Pairing-Dependent Plasticity in a Dissected Fly Brain Is Input-Specific and Requires Synaptic CaMKII Enrichment and Nighttime Sleep

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In Drosophila, in vivo functional imaging studies revealed that associative memory formation is coupled to a cascade of neural plasticity events in distinct compartments of the mushroom body (MB). In-depth investigation of the circuit dynamics, however, will require an ex vivo model that faithfully mirrors these events to allow direct manipulations of circuit elements that are inaccessible in the intact fly. The current ex vivo models have been able to reproduce the fundamental plasticity of aversive short-term memory, a potentiation of the MB intrinsic neuron (Kenyon cells [KCs]) responses after artificial learning ex vivo. However, this potentiation showed different localization and encoding properties from those reported in vivo and failed to generate the previously reported suppression plasticity in the MB output neurons (MBONs). Here, we develop an ex vivo model using the female Drosophila brain that recapitulates behaviorally evoked plasticity in the KCs and MBONs. We demonstrate that this plasticity accurately localizes to the MB α’3 compartment and is encoded by a coincidence between KC activation and dopaminergic input. The formed plasticity is input-specific, requiring pairing of the conditioned stimulus and unconditioned stimulus pathways; hence, we name it pairing-dependent plasticity. Pairing-dependent plasticity formation requires an intact CaMKII gene and is blocked by previous-night sleep deprivation but is rescued by rebound sleep. In conclusion, we show that our ex vivo preparation recapitulates behavioral and imaging results from intact animals and can provide new insights into mechanisms of memory formation at the level of molecules, circuits, and brain state.

Key words: brain explant; CaMKII; Drosophila mushroom body; learning and memory circuit; neural plasticity; sleep

Significance Statement

The mammalian ex vivo LTP model enabled in-depth investigation of the hippocampal memory circuit. We develop a parallel model to study the Drosophila mushroom body (MB) memory circuit. Pairing activation of the conditioned stimulus and unconditioned stimulus pathways in dissected brains induces a potentiation pairing-dependent plasticity (PDP) in the axons of α’β’ Kenyon cells and a suppression PDP in the dendrites of their postsynaptic MB output neurons, localized in the MB α’3 compartment. This PDP is input-specific and requires the 3’ untranslated region of CaMKII. Interestingly, ex vivo PDP carries information about the animal’s experience before dissection; brains from sleep-deprived animals fail to form PDP, whereas those from animals who recovered 2 h of their lost sleep form PDP.

Introduction

A neutral experience (conditioned stimulus [CS]) can be remembered as positive or negative if closely followed by rewarding or punishing reinforcement (unconditioned stimulus [US]). The ability to form this type of "associative" memory is phylogenetically conserved; Drosophila form robust associative memories (Tully and Quinn, 1985), most of which are encoded and stored in the mushroom body (MB) (de Belle and Heisenberg, 1994). The MB is a higher brain structure made of 15 distinct compartments. Each compartment is built on a scaffold of axons of one of the three main types of Kenyon cells (KCs; αβ, α’β’, and γ). The KCs connect to MB output neurons (MBONs), which project out of the MB to bias behavior (Aso et al., 2014a,b; F. Li et al., 2020). The KC→MBON synapses are modulated by dopaminergic neurons.
During aversive olfactory associative learning, an odor (the CS) activates a sparse group of KCs, such that this odor identity is represented across all MB compartments (Turner et al., 2008; Lin et al., 2014). Simultaneously, dopaminergic neurons from the protocerebral posterior lateral (PPL1) cluster are activated by the US, encoding negative prediction errors in MB compartments (Riemensperger et al., 2005; Scroli et al., 2006; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2012). When KC activation and the dopaminergic signal coincide within a compartment, the KC-MBON synapses in that compartment are depressed, biasing the circuit output to aversion (Sejourne et al., 2011; Cohn et al., 2015; Hige et al., 2015; Oswald and Waddell, 2015; Oswald et al., 2015).

Many studies have investigated the properties of this circuitry using in vivo calcium imaging in intact animals (for review, see Adel and Griffith, 2021). In contrast, explanted brains have been used mostly for establishing connectivity between neurons or interrogating a specific biochemical pathway; only a few studies have attempted to understand memory circuit logic ex vivo (Wang et al., 2008; Ueno et al., 2013, 2017; Suzuki-Sawano et al., 2017). In the best-developed paradigm, Ueno et al. (2013) observed a potentiation of KC responses in the tips of the MB vertical lobes which they termed "long-term enhancement" (LTE). This laid the groundwork for developing ex vivo models of this circuit, but there were major differences between LTE and associative memory observed in intact animals. The most significant were that the specificity was not specific to the α′ β′ KCs and that dopamine release by the US was not observed; it was only seen after CS + US coincidence (Ueno et al., 2013, 2017).

In this study, we establish an ex vivo paradigm that resolves these discrepancies and exhibits the cardinal features of associative learning. We show that pairing odor and punishment pathway activation in dissected brains results in a localized potentiation of the α′ β′ KCs and suppression of their postsynaptic MBONs in the α′ compartment. Because both KC potentiation and MBON suppression are strictly dependent on temporal coincidence of the CS and US, we term this paradigm "pairing-dependent plasticity" (PDP). We show that, like the CS specificity of associative memories, PDP is specific to the subset of odor-representing projection neurons activated during the artificial training. We also provide evidence that dopamine is released by activation of the US pathway and does not require CS + US coincidence.

This ex vivo paradigm can be used for obtaining new mechanistic insight into memory formation at the molecular and circuit levels. We present data indicating that the 3′UTR of the CaMKII gene is critical for short-term memory (STM) formation and that the primacy of α′ compartment plasticity in learning is because of differences in input/response relationships between α and α′. Finally, we demonstrate that the ability of the ex vivo brain to be plastic can be influenced by prior in vivo experience, as we report that brains of sleep-deprived flies fail to form PDP, but as little as 2 h of recovery sleep rescues this learning impairment.

Materials and Methods

Fly husbandry. All fly stocks were cultured on standard food at room temperature. Experimental flies were kept at 25°C and 70% relative humidity on a 12 h light, 12 h dark period. Fly lines used in this study include VlT030559-GAL4, MB027B split-GAL4 (Aso et al., 2014a), GH146-GAL4, UAS-CaMMP6f, 20x UAS-GCaMP6f, UAS-jRGCaMP1a, LexAop-P2X2, TH-lexA was gifted to us from the Davis laboratory (Berry et al., 2015) and UAS-GRABDA2m was gifted from the Li laboratory (Sun et al., 2020). CaMKIIαD1164 flies are described by Chen et al. (2022).

