Dopamine neurons promote arousal and wakefulness via Dop1R receptor in the Drosophila mushroom body

Authors: Margaret Driscoll[1], Victoria Coleman[1], Morgan McLaughlin[1], Amanda Nguyen[1], and Divya Sitaraman[1,2*]

1. Department of Psychological Sciences, College of Arts and Sciences, 5998 Alcala Park, University of San Diego, San Diego, CA 92110

2. Department of Psychology, College of Science, 25800 Carlos Bee Blvd, California State University, Hayward, CA 94542

Correspondence should be addressed to: dsitaramanlab@gmail.com or divya.sitaraman@csueastbay.edu

Abstract:
Diseases associated with impaired sleep regulation negatively impact motor skills, emotional states, and cognitive performance. Neural circuits involved in sleep homeostasis play a critical role in sleep-wake transition but the precise circuit mechanisms underlying persistence of sleep, arousal and wakefulness remain unclear. The fruit fly, *Drosophila melanogaster*, provides a powerful model system for the study of sleep and circuit mechanisms underlying state transitions and persistence of states to meet the organisms motivational and cognitive needs. Here we investigate the role of specific subsets of dopamine neurons innervating the mushroom body (MB), a region implicated in sleep and associative learning in *Drosophila*. We find that inhibition of specific subsets of PAM and PPL1 dopamine neurons projecting to the MB increase sleep in the presence of strong wake-inducing stimuli. The wakefulness induced by activity of the PAM neurons is mediated by both Dop1 R1 and Dop1R2 receptors located in downstream Kenyon cells and mushroom body output neurons (MBONs). Further, we find that unlike the activity of the MBONs, whose activity is modulated by sleep-need and sleep-debt, the dopamine neuronal activity is independent of sleep-need. The sleep-regulating dopamine neurons characterized in these studies are also involved in mediating short-term courtship memories, appetitive memories and memory extinction processes. Taken together, we have identified and characterized the dopamine circuit and cellular mechanisms that link motivation-dependent behavioral output and suppression of sleep to coordinate motivated processes with wakefulness.

Introduction:

Sleep, wakefulness and arousal represent internal states that control multiple physiological and behavioral processes. Transition between these states and persistence of these individual states involves neural circuits that are dispersed throughout the brain and interact with systems involved in controlling hunger, goal-directed behavior and memory formation.

Sleep and wakefulness are regulated by two processes, known as process S and process C, where Process S is a homeostatic process by which sleep pressure increases during wakefulness and decreases during sleep, and process C is a circadian process that is controlled by circadian pacemaker circuits of the brain and determines the propensity to sleep during specific times of the day and night.[1,2] A third process has also been widely studied, that affects sleep and arousal by motivational or cognitive processes that modulate transition and persistence of these states[3,4]. At the behavioral level motor output, attention, motivation, reward, and feeding all function on the basis of wakefulness and represent different levels of arousal and involve conserved neuromodulators like dopamine, serotonin and histamine. Neuromodulators can target synapses and by altering excitability of neurons generate variable output from defined circuits[5,6]

Dopamine is a wake-inducing neuromodulator in flies and mammals[7-11]. The ability to manipulate genes and genetically defined neural circuits has allowed identification of distinct sleep-wake microcircuits and their modulation by dopamine in flies and rodents[3,4,7-9,12-15]. Broad manipulations of dopamine neurons using genetic and pharmacological approaches shows decreases in sleep, supported by increased sleep in receptors and transporters mutants[12,15]. In the most well-defined dopamine mediated wake circuit in flies, a cluster of neurons within the central complex innervating the dorsal fan shaped body is critical for sleep homeostasis. The dorsal fan shaped body dependent sleep switch is inhibited by dopamine input via Dop1R2 signaling and altered potassium conductance.[13,16,17] Targeted activation of most dopamine neurons projecting to regions outside CX (central complex) are also strongly wake-promoting suggesting that distinct dopamine clusters induce wakefulness via distinct neural structures[18,19]. However, it's unclear if dopamine projections outside CX are also involved in sleep homeostasis and sleep-wake switching mechanisms.

The largest cluster of dopamine neurons (~ 130-150 neurons, PAM and PPL) in the fly brain projects to a region called the mushroom body (MB) and their role in sleep regulation is not well defined. Here we identify the precise circuit, cellular and molecular basis of dopamine innervations to the MB using genetic tools to divide cellular sub-classes and knockdown specific receptors within downstream cell types (Kenyon cells and Mushroom body output neurons, MBONs) in the context of wakefulness. In published screen MB DAN cell types, we find that artificial activation of DANs projecting to lobe compartments containing the dendrites of wake-promoting MBONs are wake-promoting. Further, artificial and transient activation of DANs projecting to the γ5 and β′2 compartments involved in sleep homeostasis induces activation of wake-promoting MBON-γ5β′2a/β′2mp, that is suppressed by inhibitor to Dop1R antagonist SCH 23390[19]

Here we investigate into the specific PAM and PPL cell types and receptor mechanisms underlying wakefulness induced by MB-DANs and core MB sleep circuits. We show DANs projecting to γ5β′2, γ4> γ2,1, α'1, α'2 and α2 is required for sleep initiation and persistence and activity of the strongest sleep-regulating PAM cluster neurons is not directly modulated by sleep-need. Furthermore, we show that these effects are mediated by two of the four dopamine receptors, Dop1R1 and Dop1R2 that function within the KCs and MBON-γ5β′2a/β′2mp in sleep initiation and persistence.

These studies characterize the PAM mediated dopaminergic sleep control mechanism that functions by modulation of the mushroom bodies, and provides evidence for cellular and molecular mechanisms mediated by Dop1R1 and Dop1R2 for control of sleep by the MB associative network.

The PAM DAN network is particularly critical in understanding wakefulness induced by competing motivational and cognitive demands as these neurons have been shown to be responsive to external stimuli such as sugar reward or electric shock used to reinforce memories in a cell-type specific manner[20-22]. Therefore, both the internal wake states and external stimuli are integrated by the same DANs, each of which function by modulating specific domains within the MB.

Materials and Methods

Fly stocks

Flies were maintained on conventional cornmeal-agar-molasses medium in 12 h light: 12 h dark conditions at 21°C (for dTRPA1 experiments) or 25°C (for immunohistochemistry), with ambient humidity of 60–70%. 3-7-day old flies were collected and tested for sleep, feeding and immunohistochemistry. The following stocks used in the experiments were obtained from Bloomington Drosophila Resource Center:

26263 +, +, UAS-dTrpA1 attp16

31765 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM04077}attP2

55239 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC02344}attP2/TM3, Sb[1]

62193 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05200}attP40

26018 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02043}attP2

51423 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC02893}attP2

65997 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC06293}attP2

26001 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02025}attP2

36824 y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01057}attP2

50621 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMC02988}attP40

31981 y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF03415}attP2

32194 w[*]; P{y[+t7.7] w[+mC]=20XUAS-IVS-mCD8::GFP}attP2

Split-Gal4 lines: MB054B, MB312B, MB196B, MB194B, MB213B, MB060B, MB209B, MB060B, MB308B, MB438B, MB011B, MB010B, MB107B, MB298B, pBDGAL4, 58E02-Gal4, 48B04-Gal4 and 15A04-Gal4 were obtained from Yoshi Aso and Gerry Rubin and have been described in [19,23-25]. Deletion mutants DopR1 ($DopR1^{attp}$) and DopR2 ($DopR2^{attp}$) were donated by Yoshi Aso and first described in [26] and UAS-CaLexA flies were obtained from Dr. Jing Wang (UCSD) and first described in [27].

Sleep assays

Split-GAL4 flies were crossed to flies bearing *dTRPA1* (in attP16) or *Shibire* and maintained at 21°C, 24-64 male progeny, 0-7 days post-eclosion, were placed in 65 mm × 5 mm transparent plastic tubes with standard cornmeal dextrose agar media, placed in a Drosophila Activity Monitoring system (Trikinetics), and locomotor activity data were collected in 1 min bins.

Activity monitors were maintained in a 12 h:12 h light-dark cycle at 65% relative humidity, and tested flies were given 48 hours to acclimate to the light/dark cycle of the incubator. Total 24-h sleep quantity (daytime plus nighttime sleep) was extracted from locomotor activity data as described: sleep is defined as a contiguous period of inactivity lasting 5 min or more. Sleep profiles were generated depicting average sleep (minutes per 30 min) for day 1 (baseline), day 2 (activation), and day 3 (recovery).

In addition to permissive temperature controls other genotypic controls were used for hit detection as indicated. For all screen hits, waking activity was calculated as the number of beam crossings/min when the fly was awake. Statistical comparisons between experimental and control genotypes were performed using Prism (Graphpad Inc, CA).

Carbamazepine and Caffeine Feeding

CBZ (Sigma-Aldrich, C4024) was dissolved in 45% (2-hydroxypropyl)-beta-cyclodextrin (Sigma-Aldrich, H107) as described in to prepare a stock solution. For CBZ experiments, flies were loaded in tubes containing 2% agarose (Sigma, A9539) and 5% sucrose (S0389, Sigma-Aldrich) with 0.1 mg/ml CBZ. For caffeine feeding experiments Caffeine (Sigma-Aldrich, C0750) was mixed into melted sucrose/agar food at a concentration of 1mg/ml.

IHC

Dissection and immunohistochemistry of fly brains were performed as previously described with minor modifications (https://www.janelia.org/project-team/flylight/protocols). Brains of 3–7day

old male flies were dissected in 1X PBS medium (BPP3920, Fisher Sci) and fixed in 2% paraformaldehyde in PBT for 60 min at room temperature (RT). After washing in PBT (0.5% Triton X-100 in PBS), brains were blocked in 5% normal goat serum (NGS) in PBT overnight. Brains were then incubated in primary antibodies diluted in NGS, nutated for 4 hours at room temperature, then transferred to 4°C for 2 days, washed three times in PBT for 30 min, then incubated in secondary antibodies diluted in NGS, nutated for 4 hours at room temperature, then transferred to 4°C for 2 days. Brains were washed thoroughly in PBT three times for 30 min and mounted in Vectashield (Vector laboratories, CA) for imaging.

