A single pair of neurons links sleep to memory consolidation in *Drosophila melanogaster*

Paula R. Haynes¹, Bethany L. Christmann¹ and Leslie C. Griffith*

Department of Biology, Volen National Center for Complex Systems and National Center for Behavioral Genomics, Brandeis University, Waltham, MA 02454-9110

¹These authors contributed equally to this work

*Corresponding author:

Leslie C. Griffith
Dept. of Biology MS008
Brandeis University
415 South St.
Waltham, MA 02454-9110

Tel: 781 736 3125
FAX: 781 736 3107
Email: griffith@brandeis.edu
Abstract

Sleep promotes memory consolidation in humans and many other species, but the physiological and anatomical relationships between sleep and memory remain unclear. Here we show the dorsal paired medial (DPM) neurons, which are required for memory consolidation in Drosophila, are sleep-promoting inhibitory neurons. DPMs increase sleep via release of GABA onto wake-promoting mushroom body (MB) $\alpha'/\beta'$ neurons. Functional imaging demonstrates that DPM activation evokes robust increases in chloride in MB neurons, but is unable to cause detectable increases in calcium or cAMP. Downregulation of $\alpha'/\beta'$ GABA$_A$ and GABA$_B$R3 receptors results in sleep loss, suggesting these receptors are the sleep-relevant targets of DPM-mediated inhibition. Regulation of sleep by neurons necessary for consolidation suggests that these brain processes may be functionally interrelated via their shared anatomy. These findings have important implications for the mechanistic relationship between sleep and memory consolidation, arguing for a significant role of inhibitory neurotransmission in regulating these processes.

These results argue for a significant role of inhibitory neurotransmission in memory consolidation and its regulation by sleep.

Introduction

Accumulating evidence suggests that sleep plays a role in promoting the consolidation of memory (Stickgold, 2005; Diekelmann and Born, 2010; Mednick et al., 2011; Abel et al., 2013; Rasch and Born, 2013). Sleep deprivation following an associative learning task impairs consolidated memory in Drosophila, rodents and humans whereas sleep immediately after a learning task actually improves consolidated memory across the same broad range of organisms (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2011; Rasch and Born, 2013; Diekelmann, 2014). It is not, however, clear exactly how sleep promotes memory consolidation: it may simply be a permissive state generated by other brain regions that prevents sensory interference with memory circuits, or alternatively the memory circuitry itself may actively participate in sleep promotion as an integral aspect of the consolidation process. To begin to probe these issues, we have investigated the role of
the dorsal paired medial (DPM) neurons, which are critical to memory consolidation in
*Drosophila melanogaster*, in the regulation of sleep.

The *Drosophila* learning and memory circuitry has been well characterized and
provides an excellent system in which to study cellular interactions between sleep and
memory consolidation. The mushroom bodies (MBs) are a set of ca. 5000 neurons in the
*Drosophila* brain, organized into 5 distinct lobular neuropils, which are required for odor
memory acquisition, consolidation and retrieval. Although the anatomy involved in
memory consolidation in mammals is highly complex and distributed, in the fly it is quite
compact: the DPM neurons, a single pair of neurons innervating all of the MB lobes, are the
mediators of consolidation for odor memories (Waddell, 2000; Keene et al., 2004; Yu et al.,
2005; Keene et al., 2006; Krashes and Waddell, 2008). Like mammals, *Drosophila*
consolidate memories at the systems level. Critical memory information is transferred from
short-term storage in neurons required for initial acquisition to anatomically and
physiologically distinct long-term storage sites (Yu et al., 2005; Krashes et al., 2007; Wang
et al., 2008; Cervantes-Sandoval et al., 2013; Dubnau and Chiang, 2013). DPM neurons,
along with the α’/β’ subset of MB neurons, are required for early phases of this memory
information transfer (Keene et al., 2004; Keene et al., 2006; Krashes et al., 2007; Krashes
and Waddell, 2008).

The MB memory circuit has also been implicated in the regulation of sleep by a
number of studies (Joiner et al., 2006; Pitman et al., 2006; Yuan et al., 2006; Yi et al., 2013).
Loss of MB 5HT_{1A} receptors (Yuan et al., 2006) as well as alterations in MB PKA activity
(Joiner et al., 2006) and neurotransmitter release (Pitman et al., 2006) have been shown to
affect sleep in *Drosophila* in a lobe-specific manner. Mutation of the *amnesiac (amn)* gene,
which encodes a putative neuropeptide expressed in DPM neurons (Waddell, 2000), results
in fragmented sleep and impaired sleep rebound following deprivation, suggesting a role
for these cells (Liu et al., 2008). While the molecular and cellular requirements for sleep
and memory clearly overlap, whether the circuit that regulates sleep is identical to that
required for memory is not clear and this is a question that bears directly on the functional
interrelationship between sleep and memory consolidation.

The primary question addressed in this study is the role of the DPM neurons and
their outputs in regulation of sleep. The DPM contribution to memory consolidation had
been suggested to occur due to the release of acetylcholine (ACh) (Keene et al., 2004) and the product of the amn gene (Waddell, 2000) enhancing MB potentiation via an excitatory feedback loop (Yu et al., 2005; Keene and Waddell, 2007) similar to what has been proposed to occur in the mammalian hippocampus (Hebb, 1949; Hopfield, 1982; Amit, 1989; Treves and Rolls, 1994; Battaglia and Treves, 1998; Lisman, 1999). Recently, however, DPM release of serotonin (5HT) has been shown to promote anesthesia resistant memory, a form of consolidated memory, by acting on G\(\alpha_5\)-coupled 5HT\(_{1A}\) receptors in the \(\alpha/\beta\) lobes of the MBs (Lee et al., 2011). The involvement of a potentially inhibitory receptor, 5HT\(_{1A}\), in consolidation suggests that a simple positive feedback model for consolidation is unlikely to be completely correct, and it highlights the fact that there is currently no information on the functional nature of the synapses between DPM neurons and MBs. An understanding of this synapse is critical for elucidating DPM’s role in sleep. To address this aspect of DPM function, we have investigated the nature of their connection to the MBs.

Here we show that the DPM neurons promote sleep via the release of 5HT and the inhibitory neurotransmitter GABA. We find that DPM activation results in inhibitory chloride influx into post-synaptic MB neurons and find no evidence that DPM neuron activation has an excitatory effect on post-synaptic MB neurons. We suggest a model in which post-synaptic MB \(\alpha'/\beta'\) neurons are wake-promoting and inhibition by DPM neuron GABA and 5HT release during memory consolidation results in increased sleep. These findings provide new insight into the functional relationship between sleep and memory consolidation, and suggest an important role for inhibitory neurotransmission in regulating these processes.

Results

DPM activity promotes sleep

In order to determine whether DPMs play a role in regulating sleep, we acutely activated these neurons by driving the warmth-sensitive cation channel, dTrpA1 (Hamada et al., 2008) with NP2712-GAL4, a driver with relatively specific and strong DPM expression
(Figure 1, supplement 1). A temperature shift at ZT0 from 22°C, a temperature at which
dTrpA1 is inactive, to 31°C, where it is open and can depolarize DPMs, produced an
immediate and dramatic increase in sleep (Figure 1A). dTrpA1 activation with a weaker,
but even more specific DPM driver line, VT64246-GAL4, also resulted in immediate and
significant increases in sleep (Figure 1, supplement 2A and C) indicating the effect is due to
activation of DPM neurons and not other neurons in the NP2721-GAL4 expression pattern.
Activating DPMs with dTrpA1 did not alter the level of locomotor activity during waking
periods (light period of first day: \( P_{\text{GAL4}} = 0.57 \), \( P_{\text{UAS}} < 0.0001 \) and for the dark period of first
day: \( P_{\text{GAL4}} = 0.5 \), \( P_{\text{UAS}} = 0.13 \)), suggesting that this treatment does not cause locomotor
impairment. Additionally, video recordings at 0-2 min and >2 h after DPM activation at 31-
32°C show that flies are immediately arousable by gentle tapping and have normal geotaxis
and locomotion, consistent with DPM activation inducing a sleep state rather than paralysis
or locomotor dysfunction (Movie 1).

Upon cessation of dTrpA1 activation, after two days of activation at 31°C, flies
showed decreased sleep. The negative sleep rebound after release is consistent with the
presence of strong compensatory homeostatic mechanisms counteracting excessive sleep
(Shang et al., 2013) and/or excessive DPM activity. Since DPM neurons are normally
activated in the first 0-3 h following training (Yu et al., 2005; Cervantes-Sandoval and Davis,
2012), it is unlikely that these neurons would ever naturally exhibit such high levels of
activity for the length of time we have imposed artificially. The immediate increase in sleep
upon dTrpA1 activation is most likely to be indicative of normal DPM function. The idea
that DPMs can act acutely is in agreement with the literature that shows there is a
temporally circumscribed window during which they are required for consolidation (Keene
et al., 2004; Yu et al., 2005; Keene et al., 2006) and with the finding that a short period (45
min) of TrpM8 activation of DPM neurons following a learning task rescues age-induced
memory impairment (Tonoki, 2012).

Since activation of DPM neurons acutely induces sleep in flies, we wanted to
determine if DPM activity also played a role in the maintenance of baseline sleep. In order
to assess this, we used the NP2721-GAL4 line to drive expression of a temperature-sensitive,
dominant negative Dynamin, Shibire* (Shi\(^*\)) to block vesicle recycling in DPMs. Following
a shift at ZT0 from the permissive temperature of 18°C, to the restrictive temperature of
31°C, flies showed a small but significant decrease in levels of nighttime sleep relative to
the baseline sleep of each genotype at 18 °C (Figure 1B1). Because the temperature shift
protocol induced changes in the baseline nighttime sleep of control flies (compare
“baseline” and “recovery” days in panel B1) we asked if the ability of DPM inhibition to
decrease nighttime sleep was independent of baseline by doing a second round of
temperature shift (Figure 1B2). We found that DPM inhibition decreased sleep regardless
of the starting baseline. We obtained a similar result using another DPM line, C316-GAL4 to
drive Shi\textsuperscript{ts} (Figure 1, supplement 3). Additionally, when the weaker, but cleaner, VT64246-GAL4
driver line was used with the temperature-sensitive repressor, Tubulin-GAL80\textsuperscript{ts}, to
produce acute expression of the hyperpolarizing potassium channel Kir2.1 in DPM neurons
nighttime sleep was also reduced (Figure 1, supplement 2B and D).

