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Notch signaling modulates sleep homeostasis and learning after sleep deprivation in *Drosophila*

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Summary

The role of the trans-membrane receptor *Notch* in the adult brain is poorly understood. Here, we provide evidence that *bunched*, a negative regulator of *Notch* is involved in sleep homeostasis. Genetic evidence indicates that interfering with *bunched* activity in the mushroom bodies (MBs) abolishes sleep homeostasis. Combining *bunched* and *Delta* loss-of-function mutations rescued normal homeostasis, suggesting that *Notch* signaling may be involved in regulating sensitivity to sleep loss. Preventing the down regulation of *Delta* by over-expressing a wild-type transgene in MBs reduces sleep homeostasis and, importantly, prevents learning impairments induced by sleep deprivation. Similar resistance to sleep loss is observed with the $Notch^{spl\text{-}1}$ gain-of-function mutants. Immunohistochemistry reveals that the *Notch* receptor is expressed in glia, whereas *Delta* is localized in neurons. Importantly the expression of the intracellular domain of *Notch*, a dominant activated form of the receptor, in glia is sufficient to prevent learning deficits after sleep deprivation. Together these results identify a novel neuronal-glia signalling pathway dependent on *Notch* and regulated by *bunched*. These data highlight the emerging role of neuron-glia interactions in regulating both sleep and learning impairments associated with sleep loss.

Results and discussion

Mutations in *bunched*, a regulator of *Notch* signaling, affect sleep homeostasis

We, and others, have used microarrays to identify genes that are induced following sleep deprivation [1, 2]. Among the genes that have been identified, we found that *bunched*, a transcription factor regulating *Notch* activity in follicle cells [3], is up-regulated after sleep deprivation in fly heads (Figure 1A, left). *bunched* mRNA was also elevated in response to mechanical stimulation and exposure to oxidative stress suggesting that it is sensitive to physiological challenges in general (Figure 1A, left). Interestingly, mRNA for the *bunched* human homolog *TSC-22D* is detected in human saliva and is also up-regulated in sleep deprived healthy subjects (Figure 1A, right). In addition, TSC22D3 transcripts have been

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The supplemental information contains two Supplemental tables and four Supplemental Figures as well as Supplemental Experimental Procedures, and Supplemental References:

found to be up-regulated in the brain of sleep deprived mice [4]. Together, these results suggest that *bunched* function in response to sleep deprivation may be phylogenetically conserved. Nevertheless, it is important to note that gene profiling experiments are correlative in nature and do not necessarily reflect a change in gene activity. To determine whether *bunched* might, in turn, influence the response to sleep loss, we evaluated sleep homeostasis in several viable P element lines inserted in the *bunched* locus: $bun^{BG01623}$, $bun^{KG06590}$, $bun^{KG00456}$ and $bun^{KG00392}$. Sleep homeostasis is defined as a compensatory increase in sleep above baseline that occurs in the days following sleep loss [5-8]. The increase in sleep is highest immediately following sleep deprivation and progressively returns to pre-deprivation levels [5-8]. For comparisons across genotypes, individual flies are required to lose >90% of their nighttime sleep quota and the percentage of sleep recovered is calculated by dividing the minutes of sleep reclaimed during 48 h of recovery by the minutes of sleep lost; this metric represents a sensitive and reliable mean to assess sleep homeostasis [5, 8-10]. As seen in Supplemental Figure S1, all the *bunched* mutant lines showed a reduced homeostatic response to sleep deprivation. Excision of the P element in one of these lines, $bun^{BG01623}$, restored normal homeostasis, indicating that the phenotype is due to the P element insertion (Figure 1B).