The following antibodies were used: rabbit polyclonal anti-GFP (1:1000; Life Technologies), mouse anti-nc82 (1:50; Developmental Studies Hybridoma Bank, Univ. Iowa), and cross-adsorbed secondary antibodies to IgG (H+L): goat Alexa Fluor 488 anti-rabbit (1:800; Life Technologies) and goat Alexa Fluor 568 (1:400; Life Technologies).

For CaLexA experiment, images were taken under 40X magnification and analyzed using Fiji/ImageJ (https://imagej.net/Fiji). To measure signal intensities, a maximal intensity projection of all the slices obtained from a z-stack comprising the PAM neuron (innervation patterns) was generated for control and experimental groups (sleep-deprived, carbamazepine and caffeine treated). Comparison groups were scored blind and fluorescence intensity was used for quantifying CaLexA signal.

Statistical Analysis

Different sleep measures (sleep amount, latency etc.) are presented as bar graphs or scatter plots showing mean ± SE or. A one-way ANOVA was used for comparisons between two or more treatments or two or more genotypes and post hoc analyses were performed using Bonferroni correction. For sleep latency measures so we used non-parametric analysis using Kruskal Wallis one-way ANOVA. For comparisons of calcium levels between genotypes or treatments, t test (two-tailed) were used. All statistical analyses and graphing were performed using Prism software (GraphPad Software 7.04; San Diego, California).

Results:

3.1 Silencing of specific dopamine neurons has differential effects on daytime and nightime sleep.

The 130 dopamine neurons, DANs project to and represent the primary modulatory input to MB and subdivided into PAM, PPL1 and PPL2 neurons. PAM neurons specifically project to the horizontal lobes (β, β′, and γ); PPL1 neurons innervate the vertical lobes (α and α′), heel, and peduncle; and PPL2ab neurons project to the calyx. In an unbiased screen of all DANs projecting to the MB using a cell specific split-GAL4 library we found that transient thermogenetic activation using the dTRPA1 temperature-gated depolarizing cation channel reveals multiple classes of PAM and PPL1 DANs suppresses sleep[18,19]

To investigate the role of compartment specific dopamine release by neurons identified in the published activation screen of subsets of PAM and PPL neurons projecting to the mushroom body we inhibited the sleep-regulating DANs by expressing temperature sensitive dominant negative mutation of dynamin, Shibire^{ts1}. We blocked synaptic transmission from the sleep-regulating DANs in the presence of 0.1mg/ml CBZ (carbamazepine). CBZ, is a pharmacological agent that functions as an antagonist of the GABA receptor *Rdl* and suppresses both daytime and nighttime sleep by modulating Pdf neuron [28]. We found that blocking dopamine release from smaller subsets of PPL1 neurons labelled by MB308B and MB438B increased both daytime and nighttime sleep, while a broader PPL1 driver MB060B had effects specific to daytime sleep.

In comparison to the PPL1 neuronal inhibition, blocking dopamine release by specific PAM neurons (MB054B, and MB312B) resulted in a stronger suppression of the wake-inducing effects of CBZ (Figure 1A, C and D). Broader PAM lines (MB196B and MB194B) had effects comparable to the PPL1 lines and MB213B and MB189B did not suppress wakefulness induced by CBZ (Figure 1A-D). We also found that blocking dopamine release from PAM and PPL1 neurons decreased CBZ induced latency or time to sleep after lights off at ZT 12 (Figure S1). Taken together, these data show that compartment specific sleep-regulating DANs have differential effects on sleep-suppression and wakefulness.

Sleep measurements were conducted using the well-established infrared beam-crossing locomotor assay first described in [29,30]. Statistical significance of total sleep, daytime and

nighttime sleep was determined by performing one-way ANOVA followed by Bonferroni post-hoc analysis [for Total Sleep $F(10, 567) = 8.814$, $p<0.0001$, for daytime sleep $F(10, 598) = 10.11$ and for nighttime sleep $F(10, 567) = 11.19$]. For sleep latency data were analyzed using Kruskal Wallis one-way ANOVA followed by post-hoc Dunns test ($H=179.6$, df:10, $p<0.0001$). The expression patterns of these specific and broad sleep-regulating dopamine drivers were first described in [19,23] and were confirmed by expression of UAS-mCD8-GFP (Figure 1E and 1F).

While, the effects of CBZ on sleep in flies are thought to be specific to GABAergic modulation of the Pdf neurons, it's conceivable that the drug directly impacts other GABA neurons in the MB network and influence the activity of sleep-regulating dopamine neurons[31 32]. To test this possibility, we directly imaged activity in sleep-regulating PAM dopamine neurons MB196B and MB054B by expressing CaLexA[27]. We focused our attention on these neurons as PAM neurons had a stronger effect on suppressing sleep duration and latency effects induced by CBZ and our work has previously identified downstream targets of these neurons allowing for a functional dissection of PAM mediated sleep regulation within the MB network[18,19,23,25]. Furthermore, dendrites of wake-promoting MB output neurons innervate γ5, β′2 compartments and γ4 of the mushroom body lobes that are innervated by PAM neurons labelled by MB054B, MB312B and MB196B[18,19,23,25].

CalexA or Calcium dependent nuclear import of LexA is a neural activity reporter that functions by Ca^{++} dependent nuclear import of a transcription factor which then drives GFP expression[27]. Hence, GFP levels in fixed tissue were used as a marker for neuronal activity to compare DAN activity in flies that were fed CBZ. We found no differences in calcium dependent GFP levels in sleep-regulating PAM DAN innervations in the absence and presence of CBZ suggesting that exposure to the drug does not alter the activity of these neurons (Figure S2). The control and CBZ fed flies were compared using unpaired t-test for MB196B ($t=0.56$, $df=19$ and $p=0.56$) and for MB054B ($t=0.37$, $df=18$ and $p=0.7$)

The CaLexA system is well suited for visualizing neural activity in intact animals for long but not short time scales and used extensively in identifying sleep and arousal-related activity within MB, CX (central complex) and clock neurons [14,33,34]. To ensure that the expression levels in split-GAL4 lines is strong enough to detect changes, we measured calcium dependent GFP levels in sleep-regulating PAM DAN's in the absence and presence of caffeine. We found that

16-hour administration of 1mg/ml caffeine, increases activity of PAM neurons as previously published[35]. The control and caffeine fed flies were compared using unpaired t-test (for MB196B t=2.66, df=10 and p=0.023)

These data show that thermogenetic inhibition of sleep-regulating PAM and PPL1 dopaminergic neurons in the presence of strongly wake-promoting state induced by CBZ has differential affects on daytime and nighttime sleep, effects that were not observable in the activation screen suggesting that activity in these neurons is necessary for wakefulness.

3.2: PAM Dopamine neurons activity is not altered by sleep need.

The activation and inhibition experiments clearly indicate a role for specific PAM neurons in sleep regulation. One of the PAM neurons labelled by MB054B projects to the γ5 and β′2 compartments of the mushroom body. We recently showed that MB output neurons within this compartment shows decreased electrical activity post sleep-deprivation when there is an increased sleep-need suggesting that neural activity within this compartment of the MB network is critical for wakefulness[18].

To test if the dopamine neurons projecting to wake-active γ5, β′2 and γ4 compartments of the MB alter their neural states as a function of sleep need, we expressed CalexA in these dopamine neurons (MB054B and MB196B) and measured neuronal activity post sleep-deprivation induced by 16-hour mechanical shaking as described in [18]. Sibling flies of the same genotype placed in the same temperature-controlled environment were used as sleep-replete controls (Figure 2A). There were no significant differences between sleep-deprived and sleep-replete conditions suggesting that dopamine neurons activity is not altered by deprivation induced sleep-need. CalexA signal was compared between sleep-deprived and sleep-replete groups using unpaired t-test for MB054B (t=0.75, df=16 and p=0.46) and MB196B (t=0.68, df=14 and p=0.504). Since, GFP was visible in both conditions it is possible that there is basal activity in these neurons that does not change with sleep-deprivation. Additionally, the changes in activity could be very small or undetectable because split-Gal4 lines are weaker drivers of UAS-transgenes.

To address this possibility, we used three additional GAL4 lines (15A04, 48B04 and 58E02) that label these sleep-regulating PAM dopamine neurons. Like, split-GAL4 lines, some basal activity

was detected in both sleep-deprived and sleep-replete flies but were not significantly different (Figure 2B and C). CalexA signal was compared between sleep-deprived and sleep-replete groups using unpaired t-test for 15A04-GAL4 ($t=0.24$, $df=24$ and $p=0.81$), 48B04-GAL4($t=1.8$, $df=28$ and $p=0.08$) and 58E02-GAL4 ($t=0.91$, $df=22$ and $p=0.38$).

Together these data suggest that while dopamine release regulates sleep and wakefulness, the activity of these neurons is not directly regulated by homeostatic mechanisms. These findings are consistent with previously published results where TrpA1-based stimulation of sleep-regulating PAM neurons (MB054B, MB312B, MB196B) caused a rapid and robust decrease in nighttime sleep, but were not followed by a positive sleep rebound the day after stimulation[19]

3.3: Sleep regulating dopamine neurons signal via DopR1 and DopR2.

To further explore how these specific subsets of dopamine neurons regulate sleep we sought to identify the receptors and downstream targets within the MB. Four dopamine receptors have been identified in the Drosophila genome: DopR1, DopR2, D2R and DopEcR.
Two of these dopamine receptors, DopR1 and DopR2 are co-expressed in the populations of Kenyon Cells (KCs)[36] and known to play distinct role in learning, memory formation and forgetting pathways[37-39]. While, the role of these dopamine receptors in sleep regulation has been identified within central complex, the identity of dopamine receptors involved in MB specific sleep regulation is unknown [10,11].