These data demonstrate that sleep loss after inhibition of DPM activity is both cell-
specific and independent of the particular method used to suppress DPM activity. The
limitation of the DPM loss-of-function phenotype to nighttime sleep implies there is a
baseline function of DPM activity that occurs even in isolated animals in a relatively
stimulus-poor environment, but the small magnitude of these changes suggests that DPMs
are not the major driver of baseline sleep. The DPM-dependent gain-of-function
experiments with dTrpA1, however, indicate that significant changes in both nighttime and
daytime sleep can be produced with acute activation of DPM neurons, as might perhaps
naturally occur secondary to some type of experience.

The DPM neurons are coupled via gap junctions to second pair of neurons
innervating the MB, the anterior paired lateral (APL) neurons (Wu et al., 2011). It was
possible that sleep gains resulting from dTrpA1-mediated activation of DPMs were not due
to neurotransmitter release from DPMs themselves, but instead were a secondary result of
gap-junction coupled APL activation and neurotransmitter release. In order to distinguish
between these possibilities we coexpressed dTrpA1 and the temperature-sensitive
Dynamin mutant, Shi\textsuperscript{ts}, in DPM neurons. Since Shi\textsuperscript{ts} protein and mRNA are unlikely to pass
through gap junctions, DPM-Shi\textsuperscript{ts} expression should prevent neurotransmitter release in a
cell-autonomous manner from DPM, but not affect APL neurons. Thus, if dTrpA1-mediated
sleep gains are the result of DPM, but not APL neurotransmitter release, they should be
blocked by the coexpression of Shi\textsuperscript{ts} in DPMs at high temperature. Conversely, if APL
neurotransmitter release is responsible for sleep gains resulting from DPM activation, DPM expression of Shil should have no effect on dTrpA1-evoked sleep. We found that coexpression of Shil completely blocked activity-induced sleep gains (Figure 1C). This was not due to dilution of GAL4-mediated expression since the coexpression of a neutral second UAS transgene (UAS-GCaMP6) did not block dTrpA1-stimulated sleep (Figure 1, supplement 4). This suggests that sleep from dTrpA1-mediated DPM activation is the result of release of neurotransmitter from DPMs, not APLs.

Thus, we find that DPMs are capable of acutely promoting sleep and have an additional role in mediating baseline sleep during the night in stimulus-poor conditions (single flies in sleep tubes). How this function of DPMs is regulated is unknown. Given their role in memory consolidation, however, it is likely DPMs are chiefly active in acute sleep regulation when they are recruited to promote sleep following stimulus-rich experiences such as learning.

**α’/β’ activity promotes wakefulness**

MB α’/β’ neurons are thought to be a key postsynaptic target of DPM neurons (Keene et al., 2006; Krashes et al., 2007; Pitman et al., 2011). Both DPM and α’/β’ activity are required during the memory consolidation period 0-3 h after training for the storage of subsequent 24 h long-term memory (Krashes and Waddell, 2008). Recently, multiple groups have posited that *Drosophila* experiences a form of systems consolidation, similar to that of mammals, in which memories are transferred from a set of neurons serving as a short-term storage site (e.g. the hippocampus in mammals and γ and α’/β’ lobes in flies) to a different set of neurons which store the memory in a more stable long-term state (e.g. the cortex in mammals, α/β lobes and MB output neurons in flies) (Cervantes-Sandoval et al., 2013; Dubnau and Chiang, 2013). Since systems consolidation in *Drosophila* requires DPM and α’/β’ activity and is known to be promoted by sleep in other organisms, we reasoned that α’/β’ activity may also play a role in promoting sleep. While it has been shown previously that the *Drosophila* MB can promote sleep (Joiner et al., 2006; Pitman et al., 2006; Yi et al., 2013), a specific role for the α’/β’ lobes has not been reported.
To address this issue, we acutely activated these neurons with an MB-restricted version of the α'/β' driver line c305a-GAL4 and the warmth-sensitive cation channel, dTrpA1. If DPM neurons act to excite α'/β', as postulated by models of consolidation, we would expect this manipulation to increase sleep. Instead, we see a strong decrease in nighttime sleep. This α'/β'-dependent nighttime sleep loss remained stable throughout 48 h of dTrpA1 activation and was accompanied by increasing daytime sleep loss which continued even after release from dTrpA1 activation (Figure 2A and C). This unusual pattern exactly matches the phenotype seen in flies expressing Shi<sup>ts</sup> in DPM neurons. Thus DPM and α'/β' activity have opposing roles in the regulation of sleep: DPM activity promotes sleep whereas α'/β' activity is wake-promoting. These data suggest that DPM neurons may inhibit MB neurons.

**DPMs contain 5HT and GABA, but not ACh or dopamine**

The shared requirement for activity during memory consolidation (Krashes et al., 2007; Krashes and Waddell, 2008) as well as the extensive physical connectivity as determined by membrane-localized GRASP (Pitman et al., 2011) strongly suggests that DPM and MB neurons are synaptically connected. Given the lack of information on the functional nature of the connections, we set out as a first step to determine what neurotransmitters are present in in DPM neurons. Colocalization of mCD8-GFP expression in DPM cell bodies with staining against a panel of neurotransmitters shows that DPM neurons contain both GABA (Figure 3A) and 5HT (Figure 3, supplement 1A). DPM cell bodies also stain positively for Gad1 (Figure 3C), the GABA synthetic enzyme. We found no evidence for expression of ChAT, the ACh synthetic enzyme (Figure 3C), or tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis (Figure 3, supplement 1B). These combined results suggest that the DPM neurons release GABA and 5HT, but not ACh or dopamine.

Although GABA release is known to be inhibitory, there are both stimulatory and inhibitory 5HT receptors in the *Drosophila* brain and it is unknown whether Gα<sub>s</sub>-coupled 5HT receptors such as 5HT7 are expressed in the MBs. To test whether 5HT could be stimulatory in the MBs, we used 5HT7-GAL4 (Becnel et al., 2011) to drive expression of
Epac1-camps (EPAC) (Nikolaev et al., 2004; Shafer et al., 2008), a FRET-based cyclic nucleotide sensor. There was no expression evident in the MBs, although there was strong fluorescence in the central complex as reported previously (Becnel et al., 2011). We bath-applied 5HT and saw increased cAMP in the labeled cells of the ellipsoid body, confirming the efficacy of the drug as well as the positive coupling to cyclase in 5HT7-GAL4+ cells (Figure 3, supplement 2). Because the lack of 5HT7-GAL4 expression in MB does not necessarily mean there is not a stimulatory 5HT receptor expressed there, we also bath-applied 5HT to brains with EPAC driven by MB247-lexA to determine if there was an excitatory response from this structure. We saw no increase in cAMP (Figure 3 supplement, 2). Because EPAC may not be not effective at reporting inhibition (e.g. if there is no basal activation of cyclase), we cannot rule out inhibitory effects of 5HT via 5HT1A, which is known to be expressed in MBs (Yuan et al., 2006; Lee et al., 2011).

DPMs project throughout the MBs and have both pre- and postsynaptic markers comingled in all lobes (Waddell, 2000; Wu et al., 2013) To investigate where DPM GABA might be released, we examined colocalization of a DPM-expressed presynaptic marker, Bruchpilot-short-GFP (Schmid et al., 2008; Fouquet et al., 2009) with immunostaining against VGAT, the vesicular GABA transporter. Colocalization was prominent in the MB α'/β' lobes and MB peduncles (Figure 3E). This is consistent with a role of DPM neurons in inhibiting α'/β' neurons in order to promote sleep and opens up the interesting possibility that there may be branch-specific neurotransmission from DPM neurons (Yu et al., 2005; Cervantes-Sandoval and Davis, 2012; Samano et al., 2012). We found little to no colocalization between DPM presynaptic sites and the cholinergic marker ChAT (Figure 3D), again suggesting that DPM neurons do not release ACh.

**DPM activation has an inhibitory effect on MB neurons**

These results suggest that DPM neurons might be inhibitory rather than excitatory, and are inconsistent with a role for DPM neurons in directly enhancing potentiation. To test the sign of the connection, we first used functional imaging techniques to determine if DPM activation could stimulate postsynaptic MB neurons (Figure 4A). We expressed the mammalian ATP-gated P2X2 receptor (Lima and Miesenbock, 2005; Yao et al., 2012) in DPMs using NP2721-GAL4 or c316-GAL4, and activated these cells by applying ATP to a
dissected adult *Drosophila* brain. We first confirmed that bath-applied ATP was sufficient to activate the P2X2 receptors in DPMs by co-expressing genetically-encoded fluorescent sensors and using functional imaging to observe changes in fluorescence indicating a response. Using c316-GAL4 to drive UAS-GCaMP3.0 (Tian et al., 2009), UAS-Arclight (Cao et al., 2013), and UAS-Synapto-pHlorin (SpH) (Meisenbock et al., 1998), we found that P2X2-mediated stimulation effectively activated DPM neurons, evoking increases in intracellular calcium, membrane voltage, and vesicle fusion, respectively (Figure 4B). It should be noted that these responses were observed in the DPM projections to the MBs, not the DPM cell bodies, demonstrating that this technique successfully activates the DPMs and causes them to release neurotransmitter from their projections onto downstream targets in the MB neuropil. We also co-expressed P2X2 receptors and UAS-GCaMP3.0 in the DPMs using NP2721-GAL4 to confirm that this technique was effective with the weaker driver (Figure 4, supplement 1A).

To determine the effect of DPM activation on the MBs, we expressed P2X2 receptors in DPMs using the UAS/GAL4 binary expression system and either GCaMP3.0 or EPAC in the MBs using the *lexA/lexAop* binary expression system to observe changes in intracellular calcium or cAMP, respectively (Figure 4A). We found that activation of DPM neurons had no excitatory effects on the MBs when using either NP2721-GAL4 (Figure 4, supplement 1B and C) or the stronger c316-GAL4 with eyeless-GAL80 and MB-GAL80 to restrict expression to DPM neurons (Figure 4C and D). To confirm that these negative results were not due to lack of drug efficacy or some systematic problem, positive controls (P2X2 and GCaMP3.0 or EPAC expressed in the same cell type) were performed concurrently with every experiment (data not shown). We also did a separate set of experiments using dTrpA1 and a temperature step to activate DPM neurons, but failed to see any MB calcium responses (data not shown).

Although we demonstrated that P2X2 is capable of activating DPM neurons and causing vesicle fusion, we needed to rule out the possibility that MBs were simply incapable of responding to excitatory synaptic inputs. We first examined the response of the MBs to bath-applied carbachol (CCh), a cholinergic agonist. Tetrodotoxin (TTX) was present to block action potentials so that we could isolate direct effects on MBs. We
observed robust increases in calcium and cAMP (Figure 4, supplement 2), indicating that MB neurons are capable of responding to ACh in an excitatory manner.