The MBs have been shown to regulate sleep [11, 12], Thus, to determine if *bunched* is required in the MB for the regulation of sleep homeostasis, we used the 247-GAL4 driver to express a wild-type copy of the *bunched* coding sequence in a *bunched* mutant background. $bun^{BG01623}$ bears a GAL4 driver and could not be used. The $bun^{KG06590}$ allele was used instead. Similarly to $bun^{BG01623}$, $bun^{KG06590}$ homozygous flies display a total lack of sleep homeostasis (Figure 1C, Supplemental Figure S1). The phenotype persists and was even enhanced if the allele is placed over a deficiency covering *bunched* (Figure 1C). This enhanced phenotype may be consequence of the inactivation of other genes deleted in the deficiency or may reflect the hypomorphic nature of $bun^{KG06590}$ which results from a P element insertion in the last intron of *bun* (Figure S1). As shown in Figure 1D, $bun^{KG06590}$; *247/+* and $bun^{KG06590}$; *UAS-bun2/+* had an increased homeostatic response compared to the $bun^{KG06590}$ strain. However, $bun^{KG06590}$;*247/UAS-bun2* flies displayed a dramatically increased homeostatic response compared to both $bun^{KG06590}$; *247/+ and* $bun^{KG06590}$; *UAS-bun2/+* control lines, suggesting that activating *bunched* function in the MB is sufficient to increase the sleep homeostatic response. To confirm the requirement of *bunched* function in the MB, we expressed a dominant negative *bunched* construct, *UAS-bunX* [13], in the MB. Driving *UAS-bunX* in MB with two different GAL4 drivers resulted in a reduction in the homeostatic response to sleep loss compared to genetic controls, as anticipated (Figure 1E-F). Normal sleep rebound was observed when *bunched* was disrupted in the adult by feeding *MB-Switch/UAS-bunX* flies RU486 (not shown, [14]). The normal sleep rebound could reflect either an insufficient level of induction using this driver, or that disrupting *bunched* during development disrupts circuits that influence sleep regulation.

Sleep homeostasis and learning impairments after sleep loss in mutations affecting the Notch signaling

During oogenesis, *bunched* regulates *Notch* signaling [3], such that a loss of *bunched* function results in an up-regulation of the *Notch* pathway. Consistent with this idea, combining $bun^{KG06590}$ with the amorphic $Delta^{X}$ mutant rescued normal homeostasis (Figure 2A), suggesting that the *bunched* sleep homeostasis phenotype is linked to increased activation of *Delta*. Since expressing a dominant negative allele of *bunched* in the MBs reduces sleep homeostasis, we asked whether the activation of the Notch pathway in the MBs could protect against the negative effects of sleep loss. To begin, we first over-expressed the ligand *Delta* locally in the MB. Interestingly, over-expressing *Delta* in the MBs using the MB specific *247*-GAL4 driver and a previously validated *UAS-Delta*

construct (M.H insertion [15]) was sufficient to block the homeostatic response to sleep loss while both parental lines (*247/+* and *UAS-Delta/+*) displayed a wild-type sleep rebound (Figure 2B). Similar results were obtained with another *UAS-Delta* construct (T.J insertion on the X chromosome [16], data not shown). Similar results were obtained when *UAS-Delta* was induced only at the adult stage using the *MB-Switch* GAL4 driver ([14], Figure 2C).

The lack of a homeostatic response may indicate that the animal is better able to withstand the negative effects of waking, or it may simply reflect a physiological impairment that globally disrupts sleep regulatory processes. To distinguish between these two possibilities we evaluated learning using Aversive Phototaxic Suppression (APS) [17]. We have recently shown that APS is sensitive to both sleep loss and sleep fragmentation [18]. Although both parental lines were impaired following 12 h of sleep deprivation, *UAS-Delta/+*; *247/+* flies maintained their ability to learn (Figure 2D). The Phototaxis Index (PI) and Quinine Sensitivity Index (QSI) for all lines were in the normal range of wild-type flies previously reported indicating that differences in performance cannot be attributed to changes in sensory thresholds (Supplemental Table S2) [1, 18, 19].

