

# Amnesiac Is Required in the Adult Mushroom Body for Memory Formation

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It was proposed that the *Drosophila amnesiac* gene (*amn*) is required for consolidation of aversive memory in the dorsal paired medial (DPM) neurons, a pair of large neurons that broadly innervate the mushroom bodies (MB), the fly center for olfactory learning and memory (Waddell et al., 2000). Yet, a conditional analysis showed that it was not possible to rescue the memory deficit of *amn*<sup>X8</sup> null mutant flies when *amn* expression was restored only in the adult (DeZazzo et al., 1999), which led the authors to suggest that *amn* might be involved in the development of brain structures that normally promote adult olfactory memory. To further investigate temporal and spatial requirements of Amnesiac (AMN) peptide in memory, we used RNA interference in combination with conditional drivers. Experiments were conducted either in both sexes, or in either sexes. Our data show that acute modulation of *amn* expression in adult DPM neurons does not impact memory. We further show that *amn* expression is required for normal development of DPM neurons. Detailed enhancer trap analyses suggest that *amn* transcription unit contains two distinct enhancers, one specific of DPM neurons, and the other specific of  $\alpha/\beta$  MB neurons. This prompted us to investigate extensively the role of AMN in the adult MB. Together, our results demonstrate that *amn* is acutely required in adult  $\alpha/\beta$  MB neurons for middle-term and long-term memory. The data thus establish that *amn* plays two distinct roles. Its expression is required in DPM neurons for their development, and in adult MB for olfactory memory.

**Key words:** amnesiac; dorsal paired medial neurons; *Drosophila*; mushroom body; olfactory memory

## Significance Statement

The *Drosophila amnesiac* gene encodes a neuropeptide whose expression was proposed to be required for consolidation of aversive memory in the dorsal paired medial (DPM) neurons, a pair of large neurons that broadly innervate the mushroom bodies (MB), the olfactory memory center. Here, we investigated *amnesiac* temporal and spatial requirement using conditional tools that allowed us to manipulate its expression in selected neurons. This work leads to a complete reassessment of the role of *amnesiac* in brain development and memory. We show that *amnesiac* is required for two distinct processes: for normal development of DPM neurons, and in adult MB for memory.

## Introduction

*Drosophila* is well suited to studying memory formation. The mushroom bodies (MB) constitute the central integrative brain

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structure for olfactory memory (de Belle and Heisenberg, 1994). They are composed of 4000 intrinsic neurons, called Kenyon cells (KC), classed into three subtypes whose axons form two vertical ( $\alpha$  and  $\alpha'$ ) and three medial ( $\beta$ ,  $\beta'$ , and  $\gamma$ ) lobes (Crittenden et al., 1998). Using a classical conditioning paradigm in which an odorant is paired with the delivery of electric shocks, the fly is capable of forming six discrete memory phases reflected at the neural network level (Bouzaiane et al., 2015). Memory measured 2 h after a single conditioning is a composite memory formed of labile middle-term memory (MTM) encoded in the  $\alpha/\beta$  neurons, and middle-term anesthesia-resistant memory encoded in  $\gamma$  KC. The fly can also produce two exclusive forms of consolidated memory measured 24 h after training (Isabel et al., 2004): long-term anesthesia-resistant memory (LT-ARM) is formed after five massed cycles of conditioning, whereas the robust long-term

memory (LTM) is only formed after five spaced cycles. Crucially, LTM is the only memory phase dependent on *de novo* protein synthesis (Tully et al., 1994). LT-ARM and LTM are encoded in  $\alpha'/\beta'$  and  $\alpha/\beta$  KC, respectively.

The *Drosophila amnesiac* (*amn*) mutant was first isolated in a behavioral screen for memory defects (Quinn et al., 1979). The *amn* gene encodes a predicted neuropeptide precursor with a signal sequence (Feany and Quinn, 1995). Several reports have implicated AMN in activation of the cAMP pathway (Moore et al., 1998; Bhattacharya et al., 2004). Although the mature products have not been identified, sequence analyses point to three neuropeptides, one of which is homologous to the mammalian pituitary adenylate cyclase-activating polypeptide (PACAP) (Feany and Quinn, 1995; Hashimoto et al., 2002). PACAP is a neuropeptide that is widely expressed throughout the brain and acts as a neuromodulator or neurotrophic factor through activation of G-protein-linked receptors. It has been shown to regulate a variety of physiological processes through stimulation of cAMP production (Miyata et al., 1989; Arimura, 1998; Vaudry et al., 2000). As in mammals, the fly cAMP/PKA pathway plays a key role in associative memory, the adenylate cyclase Rutabaga being thought to act as a coincidence detector in the MB to associate the olfactory and electric-shock pathways (Levin et al., 1992; Tomchik and Davis, 2009; Gervasi et al., 2010).

The Amnesiac (AMN) peptide is expressed in dorsal paired medial (DPM) neurons, a pair of large serotonergic neurons that intensively innervate all lobes of the MB (Waddell et al., 2000; Lee et al., 2011). DPM output was shown to be required during the consolidation phase for MTM, prompting the notion that DPM neurons might release the AMN modulatory neuropeptide which alters the physiology of MB neurons to help stabilize or consolidate odor memories (Keene et al., 2004). Despite many studies addressing AMN role in memory, its function has remained unclear. Noteworthy, memory rescue experiments succeeded when a wild-type *amn* transgene was expressed throughout development including the adult stage (DeZazzo et al., 1999; Waddell et al., 2000), but not when its expression was limited to the adult stage (DeZazzo et al., 1999), suggesting that *amn* is required for development.

Here, to clarify the role of *amn* in memory, we used conditional RNA interference (RNAi) to manipulate *amn* expression with the spatial and temporal control allowed by Gal4 drivers. As expected, constitutive expression of *amn*-specific RNAi in DPM neurons mimicked memory phenotypes previously described for *amn* mutants. In sharp contrast, conditional expression in adult DPM neurons did not impact memory. Furthermore, we observed abnormal DPM morphology in *amn*-deficient flies. Together, the data show that *amn* expression in DPM neurons is required for neuronal development. Strikingly, we next show that AMN plays a key role in adult  $\alpha/\beta$  neurons for MTM and LTM formation. Thus AMN plays two distinct roles: it is required for DPM neuronal development, and in the adult MB for memory.

## Materials and Methods

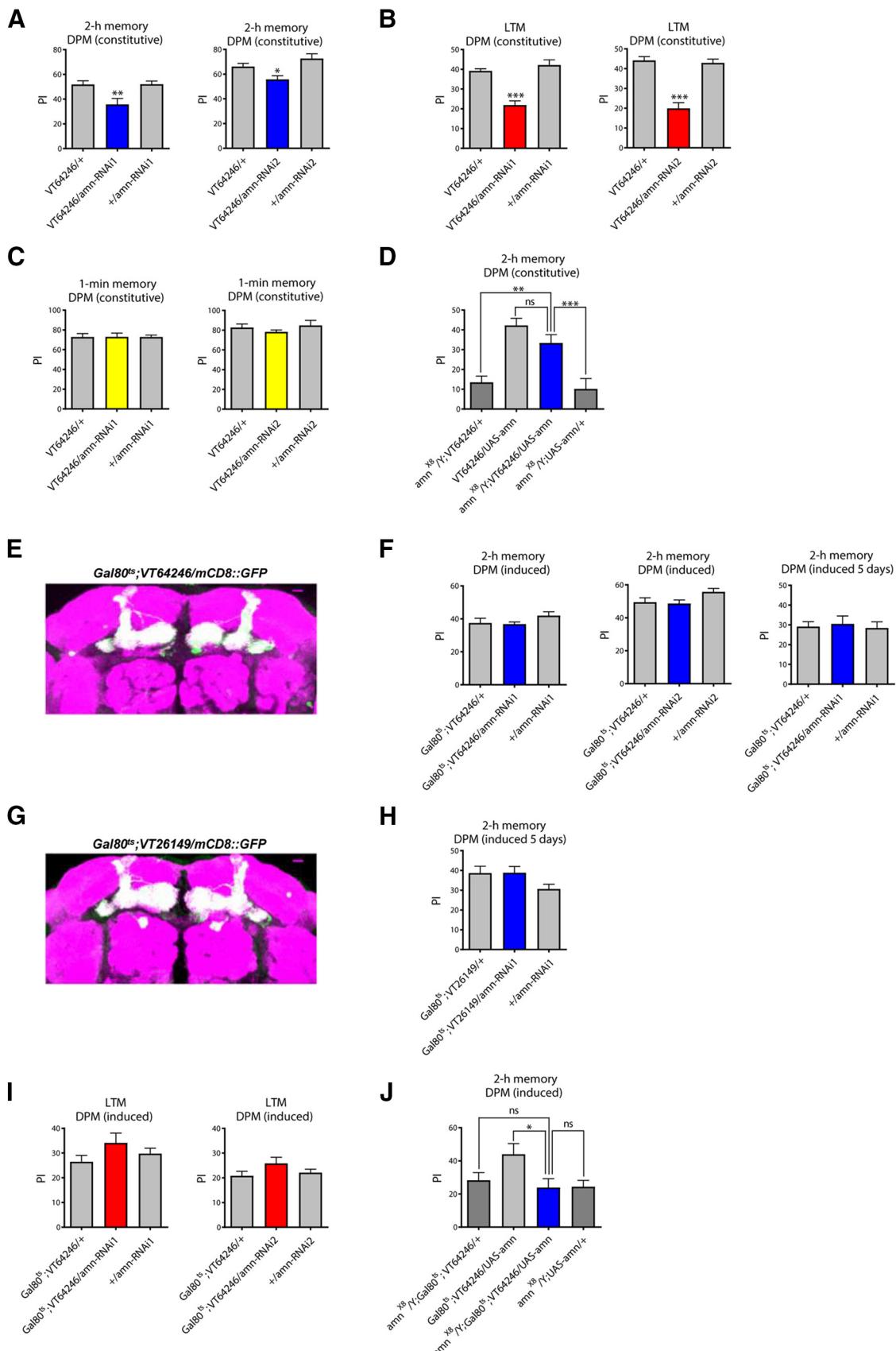
**Drosophila stocks.** *Drosophila melanogaster* wild-type strain Canton-Special (Canton-S) and mutant flies were raised on standard medium at 18°C in 60% humidity in a 12 h light/dark cycle. All strains used for memory experiments were outcrossed to the Canton-S background. *amn-RNAi* lines were obtained from the Vienna Drosophila Resource Center (VDRC; *amn-RNAi1*, 5606) and from the Bloomington Drosophila Stock Center (Indiana University; *amn-RNAi2*, 25797). GAL4 drivers used to achieve RNAi expression were *VT64246* (VDRC, 264246) and *VT26149* (VDRC, 204563) for expression in DPM neurons, 238Y, *c747* and *VT30559* (VDRC, 206077) for expression in the MB, *c739* for

expression in  $\alpha/\beta$  neurons, *VT30604* (VDRC, 200228) for expression in  $\alpha'/\beta'$  neurons, and *VT49483* (VDRC, 206419) for expression in  $\gamma$  neurons. To specifically induce RNAi expression in adults, we took advantage of the TARGET system (McGuire et al., 2003). To achieve RNAi induction, flies were kept at 30°C for 3 or 5 d before conditioning, and also until memory test for LTM and LT-ARM analyses. To specifically block RNAi induction in the MB, *MB247-Gal80* (MB-Gal80; Krashes et al., 2007) was used. Rescue experiments were conducted with the *UAS-amn* construct described by Waddell et al. (2000).