To show MBs could respond to activation of upstream stimulatory neurons using the P2X2 technique, we expressed P2X2 receptors in the PAM cluster of dopaminergic neurons using R58E02-lexA and EPAC in the MBs using MB247-GAL4. Previous reports have demonstrated that the PAM cluster of dopamine neurons signals to the MBs by activation of Gαs-coupled receptors (Liu et al., 2012). As expected, we observed an increase in cAMP in the MBs in response to PAM neuron activation (Figure 4E), indicating that MBs are capable of responding to stimulatory inputs.

These results clearly demonstrate that DPM neurons are not excitatory; however, these fluorescent sensors may not be effective at reporting inhibition unless there is some basal level of activity in the circuit. Therefore, to determine if DPM activation has an inhibitory effect on MBs, we used P2X2 receptors to activate the DPMs and expressed the fluorescent intracellular chloride sensor SuperClomeleon (Grimley et al., 2013) in the MBs. We found that DPM activation evoked an increase in chloride in the MBs which could be almost completely blocked by bath-application of picrotoxin (Figure 5A). To determine if these results could be caused by DPM GABA release, we bath-applied GABA and observed similar MB SuperClomeleon responses in the presence of TTX, which could be completely blocked by picrotoxin (Figure 5B). These results demonstrate that DPM neurons inhibit the MBs via activation of GABA_A receptors.

**GABA and 5HT mediate the sleep-promoting effects of DPMs**

To determine if GABA- and/or 5HT-mediated inhibition was playing a role in the ability of DPMs to promote sleep, we manipulated transmitter in DPM neurons and receptors in α'/β' neurons. In order to assess whether DPM GABA release promotes sleep we expressed VGAT-RNAi to knock down the vesicular GABA transporter, using two different DPM lines, c316-GAL4 and NP2721-GAL4. Knockdown of VGAT in DPM neurons results in the loss of a large proportion of nighttime sleep (Figure 6A and B). Nighttime sleep loss is not due to hyperactivity since flies exhibit normal levels of nighttime activity.
while awake when compared to controls (for \textit{c316-GAL4} with \textit{VGAT-RNAi} P_{GAL4}=0.83, 
P_{UAS}=0.62 and for \textit{NP2721-GAL4} with \textit{VGAT-RNAi} P_{GAL4}=0.84, P_{UAS}=0.69).

As noted above, DPM neurons are coupled via gap junctions to the Anterior Paired Lateral (APL) neurons (Wu et al., 2011), which densely innervate the MB and synthesize the neurotransmitters GABA (Liu and Davis, 2009) and octopamine (Wu et al., 2013). Small molecules including neurotransmitters can pass through gap junctions (Vaney et al., 1998) and, for some subtypes of mammalian connexins, even RNAi fragments can pass (Valiunas et al., 2005). This raised the possibility that DPM sleep phenotypes could be dependent on APL-synthesized GABA and that manipulation of VGAT in DPMs might be indirectly acting by inhibition of APL GABA packaging. In order to assess this, we expressed \textit{VGAT-RNAi} using three different APL \textit{GAL4} lines: \textit{GH146, NP5288, NP2631} (Figure 6, supplement 1). We never saw sleep loss with \textit{NP2631-GAL4}. With \textit{GH146-} and \textit{NP5288-GAL4}, we saw weak nighttime sleep loss. When it was observed, nighttime sleep loss due to APL-driven expression of VGAT-RNAi was generally accompanied by an increase in activity while awake indicating an effect on locomotion; this is never seen with VGAT-RNAi in DPMs. Thus while APL GABA may be weakly sleep-promoting, its effects are qualitatively and quantitatively distinct from GABA released from DPMs. This also implies that the contribution of APLs to phenotypes seen after DPM activation or hyperpolarization are likely to be minimal. Along these lines it is also important to note that driving \textit{Shi}\textsuperscript{ts} in DPMs reduces baseline sleep and completely blocks dTrpA1-induced sleep gains. These manipulations, using DPM-expressed \textit{Shi}\textsuperscript{ts}, would not be expected to influence APL activity in any way, again supporting the idea that DPMs promote sleep in a distinct and cell-autonomous manner.

While there are two known types of GABAergic neurons innervating the MB, DPMs are likely the sole source of MB lobe 5HT (Lee et al., 2011). MB expression of inhibitory, \textit{G\alpha}_{i}-coupled 5HT\textsubscript{1A} receptors are necessary for anesthesia-resistant memory (Lee et al., 2011), and are also known to promote sleep (Yuan et al., 2006). 5HT from DPMs, however, has not actually been shown to promote sleep. Knockdown of 5HT synthesis in DPM neurons with RNAi targeted against tryptophan hydroxylase (Trh) using the most strongly expressing DPM line, \textit{c316-GAL4}, results in a significant loss of nighttime sleep (Figure 6C).
This nighttime sleep loss is also apparent, but milder, with Trh-RNAi being driven by the somewhat weaker NP2721-GAL4 (Figure 6D). No difference is seen in nighttime activity during waking periods vs. controls (for c316-GAL4 with Trh-RNAi \( P_{GAL4}=0.09, P_{UAS}=0.17 \)) indicating the sleep loss is not an artifact of hyperactivity.

**DPM GABA acts on \( \alpha'/\beta' \) lobes to promote sleep**

GCaMP, EPAC and Arclight experiments demonstrate a lack of excitatory transmission from DPM neurons to the MBs and SuperClomeleon experiments demonstrate that the DPMs are capable of inhibiting MB neurons. The wake-promoting phenotype of MB \( \alpha'/\beta' \) neurons as well as a shared temporal role in memory consolidation suggest these neurons could be the targets of sleep-promoting DPM GABA release. In order to test this possibility, we expressed RNAi against *Drosophila* GABA receptors in MB \( \alpha'/\beta' \) neurons. It has previously been shown that the *Drosophila* ionotropic GABA\(_A\) receptor, Rdl, is highly expressed in all lobes of the MBs (Liu et al., 2007). Consistent with the phenotype of DPM VGAT knockdown, we observe decreased nighttime sleep with knockdown of either Rdl (Figure 7A), or GABA\(_B\)-R3 (Figure 7B) in MB \( \alpha'/\beta' \) neurons. In both cases sleep loss is the result of a decrease in the duration of nighttime sleep episodes. Knockdown of Rdl results in less total sleep loss since an increase in the total number of nighttime sleep episodes partially compensates for the decrease in mean sleep episode duration. Importantly, concurrent expression of the *MB-GAL80* transgene, which blocks GAL4-mediated expression of receptor RNAs, greatly suppresses sleep loss and fragmentation phenotypes showing that the effects are specific to the MB \( \alpha'/\beta' \) lobes (Figure 7, supplement 1).

Interestingly, experiments to determine the lobe-specific role of 5HT\(_{1A}\) receptors suggest that 5HT acts generally in the MB, not just on the \( \alpha'/\beta' \) lobes (data not shown), suggesting that these two transmitters may play somewhat different roles at the circuit level in sleep and memory consolidation.

**Discussion**

The inhibitory neurotransmitter, GABA, is known to promote sleep in both mammals (Rasch and Born, 2013) and *Drosophila* (Agosto et al., 2008), but specific sleep-promoting GABAergic neurons have not been identified in the fly. Additionally, it is known
that sleep promotes memory consolidation in *Drosophila* (Donlea et al., 2011) and other
animals (Rasch and Born, 2013; Diekelmann, 2014), but it is unclear how sleep and
memory circuits interact to facilitate memory consolidation. Here we find a shared
anatomical locus of memory consolidation and GABAergic sleep-promotion in the DPM
neurons. We show that α′/β′ neurons, postsynaptic targets of the GABAergic DPM
processes, are wake-promoting. The specific involvement of neurons required for memory
consolidation (and their memory-relevant post-synaptic targets) in the regulation of sleep
suggests that generation of sleep by activation of learning circuits is an intrinsic property of
the circuit, not an extrinsically imposed phenomenon. Further, our finding that the
memory-consolidation specific DPM neurons are inhibitory suggests that inhibitory
neurotransmitters may play an as-of-yet uncharacterized role in memory consolidation in
*Drosophila*.

**The role of DPM vs. APL neurons in regulation of sleep**

Previous studies on the role of GABA in the *Drosophila* learning circuit have focused
on the role of the APLs, a pair of GABAergic neurons which densely innervate the MBs and
are coupled to DPMs by gap junctions. APL GABA has been shown to inhibit acquisition (Liu
and Davis, 2009), perhaps by acting at the level of olfactory coding (Lin et al., 2014). APLs
have also been shown to be critical for a labile component of anesthesia-sensitive
intermediate-term memory but not for consolidation to long-term memory (Pitman et al.,
2011). Because of the gap junction coupling, it was formally possible that GABA found in
DPMs could be coming from APLs and that sleep loss due to DPM VGAT-RNAi expression
was the result of reductions in APL VGAT levels (Vaney et al., 1998; Valiunas et al., 2005). It
was also possible that phenotypes seen after manipulation of DPM electrical activity were
secondary to changes in APL activity. However, a number of lines of evidence suggest that
DPM neurons are intrinsically GABAergic and sleep-promoting independent of APLs. First,
we find that DPMs stain positively for the GABAergic markers Gad1 and VGAT, meaning
that DPMs intrinsically possess the ability to synthesize and release GABA. Second, we find
that direct expression of *VGAT-RNAi* in APL neurons has a relatively minor effect on sleep
as compared to phenotypes seen with expression in DPMs, indicating that GABA
endogenous to DPMs is a more significant regulator of sleep than GABA from APLs. Third,
we find that sleep loss due to VGAT-RNAi expression in APLs, when it is seen, is accompanied by increases in nighttime activity while awake, which is never seen with DPM-driven VGAT-RNAi expression. This indicates that although APL GABA may modestly promote sleep in its own right, the APL VGAT-RNAi sleep loss phenotype is distinct from that of DPMs. Fourth, expression of Shi$^r$ in DPMs, a manipulation that should have no effect on APL activity since it does not alter the electrical properties of DPMs, results in the same nighttime sleep loss as DPM-driven VGAT-RNAi and Kir2.1. Fifth, coexpression of Shi$^r$ along with dTrpA1 in DPMs, a manipulation that should not affect APL neurotransmitter release, results in a complete blockade of activation-induced sleep gains. The most parsimonious explanation for all of these data is that the relevant effect of these manipulations is a change in transmitter release specifically from DPMs and that this bidirectionally modulates sleep. Thus, while it remains possible that APL neurons are modestly sleep-promoting in their own right, we find strong evidence for an independent and significant role in regulation of sleep by DPM neurons.

