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Report

# Heterotypic Gap Junctions between Two Neurons in the *Drosophila* Brain Are Critical for Memory

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## Summary

**Gap junctions play an important role in the regulation of neuronal metabolism and homeostasis by serving as connections that enable small molecules to pass between cells and synchronize activity between cells [1–3]. Although recent studies have linked gap junctions to memory formation [4, 5], it remains unclear how they contribute to this process [1, 5]. Gap junctions are hexameric hemichannels formed from the *connexin* and *pannexin* gene families in chordates and the *innexin* (*inx*) gene family in invertebrates [6, 7]. Here we show that two modulatory neurons, the anterior paired lateral (APL) neuron and the dorsal paired medial (DPM) neuron, form heterotypic gap junctions within the mushroom body (MB), a learning and memory center in the *Drosophila* brain. Using RNA interference-mediated knockdowns of *inx7* and *inx6* in the APL and DPM neurons, respectively, we found that flies showed normal olfactory associative learning and intact anesthesia-resistant memory (ARM) but failed to form anesthesia-sensitive memory (ASM). Our results reveal that the heterotypic gap junctions between the APL and DPM neurons are an essential part of the MB circuitry for memory formation, potentially constituting a recurrent neural network to stabilize ASM.**

## Results

### The APL and DPM Neurons Are Dye Coupled

*Drosophila* can be trained to form a Pavlovian association between an odor and electric shock. Flies naturally avoid electric shock, which serves as the unconditioned stimulus (US). When this shock is paired with delivery of a particular odor, the conditioned stimulus (CS), flies learn this association and subsequently avoid the odor [8]. Coincident US and CS input is registered in the mushroom body (MB), a brain area composed of approximately 2500 Kenyon cells (KCs) [9–11]. The dendrites of the KCs form the cap or calyx of the MB, and their axons project anteriorly to form the stalk or peduncle before those axons terminate in one or more lobes, termed $\alpha\beta$, $\alpha'\beta'$, and $\gamma$ lobes. KCs receive olfactory input from the projection neurons (PNs) of the antennal lobe, which contact KC dendrites in the calyx. KCs respond very selectively to odor, which is thought to be a useful property for forming accurate memories [12].

Dorsal paired medial (DPM) neurons express the *amnesiac* (*amn*) gene, which encodes a neuropeptide transmitter. DPM neurons innervate all of the lobes of the MB and part of the peduncle but do not send processes anywhere else, including the calyx [13] (see also Figure S1C available online). This anatomical arrangement suggests that DPM and KCs are recurrently connected; that is to say, DPM likely receives direct KC input and releases its neuropeptide transmitter back to the KC axons [14]. Consistent with the notion that the DPM neuron pools inputs from many KCs, the neuron responds both to shock and, as expected, to a wide range of different odors [15] (see also Figures S1A and S1B).

The anterior paired lateral (APL) neuron has a morphology that partly overlaps with DPM. Its cell body is located in the lateral protocerebrum, where it gives rise to a primary neurite that projects medially and bifurcates to innervate the entire MB, one branch entering the calyx and another branch entering the vertical lobe [16] (see also Figure S1D). The APL neuron also responds to the US shock [16], which is mediated through two clusters of dopaminergic neurons acting on KCs at the MB lobes [17–19]. Using functional imaging to monitor intracellular calcium changes, we found that the APL neuron is also an odor generalist responsive to at least eight tested odorants (Figures S1A and S1B). The APL neuron has been reported to suppress associative olfactory learning by releasing the inhibitory neurotransmitter GABA; training attenuates its response to the conditioned odor [16]. On the other hand, the DPM neuron and its *amnesiac* gene product are dispensable during the acquisition phase of learning but are critical for a delayed memory trace and a robust intermediate-term memory [13, 15, 20, 21].

The overlapping projections of DPM and APL neurons, along with their similar odor response properties, prompted us to examine whether information might be exchanged between these two neurons. Whole-cell patch-clamp recordings from the DPM cell body were used to dye fill the DPM neuron. We observed that, in addition to labeling the DPM cell body and its processes in the lobes, staining extended through the entire peduncle and calyx to the APL cell body (Figure 1A; Figures S1C and S1D). Dye coupling is indicative of gap-junctional connections between neurons [22]. To further resolve the site of DPM-APL contact, we labeled the APL and DPM neurons with two different colors of fluorescent proteins using the dual binary systems of GAL4 and LexA (Figure 1B; Figures S1F and S1G). We found that although both neurons innervate all lobes of the MB, they contact preferentially in the $\alpha'\beta'$ lobes (Figure 1C).