**Behavioral experiments.** Flies were trained with classical olfactory aversive conditioning protocols as described by Pascual and Préat (2001). Training and testing were performed at 25°C in 80% humidity. Conditioning was performed on samples of 25–35 flies aged between 3 and 4 d with 3-octanol (~95% purity; Sigma-Aldrich) and 4-methylcyclohexanol (99% purity; Sigma-Aldrich) at 0.360 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 sum of flies in both arms. The average of two reciprocal experiments gave a performance index (PI). For analyses of immediate memory, flies were tested immediately after a single training cycle (1 min memory). To assess MTM, flies were submitted to one-cycle training, and memory was tested 2 h later. Cold anesthesia was achieved by a 2 min cold shock performed 1 h before the test. For LTM analyses, flies were trained with five cycles spaced at 15 min rest intervals, and tested 24 h later, and for LT-ARM analyses, flies were submitted to five massed conditioning cycles and memory was tested 24 h later. For odor avoidance tests after electric shock and response to electric shock, flies were treated as described by Pascual and Préat (2001).

**Immunohistochemistry experiments.** *Gal80<sup>ts</sup>;VT64246/UAS-mCD8::GFP* and *Gal80<sup>ts</sup>;VT26149/UAS-mCD8::GFP* flies were incubated for 3 d at 30°C before brain dissection. For *amn-RNAi* analyses, *VT64246;UAS-mCD8::GFP* female flies were crossed to *amn-RNAi1* males, and brains of female F1 progenies were analyzed. For *amn<sup>X8</sup>* analyses, *amn<sup>X8</sup>;MB-Gal80* were crossed to *VT64246;UAS-mCD8::GFP* or *VT26149;UAS-mCD8::GFP* males, and brains of male F1 progenies were analyzed. For *amn<sup>X8</sup>* rescue analyses, *amn<sup>X8</sup>;UAS-amn* female flies were crossed to *c739* males, and brains of male F1 progenies were analyzed. Flies were collected 3–4 d after hatching (at 25°C). Brains were dissected and fixed for 20 min at room temperature in 4% formaldehyde in PBT (PBS containing 1% Triton X-100). Samples were then rinsed three times for 20 min in PBT, blocked with 2% bovine serum albumin in PBT for 2 h, and further incubated at 4°C overnight with primary antibodies [rabbit anti-GFP at 1:400 (ThermoFisher Scientific) or rabbit anti-5HT at 1:500 (Sigma-Aldrich) and mouse anti-nc82 at 1:100 (DSHB)] in the blocking solution. Brains were washed three times for 20 min in PBT and then incubated at 4°C overnight with secondary antibodies at 1:400 [anti-rabbit conjugated to AlexaFluor 488 (ThermoFisher Scientific) and anti-mouse IgG (H+L) conjugated to AlexaFluor 488 (Life Technologies, A11029), or anti-mouse IgG (H+L) conjugated to AlexaFluor 633 (Life Technologies, A21126) for *amn<sup>X8</sup>* analyses] in the blocking solution. After three washes (20 min), brains were mounted in the Prolong Mounting Medium (Life Technologies) for microscopy analysis. Images were acquired with a Nikon A1R confocal microscope. Confocal Z-stacks were acquired in 1  $\mu$ m (*amn<sup>X8</sup>* analyses) or 1.5  $\mu$ m slices (*amn-RNAi1* analyses), and imported into NIH ImageJ for analyses. For axonal length Fiji's plugin Simple Neurite Tracer (Longair et al., 2011) was used to determine the trajectory and length of the axone along all plans in stack images. For soma volume, we used Fiji's plugin Volumest (Merzin, 2008).

**Quantitative PCR analyses.** Flies were raised at 25°C. 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 Minilute Cleanup kit (Qiagen), and reverse-transcribed with oligo(dT)20 primers using the SuperScript III First-Strand kit (Life Technologies) according to the manufacturer's instructions. The *amn* oligonucleotides used were as follows: forward:



**Figure 1.** Acute modulation of *amn* expression in adult DPM neurons does not impact memory. **A**, Constitutive expression of *amn-RNAi* in DPM neurons leads to a 2 h memory deficit. Flies expressing *amn-RNAi1* or *amn-RNAi2* under the control of the constitutive *VT64246* driver exhibit significantly lower scores than genetic controls (*amn-RNAi1*:  $F_{(2,29)} = 6.793$ , \*\* $p = 0.004$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,39)} = 7.415$ , \*\* $p = 0.002$ ,  $n \geq 13$ ). **B**, Constitutive expression of *amn-RNAi* in DPM neurons leads to a LTM deficit. Flies expressing *amn-RNAi1* or *amn-RNAi2* under the control of the *VT64246* driver exhibit significantly lower scores than genetic controls (*amn-RNAi1*:  $F_{(2,34)} = 29.670$ , \*\*\* $p < 0.0001$ ,  $n \geq 11$ ; *amn-RNAi2*:  $F_{(2,34)} = 35.47$ , \*\*\* $p < 0.0001$ ,  $n \geq 11$ ). **C**, Immediate memory is not altered by constitutive expression of *amn-RNAi* in DPM neurons. *VT64246/amn-RNAi1* and *VT64246/amn-RNAi2* flies show similar scores to genetic controls (Figure legend continues).

**Table 1.** Shock reactivity and olfactory acuity of flies expressing *amn-RNAi* in DPM neurons

| Genotype                  | Shock reactivity | Olfactory acuity |                    |
|---------------------------|------------------|------------------|--------------------|
|                           |                  | Octanol          | Methylcyclohexanol |
| VT64246/+                 | 71.80 ± 4.36     | 58.30 ± 2.77     | 60.50 ± 5.15       |
| VT64246/ <i>amn-RNAi1</i> | 65.30 ± 2.87     | 73.30 ± 4.60     | 59.80 ± 4.78       |
| +/ <i>amn-RNAi1</i>       | 69.70 ± 2.83     | 61.90 ± 4.53     | 51.20 ± 4.01       |
| VT64246/+                 | 69.50 ± 4.11     | 59.80 ± 4.45     | 67.70 ± 4.39       |
| VT64246/ <i>amn-RNAi2</i> | 67.20 ± 3.65     | 60.40 ± 3.35     | 63.60 ± 5.19       |
| +/ <i>amn-RNAi2</i>       | 74.00 ± 4.39     | 58.40 ± 5.31     | 62.20 ± 5.10       |

Data are shown as mean ± SEM. VT64246/*amn-RNAi1* and VT64246/*amn-RNAi2* flies exhibit normal shock reactivity (*amn-RNAi1*:  $F_{(2,29)} = 0.938$ ,  $p = 0.406$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.725$ ,  $p = 0.493$ ,  $n = 10$ ) and normal olfactory acuity for octanol (*amn-RNAi1*:  $F_{(2,29)} = 3.726$ ,  $p = 0.037$ ,  $n = 10$ , post hoc Newman–Keuls test; VT64246/*amn-RNAi1* versus VT64246/+; \* $p < 0.05$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.053$ ,  $p = 0.948$ ,  $n = 10$ ) and methylcyclohexanol (*amn-RNAi1*:  $F_{(2,29)} = 1.230$ ,  $p = 0.308$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.352$ ,  $p = 0.707$ ,  $n = 10$ ).

5'-ACATGCGCAGTTTTGTTGT-3'; reverse: 5'-AACAGTAATAACGC AGTGCAACG-3'. Level of the target cDNA was compared against level of  $\alpha$ -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).

**Statistical analyses.** Comparisons of the data series between two conditions were achieved by a two-tailed unpaired Student's *t* test. Results of *t* tests are given as the value  $t_{(x)}$  of the *t* distribution with  $x$  degrees of freedom obtained from the data. Comparisons between more than two distinct groups were made using one-way ANOVA followed, if significant at  $p \leq 0.05$ , by Newman–Keuls multiple-comparisons 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 total number of degrees of freedom for the distribution. Asterisks on the figure denote the least significant of the pairwise *post hoc* comparisons between the genotype of interest and its controls, following the usual nomenclature. Statistical tests were performed using GraphPad Prism 5 software. All data are displayed as mean ± SEM.