To confirm that activation of the *Notch* pathway regulates sensitivity to sleep deprivation and sleep homeostasis we evaluated sleep homeostasis in Notch gain-of-function mutants (N^{Ax59b}, $N^{spl\text{-}1}$). N^{Ax59b} flies were tested as heterozygous and exhibited a wild-type homeostatic response to sleep deprivation (Supplemental Table S1) presumably because the presence of wild type Notch reduces the dominant phenotype of this allele [20, 21]. However, the $N^{spl\text{-}1}$ allele did not compensate for lost sleep by initiating a homeostatic response (Figure 2E). Moreover, $N^{spl\text{-}1}$ flies maintained their ability to learn after sleep deprivation suggesting that the $N^{spl\text{-}1}$ mutation is protecting flies from the effects of sleep loss (Figure 2F). PI and QSI were in the normal range for $N^{spl\text{-}1}$ flies indicating that the changes in performance were not due to alterations in sensory thresholds (Supplemental Table S1). $N^{spl\text{-}1}$ encodes a Notch receptor with a single amino acid substitution in the extracellular domain, introducing a new O-fucosylation site [22, 23]. The effect of the $N^{spl\text{-}1}$ is context specific [21, 23]. A study of the effect of $N^{spl\text{-}1}$ on R8 photoreceptor development suggested that the mutation is increasing Notch sensitivity to the ligand Delta, leading to an ectopic activation of Notch in the R8 precursors cells [23].

We next tested several hypomorph/loss of function alleles (N^{nd2}, N^{nd1}, N^{5419}, N^{Co}). Except N^{nd1}, these alleles are homozygous lethal and were tested as heterozygotes. Surprisingly, all of these alleles showed a wild-type homeostatic response to sleep loss (Supplemental Table S2). The presence of a wild type *Notch* allele may have been sufficient to compensate for the effect of the mutation. Alternatively, the observed phenotypes could be attributed to developmental defects. Thus, we evaluated learning using the temperature-sensitive *Notch* allele N^{ts1} [24]. N^{ts1} flies showed normal APS performance at 23°C (Figure 2G, right graph). In contrast siblings that were tested at 31°C showed severe learning impairments. Genetic control flies (*y v*) showed normal performance both at 23°C and 31°C (Figure 2G, left graph). Importantly, N^{ts1} learning impairments at 31°C were not due to deficits in PI and QSI, which were in the normal range (Supplemental table S2), and could be rescued by introducing a duplication of the *Notch* locus in the genetic background (Figure 2G, right graph). In addition, similar learning impairments were observed with another thermo-sensitive allele: N^{ts2} (Supplemental Figure S3A). These results indicate that *Notch* is required for learning in the adult fly, in the absence of developmental defects. Given that temperature alters sleep homeostasis [10], we did not evaluate sleep rebound in N^{ts1} mutants.

Notch and Delta immuno-localization in the adult brain

Whole brain immuno-histochemistry revealed different patterns of localization for Notch and Delta proteins (Figure 3A-I). Delta is expressed in a punctuate pattern throughout the brain cortex (Figure 3A, Supplemental Figure S3B shows an overall view) and is clearly detected in the cell bodies of Kenyon cells (Figure 3B-C). On the other hand, the Notch intracellular domain is predominantly detected in membranes surrounding the brain neuropiles and the cell bodies in the cortex, a pattern overlapping with glial cell membranes (Figure 3D-F, Supplemental Figure S3B shows an overall view). These results indicate that Notch and Delta could mediate neuron-glia signalling through cell-cell contacts. Such a possibility had already been suggested for the larval brain [25-27]. To further localize Notch activity in the adult brain, we have evaluated the expression of a Notch-reporter construct: *Su(H)Bs-lacZ* [28]. In this construct, *lacZ* is under the control of a promoter containing several *Suppressor of Hairless* (*Su(H)*) binding sites, and is induced following Notch activation. As shown in Figure 3J-L, *Su(H)Bs-lacZ* is specifically expressed in a subset of repo-positive glial cells, thus indicating that the Notch receptor is activated in glia. Similar results were obtained with a related Notch reporter construct driving EGFP expression ([29]; not shown).

Activating the Notch in glia abolishes sleep homeostasis and learning impairments after sleep loss

Upon binding to its trans-membrane ligand, Notch undergoes three rounds of cleavages leading to the shedding of the extracellular domain and the release of the intracellular domain into the cytoplasm. The intracellular domain associates with the transcription factor *Su(H)* and activates the transcription of targets genes such as members of the *Enhancer of split* complex [30]. Thus expressing the intracellular domain of *Notch* is sufficient to activate the pathway [31, 32]. We expressed the intracellular domain of Notch using a *UAS-NICD* (Notch Intra-Cellular Domain) and the glia-specific *Eaat1-GAL4* driver [33]. As shown in Figure 4A, *UAS-NICD/+*; *Eaat1-GAL4/+* flies did not show a homeostatic response to sleep deprivation. In addition, *UAS-NICD/+; Eaat1-GAL4/+* flies showed normal learning after sleep deprivation (Figure 4B). In contrast, the genetic background controls showed a sleep rebound and learning impairments after sleep deprivation. Expressing *UAS-NICD* in neurons using *elav-Switch* did not alter sleep homeostasis (Supplemental Figure S4). Altogether, the data suggest that activating *Notch* signaling in glia can modulate the response to sleep loss as measured by sleep homeostasis and learning.

