

# Amyloid Precursor Protein in *Drosophila* Glia Regulates Sleep and Genes Involved in Glutamate Recycling

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Amyloid precursor protein (App) plays a crucial role in Alzheimer's disease via the production and deposition of toxic  $\beta$ -amyloid peptides. App is heavily expressed in neurons, the focus of the vast majority of studies investigating its function. Meanwhile, almost nothing is known about App's function in glia, where it is also expressed, and can potentially participate in the regulation of neuronal physiology. In this report, we investigated whether *Appl*, the *Drosophila* homolog of *App*, could influence sleep–wake regulation when its function is manipulated in glial cells. *Appl* inhibition in astrocyte-like and cortex glia resulted in higher sleep amounts and longer sleep bout duration during the night, while overexpression had the opposite effect. These sleep phenotypes were not the result of developmental defects, and were correlated with changes in expression in glutamine synthetase (GS) in astrocyte-like glia and in changes in the gap-junction component innexin2 in cortex glia. Downregulating both GS and innexin2, but not either one individually, resulted in higher sleep amounts, similarly to *Appl* inhibition. Consistent with these results, the expression of GS and innexin2 are increased following sleep deprivation, indicating that GS and innexin2 genes are dynamically linked to vigilance states. Interestingly, the reduction of GS expression and the sleep phenotype observed upon *Appl* inhibition could be rescued by increasing the expression of the glutamate transporter dEaat1. In contrast, reducing dEaat1 expression severely disrupted sleep. These results associate glutamate recycling, sleep, and a glial function for the App family proteins.

**Key words:** amyloid precursor protein; *Drosophila*; glia; glutamate; sleep/wake

## Significance Statement

The amyloid precursor protein (App) has been intensively studied for its implication in Alzheimer's disease (AD). The attributed functions of App are linked to the physiology and cellular biology of neurons where the protein is predominantly expressed. Consequences on glia in AD are generally thought to be secondary effects of the pathology in neurons. Researchers still do not know whether App plays a role in glia in nonpathological conditions. We report here that glial App plays a role in physiology and in the regulation of sleep/wake, which has been shown recently to be involved in AD pathology. These results also associate glutamate recycling and sleep regulation, adding further complexity to the physiological role of App and to its implication in AD.

## Introduction

Since the identification 20 years ago of *App*, the gene for amyloid precursor protein (App), the accumulating evidence of its in-

volvement in Alzheimer's disease (AD), the main cause of age related dementia worldwide, has attracted much attention. The production of particular  $\beta$ -amyloid peptides in neuronal cells by sequential cleavage of App, and their deposition in senile plaques, has been shown to have toxic consequences in a diversity of cell culture and *in vivo* model systems, including *Drosophila*. Age, genetic, and environmental factors are known to increase the severity of the pathophysiological processes triggered by  $\beta$ -amyloid peptides. In recent years, the hypothesis that sleep plays a critical and potentially bidirectional role in this context has received increasing support (Ju et al., 2013). Sleep is significantly disrupted in AD patients, and the excessive production of  $\beta$ -amyloid peptides has been shown to disrupt sleep in animal models (Ju et al., 2014; Tabuchi et al., 2015; Gerstner et al., 2016). Conversely, the clearance of  $\beta$ -amyloid peptides from the interstitial fluid and CSF is facilitated during sleep (Kang et al., 2009; Xie et al., 2013). In addition, a recent report using *Drosophila*

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suggests that accumulation of  $\beta$ -amyloid peptides is increased following sleep deprivation and is linked to neuronal hyperactivity (Tabuchi et al., 2015). App, as well as the paralogs Aplp1 and Aplp2, are evolutionary conserved cell-surface proteins that have been attributed multiple functions, including cell adhesion, interactions with the extracellular matrix, cell signaling, and the regulation of vesicular trafficking. In mouse knock-out models as well as in *Drosophila*, where a single App-like protein is expressed (App); Luo et al., 1990; Martin-Morris and White, 1990), studies have shown that these proteins modulate intracellular signaling, gene expression, neurite outgrowth, synaptic function, and plasticity (Allebrandt et al., 2013). Inhibition of *Appl* expression in *Drosophila* mushroom body neurons, a central brain structure that plays a key role in learning/memory as well as in sleep regulation, results in long-term memory deficits (Goguel et al., 2011; Bourdet et al., 2015a), which is similar to previous findings obtained in mouse *App* knock-out models (Dawson et al., 1999; Phinney et al., 1999). These results suggest that the loss of *App* function could contribute to the memory loss observed in AD patients and may be in synergy with the accumulation of abnormal  $\beta$ -amyloid peptides (Bourdet et al., 2015b). Nevertheless, the physiological functions of the App family of proteins and the extent to which these functions are disrupted in AD by toxic  $\beta$ -amyloid peptides remain unclear (Phinney et al., 1999). In the brain, *App* is heavily expressed in neurons, the focus of most studies investigating *App*'s function and the location of the majority of *App*'s molecular partners identified so far (Martin-Morris and White, 1990; Apelt et al., 1997; Allebrandt et al., 2013). In contrast, almost nothing is known about the role of *App* in glia, where it is also expressed and where it can potentially participate in the regulation of neuronal physiology (Grolla et al., 2013), with consequences on behavior. In this study, we investigated the potential role of endogenous glial *Appl* in sleep/wake regulation, and glial gene expression changes linked to glutamate recycling and gap junctions. These results extend the functional complexity of the role played by the App family of proteins.

## Materials and Methods

**Fly stocks.** The following fly stocks were used: *CantonS* (*Cs*), *Inx2<sup>BG02429</sup>* (RRID:BDSC\_12834), *NP2222-Gal4* (RRID:DGGR\_112830), *UAS-Appl-RNAi<sup>JF02878</sup>* (RRID:BDSC\_28043), *UAS-Appl-RNAi<sup>HMS01931</sup>* (RRID:BDSC\_39013), *repo-Gal4* (RRID:BDSC\_7415), *Tub-Gal80<sup>ts</sup>* (RRID:DGGR\_130452), *UAS-dicer2*, *dEaat1(GMR75H11)-Gal4* (RRID:BDSC\_39914), *UAS-GS2-RNAi<sup>HMS02197</sup>* (Bloomington *Drosophila* Stock Center), *UAS-Appl-RNAi<sup>KK102543</sup>* (RRID:FlyBase\_FBst0480124), *UAS-Appl-RNAi<sup>GD3170</sup>* (RRID:FlyBase\_FBst0464704; Vienna *Drosophila* Stock Center), *mz0709-Gal4*, *alrm-Gal4* (M. Freeman, University of Massachusetts), *9-137-Gal4* (U. Heberlein, Janiela Research Campus), *repo-Gal80* (V. Morel, Centre National de la Recherche Scientifique, Lyon), *UAS-Appl* (T. Preat, Centre National de la Recherche Scientifique, Paris), *UAS-inx2* (R. Bauer, University of Bonn), *dEaat1-Gal4*, *UAS-dEaat1* (RRID:BDSC\_8202), *UAS-dEaat1-RNAi<sup>JRX</sup>*, *UAS-dEaat1-RNAi<sup>JR2</sup>* (S. Birman, Centre National de la Recherche Scientifique, Paris), *UAS-GFP* (B. Mollereau, École normale supérieure de Lyon), *UAS-RFP:inx2*, *UAS-inx2:RFP* (A. Brand, University of Cambridge). Stocks were outcrossed  $\geq 3$  times to wild-type flies before being used for experimentation. Unless otherwise stated, *UAS-dicer2* was always associated with *UAS-Appl-RNAi* to increase the efficiency of the knock down. Flies were reared at 25°C, 50–60% humidity, in a 12 h light/dark cycle in a standard food containing inactivated yeast, molasses, sucrose, and agar.

**Sleep monitoring.** Freshly eclosed females were collected from culture vials under CO<sub>2</sub> anesthesia. Adult 3–5-d-old flies were transferred individually to 65 mm glass vials for continuous sleep recordings in Trikinetiks locomotor activity monitors for  $\geq 5$  d. Sleep parameters were evaluated as described previously (Shaw et al., 2000). Sleep deprivation

was performed with a sleep-nullifying apparatus system as previously described (Shaw et al., 2000, 2002). Flies were sleep deprived for 25 h starting at lights on. White area in baseline sleep graphs shows the photophase; the shaded area shows the scotophase.

**Immunofluorescence in whole-mount brains.** Brains were dissected in cold PBS, fixed for 20 min in a 4% paraformaldehyde PBS, washed in 0.3% Triton X-100 PBS (PBS-T) and blocked for  $\geq 45$  min in 3% goat serum in PBS-T, before overnight incubation in the corresponding primary antibody: rabbit anti-GFP (1:2000; Invitrogen, catalog #A6455; RRID:AB\_221570), rabbit anti-inx2 (1:50; GenScript), mouse anti-glutamine synthetase (anti-GS; 1:250; Millipore, catalog #MAB302; RRID:AB\_2110656). After primary antibody incubation, brains were washed in PBS-T and incubated with Alexa 488-conjugated and Alexa 633-conjugated secondary antibodies (Thermo Fisher Scientific, catalog A11008; RRID:AB\_143165; catalog #A21050 RRID:AB\_141431). Brains were mounted in hard-set Vectashield with DAPI (Vector Laboratories, catalog #H-1500; RRID:AB\_2336788) and imaged using a confocal microscope (Leica, TCS-SP5 X). ImageJ (RRID:SCR\_003070) was used for image processing and fluorescence quantification. Mean fluorescence of GS was measured within the calyx. Innexin2 (*Inx2*) levels were measured by quantifying mean fluorescence levels in the calyx and cortex region. In all cases, quantification was done averaging 10–12 Z planes of  $\geq 3$  brains for each condition. Maximal projection images are shown.

