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Neurexin-1 regulates sleep and synaptic plasticity in *Drosophila melanogaster*

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Abstract

Neurexins are cell adhesion molecules important for synaptic plasticity and homeostasis, though links to sleep have not yet been investigated. We examined effects of neurexin-1 perturbation on sleep in *Drosophila*, showing that neurexin-1 nulls display fragmented sleep and altered circadian rhythm. Conversely, over-expression of neurexin-1 can increase and consolidate night-time sleep. This is not solely due to developmental effects as it can be induced acutely in adulthood, and is coupled with evidence for synaptic growth. Timing of over-expression can differentially impact sleep patterns, with specific night-time effects. These results show that neurexin-1 is dynamically involved in synaptic plasticity and sleep in *Drosophila.* Neurexin-1 and a number of its binding partners have been repeatedly associated with mental health disorders, including autism spectrum disorders, schizophrenia and Tourette syndrome, all of which are also linked to altered sleep patterns. How and when plasticity-related proteins such as neurexin-1 function during sleep can provide vital information on the interaction between synaptic homeostasis and sleep, paving the way for more informed treatments of human disorders.

Introduction

During brain development, a vast diversity of neurons synapse precisely with each other to form complex networks. Even after these initial connections are made, the circuitry is plastic and altered to reflect an animal's experiences. Neurexins encode transmembrane cell adhesion molecules (Ushkaryov et al. 1992) that are vital for the formation, maturation and specification of synapses (Dean and Dresbach 2005). Extensive alternative splicing and variable interactions of neurexins with their respective postsynaptic partners, such as the neuroligins, appear to comprise a code that can direct synapse development towards excitation or inhibition (M Missler and Südhof 1998; Graf et al. 2004; Ben Chih, Engelman,

and Scheiffele 2005). Furthermore, neurexin may be essential for activity-dependent synaptic plasticity, rather than simply acting as a synaptogenic agent (Biswas et al. 2010; Choi et al. 2011). Dysfunction of these proteins may therefore impair synaptic function and alter homeostasis, leading to improper brain function (Thomas C Südhof 2008). Reflecting their importance, altered neurexins and their binding partners are associated with a number of neurodevelopmental and neuropsychiatric disorders, including schizophrenia and autism spectrum disorders (ASD) (Reichelt, Rodgers, and Clapcote 2011).

Sleep is a state of decreased arousal thought to be vital for mechanisms underlying synaptic plasticity and homeostasis. Sleep disturbances are common in a number of cognitive disorders, including ASD (Kotagal and Broomall 2012) and schizophrenia (Wilson and Argyropoulos 2012). Though the functions of sleep are still unclear, it is suggested that waking periods lead to a net overall increase in synaptic strength, and sleep could provide a defined period of synaptic plasticity to downscale, refine, consolidate or strengthen particular synapses and circuitry (Tononi and Cirelli 2014). Plasticity-related genes have been linked to sleep regulation (Mongrain et al. 2010), including neuroligin-1 (Helou and Bélanger-Nelson 2013) and neuroligin-4 (Y. Li et al. 2013), though neurexin has not been directly investigated.

Drosophila melanogaster is a useful system in which to study neurexin due to the wide array of genetic tools with temporal and spatial control, along with the reduced complexity and highly conserved nature of neurexin in invertebrates (Tabuchi and Südhof 2002; Biswas et al. 2008). Previous work has shown that perturbation of neurexin and neuroligins in *Drosophila* leads to a variety of synaptic alterations at the neuromuscular junction (NMJ) (Knight, Xie, and Boulianne 2011). In *Drosophila*, sleep deprivation (SD) and enriched environments are

linked to increases in synaptic proteins and growth in the brain, followed by decreases in expression of synaptic proteins after rebound sleep (Gilestro, Tononi, and Cirelli 2009; J. M. Donlea, Ramanan, and Shaw 2009; Bushey, Tononi, and Cirelli 2011). Sleep in *Drosophila* and humans shares a number of common features, including: 1) homeostatic regulation (Huber et al. 2004); 2) cycling arousal states (van Alphen et al. 2013) and 3) interaction with circadian rhythms and life history, including age and waking experience (P J Shaw et al. 2000; Ganguly-Fitzgerald, Donlea, and Shaw 2006).

Due to the previously discussed importance for neurexin in synaptic plasticity and homeostasis, along with more recent evidence linking neuroligins and sleep in mice (Helou and Bélanger-Nelson 2013) and *Drosophila* (Y. Li et al. 2013), we predicted that alteration of neurexin would result in sleep and plasticity phenotypes. Here, we provide evidence that flies lacking neurexin-1 display fragmented sleep and perturbed circadian rhythms. Conversely, flies over-expressing neurexin-1 show more consolidated sleep and evidence of synaptic development. Interestingly, the effects of neurexin-1 overexpression are relatively specific to night-time sleep, suggesting that neurexin-mediated plasticity may support functions associated with sleep at night in *Drosophila*.

Materials and methods

Fly stocks

Drosophila melanogaster were cultured on standard medium (agar, sugar, yeast) at 25ºC, 50-60% humidity, under a 12:12 light/dark cycle (lights on at 8:00 AM). *UAS-dnrx*, $dnrx^{273}$*/TM3KR:GFP* and $dnrx^{241}$*/TM6TbsbYFP* were provided by Manzoor Bhat (J. Li et al.

2007) and *dnrx*^{313}*/Tm6TbSbYFP* was provided by David Featherstone (K. Chen et al. 2010). Remaining stocks **(including Bloomington Stock #5905, w1118)** are available from the Bloomington *Drosophila* Stock Center (BDSC) at Indiana University.