We used a pan-neuronal driver nsyb-GAL4 with dicer expression and targeted all four dopamine receptors using validated UAS-RNAi lines in the context of sleep regulation. Given the wide variety of RNAi of lines available to downregulate receptor transcripts we picked at least two transgenic lines for each receptor that have been previously validated by quantitative RT PCR [40-44]. Male flies with reduced receptor levels were tested and we found that downregulation of DopR1 and DopR2 specifically increased total sleep without altering the locomotor activity measured by beam counts/minute during wake-period (Figure 3A and 3D). Both these receptors had differential effects on daytime and nighttime sleep such that DopR1 affected both daytime and nighttime sleep while DopR2 effects were restricted to nighttime sleep (Figure 3B and 3C).

Statistical significance of total sleep, daytime and nighttime sleep was determined by performing one-way ANOVA followed by Bonferroni post-hoc analysis [for Total Sleep= $F(9, 938) = 13.64$, $p<0.0001$, for daytime sleep $F(9, 938) = 28.45$ and for nighttime sleep $F(9,938) = 15.65$]. For

waking activity, beam counts/minute were analyzed using Kruskal Wallis one-way ANOVA followed by post-hoc Dunns test (H=74.46, df:8).

3.4 DopR1 and DopR2 differentially regulate nighttime sleep and sleep latency by influencing mushroom body circuit

Since, pan-neuronal manipulations effect receptor levels outside of MB we repeated these experiments with validated RNAi lines that increased sleep (Figure 3) by targeting them to MB neuronal populations that are potentially downstream to the to wake-active γ5, β′2 and γ4 PAM dopamine neurons using highly specific split-Gal4 lines that were first described in[23,25].

Specifically, we targeted two MB output neurons (MBONs) projecting to the γ5 (MB011B), β′2 (MB011B) and γ4 (MB298B) compartments and Kenyon cell populations projecting to these lobes (MB010B- all KCs, MB107B- α'β' KCs. One of the RNAi lines targeting Dop1R1 transcripts increased total sleep when expressed in γ5 β′2 (amp) MBONs, α'β' KCs and all KCs as compared to pBDG4U (empty Gal4) suggesting that suppression of dopamine signaling in these regulated sleep duration [$F (5, 201) = 18.79$, $p<0.0001$] (Figure 4A).

A second RNAi line targeting the same receptor increased total sleep when expressed in all KCs and γ5 β′2 (amp) MBONs [$F (5, 225) = 23.44$, $p<0.0001$] without altering total sleep in α'β' KCs and γ4 MBONs (Figure 4B). In addition, we looked at sleep duration during nighttime when sleep is consolidated and more consistent within genotypes and find that both RNAi lines increase nighttime sleep, a result consistent with the published role of MB dopamine neurons in nighttime sleep[19]. Further, we find that the increases in nighttime sleep resulted from decreased sleep latencies (duration between lights off and 1st sleep bout) in flies where the Dop1R1 levels were downregulated specifically in all Kenyon cells and γ5 β′2 (amp) MBONs.

Downregulating Dop1R2 receptor function wake-regulating MBON compartments and Kenyon cell populations increased total sleep when expressed in γ5 (MB011B), β′2 (MB011B) and Kenyon cell populations projecting to these lobes (MB010B- all KCs, MB107B- α'β' KCs) but not in γ4 (MB298B) MBONs (Figure 5 A-D) [$F (5, 233) = 60.09$, $p<0.0001$].

As in the case of Dop1R1 receptor knockdown, the effects were specific to nighttime sleep [$F(5, 233) = 86.04$, $p<0.0001$] and decreased sleep latency (Figure 4D) [$F(5, 233) = 111.1$, $p<0.0001$] indicating a role for MB dopamine signaling in sleep initiation. Taken together, these results show that PAM dopamine signaling is mediated by Dop1R1 and Dop1R2 receptors specifically via the wake-regulating KCs or γ5 β′2 MBONs or both.

3.5 PAM γ5 signal via DopR1 and DopR2 to regulate daytime, nighttime sleep and latency

In order to directly address and test the role of PAM γ5 signaling through Dop1R1 and Dop1R2 receptor in wake regulation we specifically activated PAM γ5 neurons using a temperature sensitive cation channel dTrpA1 in Dop1R1 and Dop1R2 hypomorph backgrounds [26,45].

Activation of PAM γ5 in an isogenized background decreases sleep amount as shown in the sleep plots and quantified in Figure 6. The sleep suppression caused by PAM γ5 were reversed in the DopR1 and DopR2 hypomorph in isogenized background. Total sleep loss caused by PAM γ5 activation was reversed by the manipulation of the DopR1/R2 receptors consistent with our findings of direct manipulation of receptor levels using RNAi lines [$F(3, 179) = 19.6$, $p<0.0001$]. The hypomorphs had different effects on daytime and nighttime sleep. Both receptors suppressed nighttime sleep suppression induced by PAM γ5 activation but Dop1R2 did not seem to alter daytime sleep suppression [$F(3, 179) = 76.2$, $p<0.0001$] for daytime sleep and [$F(3, 179) = 71.76$, $p<0.0001$] for nighttime sleep].

The increase is nighttime sleep was accompanied by a decrease in sleep latency consistent with RNAi mediated receptor manipulation of MBONs and KCs [$F(3, 179) = 24.41$, $p<0.0001$]

Discussion

The mushroom body is a key structure in the regulation of motivational and cognitive processes[46-53]. The core-structure of the MB is identified by KC (Kenyon cell)-MBON (output neuron) synapses which are modulated by dopamine, octopamine, sNPF, and SIFamide [23,25,54]. The mushroom body lobes are tiled by discrete anatomic compartments defined by the axons of a specific subset of DANs and the dendrites of one or two mushroom body output neurons (MBONs). This anatomical arrangement positions the DANs strategically to convey positive and negative reinforced information by changing the synaptic weight of KC-MBONs in producing aversive and appetitive responses[23,25]. While, the most in-depth analysis of these synapses and distinct DAN-KC-MBON connectivity and behavioral output comes from studies of olfactory conditioning, there is evidence that these synapses play a critical role in innate behaviors like feeding and sleep [19,41].

Our previous findings show that two segregated excitatory synaptic microcircuits propagate homeostatic sleep information from different populations of intrinsic MB “Kenyon cells” (KCs) to specific sleep-regulating MBONs: sleep-promoting KCs increase sleep by preferentially activating the cholinergic MBONs (γ2α’1), while wake-promoting KCs decrease sleep by preferentially activating the glutamatergic MBONs (γ5β’2 and γ4>γ1γ2). Importantly, activity of the sleep-promoting MB microcircuit is increased by sleep deprivation and is necessary for homeostatic rebound sleep (i.e., the increased sleep that occurs after, and in compensation for, sleep lost during deprivation)[18]

Our unbiased screen of DANs projecting to the all 15 compartments of the MB reveals that inputs into both wake- and sleep- promoting microcircuits, suppress sleep[19]. Further, we also found populations of wake-promoting DANs that project to MB compartments whose constituent KCs and MBONs did not affect sleep.

To understand the requirement of dopamine in MB-dependent sleep regulation we blocked release from DANs identified in the screen in the presence of a strong wake-inducing drug CBZ. Blocking of dopamine neurons in the absence of CBZ did not produce an increase in sleep because of ceiling effects and inability to see increases in sleep during nighttime when sleep is most consolidated. To address the possibility that CBZ itself does not alter the activity of DANs, we imaged Calcium in these neurons using CaLexA and find that prolonged exposure to CBZ as

used in our sleep-studies does not alter activity of the DANs projecting to the MB. To further test the ability of CaLexA to probe calcium in PAM-DANs, we treated flies with caffeine and detected changes in activity as previously reported [35]. Blocking release from specific PAM clusters projecting to γ5 and γ4>γ1γ2 MBONs increased sleep (during nighttime) and decreased latency, while PPL1 clusters had effects on either daytime or nighttime sleep. We focused our attention to the PAM clusters as the effects were stronger and reproducible my multiple split-GAL4 lines used in the study.

Our previous findings show that the γ5 β'2 MBONs show reduced activity post-sleep deprivation, indicating that their activity is negatively correlated to an increase sleep drive during rebound period. We probed the activity of γ5 and γ4>γ1γ2 MBONs using sparse split-GAL4 drivers and broad GAL4 lines for stronger expression and find that their activity is not altered by sleep-deprivation suggesting that DANs synaptically communicating with MBONs, signal arousal independent of homeostatic sleep drive.

In addition to probing the release and activity of these PAM-DANS we also explored the dopamine receptors and their location within the MB in signaling arousal. To this end we expressed validated RNAi lines in subsets of KCs and MBONs and find that Dop1R1 and Dop1R2 are critical in mediating the arousal signal via KCs and γ5β'2 MBONs. To directly link the dopamine release from PAM γ5 to Dop1R signaling via the MB, we activated the PAM γ5 in Dop1R1 and Dop1R2 null background and find that the loss of receptors rescues sleep-suppression induced by γ5 activation.

Dopamine neurons within MB, modulate the functioning of KC-MBON compartments in multiple ways by recruiting specific classes of dopamine receptors. These receptors have been studied extensively in the context of associative olfactory memory where Dop1R1 is necessary for memory formation, while Dop1R2 signaling is critical for erasing memories [37,55]. Whether, this mechanism of producing different outcomes by differential recruitment of receptors in response to the same DANs extends to sleep-regulating PAM remains unexplored. Our work reveals that DopR1 and DopR2 induce wakefulness in response to PAM γ5 and likely work synergistically in suppressing sleep.

The sleep-regulating PAM γ5 DANs identified in our study also mediate punishment and reward associated experiences suggesting that the activity of these neurons in the absence of

reinforcing stimuli mediates wakefulness that primes the MB structure to be responsive to the environment. A recent study using EM dataset of a Full Adult Fly Brain (FAFB) mapped the inputs into the PAMγ5 DANs and identified that at least one PAM-γ5 DAN subtype (named γ5fb) receives recurrent synaptic input from γ5β´2a mushroom body output neurons (MBONs). In addition to this input the PAMγ5 could be receiving inputs from gustatory interneurons and lateral horn output neurons mediating sensory information[56]. Whether, these inputs play a role in wakefulness is unclear but suggests that the PAMγ5 is heterogenous group and could serve as a key link between sensory inputs and core sleep regulating circuitry within the MB.

