Neurofibromin 1 regulates early developmental sleep in *Drosophila*

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ABSTRACT

Sleep disturbances are common in neurodevelopmental disorders, but knowledge of molecular factors that govern sleep in young animals is lacking. Evidence across species, including *Drosophila*, suggests that juvenile sleep has distinct functions and regulatory mechanisms in comparison to sleep in maturity. In flies, manipulation of most known adult sleep regulatory genes is not associated with sleep phenotypes during early developmental (larval) stages. Here, we examine the role of the neurodevelopmental disorder-associated gene *Neurofibromin 1* (*Nf1*) in sleep during numerous developmental periods. Mutations in *Neurofibromin 1* (*Nf1*) are associated with sleep and circadian disorders in humans and adult flies. We find in flies that *Nf1* acts to regulate sleep across the lifespan, beginning during larval stages. *Nf1* is required in neurons for this function, as is signaling via the Alk pathway. These findings identify *Nf1* as one of a small number of genes positioned to regulate sleep across developmental periods.

1. Introduction

Sleep plays a critical role in the developing brain. Disruptions to sleep during development can have long-lasting neurobehavioral effects (Kayser and Biron, 2016; Mirmiran et al., 1981; Frank, 2011; Jones et al., 2019; Halbower et al., 2006). However, an understanding of genes and circuits regulating sleep during early developmental periods is lacking. Across species, sleep duration and depth are elevated during the juvenile period (Roffwarg et al., 1966; Jouvet-Mounier et al., 1970; Frank and Heller, 1997; Shaw et al., 2000; Kayser et al., 2014; Blumberg et al., 2022). Studies in *Drosophila melanogaster* have yielded important insights into the molecular and neural circuit basis of sleep, and like other animals, fruit flies exhibit developmental changes in sleep (sleep ontogeny) (Shaw et al., 2000; Kayser et al., 2014). Specifically, juvenile adult flies exhibit increased sleep amount, are harder to sleep deprive, and spend more time in a deep sleep state (Shaw et al., 2000; Kayser et al., 2014; Dilley et al., 2018; Gong et al., 2022). Interestingly, genetic mechanisms governing sleep in mature adulthood appear at least partially separable from those controlling the process of sleep ontogeny itself. For example, all known adult short-sleeping mutants still sleep more when young (Dilley et al., 2018). Conversely, specific genes have

been identified that regulate the juvenile sleep state but are not associated with a mature adult sleep phenotype (Chakravarti Dilley et al., 2020).