Ex vivo imaging and electrical stimulations. Brains from 4- to 8-d-old female flies were dissected in ice-cold HL3 medium (70 mM NaCl, 115 mM sucrose, 5 mM KCl, 20 mM MgCl2, 1.8 mM CaCl2, 10 mM NaHCO3, 5 mM trehalose, 5 mM HEPES, osmolality: 395.4 mOsm; pH 7.3) (Stewart et al., 1994). Brains were then transferred to an imaging chamber containing fresh HL3 saline and immobilized using tungsten pins over the optic lobes. In the case of paired stimulations of the antennal lobe (AL) and the ascending fibers of the ventral nerve cord (AFV), the brain was dissected with the ventral nerve cord attached. The ventral nerve cord was then cut using sharp scissors near its base, leaving one end of the AFV free. Glass suction microelectrodes were used to apply the electrical stimulation to either the AL or the AFV. Because odor information is randomly encoded in the MB (Bhandawat et al., 2007; Ito et al., 2008; Turner et al., 2008; Honegger et al., 2011; Lei et al., 2013), we did not target specific AL glomeruli across different animals. However, the properties of the stimulation and the electrode were kept the same across all experiments unless noted otherwise. Based on the size of the AL electrode tip and the distribution of AL calcium responses to AL stimulation (see Fig. 3B–D), we estimate that 20%–25% of the ipsilateral AL projection neurons are activated with our AL stimulation protocol. Brains were always perfused with fresh saline throughout the experiment with a flow rate of ~2 drops per second.

In pre-induction and post-induction AL stimulations, 5 trains of 20 pulses at 100 Hz were applied. Pulse width is 1 ms and interpulse interval is 2 ms. Stimulation intensity (stimulation voltage when performing AL pairing stimulation) or 200 (high input stimulation) µAmps. During induction, 12 AL stimulation trains were applied with 5 s intertrain interval. Regarding the AL electrode size, we noticed a relationship between the diameter of the AL electrode tip and the input current such that applying a 100 µAmps input stimulation via a large electrode tip diameter has a similar effect on baseline KC calcium responses and on the resultant plasticity as applying a 200 µAmps via a small electrode tip diameter. For example, with a 100 µAmps input stimulation, PDP can be formed in the MB α3 compartment if the diameter of the AL electrode is significantly enlarged. Therefore, to minimize variability within the same dataset, all electrodes used in any experiment in this study were made at the same time using a p-97 micropropette puller (Sutter Instruments) before data collection. To minimize variability across the different experiments, we kept the AL electrode size approximately one-fourth of the AL size.

AFV stimulation was similar to AL stimulation, but the diameter of the AFV stimulation electrode was large enough to suck in the free end of the AFV. In GRABDA2m experiments, AFV stimulation strength was adjusted to be above the threshold of KC GRABDA2m responses; it varied between 500 and 1000 µAmps. We noted that AFV stimulation under these conditions did not elicit GRABDA2m responses in the AL projection lobes, suggesting that, while PPL1 neurons were stimulated, PAM neurons were not. Whether this reflects differences in circuitry or differences in relative excitability (we are using HL3) is unknown. In AL pairing experiments, we noted in tests of the AFV electrode that there were MB GCaMP responses in some animals, but not all (2 of 8 had no response). In no case, however, did AFV stimulation alone cause PDP, suggesting that AFV-stimulated MB calcium increases are not able to act as a CS. Plasticity has been observed to be dependent on calcium entry pathway previously in mammalian brain (Deisseroth et al., 1998).

Images were captured using an ORCA Flash4.0 V3 sCMOS camera at 200 frames per second (except in the MBON imaging experiment using MB027B split-GAL4 > 20x – UAS-GCaMP6f, frame rate was reduced to 2 frames per second) with a 40× water immersion lens on an Olympus upright microscope BX50W1. Images were collected as 512 x 512 resolution and a binning factor of 2 x 2. Imaging was done using the HCImage Live software. Excitation of the used fluorescent sensors was done using the CoolLED eP-4000 LED source. For GCaMP and GRABDA2m the 470 nm LED channel was used with an excitation filter Chroma 450/50 and emission filter FF01-525/45-25, jRcAMP excitation was done using the 550 nm LED channel and the excitation filter FF01-530/43-25 and an emission filter FF01-607/36-25. Calcium traces at every frame were calculated as ΔF/Fo = (F – F0)/F0, where F is
the florescence value at a given frame and \(F_0\) is the florescence value at baseline. Peak response to a stimulation was calculated by subtracting the average \(AF/F_0\) during the last second before a stimulation from the peak \(AF/F_0\) during stimulation. The responses to AL stimulations before and 15 min after induction were averaged to calculate the pre- and post-responses, respectively. PDP values or \(\Delta F/F\) relative change was calculated as (post – pre)/pre.

The isolated pulse stimulator model 2100 and the perfusion system ValveLink 8.2 were triggered using a custom program written and controlled by the pClamp 11 software and the Digitiga 1550A digitizer.

Behavioral experiments. Aversive learning experiments were performed in an environmental room in red light at 25°C with 65% humidity. Flies (mixed males and females) were between 4- and 14-d-old. Flies were given at least 10 min acclimation period in room before training or testing. Data for each experiment were pooled from at least three independent experimental days. The learning assays were performed as described by the Quinn laboratory (Tully and Quinn, 1985). The US was provided as 12 l 1 s 90 or 60 V shocks during the 1 min CS-US pairing; 10% 4-methylcyclohexanol and 3-octanol OCT were used as the CS odors. Flies were then given a 2 min rest. Testing involved 2 min of simultaneous exposure to CS odors, after which flies choosing either odor were counted. A performance index was calculated for each trial as (number of flies choosing the conditioned odor) – (number of flies choosing the not-conditioned odor))/total number of flies. This performance index was averaged between reciprocal trials where one of the odors was conditioned in one trial and the other odor was conditioned in the other. The learning index was calculated as the CaMKII band /Cy3 band (Invitrogen) overnight, then washed 3 × 30 min.