### Gap Junctions between APL and DPM Neurons Are Necessary for Normal Three-Hour Memory

Next, we asked which types of gap junctions exist between APL and DPM neurons and what role they play in memory formation. There are eight *innexin*-encoding loci in the

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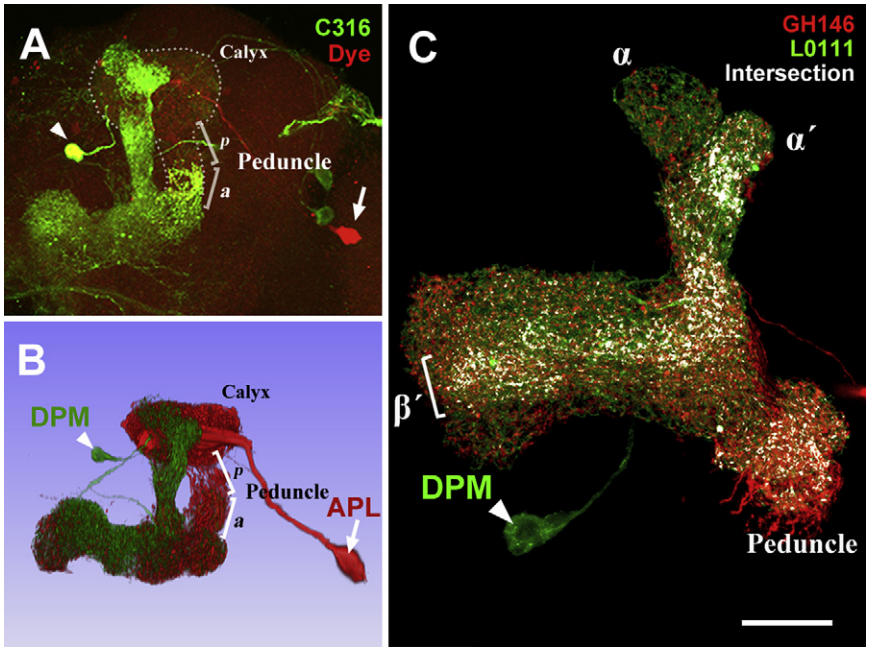


Figure 1. Dye Coupling between DPM and APL Neurons

(A) Biocytin dye loaded into the soma of a GFP-labeled DPM neuron (arrowhead) via whole-cell patch-clamp recording appeared in the soma of APL neuron (arrow) and its fibers (red) projecting into the entire mushroom body (MB) including calyx and posterior (p) peduncle (dotted line). The DPM neuron innervates all MB lobes and anterior (a) peduncle. Genotype shown is *UAS-mCD8::GFP;C316-GAL4.*

(B) Anterior view of DPM and APL neurons labeled by GFP (green) and mKO (red), respectively. The volume image is processed from Figure S1F after removing other neurons. Genotype shown is *GH146-GAL4,UAS-mKO,UAS-mKO/L0111-LexA::GAD,lexAop-rCD2::GFP;lexAop-rCD2::GFP/+.*

(C) The intersection (white) between APL neuron (red) and DPM neuron (green). The image is a maximum projection of (B) after removing the calyx. Voxels occupied by APL and DPM neurons are assigned as the white channel. Genotype shown is the same as in (B). Scale bar represents 20 μm.

In this and all following images, arrowhead indicates DPM cell body, arrow indicates APL cell body, and brain is counterstained with DLG antibody immunostaining (magenta). See also Figure S1.

*Drosophila* genome [23]. Taking advantage of a *Drosophila* RNA interference (RNAi) library [24] under control of the GAL4 expression system, we knocked down each of the eight *innexin* genes (Table S1) in both the APL and DPM neurons using a combination of two GAL4 drivers, *GH146-GAL4* and *C316-GAL4*. The effectiveness of each $inx^{RNAi}$ was validated via quantitative PCR (Figure S2A). We then tested flies for memory retention 3 hr after associative conditioning, a phase of memory referred to as intermediate-term [25]. We found that the *inx6* or *inx7* products in APL and DPM neurons are necessary for normal 3 hr memory (Figure 2A; Figure S2B). We confirmed the specificity of knockdown using quantitative immunostaining to show that $inx6^{RNAi}$ or $inx7^{RNAi}$ expression in both *GH146-GAL4* and *C316-GAL4* neurons effectively decreases the levels of the respective proteins within the MB lobes without affecting their levels in other brain regions (Figure 2B). Immunocytochemistry also indicated that INX6 and INX7 are widely expressed in the fly brain but are not always colocalized. In the central complex, strong immunopositive signals were observed for INX6 and INX7 in the fan-shaped body and ellipsoid body, respectively.

Within the MB, INX6 and INX7 colocalized preferentially in the $\alpha'\beta'$ lobes and the anterior half of peduncle (Figures 2C and 2D), whereas the posterior half of the peduncle was immunonegative (data not shown). The preferential localization of INX6 and INX7 subunits to the $\alpha'\beta'$ lobes, together with the fact that the anatomical overlap between APL and DPM neurons is most extensive in the $\alpha'\beta'$ lobes, strongly suggests that this is the site of gap-junctional coupling between these two neurons.

