors were diluted in paraffin oil (VWR International). Memory tests were performed with a T-maze apparatus (Tully and Quinn, 1985). Flies were given 1 min to choose 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 number in both. The average of two reciprocal experiments gave a performance index. For learning analyses, flies were tested immediately after a single training cycle. To assess MTM, flies underwent one-cycle training, and memory was tested 2 h later. For LTM analyses, flies were trained with 5 cycles spaced at 15 min rest intervals, and tested 24 h later. For odor avoidance tests after electric shock and response to electric shock, flies were treated as described by Pascual and Preat (2001). For experiments including genotypes with a construct on the X chromosome (Nep1^{EY21255} and dBACE), only females were analyzed.

qPCR analyses. For *elav* genotypes, flies were raised at 25°C. *Gal80^{ts}*; VT64246 flies were raised at 18°C, and GAL4-transcription was induced for 3 d at 30°C before RNA extraction. Total RNA was extracted from 60 female heads using the RNeasy Plant Mini Kit (QIAGEN). Preparations underwent a DNaseI treatment (Biolabs) for 15 min at 37°C. DNase was heat-inactivated with EDTA (10 mM). Samples were cleaned with the RNA Minielute Cleanup kit (QIAGEN), and reverse-transcribed with oligo(dT)20 primers using the SuperScript III First-Strand kit (Invitrogen) according to the manufacturer's instructions. Level of the target cDNA was compared against level of α -Tub84B (Tub, CG1913) cDNA used as a reference. Amplification was performed using a LightCycler 480 (Roche) and the SYBR Green I Master mix (Roche). Reactions were performed in triplicate. Specificity and size of amplification products were assessed by melting curve analyses and agarose gel electrophoresis, respectively. Expression relative to reference is expressed as a ratio ($2^{-\Delta C_p}$, where C_p is the crossing point).

Western blot analyses. To analyze dA β level, we used a *tub-Gal80^{ts}*; *elav* pan-neuronal driver. Proteins were extracted from 50 female fly heads after 48 h of induction at 30°C. Samples were prepared as in Burnouf et al. (2015). Soluble proteins were separated using SDS-PAGE gels containing a gradient of 10%–20% acrylamide (Novex 10%–20% Tricine, Invitrogen) and transferred to nitrocellulose membrane (GE Healthcare). Membranes were cut into two parts: the lower one was probed with anti-HA (ab130275, Abcam, 1:10,000 dilution), and the upper one was probed with anti- α -Tub (T6199; Sigma-Aldrich, 1:40,000 dilution). Western blots were routinely developed using the ECL system (Invitrogen). Protein levels were quantified using ImageQuant TL software (GE Healthcare) and expressed as a ratio relative to α -Tub.

Experimental design and statistical analyses. For behavioral experiments, memory scores were displayed as mean $\pm$ SEM. Scores resulting from all genotypes were analyzed using one-way ANOVA followed, if significant at $p \leq 0.05$, by Newman–Keuls multiple-comparison tests. Overall, ANOVA p value is given in the legends along with the value of the corresponding Fisher distribution $F_{(x,y)}$, where x is number of degrees of freedom for groups and y is the total number of degrees of freedom for the distribution. Asterisks in the figure denote the least significant of the pairwise *post hoc* comparisons between the genotype of interest and its controls, following the usual nomenclature. Quantitative mRNA measurements were analyzed from $2^{-\Delta C_p}$ using Student's t tests, with significance threshold set at $p \leq 0.05$. Western blot quantification was analyzed using Student's one-sample t test.

Results

Modulation of Nep1 expression in the adult MB does not impact memory of flies expressing dA β

We previously showed that Nep1 expression is required in the α/β neurons of the MB for MTM (Turrel et al., 2016). Using a

Figure 1. Modulation of Nep1 expression in the adult MB does not impact memory of flies expressing dA β . **A**, dA β expression in α/β neurons does not alter MTM ($F_{(2,35)} = 0.7471, p = 0.4816, n = 12$). **B**, dA β expression in α/β neurons does not aggravate the MTM defect caused by RNAi-Nep1A expression. $Gal80^{ts};c739/dA\beta;RNAi-Nep1A$ flies exhibit an MTM deficit that is not significantly different from that of $Gal80^{ts};c739/RNAi-Nep1A$ flies ($F_{(4,69)} = 5.086, p = 0.0013, n \geq 14$, *post hoc* Newman–Keuls test, $***p < 0.01$, $Gal80^{ts};c739/RNAi-Nep1A$ versus $Gal80^{ts};c739/+$. $*p < 0.05$, $Gal80^{ts};c739/RNAi-Nep1A$ versus $+RNAi-Nep1A$. $p > 0.05$, $Gal80^{ts};c739/RNAi-Nep1A$ versus $Gal80^{ts};c739/dA\beta;RNAi-Nep1A$). **C**, qPCR analysis of *Nep1* mRNA targeting by the RNAi-Nep1C construct. Total RNA was extracted from *elav/+* and *elav/RNAi-Nep1C* fly heads, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using *Tub* expression as a reference. Results are shown as ratios to the reference (unpaired *t* test, $t_{(6)} = 11.07, ***p < 0.0001, n = 4$) (4 values from two independent RNA

Table 1. Shock reactivity and olfactory acuity of flies expressing dA β in adult α/β neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
<i>Gal80^{ts};c739/+</i>	71.89 $\pm$ 4.101	44.18 $\pm$ 6.630	55.58 $\pm$ 8.198
<i>Gal80^{ts};c739/dAβ</i>	68.56 $\pm$ 2.799	36.45 $\pm$ 6.742	49.25 $\pm$ 7.248
<i>+dAβ</i>	63.11 $\pm$ 3.405	37.00 $\pm$ 6.520	45.27 $\pm$ 10.54

^aData are mean $\pm$ SEM. After 3 d of induction, neither shock reactivity ($F_{(2,26)} = 1.625, p = 0.2179, n = 9$) nor olfactory acuity for octanol ($F_{(2,32)} = 0.4229, p = 0.6590, n = 11$) and methylcyclohexanol ($F_{(2,34)} = 0.3573, p = 0.7, 24, n \geq 11$) is impaired in flies expressing dA β under the control of the *Gal80^{ts};c739* driver.

classical conditioning paradigm in which an odorant is paired with the delivery of electric shocks, the fly is able to form six discrete aversive memory phases reflected at the neural network level (Bouzaiane et al., 2015). Learning is measured immediately after a single conditioning, whereas MTM is assessed 1–3 h later. The fly can also produce two antagonistic forms of consolidated memory (Isabel et al., 2004). Among them, the robust LTM is only formed after multiple conditioning cycles spaced by rest intervals. Crucially, LTM is the only memory phase-dependent on *de novo* protein synthesis (Tully et al., 1994).