Before reaching adulthood, *Drosophila* develop through an embryonic stage, three larval stages, followed by pupation. Sleep in *Drosophila* had not been examined during pre-adult periods until recent work describing sleep during the 2nd instar larval stage (Szuperak et al., 2018; Churgin et al., 2019). Larval sleep shares behavioral features with sleep across phylogeny, including behavioral quiescence associated with reduced arousability and sleep rebound following sleep deprivation. As with juvenile adult sleep, findings support the idea that distinct genes and circuits coordinate sleep during early development. For example, well-characterized adult fly sleep circuits do not exist at this stage in the larval brain (Chakravarti Dilley et al., 2020). Moreover, most adult mutants with severe short sleep phenotypes have normal sleep during larval stages (Szuperak et al., 2018). Finally, dopamine, a major arousal promoting neurotransmitter in adulthood of the fly (Kume et al., 2005; Wu et al., 2008; Van Swinderen and Andretic, 2011), has no apparent effect on arousal in larvae (Szuperak et al., 2018). Given these major differences in regulation of sleep across development, a more thorough examination of genes regulating sleep early in development is
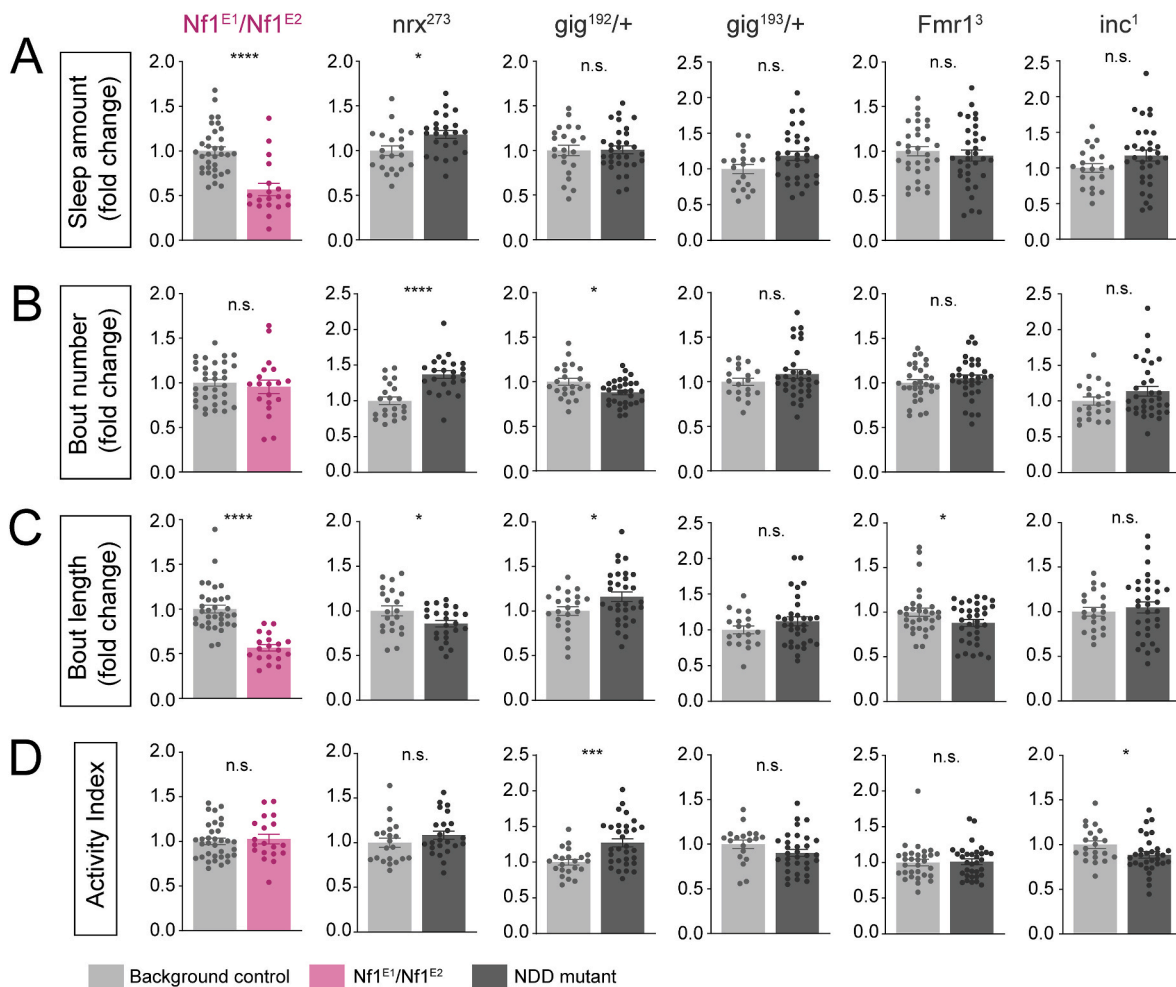
Fig. 1. Characterization of early life sleep in fly mutants associated with neurodevelopmental disorders. Sleep and activity in the following mutants (columns from left to right): $Nf1^{E1}/Nf1^{E2}$, nrx^{273}, $gig^{192}/+$, $gig^{193}/+$, $Fmr1^3$, inc^1. All values are normalized to background control. **A)** Quantification of total sleep time calculated over 6 h of recording. **B)** Quantification of sleep bout number over 6 h of recording. **C)** Quantification of average sleep bout length for individual larvae over the course of 6 h recording. **D)** Quantification of waking activity. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by unpaired *t*-test (A–D). From left to right in A-D, n = 34, 19, 21, 24, 22, 31, 19, 32, 31, 33, 21, 32 larvae per genotype, respectively.

warranted.