←

(Figure legend continued.) (*amn-RNAi1*:  $F_{(2,29)} = 0.0003$ ,  $p = 0.100$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,14)} = 0.778$ ,  $p = 0.481$ ,  $n = 5$ ). **D**, *amn* expression in DPM neurons rescues *amn*<sup>X8</sup> memory defects. *amn*<sup>X8</sup>/Y;VT64246/UAS-*amn* flies expressing *amn* in DPM neurons show higher MTM scores than their *amn*<sup>X8</sup>/Y genetic controls, and similar scores to normal flies expressing *amn* in DPM neurons ( $F_{(3,57)} = 13.79$ , \*\*\* $p < 0.0001$ ,  $n \geq 13$ ). Only males were analyzed. **E**, Analysis of induced expression of the *Gal80*<sup>ts</sup>;VT64246 driver. *Gal80*<sup>ts</sup>;VT64246/mCD8::GFP flies were incubated for 3 d at 30°C. Dissected brains were used for immunostaining (green, mCD8::GFP; magenta, nc82). Scale bar, 10  $\mu$ m. **F**, Acute inhibition of *amn* expression in DPM neurons does not impact 2 h memory. After 3 d of induction, *amn-RNAi*-expressing flies show similar scores to genetic controls (*amn-RNAi1*:  $F_{(2,47)} = 1.479$ ,  $p = 0.239$ ,  $n = 16$ ; *amn-RNAi2*:  $F_{(2,46)} = 2.906$ ,  $p = 0.065$ ,  $n \geq 15$ ). After 5 d of induction, *Gal80*<sup>ts</sup>;VT64246/*amn-RNAi1* flies show normal 2 h memory ( $F_{(2,38)} = 0.103$ ,  $p = 0.903$ ,  $n = 8$ ). **G**, Analysis of induced expression of the *Gal80*<sup>ts</sup>;VT26149 driver. *Gal80*<sup>ts</sup>;VT26149/mCD8::GFP flies were incubated for 3 d at 30°C. Dissected brains were used for immunostaining (green, mCD8::GFP; magenta, nc82). Scale bar, 10  $\mu$ m. **H**, Acute inhibition of *amn* expression for 5 d using the *Gal80*<sup>ts</sup>;VT26149 driver does not impact 2 h memory ( $F_{(2,38)} = 0.119$ ,  $p = 0.102$ ,  $n = 8$ ). **I**, Acute inhibition of *amn* expression in DPM neurons does not affect LTM. Flies were incubated for 3 d at 30°C before conditioning, and also until memory test. *amn-RNAi*-expressing flies show similar scores to genetic controls (*amn-RNAi1*:  $F_{(2,24)} = 1.693$ ,  $p = 0.2070$ ,  $n \geq 8$ ; *amn-RNAi2*:  $F_{(2,29)} = 1.711$ ,  $p = 0.200$ ,  $n = 10$ ). **J**, *amn* expression in adult DPM neurons does not rescue *amn*<sup>X8</sup> memory defects. After 3 d of induction, *amn*<sup>X8</sup>/Y; *Gal80*<sup>ts</sup>;VT64246/UAS-*amn* flies show similar MTM scores to their *amn*<sup>X8</sup>/Y genetic controls, and lower scores than normal flies expressing *amn* in adult DPM neurons ( $F_{(3,50)} = 3.49$ , \* $p = 0.023$ ,  $n \geq 12$ ). Only males were analyzed. Error bars indicate SEM.

## Results

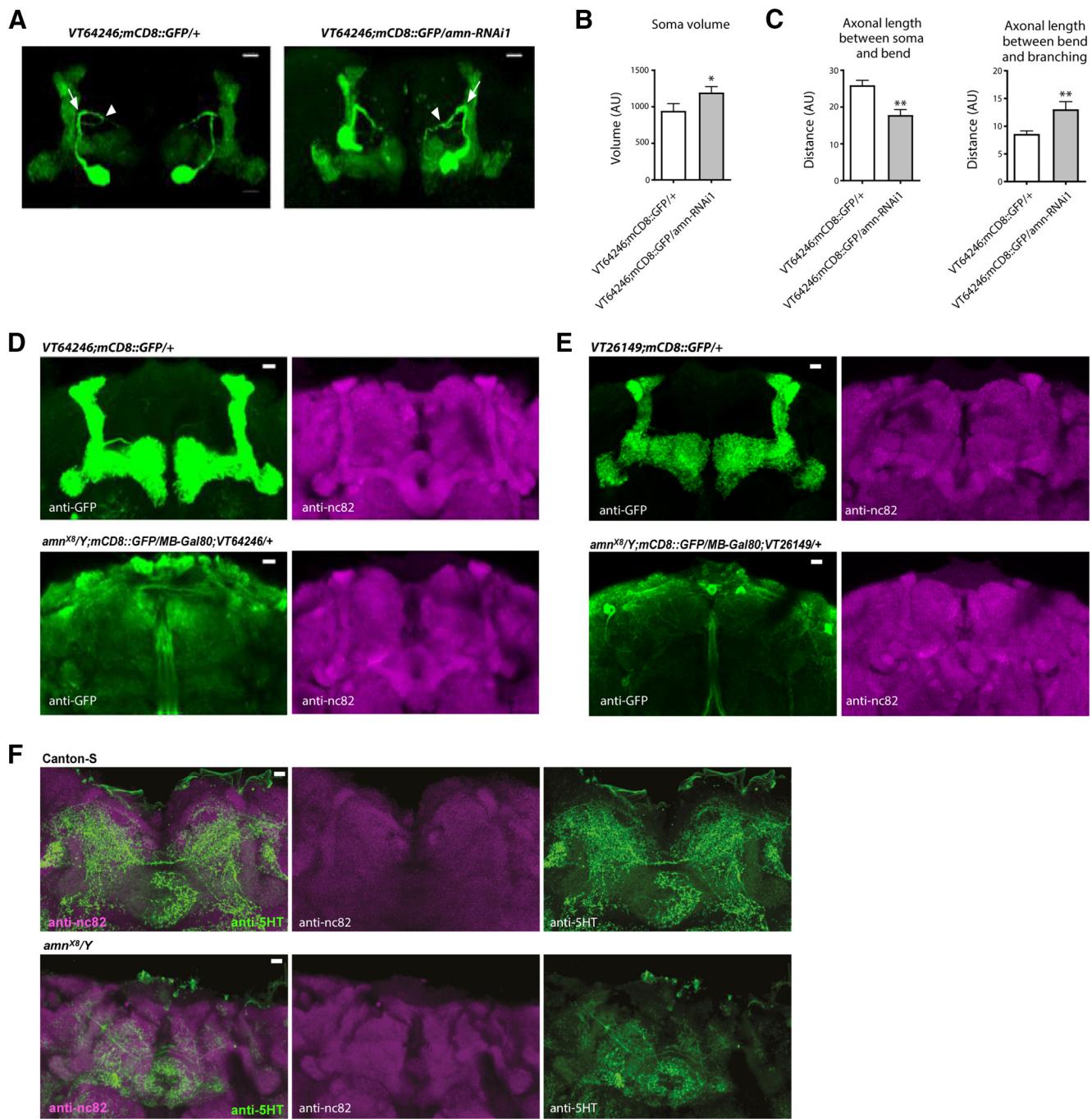
### Acute modulation of *amn* expression in adult DPM neurons does not impact memory

*Amn* memory studies conducted to date have mainly used constitutive *amn* mutants, and therefore have not been able to address temporal requirement. To study the temporal involvement of *amn* in olfactory memory, we used RNAi and conditional tissue-specific drivers. We targeted *amn* expression with a UAS-RNAi construct (*amn-RNAi1*) that was previously shown to phenocopy the thermal nociceptive defect of *amn* mutants (Aldrich et al., 2010). Memory was analyzed using the classical conditioning paradigm of odor-avoidance response. In this paradigm, groups of flies are successively exposed to two distinct odors, only one of which is associated with electric shocks. Following a single cycle of conditioning, we measure immediate memory (1 min memory) and 2 h memory. For consolidated memory phases, LT-ARM is assessed 24 h after five massed conditioning cycles whereas LTM is assessed 24 h after five spaced conditioning cycles.

We first analyzed the 2 h memory of flies constitutively expressing *amn-RNAi1* in DPM neurons. To this end, we used the VT64246 GAL4-driver that specifically labels DPM neurons (Lee et al., 2011). As expected, VT64246/*amn-RNAi1* flies showed a significant 2 h memory deficit (Fig. 1A), and this result was confirmed with a second non-overlapping *amn-RNAi* construct (*amn-RNAi2*; Fig. 1A). To address the specificity of the memory phenotype, we next analyzed LTM and observed a strong LTM deficit (Fig. 1B). In contrast, VT64246/*amn-RNAi1* and VT64246/*amn-RNAi2* flies displayed normal scores when memory was assessed immediately after a single cycle of conditioning (Fig. 1C). The ability of these flies to avoid electric shocks and their olfactory acuity to each odor after electric shock was unaffected (Table 1), showing that they displayed normal perception of the conditioning stimuli. We conclude that *amn-RNAi* expression specifically in DPM neurons during development and in adulthood mimics memory phenotypes described for *amn*-mutant flies (Quinn et al., 1979; Yu et al., 2006; Lee et al., 2011), thus confirming *amn-RNAi* is an efficient tool for studying the effect of *amn* inhibition on memory.

Waddell et al. (2000) previously showed that reestablishing *amn* expression in DPM neurons was sufficient to rescue the memory of *amn* mutant flies. However, the *c316* driver used in their study was later shown to also label, although to a lesser extent, other cells including KC (Wu et al., 2011). To better assess the spatial requirement of *amn* in memory, we aimed to reestablish *amn* expression specifically in DPM neurons of *amn*<sup>X8</sup> null mutant flies. As expected, *amn*<sup>X8</sup>/Y control flies expressing the driver (*amn*<sup>X8</sup>/Y;VT64246/+ or containing the UAS-*amn* construct alone (*amn*<sup>X8</sup>/Y;UAS-*amn*/+)) showed strong 2 h memory deficits (Fig. 1D). *amn*<sup>X8</sup>/Y;VT64246/UAS-*amn* flies expressing *amn* specifically in DPM neurons showed significantly higher MTM scores than *amn*<sup>X8</sup>/Y control groups (Fig. 1D), indicating a functional rescue.

We next investigated *amn* function specifically in adult DPM neurons. To restrict *amn-RNAi* expression to adulthood, we took advantage of the TARGET system (McGuire et al., 2003) that relies on a conventional GAL4 activation system and a temperature-sensitive Gal80 inhibitor (Gal80<sup>ts</sup>). Gal80<sup>ts</sup> represses GAL4 transcriptional activity at low temperature (18°C) but this inhibition is abolished at high temperature (30°C), allowing GAL4-mediated transgene expression. Experiments were performed with a *tub-Gal80*<sup>ts</sup>;VT64246 line that ubiquitously expresses Gal80<sup>ts</sup> (*Gal80*<sup>ts</sup>;VT64246). Using an UAS-mCD8::GFP transgene



**Figure 2.** *amn* is involved in the development of DPM neurons. **A–C**, Constitutive expression of *amn-RNAi1* in DPM neurons alters their morphology. DPM neurons were revealed using anti-GFP staining. AU, Arbitrary unit. **A**, A representative brain of each genotype is shown. Scale bar, 10  $\mu$ m. **B**, DPM cell body volume is increased in *VT64246; mCD8::GFP/amn-RNAi1* compared with *VT64246; mCD8::GFP/+* control flies ( $t$  test,  $t_{(22)} = 2.177$ ,  $*p = 0.041$ ,  $n = 12$ ). **C**, Axon measurements. Axonal length between the cell body and the major axonal bend (arrow) is reduced in *VT64246; mCD8::GFP/amn-RNAi1* compared with *VT64246; mCD8::GFP/+* control flies ( $t$  test,  $t_{(22)} = 3.756$ ,  $**p = 0.001$ ,  $n = 12$ ), whereas axonal length between the bend and the DPM axonal bifurcation point (arrowhead) is increased ( $t$  test,  $t_{(22)} = 2.952$ ,  $**p = 0.007$ ,  $n = 12$ ). **D–F**, Analyses of *amn<sup>X8</sup>* null mutant fly brains. **D**, *VT64246; mCD8::GFP/+* control brains ( $n = 11$ ) and *amn<sup>X8/Y</sup>; mCD8::GFP/MB-Gal80; VT64246/+* mutant brains ( $n = 13$ ) were analyzed with anti-GFP (left) and anti-nc82 (right) primary antibodies. Identical Z-stack projections are shown on the left and right. A representative brain of each genotype is shown. Scale bar, 10  $\mu$ m. **E**, *VT26149; mCD8::GFP/+* control brains ( $n = 7$ ) and *amn<sup>X8/Y</sup>; mCD8::GFP/MB-Gal80; VT26149/+* mutant brains ( $n = 10$ ) were analyzed with anti-GFP (left) and anti-nc82 (right) primary antibodies. Identical Z-stack projections are shown on the left and right. A representative brain of each genotype is shown. Scale bar, 10  $\mu$ m. **F**, Canton-S control brains ( $n = 8$ ) and *amn<sup>X8/Y</sup>* mutant brains ( $n = 8$ ) were analyzed with anti-nc82 and anti-5HT primary antibodies. Identical Z-stack projections are shown for each panel. A representative brain of each genotype is shown. Scale bar, 10  $\mu$ m. Error bars indicate SEM.