Conclusion

The evidence presented here suggests that *Notch* signaling controls factors that reduce the negative consequences of waking as measured by an attenuated sleep rebound and intact learning following 12 h of sleep deprivation. Although it is tempting to speculate that the intact learning seen following sleep loss is simply due to the flies not being sleepy, our previous studies have shown that sleepiness does not result in performance impairments in the APS [18]. Thus, Notch activation may preserve learning by preventing neuronal over-stimulation during extended waking. Reducing neuronal stimulation may also prevent the build-up of sleep debt and thus explain the lack of sleep rebound. Canonical *Notch* signaling leads to *Su(H)*-dependent changes in transcription but several other downstream pathways have been identified [30, 34], thus, further work is required to determine which pathway downstream of the receptor is effectively involved in this context. Our results suggest that *Notch* is mediating a neuron-glia signaling mechanism. These data provide additional support to recent work showing an involvement of glia in sleep homeostasis and cognitive impairments [35]. In mammals, adenosine released by glia appears to play a critical role [35]. Given that mutants for the only known *Drosophila* adenosine receptor have normal

sleep homeostasis [36] other factors are likely to be involved. It is interesting to note in this context that expression of the cell adhesion molecule klingon, required for long term memory and controlled by *Notch* in the adult brain, has been reported to be expressed in the glia [37]. It should be noted that Notch localization and activation in glia may seem at odds with reports showing a requirement for *Notch* as well as the downstream effector *Su(H)* in MB neurons for memory consolidation [24, 38]. Our data do not exclude a low level of Notch expression in neurons. In fact, it would not be surprising that *Notch* is expressed in both cell types and mediate a two-way signaling between adjacent cells, as it occurs commonly during developmental processes [39].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Mutations in bunched, a regulator of Notch, affect sleep homeostasis
- Notch signaling can alter sleep homeostasis and sleep loss induced learning deficits
- Notch is present in glial processes while Delta is predominantly in neuronal cell bodies
- Results suggest that Notch mediate a neuron-glia pathway involved in sleep and learning

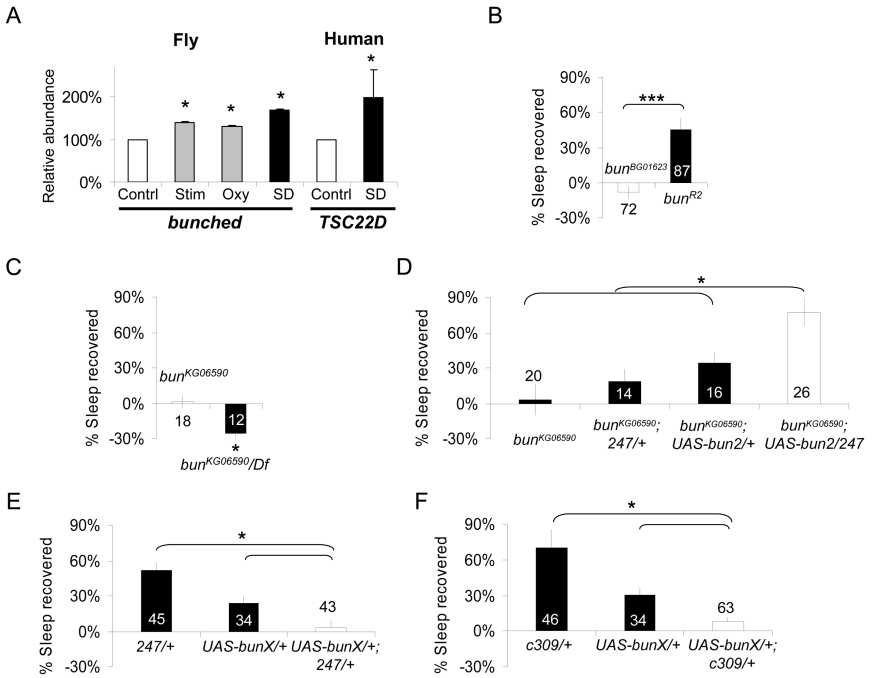

Figure 1. *bunched* is induced by sleep deprivation and regulates sleep homeostasis