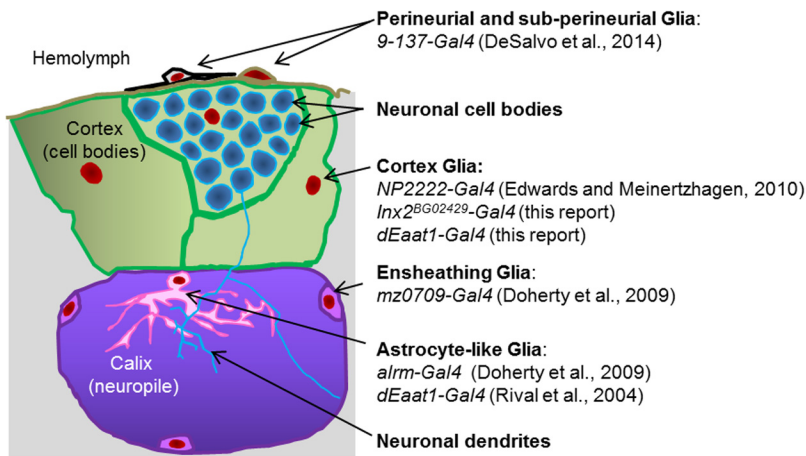
**Quantitative real-time PCR.** Fly heads were collected (four independent samples of 20 heads for each group) and frozen immediately in dry ice until further processing. Total RNA was extracted using RNeasy Minikit with DNase treatment (Qiagen). Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Finally, quantitative real-time PCR was performed on a 7900HT Fast Real-Time PCR System using FAST SYBR Green Master Mix (Applied Biosystems). *RP49* transcript levels were used to normalize results between groups. For each gene, the relative expression ratio was determined using the  $2^{-\Delta\Delta Ct}$  calculation method. The following primers were used: *RP49* primers: 5'-acgacaatctccttgcctct-3' and 5'-atcggttacggatcgaacaa-3'; *dEaat1* primers: 5'-tcggcattcagcctatcac-3' and 5'-tcaatgctcactggatcgc-3'.

**Statistical analysis.** Distribution and homogeneity as well as statistical group comparisons were tested using Microsoft Excel plugin software Statel. In all cases averages with SEM are plotted. The following corresponding statistical and *post hoc* test values are reported in the figure legends: Mann–Whitney (see Z values); ANOVA with Tukey's *post hoc* (see F values); or Kruskal–Wallis (see K–W values) with a posteriori test; one-tailed *t* tests were used to evaluate transcript levels by quantitative real-time PCR (\**p* < 0.05; \*\**p* < 0.001). *N* number is listed for each group in the following order: Gal4 control, UAS control, and experimental group.

## Results

### Modulation of *Appl* expression in cortex and astrocyte-like glia disrupts sleep

To determine whether glial *Appl* can affect sleep/wake regulation, we used the Gal4/UAS system to reduce or increase *Appl* expression in different glial subtypes of the adult brain (Fig. 1). First, we tested specific glial Gal4 drivers combined with *Appl-RNAi<sup>JF02878</sup>*, an *Appl-RNAi* construct targeting the terminal region of the *Appl* transcript common to all *Appl* isoforms reported (Ni et al., 2009; Flybase; Fig. 2A). All Gal4 constructs driving expression in perineurial, subperineurial, cortex, unsheathing, and astrocyte-like glia, or a combination of subtypes, lead to viable adults, with no obvious morphological or behavioral defects. Two Gal4 constructs that share expression in cortex glia resulted in significantly higher sleep amounts upon driving expression of *Appl-RNAi* (Fig. 2C–H). The most severe phenotype was observed with the *dEaat1-Gal4* driver (Fig. 2C,I), which is expressed in both astrocyte-like and cortex glia (Fig. 3A). However, no effect on sleep was observed upon expression of *Appl-RNAi* in astrocyte-like glia (*alrm-Gal4* driver; Fig. 2I), suggesting that *Appl* is required in cortex glia for sleep/wake regulation. Combining the



**Figure 1.** The different glial subtypes of the adult *Drosophila* brain. The schematic represents the area surrounding the calyx neuropile, which includes cell bodies and neurites of the mushroom bodies' neurons (Kenyon cells). Each glial cell type can be targeted with the listed Gal4 drivers.

*dEaat1-Gal4* driver with pan-glial expression of the Gal4 inhibitor Gal80 restored a control-like sleep/wake phenotype, demonstrating that the effect on sleep/wake is not due to driving *Appl*-RNAi in nonglial cells (Fig. 2*E,I*). To further confirm this set of results and exclude the possibility that it could originate from off-targets of the *Appl*-RNAi<sup>JF02878</sup> construct, we replicated the experiment using another *Appl*-RNAi construct that targets the first, untranslated exons of the *Appl* transcript (*Appl*-RNAi<sup>HMS01931</sup>), as well as another Gal4 driver expressed in cortex glia, *Inx2*<sup>BG02429</sup>-Gal4, and obtained similar results (Fig. 2*F–I*). Expressing two other different *Appl*-RNAi constructs under the control of *Inx2*<sup>BG02429</sup>-Gal4 also resulted in higher sleep amounts (Table 1). In almost all experimental genotypes tested, high daily sleep amounts were due to larger amounts of night sleep. Elevated night sleep was associated with longer average night-bout duration (Table 1). These sleep/wake phenotypes were not due to the use of UAS-dicer2 to enhance the efficiency of RNAi constructs (see Materials and Methods), since sleep/wake was unaffected when dicer2 alone was overexpressed (total sleep: *dEaat1-Gal4*, 715 ± 30 min; *dEaat1-Gal4* > UAS-dicer2, 718 ± 26 min).

In contrast, overexpressing full-length *Appl* with the *dEaat1-Gal4* driver resulted in the reverse phenotype: reduced sleep amounts, during both day and night (Fig. 2*J,K*). Compared with the controls, the latency between lights off and the first sleep bout of the night was higher (76 ± 9 vs 46 ± 5 and 50 ± 4 min for the Gal4/+ and UAS/+ controls respectively  $p < 0.05$ ), suggesting difficulty to initiate sleep at night. Sleep bout duration during the night was not significantly affected, however (Table 1). Altogether, these data show that manipulating *Appl* expression in glia can affect sleep/wake regulation in a bidirectional way.

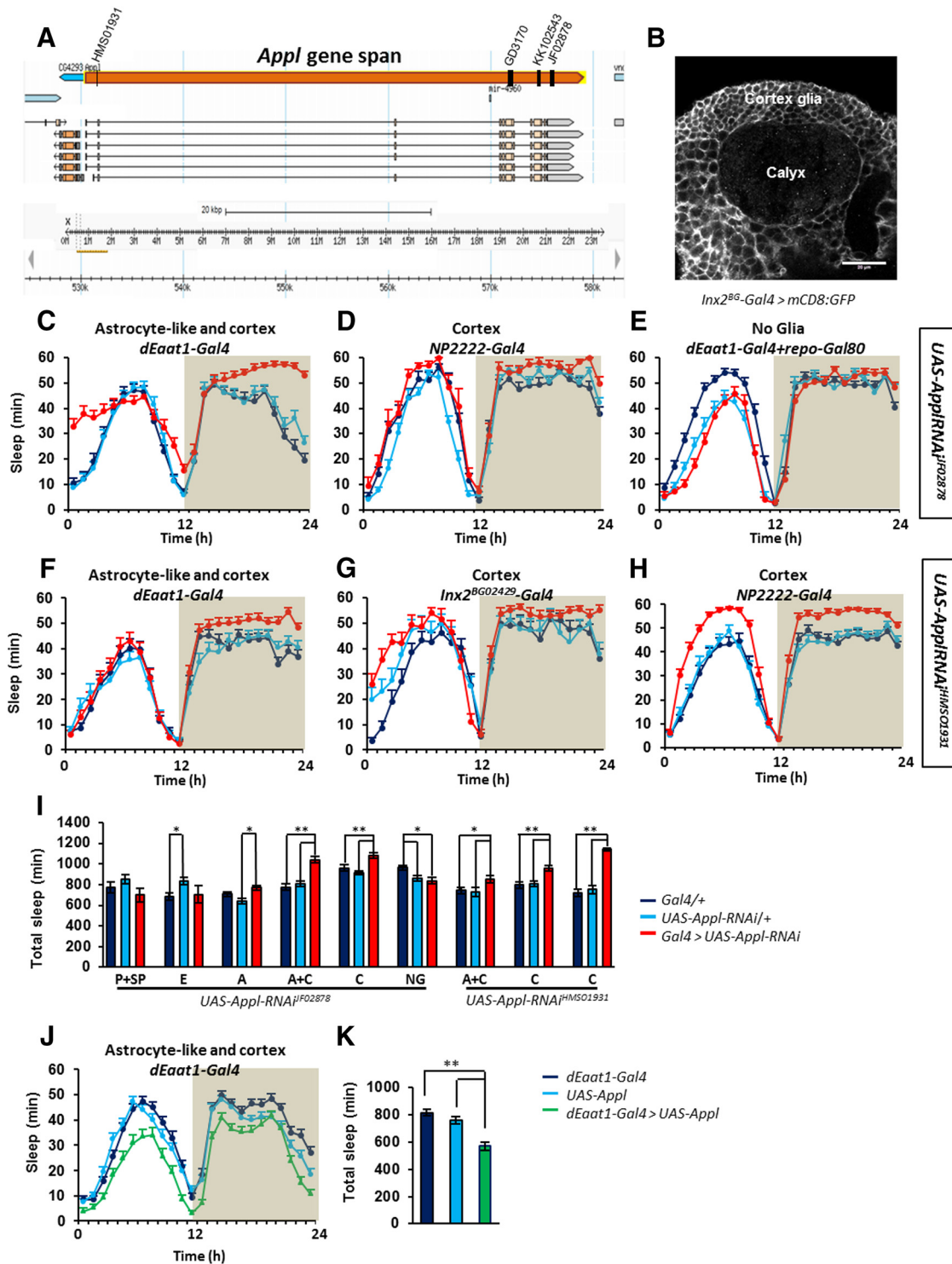
### Inhibition of *Appl* in cortex glia is associated with changes in dEaat1, GS, and innexin 2 expression