(*mCD8::GFP*), we first verified that the *Gal80<sup>ts</sup>; VT64246* driver generated a high level of expression after 3 d of induction at 30°C (Fig. 1E), consistently with recent data showing that it leads to efficient RNAi expression after 3 d of induction (Turrel et al., 2016). Surprisingly, *Gal80<sup>ts</sup>; VT64246/amn-RNAi1* and *Gal80<sup>ts</sup>*;

*VT64246/amn-RNAi2* induced flies both showed normal 2 h memory (Fig. 1F). Even after 5 d of induction, *Gal80<sup>ts</sup>; VT64246/amn-RNAi1* flies displayed normal 2 h memory (Fig. 1F). To confirm these data, we repeated the experiment with *tub-Gal80<sup>ts</sup>; VT26149*, another DPM-specific line that drives efficient expres-

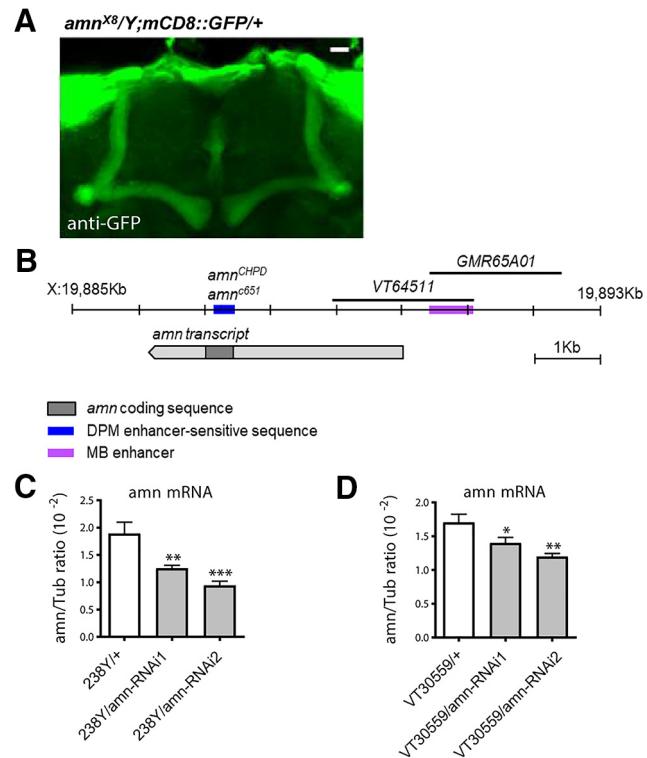
sion after induction (Fig. 1G). After 5 d of RNAi induction, *Gal80<sup>ts</sup>;VT26149/amn-RNAi1* flies displayed normal 2 h memory (Fig. 1H). We next analyzed LTM and found that neither *amn-RNAi1* nor *amn-RNAi2* expression in adult DPM neurons were able to induce a LTM deficit (Fig. 1I). In conclusion, constitutive inhibition of *amn* expression in DPM neurons leads to memory impairment whereas acute inhibition in adult DPM does not.

We next restored *amn* expression in DPM neurons of adult *amn<sup>X8</sup>* null mutant flies. *amn<sup>X8</sup>/Y;Gal80<sup>ts</sup>;VT64246/UAS-amn* flies showed 2 h memory that was not different from *amn<sup>X8</sup>/Y* mutant genotypes, and significantly lower than control flies (Fig. 1J). Thus restoration of *amn* expression in adult DPM neurons does not rescue *amn<sup>X8</sup>* memory impairment. Together, the findings suggest that *amn* expression is required in DPM neurons during developmental stages, and not acutely during adulthood.

### *amn* is involved in the development of DPM neurons

To investigate the potential influence of *amn* on the development of DPM neurons, we analyzed the morphology of these neurons in flies expressing mCD8::GFP and *amn-RNAi1* under the control of the VT64246 driver. As expected, we observed extensive arborization of DPM neurons onto the MB lobes. However, flies expressing *amn-RNAi1* in DPM neurons exhibited DPM neurons that were morphologically abnormal on several aspects (Fig. 2). DPM cell bodies exhibited irregular shape and less accurate localization relative to the MB (Fig. 2A) and they were bigger in flies expressing *amn-RNAi1* in DPM neurons compared with controls (Fig. 2B). In addition, the DPM axonal path was modified in *amn*-deficient flies (Fig. 2C). Together, the data indicate that *amn* expression in DPM neurons is required for their own normal development.

To further assess *amn* involvement in the development of DPM neurons, we next aimed at analyzing the morphology of DPM neurons in *amn* null mutant flies. The *amn<sup>X8</sup>* null mutant line results from the excision of a GAL4-encoding P-element inserted in the *amn<sup>28A</sup>* line (Moore et al., 1998). Yet, the *amn<sup>28A</sup>* line contained two insertions and only one was excised in the *amn<sup>X8</sup>* flies (Moore et al., 1998). It was later mentioned that *amn<sup>X8</sup>* flies had retained GAL4 activity localized in the MB (Keene et al., 2004). To be able to analyze GFP expression specifically driven in DPM neurons, we constructed a line expressing Gal80 in the MB of *amn<sup>X8</sup>* flies using the MB247-Gal80 transgene (MB-Gal80; Krashes et al., 2007). Strikingly, we did not observe immunostaining on the MB lobes in *amn<sup>X8</sup>/Y;mCD8::GFP/MB-Gal80;VT64246/+* fly brains (Fig. 2D). We verified that MB did not display any gross alteration of their morphology using the nc82 antibody to label synapses (Fig. 2D). To rule out the possibility that the loss of expression might be due to a change of the VT64246 expression pattern in an *amn* background, we analyzed GFP expression with the DPM-specific driver VT26149. We did not observe immunostaining on the MB lobes in *amn<sup>X8</sup>/Y;mCD8::GFP/MB-Gal80;VT26149/+* fly brains (Fig. 2E). Finally, to further confirm that the absence of staining was due to an abnormal development of the DPM neurons, we took advantage of the fact that DPM neurons can be revealed by serotoninergic (5HT) staining (Lee et al., 2011). In Canton-S control fly brains, we observed numerous ramifications coming from DPM neurons onto the MB (Fig. 2F). In contrast, in *amn<sup>X8</sup>* mutant flies, these ramifications were mostly absent, confirming an abnormal development of DPM neurons (Fig. 2F). We conclude that in the absence of *amn* expression, DPM neurons do not develop normally.