The combination of genetic tools to manipulate receptors, neurons and genetically identified neural circuits reveals that PAM γ5 and downstream responses mediated by both Dop1R1 and Dop1R2 receptors in the KCs and MBONs identifies a circuit within the MB associative network that likely underlies the mechanistic relationship between motivation-dependent behavioral output and suppression of sleep to coordinate motivated processes with wakefulness.

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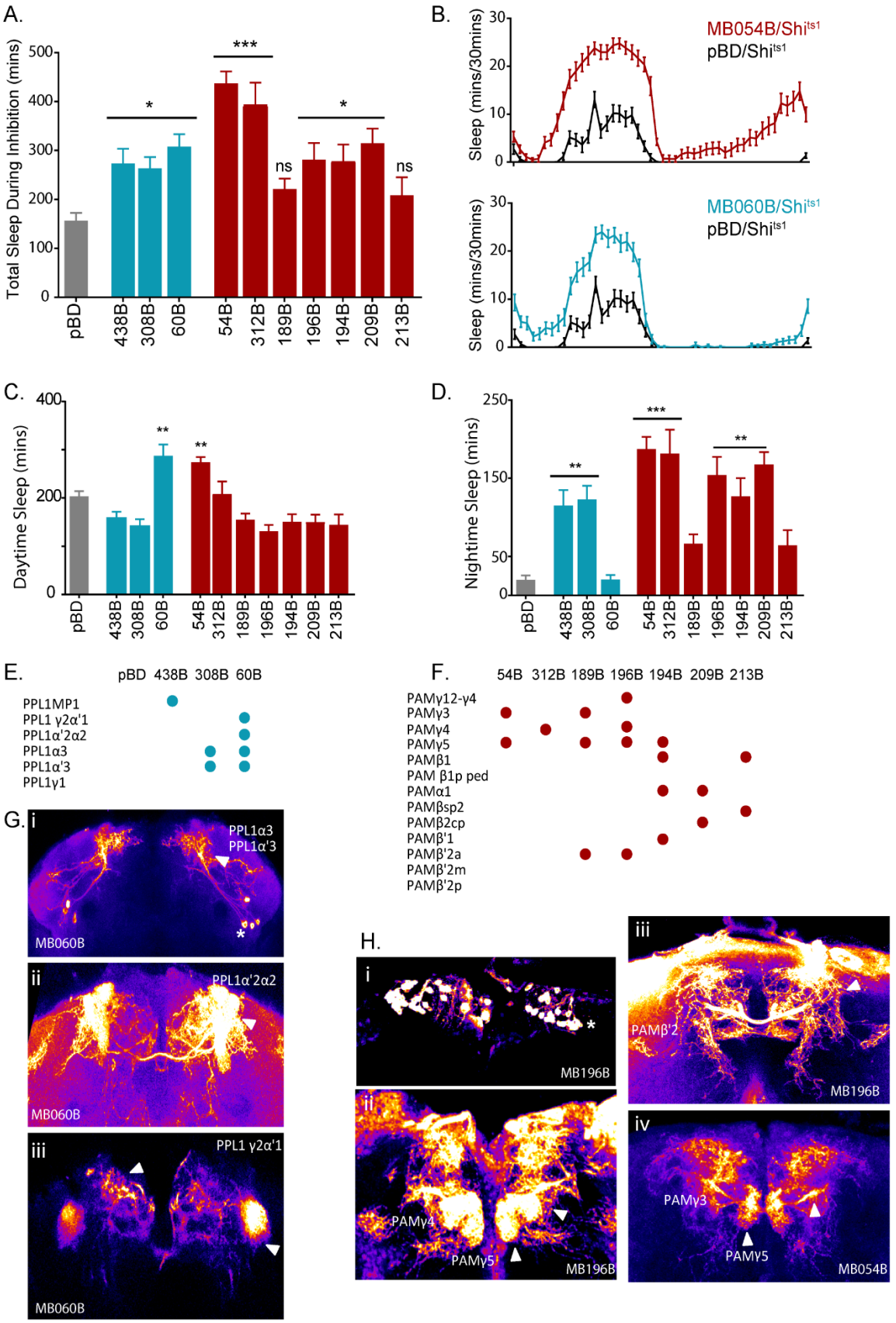
A.
Total Sleep During Inhibition (mins)
500
400
300
200
100
0
*
***
*
ns
ns
pBD
438B
308B
60B
54B
312B
189B
196B
194B
209B
213B
B.
Sleep (mins/30mins)
30
20
10
0
MB054B/Shi^{ts1}
pBD/Shi^{ts1}
MB060B/Shi^{ts1}
pBD/Shi^{ts1}
C.
Daytime Sleep (mins)
400
200
0
**
**
D.
Nightime Sleep (mins)
250
150
50
0
**
***
**
E.
pBD 438B 308B 60B
PPL1MP1
PPL1 γ2α'1
PPL1α'2α2
PPL1α3
PPL1α'3
PPL1γ1
F.
54B 312B 189B 196B 194B 209B 213B
PAMγ12-γ4
PAMγ3
PAMγ4
PAMγ5
PAMβ1
PAM β1p ped
PAMα1
PAMβsp2
PAMβ2cp
PAMβ'1
PAMβ'2a
PAMβ'2m
PAMβ'2p
G.
i
PPL1α3
PPL1α'3
MB060B
ii
PPL1α'2α2
MB060B
iii
PPL1 γ2α'1
MB060B
H.
i
MB196B
ii
PAMγ4
PAMγ5
MB196B
iii
PAMβ'2
MB196B
iv
PAMγ3
PAMγ5
MB054B

Figure 1: Wakefulness requires MB dopamine neurons. (A) Inhibition of specific sleep-regulating MB-DANs increases total sleep during a 24-hour period in flies with increased wakefulness. Sleep was measured using the Drosophila activity monitoring system in tubes containing sleep-suppressing agent carbamazepine at 29 degrees. Neural activity was suppressed by over-expressing temperature sensitive dominant negative dynamin mutation, Shi^{ts1} (B) Representative sleep profile of flies with targeted inhibition of specific PAM (MB054B, red, top panel) and PPL1 (MB060B, blue, bottom panel) neurons with empty-GAL4 control (pBD, black). (C) Daytime sleep during the first 12-hour period during inhibition of specific PPL1 (blue), PAM (red) neurons as compared to empty-GAL4 control (pBD, grey). (D) Nighttime sleep during inhibition of specific PPL1 (blue), PAM (red) neurons as compared to empty-GAL4 control (pBD, grey). (E and F) MB lobe specific projections of the dopamine drivers used in the experiments. (G, i-iii) GFP expression in sleep-regulating PPL1 dopamine neurons showing MB innervation, asterisks indicate cell bodies and arrowheads represent lob specific projections. (H, i-iv) GFP expression in sleep-regulating PAM dopamine neurons showing MB innervation, asterisks indicate cell bodies and arrowheads represent lobe specific projections.

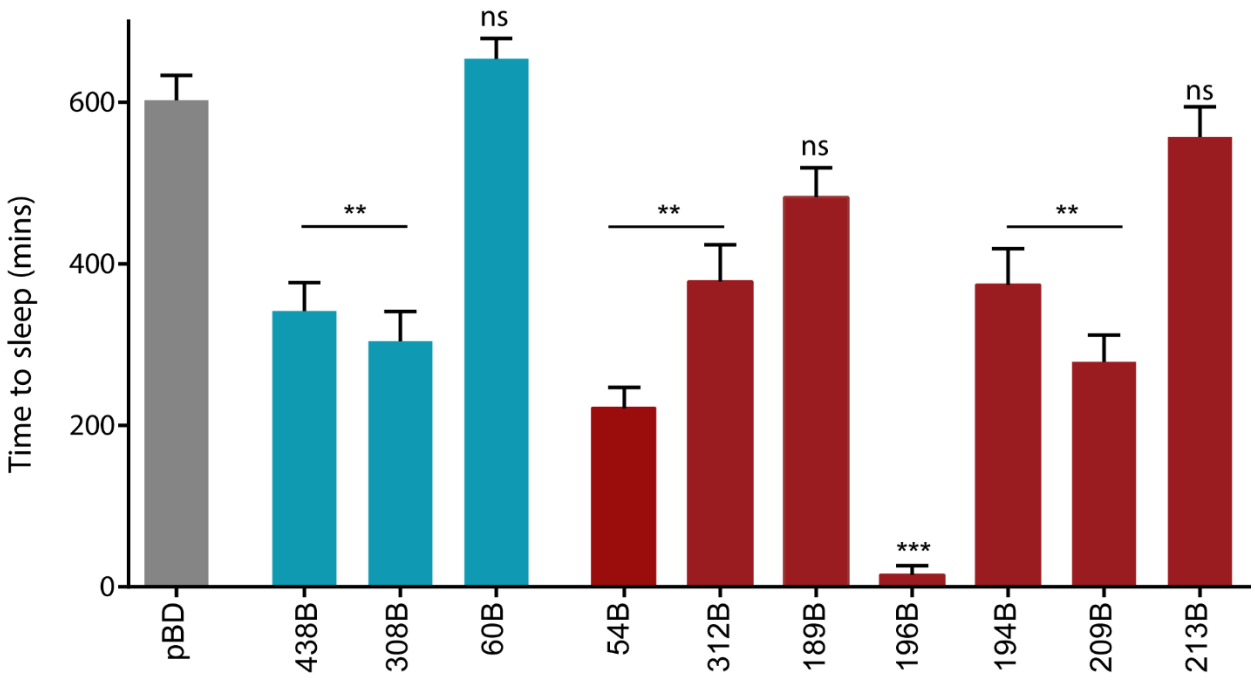

Figure S1: PAM and PPL1 dopamine inhibition decreases sleep-latency. (A) Sleep latency or time to sleep (minutes) was calculated as the time gap between lights off and initiation of the first sleep bout. Blocking dopamine release from specific PAM and PPL1 neurons driven by MB438B, MB308B, MB054B, MB312B, MB196B, MB194B and MB 209B-GAL4s reduces latency and sleep initiation post lights off at ZT 12. Sleep latency data was analyzed using Kruskal Wallis one-way ANOVA followed by post-hoc Dunns test, ** indicates P<0.01 and *** indicates P<0.0001.