## Gap Junctions Are Critical for Anesthesia-Sensitive but Not Anesthesia-Resistant Memory

Because both APL and DPM neurons play critical but distinct roles in associative olfactory learning and memory [13, 16, 20, 21], we next examined the role of the APL-DPM gap junctions at various stages of memory formation and retention. Surprisingly, we found that RNAi-mediated knockdown of either INX6 or INX7 in APL and DPM neurons impairs only intermediate-term memory, whereas initial learning is normal (Figures 3A and 3B). Intermediate-term memory in flies comprises two distinct components, anesthesia-sensitive and anesthesia-resistant memory (ASM and ARM), each accounting for about half of the total retention level when tested 3 hr after one session of associative conditioning [10, 26, 27]. Using cold-shock anesthetization at 2 hr after training to abolish ASM [28–32], we found that ARM was normal in flies with knockdown of INX6 (Figure 3A) or INX7 (Figure 3B) proteins in both APL and DPM neurons. This result was verified with an independent $inx6^{RNAi}$ line (v8638) targeting a distinct sequence with a different insertion site (Table S1) to eliminate the possibility of an off-target effect (Figure S3).

These experiments utilized chronic knockdown of INX6 and INX7 in the APL and DPM neurons. The gross anatomy of APL and DPM neurons in $inx^{RNAi}$ flies was not different from that in control flies (Figure 3C), suggesting that their development was not affected by the chronic knockdown. Nevertheless, considering the critical roles of INX6 and INX7 in neural development [33, 34], we used an inducible knockdown strategy to reduce INX6 and INX7 expression specifically in adult flies. By inactivating the temperature-sensitive repressor GAL80, we selectively induced RNAi knockdown with a temperature shift 5 days before associative conditioning. Inducible knockdown of INX6 or INX7 still significantly impaired 3 hr memory (Figures 3D and 3E). Thus, the gap-junctional communication between APL and DPM neurons is critical for the formation of ASM but not ARM.

## Gap Junctions between APL and DPM Neurons Are Heterotypic

Although the *C316-GAL4* driver is expressed in DPM neurons, the expression pattern includes many other cells (Figure 4A;