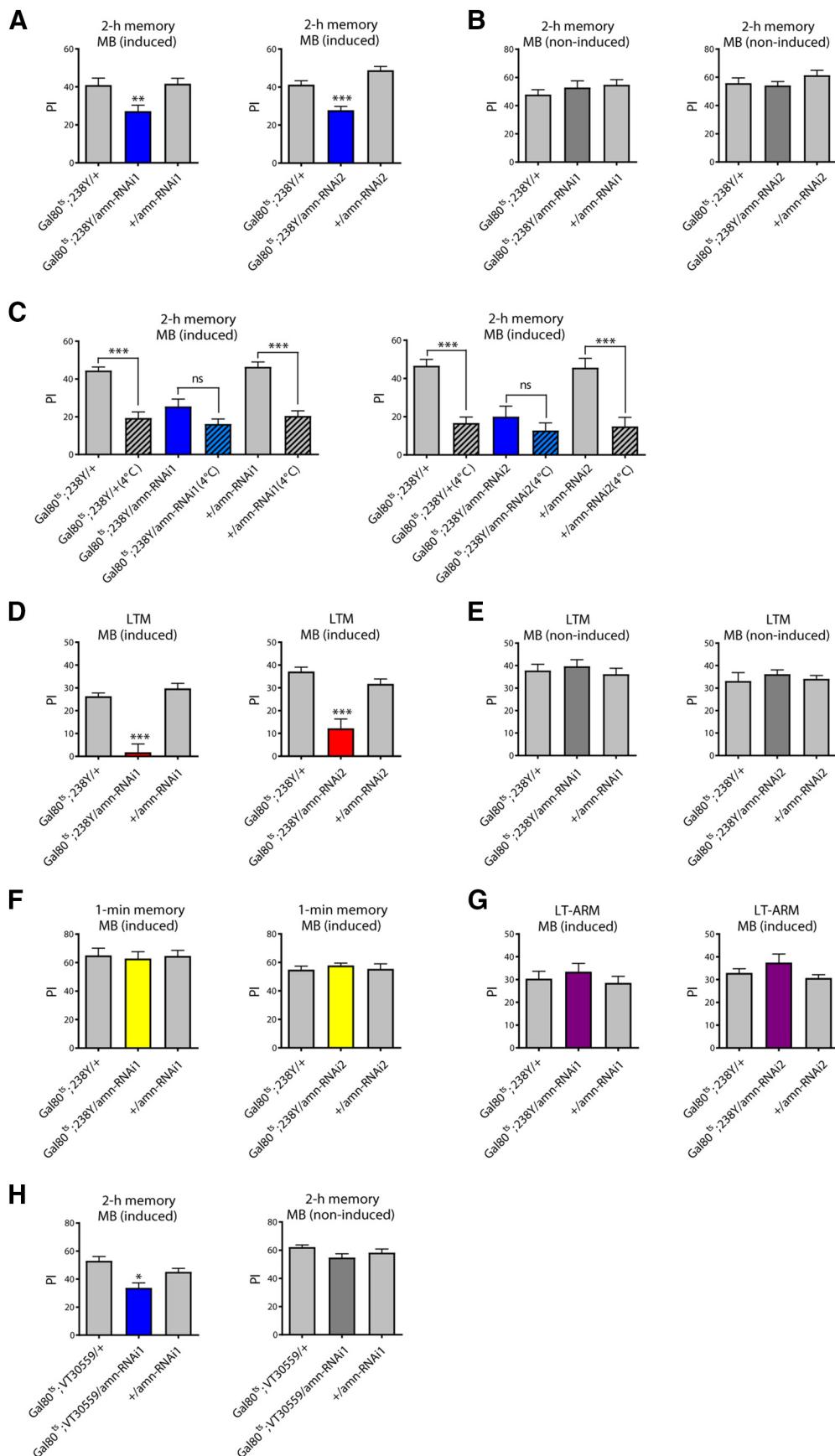


**Figure 3.** *amn* expression in the MB. **A**, *amn<sup>X8</sup>* flies express GAL4 in the MB  $\alpha/\beta$  neurons. Dissected brains were used for immunostaining (green, mCD8::GFP). **B**, Schematic representation of the *amn* transcript with the localization of a MB enhancer sequence and of DPM enhancer-sensitive sequence. **C, D**, qPCR analyses of *amn* expression levels. Total RNA extracted from female heads were reverse-transcribed and further quantified by PCR. Expression relative to reference is expressed as a ratio ( $2^{-\Delta C_p}$ , where  $C_p$  is the crossing point). **C**, 238Y/*amn-RNAi1* and 238Y/*amn-RNAi2* show 33% and 50% decrease in *amn* mRNA compared with the 238Y/+ control. Each bar corresponds to six measures from three independent experiments ( $F_{(2,17)} = 13.16$ , \*\*\* $p = 0.0005$ ,  $n = 6$ ). **D**, VT30559/*amn-RNAi1* and VT30559/*amn-RNAi2* show 18% and 30% decrease in *amn* mRNA compared with the VT30559/+ control. ( $F_{(2,23)} = 8.522$ , \*\* $p = 0.002$ ,  $n = 8$ ). Each bar corresponds to eight measures from four independent experiments. Error bars indicate SEM.

### *amn* expression in the MB

As we could not evidence any memory impact upon acute modulation of *amn* expression in the adult, and as we observed that *amn* is required for the proper development of neurons involved in memory, the next question to address was whether AMN expression plays a role in the adult brain for memory.

As mentioned in the previous section, Keene et al. (2004) reported as data not shown that *amn<sup>X8</sup>* flies expressed GAL4 activity in the MB. Indeed, when we analyzed GFP expression driven by *amn<sup>X8</sup>*, we observed immunostaining of the MB  $\alpha$  and  $\beta$  lobes (Fig. 3A), confirming that *amn<sup>X8</sup>* flies had retained a GAL4-coding sequence, and suggesting the presence of a MB-specific enhancer in the *amn* gene. Analysis of GAL4-expression patterns in the adult brain points, in addition to a DPM enhancer-sensitive sequence, to the presence of a second enhancer located upstream of the *amn* transcript that leads to specific expression in the MB (Fig. 3B). GAL4 inserted in a region located at the start of the *amn* coding sequence (blue box) lead to expression in DPM neurons (*amn<sup>c651</sup>* and *amn<sup>CHPD</sup>*; Waddell et al., 2000). A fragment of genomic DNA covering a region upstream of the *amn* transcription unit is linked to GAL4 expression in the MB  $\alpha/\beta$  neurons (GMR65A01; Jenett et al., 2012; expression pattern available from <http://flybase.org/reports/FBtp0061966.html>). Similarly, the Vienna Tile VT64511 covering



**Figure 4.** Acute inhibition of *amn* expression in adult MB neurons leads to memory defects. **A, B**, *amn* inhibition in adult MB neurons induces MTM defects. **A**, Flies expressing *amn-RNAi* in the adult MB exhibit a 2 h memory deficit (*amn-RNAi1*:  $F_{(2,35)} = 7.34$ , \*\*\* $p = 0.002$ ,  $n = 12$ ; *amn-RNAi2*:  $F_{(2,39)} = 27.81$ , \*\*\* $p < 0.0001$ ,  $n \geq 13$ ). **B**, Non-induced flies display normal 2 h memory scores (*amn-RNAi1*:  $F_{(2,29)} = 0.822$ ,  $p = 0.451$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,34)} = 1.242$ ,  $p = 0.302$ ,  $n \geq 11$ ). **C**, When indicated (4°C), flies were submitted to 2 min cold (Figure legend continues).

**Table 2.** Shock reactivity and olfactory acuity of flies expressing *amn-RNAi* in adult MB neurons

| Genotype                                       | Shock reactivity | Olfactory acuity |                    |
|--|------------------|------------------|--------------------|
|  |                  | Octanol          | Methylcyclohexanol |
| Gal80 <sup>ts</sup> ;238Y/+                    | 79.75 ± 3.92     | 75.10 ± 3.79     | 50.70 ± 0.92       |
| Gal80 <sup>ts</sup> ;238Y/ <i>amn-RNAi1</i>    | 73.13 ± 3.83     | 70.50 ± 3.09     | 57.00 ± 4.10       |
| +/ <i>amn-RNAi1</i>                            | 72.00 ± 4.67     | 73.50 ± 3.62     | 52.00 ± 1.65       |
| Gal80 <sup>ts</sup> ;238Y/+                    | 83.70 ± 4.42     | 53.50 ± 5.43     | 55.50 ± 6.57       |
| Gal80 <sup>ts</sup> ;238Y/ <i>amn-RNAi2</i>    | 81.20 ± 2.84     | 58.50 ± 6.82     | 46.10 ± 4.15       |
| +/ <i>amn-RNAi2</i>                            | 71.40 ± 1.82     | 44.20 ± 4.59     | 49.40 ± 2.90       |
| Gal80 <sup>ts</sup> ;VT30559/+                 | 74.88 ± 5.11     | 69.38 ± 4.56     | 66.25 ± 7.01       |
| Gal80 <sup>ts</sup> ;VT30559/ <i>amn-RNAi1</i> | 77.38 ± 2.10     | 66.38 ± 3.82     | 70.88 ± 5.27       |
| +/ <i>amn-RNAi1</i>                            | 77.88 ± 3.74     | 60.88 ± 3.57     | 55.00 ± 4.83       |

Data are shown as mean ± SEM. Gal80<sup>ts</sup>;238Y/*amn-RNAi1* and Gal80<sup>ts</sup>;238Y/*amn-RNAi2*-induced flies exhibit normal shock reactivity (*amn-RNAi1*:  $F_{(2,23)} = 1.015$ ,  $p = 0.380$ ,  $n = 8$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.992$ ,  $p = 0.384$ ,  $n = 10$ ) and normal olfactory acuity for octanol (*amn-RNAi1*:  $F_{(2,29)} = 0.442$ ,  $p = 0.647$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 4.113$ ,  $p = 0.028$ ,  $n = 10$ ) and methylcyclohexanol (*amn-RNAi1*:  $F_{(2,29)} = 1.627$ ,  $p = 0.215$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 1.628$ ,  $p = 0.215$ ,  $n = 10$ ). Gal80<sup>ts</sup>;VT30559/*amn-RNAi1*-induced flies exhibit normal shock reactivity ( $F_{(2,23)} = 0.174$ ,  $p = 0.842$ ,  $n = 8$ ) and normal olfactory acuity for octanol ( $F_{(2,23)} = 1.156$ ,  $p = 0.334$ ,  $n = 8$ ) and methylcyclohexanol (*amn-RNAi1*:  $F_{(2,23)} = 1.993$ ,  $p = 0.161$ ,  $n = 8$ ).

a region upstream and at the start of the *amn* transcription unit leads to GAL4 expression in the MB  $\alpha/\beta$  neurons.

We could not directly assess *amn* expression in the MB with immunochemistry experiments because AMN antibodies are not available. We tried to perform *in situ* experiments, but probably because *amn* RNA is expressed at very low levels (Feany and Quinn, 1995; Chintapalli et al., 2007; Affymetrix data available from <http://flyatlas.org/atlas.cgi?name=FBgn0000076>), we did not observe specific staining. Thus, to assess *amn* expression in MB neurons, we analyzed the effect of *amn-RNAi* expression under the control of the 238Y driver known to label MB neurons (Aso et al., 2009). qPCR analyses revealed a decrease of *amn* mRNA level in 238Y/*amn-RNAi1* and 238Y/*amn-RNAi2* fly heads compared with controls (Fig. 3C). We next used the VT30559 driver that labels KC more specifically than the 238Y driver (Plaçais et al., 2017). VT30559/*amn-RNAi1* and VT30559/*amn-RNAi2* fly heads also expressed less *amn* mRNA than controls (Fig. 3D). Together, the data strongly suggest that *amn* is indeed expressed in the adult MB.

### Acute inhibition of *amn* expression in adult MB neurons leads to memory defects

We then investigated the involvement of *amn* in memory in the MB. Flies expressing *amn-RNAi* in all adult MB neuronal types with a conditional *tub-Gal80<sup>ts</sup>;238Y* driver (*Gal80<sup>ts</sup>;238Y*)

&lt;

(Figure legend continued.) shock 1 h before the memory test. Cold-shock treatment induces a memory decrease in control flies but not in *Gal80<sup>ts</sup>;238Y/amn-RNAi* flies (*amn-RNAi1*:  $F_{(5,75)} = 21.99$ , \*\*\* $p < 0.0001$ ,  $n \geq 12$ ; *amn-RNAi2*:  $F_{(5,71)} = 12.98$ , \*\*\* $p < 0.0001$ ,  $n = 12$ ). **D, E**, *amn* inhibition in adult MB neurons induces LTM defects. **D**, *Gal80<sup>ts</sup>;238Y/amn-RNAi*-induced flies show LTM impairment (*amn-RNAi1*:  $F_{(2,24)} = 35.70$ , \*\*\* $p < 0.0001$ ,  $n \geq 8$ ; *amn-RNAi2*:  $F_{(2,34)} = 20.22$ , \*\*\* $p < 0.0001$ ,  $n \geq 11$ ). **E**, In the absence of induction, *Gal80<sup>ts</sup>;238Y/amn-RNAi* flies show normal LTM (*amn-RNAi1*:  $F_{(2,29)} = 0.393$ ,  $p = 0.679$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.363$ ,  $p = 0.699$ ,  $n = 10$ ). **F**, Immediate memory is not affected by *amn* inhibition in the adult MB (*amn-RNAi1*:  $F_{(2,23)} = 0.060$ ,  $p = 0.942$ ,  $n = 8$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.320$ ,  $p = 0.729$ ,  $n = 10$ ). **G**, LT-ARM is not affected by *amn* inhibition in the adult MB (*amn-RNAi1*:  $F_{(2,29)} = 0.565$ ,  $p = 0.575$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,46)} = 1.709$ ,  $p = 0.193$ ,  $n \geq 15$ ). **H**, MTM analyses with the *Gal80<sup>ts</sup>;VT30559* driver. *Gal80<sup>ts</sup>;VT30559/amn-RNAi* flies show impaired MTM ( $F_{(2,35)} = 9.953$ , \*\*\* $p = 0.0004$ ,  $n = 12$ ). In absence of induction, *Gal80<sup>ts</sup>;VT30559/amn-RNAi* flies show normal memory ( $F_{(2,35)} = 2.547$ ,  $p = 0.936$ ,  $n = 12$ ). Error bars indicate SEM.