Glia in mammals and in flies is known to be involved in neurotransmitter recycling functions (Rival et al., 2006; Chaturvedi et al., 2014). The reuptake and recycling of glutamate, an important neurotransmitter for learning and memory, synaptic plasticity, energy metabolism, and sleep is indeed a crucial glial function, requiring identified transporters and enzymes. These functions are impaired in AD (Shrivastava et al., 2013; Osborn et al., 2016), but it remains unclear whether this effect is a consequence of neuronal dysfunction of astroglial following expo-

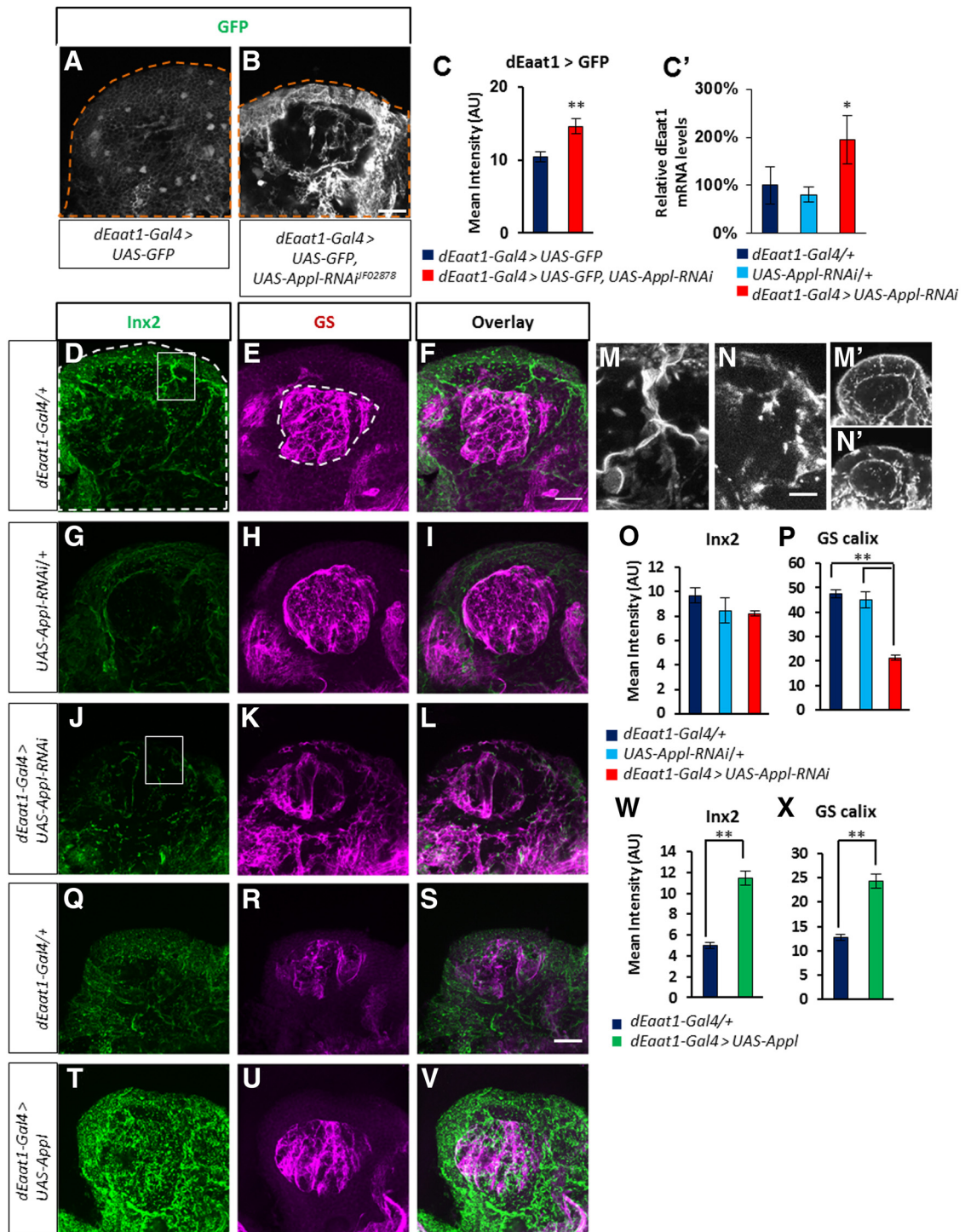
sure to toxic protein products, or of a failure of *Appl* function. The recycling involves the reuptake of synaptically liberated glutamate by the transporter dEaat1, and the conversion of glutamate to glutamine by GS. The genes for dEaat1 and GS are expressed in glia in *Drosophila*, similarly to mammals (Freeman et al., 2003; Lievens et al., 2005). To monitor glutamate transporter expression, we measured UAS-GFP expression driven by the *dEaat1-Gal4* construct (*dEaat1*>GFP) in whole-mount brain. Inhibition of *Appl* expression resulted in an increase in *dEaat1*>GFP expression, indicating that the transcription of *dEaat1* is upregulated (Fig. 3*A–C*). The expression is notably increased toward the periphery of the brain, where a subset of cortex glial cells is in direct contact with subperineurial glia, the *Drosophila* equivalent of the blood–

brain barrier. Quantitative PCR evaluation of mRNA levels confirmed that *dEaat1* is transcriptionally upregulated in *Appl* mutants (Fig. 3*C'*). Additionally, we evaluated GS protein using immunofluorescence in the same conditions (Fig. 3*D–L*) and observed a dramatic reduction of GS expression within the brain neuropiles, where the protein is normally very abundant (Fig. 3*E,H,K,P*). In the cortex region, GS levels were slightly increased upon inhibition of *Appl* expression (Fig. 3*K*, legend). In contrast, *Appl* overexpression in glia resulted in higher GS expression in the neuropiles, without any effect in the cortex region (Fig. 3*R,U,X*).

Gap junctions between astrocytes (Rouach et al., 2008) and between different glial cell types in *Drosophila* (Chaturvedi et al., 2014) have been shown to play a critical role in intercellular communications required for neurotransmitter reuptake and recycling (Giaume et al., 2010). Therefore, we used immunofluorescence to assess expression and localization of Inx2, a major glial gap junction component in *Drosophila* (Holcroft et al., 2013; Chaturvedi et al., 2014; Spéder and Brand, 2014). Inx2 is expressed at the boundary between astrocyte-like glia and cortex glia and between cortex glia and subperineurial glia, and along lines in the cortex region that presumably correspond to the limits of each cortex glial cell (Fig. 3*D,M,M'*). The expression of Inx2 within the cortex area was strongly upregulated upon *Appl* overexpression (Fig. 3*Q,T,W*). Inhibition of *Appl* expression using *Appl*-RNAi<sup>JF02878</sup> did not result in a significant reduction of Inx2 expression (Fig. 3*D,G,J,O*), but changes in the cellular distribution of Inx2 were observed. In mutant flies, the continuous expression of Inx2 along cellular boundaries was lost, becoming interrupted and clumpier (Fig. 3*M,M',N,N'*). In addition, we further replicated these findings with the *Appl*-RNAi<sup>HMS01931</sup> transgene, which targets a different segment of the *Appl* transcript (Fig. 2*A*). With this construct, Inx2 cellular distribution was not only disrupted but also significantly reduced compared with controls (*dEaat1-Gal4*, 23.71 ± 1.77; UAS-*Appl*-RNAi<sup>HMS01931</sup>, 19.19 ± 0.88; *dEaat1-Gal4* > UAS-*Appl*-RNAi<sup>HMS01931</sup>, 10.70 ± 0.75; K-W, 46.23;  $p < 0.00001$ ;  $N = 5$  brains each group) and GS levels in calyx were similarly reduced (*dEaat1-Gal4*, 57.00 ± 2.90; UAS-*Appl*-RNAi<sup>HMS01931</sup>, 39.35 ± 1.98; *dEaat1-Gal4* > UAS-*Appl*-RNAi<sup>HMS01931</sup>, 29.87 ± 1.94; K-W, 45.54;  $p < 0.00001$ ;  $N = 3, 3, 4$  brains). The effect GS and Inx2 expression were not due to the use of UAS-dicer2 to enhance the efficiency of RNAi constructs (see Materials and Methods), since their expressions remain un-



