Behavioral/Cognitive

**Drosophila** Middle-Term Memory: Amnesiac is Required for PKA Activation in the Mushroom Bodies, a Function Modulated by Neprilysin 1

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In *Drosophila*, the mushroom bodies (MB) constitute the central brain structure for olfactory associative memory. As in mammals, the cAMP/PKA pathway plays a key role in memory formation. In the MB, Rutabaga (Rut) adenylate cyclase acts as a coincidence detector during associative conditioning to integrate calcium influx resulting from acetylcholine stimulation and G-protein activation resulting from dopaminergic stimulation. Amnesiac encodes a secreted neuropeptide required in the MB for two phases of aversive olfactory memory. Previous sequence analysis has revealed strong homology with the mammalian pituitary adenylate cyclase-activating peptide (PACAP). Here, we examined whether amnesiac is involved in cAMP/PKA dynamics in response to dopamine and acetylcholine co-stimulation in living flies. Experiments were conducted with both sexes, or with either sex. Our data show that amnesiac is necessary for the PKA activation process that results from coincidence detection in the MB. Since PACAP peptide is cleaved by the human membrane neprilysin hNEP, we searched for an interaction between Amnesiac and Neprilysin 1 (Nep1), a fly neprilysin involved in memory. We show that when Nep1 expression is acutely knocked down in adult MB, memory deficits displayed by amn hypomorphic mutants are rescued. Consistently, Nep1 inhibition also restores normal PKA activation in amn mutant flies. Taken together, the results suggest that Nep1 targets Amnesiac degradation to terminate its signaling function. Our work thus highlights a key role for Amnesiac in establishing within the MB the PKA dynamics that sustain middle-term memory (MTM) formation, a function modulated by Nep1.

Key words: Amnesiac; coincidence detection; Drosophila olfactory memory; mushroom body; Neprilysin; PKA

**Significance Statement**

The *Drosophila amnesiac* gene encodes a secreted neuropeptide whose expression is required for specific memory phases in the mushroom bodies (MB), the olfactory memory center. Here, we show that Amnesiac is required for PKA activation resulting from coincidence detection, a mechanism by which the MB integrate two spatially distinct stimuli to encode associative memory. Furthermore, our results uncover a functional relationship between Amnesiac and Neprilysin 1 (Nep1), a membrane peptidase involved in memory and expressed in the MB. These results suggest that Nep1 modulates Amnesiac levels. We propose that on conditioning, Amnesiac release from the MB allows, via an autocrine process, the sustaining of PKA activation-mediating memory, which subsequently is inactivated by Nep1 degradation.

**Introduction**

Associative learning, which temporally pairs a conditioned stimulus (CS) to an unconditioned stimulus (US), is a powerful way of acquiring adaptive behavior. At the molecular and cellular levels, the association between CS and US is mediated by coincidence detection mechanisms that reflect the superadditive activation of a molecular pathway in the presence of both stimuli. One of the major coincidence detectors is the cAMP/PKA pathway, which depends on Type-I adenylate cyclases stimulated by both calcium/calmodulin, via acetylcholine signaling representing the CS, and G-protein coupled to dopamine metabotropic receptors activated by dopaminergic neurons encoding the US (Mons et al., 1999).
In *Drosophila*, the mushroom bodies (MB) constitute the central integrative brain structure for olfactory memory (de Belle and Heisenberg, 1994). The MB are composed of 4000 intrinsic neurons called Kenyon cells (KC), and classed into three subtypes whose axons form two vertical (α and α′) and three mediolateral (β, β′, and γ) lobes (Crittenden et al., 1998). Using a classical conditioning paradigm in which an odorant (CS) was paired to electric shocks (US), Bouzaiane et al. (2015) revealed that flies are capable of forming six discrete memory phases reflected at the neural network level. Among these phases are middle-term memory (MTM) and long-term memory (LTM), which are both encoded in αβ KC. As in mammals, the fly cAMP/PKA pathway plays a key role in associative memory, wherein the adenyl cyclase Rutabaga (Rut) acts as a coincidence detector in the MB to associate the CS and US pathways (Levin et al., 1992; Tomchik and Davis, 2009; Gervasi et al., 2010).

The amnesiac *Drosophila* mutant (*amn*) was isolated in a memory defect behavioral screen (Quinn et al., 1979). As with other components of the cAMP/PKA pathway involved in *Drosophila* memory, *amn* is expressed in the MB (Shih et al., 2019). We recently showed that *amn* expression in the MB is specifically required for MTM and LTM (Turrel et al., 2018). *amn* encodes a neprilysin precursor with a signal sequence (Feany and Quinn, 1995). Sequence analyses suggest the existence of three peptides, with one of them homologous to mammalian pituitary adenylate cyclase-activating peptide (PACAP) (Feany and Quinn, 1995; Hashimoto et al., 2002). PACAP is widely expressed throughout the brain, acting as a neuromodulator or neurotrophic factor through activation of G-protein-linked receptors to regulate a variety of physiological processes through stimulation of cAMP production (Miyata et al., 1989; Arimura, 1998; Vaudry et al., 2000). Furthermore, PACAP may exert a role in learning and memory (Sacchetti et al., 2001; Hashimoto et al., 2002; Matsuyama et al., 2003; Schmidt et al., 2015).