**Evidence for DPM inhibition, but not excitation of $\alpha'/\beta'$ lobes**

Our finding that DPM neuron activation has an inhibitory effect on post-synaptic MB neurons is in apparent disagreement with models of memory consolidation that posit recurrent excitatory feedback between DPM and $\alpha'/\beta'$ neurons (Yu et al., 2005; Keene and Waddell, 2007). Our observations using SuperClomeleon demonstrate that DPMs evoke a chloride increase in MB neurons, but we did not see decreases in calcium, membrane voltage or cAMP after DPM activation using fluorescent sensors specific for those cellular parameters. This failure is not surprising for two reasons. First, in order to see an inhibitory response it is likely that there has to be some activity or tone in the system. In cases where MB inhibition has been seen with GCaMP it is always in context of a temporally controlled acute activation of the system (Lei et al., 2013; Lin et al., 2014; Masuda-Nakagawa et al., 2014). Second, the nature of our DPM activation (bath application of ATP) would make it difficult to see small inhibitory changes over noise in averaged data since our activation of cells is not cleanly time-locked due to differences in diffusion of ATP into the brain between experiments. This is particularly critical with a sensor like Arclight
where the expected hyperpolarization induced by inhibition might only be a few millivolts as opposed to 10-40 mV for depolarization by an action potential.

An additional critical test of the sign of this synapse is to ask if there is evidence of inhibition in the functional output of the circuit. Our finding that DPM and \( \alpha'/\beta' \) activity have opposite roles in the regulation of sleep to the extent that suppression of DPMs with either Shi\textsuperscript{ts} or Kir2.1 almost exactly phenocopies the increasing levels of nighttime sleep loss that results from dTrpA1 activation of \( \alpha'/\beta' \) strongly suggests an inhibitory connection. Further, we find that decreases in DPM VGAT result in similar nighttime sleep loss phenotypes as \( \alpha'/\beta' \) Rdl or GAB\textsubscript{a}R3 knockdown. Thus, suppression of DPM synaptic release (Shi\textsuperscript{ts}), DPM electrical activity (Kir2.1) and DPM GABA release (VGAT-RNAi) all result in nighttime sleep loss phenotypes nearly identical to dTrpA1 activation of \( \alpha'/\beta' \) or loss of \( \alpha'/\beta' \) GABA receptors. All of these results are consistent with a model in which DPMs act to inhibit \( \alpha'/\beta' \) neurons by release of GABA.

**Inhibition of MBs and sleep**

MBs are a sensory integration center in the insect brain. They have been shown, using many different behavioral paradigms, to be critical nodes for attention and arousal (Xi et al., 2008; van Swinderen et al., 2009; Chow et al., 2011). Paying attention to the right features of one’s environment, whether naively or as a learned response, has high survival value. The fact that MBs are important sites of plasticity is likely related to this role in attention. The linkage of attention and arousal to MB output is consistent with our finding that suppression of arousal-promoting MB subsets increases sleep.

The involvement of DPMs, a neuron type previously believed to function exclusively as a regulator of memory consolidation, in control of sleep raises several interesting questions. First, are DPMs the only sleep-inducing regulators of MB activity? This seems unlikely, since memory consolidation is but one function of the MBs and there are other behavioral situations in which an animal might want to modulate MB-regulated arousal such as during courtship/aggressive behaviors (Baier et al., 2002; Sakai and Kitamoto, 2006), or more generally during the integration of internal and external cues and decision making (Zhang et al., 2007; Neckameyer and Matsuo, 2008; Krashes et al., 2009; Donlea et al., 2012; Bracker et al., 2013). A second question is whether DPMs might also have a role in
regulating MB output in contexts other than during memory storage. Our data suggest a role for DPMs in maintaining basal levels of nighttime sleep indicating that they may be responsive to other types of input. An understanding of the regulation of DPMs and their \textit{in vivo} activity patterns will be required to gain insight into these issues.

\textbf{Inhibition in memory consolidation}

A potential role for GABA-mediated inhibition in memory consolidation is novel. Long-term memory storage in mammals is believed to involve a transfer of information from one brain region to another. In \textit{Drosophila}, it is associated with sequential potentiation of activity in specific MB neuropils. Elegant studies using conditional inhibition of transmitter release have provided a temporal ordering of transfer (Keene and Waddell, 2007; Cervantes-Sandoval et al., 2013; Dubnau and Chiang, 2013) leading to the idea that the role of DPM neurons is to facilitate the movement of memory from $\alpha'/\beta'$ lobes, one initial site of memory storage, to a more permanent home in $\alpha/\beta$ lobes and perhaps other neurons (Chen et al., 2012). Given the clear requirements for molecular pathways associated with synaptic potentiation, and the need for synaptic transmission from both DPMs and $\alpha'/\beta'$ neurons after acquisition, the view that information transfer involves a positive feedback loop between these cell types makes sense.

The data presented here, however, strongly suggest that the DPM neurons are inhibitory. It is clear that potentiated output from $\alpha'/\beta'$ neurons is required for consolidation, so how could their inhibition facilitate this process? Our findings suggest DPMs are unlikely to participate directly in the excitatory arm of a recurrent feedback loop. However, both models and physiological data related to mammalian cortical/hippocampal recurrent feedback circuits involved in the maintenance of stable memory states also require the presence of inhibition (Buzsaki and Chrobak, 1995; Hasselmo et al., 1995; Battaglia and Treves, 1998; Chance and Abbott, 2000). Interestingly, the coordination of excitatory activity amongst diverse functional sub-circuits in the mammalian hippocampus and neocortex is regulated by the activity of broadly-projecting, gap-junction coupled inhibitory neurons/networks (Freund and Antal, 1988; Gibson et al., 1999; Tamas et al., 2000; Baude et al., 2007; Jinno et al., 2007) which have been proposed to potentially
control the timing of memory replay events during sleep/memory consolidation (Viney et al., 2013). The DPM-APL network may represent an analogous set of neurons in *Drosophila* which function to coordinate the temporal stabilization, gating and transfer of different memory stages between different sub-circuits within the MBs. While APL neurons have been shown to broadly inhibit recurrent feedback from all MB Kenyon cells to all MB Kenyon cells (Lin et al., 2014), it has been proposed that DPMs may impose a directionality on internal MB feedback which would allow for memory transfer and consolidation (Yu et al., 2005). Although, inhibition was not previously considered for such a role, it may be capable of coordinating the timing of prime lobe output in a way that is not possible via excitation. Thus, our data are not inconsistent with the presence of an excitatory recurrent feedback loop within the MB α'/β' lobes, but rather provide information that constrains future models in a new way and suggests new possibilities for the role of DPMs that may not have been considered previously.

What kind of role could inhibitory neurons play? While α'/β' neurons need to be active during acquisition, DPMs do not and this temporal difference suggests some testable possibilities for the role of DPM-mediated inhibition in consolidation. One idea is that temporally-regulated inhibition of potentiated α'/β' output could serve as a way of sharpening the transition of memory from one neuropil to another by suppressing activity in the brain area from which information has already been transferred. A second possibility is that a period of inhibition during consolidation is a way of preventing new, potentially interfering, information from being encoded in α'/β' before the first memory is transferred to α/β. A third possibility is that the function of inhibition is actually to provide precisely timed rebound excitation to α'/β'. This would not have been seen in our imaging experiments due to the slow kinetics of ATP washout, but could actually result in feedback excitation of MB neurons.

All of these models imply that there is a very tight temporal ordering of activity within the circuit, with DPM neurons suppressing α'/β' neuron activity in a narrow window either before or after their output function has been completed. It is important to note that none of these ideas is inconsistent with the demonstrated requirement for α'/β' activity during the post-training consolidation period. GABA- and 5HT-mediated inhibition is not equivalent to the action of *Shi*<sup>is</sup>, which completely shuts off neurotransmission. Inhibition is
often modulatory rather than switch-like and can even be compartment-specific—for example, it could serve to alter the ratio of activity in $\alpha'/\beta'$ to that in $\alpha/\beta$. More precise mapping of the connectivity and branch-specific activity in the MB neuropil will be required to develop a more detailed model.

Coupling sleep and memory consolidation at the cellular level

Although investigators have speculated that memory consolidation and sleep interact, an actual understanding of how they are related at the circuit level has been elusive. This study is the first demonstration of a cellular- and circuit-level mechanism for the coupling of sleep and consolidation. The fact that memory and sleep are behaviorally linked even in the insect (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2011) is evidence of the evolutionary importance of coupling these two processes. The simplicity of the cellular mechanism in *Drosophila*, using a single pair of neurons to carry out both functions, provides an example of how coupling can occur in a small nervous system and suggests a template for understanding it in larger brains.
Acknowledgments

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Materials and methods

Fly stocks

Fly stocks were raised on modified Brent and Oster cornmeal-dextrose-yeast agar media (Brent, 1974) Per batch: 60L H₂O, 600g Agar, 1,950g flaked yeast, 1,451g cornmeal, 6,300 g dextrose, 480g NaKT, 60g CaCl₂, 169g Lexgard dissolved in ethanol. Flies were raised under a 12:12 light:dark cycle at 25°C except for animals carrying UAS-dTrpA1 which were raised at 22°C or flies carrying either pTub-GAL80ts or UAS-IVS-Syn21-ShiTs which were raised at 18°C. UAS-P2X2 (Lima and Miesenbock, 2005), lexAop-P2X2, lexAop-Epac1-camps (1A), lexAop-GCaMP3.0 (Yao et al., 2012), and UAS-Epac1-camps(55A) (Shafer et al., 2008), flies were kindly provided by Dr. Orie Shafer. UAS-Arclight (Cao et al., 2013) was a gift from Dr. Michael Nitabach, and the SHT7-GAL4 flies (Becnel et al., 2011) were a gift from Dr. Charles Nichols. The following lines have also been previously described:

20xUAS-IVS-Gcamp6m (Akerboom et al., 2012), UAS-Synapto-pHlorin (SpH) (Meisenbock et al., 1998), UAS-GCaMP3.0 (Tian et al., 2009), UAS-dTrpA1 (chromosome 2 insertion site) (Hamada et al., 2008), UAS-Rdl-RNAi 8-10J (Liu et al., 2007; Liu et al., 2009), 20xUAS-IVS-Syn21-ShiTs (Pfeiffer et al., 2012), UAS-mCD8-GFP (Lee, 1999), UAS-Brp-short-GFP (Schmid et al., 2008; Fouquet et al., 2009), UAS-Kir2.1 (Baines et al., 2001), c316-GAL4 (Waddell, 2000), c305a-GAL4 (Krashes et al., 2007), NP2721-GAL4 (Wu et al., 2011), VT64246-GAL4 (Lee et al., 2011), MB247-GAL4 (Zars, 2000), MB247-lexA (Pitman et al., 2011), R58E02-lexA (Liu et al., 2012), MB-GAL80 (Thum et al., 2007), eyeless-GAL80 (Chotard et al., 2005), ptub-GAL80Ts (McGuire et al., 2003), ptub>GAL80> (Gordon and Scott, 2009), LexAOP-Flp (Shang et al., 2008), and GH146-GAL4, NP5288-GAL4, NP2631-GAL4 (Tanaka et al., 2008). The following RNAi lines were obtained from the VDRC (Dietzl et al., 2007): VGAT-RNAi (X-stock #45917), VGAT-RNAi (II- stock #45916), Trh-RNAi (II-#105414), GABAaR3-RNAi (III-#50176). The following RNAi lines have been functionally verified previously: Rdl-RNAi (Liu et al., 2007), GABAaR3-RNAi (Dahdal et al., 2010).