Samples were then incubated in AlexaFluor-633 anti-mouse antibody (Invitrogen) for 3 d. CaMKII antibody solutions were diluted in 0.5% Triton-PBS (PBST) solution, blocked in 10% normal goat serum solution for 1 h, and incubated in mouse anti-CaMKII antibody (1:10 000, Cosmo) for 3 d. CaMKII antibody solutions were removed, and samples washed in PBST solution for 3 × 30 min. Samples were then washed in AlexaFluor-633 anti-mouse antibody (Invitrogen) overnight, then washed 3 × 30 min in PBST solution and mounted in the Vectashield mounting medium. Images were taken under a 20× objective lens with the same settings using a Leica SP5 confocal microscope. The images were analyzed by ImageJ software. For the intensity of MB lobes was quantified.

Immunostaining. Adult fly brains were dissected in cold Schneider’s Insect Medium (Sigma, S0146), and then fixed in 4% PFA solution for 30 min at room temperature. Fixed brains were washed 3 × 30 min in 0.5% Triton-PBS (PBST) solution, blocked in 10% normal goat serum solution for 1 h, and incubated in mouse anti-CaMKII antibody (1:10 000, Cosmo) for 3 d. CaMKII antibody solutions were removed, and samples washed in PBST solution for 3 × 30 min. Samples were then washed in AlexaFluor-633 anti-mouse antibody (Invitrogen) overnight, then washed 3 × 30 min in PBST solution and mounted in the Vectashield mounting medium. Images were taken under a 20× objective lens with the same settings using a Leica SP5 confocal microscope. The images were analyzed by ImageJ software. For the intensity of MB regions, the middle slices were selected as the representative pictures, and mean intensity of all MB lobes was quantified.

Immunoblotting. A total of 100 5-d-old Canton-S WT or CaMKII\textsuperscript{UUDel} flies (mixed males and females) were frozen on dry ice and vortexed to remove heads. Fly heads were separated from the fly bodies using a sieve. The heads were then homogenized in loading buffer (4× Bolt LDS, Invitrogen, Novex with 5% β-mercaptoethanol added) and heated for 10 min. Proteins were separated by SDS-PAGE (Bolt, BioTris Protein Gels, Invitrogen) and transferred to a nitrocellulose membrane (GE Healthcare). Membrane was blocked (Blocking Buffer for Fluorescent Western Blotting, Rockland Immunoc hemicals) and then incubated with Anti-dCaMKII Clone 18 (1:1000, CosmoBio) and anti-actin mAb C4 (1:1000, Millipore). The secondary antibody used was DyLight 680 mouse. Membrane was imaged using ChemiDoc system from Bio-Rad. Intensity of bands was calculated using Adj. Volume (Int) within the ImageLab 6.0 software. Intensity of the CaMKII band was normalized to that of actin in the same lane.

Experimental design and statistical analysis. Female flies were used in all imaging experiments because of their larger size. Both males and females aged 4-8 d were used in other experiments unless noted otherwise. All statistical analyses were performed in Prism 9 software. All tests were two-tailed, and confidence levels were set at \(\alpha = 0.05\). Normality of statistical data was determined via the Shapiro–Wilk test (\(\alpha = 0.05\)). Parametric tests were used for all experiments, except the immunoblotting experiment where nonparametric tests were used. The statistical tests, \(p\) values, sample sizes, and other statistical information for each experiment are listed in the figure legends. Post hoc analysis information for Figures 2G and 5F is listed in Tables 1 and 2, respectively.

Results

Artificial aversive training induces a potentiation PDP in KCs

Previous studies had demonstrated that enhanced calcium responses similar to those reported in the MB in vivo after aversive training can be achieved in dissected brains (Wang et al., 2008; Tomchik and Davis, 2009; Ueno et al., 2013). To investigate whether this plasticity recapitulates the formation of aversive memory, and to optimize our protocol, we dissected the CNS (brain with attached ventral nerve cord) of 4- to 8-d-old mated female flies expressing the Ca\(^2+\) indicator GCaMP6f in the MBs using the KC driver (VT030559-GAL4). Dissected brains were pinned in an imaging bath chamber, and the cervical connective toward the base of the ventral nerve cord was cut to allow electrical stimulation of the AFV connecting the ventral nerve cord to the brain (Fig. 1A). We used glass suction electrodes to stimulate the AL projection neurons and the AFV to activate the odor pathway and the electric shock pathway, respectively (Fig. 1B). Given that in vivo aversive STM formation is correlated with enhanced calcium responses in the α' 3 MB compartment but not in the α3 compartment (Krashes et al., 2007; Wang et al., 2008; Cervantes-Sandoval et al., 2013; Zhang et al., 2019), we decided to focus our calcium imaging on these two regions (Fig. 1C). We found that both α' and α3 compartments respond only to ipsilateral AL stimulation, but AFV stimulation could generate a calcium response in both compartments of both MBs. Therefore, we imaged the MB ipsilateral to the stimulated AL.

To induce the formation of an aversive STM trace, we paired 12 trains of stimulation of both the AL electrode and the AFV electrode, thus activating the CS and the US pathways at the same time (paired induction). As controls, we repeated the same induction paradigm but separated the AL stimulation from the AFV stimulation by 30 s (unpaired induction) or omitted the AFV stimulation (AL alone induction) or omitted the AL stimulation (AFV alone induction) or simply allowed the brain to rest for 15 min (no induction). We compared the change in calcium response to AL stimulation before induction and 15 min after induction (Fig. 1D). We predicted that, if this ex vivo model truly recapitulated short-term aversive learning, only the paired induction should produce an enhancement of the calcium responses in the α' and none of the stimuli should potentiate α3 (Krashes et al., 2007; Wang et al., 2008; Davis, 2011; Cervantes-Sandoval et al., 2013). Indeed, this was the result we obtained (Fig. 1E,F). Interestingly, we noticed that the repetitive unpaired activation of KCs results in suppression of their calcium responses (Fig. 1F). This is reminiscent of desensitization or habituation; a nonassociative plasticity/ memory described as a decrement in neural responses to uninteresting, frequently encountered stimuli whose predictive value is negligible (Wilson and Linster, 2008; Wilson, 2009). These nonassociative processes have been documented in insects (Cho et al., 2004; Das et al., 2011; Semelidou et al., 2018), rodents
Dopamine replaces the artificial US stimulus but does not replace the CS