**Figure 2.** Manipulation of *Appl* expression in cortex and astrocyte-like glia modifies sleep. Targeting different segments of the *Appl* transcript with independent RNAi constructs results in higher sleep amounts during the night. **A**, Graphical representation of the *Appl* gene and localizations of the areas targeted by the different *Appl* RNAi constructs. Each targeted region is shared by all *Appl* transcripts (adapted from Flybase). **B**, mCD8-GFP expression driven by *Inx2<sup>BG02429</sup>-Gal4* highlighting cortex glia around the calyx neuropile. Baseline sleep (minutes of sleep per hour) upon expression of *UAS-App/RNAi<sup>JF02878</sup>* driven in (**C**) astrocyte-like and cortex (*dEaat1-Gal4*) K-W = 29.85,  $p < 0.00001$ ;  $N = 39, 39, 35$ , (**D**) cortex (*NP2222-Gal4*) K-W = 14.58,  $p < 0.00068$ ;  $N = 21, 24, 8$ , (**E**) no glia (*repo-Gal80, dEaat1-Gal4*)  $F = 6.606$ ,  $p < 0.0019$ ;  $N = 43, 44, 43$ . **F–H**, *UAS-App/RNAi<sup>HMS01931</sup>* replicated the phenotype when driven in (**F**) cortex and astrocyte-like glia with *dEaat1-Gal4* (K-W, 6.689;  $p < 0.035$ ;  $N = 40, 41, 41$ ) and in (**G**) cortex glia with *Inx2<sup>BG02429</sup>-Gal4* ( $F = 10.9$ ;  $p < 0.000035$ ;  $N = 56, 56, 60$ ) or (**H**) with *NP2222-Gal4* (*NP2222-Gal4*; K-W, 50.78;  $p < 0.00001$ ;  $N = 41, 43, 43$ ). **I**, Total daily sleep for each genotype. Peri-neurial and sub-perineurial (P+SP; *9-137-Gal4*); ensheathing (E; *mz0709-Gal4*); astrocyte-like (A; *alm-Gal4*); astrocyte-like and cortex (A+C; *dEaat1-Gal4*); cortex [C; *NP2222-Gal4* with *UAS-App/RNAi<sup>JF02878</sup>*, *NP2222-Gal4* (left) and *Inx2<sup>BG02429</sup>-Gal4* (right) with *UAS-App/RNAi<sup>HMS01931</sup>*] and no glia (NG; *repo-Gal80, dEaat1-Gal4*). Dark blue, Glia *Gal4/+* controls; light blue, *UAS-App/RNAi/+* controls; red, corresponding glial driver controlling *UAS-App/RNAi*. **J**, Overexpression of *Appl* in cortex and astrocyte-like glia (*dEaat1-Gal4 > UAS-App*) reduces sleep levels. **K**, Comparison of total daily sleep for each genotype ( $F = 24.4$   $p < 0.00001$ ;  $N = 63, 61, 62$ ).



**Figure 3.** *App* knock-down is associated with changes in genes linked to glutamate recycling. **A, B**, Confocal images of whole-mount brains expressing GFP (green, immunolabeling) driven by *dEaat1-Gal4* in cortex and astrocyte-like glia: (**A**) control *dEaat1-Gal4 > UAS-GFP* and (**B**) glial *App* knock-down *dEaat1-Gal4 > UAS-GFP, UAS-App1-RNAi<sup>F02878</sup>*. **C**, Quantification of GFP immunofluorescence reflecting *dEaat1* expression levels shows increased expression in *App* mutants. GFP is not transported efficiently to the neuropiles (Rival et al., 2004) and was quantified in the cortex area ( $Z = 3.53, p < 0.00041, N = 4$  each group). **C'**, Quantitative PCR evaluation of *dEaat1* transcripts in whole heads ( $p < 0.05$ , 1-tailed *t* test). **D–L**, Immunolabeling of *Inx2* (**D, F, G, I, J, L**; green) and *GS* (**E, F, H, I, K, L**; magenta) in *dEaat1-Gal4/+* (**D–F**) and in *UAS-App1-RNAi<sup>F02878</sup>/+* (**G–I**) controls, and in *dEaat1-Gal4 > UAS-App1-RNAi<sup>F02878</sup>* mutants (**J–L**). **M, N**, Close-up corresponding to the insets in **D** and **J**, showing *Inx2* signal in cortex glia in (**M**) *dEaat1-Gal4/+* control and abnormal distribution of the protein in (**N**) *dEaat1-Gal4 > UAS-App1-RNAi<sup>F02878</sup>* mutant. **M', N'**, *Inx2* signal in single-plane confocal images of the whole calyx area shown in **D** and **J**. *dEaat1-Gal4/+* control (**M')** shows lines of *Inx2* expression at the boundaries of the cortex glial cells area, while the *dEaat1-Gal4 > UAS-App1-RNAi<sup>F02878</sup>* mutant (**N')** shows more clumpy distribution. **O, P**, Quantification of *Inx2* immunofluorescence (**O**;  $K-W, 17.33; p < 0.00017; n = 5$  each group) and of *GS* immunofluorescence in the calyx (**P**;  $K-W, 75.53; p < 0.00017; n = 5$  each group). *GS* immunofluorescence in the cortex area surrounding the calyx was significantly enhanced in *dEaat1-Gal4 > UAS-App1-RNAi<sup>F02878</sup>* flies compared with controls (*dEaat1-Gal4*,  $23.06 \pm 0.41$ ; *UAS-App1-RNAi<sup>F02878</sup>*,  $22.29 \pm 0.72$ ; *dEaat1-Gal4 > UAS-App1-RNAi<sup>F02878</sup>*,  $29.16 \pm 0.63$ ;  $K-W, 21.83; p < 0.00018; N = 5$  each group). Dark-blue bar, *Gal4/+* controls; light-blue bars, *UAS/+* controls; red bars, *Gal4 > UAS* group. **Q–V**, Immunolabeling of *Inx2* (**Q, T**) and *GS* (**R, U**) in brains of flies overexpressing glial *App*. **W, X**, Quantification of immunofluorescence shows higher *Inx2* levels (**W**;  $Z = 6.98; p < 0.00001; n = 3$  controls and  $n = 4$  experimental group) and higher *GS* expression in calyx (**X**;  $Z = 5.96; p < 0.00001; n = 3$  controls and  $n = 4$  experimental group) but not in cortex glia surrounding the calyx ( $Z = 1.039; p = 0.3; N = 5$  each group), upon *App* overexpression. Dark-blue bars, *Gal4/+* controls; green bars, the experimental group overexpressing *App*. Scale bars: (in **B, F, S, A–L, Q–V**) 20  $\mu\text{m}$ ; (in **N, M, N'**) 10  $\mu\text{m}$ .

**Table 1. Sleep parameters evaluated in all *Appl* misexpression experiments**

Genotype	Glia	UAS	Total sleep	Night Sleep	Night bout <sup>d</sup>	Maximal night bout <sup>b</sup>	Night bout # <sup>a</sup>	Day sleep	Day bout <sup>d</sup>	Maximal day bout <sup>b</sup>	Day bout # <sup>a</sup>	Latency <sup>c</sup>
<i>UAS-dicer2/+; repo-Gal80, Eaat1-Gal4/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	No glia	JF02878	U	U	U	U	U	U	U	U	U	U
<i>UAS-dicer2/+; 9-137-Gal4/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	Perineurial and subperineurial	JF02878	U	U	U	U	U	U	U	U	U	U
<i>UAS-dicer2/+; mz0709-Gal4/UAS-Appl-RNAi<sup>JF02878</sup></i>	Ensheathing	JF02878	U	U	U	U	U	U	U	U	U	U
<i>UAS-dicer2/+; Alrm-Gal4/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	Astrocyte-like	JF02878	U	U	U	U	U	U	U	U	U	U
<i>UAS-dicer2/+; dEaat1-Gal4/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	Astrocyte-like and cortex	JF02878	H,ss	H,ss	H,ss	H,ss	L,s	H,ss	L,s	U	H,ss	L,ss
<i>UAS-dicer2/+; NP2222-Gal4/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	Cortex	JF02878	H,s	H,ss	H,ss	H,ss	L,s	L,s	U	H,ss	U	U
<i>Inx2<sup>BG02429</sup>/UAS-dicer2; Tub-Gal80<sup>ts</sup>/+; UAS-Appl-RNAi<sup>JF02878</sup>/+</i>	Cortex (acute)	JF02878	H,s	H,ss	H,ss	H,ss	U	L,ss	L,s	L,s	L,s	U
<i>UAS-dicer2/+; dEaat1-Gal4/UAS-Appl-RNAi<sup>HMS01931</sup></i>	Astrocyte-like and cortex	HMS01931	H,ss	H,ss	H,ss	H,s	L,s	U	U	U	U	U
<i>Inx2<sup>BG02429</sup>/UAS-dicer2; UAS-Appl-RNAi<sup>HMS01931</sup>/+</i>	Cortex	HMS01931	H,ss	H,ss	H,s	H,ss	L,s	U	H,s	U	L,s	L,s
<i>UAS-dicer2/+; UAS-Appl-RNAi<sup>HMS01931</sup>/NP2222-Gal4;</i>	Cortex	HMS01931	H,ss	H,ss	H,ss	H,ss	L,ss	H,ss	H,ss	H,ss	L,s	U
<i>UAS-dicer2/+; dEaat1-Gal4/+; UAS-Appl-RNAi<sup>GD3170</sup>/+</i>	Astrocyte-like and cortex	GD3170	U	U	H,s	H,s	L,s	U	H,s	H,s	L,ss	H,ss
<i>Inx2<sup>BG02429</sup>/UAS-dicer2; UAS-Appl-RNAi<sup>GD3170</sup>/+</i>	Cortex	GD3170	H,ss	H,ss	U	H,ss	U	H,s	U	U	U	L,ss
<i>UAS-dicer2/+; dEaat1-Gal4/UAS-Appl-RNAi<sup>KK102543</sup></i>	Astrocyte-like and cortex	KK102543	H,s	U	U	U	U	H,s	H,ss	H,ss	L,ss	U
<i>Inx2<sup>BG02429</sup>/UAS-dicer2; UAS-Appl-RNAi<sup>KK102543</sup>/+</i>	Cortex	KK102543	H,ss	H,ss	H,s	U	L,s	H,ss	U	U	H,ss	L,s
<i>; dEaat1-Gal4/UAS-Appl;</i>	Astrocyte-like and cortex	<i>Appl</i>	L,ss	L,ss	L,s	L,s	U	L,ss	U	L,ss	L,ss	H,ss