The genetic intersectional method used to restrict expression to the MB is described in Shang et al 2008. MB restriction to the prime lobes with c305a is shown in (Perrat et al., 2013).

SuperClomeleon Flies
13xLexAOP-IVS-Syn21-SuperClomeleon expressing flies were generated using the SuperClomeleon construct designed by Grimley et al. (Grimley et al., 2013). Gateway cloning (Invitrogen) was used to create the entry vectors pDonr221-13xLexAOP and pDonrP2rP3-Syn21-SuperClomeleon-p10. A modified version of pBPGUw (Pfeiffer et al., 2008), pBPGUw-R1R3-p10 was used for gateway recombination and injection into flies where it was targeted to the attP40 landing site on the second chromosome. pBPGUw-R1R3-p10 was generated from the following modifications to pBPGUw: removal the DSCP (Drosophila Synthetic Core Promoter), replacement of the attR2 Gateway recombination site with attR3, and replacement of the weaker Hsp70 terminator with the stronger p10 terminator sequence (Pfeiffer et al., 2012).

The entry vector, pDonr221-13XLexAOP, was generated by PCR and Gateway cloning of the 13XLexAOP-Hsp70 TATA-IVS sequence from pJFRC19 (Pfeiffer et al., 2010), into the pDonr221 Gateway entry vector. The pDonrP2rP3-SuperClomeleon entry vector was generated by PCR and Gateway cloning of the SuperClomeleon sequence from pUC19-SuperClomeleon (Grimley et al., 2013) into the pDonrP2rP3 Gateway entry vector. Primers for the pDonr221 construct were designed by fusing Gateway attB1 and attB2 sequences upstream and downstream, respectively, of the 13xLexAOP and IVS sequences.

Primers for pDonrP2rP3-SuperClomeleon were designed by fusing Gateway attB2r and attB3 sequences upstream and downstream, respectively, of the SuperClomeleon sequence. To enhance expression, the 21bp Syn21 sequence (Pfeiffer et al., 2012) was added to the forward primer just upstream of SuperClomeleon. All PCRer and ligated sequences were verified by sequencing before injection into flies.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Primers (5’-3’)</th>
<th>Sequence (Gateway sequences are in caps and vector sequences are lower case)</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB1-13xLexAOP forward</td>
<td>GGGGACAAGTTTTGTACAAAAAAAGCAGGCTATgcatgctgcaggttactgtac</td>
</tr>
<tr>
<td>attB2-IVS reverse</td>
<td>GGGGACCACTTTTGTCAAAGAAAGCTGGGTAggccgctgaagtaggataag</td>
</tr>
<tr>
<td>attB2r-Syn21-SuperClomeleon</td>
<td>GGGGACAGCTTTCTTGTACAAAAAGTGAAAACCTTAAAAAAAAAAAAATCAA Aatggtgagcaagggcagg,</td>
</tr>
</tbody>
</table>
Behavioral analysis

Individual virgin female flies were housed separately in 65 mm × 5 mm glass tubes (Trikinetics, Waltham, MA) containing 5% agarose with 2% sucrose. Parafilm with pinholes poked in it was used to cover the open end of each tube. Two- to five-day old flies were entrained under standard light-dark conditions, with a 12 h light phase and followed by 12 h dark phase for 3–4 days prior to collection of data for sleep analysis. Locomotor activity was collected with DAM System monitors (Trikinetics) in 1 min bins as previously described (Agosto et al., 2008). Sleep was defined as bouts of uninterrupted inactivity lasting for five or more minutes (Hendricks, 2000; Shaw et al., 2000). Sleep/activity parameters (total sleep, mean sleep episode duration, maximum sleep episode duration, number of sleep episodes and activity while awake) were analyzed for each 12 h period of light or dark conditions and averaged across three days. Sleep analysis was conducted using an in-house Matlab program described previously (Donelson et al., 2012). Since we found that some, but not all sleep data were not normally distributed, we chose to use the less powerful, but more conservative Mann-Whitney/Wilcoxon ranked sum test rather than ANOVA and Tukey post-hoc tests, which assume data are normally distributed. As the Mann-Whitney/Wilcoxon ranked sum test is a pairwise test, this generates two P values, one for experimental vs. the UAS control and one for the experimental vs. GAL4 control line. In all figures only the most conservative/numerically greatest P value is reported.

For temperature-shift experiments, flies expressing either Shi⁰⁸ or Tub-GAL80⁰⁸ were raised at 18°C, whereas flies expressing dTrpA1 were raised at 22°C. In all cases baseline data was recorded at the respective rearing temperature (18°C or 22°C) and compared to sleep at the activation (dTrpA1) or suppression (Tub-GAL80⁰⁸ and Shi⁰⁸) temperature of 31°C. The effect of heat on sleep is highly sensitive to genotype. In order to assess heat-induced changes in sleep, first, the baseline sleep of each fly at either 18°C or 22°C was
subtracted from sleep of the same fly at 31°C and this difference was averaged together
across flies of the same genotype. Following baseline subtractions for each genotype,
average GAL4 or UAS values were then subtracted from average experimental group values
to obtain minutes of sleep gained or lost versus controls.

**Immunohistochemistry**

For immunostaining, a standard fixation and staining protocol was used. Briefly,
brains were dissected in ice-cold PBS and were fixed immediately after dissection for 15
min at room-temperature in 4% paraformaldehyde (vol/vol). Brains were incubated in PBS
containing 0.5% Triton X-100, 10% NGS and primary or secondary antibodies for one night
each with 3 x 15 min washes between each incubation. Brain samples were then mounted
using Vectashield and were visualized by a Leica TCS SP5 confocal microscope with a 20x,
40x or 63x objective lens. All images were taken sequentially to prevent bleed-through
between channels. For colocalization, either mouse anti-GFP (1:200, Roche Applied
Biosciences) or rabbit anti-GFP (1:1,000, Invitrogen A11122) was used together with
transmitter-specific primary antibodies as follows: rabbit anti-dVGAT (1:400, (Fei et al.,
2010), a kind gift from Dr. D.E. Krantz), rabbit anti-GABA (1:200, Sigma; Cat. No. A2052),
rabbit anti-Gad1 (1:500, kind gift from Dr. F.R. Jackson), mouse anti-choline
acetyltransferase (ChAT) (1:200, code 4B1 Developmental Studies Hybridoma Bank;
(Takagawa, 1996; Yasuyama, 1996)), rabbit anti-serotonin (1:1,000, Sigma S5545) and
mouse anti-tyrosine hydroxylase (TH) (1:500, Immunostar 22941). Alexa Fluor 488 and
635 anti-mouse or anti-rabbit secondary antibodies (1:200, Invitrogen) were used to
visualize staining patterns. Alexa 488 was always used to label GFP so that any residual
endogenous GFP fluorescence would be of a similar wavelength as the dye and would not
bleed through to the 633 wavelength channel.

**Image processing and intensity correlation analysis (ICA)**

All image processing was done using the freely available FIJI (IMAGEJ) software and
plugins (Schindelin et al., 2012). Background was subtracted from all confocal stacks prior
to further processing. All images are sums or maximum intensity Z-projections of the
relevant confocal slices. Quantification of cells with positive/negative staining was done by
visually comparing colocalization of GFP and antibody staining in multiple individual Z-
slices. Cell bodies/MB lobe sets where high background prevented interpretation of
staining were excluded. For the images presented in figure 3, Intensity Correlation
Analysis (ICA) was also performed to assess spatial colocalization of staining between
channels (Li et al., 2004). Rather than only comparing visible overlap of the absolute
fluorescence intensity in each channel (red plus green equals yellow), which is subject to
viewer bias and differences in staining intensity between channels, this method determines
whether changes in staining intensity covary or are correlated between channels. This
provides an objective and spatially specific representation of colocalization. ICA analysis
generates a correlation/cocolocalization value for each pixel defined by the Product of the
Differences from the Mean (PDM) i.e. PDM= (red intensity- mean red intensity)×(green
intensity – mean green intensity). PDM values for each pixel can then be visualized as an
image showing positive intensity correlation (PDM>0) and negative intensity correlation
(PDM<0). Relative PDM value scales are shown on each figure generated from ICA analysis.

**Functional fluorescence imaging**

Adult hemolymph-like saline (AHL) consisting of (in mM): 108 NaCl, 5 KCl, 2 CaCl₂,
8.2 MgCl₂, 4 NaHCO₃, 1 NaH₂PO₄·H₂O, 5 trehalose, 10 sucrose, 5 HEPES; pH 7.5 (Wang,
2003) was used to bathe the brain, as previously described (Wang, 2003; Shang et al.,
2011). For SuperClomeleon experiments, the AHL pH was increased to 7.7 to optimize
response magnitude. Test compounds adenosine 5’-triphosphate magnesium salt (ATP),
carbamoylcholine chloride (CCh), picrotoxin (PTX), and γ-aminobutyric acid (GABA) were
purchased from Sigma-Aldrich (St Louis, MO), serotonin hydrochloride (5HT) was
purchased from Tocris Bioscience (Minneapolis, MN), and tetrodotoxin (TTX) was
purchased from Abcam Biochemicals (Cambridge, England). ATP, CCh, and TTX were
dissolved in milliQ water and frozen as aliquot stocks, which were then prepared for
experiments by dilution in AHL. PTX was dissolved and frozen in DMSO aliquots, which
were diluted in AHL for experiments. All solutions used in experiments with PTX were
prepared with the same percentage of DMSO. GABA and 5HT were dissolved directly in
AHL immediately prior to the experiment, and 5HT was kept in light-shielded containers to
prevent degradation.
Imaging experiments were performed using a naked brain preparation. Flies were anesthetized on ice and brains were dissected into cool AHL. Dissected brains were then pinned to a layer of Sylgard (Dow Corning, Midland, MI) silicone under a small bath of AHL contained within a recording/perfusion chamber (Warner Instruments, Hamden, CT). Brains expressing GCaMP3.0, Arclight, and SpH were allowed to settle for 5 min after dissection to reduce movement. These brains were then exposed to fluorescent light for approximately 30 s before imaging to allow for baseline fluorescence stabilization, while brains expressing the FRET sensors Epac1-camps or SuperClomeleon were exposed an extra 5 min to minimize differences in photobleaching rates between the CFP and YFP fluorophores, as YFP has been described to photobleach more slowly than CFP (Shafer et al., 2008; Pirez et al., 2013). Perfusion flow was established over the brain with a gravity-fed ValveLink perfusion system (Automate Scientific, Berkeley, CA). ATP, CCh, GABA, or 5HT were delivered by switching perfusion flow from the main AHL line to another channel containing diluted compound after 30 s of baseline recording for desired durations followed by a return to AHL flow. To control for the effects of switching channels, a vehicle control trial was performed by switching to another line containing AHL for the same duration as the experimental trial.