Children with neurodevelopmental disorders (NDDs) commonly experience sleep disturbances and disruption (Robinson-Shelton and Malow, 2016; D'Souza et al., 2020; Kamara and Beauchaine, 2020; Reynolds et al., 2019). In fact, many NDDs include sleep problems as part of their diagnostic criteria (Shelton and Malow, 2021). *Drosophila* has been extensively utilized towards mechanistic insights in NDDs (Gatto and Broadie, 2011; Coll-Tané et al., 2019), and recent work has used flies to understand interactions between sleep and NDDs (Larkin et al., 2015; Bushey et al., 2009; Doldur-Balli et al., 2022; Gong et al., 2021; Coll-Tané et al., 2021). Here, we set out to characterize developmental sleep changes in fly models of NDDs and NDD-associated genes with known sleep phenotypes in mature adulthood. Loss of one of the genes of interest, *Neurofibromin 1* (*Nf1*), had profound effects on sleep from early developmental stages throughout adulthood. Neurofibromatosis type 1 (NF1) is a neurodevelopmental disorder caused by loss-of-function mutations in *NF1*. The disease is predominantly characterized by neurofibromas and other tumors of the nervous system, but broad deficits in neurocognitive function significantly degrade quality of life. Children with NF1 have greatly increased rates of autism spectrum disorder (ASD), often accompanied by social and communicative difficulties (Morris et al., 2016). Moreover, both children and adults with NF1 exhibit disturbed sleep and poor sleep quality (Licis et al., 2013; Johnson et al., 2005; Leschziner et al., 2013). Studies in *Drosophila Nf1*

mutants in mature adulthood have likewise demonstrated disruptions to both sleep and rest:activity circadian rhythms (Williams et al., 2001; Bai et al., 2018; Bai and Sehgal, 2015; Brown et al., 2022; Machado Almeida et al., 2021). Specifically, adult *Nf1* mutants exhibit loss of rest:activity rhythms under constant conditions, and a reduction in sleep duration with associated sleep fragmentation. Here, we demonstrate that, in contrast to loss of other examined NDD-associated genes, loss of *Nf1* impairs sleep across developmental periods. Thus, *Nf1* is one of the first known genes to regulate sleep across developmental, juvenile, and adult stages in the fly.