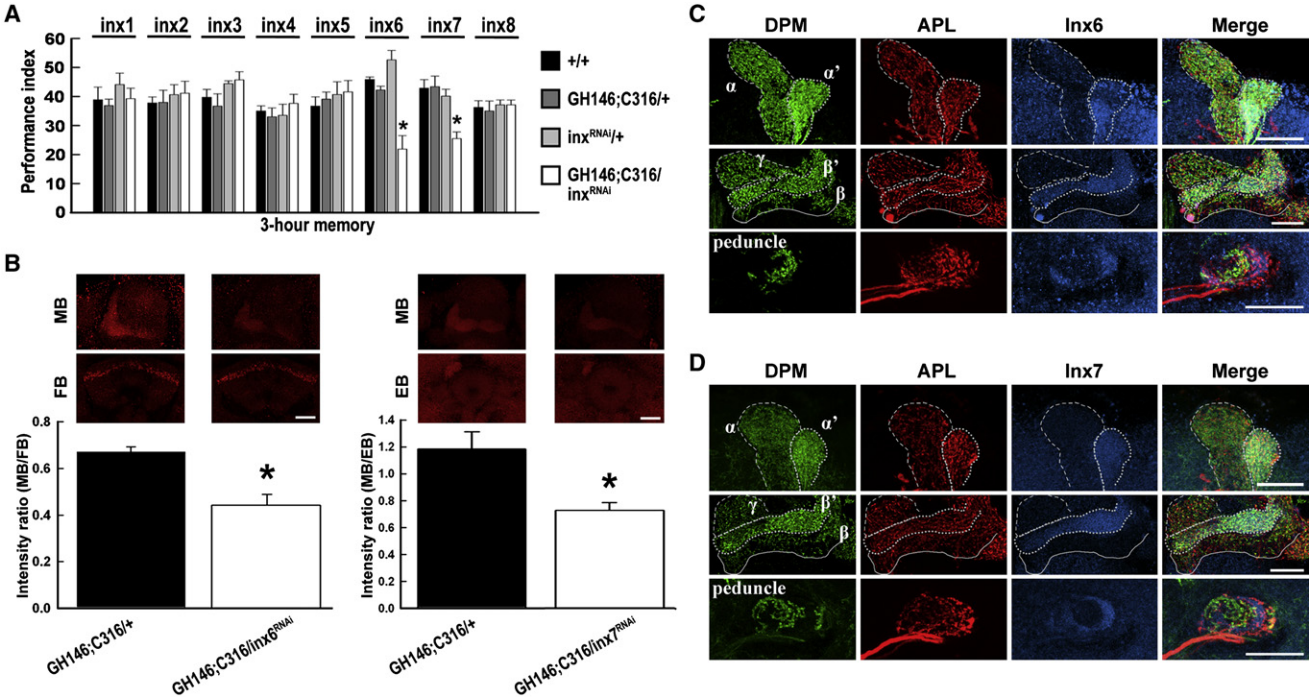


Figure 2. Downregulation of INX6 or INX7 Impairs Three-Hour Memory

(A) Three-hour memory (performance index) in flies carrying one of the eight *UAS-inx*$^{RNAi}$ effectors driven by the double drivers *GH146-GAL4* and *C316-GAL4*. Each datum represents mean ± standard error of the mean (SEM) (n = 7–12). *p < 0.05. Genotypes shown are as follows: "+/+," wild-type; "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "inx$^{RNAi}$/+," *+/inx*$^{RNAi}$*;+/+*; "GH146; C316/inx$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4, UAS-mCD8::GFP/inx*$^{RNAi}$*;C316-GAL4/+* ("*inx*$^{RNAi}$": see Table S1).

(B) Quantitative immunostaining. Each image is a single optical section. All images were taken under the same recording conditions. For INX6 antibody staining, the intensity ratio represents difference between MB and fan-shaped body (FB). For INX7 antibody staining, the intensity ratio represents difference between MB and ellipsoid body (EB). Each datum represents mean ± SEM (n = 4–6). *p < 0.05. Genotypes shown are as follows: "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "GH146;C316/inx6$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx6*$^{RNAi}$ (v46398);*C316-GAL4/UAS-Dcr-2*; "GH146;C316/inx7$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx7*$^{RNAi}$ (v103256);*C316-GAL4/+*.

(C) INX6 antibody immunostaining. Genotype shown is the same as in Figure 1C.

(D) INX7 antibody immunostaining. Genotype shown is the same as in Figure 1C.

For all images, scale bars represent 20 μm. See also Figure S2 and Table S1.

Figure S1E), among them a portion of the MB KCs (Figure 4B). We used *2721-GAL4* as a more specific DPM neuron driver (Figure 4C) that does not express in KCs (Figure 4D) to show that INX6 is required in the DPM neuron but not the APL neuron (Figure 4E). INX7 was required in the APL neuron but not the DPM neuron for the formation of ASM (Figure 4H); learning remained normal in all cases (Figure S4). These results suggest that these gap junctions are heterotypic, a possible indication that they do form asymmetric gap junctions.

We also refined the expression pattern of *GH146-GAL4*, which is expressed in many olfactory PNs in addition to APL neurons [35] (Figure 4F). By combining *GH146-GAL4* with *Cha-GAL80* [36], which expresses a transcriptional repressor in many neurons, we could reduce expression to undetectable levels in APL neurons, leaving visible expression in only a subset of PNs (Figure 4G). Knockdown of INX7 in these remaining PNs did not affect 3 hr memory formation (Figure 4I), suggesting that knockdown in APL neurons is required for the memory defect.

## Discussion

The key finding of our study is that two MB modulatory neurons, the APL and DPM neurons, form heterotypic gap junctions that are specifically required for anesthesia-sensitive intermediate-term memory of aversive olfactory conditioning in *Drosophila*. This conclusion is supported by three independent lines of evidence. First, APL and DPM neurons are dye coupled (Figure 1), a diagnostic feature of cells connected via gap junctions. Second, anatomically the two neurons intersect preferentially in the α′β′ lobes and anterior peduncle, which coincides with INX6 antibody and INX7 antibody immunostaining results (Figure 1; Figure 2). Third, intermediate-term ASM is impaired by inducible knockdown of either INX6 in DPM neurons or INX7 in APL neurons, but not the converse (Figure 3; Figure 4). This suggests that the APL-DPM gap junctions are heterotypic and raises the possibility that the connections are in some way asymmetric. Interestingly, both APL and DPM neurons respond to electric shock and to multiple odorants [15, 16] (Figures S1A and S1B). The overlapping anatomy of these two neurons, together with INX immunostaining results, suggests that sensory information about the US, CS, or both can be transferred between APL and DPM neurons through gap junctions in the α′β′ lobes. Although it remains to be determined which of the two dye-coupled partners receives the olfactory signals first, the APL neuron is the more likely candidate because it has calyx-wide dendritic arborizations where it could receive PN input (Figure 1; Figure S1).