After its release, a neurotransmitter’s action is terminated either by diffusion, re-uptake by the presynaptic neuron, or enzymatic degradation. In contrast, neprilyspeptidase signaling is exclusively terminated by enzymatic degradation (Russo, 2017). Possible enzyme candidates include neprilysins, type 1 metalloproteinases whose main function is the degradation of signaling peptides at the cell surface (Turner et al., 2001). Indeed, the human neprilysin hNEP is capable of cleaving a PACAP neuropeptide (Gourlet et al., 1997). *Drosophila* express four neprilysins (Bland et al., 2008; Meyer et al., 2011) that are all required for MTM and LTM, establishing that neprilysin degradation is a central process for memory formation (Turrel et al., 2016). Among the four neprilysins, Neprilysin 1 (Nep1) is the only one whose expression is required for MTM in the MB (Turrel et al., 2016).

Here, we aimed to confirm whether AMN intervenes in memory by modulating cAMP concentration, as suggested by its sequence homology. For this, we analyzed PKA dynamics in the MB vertical lobes. Our results show that Nep1 mutant brains fail to display PKA activation in the α lobe in response to co-application of dopamine and acetylcholine. We then searched whether Nep is involved in terminating AMN action. Using RNAi, we show that Nep1 knock-down restores normal MTM and normal PKA dynamics in *amn* mutants, establishing a functional interaction between Nep1 and AMN in the MB.

**Materials and Methods**

*Drosophila* stocks

*Drosophila melanogaster* wild-type Canton Special (CS) and mutant flies were raised on standard medium at 18°C in 60% humidity in a 12/12 h light/dark cycle. *UAS-RNAi* lines were obtained from the Vienna *Drosophila* Resource Center (VDRC; *amnRNAi*: ID 5606; *Nep1RNAi*: ID 108860) and from the National Institute of Genetics (NIG; *Nep1RNAi*: ID 5894-R3). Ga4 drivers were used to achieve RNAi expression. The 2387 and 2739 drivers were used for expression in the MB, and in αβ neurons, respectively. *tubulin-Gal80* (Gal80*) was used for conditional expression in the adult. The *amn*Δ9-null mutant line is described in Moore et al. (1998). To specifically induce RNAi expression in adults, Gal80* was inactivated by keeping flies at 30°C for 3 d before conditioning, as described by McGuire et al. (2003). Rescue experiments were conducted with the *UAS-amn* construct (*amn*) described in Waddell et al. (2000). For Nep1 overexpression, a KpnI-PCR fragment (PfuUltra HF DNA polymerase, Agilent) encompassing the Nep1 coding sequence was generated using the GH03315 Nep1 cDNA (Rubin et al., 2000). The following oligonucleotides were used: 5′-CCCCCGGTACCCCTATAGTA GAAGATGTCG -3′ (forward) and 5′-CCCCCGGTACCCCGGGCG GAACTACCAC -3′ (reverse) where KpnI sites are underlined. The PCR fragment was subjected to Kpn1 digestion (New England Biolabs) and purified from agarose gel (QIAquick Gel Extraction kit, QIAGEN) before cloning into the pUAST vector (Brand and Perrimon, 1993) digested by Kpn1. Resulting clones were verified by multiple digestion and sequencing. The pUAST-Nep1 plasmid was injected into the CS line (Rainbow Transgenic Flies, Inc) to create the *UAS-Nep1* line (*Nep1*).**

**Behavioral experiments**

Flies were trained with classical olfactory aversive conditioning protocols as described by Pascual and Prétat (2001). Training and testing were performed at 25°C in 80% humidity. Conditioning was performed on samples of 25–35 flies aged 3–4 d using 0.360 mM 3-octanol (95–95% purity, Sigma-Aldrich) and 0.325 mM 4-methylcyclohexanol (99% purity, Sigma-Aldrich). 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). To assess MTM, flies were submitted to one-cycle training, and memory was tested 2 h later.

**Quantitative PCR analyses**

Flies were raised at 25°C and further incubated for 3 d at 30°C in the case of Ga4 induction. Total RNA was extracted from 60 female heads using the RNeasy Plant Mini kit (QIAGEN). Preparations were treated with DNaseI (Biolabs) for 15 min at 37°C. DNase was heat-inactivated with EDTA (10 mM). Samples were cleaned with the RNeasy MiniElute 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 were as previously described (Turrel et al., 2018). The target cDNA level was compared against the level of α-Tub48B (Tub, CG1913) cDNA, which was used as a reference. Amplification was performed using a LightCycler 480 (Roche) and the SYBR Green I Master mix (Roche). Reactions were conducted in triplicate. Specificity and size of amplification products were assessed by melting curve analyses and agarose gel electrophoresis, respectively. Expression relative to the reference was expressed as a ratio (2^−ΔCp, where Cp is the crossing point).