To find out whether amyloid peptide was a target for Nep1 for memory processes, we proceeded to modulate Nep1 expression in transgenic flies expressing *Drosophila* amyloid peptides. To this end, we used a construct allowing expression of a dA β derived from APPL (Carmin-Simmen et al., 2009). dA β ectopic expression in the MB was achieved with the *c739* driver that labels the α/β KC (Yang et al., 1995). To restrict GAL4 expression to adulthood, we used a *tub-Gal80^{ts};c739* line (*Gal80^{ts};c739*) that ubiquitously expresses a thermosensitive GAL4 inhibitor (*Gal80^{ts}*) inactive at 30°C (McGuire et al., 2003). As previously shown (Bourdet et al., 2015b), dA β expression in the adult α/β KC did not impair MTM (Fig. 1A). To determine whether the MTM deficit displayed by Nep1-knockdown flies (Turrel et al., 2016) was exacerbated by dA β expression: if Nep1 degrades dA β , then Nep1 knockdown should lead to an increase in dA β level that might impair MTM formation.

To reduce Nep1 expression, we used specific RNAi constructs. The RNAi-Nep1A construct leads to a 75% decrease in Nep1 expression in *elav/RNAi-Nep1A* flies and caused an MTM deficit when expressed in adult α/β KC (Turrel et al., 2016). Concomitant dA β overexpression did not further affect MTM of RNAi-Nep1A-expressing flies (Fig. 1B). We next used a second nonoverlapping construct, RNAi-Nep1C. qPCR experiments showed that *elav/RNAi-Nep1C* flies exhibited a 63% decrease in

extractions). **D**, dA β expression in α/β neurons does not alter MTM of RNAi-Nep1C-expressing flies. $Gal80^{ts};c739/RNAi-Nep1C$ and $Gal80^{ts};c739/dA\beta;RNAi-Nep1C$ show normal MTM ($F_{(4,71)} = 2.354, p = 0.0626, n \geq 14$). **E**, Flies expressing dA β in α/β neurons present an LTM deficit that is not alleviated by Nep1 overexpression. $Gal80^{ts};c739/dA\beta$ flies exhibit an LTM deficit ($F_{(6,83)} = 17.86, p < 0.0001, n = 12$, *post hoc* Newman–Keuls test, $***p < 0.001$, $Gal80^{ts};c739/dA\beta$ versus $Gal80^{ts};c739/+$. $***p < 0.001$, $Gal80^{ts};c739/dA\beta$ versus $+dA\beta$). Flies overexpressing Nep1 in the α/β neurons show normal LTM ($p > 0.05$, $Gal80^{ts};c739/Nep1^{EY21255}$ versus $Gal80^{ts};c739/+$. $p > 0.05$, $Gal80^{ts};c739/Nep1^{EY21255}$ versus $+Nep1^{EY21255}$). $Gal80^{ts};c739/dA\beta;Nep1^{EY21255}$ flies show an LTM deficit similar to that of $Gal80^{ts};c739/dA\beta$ ($***p < 0.001$, $Gal80^{ts};c739/dA\beta;Nep1^{EY21255}$ versus $Gal80^{ts};c739/+$. $***p < 0.001$, $Gal80^{ts};c739/dA\beta;Nep1^{EY21255}$ versus $+dA\beta;Nep1^{EY21255}$. $p > 0.05$, $Gal80^{ts};c739/dA\beta$ versus $Gal80^{ts};c739/+$). In the absence of induction, all genotypes exhibit normal LTM scores ($F_{(4,59)} = 0.7078, p = 0.5901, n = 12$). **F**, qPCR analysis of *Nep1* overexpression using the *Nep1^{EY21255}* line. Total RNA was extracted from *elav/+* and *elav/Nep1^{EY21255}* fly heads, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using *Tub* expression as a reference. Results are shown as ratios to the reference (unpaired *t* test, $t_{(14)} = 2.761, *p = 0.0153, n = 8$) (8 values from four independent RNA extractions).

Figure 2. $dA\beta$ expression is detrimental to MTM formation when combined with Nep1 knockdown in adult DPM neurons. **A**, Flies expressing $dA\beta$ in adult DPM neurons show normal MTM ($F_{(2,32)} = 0.0550$, $p = 0.9466$, $n = 11$). **B**, $dA\beta$ expression in Nep1-knockdown flies exacerbates their MTM deficit. *Gal80^{ts};VT64246/RNAi-Nep1A* flies exhibit an MTM deficit ($F_{(4,69)} = 17.62$, $p < 0.0001$, $n \geq 13$, post hoc Newman–Keuls test, $*p < 0.05$, *Gal80^{ts};VT64246/RNAi-Nep1A* versus *Gal80^{ts};VT64246/+*. $**p < 0.01$, *Gal80^{ts};VT64246/RNAi-Nep1A* versus *+RNAi-Nep1A*), which is aggravated by concomitant expression of $dA\beta$ ($***p < 0.001$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1A* versus *Gal80^{ts};VT64246/+*. $***p < 0.001$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1A* versus *+dAβ;RNAi-Nep1A*. $***p < 0.001$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1A* versus *Gal80^{ts};VT64246/RNAi-Nep1A*). In the absence of induction, all genotypes exhibit normal MTM scores ($F_{(4,54)} = 0.6991$, $p = 0.5962$, $n = 11$). **C**, Learning is not affected by $dA\beta$ expression and concomitant Nep1 knockdown in DPM neurons ($F_{(2,23)} = 1.251$, $p = 0.3066$, $n = 8$). **D**, Flies expressing RNAi-Nep1C do not exhibit an MTM deficit ($F_{(4,80)} = 8.951$, $p < 0.0001$, $n \geq 16$). In contrast, RNAi-Nep1C coexpression with $dA\beta$ in adult DPM neurons induces an MTM deficit ($**p < 0.01$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1C* versus *Gal80^{ts};VT64246/+*. $***p < 0.001$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1C* versus *+dAβ;RNAi-Nep1C*. $**p < 0.01$, *Gal80^{ts};VT64246/dAβ;RNAi-Nep1C* versus *Gal80^{ts};VT64246/RNAi-Nep1C*). In the absence of induction, all genotypes exhibit normal MTM scores ($F_{(3,66)} = 3.655$, $p = 0.0170$, $n = 17$). **E**, $dA\beta$ level is increased when Nep1 is knocked down. Western blot analyses. Left, Membranes were cut in two halves: the upper part was stained using an α -Tub antibody, and the lower part was stained using an HA-specific antibody. Right, Quantification. $dA\beta$ is expressed as a ratio to α -Tub, relative to the *Gal80^{ts};elav/dAβ* control (one-sample t test, $t_{(7)} = 2.712$, $*p = 0.0301$, $n = 8$) (8 values from eight independent extractions). Error bar indicates SEM.