Imaging was performed using an Olympus BX51WI fluorescence microscope (Olympus, Center Valley, PA) under an Olympus x40 (0.80W, LUMPlanFl) or x60 (0.90W, LUMPlanFl) water-immersion objective, and all recordings were captured using a charge-coupled device camera (Hamamatsu ORCA C472-80-12AG). For GCaMP3.0, Arclight, and SpH imaging, we used the following filter set (Chroma Technology, Bellows Falls, VT): excitation, HQ470/x40; dichroic, Q495LP; emission, HQ525/50m. For EPAC, a 86002v1 JP4 (436; Chroma Technology) excitation filter was used, and emitted light from the CFP and YFP fluorophores was separated using a splitter (Photometrics DV² column) with the emissions filters D480/30m and D535/40m (Photometrics, Tucson, AZ), which allowed for simultaneous collection from both fluorescence channels. Frames were captured at 2 Hz with 4x binning for either 2 min or 4 min using μManager acquisition software (Edelstein et al., 2010). Neutral density filters (Chroma Technology) were used for all experiments to reduce light intensity to limit photobleaching.
Although there are tools for temporally-controlled activation such as dTrpA1 or Channelrhodopsin (ChR2) that are well-characterized, we utilized P2X2 receptors for the majority of our experiments. Activation wavelengths for ChR2 overlap with those of many fluorescent sensors such as GCaMP and EPAC, so ChR2 could not be used in this circuit due to the close proximity of the cells and processes. Applying heat to activate dTrpA1 causes changes in refractive index, which disrupt focus, necessitating manual focus correction in the cases where we used this technique.

Regions of interest (ROIs) were selected within the horizontal or vertical lobes of the MBs or, in the case of DPM neurons, over the horizontal projections to the MBs. Figures depict responses in horizontal lobes or projections; however, similar results were observed in the vertical lobes when noted in the figure captions. For recordings using GCaMP3.0, Arclight, and SpH, ROIs were analyzed using custom software developed in ImageJ (Schindelin et al., 2012) and National Institute of Health, Bethesda, MD). Briefly, the percent change in fluorescence over time was calculated using the following formula: $\Delta F/F = (F_n - F_0)/F_0 \times 100\%$, where $F_n$ is the fluorescence at time point $n$, and $F_0$ is the fluorescence at time 0. For GCaMP3.0 and SpH, maximum fluorescence change values were determined as the maximum percentage change observed for each trace over the entire duration of each imaging experiment. For Arclight, because increases in voltage are represented as decreases in fluorescence, maximum fluorescence change values were determined as the minimum percentage change. Maximum values for each group were then averaged to calculate the mean maximum change from baseline.

For recordings using EPAC or SuperClomeleon, ROIs were analyzed using custom software developed in MATLAB (The MathWorks, Natick, MA). This analysis package is provided as Source Code Files. Briefly, identical ROIs were selected from both the CFP and YFP emissions channels, and the fluorescence resonance energy transfer (FRET) signal (YFP/CFP ratio) was calculated for each time point and normalized to the ratio of the first time point. The relative cAMP changes were determined by plotting the normalized CFP/YFP ratio (percentage) over time. As with GCaMP3.0 and SpH, the average maximum percent change values were determined as the mean maximum values for each group.

Statistical analyses were performed using MATLAB (The MathWorks). A Kruskal-Wallis one-way ANOVA was used to determine statistical significance between the experimental group...
and the two negative controls. In cases in which there was significance in the ANOVA, a Mann-
Whitney U test (also known as Wilcoxon rank-sum test) was used to determine the significance
between the experimental group and each negative control. In all figures only the most
conservative/numerically greatest P value is reported. Results are expressed as means + standard
error of the mean (SEM).

References

from molecules to whole-brain networks." Curr Biol 23(17): R774-788.
"Modulation of GABAA receptor desensitization uncouples sleep onset and
Akerboom, J., T. W. Chen, T. J. Wardill, L. Tian, J. S. Marvin, S. Mutlu, N. C. Calderon, F. Esposti,
B. G. Borghuis, X. R. Sun, A. Gordus, M. B. Orger, R. Portugues, F. Engert, J. J. Macklin,
Baier, L. Lagnado, S. S. Wang, C. I. Bargmann, B. E. Kimmel, V. Jayaraman, K. Svoboda,
Amit, D. J. (1989). Modeling brain function- the world of attractor neural networks,
Cambridge University Press.
Bailer, A., B. Wittek and B. Brembs (2002). "Drosophila as a new model organism for the
Properties in Drosophila Neurons Developing without Synaptic Transmission." The
Journal of Neuroscience 21(5): 1523-1531.
Battaglia, F. P. and A. Treves (1998). "Stable and rapid recurrent processing in realistic
autoassociative memories." Neural Comput 10(2): 431-450.
"Immuneactivity for the GABAA receptor alpha1 subunit, somatostatin and
Connexin36 distinguishes axoaxonic, basket, and bistratified interneurons of the rat
hippocampus." Cereb Cortex 17(9): 2094-2107.
receptor is expressed in the brain of Drosophila, and is essential for normal
Grunwald Kadaw (2013). "Essential role of the mushroom body in context-


Viney, T. J., B. Lasztoczi, L. Katona, M. G. Crump, J. J. Tukker, T. Klausberger and P. Somogyi
(2013). "Network state-dependent inhibition of identified hippocampal CA3 axo-


"Heterotypic gap junctions between two neurons in the drosophila brain are critical

circuit modulates the formation of anesthesia-resistant memory in Drosophila."
Cur Biol 23(23): 2346-2354.

Xi, W., Y. Peng, J. Guo, Y. Ye, K. Zhang, F. Yu and A. Guo (2008). "Mushroom bodies modulate
1441-1451.

"Dynamin 2 interacts with connexin 26 to regulate its degradation and function in

684-696.

Acetyltransferase Expression in Adult Drosophila melanogaster Brain." Journal of

Yi, W., Y. Zhang, Y. Tian, J. Guo, Y. Li and A. Guo (2013). "A subset of cholinergic mushroom
body neurons requires go signaling to regulate sleep in Drosophila." Sleep 36(12):
1809-1821.

neurons form a delayed and branch-specific memory trace after olfactory classical


672-675.

Figure Legends

Figure 1. DPM activity and synaptic release are sleep promoting in a cell-autonomous manner.

(A) Flies exhibit large gains in sleep when DPM neurons are activated with dTrpA1 at 31°C (w-; NP2721-GAL4/ UAS-dTrpA1-II). Compensatory sleep loss is apparent during recovery following two days of dTrpA1 activation. (B1) Flies show small but significant sleep loss when DPM synaptic release is inhibited with ShiTs after shift to 31°C (w-; NP2721-GAL4; 20xUAS-IVS-Syn21-ShiTs).Continuing sleep loss is apparent during the first 12 h of recovery following ShiTs inhibition of DPM synaptic release. (B2) Sleep loss can be seen over multiple cycles of temperature shift when DPM synaptic release is inhibited with ShiTs (w-; NP2721-GAL4; 20xUAS-IVS-Syn21-ShiTs). For quantification in B2, Day 1 was used as a baseline to calculate Day 2 sleep changes and Day 3 was used as a baseline to calculate Day 4 sleep changes. (C) Sleep gains resulting from dTrpA1 activation are fully blocked when DPM synaptic release is inhibited with ShiTs at 31°C (w-; NP2721-GAL4/ UAS-dTrpA1-II; 20xUAS-Tts-ShiTs). Left plots: show sleep in 30-min bins during a baseline day (22°C for dTrpA1 alone, 18°C for ShiTs or combined UAS experiments), followed by 1-2 days of DPM hyperactivation or inhibition (31°C) and one day of recovery (22°C for dTrpA1 alone, 18°C for ShiTs or combined UAS experiments). Right plots: show a quantification of the 30-min data in 12-h bins across 1 or 2 days of heating and one day of recovery. Sleep change is quantified as the minutes of sleep gained or lost by the experimental genotype in comparison to either the UAS or GAL4 control genotypes during heating and recovery periods. Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the Materials and Methods section.

Figure 2. MB α’β’ neuron activity promotes wakefulness.

The α’β’ c305a-GAL4 driver line was crossed to UAS-dTrpA1-II (with c305a expression restricted to the MB) to determine effects on sleep of α’β’ activation. (A) Shows sleep in 30-min bins during a baseline day (22°C), followed by two days of DPM hyperactivation (31°C)
and one day of recovery (22℃). (B) Shows minutes of sleep gained or lost by the experimental genotype in comparison to either the UAS or GAL4 control genotypes during heating and recovery periods. MB-restricted genotypes are: 1) UAS-dTrpA1, ptub>GAL80>/c305a-GAL4; MB-LexA/LexAop-Flp, 2). c305a-GAL4; MB-LexA (GALA/LexA control), and 3). UAS-dTrpA1, ptub>GAL80>; LexAop-Flp (UAS/LexAOP control). Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the Materials and Methods section.

Figure 3. DPMs are GABAergic, but not cholinergic.