<sup>a</sup>Day bout # and Night bout # indicate the number of sleep bouts in photophase and scotophase respectively.

<sup>b</sup>Maximal day bout and Maximal night bout indicate the longest sleep bout for each corresponding light phase.

<sup>c</sup>Time between lights off and the first sleep event.

<sup>d</sup>Refers to average sleep bout duration during the considered period.

<sup>U</sup>Unchanged values compared to both parental controls,  $p > 0.1$ .

<sup>H</sup>Higher values in the mutant compared to the controls.

<sup>L</sup>Lower values in the mutant compared to the controls.

<sup>s</sup> $p < 0.05$ .

<sup>ss</sup> $0.05 < p < 0.1$ .

changed when *dicer2* alone was overexpressed (data not shown). The quantifications shown in Figure 3 are centered on the calyx, the input neuropile of the mushroom-body neurons (Kenyon cells), which play critical roles in behavior, learning, and memory, as well as in sleep regulation. The cell bodies of Kenyon cells are in the cortex surrounding the calyx (Fig. 1). The expression of *Inx2* and *GS* was similarly affected in other regions of the central brain.

To exclude that potential developmental effects could explain the phenotypes observed, and to further control the time course of *Appl* manipulation, we used the TARGET (Temporal and Regional Gene Expression Targeting) system (McGuire et al., 2004). The *Inx2<sup>BG02429</sup>-Gal4* driver, which is restricted to cortex glia (Fig. 2B) was used in these experiments. Flies were raised at 18°C (permissive temperature) until the first days of adulthood and then transferred to 30°C to allow the expression of *Appl-RNAi*. We observed no difference in sleep between the *Inx2<sup>BG02429</sup>-Gal4 > Appl-RNAi* flies and the genetic controls at 18°C, as expected in the presence of a functional *Gal80* (Fig. 4A,C,D). Similarly, expression of *GS* was unchanged in *Inx2<sup>BG02429</sup>-Gal4 > Appl-RNAi* compared with controls (data not shown) while *Inx2* was slightly upregulated ( $23.6 \pm 1.6$  vs  $16.8 \pm 0.9$  for the controls,  $p < 0.5$ ). In contrast, *Inx2<sup>BG02429</sup>-Gal4 > Appl-RNAi* flies displayed higher sleep amounts than the controls during the first day of induction at 30°C, and this change remained stable afterward. The increase in sleep occurred during the night, a period during which chronic *Appl* inhibition produced consistently the biggest changes in sleep (Figs. 2, 4B–D). The change in sleep was associated with a conspicuous decrease of *GS* immunofluorescence in the calyx neuropile, replicating the phenotype observed upon chronic inhibition of *Appl*, as shown in Figure 4F,I,L. *Inx2* expression was also significantly downregulated but its cellular distribution was similar to that of controls, suggesting that the clumpy distribution observed in chronic *Appl* inactivation may be due to a longer inhibition and/or secondary effects (Fig. 4E,H,K). These results provide evidence that

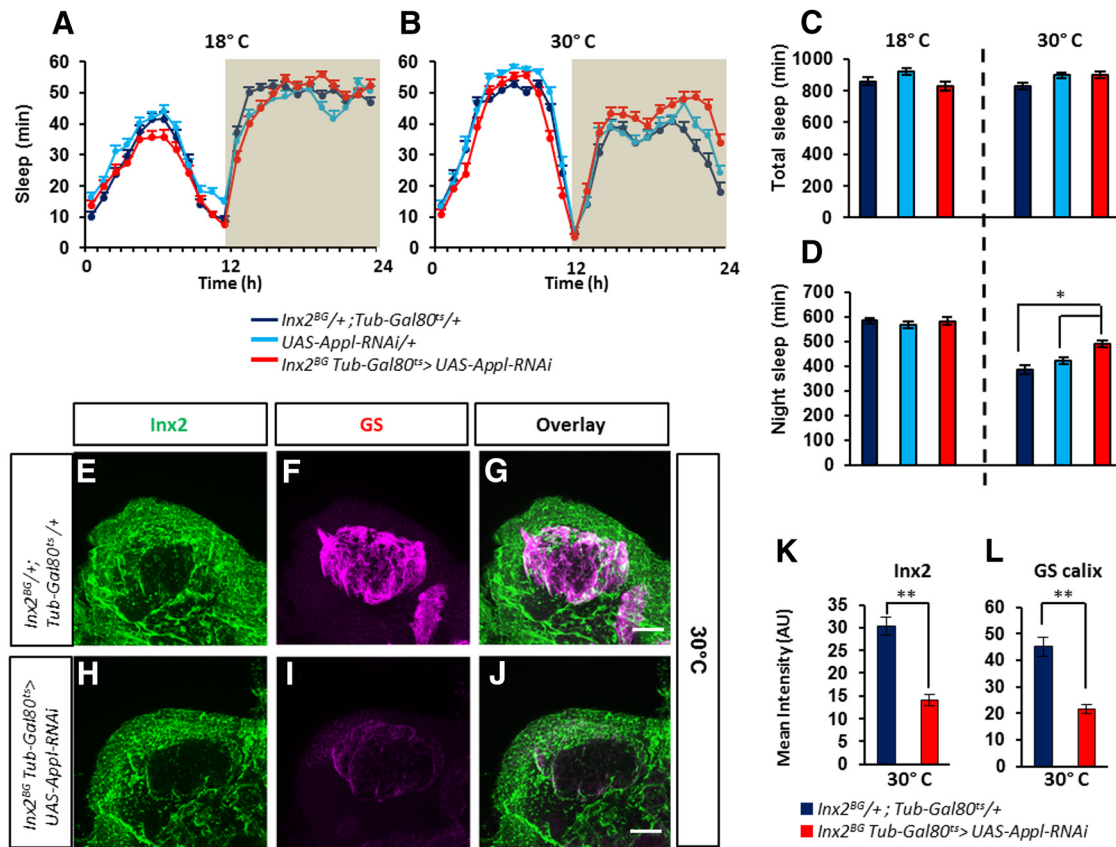
the consequences of *Appl* inhibition on sleep and *GS/Inx2* expression are strongly correlated, and can occur in a relatively short time course at the adult stage. Since the expression of the *Inx2<sup>BG02429</sup>-Gal4* driver used in these experiments is restricted to cortex glia, these results further indicate that *Appl* inhibition in one part of the glial network (cortex glia) may affect other, functionally connected compartments (astrocyte-like glia). Altogether, these observations suggest that *Appl* function in glial cells influences glutamate recycling. Further, it indicates that the communications between different glial compartments (i.e., astrocyte-like, ensheathing, and cortex glia) is abnormal in the mutant condition.

### ***Inx2* and *GS* expression are functionally linked to sleep/wake regulation**

The close association between sleep and *GS/Inx2* expression phenotypes observed upon *Appl* inhibition suggests that they could be functionally connected. To test this hypothesis, we downregulated *GS* expression and/or expressed a dominant-negative form of *Inx2* (*UAS-RFP:Inx2*; Spéder and Brand, 2014) in glia. Individually, either of these two manipulations failed to affect sleep, whereas in combination they resulted in an increase in sleep similar to *Appl* inhibition in the same cells (Fig. 5A,B). To further assess the link between sleep/wake and *GS/Inx2* expression, we evaluated their expression levels in wild-type flies under baseline condition and after a 25 h sleep deprivation. As shown in Figure 5C–J, *GS* and *Inx2* expression levels in the sleep-deprived flies were higher than in the flies allowed to sleep, indicating that these two genes are dynamically linked to vigilance states.