Quantitative Real-Time PCR

Flies (3 - 8 days old) were frozen, vortexed, and heads isolated using 710 µm and 315 µm sieves (Endecotts). Total RNA was extracted from ~30-50 heads using TRIzol (Invitrogen), according to the manufacturer's instructions. After treatment with DNase I, cDNA was synthesized from RNA using SuperScript III Reverse Transcriptase (Invitrogen). cDNA was mixed with primers and SYBR green (Applied Biosystems) and real-time quantitative RT-PCR assays were performs in a LightCycler 480 (Roche). *Drosophila* gene regions of interest were viewed using Artemis (Wellcome Trust Sanger Institute, UK), and Primer3 v.0.4.0 (Whitehead Institute for Biomedical Research, MA, USA) was used to design primers yielding products less than 150bp with an ideal melting temperature of 60ºC. The primers were F-5'-GTGGAATACCGTCGCTCCTA-3' and R-5'-CTTGCGGAAGGTGTAGAAGG-3' (product size 132bp) for *dnrx1*; F-5'-CCCAAGGGTATCGACAACAG-3' and R-5'-GTTCGATCCGTAACCGATGT-3' (78bp) for *Rp49*; F-5'-TGCACAAAGGAAATGAGCTG-3' and R-5'GTCGTGATGTCCCCGATAAC-3' (82bp) for *dnlg1*; F-5'- GTTCCTTGATGGGTTGCATT-3' and R-5'-GCTTCCCGCTCTTTAGCTTT-3' (103bp) for *dnlg2*; F-5'-CAGTTCCGTGCCCTCATTAT -3' and R-5'-GGGTCTACTACTGCGGTTGC-3' (130bp) for *dnlg3*; F-5'-GGAAGCCAGAGGACCCTAAG-3' and R-5'-CTACCAACTTTGGCGAGAGG -3' (112bp) for *dnlg4*; F-5'-TCCTCGCTTCAGTTTGGATT-3' and R-5'-ATAGTCCGTGAAGGCGTCTG-3' (87bp) for *dnrx4*. Primers were synthesized by

GeneWorks (Australia). Three technical repeats and three biological repeats were run for each sample and normalized to expression levels of ribosomal protein 49 (Rp49). Data was analysed using the 2-Delta Delta C (T) (2^{-DDCT}) method (Livak and Schmittgen 2001).

Immunohistochemistry

Brains of adult flies were dissected and stained based on standard protocols (Wu and Luo 2006). Briefly, dissected brains were fixed in 4% paraformaldehyde for 20 minutes on a rotator at room temperature, washed (0.2% PBS-TritonX-100, 3 x 20 minutes), blocked in 5% normal goat serum for 30 minutes, then incubated in primary antibody diluted in block overnight on a rotator at 4ºC. Primary antibodies: mouse anti-nc82 (1:10, Developmental Studies Hybridoma Bank, University of Iowa); rabbit anti-GFP (1:1000, Invitrogen). Brains were then washed (3 x 10 minutes in 0.2% PBS-T) and secondary antibodies diluted in blocking solution were incubated overnight on a rotator at 4ºC (in darkness). Secondary antibodies: AlexaFluor-488 goat anti-rabbit / AlexaFluor-647 goat anti-mouse (1:250, Invitrogen). Finally, brains were washed (3 x 10 minutes in 0.2% PBS-T) and mounted on bridge slides in Vectashield mounting medium (Vector Laboratories).

Imaging and intensity processing

Preparations were viewed with a Plan Apo 10x objective (numerical aperture of lens = 0.45) and Z-stacks scanned using a Nikon fluorescence microscope running NIS-Elements software. For nc82 (Brp) comparative analysis, images were taken using identical imaging settings. Intensity levels were then measured using ImageJ/Fiji (NIH, Bethesda), similar to protocols previously described (Zweier et al. 2009) and outlined in the Fiji documentation

(http://fiji.sc/Image_Intensity_Processing). Briefly, images were corrected for background and an averaged reference image of the Z-stack was made and used to then manually select anatomical regions of interest. The intensity of the antennal lobes was then measured throughout the Z-stack and values output into Microsoft Excel for further analysis. The average intensity of each antennal lobe throughout the Z-stack was taken, with a final single value from each brain consisting of the average intensity of both antennal lobes. Data was collected from two independent experiments and mean Brp intensity the antennal lobes of 19ºC brains (n = 7) was set as 100% for comparison to 31ºC brains (n = 8).

Behavioural analysis

Flies were briefly anaesthetized using CO_2 and separated by gender into fresh food vials, with matching numbers (~15-30) of flies of each genotype. 24 hours later, individual flies (3 - 8 days old) were placed in 65mm glass tubes (3mm diameter) containing food, sealed with wax and plugged with cotton wool. Tubes were loaded into *Drosophila* activity monitoring systems (DAMS; Trikinetics, Waltham, MA), then placed in an incubator (Tritech Research) at 25ºC. Locomotor activity was monitored via automatic counting (in 1 minute bins) whenever a fly crosses an infrared beam, with counts stored for later analysis. Uninterrupted inactivity (zero counts per minute) for over 5 minutes has previously been defined as sleep (P J Shaw et al. 2000; Huber et al. 2004); data was analysed to calculate total sleep time, bout duration and number via a custom MATLAB (Mathworks, Natick, MA) script. For sleep deprivation experiments we used a Sleep Nullifying Apparatus (SNAP) that mechanically disturbs flies every 20 seconds (Paul J Shaw et al. 2002). For circadian analysis, protocol was based on those previously described (Chiu et al. 2010) and locomotor activity data was

analysed using an ImageJ/FiJi plugin, 'ActogramJ' (Schmid, Helfrich-Förster, and Yoshii 2011).