**Live imaging**

Preparation of flies for live imaging was performed as previously described (Fiala and Spall, 2003). Untrained flies were removed from culture bottles and glued to a plastic coverslip coated with a thin transparent plastic sheet. The coverslip was then placed in the recording chamber and a drop of *Drosophila* Ringer’s solution was placed over the head (130 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 36 mM sucrose, and 5 mM HEPES-NaOH; pH 7.3). A small region of cuticle was cut away from the top of the head capsule, and tracheae were removed from the area. Flies were then placed under a Leica TCS-SPL microscope and observed with a 25× water-immersion objective (NA = 0.95, Leica). Experiments were performed at room temperature and brains were...
PKA activation in the MB vertical lobes after coincidence detection is abolished in amn-deficient flies. Either the PKA AKAR2 FRET probe or the calcium GCaMP3 probe was expressed in the MB with the 238Y Gal4 driver. Only males were analyzed. A, Bath application of either dopamine (DA; 10 μM) or acetylcholine (ACh; 10 mM) does not induce PKA activation in the vertical lobes (t test, t(18) = 1.549, p = 0.139, n = 10). B, PKA is activated on co-stimulation of DA and ACh in +/Y;238Y,AKAR2/+ control flies. In contrast, DA and ACh co-application does not lead to PKA activation in amn mutant fly brains. Mean FRET ratio changes in the vertical lobe are shown in response to co-application of DA and ACh (F(2,29) = 9.301, ***p = 0.0008, n = 10). Asterisks denote the pairwise post hoc comparisons between the genotype of interest and the +/Y;238Y,AKAR2/+ control, following the usual nomenclature. Right panel, Grouped time course of FRET ratios. Graphs were produced using the same data as for the bar graph. The arrow indicates the time of neurotransmitter application. C, DA application at high

Figure 1. PKA activation in the MB vertical lobes after coincidence detection is abolished in amn-deficient flies. Either the PKA AKAR2 FRET probe or the calcium GCaMP3 probe was expressed in the MB with the 238Y Gal4 driver. Only males were analyzed. A, Bath application of either dopamine (DA; 10 μM) or acetylcholine (ACh; 10 mM) does not induce PKA activation in the vertical lobes (t test, t(18) = 1.549, p = 0.139, n = 10). B, PKA is activated on co-stimulation of DA and ACh in +/Y;238Y,AKAR2/+ control flies. In contrast, DA and ACh co-application does not lead to PKA activation in amn mutant fly brains. Mean FRET ratio changes in the vertical lobe are shown in response to co-application of DA and ACh (F(2,29) = 9.301, ***p = 0.0008, n = 10). Asterisks denote the pairwise post hoc comparisons between the genotype of interest and the +/Y;238Y,AKAR2/+ control, following the usual nomenclature. Right panel, Grouped time course of FRET ratios. Graphs were produced using the same data as for the bar graph. The arrow indicates the time of neurotransmitter application. C, DA application at high
continuously perfused with *Drosophila* Ringer's solution. Four frames were acquired per minute during live imaging. For AKAR2-FRET experiments, two-photon excitation was obtained using a mode-locked Ti:sapphire laser (Mai Tai, Spectra Physics) set at 860 nm. The specifications of the detection channels were identical to those described in Gervasi et al. (2010). Scanning and acquisition were controlled by the LAS AF software (Leica). CFP and YFP images (512 × 512 pixels) were acquired simultaneously with a line rate of 400 Hz. To analyze the calcium response, the GCaMP3 probe was excited by the 488-nm line of an argon laser, with scanning at a line rate of 400 Hz. Fluorescence-induced light emission was collected by a photomultiplier in the 505- to 555-nm wavelength range.

**Image analyses**

To obtain FRET ratio time courses, CFP and YFP images were background subtracted at each time point, in which the background was subtracted at each time point, in which the background was accounted for by the respective channel's signal at each time point. Image analyses were conducted on a segment of acquisition time from 500 to 700 s. Photobleaching compensation, determined during the baseline, was applied for all files from each experiment.

**Statistical analyses**

Comparisons of the data series between two conditions were achieved by using one-way analysis of variance, which were followed (if significant at p ≤ 0.05) by Newman–Keuls multiple comparisons tests. The overall analysis of variance p value is provided in the legends along with the value of the corresponding Fisher distribution F(x,y), where x is the number of degrees of freedom for groups and y is the 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 the GraphPad Prism 5 software. All data are displayed as mean ± SEM.

**Results**

PKA activation following coincidence detection in MB vertical lobes is abolished in *amn* mutant flies