Nep1 expression (Fig. 1C). We further observed that reducing Nep1 expression in adult α/β KC to a smaller extent than that previously achieved with the RNAi-Nep1A construct appeared to have no impact on MTM (Fig. 1D). Concomitant $dA\beta$ overexpression did not alter MTM (Fig. 1D). Thus, reducing Nep1 level in adult α/β KC of flies expressing amyloid peptide did not reveal any functional interaction between Nep1 and $dA\beta$.

We next analyzed LTM and observed that *Gal80^{ts};c739/dAβ* induced flies displayed a severe LTM defect, whereas noninduced flies showed normal LTM scores (Fig. 1E). We analyzed the sensory-motor capacities of the *Gal80^{ts};c739/dAβ* flies, and found that they displayed normal shock reactivity and olfactory acuity (Table 1). We conclude that $dA\beta$ expression in adult α/β KC induces an LTM deficit. We thus asked whether Nep1 overexpression would alleviate the LTM defect caused by $dA\beta$ expression. To this end, we took advantage of the *Nep1^{EY21255}* line that contains a P-element inserted upstream of the first noncoding Nep1-RA exon. This P-element construct carries UAS binding sites for the GAL4 transcriptional regulator, allowing GAL4-mediated expression of genes proximate to the site of the insertion (Bellen et al., 2004). It was previously shown that this line allows Nep1 overexpression (Panz et al., 2012). We performed

Table 2. Shock reactivity and olfactory acuity of flies expressing $dA\beta$ and Nep1-RNAi in adult DPM neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
<i>Gal80^{ts};VT64246/+</i>	66.20 $\pm$ 3.133	38.40 $\pm$ 1.485	52.80 $\pm$ 7.513
<i>Gal80^{ts};VT64246/dAβ;RNAi-Nep1A</i>	65.00 $\pm$ 3.697	39.90 $\pm$ 2.900	57.50 $\pm$ 2.655
<i>+dAβ;RNAi-Nep1A</i>	70.40 $\pm$ 3.246	36.20 $\pm$ 1.825	56.00 $\pm$ 5.914
<i>Gal80^{ts};VT64246/+</i>	63.50 $\pm$ 4.856	50.60 $\pm$ 3.387	51.00 $\pm$ 2.039
<i>Gal80^{ts};VT64246/dAβ;RNAi-Nep1C</i>	64.00 $\pm$ 5.510	54.10 $\pm$ 2.938	59.30 $\pm$ 5.281
<i>+dAβ;RNAi-Nep1C</i>	55.80 $\pm$ 4.299	55.30 $\pm$ 4.828	49.10 $\pm$ 2.433

^aData are mean $\pm$ SEM. After 3 d of induction, neither shock reactivity ($dA\beta$;RNAi-Nep1A: $F_{(2,29)} = 0.7089$, $p = 0.5011$, $n = 10$; $dA\beta$;RNAi-Nep1C: $F_{(2,29)} = 0.8753$, $p = 0.4282$, $n = 10$) nor olfactory acuity for octanol ($dA\beta$;RNAi-Nep1A: $F_{(2,29)} = 0.7452$, $p = 0.4842$, $n = 10$; $dA\beta$;RNAi-Nep1C: $F_{(2,29)} = 0.4121$, $p = 0.6664$, $n = 10$) and methylcyclohexanol ($dA\beta$;RNAi-Nep1A: $F_{(2,29)} = 0.1756$, $p = 0.8399$, $n = 10$; $dA\beta$;RNAi-Nep1C: $F_{(2,29)} = 2.325$, $p = 0.1171$, $n = 10$) is impaired in flies expressing $dA\beta$ and Nep1-RNAi under the control of the *Gal80^{ts};VT64246* driver.

qPCR experiments to assess Nep1 expression level in *elav/Nep1^{EY21255}* flies. The data show that fly heads express a 2.4-fold increase in *Nep1* mRNA level (Fig. 1F).

Flies overexpressing Nep1 (*Gal80^{ts};c739/Nep1^{EY21255}*) showed normal LTM (Fig. 1E). Simultaneous overexpression of Nep1 and $dA\beta$ in adult α/β KC did not rescue the LTM deficit caused

Figure 3. Nep1 overexpression in adult DPM neurons rescues the LTM deficit caused by dAβ expression. **A**, Nep1 overexpression in adult DPM neurons rescues the LTM deficit of dAβ-expressing flies. Flies expressing dAβ in DPM neurons exhibit a strong LTM deficit ($F_{(6,74)} = 7.606$, $p < 0.0001$, $n \geq 10$, post hoc Newman–Keuls test, *** $p < 0.001$, Gal80^{ts};VT64246/dAβ versus Gal80^{ts};VT64246/+ , *** $p < 0.001$, Gal80^{ts};VT64246/dAβ versus +/dAβ). Flies overexpressing Nep1 have normal LTM ($p > 0.05$, Gal80^{ts};VT64246/Nep1^{EY21255} versus Gal80^{ts};VT64246/+ , $p > 0.05$, Gal80^{ts};VT64246/Nep1^{EY21255} versus +/Nep1^{EY21255}). Gal80^{ts};VT64246/dAβ;Nep1^{EY21255} exhibit wild-type LTM ($p > 0.05$, Gal80^{ts};VT64246/dAβ;Nep1^{EY21255} versus Gal80^{ts};VT64246/+ , $p > 0.05$, Gal80^{ts};VT64246/dAβ;Nep1^{EY21255} versus +/dAβ;Nep1^{EY21255}). In the absence of induction, all genotypes exhibit normal LTM scores ($F_{(6,73)} = 0.6718$, $p = 0.6727$, $n \geq 10$). **B**, dAβ expression is not decreased by Nep1^{EY21255} presence. qPCR analyses of dAβ mRNA levels. Total RNA was extracted from Gal80^{ts};VT64246/dAβ and Gal80^{ts};VT64246/dAβ;Nep1^{EY21255} fly heads after induction, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using Tub expression as a reference. Results are shown as ratios to the reference (unpaired t test, $t_{(5)} = 2.774$, $p = 0.4162$, $n = 6$) (6 values from three independent RNA extractions). **C**, qPCR analysis of Nep1^{mut} expression. Total RNA was extracted from elav/+ and elav/Nep1^{mut} fly heads after induction, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using Tub expression as a reference. Results are shown as ratios to the reference (unpaired t test, $t_{(10)} = 18.55$, *** $p < 0.0001$, $n = 6$) (6 values from three independent RNA extractions). **D**, Flies overexpressing Nep1^{mut}, a mutant inactive Nep1 form, in adult DPM neurons, show normal LTM ($F_{(2,35)} = 0.1371$, $p = 0.8724$, $n = 12$). **E**, Nep1^{mut} expression does not rescue the LTM deficit caused by dAβ ($F_{(4,109)} = 6.035$, $p = 0.0002$, $n = 22$, post hoc Newman–Keuls test, * $p < 0.05$, Gal80^{ts};VT64246/dAβ vs Gal80^{ts};VT64246/+ , * $p < 0.05$, Gal80^{ts};VT64246/dAβ vs +/dAβ, ** $p < 0.01$, Gal80^{ts};VT64246/dAβ;Nep1^{mut} versus Gal80^{ts};VT64246/+ , ** $p < 0.01$, Gal80^{ts};VT64246/dAβ;Nep1^{mut} versus +/dAβ;Nep1^{mut}, $p > 0.05$, Gal80^{ts};VT64246/dAβ;Nep1^{mut} versus Gal80^{ts};VT64246/dAβ). In the absence of induction, all genotypes exhibit normal LTM scores ($F_{(4,59)} = 1.575$, $p = 0.1939$, $n = 12$).