Statistical analysis

All data are presented as mean +/- standard error of the mean (S.E.M.). Statistical analysis was performed using R (R version 2.15.1, The R Foundation for Statistical Computing, 2012). Data were viewed as boxplots and tested for normality using the Shapiro-Wilks test. For normally distributed data, unpaired two-tailed Student's *t*-tests were used to compare a single variable across two groups; two-way analysis of variance (ANOVA) followed by *post-hoc* Tukey's Honest Significant Differences test was used when more than one variable (timing of over-expression, genotype) was compared across groups. Non-parametric data was assessed for significance with Wilcoxon's rank-sum test (with *post-hoc* Bonferroni correction to account for multiple comparisons). Periodogram analysis was performed within ActogramJ using the Lomb-Scargle Periodogram method (Schmid, Helfrich-Förster, and Yoshii 2011).

Results

Neurexins and neuroligins in *Drosophila*

A single neurexin-1 gene (CG7050) is present in *Drosophila* (*dnrx1*) compared to three in mammals, and *Drosophila* neurexin-1 protein is structurally similar to mammalian α-neurexin (Tabuchi and Südhof 2002; J. Li et al. 2007; Zeng et al. 2007; Figure 1a) and highly expressed in adult *Drosophila* brain (Chintapalli, Wang, and Dow 2007). Though alternative splicing of neurexin-1 in the honeybee leads to *Am-Nrx1-A* and *Am-Nrx1-B* transcripts (Biswas et al. 2008), no alternative β transcripts are apparent in *Drosophila* (Zeng et al. 2007). Neuroligins encode transmembrane proteins first identified as binding partners for neurexins and also undergo alternative splicing (Ichtchenko, Nguyen, and Südhof 1996;

Koehnke et al. 2010) (Figure 1a). *Drosophila* has four neuroligins (*dnlg1*: CG31146, *dnlg2*: CG13772, *dnlg3*: CG34127, *dnlg4*: CG34139), compared to five in humans.

The *dnrx1* knockout lines employed in this study (represented in Figure 1b) were previously generated and confirmed as effective null alleles by examining phenotypes at the larval neuromuscular junction (NMJ) (J. Li et al. 2007; K. Chen et al. 2010). $dnrx^{241}$ ('241') contains a precise deletion of the coding region, while $dnrx^{273}$ ('273') has an ~8kb deletion that removes most of the coding sequence for the extracellular *dnrx1* region (J. Li et al. 2007). $dnrx^{313}$ ('313') contains a 10bp deletion of a highly conserved sequence at intron 7 that has been suggested to represent an essential regulatory region (K. Chen et al. 2010). Homozygotes generally exhibited more lethality (data not shown), so we examined allelic combinations that survived in large numbers into adulthood, 273/313 and 313/241, as well as a deficiency ('Df') containing a chromosomal deletion including *dnrx1*.

Quantitative PCR indicates somewhat reduced levels of *dnrx1* mRNA in 313/241 flies compared to WT flies (Figure 1c, $P = 0.1005$, degrees of freedom (d.f.) = 4). 313/241 flies have previously been described as lacking DNRX1 immunoreactivity at the NMJ (K. Chen et al. 2010), so presence of *dnrx1* mRNA in the brain may be due to the qPCR primers mapping outside of the 10bp deletion in the 313 allele (see Figure 1b for qPCR primer mapping and the location of the 313 deletion). Supporting this, we investigated mRNA levels in flies containing the 241 deletion of the entire coding region over a deficiency which includes deletion of *dnrx1* and did not detect any *dnrx1* mRNA (Figure 1c right panel 'Df/241'). 313/241 flies did not display significantly affected mRNA levels of neuroligins 1 - 4 (Figure 1c). Interestingly, we see a significant increase in levels of *Drosophila* neurexin-4 (*dnrx4*, CG6827) message in 313/241 flies, possibly representing compensatory effects (Figure 1c, *P*

= 0.0066, d.f. = 4). *dnrx4*, an orthologue of the vertebrate contactin-associated protein 2 (Caspr2; Zweier et al. 2009; Einheber et al. 1997), appears to mediate neuron-glia interactions (Banerjee et al. 2006; Faivre-Sarrailh et al. 2004; Baumgartner et al. 1996) and is phylogenetically ancestrally related to *dnrx1* (J. Li et al. 2007).

Previous studies linked defective synaptic plasticity mechanisms to sleep need in *Drosophila* (Bushey, Tononi, and Cirelli 2011; Ganguly-Fitzgerald, Donlea, and Shaw 2006; J. M. Donlea, Ramanan, and Shaw 2009), leading us to investigate sleep patterns in *dnrx1* knockout male and female flies (Figure 1d). Compared to WT flies, *dnrx1* knockouts (313/241 and 273/313) display perturbed sleep patterns, most obviously as they sleep more at dusk (Figure 1d). Circadian rhythm in wild-type *Drosophila* is crepuscular, consisting of activity peaks around the equivalents of dawn (8am or ZT0, lights on) and dusk (8pm or ZT12, lights off). It has been previously established that male and female flies have different sleep patterns, but we note that *dnrx1* knockouts of both genders have a consistently different pattern compared to WT flies, with a delayed daytime sleep profile (Figure 1d). As sleep is largely shaped by the dual processes of circadian control and homeostatic drive, we further explored and deconstructed these processes in *dnrx1* knockouts of both genders.