Multiple behavioral and in vivo imaging studies have shown that dopaminergic neurons encode the US valence information in the MB, with PPLs providing aversive reinforcement and PAMs providing appetitive reinforcement (Schwaerzel et al., 2003; Riemensperger et al., 2005; Schroll et al., 2006; Kim et al., 2007; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010, 2012, 2014b; Pech et al., 2013; Yamagata et al., 2016; Handler et al., 2019). These studies suggest that the US alone is sufficient to evoke dopamine release. However, mechanistic studies using the LTE paradigm reported no dopamine release after US pathway stimulation alone. Strong dopamine release was only seen after coincident activation of both the CS and the US pathways, and it was concluded that dopamine release is downstream of the CS-US coincidence and does not encode the primary US information (Ueno et al., 2017, 2020). To directly address this discrepancy, we expressed a G-protein-coupled receptor activation-based dopamine sensor (

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**Table 2. Sidak post hoc results for Figure 5**

| Comparison            | N1 | N2 | t   | DF | p   |
|-----------------------|----|----|-----|----|-----|
| WT’:α3 unpaired       | 6  | 6  | 0.5011 | >0.9999 |
| WT’:α3 paired         | 6  | 6  | 3.417  | 0.0174 |
| WT’:α3 unpaired       | 6  | 6  | 0.7521 | 0.9993 |
| WT’:α3 paired         | 6  | 6  | 2.708  | 0.1127 |
| WT’:α3 unpaired       | 6  | 6  | 7.344  | <0.0001 |
| WT’:α3 paired         | 6  | 6  | 4.116  | 0.0022 |
| CaMKIIUDEL:α3 unpaired| 6  | 6  | 4.428  | 0.0009 |
| CaMKIIUDEL:α3 paired  | 6  | 6  | 0.6559 | 0.9988 |
| WT’:α3 unpaired       | 6  | 6  | 0.9692 | 0.9993 |
| WT’:α3 paired         | 6  | 6  | 4.198  | 0.0017 |
| CaMKIIUDEL:α3 unpaired| 6  | 6  | 0.284  | >0.9999 |
| CaMKIIUDEL:α3 paired  | 6  | 6  | 3.488  | 0.0143 |

*Statistical data for each comparison in Figure 5F. N1, Sample size of first group; N2, sample size of second group; t, value of the t distribution.*
We next asked whether dopamine perfusion can replace electrical stimulation of the AFV. We paired electrical stimulation of the AL with perfusion of different concentrations (1, 5, and 10 μM) of dopamine and analyzed the KC response to AL stimulation before and after the pairing (Fig. 2E,F). Similar to the plasticity seen after AL+AFV induction, we found that only the paired AL+DA (5 or 10 μM) induction induced an enhancement PDP in the α3; pairing with 1 μM DA was not sufficient to induce PDP (Fig. 2G; for detailed statistical data for the post hoc analysis, see Table 1). Also, in agreement with our observation that dopamine release in α3 was very weak compared with the α3 compartment on AFV stimulation, our protocol did not induce ex vivo PDP in the α3 compartment (Fig. 2G). We also found that PDP (induced by AL + 10 μM DA) lasts for at least 1 h after induction (Fig. 2H).

Although these results supported a PDP model with direct dopamine modulation of KCs, we were concerned with the lack of synaptic specificity in the AL+DA induction, as dopamine was perfused onto the whole preparation. To address this concern, we repeated the experiment but replaced dopamine perfusion with activation of the dopaminergic neurons in the PPL1 cluster, which carry the negative valence US information to the MB (Schroll et al., 2006; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010, 2012; Burke et al., 2012; Aso and Rubin, 2016). We expressed the ATP-gated channel P2X₇ (Yao et al., 2012) in the PPL1 dopaminergic cluster using the TH-LexA driver. We paired AL stimulation with application of 2.5 mM ATP to activate PPL1s (Fig. 2I,J). Again, we observed an enhancement PDP in the α3 but not in the α3 compartment (Fig. 2K).

Together, our findings agree with previous behavioral studies and in vivo imaging studies and show that dopamine is released in response to the US alone and carries US valence information to the MB to allow associative learning. Potentiation PDP induced in our ex vivo paradigm also localizes to α’ cells only, congruent with the memory traces reported in vivo with aversive STM.

**Figure 1.** Simultaneous activation of the odor and the electric shock pathways induces an enhancement PDP in the KCs in the MB α3 compartment. A, Schematic of the dissected adult fly CNS showing the MBs and the ALs in the central brain and the ventral nerve cord. The ventral nerve cord is cut at the base of the cervical connective to free the AFV. B, Schematic of the imaging chamber showing the placement of the first electrode on the AL, and the second electrode on the free end of the AFV. C, Representative image of the MB ipsilateral to the stimulated AL. The calcium indicator GCaMP6f is expressed in the KCs (driven by VT030559-GAL4). Circles represent the analyzed ROIs surrounding the α3 and the α3 compartments. Scale bar, 25 μm. D, Paired and unpaired induction protocols. Top, In the paired induction, the ipsilateral AL is activated by 5 stimulation trains with 15 s intertrain interval followed by 1 min rest (pre-induction). Twelve trains of stimulations are then delivered to both the AL and the AFV simultaneously (induction). The brain is then rested for 15 min before being tested by 5 trains of AL stimulations like those applied during the pre-induction (post-induction). Bottom, The unpaired induction protocol. Same as paired induction, except that the AL and the AFV stimulations are separated by 30 s during the induction stage. The stimulation train is 20 pulses at 100 Hz; each pulse is 1 ms with 9 ms interpulse interval. AL stimulation strength: 100 μAmps. AFV stimulation strength: 0.5-1 mAmps. E, Example of the KC calcium response in the α3 compartment pre- and post-no induction (white), AL activation alone (light gray), AFV activation alone (dark gray), unpaired AL+AFV induction (green), and paired AL+AFV induction (blue). Data are mean ± SEM. Two-way ANOVA (α = 0.05; n = 8 in each condition); lobe effects, F(3,36) = 1.096, p = 0.2987; induction effects, F(1,36) = 11.51, p < 0.0001. Sidak post hoc tests in the α3 compartment: A versus B, p > 0.05; B versus A, p < 0.05; t(10) = 3.23, p = 0.015; 5.6, p < 0.0001; 4.48, p = 0.0002; 6.319, p < 0.0001) for (paired vs no induction; paired vs AL alone; paired vs AFV alone; paired vs unpaired), respectively. No statistically significant changes across conditions in the α3 compartment; p > 0.05; t(10) = (0.0237, p > 0.9999; 2.093, p = 0.2785; 1.105, p = 0.9218; 1.884, p = 0.4094) for (paired vs no induction; paired vs AL alone; paired vs AFV alone; paired vs unpaired), respectively.