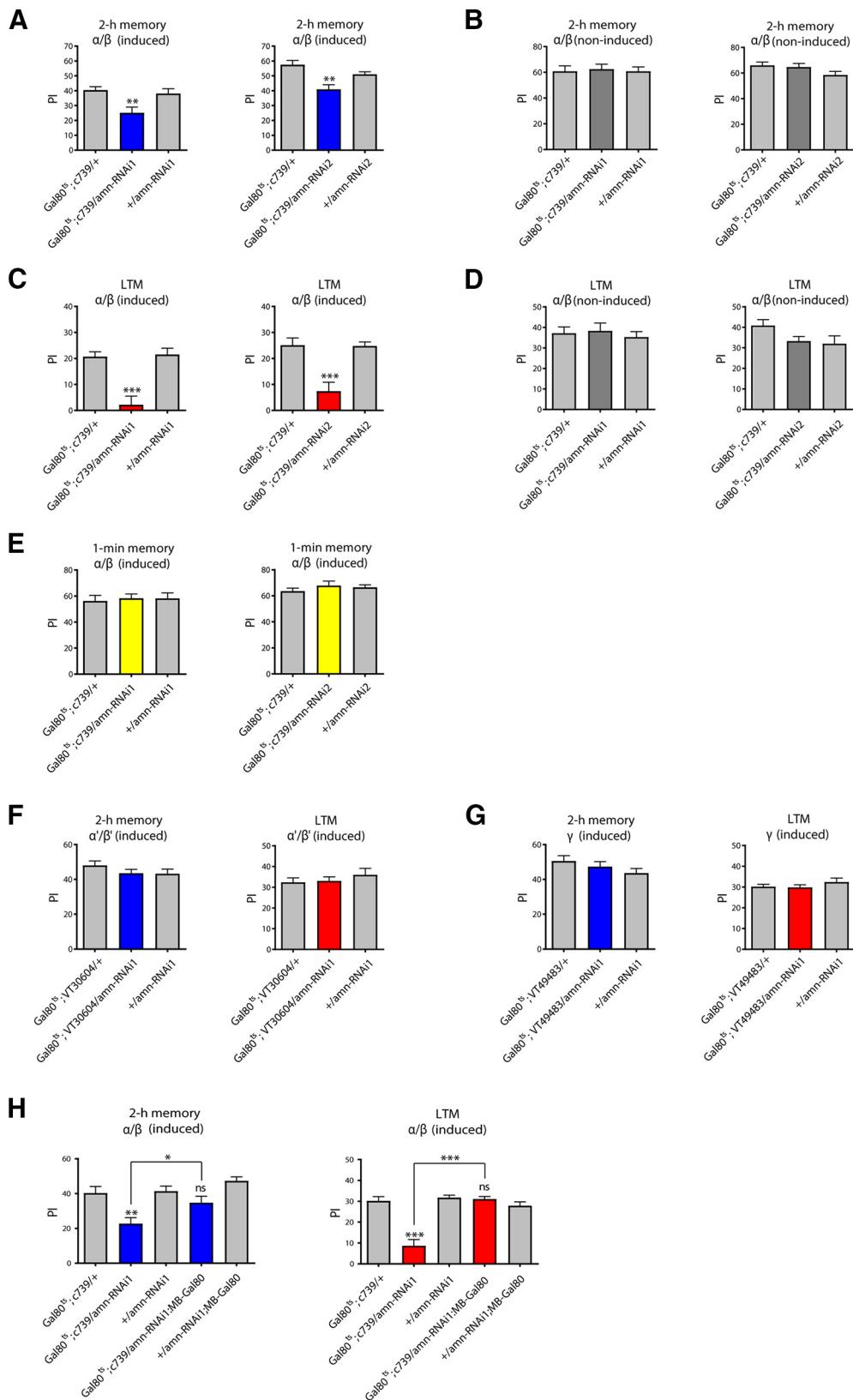
showed abnormal 2 h memory (Fig. 4A), whereas non-induced flies showed normal scores (Fig. 4B), establishing that the memory deficit is caused by RNAi induction.

Memory measured 2 h after training is composed of two distinct phases: MTM, which is a labile memory sensitive to cold anesthesia, and MT-ARM which is resistant to anesthesia (Quinn and Dudai, 1976). To examine which memory phase was affected by *amn* inhibition, we applied cold-shock treatments to the flies. The memory scores of control flies dropped whereas the memory scores of *amn-RNAi*-expressing flies remained unaffected (Fig. 4C), indicating that MTM is absent. Furthermore, after cold shock treatment, *Gal80<sup>ts</sup>;238Y/amn-RNAi* flies exhibited similar memory scores to control flies, showing that MT-ARM is not affected. We next examined LTM after spaced training and observed that *Gal80<sup>ts</sup>;238Y/amn-RNAi* flies showed a strong LTM impairment (Fig. 4D,E) but normal 1 min memory (Fig. 4F) and LT-ARM (Fig. 4G). The sensory-motor capacities of these flies were normal (Table 2). We next used a second MB driver, VT30559, to knockdown *amn* expression specifically in KC. After 3 d of induction, *Gal80<sup>ts</sup>;VT30559/amn-RNAi* flies displayed impaired 2 h memory whereas noninduced flies displayed normal 2 h memory (Fig. 4H). We verified that the ability of induced *Gal80<sup>ts</sup>;VT30559/amn-RNAi* flies to perceive conditioning stimuli was normal (Table 2). Strikingly, we conclude that inhibition of *amn* expression specifically in the adult MB recapitulates memory phenotypes previously reported for *amn*-mutant flies (Quinn et al., 1979; Yu et al., 2006; Lee et al., 2011). In short, MTM and LTM are altered, whereas STM and ARM remain unaffected.

### Acute inhibition of *amn* expression in adult $\alpha/\beta$ neurons leads to memory defects

Because MTM and LTM rely on MB  $\alpha/\beta$  neurons (Bouzaiane et al., 2015), we next aimed to specifically restrict *amn* inhibition to  $\alpha/\beta$  neurons using the inducible *tub-Gal80<sup>ts</sup>;c739* driver (*Gal80<sup>ts</sup>;c739*). Flies expressing *amn-RNAi* in adult  $\alpha/\beta$  neurons exhibited deficits in 2 h memory (Fig. 5A,B) and LTM (Fig. 5C,D) but normal 1 min memory (Fig. 5E). We verified that their responses to the stimuli used for training were unaffected (Table 3). To further characterize in which KC *amn* is required for memory, we analyzed the effect of *amn* inhibition in adult  $\alpha'/\beta'$  KC using a *tub-Gal80<sup>ts</sup>;VT30604* driver (Wu et al., 2013), and in adult  $\gamma$  KC using a *tub-Gal80<sup>ts</sup>;VT49483* driver (see Materials and Methods). Neither  $\alpha'/\beta'$  KC-driven *amn-RNAi1* expression (Fig. 5F) nor  $\gamma$  KC-driven *amn-RNAi1* expression (Fig. 5G) did impact MTM or LTM.

Together, the data show that *amn* is specifically required in the adult  $\alpha/\beta$  KC for MTM and LTM formation. To strengthen these results, we performed intersectional experiments using the MB-Gal80 transgene to inhibit RNAi expression in the MB. *Gal80<sup>ts</sup>;c739/amn-RNAi1*-induced flies showed mutant 2 h memory, as previously observed, whereas *Gal80<sup>ts</sup>;c739/amn-RNAi1;MB-Gal80*-induced flies displayed normal 2 h memory (Fig. 5H). Similarly, *Gal80<sup>ts</sup>;c739/amn-RNAi1* flies showed a strong LTM deficit whereas *Gal80<sup>ts</sup>;c739/amn-RNAi1;MB-Gal80* flies showed normal LTM (Fig. 5H). We conclude that preventing GAL4-mediated *amn-RNAi* expression in the MB with Gal80 abolishes memory deficits. In conclusion, the results demonstrate that AMN is acutely required in MB  $\alpha/\beta$  neurons for MTM and LTM formation.



**Figure 5.** Acute inhibition of *amn* expression in adult  $\alpha/\beta$  neurons leads to memory defects. **A, B**, *amn* expression in adult  $\alpha/\beta$  neurons is required for MTM. **A**, After induction,  $Gal80^ts;C739/amn-RNAi$  flies show lower MTM scores than genetic controls (*amn-RNAi1*:  $F_{(2,29)} = 6.600$ ,  $**p = 0.0046$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,42)} = 10.17$ ,  $***p = 0.0003$ ,  $n \geq 14$ ). **B**, In absence of induction,  $Gal80^ts;C739/amn-RNAi$  flies show normal MTM (*amn-RNAi1*:  $F_{(2,29)} = 0.064$ ,  $p = 0.939$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 2.141$ ,  $p = 0.137$ ,  $n = 10$ ). **C, D**, *amn* expression in the adult  $\alpha/\beta$  neurons is required for LTM. **C**,  $Gal80^ts;C739/amn-RNAi$ -induced flies show LTM deficits (*amn-RNAi1*:  $F_{(2,29)} = 16.56$ ,  $***p < 0.0001$ ,  $n \geq 9$ ; *amn-RNAi2*:  $F_{(2,29)} = 13.88$ ,  $***p < 0.0001$ ,  $n = 10$ ). **D**, In absence of induction,  $Gal80^ts;C739/amn-RNAi$  flies show normal LTM (*amn-RNAi1*:  $F_{(2,29)} = 0.220$ ,  $p = 0.804$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 2.450$ ,  $p = 0.105$ ,  $n = 10$ ). **E**, *amn* expression is not required in adult  $\alpha/\beta$  neurons for immediate memory.  $Gal80^ts;C739/amn-RNAi$  induced flies show similar scores to genetic controls (*amn-RNAi1*:  $F_{(2,29)} = 0.087$ ,  $p = 0.917$ ,  $n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.719$ ,  $p = 0.496$ ,  $n = 10$ ). **F**, *amn* expression in adult  $\alpha'/\beta'$  neurons is not required for MTM nor LTM. After induction  $Gal80^ts;VT30604/amn-RNAi$  flies (Figure legend continues.)