It was shown that a PACAP-like peptide with an apparent molecular weight compatible with AMN sequence exists in the fly (Zhong and Peña, 1995). To investigate AMN as a putative adenylate cyclase-activating peptide, we first analyzed PKA dynamics in MB neurons of *amn<sup>Y</sup>* null mutant flies that contain a genomic deletion of the *amn* open reading frame (Moore et al., 1998). In response to both dopamine (which represents the US) and acetylcholine (which mimics the CS and leads to an increase in intracellular calcium), the *Drosophila* adenylate cyclase Rut mediates coincidence detection through an increase in cAMP concentration, resulting in PKA activation in the vertical lobes (Tomchik and Davis, 2009; Gervasi et al., 2010). This PKA activation observed with artificial stimulation is used as a proxy to study coincidence detection events that are thought to occur during actual conditioning. Using a two-photon microscope, we conducted in vivo imaging of the PKA FRET probe AKAR2 (Zhang et al., 2001, 2005) expressed in the MB (Gervasi et al., 2010). As previously shown (Gervasi et al., 2010), stimulation of control brains (+/Y;238Y,AKAR2/+ ) with either 10 μM dopamine or 10 μM acetylcholine did not lead to PKA activation in the vertical lobes (Fig. 1A) whereas co-stimulation did (Fig. 1B), thus demonstrating that coincidence detection occurred. In sharp contrast, co-stimulation of *amn<sup>Y</sup>*/+ null mutant brains did not induce PKA activation in the vertical lobes (Fig. 1B), suggesting that coincidence detection did not occur. To analyze the effect of *amn* reduction in the MB, we targeted *amn* expression with a UAS-RNAi construct (amn<sub>RNAi</sub>) previously shown to phenocopy *amn* mutant defects (Aldrich et al., 2010; Turrel et al., 2018). We thus observed that like *amn<sup>Y</sup>*/+ null flies, specific inhibition of *amn* expression in the MB led to defective coincidence detection following brain co-stimulation with dopamine and acetylcholine (Fig. 1B).

Next, we analyzed the effect of brain bath application of 100 μM dopamine, a higher concentration known to activate Rut (Gervasi et al., 2010). As previously described, flies expressing the AKAR2 probe in the MB (+/Y;238Y,AKAR2/+ ) showed an increase in FRET ratio in the vertical lobes under these conditions (Fig. 1C). Stimulation with 100 μM dopamine also induced increased PKA activity in *amn<sup>Y</sup>*/+;238Y,AKAR2/+ brains (Fig. 1C). Similar results were obtained with *amn<sup>RNAi</sup>* expressing flies (Fig. 1C), showing that AMN is not required for Rut to respond to high levels of dopamine. We further analyzed whether *amn* knock-out influences calcium signaling in the MB following acetylcholine application using the GCaMP3 probe (Tian et al., 2009). We thus observed that *amn<sup>X8</sup>* null flies exhibit a normal MB calcium response (Fig. 1D). This confirms that the inability of *amn* mutant brains to respond to co-application of acetylcholine and dopamine is neither caused by a defect in calcium signaling, nor by the inability of Rut to respond to a high concentration of dopamine.

These results were reinforced by assessing PKA dynamics under adult stage-specific acute *amn* downregulation. In order to restrict *amn<sup>RNAi</sup>* expression to adulthood, we took advantage of the TARGET system (McGuire et al., 2003), which relies on a temperature-sensitive Gal80 inhibitor that represses Gal4 transcriptional activity at low temperature, while this inhibition is lifted at high temperature (30°C). Experiments were performed with a tub-Gal80<sup>t</sup> line (Gal80<sup>t</sup>) and after 3 d of induction, Gal80<sup>t</sup>*++;238Y,AKAR2/+ flies showed defective coincidence detection following brain co-stimulation with dopamine and acetylcholine (Fig. 1E).

Lastly, we investigated whether restoring *amn* expression in the MB of *amn<sup>X8</sup>* flies could restore coincidence detection. We observed that *amn<sup>Y</sup>*/+;238Y,AKAR2/+ flies responded efficiently to co-applied dopamine and acetylcholine (Fig. 1F). Thus, *amn* expression in the MB is sufficient to restore normal PKA activation.
Nep1 overexpression impairs MTM and prevents PKA activation following coincidence detection

Previously, it was shown that human PACAP peptide is degraded in vitro by hNEP (Gourlet et al., 1997). Interestingly, Drosophila Nep1 is expressed in the MB (Sitnik et al., 2014), and is involved in the adult MB in the same memory phases as AMN (Turrel et al., 2016), raising the question of whether in the fly, Nep1 interacts with AMN. We reasoned that if the Nep1 protease targets AMN peptide, Nep1 overexpression should cause the same phenotype as the amn deficiency. To assess this hypothesis, we examined MTM in flies overexpressing Nep1 in the adult MB. To this end we used a UAS-Nep1 construct (Nep1\textsuperscript{+}, Materials and Methods) in combination with the tubulin-Gal80\textsubscript{T},c739 Gal4 driver (Gal80\textsuperscript{T}, c739; Aso et al., 2009). To analyze memory, we used a classical conditioning paradigm in which an odor is paired with electric shocks. Here, groups of flies were successively exposed to two distinct odors, only one of which was associated with the delivery of voltage pulses. MTM was then assessed 2 h after a single conditioning. After induction, Gal80\textsuperscript{T},c739;Nep1\textsuperscript{+}/+ flies showed impaired MTM (Fig. 2A). In contrast, in the absence of Gal4 induction, MTM was normal (Fig. 2A). 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 Nep1 overexpression in the adult MB is deleterious for MTM.

We next examined PKA dynamics in the MB of flies overexpressing Nep1. Following co-application of dopamine and acetylcholine, we did not observe PKA activation in 238Y,AKAR2/Nep1\textsuperscript{+} brains (Fig. 2B). We verified that PKA activation on application of dopamine at high concentration was not impaired by Nep1 overexpression (Fig. 2C). Likewise, Nep1 overexpression did not alter calcium signaling (Fig. 2D). We conclude that, as is the case for amn deficiency, Nep1 overexpression in the MB abolishes PKA dynamics on coincidence detection. Taken together, the data show that Nep1 overexpression leads to phenotypes similar to that of amn deficiency, consistent with the possibility that Nep1 functionally interacts with AMN.