**PD PDP ex vivo plasticity is input-specific**

Associative memory is CS-specific. A fly that is aversively trained against an odor shows aversive behavior to that odor and closely similar odors only but does not generalize the aversion to all odors (Barth et al., 2014). Olfactory information is sparsely transmitted to KC dendrites, with the identity of the odor being determined by population coding (Marin et al., 2002; Perez-Orive et al., 2002; Wong et al., 2002; Murthy et al., 2008; Turner et al., 2008; Honegger et al., 2011). To mimic the application of two different odors to the fly brain, we placed two suction glass electrodes onto the same AL and spaced them as far as possible from each other to maximize the probability that each electrode stimulates a distinct subset of projection neurons. Theoretically, a certain degree of overlap between the two subsets could be allowed as long as the two activated populations are sufficiently distinct. To test whether the two electrodes activated distinct populations of projection neurons, we expressed the calcium indicator (GCaMP6f) in the projection neurons and divided the AL into multiple ROIs, asking whether the pattern of responses was equivalent for the two electrodes (Fig. 3A,B). We found that spacing resulted in activation of distinct populations of
projection neurons, allowing us to provide two hypothetical odors to the MB (Fig. 3C,D).

We then asked whether the observed ex vivo PDP is specific to the olfactory input activated during induction. To challenge the olfactory-input specificity of our preparation even more, we used the less specific induction method, AL + ATP, in which dopamine is impartially perfused to the whole brain to eliminate any specificity on the US pathway side (Fig. 3E). We recorded KC calcium responses to both electrode stimulations before induction (pre). Then, we delivered a train of stimulations through electrode 1, followed by a train of stimulations through electrode 2. Dopamine perfusion started 5 s before the stimulation train for only one of the two electrodes (paired) and stopped at the last pulse of the train, while normal HL3 saline was perfused during the activation of the other electrode (unpaired). We then allowed the brain to rest for 15 min and tested the KC response to electrode 2 first, then to electrode 1 to eliminate any bias because of the order of stimulation (Fig. 3F). An increased
calcium response was observed in α'3 after activation of the paired subset compared with the unpaired one (Fig. 3G,H). This result demonstrates that even with the broader bath-dopamine induction paradigm, the plasticity achieved in our paradigm is specific to the CS input activated during ex vivo training.

Artificial aversive training induces a suppression PDP in α’3 MBONs

While the α’ branches of the KCs are potentiated in response to an aversively trained odor in intact animals, responses in their postsynaptic MBONs are suppressed (Sejourne et al., 2011; Aso et al., 2014a) (Fig. 4A). We found that the α’3 MBON response to AL stimulation is suppressed after our artificial learning paradigm (Fig. 4B,C), agreeing with in vivo results. These findings show that both the enhancement and suppression PDPs achieved in our paradigm are learning-specific and are encoded as enhancement in the presynaptic KCs and a suppression in the postsynaptic MBONs.

The 3’UTR mRNA of CaMKII is important for ex vivo PDP

We next turned our attention to exploring the utility of this paradigm for understanding memory formation, choosing problems at several different levels of analysis: molecular components of the memory machinery, organizational principles of the circuitry, and the role of brain state in gating plasticity. At the molecular level, previous studies have demonstrated that CaMKII is important for synaptic plasticity and memory formation in many species (Kelleher et al., 2004; Giese and Mizuno, 2013), including Drosophila melanogaster (Griffith et al., 1993; Koh et al., 1999; Ashraf et al., 2006; Malik et al., 2013; Mitchell et al., 2021). Recently, we found that the long 3’UTR region of CaMKII mRNA is responsible for the activity-dependent synthesis of CaMKII in presynaptic terminals at the larval neuromuscular junction (Kuklin et al., 2017) and for the basal accumulation of axonal CaMKII protein in MB (Chen et al., 2022). We hypothesized that the loss of the 3’UTR would impair associative plasticity by decreasing synaptic CaMKII protein levels and disrupting the signaling machinery that is triggered by the CS+US coincidence. We tested this idea and used our ex vivo PDP preparation to gain insight into whether this effect is upstream or downstream of the CS+US coincidence.

First, we asked whether loss of the 3’UTR of CaMKII mRNA affects CaMKII levels. We used animals in which the CaMKII gene was engineered using CRISPR/Cas9 to lack the 3’UTR (CaMKII<sup>UTR<sub>Del</sub></sup>, Fig. 5A) (Chen et al., 2022). Immunostaining to quantify CaMKII protein levels in the MB neuropil in both WT and CaMKII<sup>UTR<sub>Del</sub></sup> flies showed that CaMKII levels are specifically decreased in synaptic regions (Fig. 5B). We also used Western blotting to quantify total CaMKII levels and found a substantial decrease, normalized to actin, in CaMKII<sup>UTR<sub>Del</sub></sup> flies compared with WT (Fig. 5C). This is in agreement with a previous study which found that mice expressing a mutant form of CaMKII lacking the 3’UTR show decreased levels of CaMKII in the dentritic, but not the somatic, region of hippocampal neurons (Miller et al., 2002).