by dAβ expression (Fig. 1E). In conclusion, we did not observe any functional interaction when Nep1 expression was modulated in the MB of dAβ-expressing flies.

dAβ expression impairs MTM formation when combined with Nep1 knockdown in adult DPM neurons

As Nep1 expression was also shown to be required in DPM neurons for memory formation (Turrel et al., 2016), we repeated the experiments using a *tub-Gal80^{ts};VT64246* GAL4-line (Gal80^{ts};VT64246) to drive specific expression in adult DPM neurons. We first expressed dAβ in adult DPM neurons. Like dAβ expression driven in KC, expression driven in adult DPM neurons did not impact MTM (Fig. 2A). As previously reported (Turrel et al., 2016), RNAi-Nep1A expression in adult DPM neurons led to an MTM defect (Fig. 2B). Strikingly, concomitant dAβ expression aggravated the MTM defect caused by Nep1 inhibition in DPM neurons (Fig. 2B). In the absence of GAL4 induction, all geno-

Table 3. Shock reactivity and olfactory acuity of flies expressing dAβ in adult DPM neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
Gal80 ^{ts} ;VT64246/+	71.70 ± 3.422	50.80 ± 5.092	56.20 ± 6.771
Gal80 ^{ts} ;VT64246/dAβ	63.90 ± 3.121	47.70 ± 6.035	49.90 ± 5.023
+ /dAβ	59.00 ± 4.683	48.70 ± 7.030	56.30 ± 4.522

^aData are mean ± SEM. After 3 d of induction, neither shock reactivity ($F_{(2,29)} = 2.836$, $p = 0.0762$, $n = 10$) nor olfactory acuity for octanol ($F_{(2,29)} = 0.0672$, $p = 0.9352$, $n = 10$) and methylcyclohexanol ($F_{(2,29)} = 0.4407$, $p = 0.6481$, $n = 10$) is impaired in flies expressing dAβ under the control of the Gal80^{ts};VT64246 driver.

types showed normal MTM scores (Fig. 2B). We verified that the ability of induced Gal80^{ts};VT64246/dAβ;RNAi-Nep1A flies to avoid electric shocks and their olfactory acuity to each odor after electric shock exposure, were unaffected (Table 2). We next analyzed learning capacity and observed that, like Gal80^{ts};VT64246/RNAi-Nep1A flies (Turrel et al., 2016), Gal80^{ts};VT64246/dAβ;

Figure 4. APPL expression is required in adult DPM neurons for MTM and LTM. **A**, Flies expressing APPL-RNAi in adult DPM neurons present MTM defects (RNAi-42673: $F_{(2,41)} = 7.990$, $p = 0.0012$, $n = 14$, *post hoc* Newman–Keuls test, $**p < 0.01$, $Gal80^{ts};VT64246/$

Table 4. Shock reactivity and olfactory acuity of flies expressing APPL-RNAi in adult DPM neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
<i>Gal80^{ts};VT64246/+</i>	73.92 ± 5.447	55.80 ± 5.557	47.80 ± 5.142
<i>Gal80^{ts};VT64246/RNAi-108312</i>	84.42 ± 3.526	65.30 ± 3.652	56.10 ± 6.689
<i>+ /108312</i>	75.92 ± 3.073	56.30 ± 5.365	55.50 ± 4.191
<i>Gal80^{ts};VT64246/+</i>	76.92 ± 4.853	41.25 ± 4.496	51.75 ± 4.563
<i>Gal80^{ts};VT64246/RNAi-42673</i>	66.92 ± 4.920	41.69 ± 6.970	48.08 ± 6.414
<i>+ /42673</i>	74.33 ± 3.085	46.63 ± 5.507	50.17 ± 6.297

^aData are mean ± SEM. After 3 d of induction, neither shock reactivity (RNAi-108312: $F_{(2,35)} = 1.809$, $p = 0.1797$, $n = 12$; RNAi-42673: $F_{(2,35)} = 1.411$, $p = 0.2581$, $n = 12$) nor olfactory acuity for octanol (RNAi-108312: $F_{(2,29)} = 1.175$, $p = 0.3242$, $n = 10$; RNAi-42673: $F_{(2,47)} = 0.2697$, $p = 0.7649$, $n = 16$) and methylcyclohexanol (RNAi-108312: $F_{(2,29)} = 0.7242$, $p = 0.4939$, $n = 10$; RNAi-42673: $F_{(2,35)} = 0.0999$, $p = 0.9052$, $n = 12$) is impaired in flies expressing APPL-RNAi under the control of the *Gal80^{ts};VT64246* driver.

RNAi-Nep1A flies exhibited normal learning (Fig. 2C). Thus, the association of Nep1 knockdown with dA β expression in adult DPM neurons does not perturb learning.

To confirm these results, we used RNAi-Nep1C. Similarly to its expression in KC, its expression driven in adult DPM neurons did not impact MTM (Fig. 2D), probably because this RNAi induces a smaller decrease in Nep1 than RNAi-Nep1A. Interestingly, whereas flies expressing only RNAi-Nep1C showed normal MTM, flies expressing both dA β and RNAi-Nep1C showed an MTM deficit (Fig. 2D). As expected, this deficit was not observed in the absence of GAL4 induction (Fig. 2D). We verified that the ability of induced *Gal80^{ts};VT64246/dA β ;RNAi-Nep1C* flies to perceive conditioning stimuli was normal (Table 2). We conclude that, in DPM neurons, Nep1 reduction makes dA β overexpression detrimental for MTM, revealing a functional link between Nep1 and dA β . We hypothesize that Nep1 reduction leads to a failure in dA β degradation, thus generating increased levels of dA β that impair memory.

To directly assess the effect of Nep1 inhibition on dA β levels, we used the HA tag-sequence inserted downstream of the dA β sequence (Carminé-Simmen et al., 2009). We used a *tub-Gal80^{ts};elav* line (*Gal80^{ts};elav*) to drive dA β pan-neuronal expression in adult flies. Head protein extracts were analyzed by Western blotting. Quantification revealed that dA β levels were increased when Nep1 was knocked down (Fig. 2E). The data indicate that Nep1 degrades dA β , consistent with functional analyses described above.