(A-C) Top: VT64246-GAL4 was used to drive expression of membrane-localized mCD8-GFP in DPM cell bodies, which was visualized with an anti-GFP antibody. Middle: Brains were stained using antibodies against (A) GABA (N=11/11 cell bodies with positive staining), (B) Gad1 (N=12/13 cell bodies with positive staining), and (C) ChAT (N=11/11 cell bodies had no staining). Although a number of neighboring ChAT-positive cell bodies cross over the periphery of the DPM cell bodies resulting in very localized correlation between channels (* in image), the DPMs do not show a general colocalization with anti-ChAT. Bottom: ICA was used to visualize the relative colocalization between DPM>GFP and transmitter staining in pairs of DPMs. (D-E) Left: VT64246-GAL4 was used to drive expression of a presynaptic marker, BRP-short-GFP, in DPM projections to the MB. Middle: Brains were stained with antibodies against (D) ChAT (N=16/16 MB lobe sets with negative staining) and (E) VGAT, with insets showing the MB peduncles (N=10/10 MB lobe sets with positive staining). Right: ICA was used to build false color maps of relative colocalization between DPM>Brps-GFP and transmitter staining in DPM projections. For ICA, orange indicates colocalization/correlation of pixel intensities between channels (PDM>0) and purple indicates a lack of colocalization/anticorrelation of pixel intensities between channels (PDM<0) relative to the scale shown for each image (see Materials and Methods for further details). “α” indicates the MB α’ lobe and “ped” indicates peduncles, shown in the inset.
Figure 4. DPM activation has no excitatory effect on the MBs.

(A) Schematic of genetic set-up for functional imaging experiments. P2X2 receptors were expressed in the DPM neurons, and fluorescent sensors (GCaMP shown) were expressed in the MB neurons. (B) Bath-applied ATP is effective at activating DPMs expressing P2X2 receptors. Mean maximum percentage change in GCaMP3.0 (w-, eyeless-GAL80; UAS-GCaMP3.0/+; c316-GAL4/UAS-P2X2, N = 9 with UAS-P2X2 transgene, 8 without [9, 8], P < 0.001 by Mann-Whitney U test), Arclight (w-, eyeless-GAL80; UAS-Arclight/+; c316-GAL4/UAS-P2X2, N = [11,6], P < 0.001 by Mann-Whitney U test) and Synapto-pHluorin (w-, eyeless-GAL80; UAS-Synapto-pHluorin/+; c316-GAL4/UAS-P2X2, N = [11, 6], P = 0.002 for Mann-Whitney U test) fluorescence in horizontal DPM neuron projections in response to 30 s perfusions of 2.5 mM ATP. (C-E) Black bar denotes time of perfusion of 2.5 mM ATP or vehicle. Insets are histograms summarizing the mean maximum percentage change in fluorescence of the respective sensor. The eyeless-GAL80 and MB247-GAL80 transgenes were used to restrict GAL4 driven expression to DPMs. (C) Mean GCaMP3.0 response traces of w-, eyeless-GAL80; lexAop-GCaMP3.0/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2 (green), or without the UAS-P2X2 transgene (grey), to 30 sec perfusion of 2.5mM ATP or vehicle (black). N = [10, 8], P > 0.05 for Kruskal-Wallis one-way ANOVA, histogram values are 1.9 ± 0.5% (green), 2.6 ± 0.5% (black), 2.7 ± 0.5% (grey). (D) Mean EPAC response traces of w-, eyeless-GAL80; lexAop-EPAC/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2 (orange), or without the UAS-P2X2 transgene (grey), to 30 s perfusion of 2.5 mM ATP or vehicle (black). N = [10, 8], P > 0.05 for Kruskal-Wallis one-way ANOVA, histogram values are 7.4 ± 0.8% (orange), 5.3 ± 0.8% (black), 6.6 ± 0.7% (grey). (E) MBs respond to excitatory inputs. Mean EPAC response traces of w--; R58E02-lexA/lexAop-P2X2; MB247-GAL4/UAS-EPAC (blue), or without the lexAop-P2X2 transgene (grey), to 90 s perfusion of 2.5 mM ATP or vehicle (black). N = [9, 5], P < 0.001 for Mann-Whitney U test, histogram values are 34.6 ± 3.1% (blue), 12.5 ± 1.2% (black), 13.2 ± 4.8% (grey). (C-E) Traces represent ROIs taken from horizontal sections of MB lobes. (C-D) ROIs were also taken from the vertical lobes and no change in fluorescence was seen (data not shown).

Figure 5. DPM activation has an inhibitory effect on the MBs
(A) DPM activation evokes a chloride increase in MBs via that can be reduced by picrotoxin (PTX). Mean SuperClomeleon response traces of w-, eyeless-GAL80; lexAop-SuperClomeleon/MB247-GAL80; c316-GAL4, MB247-lexA/UAS-P2X2 to perfusion of 2.5 mM ATP alone (pink, N = 12) or in bath of 10 μM PTX (green, 10). Negative controls: mean response to ATP without UAS-P2X2 transgene (grey, 8), or vehicle (black, 12). \( P < 0.001 \) between pink and negative controls, \( P = 0.001 \) between pink and green, \( P < 0.01 \) between green and negative controls for Mann-Whitney U test. Histogram values are 20.8±1.4% (pink), 13.2±0.7% (green), 9.7±0.5% (black), 9.9±0.8% (grey). (B) Bath-application of GABA in the presence of TTX evokes a chloride increase in MBs that can be blocked by PTX. Mean SuperClomeleon response traces of w--; lexAop-SuperClomeleon/+; MB247-lexA/+ to perfusion of 1.5 mM GABA alone (orange, 8) or with 10 μM PTX (blue, 8), in 1 μM TTX bath. Negative control: Mean response to vehicle 1 μM TTX bath (black, 8). \( P < 0.001 \) for Mann-Whitney U test. Histogram values are 21.9±1.6% (orange), 8.1±1.1% (blue), 7.6±1.0% (black). (A-B) Black bar denotes time of perfusion. Histograms summarize the mean maximum percent change in fluorescence of SuperClomeleon. Traces represent ROIs taken from vertical sections of MB lobes. ROIs were also taken from the horizontal lobes and similar results were seen (data not shown).

Figure 6. DPM GABA and 5HT promote nighttime sleep

DPM expression of VGAT was reduced by combining two copies of UAS-VGAT-RNAi with each of two different DPM-GAL4 drivers, c316-GAL4 (A) and NP2721-GAL4 (B). Expression levels of TRH where reduced in DPMs by driving UAS-Trh-RNAi with each of two different DPM-GAL4 drivers, c316-GAL4 (C) and NP2721-GAL4 (D). Top: shows total sleep in 30-min bins averaged across three days. Bottom: shows the same data quantified in 12-h day/night bins. In all cases, a decrease in VGAT or 5HT synthetic enzymes (TRH) in DPMs resulted in nighttime sleep loss, with no change in nighttime activity while awake, although increases in daytime activity while awake were often apparent. Grey shading indicates the dark period/night. All data are presented as mean ± SEM where * represents \( P<0.05 \), **\( P<0.001 \) and ***\( P<0.0001 \) using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the Materials and Methods section.
Figure 7. MB α’β’ GABA receptors promote nighttime sleep.
c305a-GAL4 was used to drive expression of Rdl-RNAi (A) or GABA BR3-RNAi (B) in the α’β’ neurons. **Top:** shows total sleep in 30-min bins averaged across three days. **Middle and bottom plots:** show three-day means of total sleep, mean sleep episode duration and number of sleep episodes quantified in 12-h day/night bins. α’β’>Rdl-RNAi causes mild sleep loss and increases in nighttime sleep fragmentation, whereas α’β’>GABA BR3-RNAi causes greater reductions in total sleep due to a decrease in the average sleep episode length. Grey shading indicates the dark period/night. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the Materials and Methods section.

Supplemental Figure Legends

Figure 1, Supplement 1. Comparison of DPM-expressing GAL4 lines used in experiments
**Top:** Representative maximum z-projection images through the central brain of flies from each GAL4 line with the respective promoter-GAL4 driving expression of mCD8-GFP. Asterisks denote DPM cell bodies and brain regions with ectopic GAL4 expression are labeled. **Bottom:** Relative DPM expression strength, relative degree of non-DPM expression, other brain regions with GAL4 expression and a list of experiments in which each GAL4 line was used. Ectopic expression amongst the three GAL4 lines appears to be non-overlapping, suggesting DPMs are the only common locus of expression in these lines. Although addition of ey-GAL80 greatly reduces background expression of the c316-GAL4 insertion, these flies were less healthy than other lines and thus were only used for functional imaging, but not generally for behavior experiments. Addition of the MB-GAL80 transgene was also necessary in the ey-GAL80;; c316-GAL4 line to suppress a low level of GAL4 expression in MB Kenyon cells (the brain shown above does not contain MB-GAL80). Abbreviations: OL-optic lobe, SOG-subesophageal ganglion, AL-antennal lobe, PI-pars intercerebralis.

Figure 1, Supplement 2. DPM activity regulated by a different GAL4 insertion is also sleep-promoting.
(A and C) Flies exhibit gains in sleep when DPM neurons are activated with dTrpA1 at 31°C using the weak, but very clean GAL4 driver, VT64246-GAL4, to drive dTrpA1 expression (w-; UAS-dTrpA1-II; VT64246-GAL4). (B and D) Flies show nighttime sleep loss when DPM depolarization is suppressed following the conditional expression at 31°C of the hyperpolarizing potassium channel, Kir2.1, driven by the weak, but very clean VT64246-GAL4 driver (w-, Tub-GAL80ts; UAS-Kir2.1; VT64246-GAL4). Top plots (A and B): show sleep in 30-min bins during a baseline day (22°C for dTrpA1, 18°C for Tub-GAL80ts; UAS-Kir2.1), followed by 2-3 days of DPM hyperactivation or repression (31°C). Bottom plots (C and D): show a quantification of the 30-min data (C is a quantification of data in A and D is a quantification of data in B) in 12-h bins across 2 or 3 days of heating. Sleep change is quantified as the minutes of sleep gained or lost by the experimental genotype in comparison to either the UAS or GAL4 control genotypes during heating. Grey shading indicates the dark period/night, red bars indicate increased temperature.. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the Materials and Methods section.

Figure 1, Supplement 3. Vesicle release from DPMs promotes consolidated nighttime sleep.

(A and B) Flies show nighttime sleep loss when DPM synaptic release is inhibited with ShiIs at 31°C (w-, ey3.5-GAL80; MB-GAL80; c316-GAL4/ 20xUAS-Tts-ShiIs). Continuing sleep loss is apparent during the first 12 h of recovery following release of ShiIs inhibition. (C and D) Flies exhibit highly fragmented nighttime sleep during the first night of DPM inhibition at 31°C. This impairment of sleep quality is characterized by a dramatic decrease in mean nighttime sleep bout duration (C) and an increase in the number of nighttime sleep bouts (D). Top (A): shows sleep in 30-min bins during a baseline day (18°C), followed by one day of DPM repression (31°C) and one day of recovery (18°C). Bottom plots (B, C and D): show a quantification of 30-min data in 12h-day/night bins. Sleep change, as well as changes in mean sleep episode duration and number of sleep episodes, is quantified as the minutes of sleep or number of sleep episodes gained or lost by the experimental genotype
in comparison to either the UAS or GAL4 control genotypes during heating. Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the Materials and Methods section.