### ***dEaat1* overexpression in cortex and astrocyte-like glia rescues the effect of *Appl* inhibition on sleep and *GS* expression**

To confirm the implication of genes involved in glutamate recycling in the *Appl*-dependent regulation of sleep/wake, we overex-



**Figure 4.** Time-controlled manipulation of *App1* expression in cortex glia increases night-time sleep and reduces GS and *Inx2* protein levels. **A, B**, 24 h sleep amounts under permissive (**A**) and restrictive (**B**) temperatures. The temperature-sensitive *Tub-Gal80<sup>TS</sup>* construct driven by *Inx2<sup>BG02429-Gal4</sup>* (*Inx2<sup>BG</sup>*) was used to temporally control the expression of *UAS-App1-RNAi<sup>IF02878</sup>* (*UAS-App1-RNAi*). At 30°C, sleep is reduced during the night as previously described (Ishimoto et al., 2012). **C, D**, No effect was observed at permissive temperature on either total sleep (**C**, left) or night-time sleep (**D**, left). Increasing temperature to 30°C significantly increased night-time sleep in *App1* mutant flies (**D**, right;  $F = 3.854; p < 0.024; N = 37, 38, 39$ ). **E–J**, Immunolabeling of *Inx2* (**E, H, G, J**, green) and GS (**F, I, G, J**, magenta) in brains of flies exposed to restrictive temperatures (**E–G**) *Inx2<sup>BG</sup>-Gal4/+; Tub-Gal80<sup>TS</sup> / +* and (**H–J**) *Inx2<sup>BG</sup>-Gal4 Tub-Gal80<sup>TS</sup> > UAS-App1-RNAi*. **K, L**, Quantification of immunofluorescence show lower protein levels for *Inx2* (**K**; Mann–Whitney;  $Z = 5.72; p < 0.00001; n = 3$  each group) and for GS in the calyx upon conditional inhibition of *App1* (**L**; Mann–Whitney,  $Z = 4.45; p < 0.00001; n = 3$  each group). Scale bar, 20  $\mu$ m.

pressed *dEaat1* in cortex and astrocyte-like glia together with the *App1-RNAi* transgene *App1<sup>IF02878</sup>*. To sensitize the genetic background and allow the detection of functional interactions, we drove *App1-RNAi<sup>IF02878</sup>* without the *UAS-dicer2* transgene, which significantly increases the efficiency of *App1-RNAi* constructs. A *UAS-GFP* transgene was also added to the genetic background to assess a potential dilution effect of Gal4 in the presence of two UAS transgenes. In those conditions, expressing *App1-RNAi<sup>IF02878</sup>* resulted in a mild but significant increase in sleep (Fig. 6A) together with a decrease in GS expression in the calyx compared with control (Fig. 6C, D, F). These two phenotypes were rescued completely upon coexpression of *UAS-dEaat1* (Fig. 6B–F), indicating that increasing the reuptake capacity of glial cells is sufficient to rescue both normal sleep/wake regulation and GS expression in the calyx. Overexpressing *dEaat1* did not affect sleep/wake amounts (K–W, 8.31;  $p < 0.016; N = 22, 22, 23$ ).

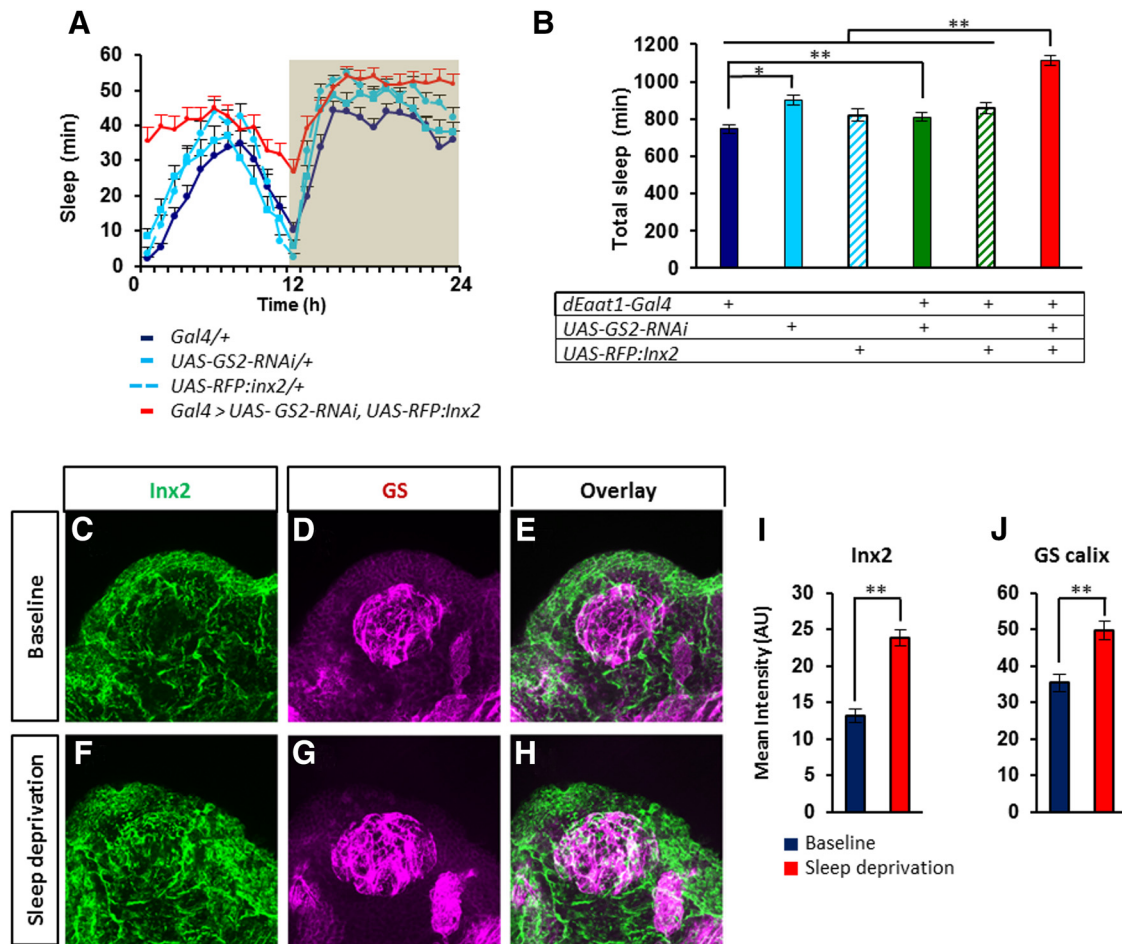
**The inhibition of *dEaat1* expression affects sleep/wake regulation**

To further test the role of glutamate reuptake in sleep/wake regulation, we proceeded to inhibit the expression of *dEaat1* in different glial-cell types and evaluated sleep–wake regulation. Higher amounts of wakefulness were observed upon inhibition of *dEaat1* in perineurial and subperineurial glia, but the most severe phenotype was observed upon inhibition in astrocyte-like and

cortex glia, using the *dEaat1-Gal4* driver, that presumably reflects endogenous *dEaat1* expression (Fig. 7A–C). Using this driver, the inhibition of *dEaat1* expression led to a striking increase in wakefulness and to a disorganization of the circadian patterning of sleep. Interestingly GS levels appeared unaffected by the reduction of *dEaat1* expression, suggesting that GS expression is not sensitive to the lack of glutamate uptake (Fig. 7D). These results demonstrate the importance of glutamate reuptake in sleep/wake regulation. They also indicate that the blocking of glutamate recycling at the level of the *dEaat1* transporter produces a phenotype distinct from the one observed upon the inhibition of *App1* expression in the same cells.

**Discussion**

The function of *App* remains elusive in glial cells (Müller and Zheng, 2013) and in other, non-neuronal cells. We report here that *App1* modulates the expression of glutamate recycling genes and regulates sleep/wake. To carry out these functions, *App1* is predominantly required in cortex glia, a cell type associated with neuronal cell bodies. Interestingly, recent reports provided evidence that calcium transients in this type of glia regulate neuronal activity (Melom and Littleton, 2013; Ma et al., 2016). Importantly, our results suggest that inhibiting *App1* in cortex glia affects GS expression in another glial cell type, astrocyte-like glia, which closely interacts with neurites and synapses. In connection



**Figure 5.** *Inx2* and *GS* protein expression are linked to sleep/wake regulation. **A**, 24 h sleep amounts showing that the combined use of *GS2-RNAi* and dominant-negative *UAS-RFP:Inx2* constructs to inhibit both *GS* and *Inx2* function result in increased sleep. **B**, Total sleep time was only increased when both constructs were coexpressed and not upon expression of either one alone ( $F = 18.37$ ;  $p < 0.00001$ ;  $N = 32, 31, 32, 27, 32, 24$ ). **C–H**, Immunolabeling of *Inx2* (**C, E, F, H**; green) and *GS* (**D, E, G, H**; magenta) in *Cs* fly whole-mount brains dissected in the first hour of lights-on either under baseline condition (**C–E**) or after 25 h of sleep deprivation (**F–H**). **I, J**, Quantification of immunofluorescence shows increased *Inx2* levels in the cortex (**I**;  $Z = 7.643$ ;  $p < 0.00001$ ;  $N = 8–9$ ), and increased *GS* levels in the calyx (**J**;  $Z = 4.035$ ;  $p < 0.000055$ ;  $N = 8–9$ ). Scale bar, 20  $\mu\text{m}$ .