Surprisingly, disrupting this heterotypic gap-junctional communication does not perturb normal learning (Figure 3;

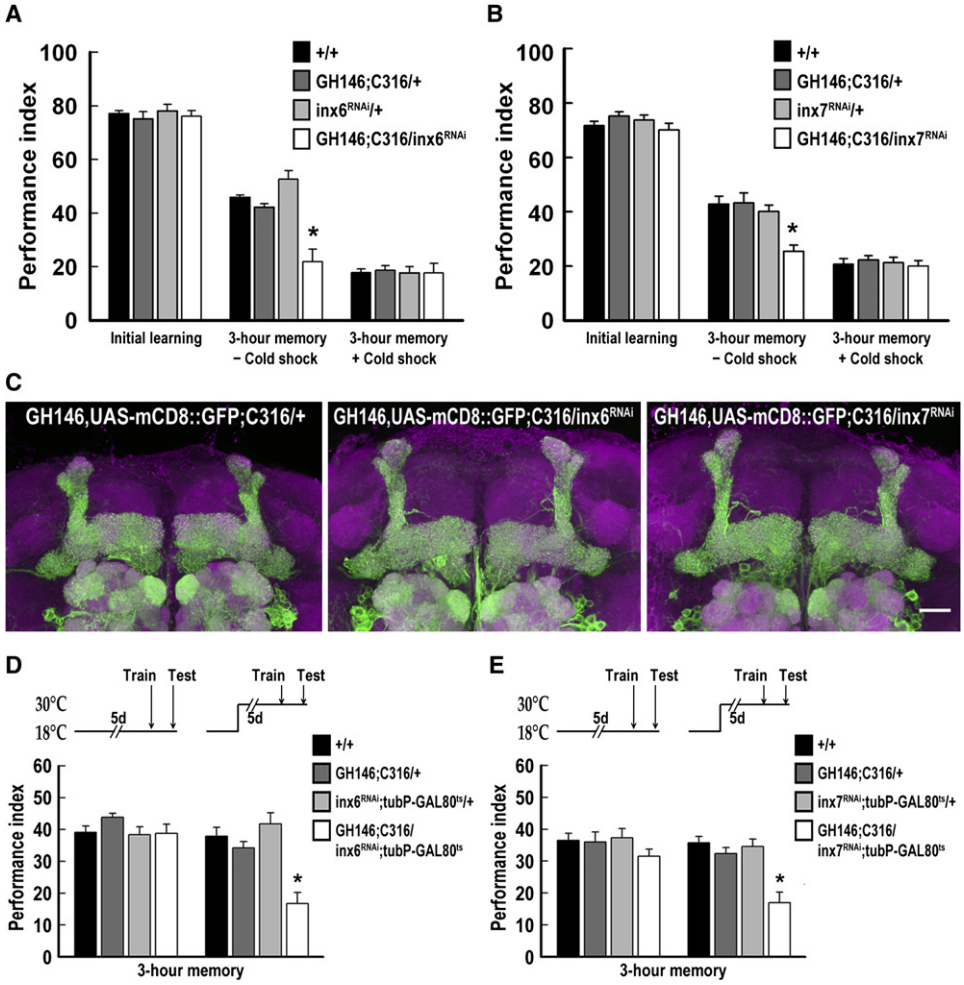


Figure 3. Downregulation of INX6 or INX7 Impairs Specifically ASM

(A) Effects of *inx6*$^{RNAi}$ (v46398) expression in both APL and DPM neurons driven by *GH146-GAL4* and *C316-GAL4*, respectively. Genotypes shown are as follows: "+/+," wild-type; "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "inx6$^{RNAi}$/+," *+/UAS-inx6*$^{RNAi}$ (v46398);*+/UAS-Dcr-2*; "GH146;C316/inx6$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx6*$^{RNAi}$ (v46398);*C316-GAL4/UAS-Dcr-2*.

(B) Effects of *inx7*$^{RNAi}$ (v103256) expression in both APL and DPM neurons driven by *GH146-GAL4* and *C316-GAL4*, respectively. Genotypes shown are as follows: "+/+," wild-type; "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "inx7$^{RNAi}$/+," *+/UAS-inx7*$^{RNAi}$ (v103256);*+/+*; "GH146;C316/inx7$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx7*$^{RNAi}$ (v103256);*C316-GAL4/+*.

(C) Gross morphology of APL and DPM neurons (green) in a control fly (left) or with constitutive expression of *inx6*$^{RNAi}$ (middle) or *inx7*$^{RNAi}$ (right) driven by *GH146-GAL4* and *C316-GAL4*. Scale bar represents 20 μm. Genotypes shown are as follows: "GH146,UAS-mCD8::GFP;C316/+," *FRT*$^{g13}$*,GH146-GAL4, UAS-mCD8::GFP/+;C316-GAL4/+*; "GH146,UAS-mCD8::GFP;C316/inx6$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx6*$^{RNAi}$ (v46398);*C316-GAL4/ UAS-Dcr-2*; "GH146,UAS-mCD8::GFP;C316/inx7$^{RNAi}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx7*$^{RNAi}$ (v103256);*C316-GAL4/+*.

(D) Effects of temporal expression of *inx6*$^{RNAi}$. Genotypes shown are as follows: "+/+," wild-type; "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "inx6$^{RNAi}$;tubP-GAL80$^{ts}$/+," *+/UAS-inx6*$^{RNAi}$ (v46398)*,UAS-Dcr-2;+/tubP-GAL80*$^{ts}$; "GH146;C316/inx6$^{RNAi}$;tubP-GAL80$^{ts}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx6*$^{RNAi}$ (v46398)*,UAS-Dcr-2;C316-GAL4/tubP-GAL80*$^{ts}$.