RNAi-42673 versus *Gal80^{ts};VT64246/+*. $**p < 0.01$, *Gal80^{ts};VT64246/RNAi-42673* versus *+ /RNAi-42673*; RNAi-108312: $F_{(2,37)} = 4.657$, $p = 0.0161$, $n \geq 12$, *post hoc* Newman–Keuls test, $*p < 0.05$, *Gal80^{ts};VT64246/RNAi-108312* versus *Gal80^{ts};VT64246/+*. $*p < 0.05$, *Gal80^{ts};VT64246/RNAi-108312* versus *+ /RNAi-108312*. **B**, Flies expressing APPL-RNAi in adult DPM neurons present LTM defects (RNAi-42673: $F_{(2,46)} = 13.85$, $p < 0.0001$, $n \geq 15$, *post hoc* Newman–Keuls test, $***p < 0.001$, *Gal80^{ts};VT64246/RNAi-42673* versus *Gal80^{ts};VT64246/+*. $**p < 0.01$, *Gal80^{ts};VT64246/RNAi-42673* versus *+ /RNAi-42673*; RNAi-108312: $F_{(2,63)} = 4.530$, $p = 0.0146$, $n \geq 21$, *post hoc* Newman–Keuls test, $*p < 0.05$, *Gal80^{ts};VT64246/RNAi-108312* versus *Gal80^{ts};VT64246/+*. $*p < 0.05$, *Gal80^{ts};VT64246/RNAi-108312* versus *+ /RNAi-108312*. **C**, In the absence of induction, all genotypes exhibit normal MTM scores (RNAi-42673: $F_{(2,29)} = 1.250$, $p = 0.3025$, $n = 10$; RNAi-108312: $F_{(2,23)} = 0.6399$, $p = 0.5373$, $n = 8$). **D**, In the absence of induction, all genotypes exhibit normal LTM scores (RNAi-42673: $F_{(2,57)} = 1.213$, $p = 0.3051$, $n \geq 19$; RNAi-108312: $F_{(2,59)} = 2.506$, $p = 0.0905$, $n \geq 19$). **E**, Learning is not affected by APPL-RNAi expression in adult DPM neurons (RNAi-42673: $F_{(2,23)} = 6.108$, $p = 0.0081$, $n = 8$, *post hoc* Newman–Keuls test, $p > 0.05$, *Gal80^{ts};VT64246/RNAi-42673* versus *Gal80^{ts};VT64246/+*. $**p < 0.01$, *Gal80^{ts};VT64246/RNAi-42673* versus *+ /RNAi-42673*; RNAi-108312: $F_{(2,23)} = 0.6399$, $p = 0.5373$, $n = 8$).

Figure 5. dBACE overexpression aggravates MTM and LTM defects caused by Nep1 knock-down in adult DPM neurons. **A**, dBACE overexpression in adult DPM neurons does not alter MTM ($F_{(2,35)} = 0.0788$, $p = 0.9244$, $n = 12$) or LTM ($F_{(2,36)} = 0.0072$, $p = 0.9928$, $n \geq 11$). **B**, dBACE overexpression in DPM neurons exacerbates the MTM deficit caused by Nep1 knock-down ($F_{(4,42)} = 14.39$, $p < 0.0001$, $n \geq 8$, *post hoc* Newman–Keuls test, $*p < 0.05$, Gal80^{ts};VT64246/RNAi-Nep1A versus Gal80^{ts};VT64246/+). $*p < 0.05$, Gal80^{ts};VT64246/RNAi-Nep1A versus +/RNAi-Nep1A. $***p < 0.001$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus Gal80^{ts};VT64246/+). $***p < 0.001$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus +/dBACE;RNAi-Nep1A. $**p < 0.01$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus Gal80^{ts};VT64246/RNAi-Nep1A. In the absence of induction, all genotypes exhibit normal MTM scores ($F_{(4,49)} = 0.3999$, $p = 0.8077$, $n = 10$). **C**, dBACE overexpression in DPM neurons exacerbates the LTM deficit caused by Nep1 knock-down ($F_{(4,66)} = 15.06$, $p < 0.0001$, $n \geq 13$, *post hoc* Newman–Keuls test, $***p < 0.001$, Gal80^{ts};VT64246/RNAi-Nep1A versus Gal80^{ts};VT64246/+). $***p < 0.001$, Gal80^{ts};VT64246/RNAi-Nep1A versus +/RNAi-Nep1A. $***p < 0.001$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus Gal80^{ts};VT64246/+). $***p < 0.001$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus +/dBACE;RNAi-Nep1A. $*p < 0.05$, Gal80^{ts};VT64246/dBACE;RNAi-Nep1A versus Gal80^{ts};VT64246/RNAi-Nep1A. In the absence of induction, all genotypes exhibit normal LTM scores ($F_{(4,51)} = 0.1327$, $p = 0.9696$, $n \geq 10$).

Table 5. Shock reactivity and olfactory acuity of flies expressing dBACE and RNAi-Nep1A in adult DPM neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
Gal80 ^{ts} ;VT64246/+	80.40 ± 4.425	68.80 ± 5.705	65.10 ± 4.233
Gal80 ^{ts} ;VT64246/dBACE;RNAi-Nep1A	78.40 ± 3.219	68.90 ± 5.248	64.00 ± 5.134
+ /dBACE;RNAi-Nep1A	80.20 ± 4.828	65.10 ± 2.799	70.70 ± 3.448

^aData are mean ± SEM. After 3 d of induction, neither shock reactivity ($F_{(4,47)} = 0.3371$, $p = 0.8515$, $n \geq 8$) nor olfactory acuity for octanol ($F_{(4,47)} = 0.3231$, $p = 0.8610$, $n \geq 8$) and methylcyclohexanol ($F_{(4,47)} = 0.5304$, $p = 0.7140$, $n \geq 8$) is impaired in flies expressing dBACE and RNAi-Nep1A under the control of the Gal80^{ts};VT64246 driver.

Nep1 overexpression in adult DPM neurons rescues the LTM defect caused by dA β expression

We next examined the Nep1–dA β interaction in LTM formation. Like expression in KC, dA β expression in DPM neurons caused a strong LTM defect (Fig. 3A). In the absence of induction, no LTM defect was observed (Fig. 3A). We tested the sensory-motor capacities of Gal80^{ts};VT64246/dA β flies, and found that they displayed wild-type shock reactivity and olfactory acuity (Table 3). We conclude that, like expression in the MB, dA β expression in adult DPM neurons induces an LTM deficit. We went on to determine whether Nep1 overexpression could overcome the negative effect of dA β expression on LTM. We observed that Gal80^{ts};VT64246/Nep1^{EY21255} flies overexpressing Nep1 in adult DPM neurons showed normal LTM (Fig. 3A). Strikingly, flies that overexpressed Nep1 in addition to dA β in adult DPM neurons showed wild-type LTM (Fig. 3A), suggesting that Nep1 enzyme could degrade dA β . We reasoned that the presence of two UAS-GAL4 could result in the decreased expression of each of them caused by GAL4 dilution. Thus, to check that the rescued phenotype observed was not caused by a decrease in dA β expression, we performed qPCR experiments to measure dA β mRNA level in Gal80^{ts};VT64246/dA β and Gal80^{ts};VT64246/dA β ;Nep1^{EY21255} fly heads. We observed that dA β expression was not decreased by the presence of Nep1^{EY21255} (Fig. 3B). We thus conclude that, in DPM neurons, Nep1 overexpression rescues the LTM deficit caused by dA β expression.