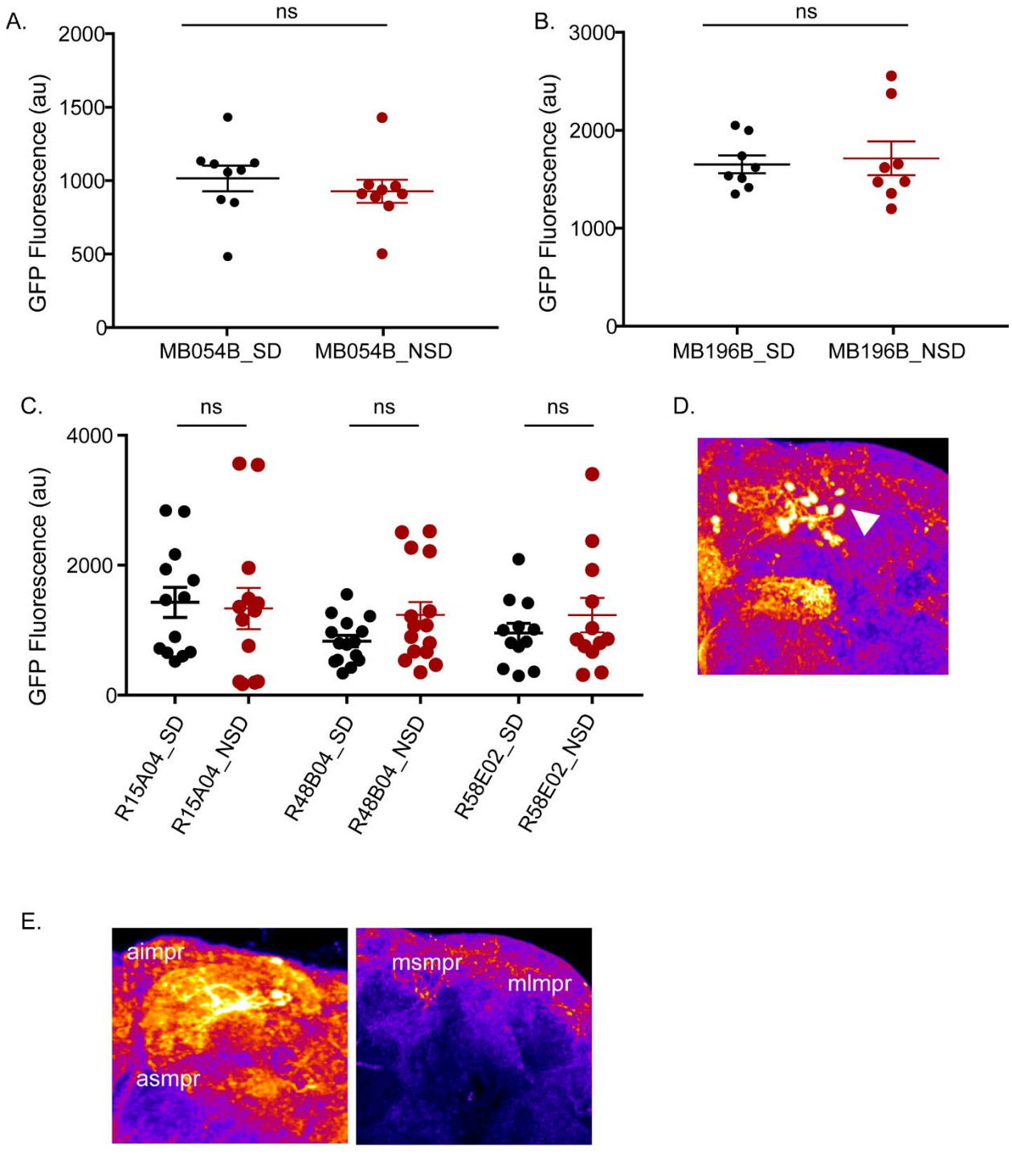
A.
ns
GFP Fluorescence (au)
2000
1500
1000
500
0
MB054B_SD
MB054B_NSD
B.
ns
GFP Fluorescence (au)
3000
2000
1000
0
MB196B_SD
MB196B_NSD
C.
ns
ns
ns
GFP Fluorescence (au)
4000
2000
0
R15A04_SD
R15A04_NSD
R48B04_SD
R48B04_NSD
R58E02_SD
R58E02_NSD
D.
E.
aimpr
asmpr
msmpr
mlmpr

Figure 2: Sleep need resulting from sleep deprivation does not alter activity and intracellular Ca^{2+} in wake-inducing PAM neurons. (A) MB054B-GAL4 expressing CaLexA brains were co-stained with antibodies against GFP and nc 82 between ZT 0-2 in flies that were sleep-deprived (for 16 hours) or sleep replete. CalexA driven GFP+ fluorescence signal was quantified and compared between sleep-deprived and sleep replete controls. (B) MB 196B-Gal4, a broad PAM driver expressing CaLexA was used and, CalexA driven GFP+ fluorescence signal was quantified and compared between sleep-deprived and sleep replete controls. (C) Broader PAM-GAL4 drivers, 15A04-GAL4, 48B04-GAL4 and 58E02-GAL4 lines were used to drive CaLexA expression and GFP fluorescence was compared between sleep-deprived and sleep-replete states in fly brains between ZT 0-2. (D and E) CaLexA induced GFP expression in 58E02-GAL4 in sleep-replete animals is shown to indicate region of interest including PAM cell bodies, msmpr, mlmpr, aimpr and asmpr regions in the protocerebral area (Aimpr, anterior inferiormedial protocerebrum; asmpr, anterior superiormedial protocerebrum; mslpr, middle superiorlateral protocerebrum; and msmpr, middle superiormedial protocerebrum). Average fluorescence intensity of sleep-deprived and sleep-replete and flies was compared using Mann-Whitney U test.

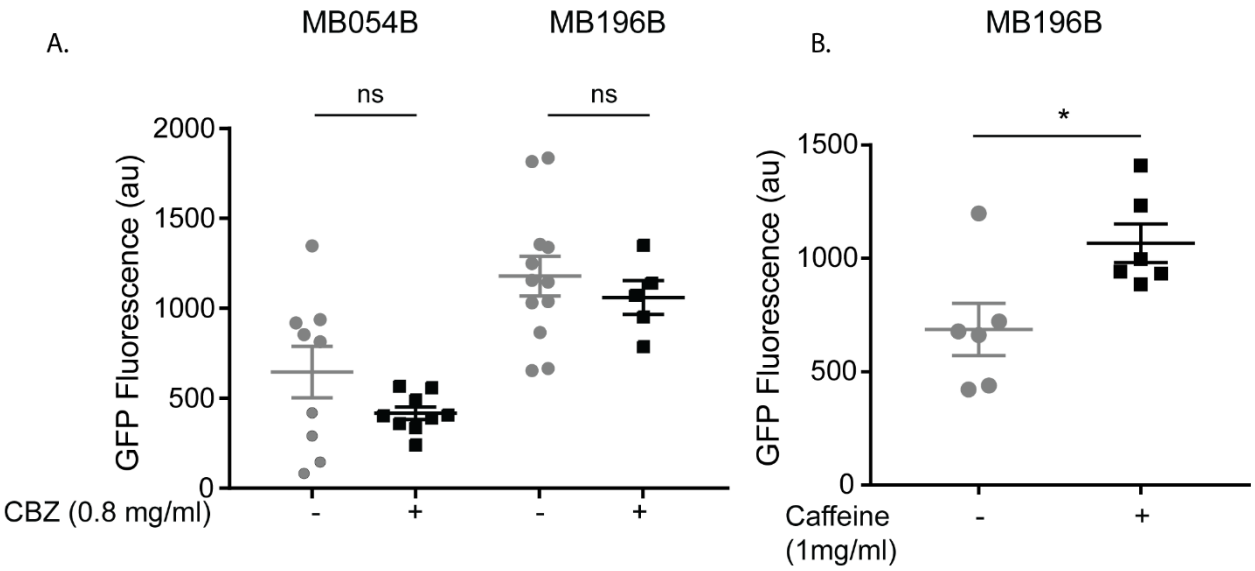

Figure S2: Carbamazepine exposure does not change activity of PAM dopamine neurons. (A) MB196-GAL4 expressing CaLexA brains were co-stained with antibodies against GFP and nc 82 between ZT 0-2 in flies that were treated with carbamazepine, CBZ (0.8 mg/ml). CalexA driven GFP+ fluorescence signal was quantified and compared between flies in CBZ and non-CBZ food. (B) MB196B-GAL4-CalexA driven GFP+ fluorescence signal was quantified and compared between flies in caffeine (1mg/ml) and non-caffeine containing food to test the ability of CalexA to report activity of dopamine neurons. * indicates $p<0.05$, average fluorescence intensity of drug treated and control flies was compared using Mann-Whitney U test, two tailed p value.