**Table 3.** Shock reactivity and olfactory acuity of flies expressing *amn-RNAi* in adult  $\alpha/\beta$  neurons

| Genotype                                 | Shock reactivity | Olfactory acuity |                    |
|--|------------------|------------------|--------------------|
|  |                  | Octanol          | Methylcyclohexanol |
| <i>Gal80<sup>ts</sup>;c739/+</i>         | 74.25 ± 4.86     | 52.70 ± 3.82     | 45.40 ± 3.78       |
| <i>Gal80<sup>ts</sup>;c739/amn-RNAi1</i> | 78.75 ± 4.46     | 56.80 ± 4.96     | 48.30 ± 4.84       |
| <i>+/amn-RNAi1</i>                       | 70.25 ± 5.36     | 59.50 ± 6.58     | 44.50 ± 3.55       |
| <i>Gal80<sup>ts</sup>;c739/+</i>         | 70.60 ± 5.56     | 52.20 ± 4.36     | 48.80 ± 4.67       |
| <i>Gal80<sup>ts</sup>;c739/amn-RNAi2</i> | 69.10 ± 4.78     | 57.60 ± 5.12     | 56.30 ± 3.92       |
| <i>+/amn-RNAi2</i>                       | 68.50 ± 4.14     | 60.90 ± 5.19     | 49.80 ± 5.33       |

Data are shown as mean ± SEM. *Gal80<sup>ts</sup>;238Y/amn-RNAi1* and *Gal80<sup>ts</sup>;238Y/amn-RNAi2*-induced flies exhibit normal shock reactivity (*amn-RNAi1*:  $F_{(2,23)} = 0.752, p = 0.484, n = 8$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.050, p = 0.952, n = 10$ ) and normal olfactory acuity for octanol (*amn-RNAi1*:  $F_{(2,29)} = 0.427, p = 0.657, n = 10; n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.744, p = 0.485, n = 10$ ) and methylcyclohexanol (*amn-RNAi1*:  $F_{(2,29)} = 0.236, p = 0.792, n = 10$ ; *amn-RNAi2*:  $F_{(2,29)} = 0.759, p = 0.478, n = 10$ ).

### Expression of the wild-type *amn* gene in MB neurons of *amn<sup>X8</sup>* mutant flies rescues their memory deficits

The next question we addressed was whether reestablishing *amn* gene expression in MB neurons of *amn* null mutant flies could rescue their memory deficits. First, we showed that overexpression of the *amn* gene in MB neurons with the 238Y, *c739*, or *c747* driver did not affect MTM nor LTM (Fig. 6A). We then performed rescue experiments. Interestingly, *amn<sup>X8</sup>/Y;238Y/UAS-amn* MTM scores were indistinguishable from 238Y/UAS-*amn* control fly scores (Fig. 6B), indicating a full rescue of the MTM deficit. Similarly, we also observed complete rescue of the LTM deficit (Fig. 6B). To confirm these results, we used the *c739*  $\alpha/\beta$ -specific driver and found that *amn* expression driven in the  $\alpha/\beta$  KC allows complete rescue of both the MTM and LTM deficit in *amn<sup>X8</sup>/Y* flies (Fig. 6C). In conclusion, *amn* constitutive expression in either DPM neurons or  $\alpha/\beta$  KC is capable of restoring functional memory.

These results seem to contradict a previous study (Waddell et al., 2000) reporting that *amn* expression in the MB failed to rescue the olfactory memory phenotypes of *amn<sup>X8</sup>* flies (Waddell et al., 2000). To resolve this apparent discrepancy, we also conducted rescue experiments with the same *c747* driver that Waddell et al. (2000) used. We observed that *c747* GAL4-driven *amn* expression had no effect on the memory deficits of *amn* null mutant flies (Fig. 6D). Thus, *amn* expression driven by either 238Y or *c739* rescues *amn<sup>X8</sup>* memory deficits whereas expression driven by *c747* does not, indicating that these drivers are functionally distinct. The fact that *c747* shows a broad brain expression (Rodan et al., 2002) may explain this difference.

Finally, to analyze whether *amn* expression in the MB of *amn<sup>X8</sup>* flies could also rescue the abnormal development of DPM neurons, we performed 5HT staining of *amn<sup>X8</sup>/Y;c739/UAS-amn* fly brains. As previously observed (Fig. 2F), *amn<sup>X8</sup>* mutant flies show a loss of specific staining of the ramifications and the soma of DPM neurons compared with Canton-S control flies (Fig. 6E). Strikingly, in *amn<sup>X8</sup>/Y;c739/UAS-amn* flies, the 5HT staining of DPM neurons was restored (Fig. 6E), indicating that expression

←

(Figure legend continued.) show normal MTM ( $F_{(2,35)} = 1.175, p = 0.321, n = 12$ ) and normal LTM ( $F_{(2,38)} = 0.602, p = 0.553, n = 13$ ). G, *amn* expression in adult  $\gamma$  neurons is not required for MTM nor LTM. After induction *Gal80<sup>ts</sup>;VT49483/amn-RNAi1* flies show normal MTM ( $F_{(2,35)} = 1.463, p = 0.246, n = 12$ ) and normal LTM ( $F_{(2,38)} = 0.856, p = 0.433, n = 13$ ). H, Intersectional analyses. *Gal80<sup>ts</sup>;c739/amn-RNAi1;MB-Gal80*-induced flies show normal MTM and LTM (MTM:  $F_{(4,72)} = 7.672, ***p < 0.0001, n \geq 14$ ; LTM:  $F_{(4,59)} = 24.12, ***p < 0.0001, n = 12$ ). Error bars indicate SEM.

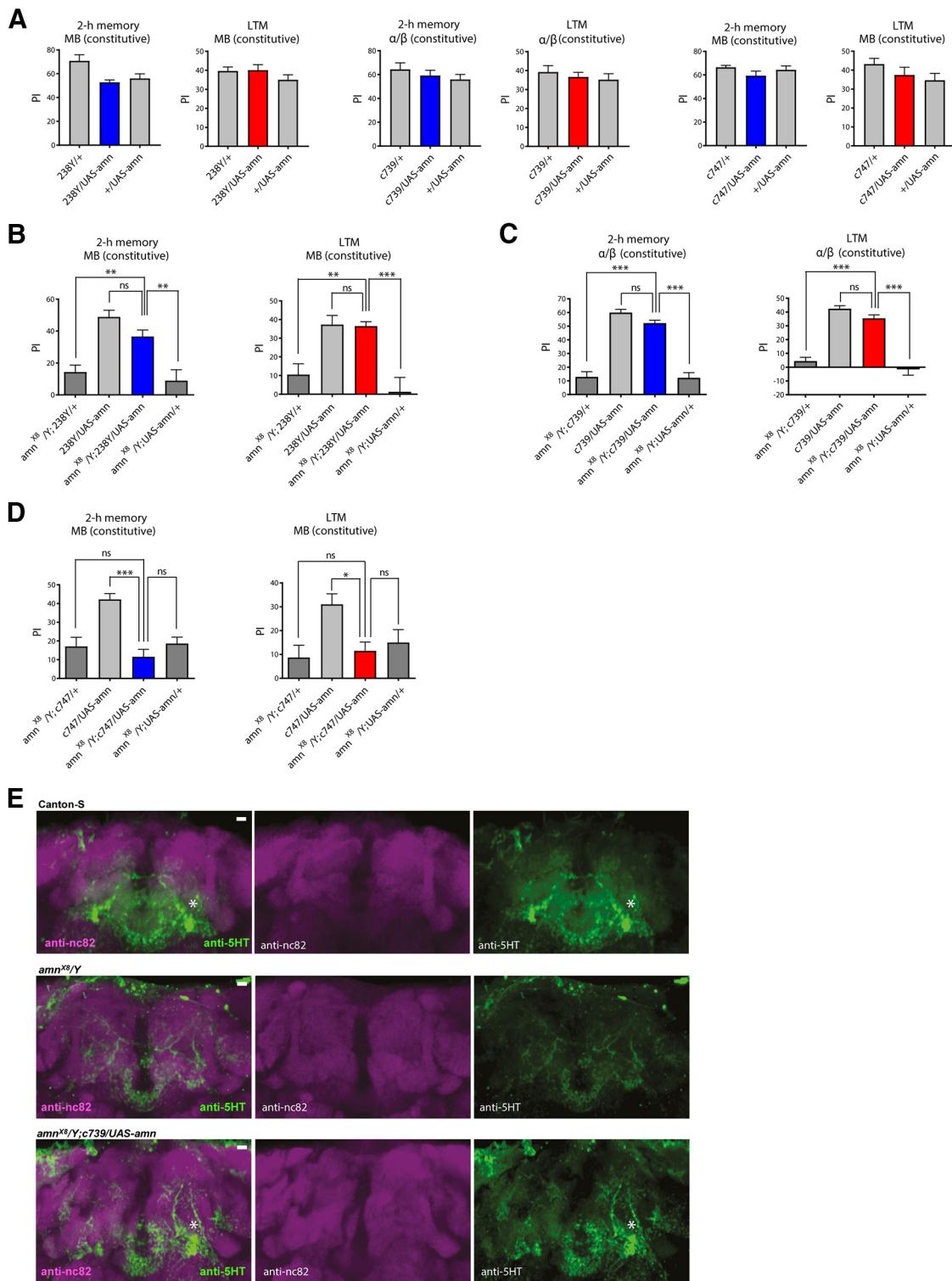
of the *amn* gene in  $\alpha/\beta$  neurons during development and adulthood is sufficient to restore normal development of DPM neurons in *amn<sup>X8</sup>* mutants.

### Discussion

Here, we show that *amn* expression in DPM neurons is required for their development. Next, using inducible inhibition of *amn* expression, we establish that AMN peptide is specifically required in adult  $\alpha/\beta$  MB neurons for MTM and LTM.