Finally, we examined whether the capacity of Nep1 overexpression to overcome dA β -induced LTM defect was mediated by its catalytic activity. To this end, we generated a Nep1 form with a mutation in its catalytic site (Devault et al., 1988). To verify the expression level of this mutated Nep1 protein (Nep1^{mut}), we performed qPCR experiments with oligonucleotides that amplify Nep1 both wild-type and mutant sequences. We observed that Nep1 mRNA levels were increased 5.7-fold in *elav*/Nep1^{mut} fly heads compared with the *elav*/+ control, showing efficient Nep1^{mut} expression (Fig. 3C). Nep1^{mut} expression in adult DPM neurons did not affect LTM (Fig. 3D). Flies expressing both dA β and Nep1^{mut} showed LTM scores similar to that of flies expressing only dA β (Fig. 3E). Thus, unlike wild-type Nep1 overexpression, that of Nep1^{mut} does not rescue the LTM defect caused by dA β expression in adult DPM neurons, indicating that the LTM rescue previously observed depends on Nep1 catalytic activity.

APPL is required in adult DPM neurons for both MTM and LTM

As we evidenced a functional interaction between ectopic dA β and Nep1 activity in adult DPM neurons, we next investigated whether DPM neurons could physiologically express the amyloid peptide. Because APPL is highly expressed in the MB, it could reveal difficult to determine with immunohistochemistry experiments staining corresponding to DPM neuritis in addition to MB

Figure 6. Nep1 overexpression rescues the memory deficit caused by APPL and dBACE coexpression in adult DPM neurons. **A**, Nep1 overexpression rescues the MTM deficit caused by APPL and dBACE coexpression in adult DPM neurons. APPL overexpression in DPM neurons does not alter MTM ($F_{(2,35)} = 1.575$, $p = 0.2222$, $n = 12$). Flies coexpressing APPL and dBACE in DPM neurons present an MTM defect ($F_{(6,70)} = 4.559$, $p = 0.0007$, $n \geq 10$, post hoc Newman–Keuls test, $***p < 0.001$, *Gal80^{ts};VT64246/*

lobes. Instead, we asked whether APPL expression, if any, was required for memory in DPM neurons. We used two nonoverlapping RNAi constructs (RNAi-108312, Bourdet et al., 2015a; RNAi-42673, Goguel et al., 2011) to specifically inhibit APPL expression in adult DPM neurons. Interestingly, *Gal80^{ts};VT64246/RNAi-42673* and *Gal80^{ts};VT64246/RNAi-108312* flies displayed both mutant MTM (Fig. 4A) and mutant LTM (Fig. 4B). Noninduced flies displayed normal MTM (Fig. 4C) and LTM scores (Fig. 4D), showing that the deficits were specifically caused by induction of APPL RNAi in adult DPM neu-

Gal80^{ts};VT64246/+. $***p < 0.001$, *Gal80^{ts};VT64246/dBACE;APPL* versus *+dBACE;APPL*. Flies overexpressing Nep1 exhibit normal MTM ($p > 0.05$, *Gal80^{ts};VT64246/Nep1^{EY21255}* versus *Gal80^{ts};VT64246/+*, $p > 0.05$, *Gal80^{ts};VT64246/Nep1^{EY21255}* versus *+Nep1^{EY21255}*). Flies coexpressing dBACE, APPL, and Nep1 present wild-type MTM ($p > 0.05$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *Gal80^{ts};VT64246/+*, $p > 0.05$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *+dBACE;APPL;Nep1^{EY21255}*, $***p < 0.01$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *Gal80^{ts};VT64246/dBACE;APPL*). In the absence of induction, all genotypes exhibit normal MTM scores ($F_{(2,29)} = 0.7477$, $p = 0.4830$, $n = 10$). **B**, Nep1 overexpression rescues the LTM deficit caused by APPL and dBACE coexpression in adult DPM neurons. APPL overexpression in DPM neurons does not alter LTM ($F_{(2,23)} = 0.7440$, $p = 0.4873$, $n = 8$). Flies coexpressing APPL and dBACE in DPM neurons present an LTM defect ($F_{(6,69)} = 7.299$, $p < 0.0001$, $n = 10$, post hoc Newman–Keuls test, $***p < 0.001$, *Gal80^{ts};VT64246/dBACE;APPL* versus *Gal80^{ts};VT64246/+*, $***p < 0.001$, *Gal80^{ts};VT64246/dBACE;APPL* versus *+dBACE;APPL*). Flies coexpressing dBACE, APPL, and Nep1 present wild-type LTM ($p > 0.05$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *Gal80^{ts};VT64246/+*, $p > 0.05$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *+dBACE;APPL;Nep1^{EY21255}*, $***p < 0.001$, *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* versus *Gal80^{ts};VT64246/dBACE;APPL*). In the absence of induction, all genotypes exhibit normal LTM scores ($F_{(2,29)} = 0.6927$, $p = 0.5089$, $n = 10$). **C**, **D**, qPCR experiments. Total RNA was extracted from *Gal80^{ts};VT64246/+*, *Gal80^{ts};VT64246/dBACE;APPL*, and *Gal80^{ts};VT64246/dBACE;APPL;Nep1^{EY21255}* fly heads after induction, and further reverse-transcribed with oligo(dT) primers. Resulting cDNA was quantified using Tub expression as a reference. Results are shown as ratios to the reference. **C**, Analysis of APPL and dBACE overexpression driven by *Gal80^{ts};VT64246* (*Appl*: unpaired t test, $t_{(18)} = 3.328$, $***p = 0.0037$, $n = 10$ (10 values from five independent RNA extractions); *dBACE*: unpaired t test, $t_{(10)} = 13.83$, $***p < 0.0001$, $n = 6$ (6 values from three independent RNA extractions)). **D**, *Appl* and *dBACE* mRNA expression is not modified by the presence of Nep1^{EY21255}. (*Appl*: unpaired t test, $t_{(10)} = 0.08343$, $p = 0.4236$, $n = 6$ (6 values from three independent RNA extractions); *dBACE*: unpaired t test, $t_{(6)} = 2.319$, $p = 0.0595$, $n = 4$ (4 values from two independent RNA extractions)). **E**, Schematic interpretation. **E1**, In a wild-type context, *dA β* is degraded by Nep1 to ensure wild-type memory. **E2**, APPL and dBACE co-overexpression leads to an increased level of *dA β* that is detrimental to memory formation. **E3**, The memory deficit caused by *dA β* overproduction is overcome by an increase in its degradation by Nep1. Blue circles represent *dA β* .