2. Materials and methods

2.1. Drosophila stocks

The following lines have been maintained as lab stocks in an iso31 background or generously provided by Drs. Amita Sehgal and Thomas Jongens: *K33*, $Nf1^{P1}$, $Nf1^{P2}$, $Nf1^{E1}$, $Nf1^{E2}$, *Alk* (), Alk^9, $Nrx-1^{273}$, gig^{192}, gig^{193}, $Fmr1^3$, inc^1, $iso31$, elav-Gal4, UAS-*Nf1*, UAS-AlkEC. inc^1 was generously provided by Dr. Nicholas Stavropoulos. *Nf1* RNAi (VDRC #13892 and #101909) and RNAi controls were obtained from Vienna Drosophila Resource Center. The $Nf1^{C1}$ mutant fly line was generated by CRISPR/Cas9 (Ren et al., 2013) using the sgRNA line: *y[1] sc[*] v[1] sev [21]; P{y[+t7.7] v[+t1.8]=TKO.GS01796}attP40* (GS01796 sgRNA
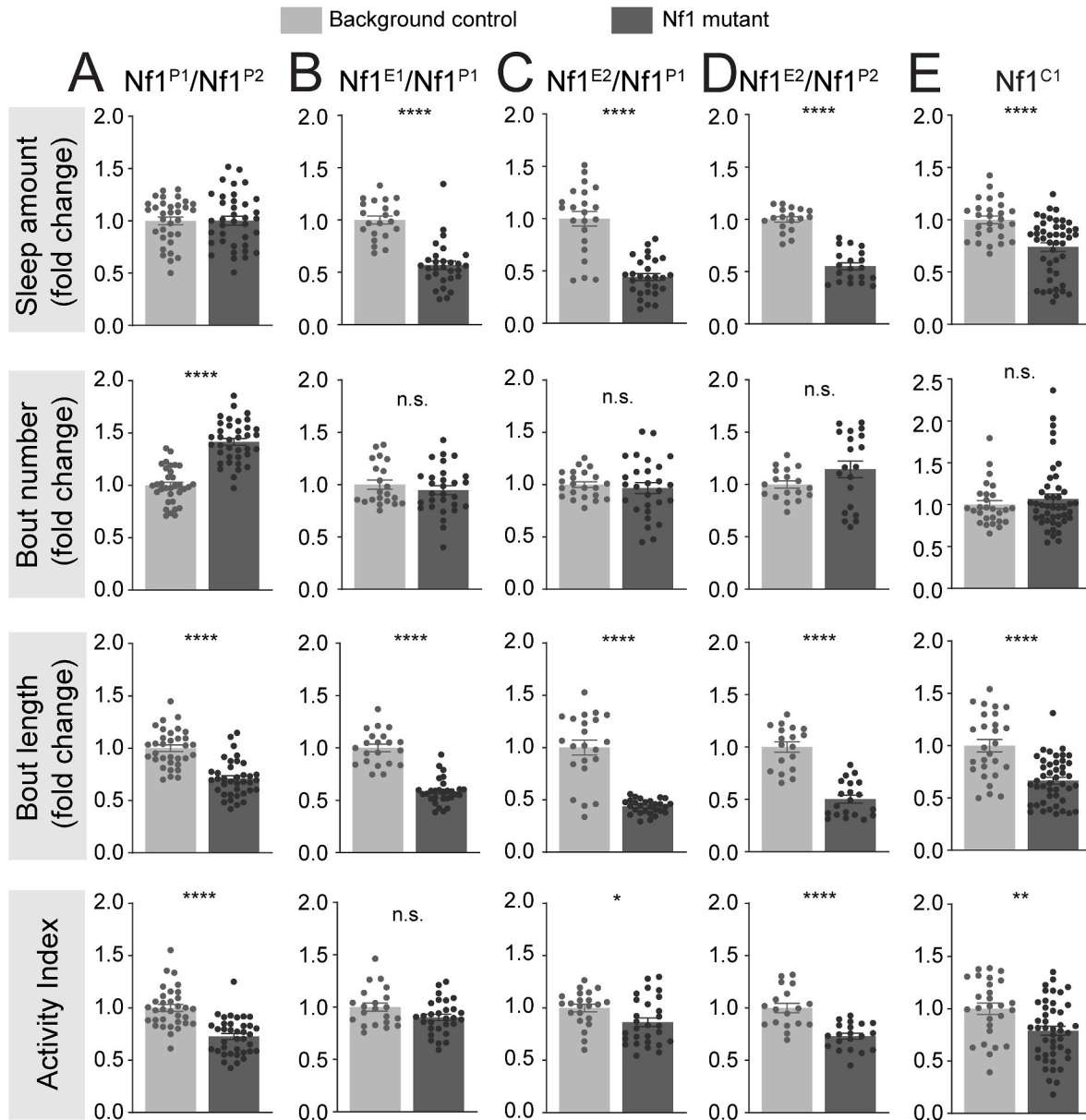
Fig. 2. Nf1 mutant larvae exhibit deficits in 2nd instar sleep duration and sleep consolidation. Sleep and activity were assessed in the following Nf1 mutants over the course of a 6 h recording: **A)** *Nf1^{P1}/Nf1^{P2}*, **B)** *Nf1^{E1}/Nf1^{P1}*, **C)** *Nf1^{E2}/Nf1^{P1}*, **D)** *Nf1^{E2}/Nf1^{P2}*, and **E)** *Nf1^{C1}*. For each genotype the following were quantified, all normalized to genetic controls run in parallel: total sleep duration, sleep bout number, sleep bout length, and wake activity. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by unpaired *t*-test. From left to right, n = 33, 37, 21, 29, 22, 28, 18, 20, 27, 45 larvae per genotype, respectively.

sequence: CGCTTCTCCCTTGTCATATC) and a germline source of Cas9: *y [1] M{w[+mC]=nos-Cas9.P}ZH-2A w[*]* (Bloomington Stock #54591). DNA sequencing of the *Nf1^{C1}* allele showed a deletion of nucleotides 160–161 (AT) in the *Nf1* coding region which results in a frame shift and premature stop codon in exon 2.