**Figure 1, Supplement 4. An additional UAS transgene does not prevent dTrpA1-induced sleep gains.**

Flies exhibit large gains in sleep when DPM neurons are activated with dTrpA1 at 31°C in the presence of an additional GAL4 target (w-; NP2721-GAL4/ UAS-dTrpA1-II; 20xUAS-IVS-GCaMP6m). **Left plot:** shows sleep in 30-min bins during a baseline day (18°C), followed by 1 day of DPM hyperactivation (31°C) and one day of recovery (18°C). **Right plot:** shows a quantification of the 30-min data in 12-h bins across 1 day of heating and one day of recovery. Sleep change is quantified as the minutes of sleep gained or lost by the experimental genotype in comparison to either the UAS or GAL4 control genotypes during heating and recovery periods. Grey shading indicates the dark period/night, red bars indicate increased temperature. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Calculation of sleep gain or loss and statistics are described in the Materials and Methods section.

**Figure 3, Supplement 1. DPMs are serotonergic, but not dopaminergic**

(A-B) **Top:** VT64246-GAL4 was used to drive expression of membrane-localized mCD8-GFP in DPM cell bodies, which was visualized with an anti-GFP antibody. **Middle:** Brains were stained with antibodies against (A) 5-HT (N=6/6 cell bodies with positive staining) and (B) TH (N=6/6 cell bodies with no staining). Although some TH-positive neurites from other neurons cross over the location of the DPM cell bodies resulting in very localized correlation between channels (* in image), the DPMs do not show a general colocalization with anti-TH **Bottom:** ICA was used to visualize the relative colocalization between DPM>GFP and transmitter staining in pairs of DPMs. For ICA, orange indicates colocalization/correlation of pixel intensities between channels and purple indicates a lack
of colocalization/anticorrelation of pixel intensities between channels relative to the scale shown for each image (see Materials and Methods for further details).

**Figure 3, Supplement 2. MB neurons do not express stimulatory serotonin receptors.**
Mean EPAC response traces of w-; 5HT7-GAL4/+; UAS-EPAC/+ (pink, N = 4), or w-; lexAop-EPAC/+; MB247-lexA/+ (green, N = 10) to 90 s perfusion of 1 mM serotonin (5HT) or vehicle (black, N = 10). Black bar denotes the time of perfusion of 5HT or vehicle. For MB247-lexA, ROIs were taken from both the horizontal and vertical lobes of the MBs. For 5HT7-GAL4, ROIs were taken from the ellipsoid body ring. P = 0.002 for Mann-Whitney U test. Histogram summarizes mean maximum percentage change in EPAC fluorescence, values are 40.6 ± 5.3% (pink), 6.7 ± 0.6% (green), 7.4 ± 0.6% (black).

**Figure 4, Supplement 1. DPM activation has no excitatory effect on the MBs.**
(A-D) Black bar denotes time of perfusion of 2.5 mM ATP or vehicle. Histograms summarize mean maximum percentage change in fluorescence of respective sensor. (A) Bath-applied ATP is effective at activating DPMs expressing P2X2 receptors. Mean GCaMP3.0 response traces of w-; UAS-GCaMP3.0/NP2721-GAL4; UAS-P2X2/MB247-lexA to 30 s perfusion of 2.5 mM ATP (pink) or vehicle (black). N = 6 for ATP responses, 3 for vehicle responses, P = 0.02 for Mann-Whitney U test, histogram values are 23.7 ± 2.1% (pink), 1.2 ± 0.7% (black). (B) Mean GCaMP3.0 response traces of w-; lexAop-GCaMP3.0/NP2721-GAL4; UAS-P2X2/MB247-lexA (green), or without UAS-P2X2 transgene (grey), to 30 s perfusion of 2.5 mM ATP or vehicle (black). N = 5 with UAS-P2X2 transgene, 5 without [5, 5], P > 0.05 for Kruskal-Wallis one-way ANOVA, histogram values are 1.7 ± 0.3% (green), 1.5 ± 0.8% (black), 1.2 ± 0.5% (grey). (C) Mean EPAC response traces of w-; lexAop-EPAC/NP2721-GAL4; UAS-P2X2/MB247-lexA (orange), or without UAS-P2X2 transgene (grey), to 90 s perfusion of 2.5 mM ATP or vehicle (black). N = [8, 6], P > 0.05 for Kruskal-Wallis one-way ANOVA, histogram values are 11.4 ± 0.9% (orange), 9.8 ± 1.0% (black), 11.4 ± 1.1% (grey). (B-C), ROIs were also taken from the vertical lobes and no change in fluorescence was seen (data not shown).
Figure 4, Supplement 2. Bath-applied carbachol (CCh) evokes an excitatory response in MBs

(A) MBs respond to CCh perfusion in the presence of TTX. Mean GCaMP3.0 response traces of w-; NP2721-GAL4/lexAop-P2X2; MB247-lexA/UAS-GCaMP3.0 to 30 s perfusion of 100 μM CCh (blue) or vehicle (black) in 1μM TTX bath. N = 8, P < 0.001 for Mann-Whitney U test, histogram values are 126.7 ± 7.5% (blue), 2.3 ± 0.5% (black). (B) Mean EPAC response traces of w-; lexAop-EPAC/+; MB247-lexA/+ to 30 s perfusion of 100 μM CCh (yellow) or vehicle (black) in 1μM TTX bath. N = 8, P < 0.001 for Mann-Whitney U test, histogram values are 90.3±5.0% (yellow), 4.4±0.6% (black). (A-B), traces represent ROIs taken from horizontal sections of MB lobes.

Figure 6, Supplement 1. APL GABA can sometimes promote nighttime sleep

APL expression levels of VGAT were reduced by combining two copies of UAS-VGAT-RNAi with each of three different APL-GAL4 drivers, NP5288-GAL4 (A), GH146-GAL4 (B) and NP2631-GAL4 (C). Top: shows total sleep in 30-min bins averaged across three days. Bottom: shows the same data quantified in 12-h day/night bins. In some, but not all cases, APL>VGAT-RNAi resulted in modest decreases in nighttime sleep, which was often accompanied by concurrent increases in nighttime activity while awake (for NP5288: P_{GAL4}<0.001, P_{UAS}<0.05). Grey shading indicates the dark period/night. All data are presented as mean ± SEM where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the Materials and Methods section.

Figure 7, Supplement 1. Sleep loss resulting from Rdl/GABAB_{R3}-RNAi is primarily due to MB αβε expression.

MB-GAL80 restricted c305a-GAL4 was used to drive Rdl-RNAi (A) or GABA_{BR3}-RNAi (B) in the c305a expression pattern outside of the MB. Top: shows total sleep in 30-min bins averaged across three days. Middle and bottom plots: show 3 day means of total sleep, mean sleep episode duration and number of sleep episodes quantified in 12-h day/night bins. Non-MB c305a driven Rdl-RNAi results in a lesser degree of sleep loss and
fragmentation and non-MB \textit{GABAB}_{2'}-RNAi results in no changes in sleep or sleep structure, indicating these phenotypes are primarily due to MB \(\alpha'\beta'\) expression of c305a\textit{-GAL4}. Grey shading indicates the dark period/night. All data are presented as mean \pm \text{SEM} where * represents P<0.05, **P<0.001 and ***P<0.0001 using the Mann-Whitney-Wilcoxon rank sum test. Statistics are described in the Materials and Methods section.

Movie 1. DPM\textgreater{} \textit{dTrpA1} activation induces sleep, but not locomotor impairment. Groups of ten individual female flies with DPM\textit{(NP2721)}\textgreater{} \textit{dTrpA1} (center), \textit{UAS-dTrpA1} (left) or DPM \textit{(NP2721)}\textit{-GAL4} (right) were kept at 31\textdegree{}C for 2 h before video recording. Flies with DPM driven dTrpA1 expression show normal locomotion when gently tapped (0:00:01), but quickly assume a stationary resting position after \(~30\) s undisturbed (0:00:35), whereas control flies remain awake and continue to explore the environment. All flies were anesthetized with CO\textsubscript{2}, counted and sorted into groups of ten one day prior to video recording and kept on food at 22\textdegree{}C prior to heating. Flies were heated at 31\textdegree{}C on food for 2 h and flipped to empty vials just prior to recording.

Source Code – Custom software developed in MATLAB
A

Baseline: 22°C  dTrpA1 activation: 31°C  Recovery: 22°C

Sleep (min/30min)

0 6 12 18 0 6 12 18 0 6 12 18 0 6 12 18

ZT (hours)

B

MB-restricted α'β' > dTrpA1
GAL4/LexA control
UAS/LexAop control

NS

Minutes of Sleep Gained or Lost vs Controls

31°C  22°C
Figure A: SuperCloFRET (CFP/YFP) over time for different conditions:
- DPM>P2X2, MB>SuperClomeleon, +ATP, -PTX
- DPM>P2X2, MB>SuperClomeleon, +ATP, +PTX
- Negative control, +P2X2, -ATP
- Negative control, -P2X2, +ATP

Figure B: SuperCloFRET (CFP/YFP) over time for different conditions:
- MB>SuperClomeleon, +GABA, -PTX
- MB>SuperClomeleon, +GABA, +PTX
- Negative control, -GABA
**A**

- **Total Sleep (min)**
  - Day Night
  - Purple: αβ > Rdl-RNAi
  - Purple: αβ'-Gal4
  - Gray: Rdl-RNAi
  - Gray: αβ'-Gal4

- **Mean Sleep Episode Duration (min)**
  - Day Night
  - Purple: αβ > Rdl-RNAi
  - Purple: αβ'-Gal4
  - Gray: Rdl-RNAi
  - Gray: αβ'-Gal4

- **Number of Sleep Episodes**
  - Day Night
  - Purple: αβ > GABA_{BR3} RNAi
  - Purple: αβ'-Gal4
  - Gray: GABA_{BR3} RNAi
  - Gray: αβ'-Gal4

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**B**

- **Total Sleep (min)**
  - Day Night
  - Purple: αβ > Rdl-RNAi
  - Purple: αβ'-Gal4
  - Gray: Rdl-RNAi
  - Gray: αβ'-Gal4

- **Mean Sleep Episode Duration (min)**
  - Day Night
  - Purple: αβ > Rdl-RNAi
  - Purple: αβ'-Gal4
  - Gray: Rdl-RNAi
  - Gray: αβ'-Gal4

- **Number of Sleep Episodes**
  - Day Night
  - Purple: αβ > GABA_{BR3} RNAi
  - Purple: αβ'-Gal4
  - Gray: GABA_{BR3} RNAi
  - Gray: αβ'-Gal4