We then tested the impact of the 3’UTR deletion on aversive STM. We found that CaMKII<sup>UTR<sub>Del</sub></sup> flies showed a significant impairment in immediate memory performance compared with WT flies (Fig. 5D). This impairment was not because of sensorimotor dysfunctions as both WT and CaMKII<sup>UTR<sub>Del</sub></sup> flies respond to electric shock (Fig. 5E). We then tested the effect of the CaMKII mRNA 3’UTR deletion on potentiation PDP in KCs. We used the previously described AL+DA induction protocol and examined KC responses to AL stimulation both before and 15 min after either paired or unpaired induction. In the α’3 compartment, although both WT and CaMKII<sup>UTR<sub>Del</sub></sup> genotypes showed statistically significant PDP relative to the unpaired induction, the PDP in the WT flies was >5 times stronger than in the CaMKII<sup>UTR<sub>Del</sub></sup> flies (Fig. 5F; for detailed statistical data for the post hoc analysis, see Table 2). We also noticed that the change in signal in the α3 compartment in WT flies after paired induction was statistically different from the unpaired induction. However, this does not translate into formation of PDP in that compartment as this change was not different from zero (one-
sample t test, \( p = 0.947 \)). This is likely because of a technical difference as the AL input stimulation in this experiment was stronger (200 \( \mu \)Amps vs 100 \( \mu \)Amp in previous experiments), which led to a stronger suppression after the unpaired AL stimulation in the unpaired induction (see also below). These results show that the 3' UTR mRNA of CaMKII is important for memory and its loss impairs learning-induced plasticity. It also indicates that the effect on the learning circuit is downstream of the CS+US coincidence and does not block the CS+US coincidence detection machinery itself.

**Stronger inputs to the MB can recruit the \( \alpha 3 \) compartment into the STM circuit**

At the circuit level, it has been hypothesized that memory in flies, like humans (McClelland et al., 1995; Dudai, 2012), undergoes systems consolidation: initial potentiation of the \( \alpha' \beta' \) lobes with a time-dependent transfer of potentiation to \( \alpha \beta \). The MB \( \alpha' \) and \( \alpha 3 \) compartments are adjacent to each other. Both respond to odor (Turner et al., 2008) and AL stimulation (Fig. 6B), and both use the same coincidence detector, rutabaga (Livingstone et al., 1984; Levin et al., 1992; Mao et al., 2004; Gervasii et al., 2010). This raises the question of how the two compartments are able to play different roles in the memory circuit and why only the \( \alpha' \beta' \) cells show immediate PDP both \textit{ex vivo} and \textit{in vivo}. To determine whether this might be because of differences in the intrinsic properties of the two classes of cells, we examined the basal responses of both compartments to a ramp of AL stimulations without any pairings (Fig. 6A). We found that \( \alpha' 3 \) axons are recruited first, with very weak AL stimulation, while \( \alpha 3 \) only starts responding at higher stimulation strengths (Fig. 6B). There was also a distinct difference in the maximum response levels: \( \alpha' 3 \) compartment responses plateau at a much lower level than \( \alpha 3 \) (Fig. 6B), likely explained by the fact that the \( \alpha 3 \) compartment receives more axons than \( \alpha' 3 \) (F. Li et al., 2020). Previous studies reported that odors activate a small percentage of KCs (\~{}5%-12%), and that the odor responses in \( \alpha' \beta' \) cells are stronger than those of \( \alpha \beta \) cells (Turner et al., 2008; Inada et al., 2017). This suggests that actual odor encoding is similar to the weak AL stimulation used in our experiments (100 \( \mu \)AmP), recruiting only \( \alpha 3 \) but insufficient to recruit \( \alpha 3 \). We hypothesized that an increase in the AL stimulation intensity during \textit{ex vivo} induction might be sufficient to recruit \( \alpha 3 \) and allow PDP in that compartment as well. We repeated both AL+AFV and AL+DA inductions, but this time with a 200 \( \mu \)Amp AL stimulation intensity. Indeed, under these conditions, we found that PDP occurred in both the \( \alpha' 3 \) and \( \alpha 3 \) compartments (Fig. 6C,D).

This result agrees with predictions from our previously published theoretical model of the associative learning circuit in which the CS+US coincidence triggers a recurrent loop between KCs and dopaminergic neurons, which increases dopamine release only onto cells receiving both inputs together (Adel and Griffith, 2021). The weak response of the \( \alpha 3 \) compartment to weak AL stimulation is subthreshold for the MB coincidence detector, and insufficient for forming PDP in \( \alpha 3 \). Stronger AL stimulation puts \( \alpha 3 \) over threshold and triggers the coincidence detector in \( \alpha 3 \) axons, thus increasing dopamine gain and forming PDP/memory.

Our model further predicts that there should be a certain dopamine concentration above which dopamine alone is sufficient to potentiate both \( \alpha' 3 \) and \( \alpha 3 \) compartments without any need for pairing, as this hypothetical high dopamine concentration bypasses the need for CS+US coincidence detector activation of the gain control machinery that increases local dopamine release. To test this prediction, we applied either 10 \( \mu \)M dopamine (used in our previous experiments) or a high dopamine concentration (200 \( \mu \)M) to the dissected brains without pairing with AL stimulation. Indeed, 200 \( \mu \)M dopamine alone was sufficient to potentiate both \( \alpha' 3 \) and \( \alpha 3 \) responses to AL stimulation (100 \( \mu \)Amps) 15 min after dopamine application (Fig. 6E). A similar effect of high dopamine was reported by Ueno et al. (2013) but interpreted differently. We show that pairing-independent plasticity resulting from very high dopamine, while possibly implemented by the same machinery responsible for associative learning, does not truly represent a memory trace as it lacks specificity to CS-activated synapses, likely representing a generalized potentiated state of the MB. In contrast, the PDP seen with lower dopamine concentrations relies on an interplay between the CS and US signals to gate dopamine gain only at the synapses activated by the CS during training.