Loss of *dnrx1* leads to sleep fragmentation and altered circadian rhythms

In order to substantiate consistent phenotypes, we examined sleep and circadian rhythm in two neurexin-1 knockout allelic combinations (313/241 and 273/313), as well as Df/313 flies, in both males (Figure 2) and females (Figure 3). In terms of total sleep duration, male *dnrx1* knockouts sleep significantly less than WT flies during the day (Figure 2a; 273/313 $P = 2.5 \times 10^{-11}$, 313/241 * $P = 0.00302$, 313/Df * $P = 0.0083$), and 313/Df males sleep significantly more than WT at night (Figure 2a; $P = 0.0032$). We next examined the duration and number

of bouts, to assess potential differences in the architecture of sleep. During the day, *dnrx1* knockouts display significantly shorter bout durations (Figure 2b; 273/313 $P = 3.1 \times 10^{-14}$, 313/241 $P = 1.1 \times 10^{-13}$, 313/Df $P = 7.1 \times 10^{-6}$) and significantly increased bout number (Figure 2c; 273/313 $P = 2.5 \times 10^{-10}$, 313/241 $P = 2 \times 10^{-16}$, 313/Df $P = 8.9 \times 10^{-4}$) compared to WT. We see similar results at night in 313/273 and 313/241 males, with significantly decreased bout duration (Figure 2b; 273/313 $P = 0.0249$, 313/241 $P = 7.6 \times 10^{-6}$) and significantly increased bout number (Figure 2c; 273/313 $P = 0.0031$, 313/241 $P = 3.5 \times 10^{-8}$) compared to WT. The architecture of sleep at night is not significantly affected in 313/Df males. Overall, sleep is more fragmented in *dnrx1* knockouts than in WT, most reliably during the day. This is best illustrated by plotting bout duration versus bout number on the same graph for individual fly data (Figure 2d): *dnrx1* knockouts have 'fragmented' sleep (many short bouts) whereas WT flies display more 'consolidated' sleep (few long bouts).

We observed earlier that *dnrx1* mutant sleep profiles appear to respond to, rather than predict, environmental light changes; this suggests potential circadian rhythm defects (Figure 1e). We chose to further examine 313/241 flies as they displayed stronger phenotypes. Consistent with perturbed circadian rhythm, we observed altered activity peaks in 313/241 males (Figure 2e). To elucidate whether the mutation was affecting circadian rhythms, we further investigated locomotor activity rhythms during dark:dark (DD) conditions. Rhythmic activity peaks seen in 12:12 hour light:dark (LD) conditions are preserved in WT flies even in continuously dark conditions, with clear anticipatory increases in locomotion during subjective dawn and dusk, indicating appropriate function of the endogenous clock (Figure 2f). In contrast, we see delayed activity peaks and abnormal circadian behaviour in 313/241 knockouts in DD conditions (Figure 2f). WT and 313/241 flies have free-running periods close to the expected 24 hours during LD (Figure 2g). During DD, WT flies retain these

rhythmic activity patterns, whereas 313/241 flies have a significantly longer free-running period length (Figure 2g; $P < 0.001$, d.f. = 62). **Though rhythm strength is reduced in dnrx1 knockouts compared to controls in DD, o**nly a small percentage of flies are arrhythmic (Fourier values < 0.04) in DD, with 94% of wild-type and 91% of *dnrx1* knockout flies retaining rhythmicity **(data not shown). Further analysis of the potential function of neurexin in core clock machinery will be important to discover possible contribution to sleep fragmentation.**

Female *dnrx1* knockout flies displayed similar defects in sleep architecture and circadian rhythms, although these were generally subtler than in males (Figure 3). This may be partly attributed to the fact that WT females generally sleep less and display more fragmented sleep than males, though it is interesting to note that neurexin-1 appears to be more highly expressed in adult males than females (Graveley et al. 2010). In terms of total sleep duration, 313/241 and 313/Df females sleep significantly more (313/241 $P = 0.00772$, 313/Df $P = 0.00826$) than WT during the day, whereas 273/313 females sleep significantly more than WT at night (Figure 3a; $P = 4.5 \times 10^{-6}$). Just as described in male *dnrx1* knockouts, we see more consistent effects on sleep bout duration (Figure 3b) and number (Figure 3c). Females display significantly decreased night bout duration in 313/241 and 313/Df flies (Figure 3b, 313/241 $P = 0.00563$, 313/Df $P = 0.00093$). Bout number is also increased significantly during the day in 313/241 and 313/Df flies (Figure 3c 313/241 $P = 4.8 \times 10^{-7}$, Df/313 $P = 0.005$) and during the night in all knockouts (273/313 $P = 0.04851$, 313/241 $P = 9.5 \times 10^{-6}$, Df/313 $P = 0.00013$). Overall, sleep appears to be more fragmented in *dnrx1* knockouts females than in WT; unlike what we see in males, effects are more obvious at night (Figure 3d). Finally, we examined circadian rhythm in 313/241 females, though activity pattern differences are initially subtler than those seen in males (Figure 3e). Rhythmic activity peaks

in WT females under LD conditions are preserved in DD conditions, whereas 313/241 knockouts display altered circadian behaviour under DD conditions (Figure 3f). WT and 313/241 females have free-running periods of around 24 hours during LD and WT flies retain these rhythmic activity patterns during DD; however, female 313/241 flies have a significantly longer free-running period length during DD (Figure 3g; $P < 0.001$, d.f. = 29).