(A) *bunched* (left) mRNA levels are increased following 12hSD (SD, dark bar), compared to untreated controls (Contrl., white bar). *bunched* mRNA levels are also increased following a mechanical stimulation that reduces sleep disruption (Stim., grey bar), or following a 12h of exposure to oxidative stress (Oxy., grey bar). For the stimulation control, flies received the same number of mechanical stimuli provided during sleep deprivation by exposing them to the SNAP at twice the speed for 30min every two hours for 24 hours. For oxydative stress, flies were fed 20 μM paraquat dissolved in 1% agar/5% sucrose overnight (12h). mRNA for the *bunched* homolog *TSC22D* is elevated in saliva following 28h of waking in humans (n=9; Wilcoxon signed rank test, p=.038) Each saliva sample collected after sleep deprivation was compared to a circadian matched baseline sample from the same subject. Levels are expressed as % of baseline expression. *p<.05.

(B) $bun^{BG01623}$ mutant flies do not display a sleep rebound following 12h of sleep deprivation, but excision of the P element in the bun^{R2} flies restores sleep rebound. *: p<0.05; Student's t-test.

(C) $bun^{KG06590}$ flies (left) show no sleep rebound after sleep deprivation. $bun^{KG06590}$ crossed to the *Df(2L)prd1.7*(Df) covering the *bunched* locus also failed to show a rebound after sleep deprivation ($bun^{KG06590}/Df$, black bar, left graph). *p<.05, Student's t-test.

(D) Expression of a *UAS-bun2* construct in the MB, using the *247-GAL4* driver is sufficient to increase sleep rebound in the $bun^{KG06590}$ mutant background. ANOVA $F_{(3,73)}=8.4$; p=7.18E-5.

(E) and (F) Expressing a dominant negative *bunched* construct (*UAS-bunX*) in the MBs using either the 247 (E) or c309 (F) GAL4 drivers (white bars in both graphs) is sufficient to reduce sleep homeostasis compared to genetic background controls (black bars). $F_{(2,121)}=12.12$; p=1.6E-05 and $F_{(2,142)}=12.65$; p=8.89E-06, respectively; *p<.05 modified Bonferroni test.

n is indicated in or beside each bar. mean±s.e.m is shown. See also Supplemental Figure S2 and Supplemental Table S1 for additional sleep data.