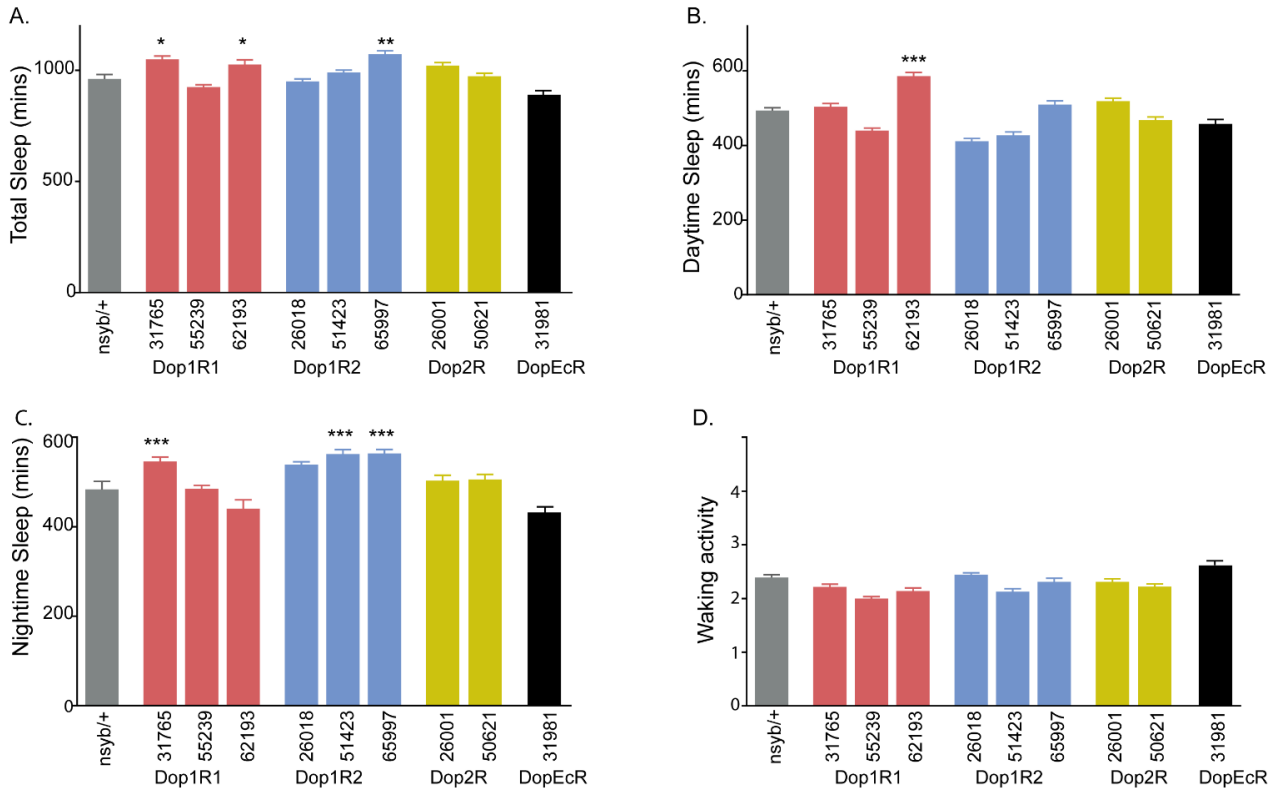
A.
Total Sleep (mins)
0
500
1000
*
*
**
nsyb/+
31765
55239
62193
26018
51423
65997
26001
50621
31981
Dop1R1
Dop1R2
Dop2R
DopEcR
B.
Daytime Sleep (mins)
0
200
400
600
***
nsyb/+
31765
55239
62193
26018
51423
65997
26001
50621
31981
Dop1R1
Dop1R2
Dop2R
DopEcR
C.
Nightime Sleep (mins)
0
200
400
600
***
***
***
nsyb/+
31765
55239
62193
26018
51423
65997
26001
50621
31981
Dop1R1
Dop1R2
Dop2R
DopEcR
D.
Waking activity
0
1
2
3
4
nsyb/+
31765
55239
62193
26018
51423
65997
26001
50621
31981
Dop1R1
Dop1R2
Dop2R
DopEcR

Figure 3: Pan-neuronal knockdown of dopamine receptors by validated RNAi lines increases sleep amount without altering walking activity during wake phase. (A) UAS-RNAi targeting Dop1R1 (3 lines), Dop1R2 (3), Dop2R (2) and DopEcR (1) were expressed using nsyb-GAL4 and assayed for changes in total sleep. (B and C) Daytime sleep (1st, 12-hour bin) and Nightime sleep (2nd, 12-hour bin) during the 24-hour period was measured for flies expressing the UAS-RNAi lines targeting the four dopamine receptors. (D) Waking activity was calculated as number of beam counts when the fly is awake. All UAS-RNAi flies had insertions on the same chromosomal site (attP2) and were injected in the same genetic background.

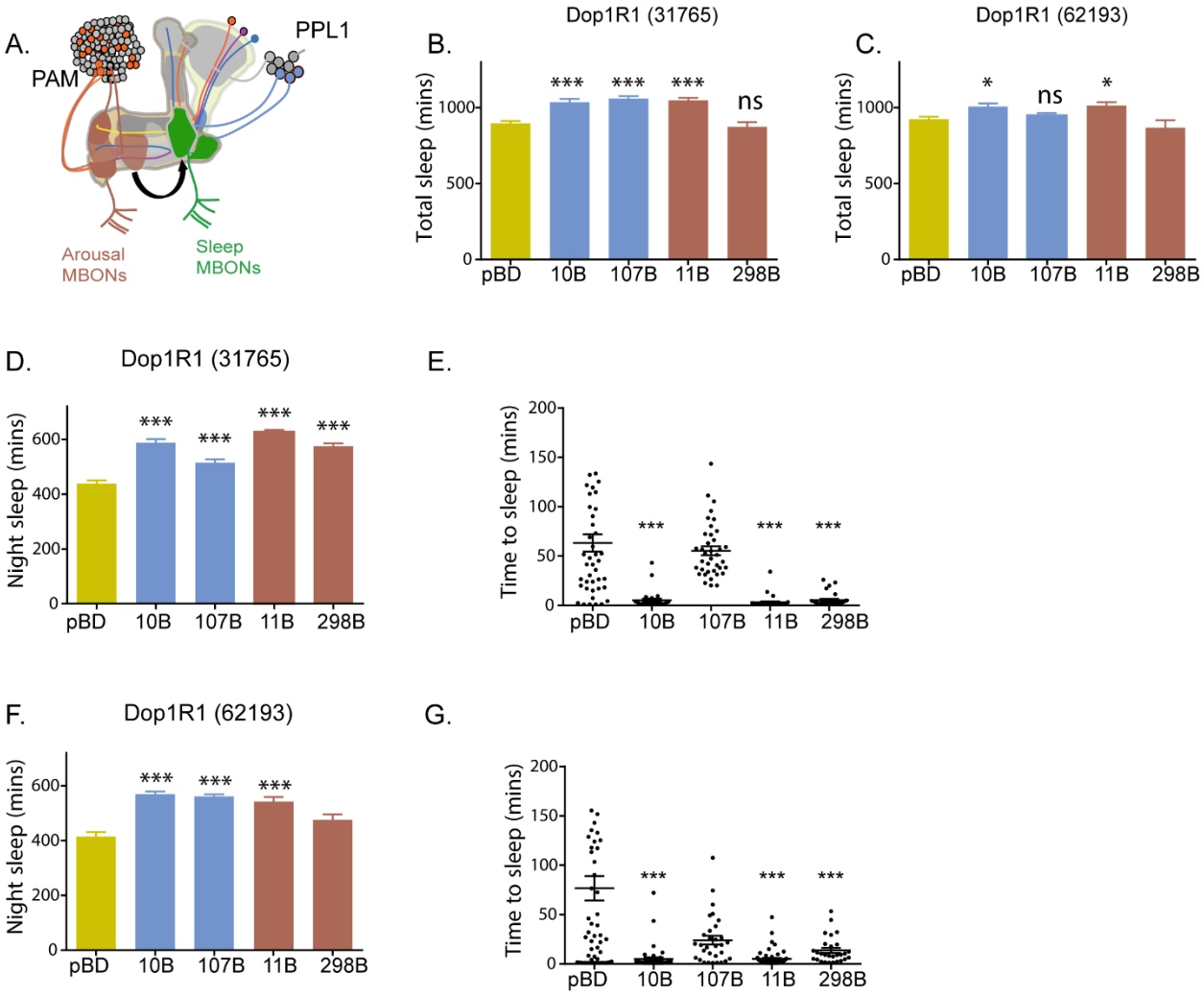
A.
PAM
PPL1
Arousal MBONs
Sleep MBONs
B.
Dop1R1 (31765)
Total sleep (mins)
0
500
1000
***
***
***
ns
pBD
10B
107B
11B
298B
C.
Dop1R1 (62193)
Total sleep (mins)
0
500
1000
*
ns
*
pBD
10B
107B
11B
298B
D.
Dop1R1 (31765)
Night sleep (mins)
0
200
400
600
***
***
***
***
pBD
10B
107B
11B
298B
E.
Time to sleep (mins)
0
50
100
150
200
***
***
***
pBD
10B
107B
11B
298B
F.
Dop1R1 (62193)
Night sleep (mins)
0
200
400
600
***
***
***
pBD
10B
107B
11B
298B
G.
Time to sleep (mins)
0
50
100
150
200
***
***
***
pBD
10B
107B
11B
298B

Figure 4: Knockdown of Dop1R1 in sleep-regulating MB-KCs and MBONs increased sleep and reduced latency. (A) Schematic of MB lobes with PAM and PPL1 innervations. (B) Total Sleep during a 24-hour period in flies expressing UAS-Dop1R1 RNAi (31765) in MB-KCs (MB010B, MB107B), MBONS (MB011B, MB 298B) and empty-GAL4 (pBD). (C) Total Sleep during a 24-hour period in flies expressing UAS-Dop1R1 RNAi (62193) in MB-KCs (MB010B, MB107B), MBONS (MB011B, MB 298B) and empty-GAL4 (pBD). (D, F) Nighttime Sleep in flies expressing UAS-Dop1R1 RNAi (31765, 62193) in MB-KCs (MB010B, MB107B), MBONS (MB-11B, MB 298B) and empty-GAL4 (pBD). (E, G) Sleep latency or time to sleep (minutes) was calculated as the time gap between lights off and initiation of the first sleep bout for flies expressing UAS-Dop1R1 RNAi (31765, 62193) in MB-KCs (MB010B, MB107B), MBONS (MB-11B, MB 298B) and empty-GAL4 (pBD).