Nep1 knock-down restores wild-type MTM and PKA activation in amn mutant flies

To investigate a potential interaction between AMN and Nep1, we wanted to knock down Nep1 expression in amn-deficient flies and analyze MTM. The amn\textsuperscript{Mx28} null mutant line results from the excision of one of two Gal4-encoding P-elements inserted in the amn\textsuperscript{RNAi} line (Moore et al., 1998), with amn\textsuperscript{Mx28} flies thus retaining Gal4 activity that is localized in the MB (Keene et al., 2004; Turrel et al., 2018). This line could thus be used in combination with Nep1 RNAi (Nep1\textsuperscript{RNAi/}) to knock down Nep1 in MB neurons in an amn-deficient context. As the amn gene is located on the X chromosome, while amn\textsuperscript{Y} hemizygous males do not express amn, amn\textsuperscript{X} heterozygous female flies express amn levels twice lower than wild-type levels.

We previously showed that amn loss of function achieved by amn\textsuperscript{RNAi} expression in the MB results in MTM impairment (Turrel et al., 2018). Consistently, we observed here that amn\textsuperscript{X} hypomorph flies exhibited an MTM deficit (Fig. 3A). Strikingly, Nep1 knock-down in amn\textsuperscript{X}/+ flies led to wild-type MTM (Fig. 3A), revealing a full rescue of the MTM deficit displayed by
Table 1. Shock reactivity and olfactory acuity of flies overexpressing Nep1 in adult α/β neurons

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Shock reactivity</th>
<th>Olfactory acuity</th>
<th>Methylcyoxalanol</th>
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</thead>
<tbody>
<tr>
<td>Gal80ts,c739/+</td>
<td>65.52 ± 9.17</td>
<td>48.25 ± 8.27</td>
<td>44.63 ± 7.03</td>
</tr>
<tr>
<td>Gal80ts,c739/+;Nep1/+</td>
<td>62.18 ± 9.52</td>
<td>54.29 ± 8.45</td>
<td>47.50 ± 8.12</td>
</tr>
<tr>
<td>Nep1/+</td>
<td>58.04 ± 9.38</td>
<td>52.63 ± 6.44</td>
<td>51.75 ± 8.73</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. After 3 d of induction, neither shock reactivity (t[22,1] = 0.163, p = 0.851, n = 12) nor olfactory acuity for octanol (t[22,1] = 0.163, p = 0.851, n = 7) and methylcyoxalanol (t[22,1] = 0.201, p = 0.819, n = 8) is impaired in flies overexpressing Nep1 under the control of the Gal80ts,c739 driver.

ammX/Y+ flies. We verified with qPCR experiments that Nep1 inhibition in ammX/Y+ flies did not result in the modification of amm mRNA levels (Fig. 3A), suggesting that the observed memory rescue is mediated at the AMN protein level. A simple hypothesis to account for these results is that Nep1 degrades the AMN peptide. In this scenario, Nep1 reduction would lead to an increase in the level of the AMN peptide, thereby restoring AMN to a level compatible with its normal function in memory. According to this hypothesis, Nep1 inhibition should not be able to rescue the memory deficit of amnX/Y null mutant flies, as they do not express AMN. Indeed, our behavior experiments show that amnX/Y; Nep1RNAI1/+ flies display an MTM deficit similar to ammX/Y flies (Fig. 3B). This result demonstrates that the memory rescue observed on Nep1 inhibition in ammX/Y+ flies is dependent on AMN expression.

We next aimed to analyze the effect of Nep1 acute inhibition in adult ammX/Y+ female flies. As expected, when Gal4 transcription was not induced, ammX/Y+;Gal80ts/+;Nep1RNAI1/+ flies exhibited an MTM deficit similar to that of ammX/Y+;Gal80ts/+ flies (Fig. 3C). In sharp contrast, when Gal4 transcription was induced, ammX/Y+;Gal80ts/+;Nep1RNAI1/+ flies exhibited normal MTM scores (Fig. 3C). We conclude that Nep1 knock-down restricted to adulthood allows the rescue of the MTM deficit displayed by amnX/Y+ hypomorph flies. Taken together, these results establish a functional interaction between amn and Nep1 in the adult fly brain.

The discovery that AMN functionally interacts with Nep1 prompted us to analyze PKA dynamics. While ammX/Y+;238Y, AKAR2/+ flies did not show PKA activation on co-application of dopamine and acetylcholine, ammX/Y+;238Y,AKAR2/Nep1RNAI flies markedly displayed PKA activation to a level similar to that of the control (Fig. 3D). This result indicates that Nep1 inhibition restores normal coincidence detection in ammX/Y+ flies.