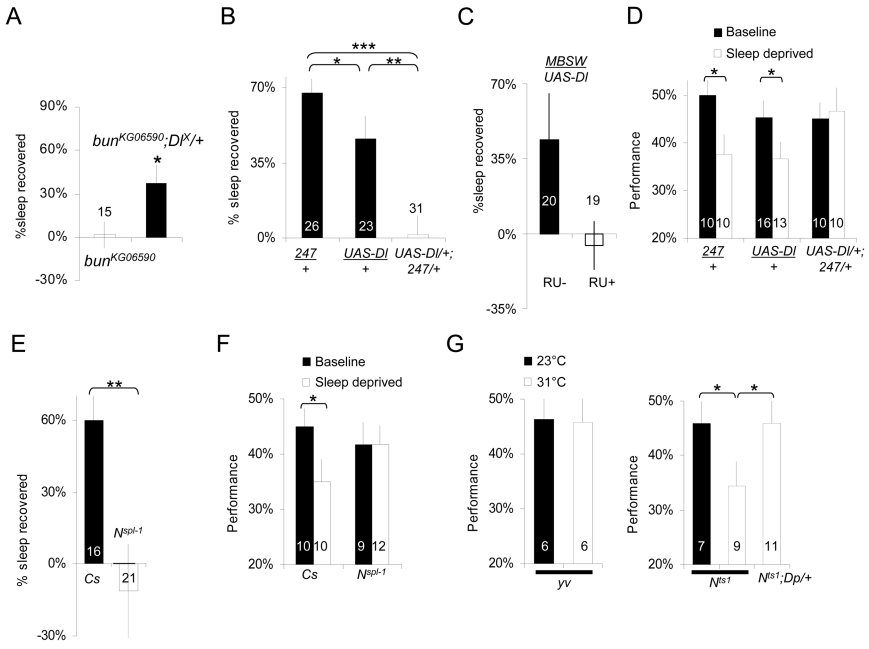

Figure 2. *Notch* regulates learning and sleep homeostasis after sleep deprivation

(A) $bun^{KG06590}$ flies (white bar, left) show no sleep rebound after sleep deprivation. Combining $bun^{KG06590}$ to $Delta^{X}$(Dl^{X}) rescues normal homeostatic response ($bun^{KG06590}$; Dl^{X}/+, black bar). *p<.05, Student's t-test.

(B) Over-expression of *Delta* using the MB specific driver *247* abolishes the sleep rebound (right, white bar). *247/+* and *UAS-Dl/+* parental controls show a wild-type sleep homeostatic responses (black bars); $F_{(2,77)}$=15.9; p=1,69E-06; *<.05, **<.005, ***<.0005 modified Bonferroni test, n is indicated in each bar. Sleep homeostasis is calculated for each individual as a ratio of the minutes of sleep gained above baseline during the 48 h of recovery divided by the total min of sleep lost during 12 h of sleep deprivation.

(C) Over-expression of *Delta* only at the adult stage using the *MB-Switch* GAL4 driver. Flies were fed RU 486 (RU+) or control food (RU-) for 48h before sleep deprivation (see methods). *p<.05 Student's t-test.

(D) Sleep deprivation does not disrupt learning in UAS-Dl/+;247/+ flies tested in the APS. In contrast both parental lines (*247/+* and *UAS-Dl/+*) show learning impairments following 12 h of sleep deprivation; *<.05 modified Bonferroni test.

(E) Flies bearing the gain of function $Notch^{spl-1}$(N^{spl-1}) allele do not show a sleep homeostatic response after 12 h of sleep deprivation compared to Cs controls; *<.05 t-test.

(F) N^{spl-1} mutant flies (right) do not show learning impairments following 12h of sleep deprivation while Cs flies show significant impairments; *p<.05 Bonferroni test.

(G) Learning is not impaired by temperature in *yv* control flies (left graph); 23°C (black) vs. 31°C (white) p>.05, t-test. *y* $Notch^{ts1}$ *v* flies (N^{ts1}, right graph) learn normally at 23°C (permissive temperature), and are impaired at 31°C (non permissive temperature). A duplication covering the *Notch* locus, *Dp (1;2) w+51b*, rescues normal learning at 31°C (N^{ts1}; *Dp/+*, right bar); $F_{(2,24)}$=5.9; p=0.008; *p<.05 modified Bonferroni test.

n is indicated in or beside each bar. mean±s.e.m is shown. See also Supplemental Figure S2 for sleep in min/h graphs and Supplemental Table S2 for control metrics.