rons. Next, we analyzed learning and observed that RNAi-APPL induced flies showed normal learning performance (Fig. 4E). We checked that induced flies showed normal shock reactivity and normal olfactory acuity to each odor after electric shock exposure (Table 4). We conclude that, in addition to expression in the MB, APPL expression in DPM neurons is required for both MTM and LTM processes. APPL being expressed in DPM neurons, dA β may possibly be produced physiologically by adult DPM neurons.

dBACE overexpression aggravates MTM and LTM defects caused by Nep1 knockdown in adult DPM neurons

We next asked whether overexpression of endogenous dA β impaired memory. The first enzyme responsible for dA β synthesis is the dBACE rate-limiting enzyme (Carmine-Simmen et al., 2009). Using an UAS-dBACE construct that encodes the fly β -secretase (Carmine-Simmen et al., 2009), we analyzed the effect on memory of dBACE overexpression in adult DPM neurons. After induction, *Gal80^{ts};VT64246/dBACE* flies showed both normal MTM and normal LTM (Fig. 5A). If dBACE overexpression results in dA β overproduction, dA β level remains insufficient to disrupt memory. We reasoned that, if Nep1 can degrade endogenous A β peptide, Nep1 knockdown should cause a further increase in dA β level that could thus reach a level where it could impair memory. Interestingly, flies expressing both RNAi-Nep1A and dBACE in DPM neurons showed an MTM deficit significantly higher than that of flies expressing only RNAi-Nep1A (Fig. 5B). Noninduced *Gal80^{ts};VT64246/dBACE;RNAi-Nep1A* flies displayed normal MTM scores (Fig. 5B). Similar results were observed for LTM: flies expressing both RNAi-Nep1A and dBACE in DPM neurons showed a significantly higher LTM deficit than flies expressing only RNAi-Nep1A, whereas in the absence of GAL4 induction, these flies showed normal scores (Fig. 5C). We verified that induced flies showed normal response to electric shock exposure and normal olfactory acuity (Table 5). Together, the data reveal a functional interaction between dBACE and Nep1, suggesting that dBACE overexpression produces a substrate for Nep1. On Nep1 knockdown, an accumulation of this substrate, presumably dA β , impairs memory.

Nep1 overexpression rescues the LTM deficit caused by APPL and dBACE coexpression in adult DPM neurons

It has been shown that dBACE constitutive neuronal coexpression with APPL generates numerous amyloid deposits in old flies (Carmine-Simmen et al., 2009). As we observed that dBACE overexpression alone did not impair memory, we analyzed dBACE coexpression with APPL. First, we assessed APPL overexpression alone. Like dBACE, APPL overexpression driven in adult DPM neurons did not impact MTM (Fig. 6A). By contrast, flies overexpressing both APPL and dBACE showed decreased MTM scores compared with control flies (Fig. 6A). In the absence of GAL4 induction, these flies showed normal MTM (Fig. 6A). We verified that induced *Gal80^{ts};VT64246/APPL;dBACE* flies displayed normal responses to the conditioning stimuli (Table 6). In conclusion, while neither APPL nor dBACE overexpression alone impacts MTM, concomitant overexpression does, and this effect is very likely caused by an overproduction of dA β . We further observed that flies overexpressing Nep1 in addition to APPL and dBACE showed normal MTM (Fig. 6A), indicating that Nep1 overexpression rescues the MTM deficit generated by APPL and dBACE coexpression. We conclude that Nep1 acts in the same pathway as APPL and dBACE.

Table 6. Shock reactivity and olfactory acuity of flies expressing dBACE and APPL in adult DPM neurons^a

Genotype	Shock reactivity	Olfactory acuity	
		Octanol	Methylcyclohexanol
<i>Gal80^{ts};VT64246/+</i>	72.70 $\pm$ 6.756	51.20 $\pm$ 2.851	55.10 $\pm$ 1.798
<i>Gal80^{ts};VT64246/dBACE;APPL</i>	81.78 $\pm$ 5.325	57.00 $\pm$ 3.864	65.30 $\pm$ 8.215
<i>+ /dBACE;APPL</i>	69.00 $\pm$ 4.415	56.70 $\pm$ 6.136	58.60 $\pm$ 5.431

^aData are mean $\pm$ SEM. After 3 d of induction, neither shock reactivity ($F_{(2,29)} = 0.0684$, $p = 0.9341$, $n = 10$) nor olfactory acuity for octanol ($F_{(2,29)} = 0.2071$, $p = 0.8142$, $n = 10$) and methylcyclohexanol ($F_{(2,29)} = 0.6896$, $p = 0.5104$, $n = 10$) is impaired in flies expressing dBACE and APPL under the control of the *Gal80^{ts};VT64246* driver.

We next analyzed LTM. Like dBACE, APPL overexpression in adult DPM neurons had no impact on LTM (Fig. 6B). In sharp contrast, APPL and dBACE coexpression caused an LTM deficit that was not observed in the absence of induction (Fig. 6B). Strikingly, concomitant overexpression of Nep1 resulted in normal LTM (Fig. 6B).

We then checked that flies coexpressing APPL, dBACE, and Nep1 expressed APPL and dBACE at similar levels to flies coexpressing only APPL and dBACE. We first examined whether APPL and dBACE overexpression could be assessed by qPCR experiments driven on cDNA synthesized from *Gal80^{ts};VT64246/APPL;dBACE* fly heads RNA. We observed a significant increase in APPL level in *Gal80^{ts};VT64246/APPL;dBACE* fly heads compared with that of the control (Fig. 6C). In addition, we observed a substantial increase in dBACE expression in *Gal80^{ts};VT64246/APPL;dBACE* flies compared with the control (Fig. 6C). We next observed that flies coexpressing APPL, dBACE, and Nep1 did not express less APPL, or less dBACE, than flies coexpressing only APPL and dBACE (Fig. 6D).

In conclusion, the data show that the negative effect on MTM and LTM of APPL and dBACE coexpression is rescued by Nep1 overexpression (Fig. 6E).

Discussion

We showed previously that Nep1 is required in KC and DPM neurons for MTM and LTM, revealing that one or several neuro-peptides need to be degraded for memory formation. Here we report that, in adult DPM neurons, Nep1 inhibition makes dA β expression detrimental to both MTM and LTM. Conversely, Nep1 overexpression rescues dA β -induced memory defects. Together, the data strongly suggest that Nep1 expressed in DPM neurons degrades dA β , raising the possibility that dA β is a Nep1 physiological substrate for memory processes.