Nep1 knock-down in the adult MB of amm mutant flies restores wild-type MTM and PKA activation

To address in greater detail the spatial relationship between amn and Nep1, we used the Gal80ts,c739 driver to achieve both amn and Nep1 inhibition in adult α/β MB neurons. Although both Nep1 expression in α/β neurons is required for MTM (Turrell et al., 2016), Gal80ts,c739/+;Nep1RNAI1/+–induced flies displayed normal MTM (Fig. 4A). This result has already been reported, and can be explained by the fact that Nep1RNAI1 does not allow inhibition of Nep1 expression at a level sufficient to alter MTM (Turrell et al., 2017). As expected (Turrell et al., 2018), ammRNAI expression in adult α/β neurons mimicked the MTM deficit observed in amnX/Y+ flies (Fig. 4A). Furthermore, concomitant expression of Nep1RNAI1 restored normal MTM (Fig. 4A). We considered that co-expression of two distinct Gal4 UAS transgenes might affect the expression level of each of them, via a Gal4 dilution effect. To rule out the possibility that the normal memory score displayed by Gal80ts,c739/ammRNAI1;Nep1RNAI1/+–induced flies was caused by a reduced level of amm inhibition, we performed qPCR experiments to assess the level of amm expression. As expected, Gal80ts,c739/ammRNAI flies head flies contained a reduced level of amm mRNA as compared with the control (Fig. 4B). Importantly, Gal80ts,c739/ammRNAI1;Nep1RNAI1/+ flies expressed a level of amm mRNA that was also reduced in comparison to the control, and which was not significantly different from flies expressing only Nep1RNAI1 (Fig. 4B). We thus conclude that in adult α/β neurons, inhibition of Nep1 expression rescues the amm MTM impairment caused by amm inhibition, without affecting amm mRNA levels, implicating an effect that depends on AMN peptide level.

To confirm these results, we repeated behavior experiments with another non-overlapping RNAI construct, Nep1RNAI2 (Turrell et al., 2016). Unlike Nep1RNAI1 expression, Nep1RNAI2 expression in adult α/β neurons caused an MTM deficit (Fig. 4C). Strikingly, Gal80ts,c739/ammRNAI1;Nep1RNAI2-induced flies showed normal MTM (Fig. 4C). We verified that co-expression of Nep1RNAI2 together with ammRNAI did not modify the level of amm mRNA (Fig. 4D). Thus, while expression of either ammRNAI or Nep1RNAI2 is deleterious for MTM, their co-expression does not impact memory. These results reveal that in this context, no other substrate than AMN needs to be targeted by Nep1 to ensure normal MTM. In conclusion, acute knock-down of Nep1 in adult α/β neurons rescues the MTM deficit displayed by amm mutant flies demonstrating that both proteins are involved in the same pathway for memory formation.

We next analyzed PKA dynamics in flies expressing concomitantly amn and Nep1 RNAI in MB neurons. As already observed, ammRNAI1+;238Y,AKAR2/+ flies did not show any PKA activation following co-application of dopamine and acetylcholine (Fig. 4E). Co-expression of either the Nep1RNAI1 or Nep1RNAI2 construct restored PKA activation to a normal level (Fig. 4E). Taken together, our data demonstrate that Nep1 knock-down in α/β neurons restores normal PKA dynamics in amm mutant flies.

Discussion

We previously showed that AMN expression is required in the MB for Drosophila memory. Here, we establish that AMN expression in the MB is necessary for the synergistic activation of PKA observed on co-stimulation by dopamine and acetylcholine in the α lobe, a process that is thought to mimic the coincidence detection event underlying memory formation. Furthermore, our data demonstrate a functional interaction between AMN and Nep1, suggesting that Nep1 targets AMN degradation, thereby terminating AMN signaling.

Six different aversive memory phases that are spatially segregated have been described in Drosophila (Bouzaiane et al., 2015). Their formation relies on distinct neuronal circuits, as well as distinct molecular and cellular mechanisms (Comas et al., 2004; Blum et al., 2009; Pagani et al., 2009; Aso et al., 2012; Bouzaiane et al., 2015; Murakami et al., 2017). rut mutants are impaired in specific memory phases (Tully and Quinn, 1985; Han et al., 1992; Blum et al., 2009; Scheunemann et al., 2013), including short-term memory (STM), encoded in γ KC, and MTM encoded in α/β KC (Scheunemann et al., 2013; Bouzaiane et al., 2015). It was previously shown that Rut expression restricted to γ KC is sufficient to restore STM, but not MTM, in rut mutant flies (Akalal et al., 2006; Blum et al., 2009). It is thus likely that
Acute inhibition of Nep1 expression restores wild-type MTM in adult amnX8/Y flies. Only females were analyzed. Left panel, mean FRET ratio changes in the vertical lobe are shown in response to co-application of dopamine and acetylcholine. Graphs were produced using the same data as for the bar graph. The arrow indicates the time of neurotransmitter application. ns, not significant.