(E) Effects of temporal expression of *inx7*$^{RNAi}$. Genotypes shown are as follows: "+/+," wild-type; "GH146;C316/+," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/+;C316-GAL4/+*; "inx7$^{RNAi}$;tubP-GAL80$^{ts}$/+," *+/UAS-inx7*$^{RNAi}$ (v103256);*+/tubP-GAL80*$^{ts}$; "GH146;C316/inx7$^{RNAi}$;tubP-GAL80$^{ts}$," *FRT*$^{g13}$*,GH146-GAL4,UAS-mCD8::GFP/UAS-inx7*$^{RNAi}$ (v103256);*C316-GAL4/tubP-GAL80*$^{ts}$.

For all behavior assays, cold-shock-induced anesthesia was performed at 2 hr posttraining and GAL80$^{ts}$ inhibition was removed by keeping flies at the restrictive temperature for 5 days before training. Each datum represents mean ± SEM (n = 8). *p < 0.05. See also Figure S3.

Figure S4), despite the observation that diminishing GABA biosynthesis in APL neurons enhances olfactory learning [16]. This suggests that the coupling between APL and DPM neurons is partially independent from the role of GABAergic transmission of APL neurons. There are several possible explanations for this separation. Because the APL neuron innervates the MB through two branches (Figure S1D), one entering the calyx and the other entering the vertical lobe, the first possible explanation is that each APL branch plays a functionally distinct role. Learning may recruit the calyceal branch of the APL neuron, controlling inhibition onto KCs [12, 16]. Later, the lobe branch of the APL neuron and the gap-junctional connection with the DPM neuron may come into play, mediating the formation of ASM in $\alpha'\beta'$ lobes. Alternatively, the heterotypic composition of the gap junctions may mean that they favor diffusion in one direction, from APL to DPM, keeping GABA release of the APL neuron unaffected by activity in the DPM neuron. This type of rectification gated by heterotypic gap junctions has been demonstrated in the *Drosophila* giant fiber system [37]. The latter hypothesis is

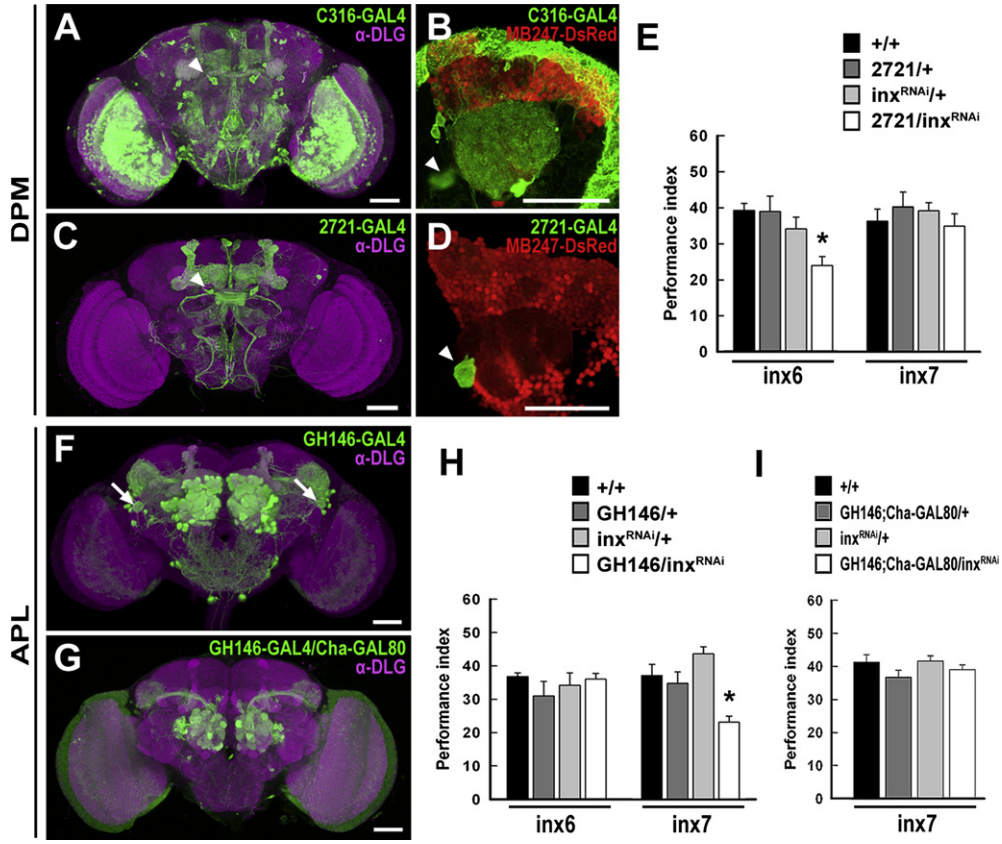


Figure 4. APL and DPM Neurons Require Different INXs

(A) *C316-GAL4* expression pattern (green). Brain surface is removed to reveal internal structures (see Figure S1E). Genotype shown is *UAS-mCD8::GFP;C316-GAL4*.