**PDP is blocked by sleep deprivation and rescued by rebound sleep**

Many of the most interesting questions about memory formation are ones that involve the interaction of plasticity with the animal’s internal state. To test the ability of this preparation to retain traces of previous experience and allow interrogation of memory mechanisms at this level, we looked at the effect of sleep on subsequent \textit{ex vivo} plasticity. Sleep is linked to memory across phyla. In \textit{Drosophila}, perturbations of sleep have been shown to impair both memory formation (Bushey et al., 2007; Seugnet et al., 2008; 2009; X. Li et al., 2009; Donlea et al., 2014; Seidner et al., 2015) and consolidation (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2009; Bushey et al., 2011; Dag et al., 2019; for review, see Goel et al., 2009; Diekelmann and Born, 2010;
**Figure 5.** The 3'UTR region of CaMKII is important for PDP formation. A, Schematic of WT (left) and CaMKII\textsuperscript{3'UTR} (right) an allele in which the 3'UTR has been replaced with an RFP reporter using CRISPR/Cas9 (right). B, Left, Representative immunostaining images showing CaMKII protein levels in the MB in WT CaMKII flies and in CaMKII\textsuperscript{3'UTR} flies. Dotted line indicates position of the MB. Scale bar, 20 μm. Right, Quantification of CaMKII levels (mean ± SEM). Unpaired t test, two-tailed; n = 20 WT flies and 22 CaMKII\textsuperscript{3'UTR} flies; ****p = 0.0001. C, Left, Western blot showing CaMKII and actin levels in WT or CaMKII\textsuperscript{3'UTR} adult female brains and in purified CaMKII samples. Right, Quantification of CaMKII immunoreactivity (mean ± SEM). Mann-Whitney test; n = 4 in each condition; sum of ranks in WT group is 10: *p = 0.0286. D, Learning index of WT and CaMKII\textsuperscript{3'UTR} flies after training with 90 or 60 V. Data are mean ± SEM. Two-way ANOVA: punishment voltage effects, F(1,572) = 18, p < 0.0001; 3'UTR effects, F(1,57) = 67.57, p < 0.0001. Sidak post hoc tests: when punishment is 60 V, ****p = 0.0001. E, WT and CaMKII\textsuperscript{3'UTR} flies can avoid electric shock. Data are mean ± SEM. Unpaired t test; data collected across 11 trials of the WT group and 20 trials of the CaMKII\textsuperscript{3'UTR} group; number of flies per group ranged between 21 and 47 with an average of 29 flies per group; **p = 0.0518. F, Mean relative change of the calcium responses in the α3 and the α3 compartments after unpaired AL + DA induction (green) and paired AL + DA induction (blue) in WT flies or CaMKII\textsuperscript{3'UTR} flies. Data are mean ± SEM. Three-way ANOVA; n = 6 in each condition: lobe effects, F(1,40) = 17.52, p = 0.0002; induction effects, F(1,40) = 68.43, p < 0.0001; 3'UTR effects, F(1,40) = 8.625, p = 0.0055; 3'UTR × lobe interaction effects, F(1,40) = 0.9631, p = 0.3323; 3'UTR × induction interaction effects, F(1,40) = 10.16, p = 0.0028; lobe × induction effects, F(1,40) = 12.25, p = 0.0012; 3'UTR × lobe × induction interaction effects, F(1,40) = 0.7388, p = 0.7872. Sidak post hoc results are listed in Table 2. Striped bars represent the CaMKII\textsuperscript{3'UTR} genotype.

**Discussion**

Drosophila neural circuits are traditionally studied by relating in vivo genetic and chemical manipulations with their consequent behavioral outcomes, from which circuit information can then be inferred (Olsen and Wilson, 2008; Simpson, 2009). More
recently, the advent of \textit{in vivo} calcium imaging allowed for tracing neural activity in actively behaving flies. Over more than a decade of such \textit{in vivo} studies, the general circuit mechanisms of associative memory have been discovered, but there are limitations imposed by imaging the brain of an active intact fly (for review, see Adel and Griffith, 2021). These include the relatively low signal-to-noise ratio, the inaccessibility of multiple brain regions because of restrictions on imaging angles, the difficulty of doing acute pharmacological studies, and the possible confounds of studying the brain of a movement-restricted fly experiencing ongoing stress. Taking inspiration from the way the LTP hippocampal slice model revolutionized our understanding of mammalian memory (Bliss and Lomo, 1973), we provide here an \textit{ex vivo} model of \textit{Drosophila} memory which can overcome these limitations and offer a powerful preparation for studying \textit{Drosophila} memory circuits. Importantly, this model provides a framework for investigating the dynamics of neural circuits in the fly brain.

Most of the previous studies investigating the associative learning circuit \textit{ex vivo} have focused on mapping connectivity (Cohn et al., 2015; Barnstedt et al., 2016; Felsenberg et al., 2017, 2018; Zhao et al., 2018) or characterizing a specific biochemical pathway (Tomchik and Davis, 2009; Handler et al., 2019; Ueno et al., 2020). Only a few \textit{ex vivo} studies (Wang et al., 2008; Ueno et al., 2013, 2017; Suzuki-Sawano et al., 2017) have focused on understanding MB circuit logic. In the LTE model developed by Ueno et al. (2013), pairing a stimulation of the CS and US pathways induced a potentiation of KC responses in the tips of the MB vertical lobes, but LTE did not fully recapitulate other characteristics of associative memory observed in intact flies. Here we develop a modified \textit{ex vivo} model that resolves these discrepancies, showing that the paired activation of odor and punishment pathways induces appropriate plasticity at multiple nodes in the circuit: potentiation of KCs and suppression of MBONs. Several mechanisms for encoding those opposite forms of the plasticity have been proposed, including spike timing-dependent plasticity and activation of distinct dopaminergic receptors (for review, see Adel and Griffith, 2021). Spike timing-dependent plasticity mechanisms appear less likely as MBON suppression was shown to not require MBON spiking (Hige et al., 2015). Perhaps the strongest model so far comes from Handler et al. (2019) who showed that differences in the order of KCs activation and dopaminergic input activate distinct dopaminergic receptors, DopR1or DopR2, which encode MBON suppression or potentiation, respectively. It is important to note that both Hige et al. (2015) and Handler et al. (2019) studied the plasticity in MB medial lobes, while our study focused on MB vertical lobes, so our paradigm may be useful in gaining greater mechanistic insight into this sign transformation in the vertical lobes.

We show that PDP is localized to the MB $\alpha'3$ compartment and not in $\alpha3$, in alignment with most imaging studies in intact flies. Importantly, in this \textit{ex vivo} preparation, punishment information is relayed to the MB through dopaminergic release from the PPL1 subset. Bath application of dopamine in our preparation does not interfere with the specificity of associative learning since PDP is exclusively formed in the cells that were active during the dopamine application. These data settle several inconsistencies between previous \textit{ex vivo} studies (Ueno et al., 2013, 2017) and the majority of \textit{in vivo} reports (Schwaerzel et al., 2003; Riemensperger et al., 2005; Schroll et al., 2006; Kim et al., 2007; Claridge-Chang et al., 2009; Mao and Davis, 2009; Aso et al., 2010, 2012, 2014b; Pech et al., 2013; Yamagata et al., 2016; Cognigni et al., 2018; Handler et al., 2019). We suggest that the
genensis of the discrepancies was not because of any inherent difference between intact and ex vivo brains but was rather a consequence of technical considerations, including stimulation strength, dopamine concentration, and the sensor tools used (for a more complete discussion, see Adel and Griffith, 2021).