Dop1R1 (65997)

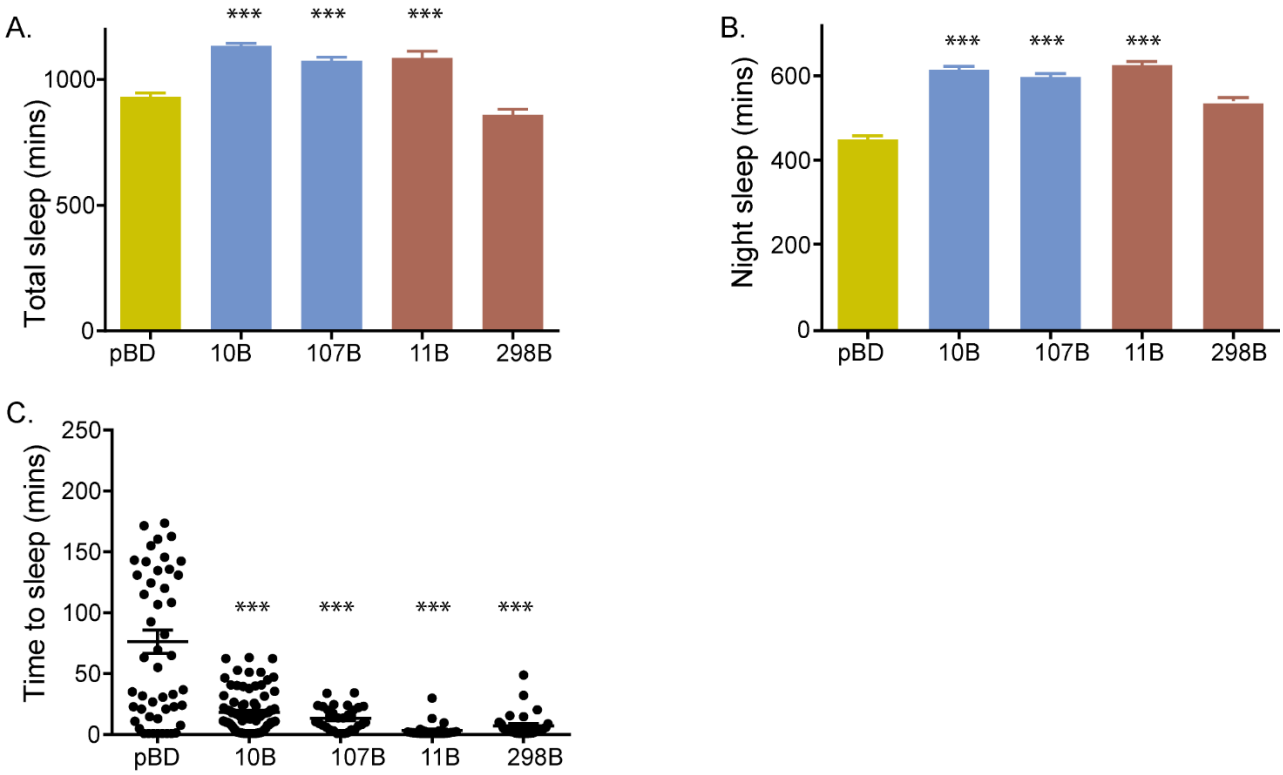

Figure 5: Knockdown of Dop1R2 in sleep-regulating MB-KCs and MBONs increased sleep and reduced latency. (A) Total Sleep during a 24-hour period in flies expressing UAS-Dop1R2 RNAi (65997) in MB-KCs (MB010B, MB107B), MBONS (MB-11B, MB 298B) and empty-GAL4 (pBD) (B) Nighttime Sleep in flies expressing UAS-Dop1R2 RNAi (65997) in MB-KCs (MB010B, MB107B), MBONS (MB-11B, MB 298B) and empty-GAL4 (pBD). (C) Sleep latency or time to sleep (minutes) was calculated as the time gap between lights off and initiation of the first sleep bout for flies expressing UAS-Dop1R2 RNAi (65997) in MB-KCs (MB010B, MB107B), MBONS (MB-11B, MB 298B) and empty-GAL4 (pBD).

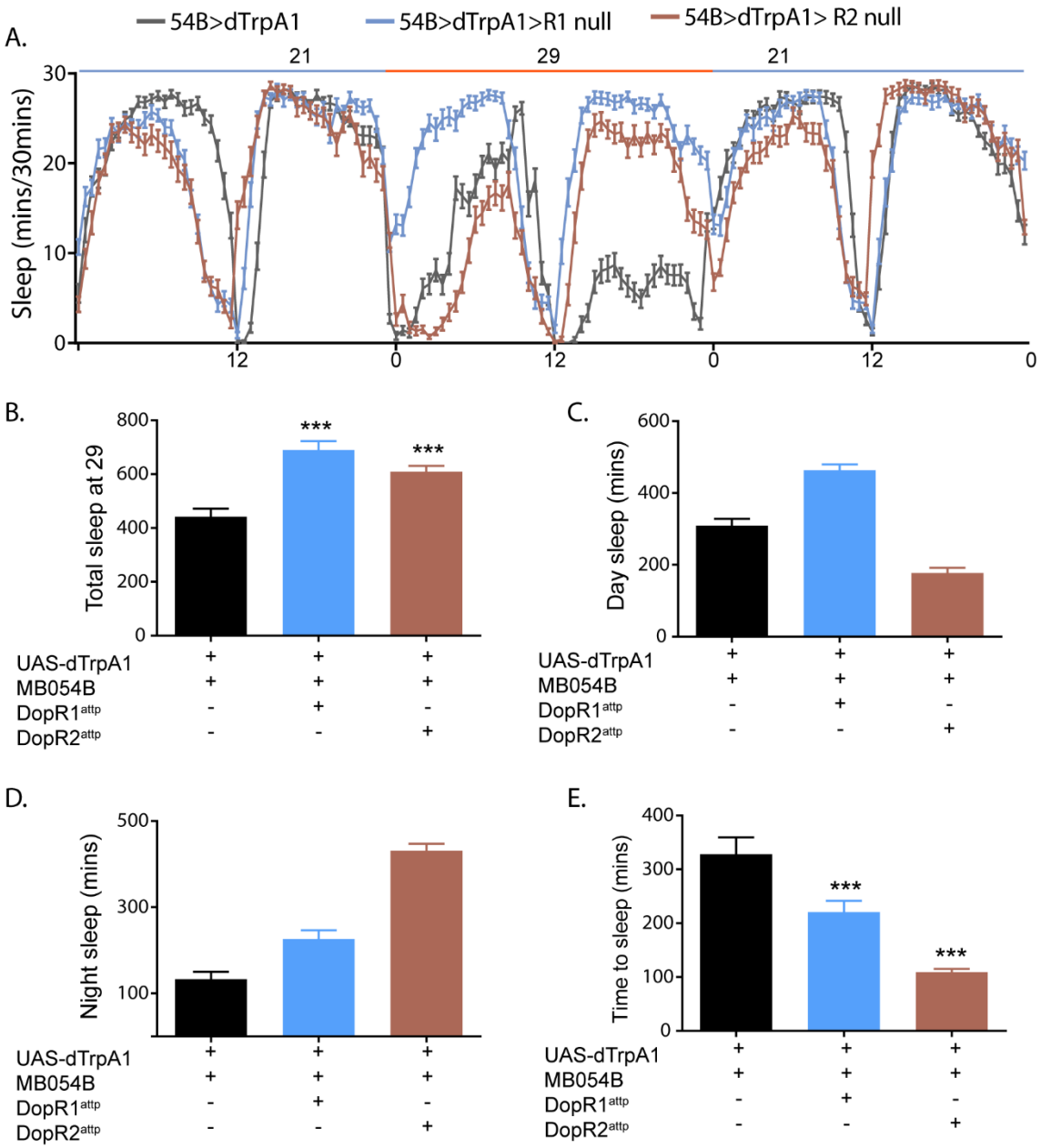
A.
54B>dTrpA1
54B>dTrpA1>R1 null
54B>dTrpA1> R2 null
21
29
21
Sleep (mins/30mins)
30
20
10
0
12
0
12
0
12
0
B.
Total sleep at 29
800
600
400
200
0
***
***
UAS-dTrpA1 + + +
MB054B + + +
DopR1attp - + -
DopR2attp - - +
C.
Day sleep (mins)
600
400
200
0
UAS-dTrpA1 + + +
MB054B + + +
DopR1attp - + -
DopR2attp - - +
D.
Night sleep (mins)
500
300
100
UAS-dTrpA1 + + +
MB054B + + +
DopR1attp - + -
DopR2attp - - +
E.
Time to sleep (mins)
400
300
200
100
0
***
***
UAS-dTrpA1 + + +
MB054B + + +
DopR1attp - + -
DopR2attp - - +

Figure 6: Wakefulness and arousal induced by PAM γ5 dopamine activation is suppressed by Dop1R1 and Dop1R2 mutations. (A) 3-day Sleep profile of flies expressing temperature sensitive cation channel dTrpA1 in PAM γ5 dopamine neurons (MB054B) in the wild type, Dop1R1 and Dop1R2 null backgrounds. Day 1 represents baseline at 21°C, Day 2 represent activation at 29°C and Day 3 represents recovery at 21°C. (B)Total sleep during Day 2 (activation, 29°C) of flies expressing dTrpA1 in PAM g5 in control and receptor null background ($DopR1^{attp}$ and $DopR2^{attp}$). (C) Daytime sleep during first 12 hours of Day 2 (activation, 29°C) for flies expressing dTrpA1 in PAM γ5 in control and receptor null background ($DopR1^{attp}$ and $DopR2^{attp}$). (D) Nighttime sleep on Day 2 (activation, 29°C) for flies expressing dTrpA1 in PAM γ5 in control and receptor null background ($DopR1^{attp}$ and $DopR2^{attp}$). (E) Sleep latency or time to sleep (minutes) was calculated as the time gap between lights off and initiation of the first sleep bout for flies expressing dTrpA1 in PAM γ5 in control and receptor null background ($DopR1^{attp}$ and $DopR2^{attp}$).