It was previously shown that acute ubiquitous *amn* expression in the adult was inefficient to rescue memory phenotypes of *amn<sup>X8</sup>* null mutant flies (DeZazzo et al., 1999). Here, induced *amn* expression in adult DPM neurons using the TARGET system also failed to rescue *amn<sup>X8</sup>* memory deficits, suggesting that *amn* expression in DPM neurons may be required for a developmental stage. We analyzed the morphology of DPM neurons in fly coexpressing *amn-RNAi* and GFP in these neurons. Although DPM arborization onto MB lobes is still visible, their abnormal cell body and axon morphology indicate that *amn* expression in DPM neurons is required to achieve their normal development. Keene et al. (2004) analyzed DPM morphology in *amn<sup>ex1</sup>* mutant fly brains using *c316* to drive GFP expression in DPM neurons. They concluded that *amn* is not essential for DPM targeting to the MB during development. Interestingly, although they did not describe it in detail, they showed a typical *amn<sup>ex1</sup>* brain that apparently displayed DPM neurons with similar subtle morphology alterations to those described in our study. We further analyzed DPM development in an *amn* knock-out context. Imaging DPM neurons in *amn<sup>X8</sup>* brains is hampered by the fact that *amn<sup>X8</sup>* flies express GAL4 in the MB (Keene et al., 2004). To circumvent this issue, we took advantage of the MB-*Gal80* transgene to inhibit GAL4 activity in the MB. Strikingly, using *VT64246* or *VT26149* to drive GFP expression in DPM neurons, we did not observe DPM arborization in *amn<sup>X8</sup>* brains, despite the presence of all MB lobes. Together the results show that *amn* plays an essential role in the development of DPM neurons, a pair of neurons involved in olfactory memory; thus raising the question of whether *amn* is involved in memory in the adult brain.

Although constitutive expression of *amn-RNAi* in DPM neurons mimics memory deficits described for *amn* mutants, *amn* inhibition restricted to adult DPM neurons does not induce any memory impairment. The absence of a memory phenotype using conditional RNAi does not demonstrate that the target gene is not involved in memory processes, as we cannot rule out that conditional RNAi expression does not drive sufficient inhibition of the target gene expression to generate a loss-of-function phenotype. However, several lines of evidence suggest that *amn* expression should be efficiently inhibited by RNAi in adult DPM neurons: (1) each of the two *amn-RNAi* constructs used here induces memory deficits when acutely expressed in the adult MB; (2) to achieve *amn* inhibition in adult DPM neurons, two distinct DPM drivers were used after up to 5 d of GAL4-induction; and (3) one of these specific drivers has been previously shown to generate strong memory deficits upon RNAi-mediated silencing (Turrel et al., 2016). Also, restoring *amn* expression in *amn<sup>X8</sup>* mutant flies during development and adulthood rescues their memory deficits, whereas expression restricted to adulthood fails to restore memory. Together, the data suggest that *amn* expression in DPM neurons is not required during adulthood for memory processes. When *amn* expression is constitutively inhibited in DPM neurons, these neurons do not form properly. As a consequence, memory is affected because DPM neurons cannot fulfill their physiological role (Keene et al., 2004), independently of



**Figure 6.** Expression of the wild-type *amn* gene in MB neurons of *amn*<sup>x8</sup> mutant flies rescues their memory deficits. **A**, MTM and LTM scores are not affected by expressing *amn* in the MB. MTM and LTM scores are not affected by expressing *amn* in the different subsets of the MB with the 238Y driver (MTM:  $F_{(2,23)} = 5.967$ , \*\*\* $p = 0.0089$ ,  $n = 8$ ; LTM:  $F_{(2,23)} = 1.178$ ,  $p = 0.3205$ ,  $n = 12$ ), the c739 driver (MTM:  $F_{(2,23)} = 0.8268$ ,  $p = 0.4512$ ,  $n = 8$ ; LTM:  $F_{(2,23)} = 0.5209$ ,  $p = 0.5988$ ,  $n = 12$ ) or the c747 driver (MTM:  $F_{(2,23)} = 1.425$ ,  $p = 0.2629$ ,  $n = 8$ ; LTM:  $F_{(2,23)} = 1.419$ ,  $p = 0.2563$ ,  $n = 12$ ). **B**, Analyses with the 238Y driver. *amn*<sup>x8</sup>/Y flies expressing *amn* show higher MTM and LTM scores than their *amn*<sup>x8</sup>/Y genetic controls and similar MTM and LTM scores to flies expressing *amn* in the MB (MTM:  $F_{(3,39)} = 14.14$ , \*\*\* $p < 0.0001$ ,  $n = 10$ ; LTM:  $F_{(3,39)} = 10.94$ , \*\*\* $p < 0.0001$ ,  $n = 10$ ). **C**, Analyses with the c739 driver. *amn*<sup>x8</sup>/Y flies expressing *amn* in the α/β KC display higher MTM and LTM scores than their *amn*<sup>x8</sup>/Y genetic controls and similar MTM and LTM scores to flies expressing *amn* in the α/β KC (MTM:  $F_{(3,59)} = 66.52$ , \*\*\* $p < 0.0001$ ,  $n = 15$ ; LTM:  $F_{(3,59)} = 53.87$ , \*\*\* $p < 0.0001$ ,  $n = 15$ ). **D**, c747 GAL4-driven *amn* expression does not rescue memory deficits of *amn*<sup>x8</sup> mutant flies. *amn*<sup>x8</sup>/Y:c747/UAS-amn flies show similar MTM and LTM scores to *amn*<sup>x8</sup>/Y genotypes (MTM:  $F_{(3,39)} = 11.83$ , \*\*\* $p < 0.0001$ ,  $n = 10$ ; LTM:  $F_{(3,23)} = 4.449$ , \* $p = 0.015$ ,  $n = 6$ ). Only males were analyzed in these experiments. **E**, Expression of the wild-type *amn* gene rescues normal 5HT staining. Canton-S control ( $n = 8$ ), *amn*<sup>x8</sup>/Y mutant ( $n = 8$ ) and *amn*<sup>x8</sup>/Y:c739/UAS-amn ( $n = 5$ ) brains were analyzed with anti-nc82 and anti-5HT primary antibodies. DPM neuron staining is indicated by a star. Identical Z-stack projections are shown for each panel. A representative brain of each genotype is shown. Scale bar, 10 μm. Error bars indicate SEM.

*amn* adult function. The fact that constitutive *amn* expression in either MB or DPM neurons can rescue memory deficits of *amn<sup>X8</sup>* flies suggests that the AMN peptide is secreted and can act non-autonomously to fulfill its roles during development and in the adult.

Analysis of GAL4-expression patterns indicates the presence of a MB enhancer located upstream of the *amn* transcription unit. Consistently, qPCR experiments show that *amn-RNAi* expression in the MB leads to a reduced level of *amn* RNA. Thus, similar to genes encoding components of the cAMP cascade, *rutabaga*, *dunce*, and *DCO*, and consistent with a role in this pathway, *amn* is expressed in the MB. Surprisingly, Waddell et al. (2000) did not report AMN expression in adult KC. They analyzed reporter-gene staining in *amn<sup>chpd</sup>* and *amn<sup>c651</sup>* fly brains, both lines that appear to contain an insertion in the DPM enhancer located in the *amn* coding sequence. Immunohistology experiments revealed AMN staining in DPM neurons at the site of protein synthesis, i.e., cell bodies (Waddell et al., 2000). AMN concentration in KC may be much lower than that observed in DPM large cell bodies, thus making difficult its observation with immunohistology experiments. Several reports indicate that *amn* is expressed at very low levels (Feany and Quinn, 1995; Chintapalli et al., 2007). A low level of expression is consistent with the fact that neuropeptides are known to display a higher receptor binding affinity than classical neurotransmitters (Merighi et al., 2011), thus eliciting their biological effects when released at lower quantities.

Remarkably, conditional inhibition of *amn* expression in the adult MB recapitulates memory phenotypes previously reported for *amn* mutant flies (Quinn et al., 1979; Yu et al., 2006; Lee et al., 2011). MTM and LTM are impaired, whereas MT-ARM and LT-ARM are normal, which is consistent with a requirement for *amn* in the  $\alpha/\beta$  neurons known to sustain MTM and LTM processes (Bouzaiane et al., 2015). In agreement with *amn* requirement in the MB, we show that *amn* expression in  $\alpha/\beta$  KC rescues *amn<sup>X8</sup>* memory deficits. These results are in apparent contradiction with a previous study showing that restoring AMN expression in the MB of *amn<sup>X8</sup>* flies did not restore normal MTM (Waddell et al., 2000). However, we also observed a lack of memory rescue when using one of the MB drivers that Waddell et al. (2000) had used, indicating that using distinct drivers to achieve AMN expression generates different results. Supporting these observations, several reports have previously described similar discrepancies among these MB drivers (Chen et al., 2008; Pavlopoulos et al., 2008; Bushey et al., 2009), probably reflecting different levels of expression and/or expression in different KC subpopulations (Rodan et al., 2002).

We report here that AMN is required for two distinct processes: its expression in DPM neurons is required for their development, and its expression in the adult MB is required for memory formation. Strikingly, *amn* memory phenotypes are rescued by AMN constitutive expression in either DPM or MB neurons. Therefore, AMN overexpression in MB neurons during development is able to compensate the absence of AMN in DPM neurons and conversely, AMN overexpression in adult DPM neurons is capable of rescuing the functional deficit in the MB. In other words, because AMN is secreted (Feany and Quinn, 1995), it could act on a nearby structure, thereby fulfilling both functions.

One of the three peptides encoded by *amn* is homologous to PACAP which is also known to be involved in brain development and function (Shen et al., 2013). Interestingly, several reports in mammals have suggested that PACAP is implicated in learning and memory (Sacchetti et al., 2001; Matsuyama et al., 2003; Schmidt et al., 2015; Cabezas-Llobet et al., 2018). An attractive

hypothesis is that AMN neuropeptide synthesized by KC would act autocrine-like to activate Rutabaga, the fly adenylate cyclase that mediates coincidence detection sustaining memory formation in the MB lobes (Levin et al., 1992; Tomchik and Davis, 2009; Gervasi et al., 2010). When overexpressed, AMN could act as a paracrine signal from a close structure. This would be possible at DPM/MB synapses, or in a longer range signaling process as most neuropeptides have multiple functions and are likely to also act as hormones (Nässel and Winther, 2010).

In conclusion, the observations reported here probably reflect tight anatomical and functional links between DPM neurons and MB lobes. In addition to its physiological role in memory, AMN is involved in the development of neuronal structures required for memory processes. Added to the fact that when overexpressed, AMN can act on a nearby structure, these features probably explain why areas of darkness have persisted for so long regarding the function of AMN. However, AMN function in adult DPM neurons remains to be determined.

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