An ex vivo preparation that recapitulates the cardinal features of the circuits underlying associative memory formation should be useful for mechanistic studies at the molecular, cellular, and systems levels. We used our model to ask a new question about the innerworkings of the circuit at each of these levels. At the molecular level, we demonstrated the importance of normal levels of CaMKII by manipulating the 3' UTR of CaMKII mRNA. Deletion of this region of the CaMKII gene drastically reduces the amount of CaMKII protein in synaptic regions and blunts the ability to form STM and to generate a potentiation PDP in KC axons. Our data argue that the role of this molecule is downstream of the CS+US coincidence detector, as we observe a much weaker PDP in CaMKII<sup>3UTR</sup>-labeled flies. Whether the behavioral defect is due solely to the KC PDP defect is not completely clear since CaMKII likely has active roles at other circuit nodes (Mitchell et al., 2021).

At the cellular level, we asked why STM and PDP form in the α3 but not the nearby α3 compartment when both compartments respond to odors (Turner et al., 2008) and AL stimulation, and both receive dopaminergic input from the same PPL1 cluster (Masek et al., 2015). Previous work found that real odors cause activity in only 5%-12% of KCs and elicit a much higher spike rate in the α3/β KCs than in the α3/β KCs. We found that low-intensity AL stimulation (100 μAmps) elicits a stronger response in the α3 than in the α3 compartment, while high-intensity AL-stimulation (200 μAmps) causes strong responses in the α3 compartment and recruits it to the learning circuit. Coupling this with our observation of lower dopamine release in α3 suggests a model in which odor presentation during associative learning causes subthreshold responses in αβ cells such that the CS+US coincidence detector is not triggered, while the stronger responses in the α3/β cells bypass this threshold, allowing plasticity in the α3/β cells only. This notion is in alignment with the previous finding that α3/β cells have a lower firing threshold than αβ cells (Inada et al., 2017). Further, it is possible that long-term memory and the enhancement memory trace in the αβ KCs after repetitive space training (Yu et al., 2019; 2020).

Figure 7. Sleep deprivation blocks PDP and rebound sleep recovers it. A, Schematic of the different sleep groups: Sleep Control, flies are undisturbed during night and tested between ZT0 and ZT24 the next day; Sleep Deprived, flies are kept on the shaker during between ZT12 and ZT24 and then tested between ZT0 and ZT2 the next day; Sleep Deprived + Recovery, same as Sleep-deprived, but flies were allowed to recover sleep between ZT0 and ZT2 and then tested between ZT2 and ZT4; Stress Control, flies are undisturbed during the night between ZT12 and ZT22 and then the shaker was turned on between ZT22 and ZT24 before testing between ZT0 and ZT2 the next day. Testing was done by the AL+DA induction described in Figure 2F. B, Total sleep of the different groups in the different ZT windows. Data are mean ± SEM. One-way ANOVA in the ZT12/ZT22 comparison, $F_{(1,40)} = 159, p < 0.0001$; Tukey’s post hoc test: control vs sleep-deprived: $q_{(40)} = 20.05, p < 0.0001$; control vs stress control: $q_{(40)} = 1.625, p = 0.4916$; sleep-deprived vs stress control: $q_{(40)} = 22.34, p < 0.0001$. In the ZT22/ZT24 comparison, $F_{(1,40)} = 66.64, p < 0.0001$; Tukey’s post hoc test: control vs sleep-deprived: $q_{(40)} = 14, p < 0.0001$; control vs stress control: $q_{(40)} = 14.47, p < 0.0001$; sleep-deprived vs stress control: $q_{(40)} = 1.114, p = 0.7136$. In the ZT0/ZT2 comparison, $t = 4.534, p < 0.0001$. C, Mean relative change of the calcium responses in the α3 (left) and the α3 (right) compartments after paired AL+DA induction. Data are mean ± SEM. Two-way ANOVA (α3 vs αα, n = 6 in each condition): lobe effects, $F_{(1,40)} = 10.10, p = 0.0029$; sleep effects, $F_{(1,40)} = 4.079, p = 0.0007$. Sidak post hoc tests: in the α3 compartment: A versus $A$, $p < 0.05$; B versus $A$, $p < 0.05$, $t_{(40)} = (0.592, p > 0.9999$. Data are mean ± SEM. One-way ANOVA (n = 6 in each condition): lobe effects, $F_{(1,40)} = 7.019, p = 0.0007$; sleep effects, $F_{(1,40)} = 0.2631, p = 0.612$; sleep induction, $F_{(1,40)} = 0.3938, p = 0.5348$. Sidak post hoc tests: in the α3 compartment: A versus $A$, $p > 0.05$; B versus $A$, $p < 0.05$, $t_{(40)} = 0.1592, p > 0.9999$. Data are mean ± SEM. One-way ANOVA (n = 6 in each condition): lobe effects, $F_{(1,40)} = 0.9308, p = 0.3438$. D, Change in F/F from individual animals plotted against the animal's time of dissection. White circles represent control animals (allowed to sleep between ZT12 and ZT24). Pink circles represent sleep-deprived animals (on the shaker between ZT12 and ZT24).
2006) require a gradual potentiation of the αβ KC responses with every training session such that the responses bypass the coincidence detection threshold after several training sessions. Whether repetition of AL+DA pairings recruits PDP in the α3 compartment remains unclear. It is also yet to be determined whether shortcircuiting the circuit and recruiting αβ cells in the first training session reduces the need for multiple spaced training sessions in long-term memory formation.

In conclusion, we looked at the ability of the effects of prior experience, or brain state, on the memory circuit to be retained in the ex vivo preparation. Excitingly, we found that sleep-deprived flies could not form PDP, but that as little as 2 h of rest before dissection allowed the brain to recover PDP formation. The complete abolition of PDP in sleep-deprived flies at first and deprived flies could not form PDP, but that as little as 2 h of rest in the experience, or brain state, on the memory circuit to be retained afterward (Fig. 7D) suggest that sleep converges on the memory circuit upstream of the CS+US coincidence detector. Whether this involves regulation of dopamine receptors in the MB during sleep remains to be determined. The ability to retain in some functional way the internal state of the brain will allow this preparation to be used to understand how memory formation is altered by global system alterations.

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