(B) Close-up view of *C316-GAL4* (green) and *MB247-DsRed* (red) expression patterns at the calyx and Kenyon cell (KC) soma. Genotype shown is *MB247-DsRed/+;MB247-DsRed/UAS-mCD8::GFP;MB247-DsRed/C316-GAL4*.

(C) *2721-GAL4* expression pattern. Genotype shown is *2721-GAL4;UAS-mCD8::GFP*.

(D) Close-up view of *2721-GAL4* (green) and *MB247-DsRed* (red) expression patterns at the calyx and KC soma. Genotype shown is *MB247-DsRed/+; MB247-DsRed/2721-GAL4;MB247-DsRed/UAS-mCD8::GFP*.

(E) Effects of *inx6* and *inx7* knockdowns in the DPM neurons driven by *2721-Gal4* on 3 hr memory. Genotypes shown are as follows: "+/+," wild-type; "2721/+," *2721-GAL4/+;+/+*; "inx$^{RNAi}$/+," *+/inx6$^{RNAi}$* (v46398);*+/UAS-Dcr-2* for "inx6" or *+/inx7$^{RNAi}$* (v103256);*+/+* for "inx7"; "2721/inx$^{RNAi}$," *2721-GAL4/inx6$^{RNAi}$* (v46398);*+/UAS-Dcr-2* for "inx6" or *2721-GAL4/inx7$^{RNAi}$* (v103256);*+/+* for "inx7."

(F) *GH146-GAL4* expression pattern. Genotype shown is *FRT$^{g13}$,GH146-GAL4,UAS-mCD8::GFP*.

(G) Expression pattern of *GH146-GAL4* subtracted by *Cha-GAL80*. Genotype shown is *GH146-GAL4/UAS-mCD8::GFP;Cha-GAL80/UAS-mCD8::GFP*.

(H) Effects of *inx6* and *inx7* knockdowns in the APL neurons driven by *GH146-Gal4* on 3 hr memory. Genotypes shown are as follows: "+/+," wild-type; "GH146/+," *GH146-GAL4/+;+/+*; "inx$^{RNAi}$/+," *+/inx6$^{RNAi}$* (v46398);*+/UAS-Dcr-2* for "inx6" or *+/inx7$^{RNAi}$* (v103256);*+/+* for "inx7"; "GH146/inx$^{RNAi}$," *GH146-GAL4/inx6$^{RNAi}$* (v46398);*+/UAS-Dcr-2* for "inx6" or *GH146-GAL4/inx7$^{RNAi}$* (v103256);*+/+* for "inx7."

(I) Knockdown of *inx7* with *GH146-GAL4;Cha-GAL80* showed normal 3 hr memory. Genotypes shown are as follows: "+/+," wild-type; "GH146;Cha-GAL80/+," *GH146-GAL4/+;Cha-GAL80/+*; "inx$^{RNAi}$/+," *+/inx7$^{RNAi}$* (v103256);*+/+*; "GH146;Cha-GAL80/inx$^{RNAi}$," *GH146-GAL4/inx7$^{RNAi}$* (v103256);*Cha-GAL80/+*.

For all images, whole-brain images are anterior view. Close-up images are posterior views. Scale bars represent 50 μm. For all behavior assays, memory performance was measured at 3 hr posttraining. Each datum represents mean ± SEM (n = 8). *p < 0.05. See also Figure S4.

also supported by the fact that blocking neurotransmission from the DPM neuron impairs intermediate-term memory without affecting learning [21].

Evidence on both the molecular and the cellular level indicates that a microcircuit of KCs and DPM neurons is involved in formation of ASM. At the molecular level, the *amn*-encoded neuropeptide, which is predominantly expressed in DPM neurons, plays an essential role in intermediate-term memory [13]. It was later shown that *amn* mutants are specifically defective in ASM, the same phase of memory that is affected in age-related memory [26]. In the MB KCs, components of the cAMP-PKA signaling pathway play specific roles in ASM [30]. The PKA catalytic subunit DCO is required for an early phase of ASM, whereas PKA-anchoring proteins are associated with defects in a later phase of ASM [30, 38]. Furthermore, knocking down NMDA receptors in KCs specifically abolishes ASM [32]. Thus, a variety of molecular changes that reside in either the KCs or DPM can affect ASM. At the cellular level, blocking synaptic release with *shibire* shows that transmission from $\alpha'\beta'$ KCs is specifically required during consolidation but not retrieval [39]. Similar experiments have established that transmission from DPM neurons is also specifically required during the consolidation period [15, 20, 21]. In contrast, transmission from $\alpha\beta$ KCs is required during retrieval but not consolidation [14, 39], indicating that KCs must act at different times during the learning and memory process. The observation that *shibire* blockade during the consolidation period impairs the memory process suggests that persistent activity of some form is

required in $\alpha'\beta'$ KC and DPM cells. Specifically, these data have led to a mnemonic model in which recurrent activity in an $\alpha'\beta'$ KC-DPM loop is required to stabilize memories formed in the $\alpha\beta$ KCs ([14]; see also [40] in this issue of *Current Biology*).