In summary, we see fragmentation of sleep in both male and female *dnrx1* knockout flies of both genders, compared to WT flies. Effects are most obvious during the day in males and during the night in females; this may be at least in part attributed to the fact that females are highly active during daytime. Effects on total sleep duration are more variable, and for this reason we focused more on effects on the architecture (sleep consolidation) rather than amount of sleep. We also noted generally stronger phenotypes in 313/241 flies. Having outlined the circadian consequences of *dnrx1* knockout, we wanted to further investigate potential homeostatic effects.

Pan-neuronal overexpression of *dnrx1* consolidates night-time sleep

Our results show *dnrx1* nulls display fragmented sleep and previous work has shown that *dnrx1* knockouts have reduced synapse numbers in the CNS (Zeng et al. 2007). Conversely, pan-neuronal over-expression of neurexin-1 or neurexin-4 increases synaptic branching and active zone number at the NMJ (Zweier et al. 2009). We thus examined whether pan-neuronal over-expression of neurexin-1 affects sleep. This was achieved through use of the binary GAL4/UAS technique, whereby a spatially restricted yeast transcription activator protein (GAL4) activates a gene downstream of the GAL4-binding upstream activation sequence (UAS) (Brand and Perrimon 1993). We used two pan-neuronal drivers, *appl-GAL4* and e*laV-GAL4* (expression patterns visualized with *UAS-mCD8-GFP* in the adult brain in

Figure 4a; *appl>GFP* and *elaV>GFP*) to drive over-expression of full length *UAS-dnrx1*. We examined effects in females in all future figures, as we rationalize that although effects on sleep were subtler than in males, less sleep in general allows more scope in detecting differences in both directions (increased or decreased).

We found that pan-neuronal overexpression of *dnrx1* has specific effects on night-time sleep only, with a significant increase in the total amount of sleep at night compared to genetic controls (Figure 4b; *appl>dnrx1* compared to *appl-GAL4/+* $P = 0.0034$ and *UAS-dnrx1/+* $P = 0.0186$, ; *elav>dnrx1* compared to *elav-GAL4/+* $P = 0.0018$ and *UAS-dnrx1/+* $P = 0.0015$). The makeup of sleep at night is also altered when *dnrx1* is over-expressed: bout duration is significantly longer in *appl>dnrx1* flies compared to controls (Figure 4c; *appl>dnrx1* compared to *appl-GAL4/+* $P = 0.0309$ and *UAS-dnrx1/+* $P = 0.0033$), and the number of bouts is significantly decreased in *elav>dnrx1* flies compared to controls (Figure 4d; *elav>dnrx1* compared to *elav-GAL4/+* $P = 0.0375$ and *UAS-dnrx1/+* $P = 0.0059$) . On the whole, flies with pan-neuronal over-expression of neurexin-1 display more consolidated sleep than controls during the night (Figure 4e). As opposed to the more fragmented sleep observed in *dnrx1* knockouts, *dnrx1* over-expression appears to specifically extend and consolidate night-time sleep. This suggests that *dnrx1* expression may be associated with sleep consolidation effects during a specific sleep stage (van Alphen et al. 2013). To investigate whether these sleep-promoting effects could be acutely engaged in adult flies, we transiently controlled *dnrx1* over-expression during adulthood, by either turning expression on during the day or during the night.

Acute overexpression of *dnrx1* affects sleep

We used the GAL4 repressor, Gal80^{ts}, to control the timing of *dnrx1* overexpression. At permissive temperatures, Gal80^{ts} inhibits transcription activation of GAL4; when shifted to restrictive temperatures it becomes non-functional, allowing GAL4 to activate constructs downstream of the UAS (Lee and Luo 1999; Zeidler et al. 2004). We employed a pan-neuronal driver (*elav^{c155}-GAL4*) in combination with a ubiquitously expressed *Tubulin-Gal80^{ts}* construct to drive neurexin-1 (*UAS-dnrx1*). As a control, we used the same GAL4 and GAL80 in combination with *UAS-mCD8-GFP*. This allowed confirmation (by visualization of GFP) that 12 hours at 31ºC was sufficient to activate GAL4-driven gene expression (Figure 5a, McGuire et al. 2003). Both genotypes (*c155^{elav}-GAL4;Tub-Gal80^{ts}/UAS-dnrx1* or *UAS-mCD8GFP*) were grown at permissive temperatures, then assessed for sleep in adulthood. After 24 hours of recording baseline activity, the incubator temperature was shifted to the restrictive 31ºC for 12 hours to allow activation of *UAS-dnrx1* and *UAS-mCD8-GFP*, then back to 19ºC for a further 24 hours. Due to potential cycling of *nrx1* levels (Shapiro-Reznik et al. 2012), we investigated whether *dnrx1* activation would have differing effects depending on timing of over-expression; thus we examined effects of day-time (Figure 5b-f) or night-time (Figure 5g-k) over-expression separately.

As expected, we did not detect any significant differences between *dnrx1* and control sleep patterns during the first 24 hours without *UAS* activation (Figure 5b-e and 5g-j, baseline values). Previous studies have noted that increasing temperature can affect circadian rhythms and sleep in flies (Ishimoto, Lark, and Kitamoto 2012; Matsumoto et al. 2003) and we similarly observe reduced total sleep in heated flies (Figure 4c and 4h 31ºC activation). Increasing the temperature leads to more fragmented sleep for both genotypes, with the

largest effect being a decrease in bout duration (Figure 5i 31ºC activation) and increased bout number (Figure 5j 31ºC activation) during heat activation at night.