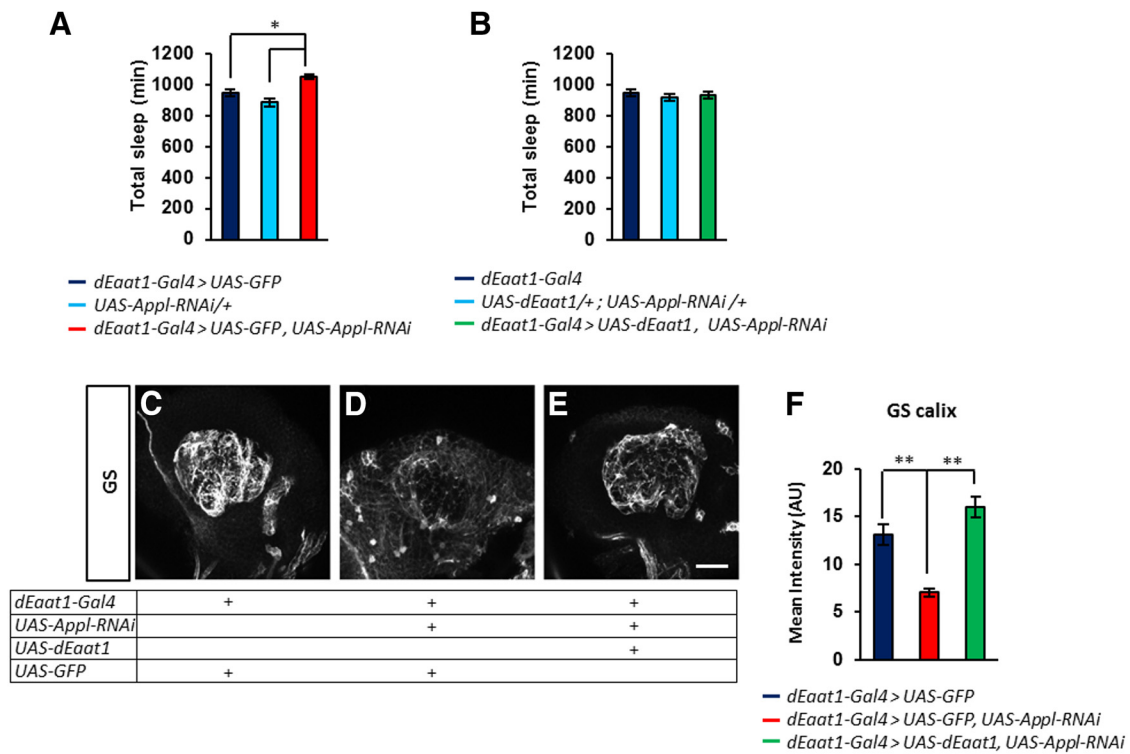
with these findings, the expression of *Inx2*, a major component of glial gap junctions at the interface between cortex and astrocyte-like glia is reduced upon *App1* inhibition in cortex glia (Fig. 7E).

In mammals, *App* and the enzymes necessary for its cleavage have been reported to be present in astrocytes (Grolla et al., 2013). Glial cells are known to be adversely impacted by AD pathology, but these defects are often thought to be consequences of neuronal dysfunction, inflammation, or other noncell autonomous processes, and therefore unrelated to *App* normal function in glial cells. It is noticeable however, that one of the main cellular phenotypes observed in this study (i.e., alteration of *GS* expression) has been reported in numerous studies in rodent AD-related models as well as in human (Robinson, 2000; Olabarria et al., 2011; Kulijewicz-Nawrot et al., 2013), suggesting that it could be linked to a disruption of *App* function within astrocytes. In AD patients and experimental models (Ju et al., 2014), including *Drosophila* (Vanderheyden et al., 2013; Tabuchi et al., 2015), the pathology is also associated with reduced and highly fragmented sleep. We find here that the inhibition of *App1* expression in glial cells results in higher sleep amounts and more consolidated sleep during the night. The sleep deficits observed in AD may thus result from both neuronal and glial dysfunction and probably neuroglial-signaling abnormalities. *App* proteins expressed in presynapses and postsynapses have been shown to

influence each other's activities and to mediate cell–cell interactions (Wang et al., 2009). Similarly, *App* could mediate interglial and/or neuroglial interactions, possibly through cell adhesion and/or cell signaling. Because of these mutual interactions, toxic amyloid peptides produced and released by neurons could impair *App* function in glia, and explain the similarity of the cellular glial phenotypes observed here with those found in AD pathology. Finally, we cannot exclude the possibility that the decrease in *GS* expression observed in this study may reflect an impact of *App1* inhibition on glial viability. However, the observation that *App1*-null mutants are viable (Luo et al., 1992) and that overexpression produces a phenotype opposite to the inhibition is more in favor of a specific regulatory role for *App1*.

*GS* plays a major role in glutamate and ammonium recycling by catalyzing the conversion of glutamate into glutamine, impacting both neurotransmission systems, as well as brain energy metabolism. The reduction of *GS* expression could lead to impaired glutamate–glutamine recycling and, possibly, to an accumulation of glutamate and excitotoxicity as hypothesized for AD. How *App* regulates *GS* expression remains unclear. In AD, it has been suggested that increased oxidation triggered by the pathology modifies the conformation of *GS*, such that it becomes non-functional and undetectable to antibodies. Alternatively, *GS* expression could be modulated by the intracellular concentra-





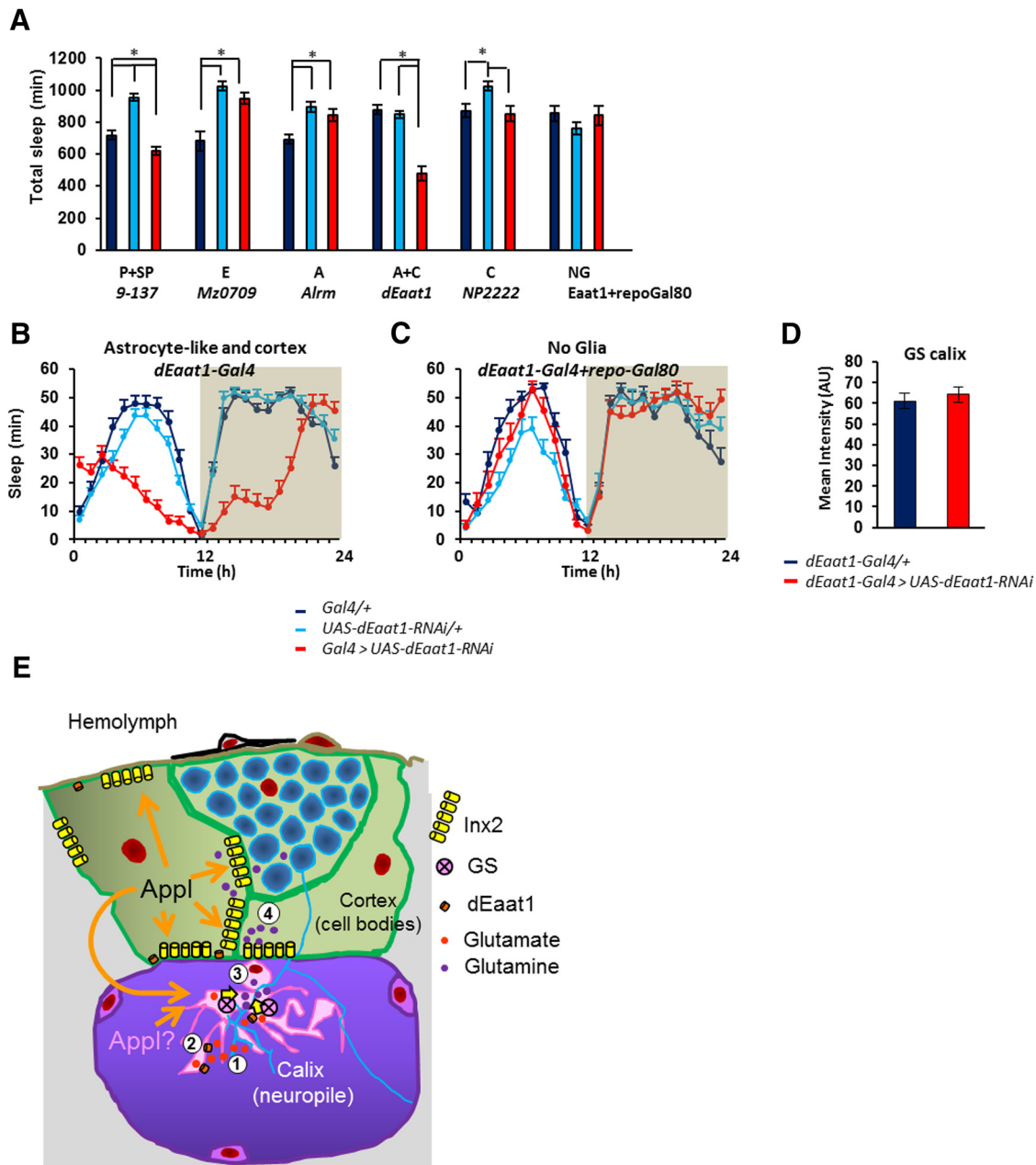
**Figure 6.** *dEeat1* overexpression in cortex and astrocyte-like glia counteracts the effect of *App1* knockdown on sleep and GS expression. **A**, Baseline total sleep in flies coexpressing *UAS-App1-RNAi*<sup>F02878</sup> and *UAS-GFP* in cortex and astrocyte-like glia under the control of *dEeat1-Gal4*. *UAS-dicer2* was not included in the genotype. *dEeat1-Gal4 > UAS-App1-RNAi*<sup>F02878</sup>, *UAS-GFP* flies display significantly higher daily sleep amounts even in the absence of *UAS-dicer2* (K-W, 29.24;  $p < 0.00001$ ;  $N = 34, 35, 37$ ). **B**, Coexpressing *UAS-dEeat1* with *UAS-App1-RNAi*<sup>F02878</sup> is sufficient to restore control-like sleep amounts, as confirmed by total daily sleep comparison between the genotypes (K-W, 0.72; not significant;  $N = 30, 31, 30$ ). Dark blue, Glia Gal4/+ controls; light blue, *UAS*+/+ controls; Red or green, corresponding experimental group. **C–E**, GS immunofluorescence quantification in calices of *Eeat-Gal4*+/+ (**C**), *dEeat1-Gal4 > UAS-App1-RNAi*<sup>F02878</sup>, *UAS-GFP* (**D**), and *dEeat1-Gal4 > UAS-App1-RNAi*<sup>F02878</sup>, *UAS-dEeat1* (**E**). **F**, GS immunofluorescence quantification in the calyx of the same genotypes (K-W, 43.13;  $p < 0.00001$ ;  $N = 4$  each group). Scale bar, 20  $\mu$ m.