Our data now link APL with the DPM-KC loop in intermediate-term ASM formation. Recent work has shown that activation of dopaminergic PPL1 neurons innervating the vertically projecting $\alpha$ and $\alpha'$ lobes and heel is sufficient to signal reinforcement for aversive odor memory [17] whereas another subset of dopaminergic MB-M3 neurons innervating the tips of the horizontal ($\beta$ and $\beta'$) lobes is specifically required for intermediate-term ASM [19]. Also, functional imaging studies monitoring changes in response to trained odor have revealed that memory traces occur ~5 min after conditioning in APL neurons [16], ~30 min in DPM neurons [15], and ~60 min in $\alpha'\beta'$ KCs [41], whereas changes in $\alpha\beta$ KCs are first detected 9 hr after learning [42]. Taking these data together with our findings here that heterotypic gap junctions exist between the APL and DPM neurons, we propose a refined mnemonic model wherein reverberatory activity in the APL-DPM-$\alpha'\beta'$ KC network evokes delayed memory traces in the $\alpha'\beta'$ KCs and DPM neuron to stabilize memories formed in the $\alpha\beta$ KCs. This reverberatory activity is likely triggered by the arrival of coincident CS-US information from olfactory PNs at the calyx [43] and shock-responsive dopaminergic PPL1 neurons at the MB lobes [17].

The evidence that output from both DPM and $\alpha'\beta'$ KCs is required during the consolidation period suggests that some type of persistent neural activity arising in these neurons supports ASM formation. Our results show that gap junctions between DPM and APL are required for ASM formation and therefore likely contribute to this ongoing neural activity. What is the possible nature of the persistent activity, and how could gap junctions contribute to the process? Persistent activity can take the form of ongoing spiking activity, but it may also reflect some more subtle aspect of neural activity. For example, presynaptic residual calcium with slow clearance kinetics has been proposed as a buffer to sustain persistent activity in a recurrent circuit as working memory [44, 45]. However, the period of ASM consolidation is much longer than the time window that working memory operates within. Prolonging the time frame when some transient markers of past activities are sustained may require more complex circuitry with a self-sustaining feedback loop. Because DPM neurons likely release both the *amn* neuropeptide and the excitatory neurotransmitter acetylcholine [20], DPM and $\alpha'\beta'$ KCs could form a reciprocally connected excitatory recurrent loop. One limitation of a feedback loop driven by purely excitatory elements is that it is potentially susceptible to runaway excitation. The gap-junctional connection between DPM and APL could guarantee that excitation from DPM is balanced by a similar magnitude of inhibition from APL. Alternatively, chemical synapses of these two types of neurons may act independently, and additional components may be required to sustain this prolonged reverberatory activity. For example, the dopaminergic MB-M3 neurons specifically required for intermediate-term ASM [19] might be another component in the recurrent network.

In conclusion, we provide both anatomical and functional evidence showing that neuronal gap junctions are required for ASM formation in a circuit that involves MB, APL, and DPM neurons. If ASM is indeed mediated by reverberatory activity within this recurrent network, gap-junctional coupling could enable APL and DPM neurons to respond synchronously throughout this period, ensuring that inhibition from APL together with neuromodulation from DPM neurons are both engaged during memory consolidation.

## Experimental Procedures

### Fly Stocks

Fly stocks were raised on standard cornmeal food at 25°C and 70% relative humidity on a 12:12 hr light:dark cycle. The lines *FRT$^{g13}$,GH146-GAL4, UAS-mCD8::GFP* and *FRT$^{g13}$,tubP-GAL80* were gifts from Tzumin Lee (Howard Hughes Medical Institute, Janelia Farm, VA). The line *UAS-G-CaMP-1.6* was a gift from Dierk Reiff [46]. 2721 (*w*;P{GawB}5015*) and 7017 (*w*;P{tubP-GAL80$^{ts}$}2/TM2*) were obtained from Bloomington Drosophila Stock Center. All of the RNAi lines and *UAS-Dcr-2* (Table S1) were obtained from the Vienna Drosophila RNAi Center [24]. *MB247-DsRed* was a gift from André Fiala [47]. The transgenic fly line *UAS-mKO* was generated by standard techniques [48] in an Canton-S *w$^{1118}$* (iso1CJ) background. *L0111-LexA::-GAD* was generated by *P* element transposition of a donor fly strain carrying *P{lexA-GAD.C}* on an X chromosome [49].

### Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.02.041.

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