We compared sleep amount and architecture in flies that had expressed either GFP or neurexin-1 in the previous 12 hours. Following 12 hours of day-time activation, flies over-expressing *dnrx1* sleep significantly more than control flies the following night and day (Figure 5c; $F_{(4, 230)} = 5.285$, recovery night $P = 1.24 \times 10^{-4}$ and recovery day $P = 2.8 \times 10^{-6}$). Bout duration during the recovery period is not affected significantly in control or *dnrx1* flies (Figure 5d recovery), though *dnrx1* flies have significantly more sleep bouts during the recovery day (Figure 5e recovery day, $F_{(4, 230)} = 4.686$, $P = 0.015$). Overall, flies that over-expressed *dnrx1* for 12 day-time hours sleep more than control flies the following day and night; this sleep may be marginally more consolidated than in controls during the recovery night but not during the recovery day (Figure 5f).

We next evaluated effects of neurexin-1 activation during the night. Flies over-expressing *dnrx1* at night sleep significantly more than control flies during the subsequent recovery night (Figure 5h recovery night, $F_{(4, 230)} = 6.359$, $P = 4 \times 10^{-7}$). Sleep during the recovery night in *dnrx1* flies also consists of significantly longer (Figure 5i recovery night, $F_{(4, 225)} = 7.087$, $P < 0.00001$) and fewer (Figure 5j recovery night, $F_{(4, 225)} = 8.869$, $P < 0.00001$) bouts than seen in controls. However, following 12 hours of night-time activation, there were no significant differences in recovery day-time sleep in *dnrx1* flies compared to controls (Figure 5h-j recovery day). Overall, flies that over-expressed *dnrx1* for 12 night-time hours display an increased amount of more consolidated sleep than controls during the recovery night (Figure 5k). It is possible that the genetic or experimental manipulation is causing an effect in control flies but not in flies overexpressing *dnrx1*, however it is still apparent that sleep is more

consolidated than in controls after *dnrx1* activation. **We have not investigated any possible effect of temperature alone.**

In conclusion, we see effects of acute 12 hour over-expression of *dnrx1* during adult-hood on sleep amount and architecture. If this over-expression happens during the day-time, flies sleep significantly more during the following night and day. If activation of *dnrx1* occurs during the night-time, recovery sleep during the following night is increased and also more consolidated than in controls. This suggests that neurexin-1 may promote particular sleep need in adult flies and may also have specific functional timing. A caveat here is that we are unsure how effectively and quickly *dnrx1* turnover is affected by 12 hours of activation.

Synaptic plasticity changes due to *dnrx1*

Sleep has been proposed to regulate synaptic connectivity (Tononi and Cirelli 2014), with changes in neuronal morphology and synapse numbers providing a readout of sleep function (Bushey, Tononi, and Cirelli 2011; J. M. Donlea, Ramanan, and Shaw 2009). Having seen that behaviourally, *dnrx1* knockouts have fragmented sleep, whereas activation of *dnrx1* for 12 hours during the night in adult-hood can lead to increased and consolidated sleep compared to controls, we investigated potential synaptic changes. An active zone component, Bruchpilot (Brp, Wagh et al. 2006), was previously visualized by antibody staining in *Drosophila* and found to be reduced in *dnrx1* larval knockouts brains (Zeng et al. 2007). On the other hand, over-expression of *dnrx1* increases Brp intensity at the NMJ (Zweier et al. 2009). To investigate potential changes to central synapses due to *dnrx1* perturbation, we quantified mean intensity of Brp in adult brains. Antennal lobes were selected as regions of interest due to their high antibody penetration efficiency and clear-cut visualization, and have

previously been reported to display robust increases in Brp staining following sleep deprivation (Gilestro, Tononi, and Cirelli 2009).

Mean fluorescent intensity in the antennal lobes of wild-type flies were set as baseline (100%), to which to compare *dnrx1* knockouts (313/241). We detect significantly decreased active zone staining in flies lacking *dnrx1*, suggesting knockouts have reduced central synapses (Figure 6a, P = 0.0122, d.f. = 8). Next, we quantified active zone staining in *elav^{c155}-GAL4;Tub-Gal80^{ts}/UAS-dnrx1* flies after 12 hours of activation at 31ºC at night (or no activation at 19ºC for controls) (Figure 6b). Mean fluorescent intensity in the antennal lobes of flies that did not have *UAS-dnrx1* activated were set as baseline (100%), to which to compare those flies that had over-expressed *dnrx1* for 12 hours. We detect increased active zone staining with Brp in the antennal lobes of flies with 12-hours pan-neuronal activation of *dnrx1* (Figure 6b P = 0.0597, d.f. = 13), suggesting that acute *dnrx1* overexpression may be associated with a recruitment of other synaptic proteins.