Figure 3. Nep1 knock-down restores wild-type MTM and PKA activation in amnX8/Y mutant flies. A, Nep1 knock-down in amnX8/Y;Gal80ts/1;Nep1RNAi1/ flies restores wild-type MTM. Only females were analyzed. Left panel, amn1 mRNA. qPCR analyses of amn mRNA levels. Total RNA extracted from female heads was reverse-transcribed and further processed by qPCR. Expression relative to the reference is given as a ratio (2^-ACq, where Cp is the crossing point). amnX8/Y; express amn mRNA levels lower than wild-type. amnX8/Y; and amnX8/Y;Nep1RNAi1/ flies show normal scores (F(2,49) = 3.12, *p = 0.0174, n ≥ 12). Right panel, Nep1 inhibition in amnX8/Y flies does not modify the amn mRNA level. Each bar corresponds to six measurements from three independent experiments. B, Nep1 inhibition does not restore wild-type MTM in amnX8/Y flies. Only males were analyzed. amnX8/Y flies exhibit lower scores than wild-type control flies while amnX8/Y;Nep1RNAi1/ flies do not show normal scores (F(2,20) = 4.12, *p = 0.0174, n ≥ 12). Right panel, amnX8/Y flies are similar to amnX8/Y;Gal80ts/1;Nep1RNAi1/ flies in the vertical lobe. This spatial restriction of dopamine action in the mushroom bodies suggests that the coincidence detector operates in parallel in γ and α/β KC, resulting in STM and MTM formation, respectively. Interestingly, mutants expressing a reduced amn level display normal STM (Turrel et al., 2018). Thus, AMN is most likely not required for the coincidence detection process that leads to STM, a process that remains to be identified. Using in vivo imaging, we previously showed that co-application of dopamine and acetylcholine induces a strong synergistic PKA response, which is Rut dependent and occurs specifically in MB vertical lobes (Gervasi et al., 2010). Here, we show that this coincident PKA activation in the α lobe is abolished in amn mutants, while neither calcium signaling nor cAMP signaling following dopaminergic stimulation alone are altered. We propose that PKA activation mimics the coincidence detection event that occurs in α/β KC during MTM formation, and that AMN intervenes in this process by enabling a sustained Rut-mediated PKA activation in the MB α lobe.

AMN might thus act at a step that ranges from the initial coincidence detection event that provokes Rut activation, to the final level of PKA activation. This is consistent with previous reports that AMN and DC0, the fly PKA catalytic subunit (Skoulakis et al., 1993), act in a common pathway, and that AMN function is upstream of DC0 function (Yamazaki et al., 2007). If AMN plays a role posterior to the coincidence detection event, it could be involved in an increase in cAMP concentration through the inhibition of phosphodiesterases that degrade cAMP. Indeed, dopamine receptors positively coupled to adenylyl cyclases are equally distributed in all MB lobes as are DC0 and Rut (Han et al., 1992; Kim et al., 2003), whereas 100 μM dopamine only induces a PKA response in the α lobe. This spatial control is achieved by the cAMP-specific phosphodiesterase Dunce (Dnc; Byers et al., 1981; Davis and Kiger, 1981; Qiu et al., 1991) which preferentially degrades cAMP in the β and γ lobes, thus restricting high dopaminergic stimulation alone. Indeed, the fact that one of the AMN peptides is
Figure 4. Nep1 knock-down in the adult MB of amn mutant flies restores wild-type MTM and PKA activation. A–D, Acute inhibition of Nep1 expression in the α/β KC of adult amn knocked down flies restores normal MTM. Flies were incubated for 3 d at 30°C to induce Nep1 and amn RNAi expression in the α/β KC under the control of the c739 Gal4 driver. A, left panel, Nep1RNAi expression does not affect MTM. Gal80ts,c739/+;Nep1RNAi1/+ flies exhibit memory scores similar to their genetic controls ($F_{2,31} = 2.677$, $p = 0.086$, $n/C21 = 10$). Right panel, Nep1RNAi acute expression in the α/β KC of adult amn knocked down flies restores normal MTM. Gal80ts,c739/amnRNAi flies exhibit memory lower scores than their genetic controls, while Gal80ts,c739/amnRNAi,Nep1RNAi1/+ flies exhibit memory scores similar to their controls, and higher than Gal80ts,c739/amnRNAi flies ($F_{4,81} = 3.133$, **$p = 0.019$, $n/C21 = 16$). B, qPCR analyses of amn expression levels. Total RNA extracted from female heads was reverse-transcribed and further quantified by qPCR. Expression relative to the reference is expressed as a ratio ($2^{-\Delta\Delta C_{p}}$, where $C_{p}$ is the crossing point). Gal80ts,c739/amnRNAi flies display a lower level of amn mRNA compared with the Gal80ts,c739/1 control. Gal80ts,c739/amnRNAi,Nep1RNAi1/+ flies show a similar level of amn mRNA compared with Gal80ts,c739/amnRNAi flies ($F_{2,27} = 4.214$, ***$p = 0.027$, $n/C21 = 8$). Each bar corresponds to 8–10 measurements from four to five independent experiments. C, left panel, Nep1RNAi2 expression leads to an MTM deficit. Gal80ts,c739/Nep1RNAi2 flies exhibit lower memory scores than their genetic controls ($F_{2,32} = 9.83$, ***$p = 0.0005$, $n/C21 = 10$). Right panel, Nep1RNAi2 acute expression in the α/β KC of adult amn knocked down flies restores normal MTM. Gal80ts,c739/amnRNAi,Nep1RNAi2/+ flies exhibit memory lower scores than their genetic controls, while Gal80ts,c739/amnRNAi,Nep1RNAi2/+ flies exhibit memory scores similar to their controls, and higher than Gal80ts,c739/amnRNAi flies ($F_{4,79} = 4.888$, **$p = 0.032$, $n/C21 = 14$). D, qPCR analyses of amn expression levels. Total RNA extracted from female heads was reverse-transcribed and further quantified by qPCR. Expression relative to the reference is expressed as a ratio ($2^{-\Delta\Delta C_{p}}$, where $C_{p}$ is the crossing point).
homologous to PACAP suggests that AMN might play a role in activating the adenylate cyclase Rut through G-protein-coupled receptors. This hypothesis fits with sequence prediction (Feany and Quinn, 1995; Hashimoto et al., 2002), and is supported by studies showing that AMN is functionally related to human PACAP. It was initially reported that Rut is activated by the application of human PACAP-38 (Zhong, 1995, 1996), and later shown that bath application of PACAP-38 rescues L-type current deficiency in amn