Drosophila is currently used to model AD, in particular A β -induced toxicity (Iijima et al., 2004; Fang et al., 2012; Lin et al., 2014; Niccoli et al., 2016; Sofola-Adesakin et al., 2016). Transgenic flies overexpressing human A β 42 in the eyes display dose- and age-dependent phenotypes that are alleviated by Nep2 upregulation (Finelli et al., 2004; Cao et al., 2008). One of the limits of these models is that ectopic human A β is expressed constitutively, and is therefore highly toxic, probably by disrupting normal development and/or cell physiology. Here we performed an inducible analysis of dA β expression in young flies, thereby providing a more physiological model. We observe identical memory phenotypes when dA β is expressed either in KC or in DPM neurons of young flies: MTM is normal, whereas LTM is disrupted. One hypothesis is that dA β expression alters general functional features of the neuron biology and that its toxicity is therefore unspecific to memory. However, dA β -expressing flies show normal reactivity to the conditioning stimuli used and display normal MTM, suggesting that such dA β production might instead interfere with specific mechanisms in play for memory

formation. One hypothesis is that dA β impacts mechanisms specific to LTM. Alternatively, dA β expression level may be an important feature for both MTM and LTM: dA β levels reached in our experiments may alter LTM but may not be sufficient to alter MTM. We favor this latter hypothesis as APPL and dBACE coexpression disrupts both MTM and LTM. Indeed, as overexpression of APPL or dBACE alone does not affect memory, we conclude that memory defects observed on coexpression result from APPL processing by dBACE, and thus very likely from dA β production.

In addition to A β , APP complex processing leads to numerous biologically active fragments that are secreted, membranous or intracellular (O'Brien and Wong, 2011; Zheng and Koo, 2011). We previously showed that a secreted APPL and a noncleaved APPL form were involved in MTM in adult α/β KC (Bourdet et al., 2015a). We now show that APPL expression is also required in DPM neurons. Interestingly, APPL conditional silencing specifically in KC or in DPM neurons leads to similar phenotypes: MTM and LTM are impaired, whereas learning remains unaffected. These data highlight the close relationship between DPM neurons and KC, a circuit thought to stabilize memories (Keene et al., 2004; Pitman et al., 2011), and suggest that APPL plays similar roles in both neuronal structures. In mammals, APP is expressed at presynaptic and postsynaptic sites (Yamazaki et al., 1995; Back et al., 2007; Hoe et al., 2009), and APP/APP transdimers have been shown to play a role in synaptic connectivity (Wang et al., 2009). It is therefore possible that APPL/APPL transdimers play a role at KC/DPM synapses. Another hypothesis is that an APPL fragment secreted from one cell type is a ligand for APPL located at the membrane of the other cell type, consistent with reports suggesting that APPL may function as a receptor (Cassar and Kretschmar, 2016).

Nep peptidases constitute the main A β -degrading enzymes in mammals. They have also been implicated in diverse pathologies, but their substrates have remained poorly described. In *Drosophila*, a nonidentified neprilysin-like neuropeptidase was shown to play a role in the regulation of the pigment-dispersing factor signaling within circadian neural circuits (Isaac et al., 2007). More recently, Nep4 was shown to be involved in the expression of a number of insulin-like peptides (Hallier et al., 2016). Our data show that an increased expression of dA β is detrimental to memory. In a context where dA β level is below a deleterious threshold, knocking down Nep1 is sufficient to raise this level above the threshold, thus leading to memory deficits. Conversely, if dA β level is above a deleterious threshold, concomitant overexpression of Nep1 restores wild-type memory. Although a noncatalytic role has been described for Nep4 intracellular domain (Panz et al., 2012), Nep1 catalytic activity is required to observe the rescue of the dA β -induced memory deficit, indicating that Nep1 degrades dA β . This conclusion is further supported by Western blot experiments showing that Nep1 inhibition results in an increase in dA β levels. Together, our data strongly suggest that Nep1, a membrane proteinase whose active site faces the extracellular compartment, cleaves and inactivates dA β in the extracellular space, consistent with the consensus that Nep function is to turn off neuropeptide signals at the synapse (Turner, 2003).

A functional interaction between Nep1 and dA β is evidenced when modulation of their expression is achieved in DPM neurons, and not in KC. One hypothesis is that Nep1 is not expressed at the synapses where dA β is secreted in α/β KC. Another possibility is that Nep1 is differently regulated in α/β KC and DPM neurons, leading to distinct specificity: Nep peptidases exhibit high substrate variability and are thus known to target numerous

peptides (Turner et al., 2001; Nalivaeva et al., 2012). A recent study suggested that Nep4 activity in the CNS affected different neuropeptide regulatory systems from the corresponding muscle-bound activity (Hallier et al., 2016). Thus, Nep1 expressed at DPM membranes could target dA β , and a distinct peptide when expressed in KC.

Learning and memory are associated with activity-dependent functional plasticity and changes in the structure and number of synapses (Korte and Schmitz, 2016). In AD patients, synaptic dysfunction has been closely correlated with cognitive decline (Querfurth and LaFerla, 2010). A large body of evidence points to a pathological role of A β , particularly as a primary cause of synaptic failure (Hardy and Selkoe, 2002; O'Brien and Wong, 2011). Importantly, A β is present in the CSF of healthy individuals, although at much lower concentrations than its neurotoxic concentrations, suggesting the possibility of a physiological role for A β (Fedele et al., 2015). In primary cultures of neurons, immunodepletion of endogenous A β causes neuronal cell death, a phenomenon rescued by addition of physiological picomolar levels of A β peptides (Plant et al., 2003). Furthermore, several reports have shown that, at very low physiological concentrations, A β modulates synaptic strength (Kamenetz et al., 2003; Abramov et al., 2009). Puzzo et al. (2008) reported that picomolar amounts of A β were able to enhance LTP and hippocampal-dependent memory in mice, whereas nanomolar concentrations of the peptide gave opposite results. Several additional studies confirmed the role of A β at low concentrations in synaptic plasticity and memory (Garcia-Osta and Alberini, 2009; Morley et al., 2010; Puzzo et al., 2011), suggesting that A β physiologically produced in the healthy brain during neuronal activity is needed for memory processes. Thus, A β appears to be a modulator of synaptic activity whose concentration needs to be strictly regulated: at the level of its production, degradation, or trafficking. We report here in *Drosophila* that memory disruption caused by an increased synthesis of dA β is rescued by Nep1 overexpression, raising the possibility that Nep-dependent degradation of dA β plays a physiological role in memory. A deficit in A β degradation would thus trigger memory deficits, the first cognitive signs of AD. Such mechanisms are consistent with evidence showing that late-onset AD is associated with impaired clearance of A β (Mawuenyega et al., 2010), and that AD brains show decreased levels of Nep expression and activity (Yasojima et al., 2001; Caccamo et al., 2005; Wang et al., 2010; Zhou et al., 2013).

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