Changes in waking experience, such as environmental enrichment or sleep deprivation, have previously been correlated with increases in synaptic density markers in *Drosophila* (Gilestro, Tononi, and Cirelli 2009; J. M. Donlea, Ramanan, and Shaw 2009; Ganguly-Fitzgerald, Donlea, and Shaw 2006). Previous work has shown that neurexin and neuroligins are dynamically regulated in response to associative learning in *Apis mellifera* (Biswas et al. 2010), neurexin is required for associative learning in *Drosophila* larvae (Zeng et al. 2007), and depletion of these proteins impairs fear learning in *Aplysia californica* (Choi et al. 2011). Behaviourally, flies are reported to display a rebound of increased total sleep in the daylight hours immediately following SD (P J Shaw et al. 2000; Hendricks et al. 2000; Paul J Shaw et

al. 2002). We investigated behavioural effects of SD in WT and 313/241 knockouts (Figure 6c). WT flies that are sleep deprived for 12 hours overnight sleep significantly more compared to baseline values in the first 3 hours following SD (Figure 6c; d.f. = 46, $P < 0.0001$). Interestingly, rebound sleep after SD in *dnrx1* knockout flies lasts longer than in WT flies: sleep is significantly increased for 6 hours compared to baseline values (Figure 6c; d.f. = 46, 0 - 3 ZT * $P < 0.0001$ and 4 - 6 ZT * $P = 0.022$). After 24 hours ('recovery'), sleep has largely returned to baseline levels, though in WT flies we see significantly (d.f. = 46, $+P = 0.024$) more sleep at ZT 4 - 6 and a decrease in sleep at ZT 10 - 12 (Figure 6c; d.f. = 46,. $P = 0.055$). This suggests that *dnrx1* mutants may be impaired in their ability to recover lost sleep, an important component of homeostasis.

Discussion

The synaptic homeostasis hypothesis suggests that sleep is important for regulation of synaptic weight, with day-time activity leading to synaptic potentiation that is then proportionally downscaled during sleep (Tononi and Cirelli 2014). If a major function of sleep is to promote synaptic plasticity, disrupting molecules required to build synapses would be predicted to alter sleep homeostasis. Several studies have shown altered synaptic phenotypes in neurexin and neuroligin mutants, but association with sleep phenotypes is less clear. Here, we show for the first time that perturbation of neurexin-1 affects sleep in *Drosophila.* Loss of *dnrx1* leads to fragmented sleep, whereas night-time over-expression results in increased and consolidated night-time sleep.

Impact of *dnrx1* on synaptic plasticity and sleep

Sleep is regulated by the interaction of circadian and homeostatic processes (Borbély and Achermann 2000), and it appears that *dnrx1* affects both of these processes. First, we discuss potential involvement of *dnrx1* in homeostatic mechanisms. *Dnrx1* null mutants have previously been described as having a reduced number of CNS synapses and exhibit associative learning defects at the larval stage (Zeng et al. 2007). Perturbation of *dnrx1, dnlg1* or *dnlg2* leads to pre- and post-synaptic defects at the NMJ, including reduced numbers of synaptic boutons and changes in the number of active zones, as well as synaptic transmission (K. Chen et al. 2010; Y. Chen et al. 2012; Banovic et al. 2010; Sun et al. 2009; Sun et al. 2011; Mozer and Sandstrom 2012; J. Li et al. 2007). In α-neurexin triple knockout mice, brain and synapse structure is generally normal though there is a reduction in dendritic spines and neurotransmission efficiency (Markus Missler et al. 2003; Zhang et al. 2005). We show that flies without neurexin-1 have more fragmented sleep, as well as reduced synaptic staining. *Dnrx1* knockouts may have reduced potential for large scale activity-dependent synaptic plasticity and so a diminished need for synaptic downscaling during night-time sleep, thus displaying fragmented sleep. Alternatively, neurexin could be required for circuits that regulate sleep.

Conversely to neurexin-1 knockouts, over-expression of neurexin-1 can lead to increased and consolidated night-time sleep as well as increased synaptic staining. Previous work has found that over-expression of Nrx1 or Nrx4 pan-neuronally leads to increased numbers of boutons, active zone density and synaptic branching at the NMJ; this was also found to be dosage dependent, whereby two copies of the GAL4 driver increases Brp staining even more than one copy (Zweier et al. 2009). Overall, increased *dnrx1* activity is important for synapse

development, possibly leading to a need for consolidated sleep in which to downscale synaptic weight; on the other hand, *dnrx1* could be a crucial element of consolidation itself. Future studies should address the cause-effect relationships between synaptic changes, learning and sleep in plasticity mutants.

The effects we see on sleep and synaptic plasticity can be induced acutely in adulthood, and thus may not merely be a consequence of developmental deficits. Previous work has shown that neurexin and neuroligins are dynamically regulated in response to sensory deprivation and associative learning in *Apis mellifera*: learning appears to upregulate nrx1, nlg1 and nlg3 mRNA (Biswas et al. 2010). In mice, 6 hours of daytime SD decreases particular nlg1 isoform transcript and protein expression in the forebrain, and lack of NLG1 leads to decreased wakefulness (Helou and Bélanger-Nelson 2013). Since *dnrx1* is likely an important synaptic and circadian molecule with complex regulation, it follows that *dnrx1* has multiple functions; arguably, these functions could be different during development of the nervous system compared to a role in adult brain plasticity.

Impact of *dnrx1* on circadian rhythm

Data from mice shows diurnal rhythms in neurexin transcripts (detecting major peaks for two transcripts at ZT6), which also appear to be coupled to excitatory/inhibitory synaptic balance (Shapiro-Reznik et al. 2012). Transcriptome data from *Drosophila* has identified transcripts that oscillate in LD or DD conditions, or that are regulated by loss of *period*: Nrx-1 transcripts were found to cycle in LD conditions, while Nlg-2 cycled in DD conditions; Nlg-2 was also found to be significantly down-regulated by *period* loss of function in LD conditions (Hughes et al. 2012). We show that flies without neurexin-1 have an extended circadian

rhythmicity. Interestingly, it has been shown that *dnlg4* is expressed in lateral clock neurons in *Drosophila*, and *dnlg4* mutants have fragmented night-time sleep and impaired GABA transmission (Y. Li et al. 2013).