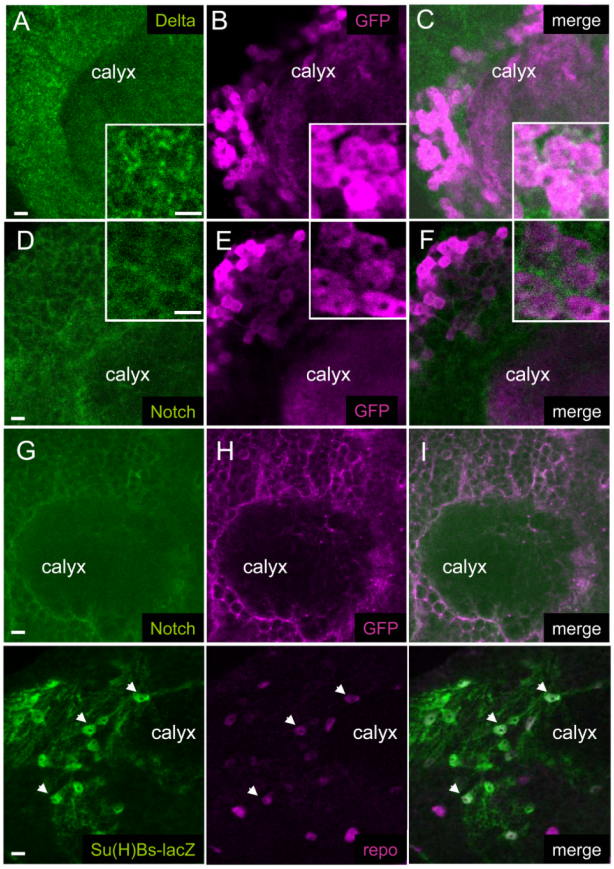

Figure 3. Notch and Delta immuno-localization in the adult brain

(A) to (I), confocal images showing the calyx, input neuropile of the MB and the surrounding neuronal cell bodies.

(A) to (F) Co-labelling of Delta (A,C, green) or Notch (D,F, green) and of GFP (B,C,E,F, magenta) in *247>UAS-GFP* brains to identify a subset of Kenyon cells. Insets show high magnification views of the cell body region. Delta is localized in punctae within neuronal cell bodies whereas Notch is localized primarily in the surrounding membranes. Both Notch and Delta are only weakly expressed in the calyx neuropile itself.

(G) to (I) Co-labelling of Notch (green) and CD8-GFP (magenta) in a *repo-GAL4>UAS-CD8-GFP* brain to reveal co-localization of Notch with glial membranes.

(J) to (L) Co-labelling for the Notch reporter Su(H)Bs-lacZ (green) and the glial-specific repo (localized in glial nuclei, magenta). Arrows show examples of co-localization. All *Su(H)Bs-lacZ* positive cells were labeled with repo.

Bar: 5μm

See also Supplemental Figure S3B for an overall view of the brain.

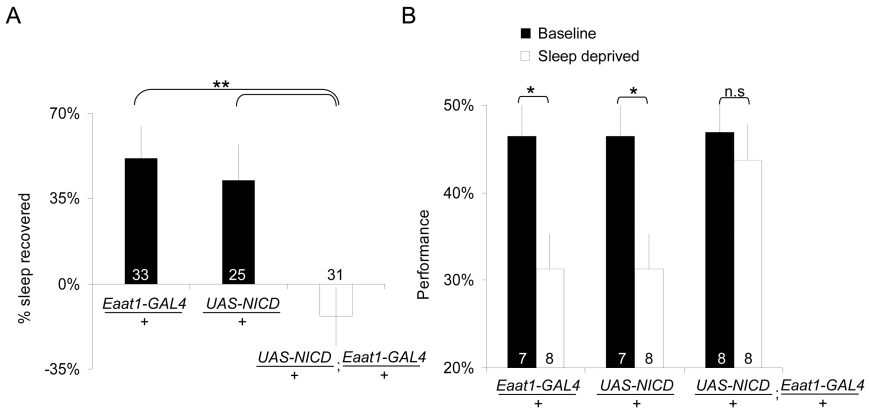

Figure 4. Expressing Notch intracellular domain in glial cells prevents impairments after sleep deprivation

(A) Expression of Notch intracellular domain *(NICD)* using the glial specific driver *Eaat1-GAL4* abolishes the sleep rebound (right, white bar). *Eaat1-GAL4/+* and *UAS-NICD/+* genetic controls show homeostatic responses comparable to wild type flies (black bars). $F(_{2,86})=7.35$; p=0.001; **p<.005 modified Bonferroni test; n is indicated in each bar.

(B) *UAS-NICD/+; Eaat1-GAL4/+* flies do not show any significant impairment in learning following 12 h of sleep deprivation while performance is significantly impaired in both parental lines (*Eaat1-GAL4/+* and *UAS-NICD/+*) after sleep loss (black vs. white); 2 way Genotype by Condition ANOVA shows main effect for condition $F(_{1,46})=12.9$; p=0.001; *p<.05 modified Bonferroni test.

n is indicated in or beside each bar. mean±s.e.m is shown. See also Supplemental Table S2 for control metrics and Supplemental Figure S2 for sleep in min/h graphs.