2.2. Larval rearing and sleep assays

Adult flies were maintained on standard molasses-based food from Lab Express (Fly Food R, recipe available at http://lab-express.com/DIS58.pdf) at 25 °C on a 12:12 light:dark (LD) cycle. For experiments performed in the subjective evening (CT12-24), adult flies were maintained on a 12:12 reverse dark:light (DL) cycle. In order to collect synchronized second and third instar larvae, adult flies were placed in an embryo collection cage (Genesee Scientific, cat#: 59–100) and eggs were laid on a petri dish containing 3% agar, 2% sucrose, and 2.5%

apple juice with yeast paste on top (for 2nd instars) or a molasses-based diet with yeast paste on top (for 3rd instars). Animals developed on this media for two days (for 2nd instars) or three days (for 3rd instars). To examine sleep in either 2nd or 3rd instar larvae at constant conditions, petri dishes were moved to constant darkness (DD) after the first day of entrainment.

Sleep assays for 2nd instar larvae were performed using the LarvaLodge and image acquisition parameters as described previously (Churgin et al., 2019). Briefly, freshly molted 2nd instar larvae were placed into individual wells of the LarvaLodge containing 120 μl of 3% agar and 2% sucrose media covered with a thin layer of yeast paste. Sleep assays in 3rd instar larvae were performed using a modified LarvaLodge, with 20 rounded wells of 20 mm diameter & 1.5 mm depth. Freshly molted 3rd instar larvae were placed into individual wells of the modified LarvaLodge containing 95 μl of 3% agar and 2% sucrose media covered with a thin layer of yeast paste. The LarvaLodge was covered
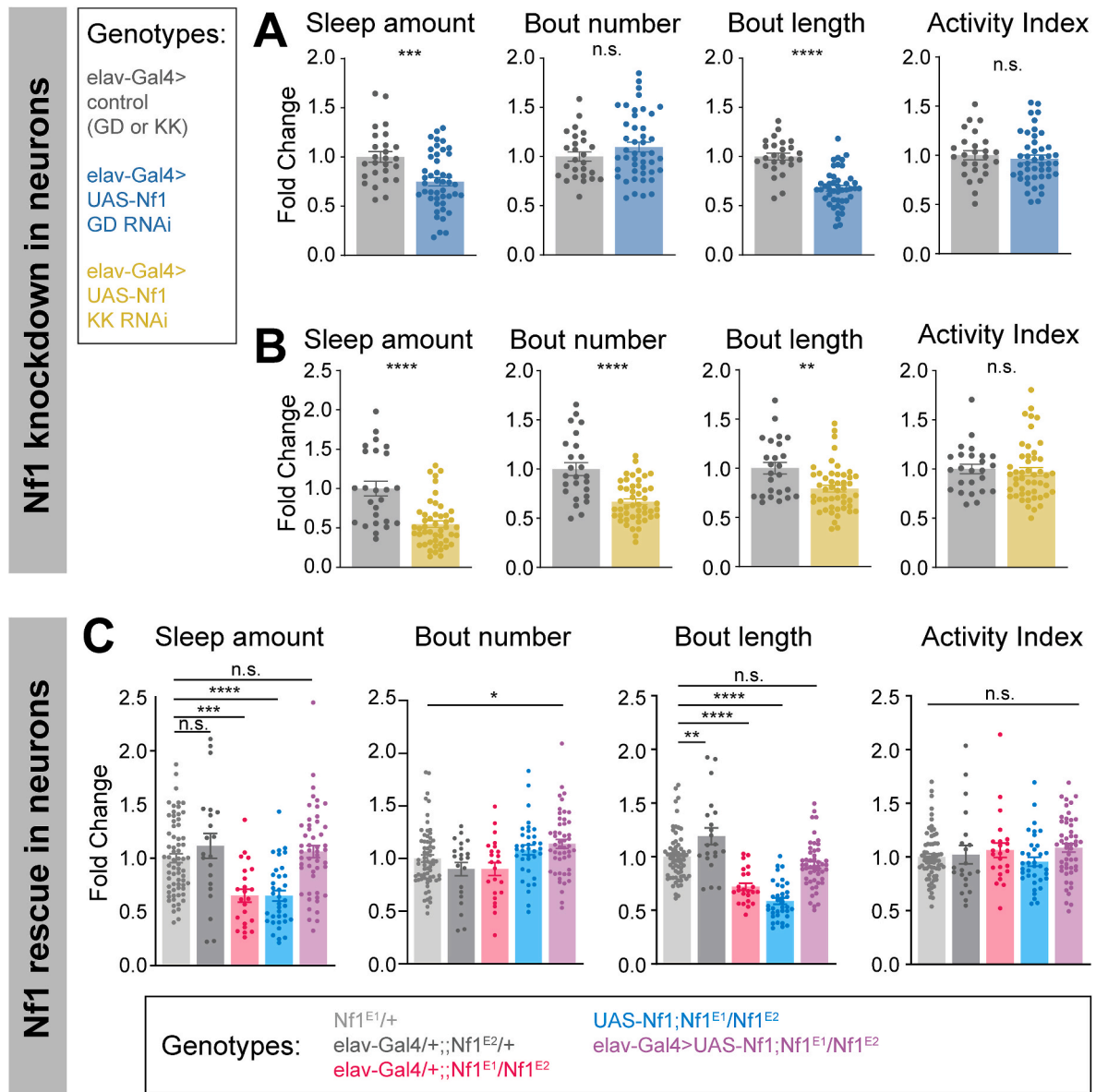