Timing of *dnrx1* activity

Interestingly, neurexin-1 may be required differently during the day compared to the night, as effects on sleep differ depending on the timing of over-expression. Following *dnrx1* over-expression throughout development and adulthood, we see an increase and consolidation of night-time sleep, and no effects on day-time sleep. If acute over-expression of *dnrx1* occurs during night-time hours, flies display more consolidated night-time sleep than WT flies; on the other hand, day-time over-expression appears to increase amount of sleep during day and night. In combination with known neurexin-1 transcript cycling, this may suggest necessity of specific timing of *dnrx1* function, or indeed differing functions depending on time of day. *Drosophila* displays differences in day- and night-time sleep, with day-time sleep appearing to be more shallow (Hendricks et al. 2000; P J Shaw et al. 2000, Figures 2 and 3) and a number of factors can differentially affect day- and night-time sleep (Ishimoto, Lark, and Kitamoto 2012). In mammals, sleep can be classified into rapid eye movement (REM) and non-REM sleep (which can be further subdivided based on electroencephalography); these distinct sleep stages appear to have differing functions (Siegel 2005). There is also evidence for deeper and lighter sleep states in *Drosophila*, with day-time up-regulation of cyclic adenosine monophosphate leading to increased night-time sleep intensity and loss of fragile-X mental retardation protein (involved in synaptic pruning) increasing day-time sleep intensity (van Alphen et al. 2013). Future work will be vital in shedding light on the potential

impact of distinct sleep states on differing biological functions, such as memory reconsolidation or redress of synaptic balance, and how neurexin-1 may be involved.

Manipulation of *dnrx1* may trigger compensatory action by *dnrx4*

Given the significant increase in *dnrx4* transcript we see in *dnrx1* knockouts, and the fact that sleep deprivation decreases transcript of both neurexins, it is likely that these genes and their binding partners work as a complex rather than as single units. Thus perturbation of one gene can have important knock-on effects for interacting genes and to some extent *dnrx4* may compensate for some effects of *dnrx1* knockout. This has also been seen in other model systems, where genetic modifications appear to be developmentally compensated in some cases and knockouts may display certain phenotypes but not others (Markus Missler, Südhof, and Biederer 2012). Indeed, subtle manipulation of these genes may have more impact than simple knockout strategies: a neuroligin-3 knock-in mouse with a point mutation relevant to the human gene reveals phenotypes not seen in the knockout (Tabuchi et al. 2007). NRX-4 is the *Drosophila* ortholog of the *CNTNAP2*-encoded protein CASPR2 and is ancestrally related to the NRX-1 family, and appears to mediate neuron-glia interactions (Baumgartner et al. 1996; Peles et al. 1997; Biswas et al. 2008). More recently, there is evidence for a role of *dnrx4* in synaptic plasticity in *Drosophila* and it may even have overlapping and/or compensatory functions in relation to *dnrx1* (Zweier et al. 2009). These compensatory effects should be carefully considered when evaluating phenotypes caused by altering expression of neurexins and neuroligins throughout development.

Relevance to human disorders

A number of disorders display altered sleep and synaptic neuronal connectivity, including ASD, schizophrenia and Tourette syndrome. Many mutations associated with ASD, including neurexin-1, relate to development and refinement of synapses (Ebert and Greenberg 2013) and a large percentage of children with autism have disrupted sleep patterns, including fragmented sleep (Cortesi et al. 2010). There is also evidence for involvement of clock genes in individuals with ASD (Bourgeron 2008) and anatomically, there appear to be local and global differences in brain connectivity (Belmonte 2004). In Tourette syndrome there is also evidence for neuroanatomical and neurochemical abnormalities (Singer and Minzer 2004), links to the neurexin complex (Clarke and Eapen 2014) and sleep problems (Ghosh et al. 2014). Finally, there is evidence linking disruption of NRX-1 to schizophrenia (Reichelt, Rodgers, and Clapcote 2011), where sleep and circadian rhythm is also abnormal (Wulff et al. 2011). Many neurodevelopmental and neuropsychiatric disorders are polygenic and heterogeneous in nature, with overlapping genetic risk factors. Identifying gene networks with common functional domains may be an effective approach to tackle the underlying biological mechanisms that are likely responsible for different behavioural aspects of disorders (Cristino et al. 2013; An et al. 2014). Atypical sleep patterns may merely reflect abnormal modulation of synaptic function and altered homeostasis, but could also contribute to the progression and pathophysiology of disorders, and so potentially offer a target for therapeutic strategies (G. Wang et al. 2011).

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Abbreviations

ASD, autism spectrum disorders; Brp, bruchpilot; Caspr2, contactin-associated protein 2; DD, dark:dark; *dnrx1*, *Drosophila* neurexin-1, *dnrx4*, *Drosophila* neurexin-4; *dnlg1*, *Drosophila* neuroligin-1; *dnlg2*, *Drosophila* neuroligin-2; *dnlg3*, *Drosophila* neuroligin-3; *dnlg4*, *Drosophila* neuroligin-4; GFP, green fluorescent protein; LD, light:dark; NMJ, neuromuscular junction; REM, rapid eye movement; SD, sleep deprivation; SNAP, sleep nullifying apparatus; WT, wild-type; ZT, zeitgeber.

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