tions of glutamate or glutamine (Robinson, 2000), and could reflect ongoing neuronal activity, as seen in this report in sleep-deprived conditions (Fig. 5). Blocking glutamate reuptake through inhibition of *dEeat1* expression results in a dramatic increase in wakefulness, consistent with an enhancement of neuronal excitability and with recent findings indicating that glutamate plays an important role in sleep homeostasis (Rival et al., 2004; Tomita et al., 2015; Liu et al., 2016; Robinson et al., 2016). However, inhibition of *dEeat1* expression does not seem to affect GS expression, suggesting that GS levels are not sensitive to intracellular glial glutamate concentration. Furthermore, inhibiting *App1* in glia results in higher sleep amounts, a phenotype opposite to the one observed upon inhibition of *dEeat1*. In parallel, *App1* inhibition results in an upregulation of *dEeat1* transcripts (Fig. 3), which appeared localized preferentially at the periphery of the brain. Increasing further *dEeat1* expression can rescue the longer sleep phenotype of *App1* mutants, while it has no effect in wild-type flies (Fig. 6). These observations together suggest that *App1* inhibition has consequences distinct from a mere blockade of glutamate recycling, but rather elicits compensatory mechanisms as a result of abnormal glutamate recycling.

In this context, *App1* appears to be mainly required in cortex glia. Cortex glia cells are associated with neuronal cell bodies and are in contact with neuropile glia (ensheathing, astrocyte-like) as well as brain-hemolymph barrier glia (perineurial and subperineurial) (Awasaki et al., 2008). Being placed at the junction between nutrient supply and synaptic activity, cortex glia cells are thus strategically located to regulate neuronal metabolism. The bulk of GS expression changes observed in this study occurred in the brain neuropiles, which receive projections of astrocyte-like

glia. It may be possible, as described for histamine in the visual system, that glutamate recycling involves long-distance interactions between several glial cell types (Chaturvedi et al., 2012). In support of this idea, we find that the expression of *Inx2*, a major glial gap junction protein, is also modulated bidirectionally by *App1* expression. *Inx2* expression is prominent at the junction between astrocyte-like/ensheathing and cortex glia, as well as between cortex glia and subperineurial glia, suggesting that it plays a key role in enabling the flow of small molecules through the different glial compartments. Both glutamate and glutamine can diffuse through gap junction (Giaume et al., 1997). Since insects lack a vascular system penetrating the brain, the network properties of glial cells should play a critical role in neurotransmitter recycling and in the distribution of nutrients. In mammals, *App* has been shown to be upregulated in glia devoid of connexin 43, a main gap junction involved in astrocyte coupling (Iacobas et al., 2004). Interestingly, astrocyte coupling is increased in mouse overexpressing *App* (Peters et al., 2009). The expression of astroglial connexins is upregulated in a mouse model of AD as well as in AD patients (Mei et al., 2010; Koulakoff et al., 2012) and alterations in gap junctions may contribute to the pathophysiology of the disease (Yi et al., 2016).

We find that both *Inx2* and GS levels are increased following sleep deprivation. Similarly, *App* transcripts have been found to increase in mouse astrocytes following sleep deprivation (Bellese et al., 2015). In addition, paradoxical sleep deprivation in rat has been reported to increase GS protein and messenger levels throughout the brain (Sallanon-Moulin et al., 1994), and in human, a Nuclear Magnetic Resonance study in sleep-deprived healthy subjects found increased glutamate and glutamine levels



**Figure 7.** Knocking down the glutamate transporter dEaat1 specifically in cortex and astrocyte-like glia disrupts sleep. **A**, Total daily sleep upon expression of *UAS-dEaat1-RNAi<sup>IR2</sup>* driven in different types of glia. P + SP, Perineurial and subperineurial (*9-137-Gal4*);  $F = 43.36$ ;  $p < 0.00001$ ;  $N = 37, 36, 38$ . E, Ensheathing (*mz0709-Gal4*);  $K-W, 18.56$ ;  $p < 0.00093$ ;  $N = 16, 16, 16$ . A, Astrocyte-like (*Alm-Gal4*);  $F = 9.526$ ;  $p < 0.00017$ ;  $N = 32, 32, 31$ . A + C, Astrocyte-like and cortex (*dEaat1-Gal4*);  $K-W, 39.58$ ;  $p < 0.00001$ ;  $N = 36, 37, 36$ . C, Cortex (*NP2222-Gal4*);  $K-W, 11.66$ ;  $p < 0.0024$ ;  $n = 14, 16, 16$ . NG, No glia (*repo-Gal80, dEaat1-Gal4*);  $F = 1.019$ ;  $p < 0.037$ ;  $N = 16, 16, 16$ . **B**, Baseline sleep (minutes of sleep per hour) of *dEaat1-Gal4 > UAS-dEaat1-RNAi<sup>IR2</sup>* flies: sleep is severely reduced and its circadian timing disrupted. **C**, Baseline sleep in *repo-Gal80, dEaat1-Gal4 > UAS-dEaat1-RNAi<sup>IR2</sup>* flies is similar to that of controls. **D**, GS protein levels in calyx upon *dEaat1* knock-down in cortex and astrocyte-like glia. Dark blue, Glia Gal4/+ controls; light blue, *UAS-dEaat1-RNAi<sup>IR2</sup>/+* controls; red, corresponding glial *Gal4 > UAS-dEaat1-RNAi<sup>IR2</sup>* knock-down (lines and bars). **E**, Model of *Appl* function in *Drosophila* glia. (1) Glutamate released at the synapses is (2) taken up by astrocyte-like glia expressing the dEaat1 transporter. Glutamate is converted to glutamine in astrocyte-like glia by GS (3) and diffuses via *Inx2* gap junctions through the glial network (4). In the cortex glutamine could be eventually converted back to glutamate in neuronal cell bodies. *Appl* modulates both *Inx2* and GS function during this process.

in selected regions of the brain (Murck et al., 2002). Together these results suggest that altered glutamatergic recycling is an evolutionary conserved consequence of sleep deprivation and may be linked to a modulation of App function, although further work is needed to confirm this model.

Altogether, these data emphasize the role of glia in sleep/wake regulation, a topic that so far has received little attention (Frank, 2013; Petit and Magistretti, 2016). Of note, the Notch transmembrane protein, which, like *Appl*, depends on  $\gamma$ -secretase for pro-

cessing, and on *mint/Spoc/spen* proteins for intracellular signaling, has also been implicated in the glia-dependent regulation of sleep/wake in *Drosophila* (Seugnet et al., 2011). Interestingly, Notch also regulates glutamate transporter as well as the expression of several innexins during development (Lechner et al., 2007; Stacey et al., 2010). In rodents, Notch signaling genes are also predominantly expressed in astrocytes (Cahoy et al., 2008). Both Notch and *App* interact with the epidermal growth factor receptor pathway, which can modulate the expression of

the dEaat1 glutamate transporter and sleep in *Drosophila* and in rodents (Kramer et al., 2001; Caillé et al., 2004; Lievens et al., 2005; Foltenyi et al., 2007). These observations suggest the existence of potentially complex interactions between multiple glial signaling pathways modulating both sleep/wake and glutamate recycling. Elucidating how these pathways interact in flexible models, such as *Drosophila*, will be an important step in understanding how they contribute to pathologies such as AD and affect sleep/wake in this context.

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