References

1. Borbely AA. A two process model of sleep regulation. *Hum Neurobiol.* 1982;1(3):195-204.
2. Borbely AA, Daan S, Wirz-Justice A, Deboer T. The two-process model of sleep regulation: a reappraisal. *J Sleep Res.* 2016;25(2):131-143.
3. Eban-Rothschild A, Appelbaum L, de Lecea L. Neuronal Mechanisms for Sleep/Wake Regulation and Modulatory Drive. *Neuropsychopharmacology.* 2018;43(5):937-952.
4. Eban-Rothschild A, Giardino WJ, de Lecea L. To sleep or not to sleep: neuronal and ecological insights. *Curr Opin Neurobiol.* 2017;44:132-138.
5. Bargmann CI. Beyond the connectome: how neuromodulators shape neural circuits. *Bioessays.* 2012;34(6):458-465.
6. Marder E. Neuromodulation of neuronal circuits: back to the future. *Neuron.* 2012;76(1):1-11.
7. Oishi Y, Lazarus M. The control of sleep and wakefulness by mesolimbic dopamine systems. *Neurosci Res.* 2017;118:66-73.
8. Eban-Rothschild A, Rothschild G, Giardino WJ, Jones JR, de Lecea L. VTA dopaminergic neurons regulate ethologically relevant sleep-wake behaviors. *Nat Neurosci.* 2016;19(10):1356-1366.
9. Dzirasa K, Ribeiro S, Costa R, et al. Dopaminergic control of sleep-wake states. *J Neurosci.* 2006;26(41):10577-10589.
10. Ueno T, Tomita J, Tanimoto H, et al. Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. *Nat Neurosci.* 2012;15(11):1516-1523.
11. Liu Q, Liu S, Kodama L, Driscoll MR, Wu MN. Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. *Curr Biol.* 2012;22(22):2114-2123.
12. Kume K, Kume S, Park SK, Hirsh J, Jackson FR. Dopamine is a regulator of arousal in the fruit fly. *J Neurosci.* 2005;25(32):7377-7384.
13. Donlea JM, Pimentel D, Talbot CB, et al. Recurrent Circuitry for Balancing Sleep Need and Sleep. *Neuron.* 2018;97(2):378-389 e374.
14. Potdar S, Sheeba V. Wakefulness Is Promoted during Day Time by PDFR Signalling to Dopaminergic Neurons in Drosophila melanogaster. *eNeuro.* 2018;5(4).
15. Deng B, Li Q, Liu X, et al. Chemoconnectomics: Mapping Chemical Transmission in Drosophila. *Neuron.* 2019;101(5):876-893 e874.
16. Pimentel D, Donlea JM, Talbot CB, Song SM, Thurston AJF, Miesenbock G. Operation of a homeostatic sleep switch. *Nature.* 2016;536(7616):333-337.
17. Donlea JM, Pimentel D, Miesenbock G. Neuronal Machinery of Sleep Homeostasis in Drosophila. *Neuron.* 2014;81(6):1442.
18. Sitaraman D, Aso Y, Jin X, et al. Propagation of Homeostatic Sleep Signals by Segregated Synaptic Microcircuits of the Drosophila Mushroom Body. *Curr Biol.* 2015;25(22):2915-2927.
19. Sitaraman D, Aso Y, Rubin GM, Nitabach MN. Control of Sleep by Dopaminergic Inputs to the Drosophila Mushroom Body. *Front Neural Circuits.* 2015;9:73.
20. Cohn R, Morantte I, Ruta V. Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in Drosophila. *Cell.* 2015;163(7):1742-1755.
21. Handler A, Graham TGW, Cohn R, et al. Distinct Dopamine Receptor Pathways Underlie the Temporal Sensitivity of Associative Learning. *Cell.* 2019;178(1):60-75 e19.
22. Waddell S. Neural Plasticity: Dopamine Tunes the Mushroom Body Output Network. *Curr Biol.* 2016;26(3):R109-112.
23. Aso Y, Hattori D, Yu Y, et al. The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife.* 2014;3:e04577.

24. Aso Y, Rubin GM. Dopaminergic neurons write and update memories with cell-type-specific rules. *Elife.* 2016;5.
25. Aso Y, Sitaraman D, Ichinose T, et al. Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *Elife.* 2014;3:e04580.
26. Keleman K, Vrontou E, Kruttner S, Yu JY, Kurtovic-Kozaric A, Dickson BJ. Dopamine neurons modulate pheromone responses in Drosophila courtship learning. *Nature.* 2012;489(7414):145-149.
27. Masuyama K, Zhang Y, Rao Y, Wang JW. Mapping neural circuits with activity-dependent nuclear import of a transcription factor. *J Neurogenet.* 2012;26(1):89-102.
28. Agosto J, Choi JC, Parisky KM, Stilwell G, Rosbash M, Griffith LC. Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. *Nat Neurosci.* 2008;11(3):354-359.
29. Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in Drosophila melanogaster. *Science.* 2000;287(5459):1834-1837.
30. Hendricks JC, Finn SM, Panckeri KA, et al. Rest in Drosophila is a sleep-like state. *Neuron.* 2000;25(1):129-138.
31. Granger P, Biton B, Faure C, et al. Modulation of the gamma-aminobutyric acid type A receptor by the antiepileptic drugs carbamazepine and phenytoin. *Mol Pharmacol.* 1995;47(6):1189-1196.
32. Kim M, Jang D, Yoo E, et al. Rogdi Defines GABAergic Control of a Wake-promoting Dopaminergic Pathway to Sustain Sleep in Drosophila. *Sci Rep.* 2017;7(1):11368.
33. Kayser MS, Yue Z, Sehgal A. A critical period of sleep for development of courtship circuitry and behavior in Drosophila. *Science.* 2014;344(6181):269-274.
34. Liu S, Liu Q, Tabuchi M, Wu MN. Sleep Drive Is Encoded by Neural Plastic Changes in a Dedicated Circuit. *Cell.* 2016;165(6):1347-1360.
35. Nall AH, Shakhmantsir I, Cichewicz K, Birman S, Hirsh J, Sehgal A. Caffeine promotes wakefulness via dopamine signaling in Drosophila. *Sci Rep.* 2016;6:20938.
36. Croset V, Treiber CD, Waddell S. Cellular diversity in the Drosophila midbrain revealed by single-cell transcriptomics. *Elife.* 2018;7.
37. Berry JA, Cervantes-Sandoval I, Nicholas EP, Davis RL. Dopamine is required for learning and forgetting in Drosophila. *Neuron.* 2012;74(3):530-542.
38. Himmelreich S, Masuho I, Berry JA, et al. Dopamine Receptor DAMB Signals via Gq to Mediate Forgetting in Drosophila. *Cell Rep.* 2017;21(8):2074-2081.
39. Kim YC, Lee HG, Han KA. D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. *J Neurosci.* 2007;27(29):7640-7647.
40. Pavlowsky A, Schor J, Placais PY, Preat T. A GABAergic Feedback Shapes Dopaminergic Input on the Drosophila Mushroom Body to Promote Appetitive Long-Term Memory. *Curr Biol.* 2018;28(11):1783-1793 e1784.
41. Tsao CH, Chen CC, Lin CH, Yang HY, Lin S. Drosophila mushroom bodies integrate hunger and satiety signals to control innate food-seeking behavior. *Elife.* 2018;7.
42. Hattori D, Aso Y, Swartz KJ, Rubin GM, Abbott LF, Axel R. Representations of Novelty and Familiarity in a Mushroom Body Compartment. *Cell.* 2017;169(5):956-969 e917.
43. Ichinose T, Aso Y, Yamagata N, Abe A, Rubin GM, Tanimoto H. Reward signal in a recurrent circuit drives appetitive long-term memory formation. *Elife.* 2015;4:e10719.
44. Sun J, Xu AQ, Giraud J, et al. Neural Control of Startle-Induced Locomotion by the Mushroom Bodies and Associated Neurons in Drosophila. *Front Syst Neurosci.* 2018;12:6.
45. Hamada FN, Rosenzweig M, Kang K, et al. An internal thermal sensor controlling temperature preference in Drosophila. *Nature.* 2008;454(7201):217-220.

46. Dubnau J. Neuroscience. Ode to the mushroom bodies. *Science.* 2012;335(6069):664-665.
47. Brembs B. Mushroom bodies regulate habit formation in Drosophila. *Curr Biol.* 2009;19(16):1351-1355.
48. Xi W, Peng Y, Guo J, et al. Mushroom bodies modulate salience-based selective fixation behavior in Drosophila. *Eur J Neurosci.* 2008;27(6):1441-1451.
49. Gronenberg W, Lopez-Riquelme GO. Multisensory convergence in the mushroom bodies of ants and bees. *Acta Biol Hung.* 2004;55(1-4):31-37.
50. Zars T. Behavioral functions of the insect mushroom bodies. *Curr Opin Neurobiol.* 2000;10(6):790-795.
51. Liu L, Wolf R, Ernst R, Heisenberg M. Context generalization in Drosophila visual learning requires the mushroom bodies. *Nature.* 1999;400(6746):753-756.
52. Davis RL. Mushroom bodies and Drosophila learning. *Neuron.* 1993;11(1):1-14.
53. Heisenberg M. Mushroom body memoir: from maps to models. *Nat Rev Neurosci.* 2003;4(4):266-275.
54. Knapek S, Kahsai L, Winther AM, Tanimoto H, Nassel DR. Short neuropeptide F acts as a functional neuromodulator for olfactory memory in Kenyon cells of Drosophila mushroom bodies. *J Neurosci.* 2013;33(12):5340-5345.
55. Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J. Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in Drosophila. *Curr Biol.* 2012;22(7):608-614.
56. Otto N, Pleijzier MW, Morgan IC, et al. Input connectivity reveals additional heterogeneity of dopaminergic reinforcement in Drosophila. *bioRxiv.* 2020:2020.2002.2019.952648.