Following the first CS/US association, initial PKA activation leads to AMN secretion that will further activate Rut. MTM is progressively building up.

Figure 5. A model for the role of AMN and Nep1 in MTM formation. A, Voltage pulse associated to odorant delivery generates coincidence detection. Initial Rut activation takes place. B, Following the first CS/US association, initial PKA activation leads to AMN secretion that will further activate Rut. MTM is progressively building up. C, AMN signaling is terminated by Nep1.

This moderate level of PKA activation does not mediate MTM formation and is below detection threshold. We hypothesize that this initial increase in PKA activity, directly or indirectly, triggers the second step of the process, namely AMN secretion, and thus generate an activation loop whereby AMN activates Rut, hence creating a much higher level of Rut activation and subsequent levels of PKA activation that is observable with the AKAR2 probe (Fig. 5B). MTM formation would rely on an AMN-dependent PKA-activation loop terminated on AMN degradation by Nep1 (Fig. 5C).

One previous study has indicated that human PACAP is a substrate for hNEP (Gourlet et al., 1997), and our present work in Drosophila describes a functional interaction between AMN and Nep1. Importantly, whereas Nep1 knock-down rescues the amn mutant memory phenotype in a genetic context where the AMN level is reduced to ~50% versus wild-type flies (heterozygous for the amn null allele), it fails to do so in a genetic context where AMN is absent (i.e., in flies hemizygous for the amn null allele). Namely, the memory rescue observed on Nep1 inhibition is dependent on the presence of AMN, suggesting that this latter is targeted by Nep1. While a biochemical confirmation of this hypothesis would be welcome, it is technically difficult to achieve. Specifically, not only are AMN antibodies not available, but amn mRNA is expressed at very low levels (Feany and Quinn, 1995; Chintapalli et al., 2007; Affymetrix data available at http://flyatlas.org/atlas.cgi?name=FBgn0000076), indicating that AMN peptide may be very scarce.

The observation that the AMN peptide may be targeted by Nep1 is in agreement with a neuromodulatory function. Once released, a signaling molecule must be removed from its site of action to prevent continued stimulation, and to allow new signals to propagate. If neurotransmitter’s action is terminated either by diffusion, re-uptake by the presynaptic neuron, or enzymatic degradation, signaling neuropeptides are specifically removed by degradation. The intensity and duration of neuropeptide-
mediated signals are thus controlled via the cleavage of these neuropeptides by peptidases like nephrilysins. Despite a few exceptions (Bland et al., 2007; Meyer et al., 2009), nephrilysins occur as integral membrane endopeptidases whose catalytic site faces the extracellular compartment (Nalivaeva and Turner, 2013). We hypothesize that on conditioning, AMN is secreted by the KC to participate in Rut activation via G-protein-coupled receptors, and is ultimately removed from the extracellular compartment by Nep1 anchored at the KC membrane. Importantly, AMN expression in the MB restores normal PKA dynamics in amn null mutant flies, suggesting that the AMN peptide secreted by the MB on conditioning should act in an autocrine-like way to sustain Rut activity in the α/β neurons. Interestingly, the effects of neuropeptide transmitters are very diverse and often long-lived (Nässel, 2009), which fits well with the specific involvement of AMN peptide in non-immediate memory phases via sustained PKA activation.

Up to date, fly nephrilysins have been involved in several behaviors: in the control of circadian rhythms, via hydrolysis of the pigment dispersing factor neurotransmitter (Isaac et al., 2007), and in the control of food intake via cleavage of insulin-like regulatory peptides (Hallier et al., 2016). In the latter study, it was shown that both Neprolisin 4 knock-down and overexpression in the larval CNS cause reduced food intake (Hallier et al., 2016). In a similar way, we show here that both Nep1 knock-down and overexpression in α/β KC impairs MTM, consistent with the need for a proper control of AMN levels. We suggest that Nep1 overexpression results in a lower loss of function, whereas Nep1 knock-down causes the prolongation of AMN action, thus generating a prolonged activation of the cAMP/PKA pathway, a process deleterious for memory. This is in agreement with a previous study demonstrating that overexpressing DC0 in the MB impairs MTM (Yamazaki et al., 2007).

In conclusion, we report here an acute role for AMN in memory formation via the PKA pathway in the α/β MB neurons, a function modulated by Nep1. Our results thus support a role for AMN as an activating adenylate cyclase peptide, much like the role of PACAP, bringing clarity to the role PACAP may play in memory consolidation in mammals.

References