Fig. 3. Nf1 acts in neurons to promote normal 2nd **instar sleep behavior. A-B)** Quantification of sleep amount, bout number, bout length, and waking activity in flies expressing two different Nf1 RNAi constructs under neuronal (elav-Gal4) control. N = 26, 45, 25, 48 for *elav-Gal4>GD control, elav-Gal4>Nf1 GD RNAi, elav-Gal4>KK control, elav-Gal4>Nf1 KK RNAi* respectively. **C)** Quantification of sleep amount, bout number, bout length, and waking activity in *Nf1* mutant flies with transgenic expression of Nf1 specifically in neurons via elav-Gal4. From left to right, n = 67, 21, 23, 36, 50 larvae per genotype in C. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by unpaired *t*-test (A–B) or One-Way ANOVA with Dunnet's multiple comparisons test (C).

with a transparent acrylic sheet and placed into a DigiTherm (Tritech Research) incubator at 25 °C for imaging. Experiments were performed in the dark.

2.3. LarvaLodge image acquisition and processing

Images were acquired every 6 s with an Imaging Source DMK 23GP031 camera (2592 X 1944 pixels, The Imaging Source, USA) equipped with a Fujinon lens (HF12.55A-1, 1:1.4/12.5 mm, Fujifilm Corp., Japan) with a Hoya 49 mm R72 Infrared Filter. We used IC Capture (The Imaging Source) to acquire time-lapse images. All experiments were carried out in the dark using infrared LED strips (Led-lightsworld LTD, 850 nm wavelength) positioned below the LarvaLodge.

Images were analyzed using custom-written MATLAB software (see Churgin et al., 2019 and Szuperak et al., 2018). Temporally adjacent images were subtracted to generate maps of pixel value intensity change. A binary threshold was set such that individual pixel intensity

changes that fell below 40 Gy-scale units within each well were set equal to zero ("no change") to eliminate noise. For 3rd instars, the threshold was set to 45 to account for larger body size. Pixel changes greater than or equal to threshold value were set equal to one ("change"). Activity was then calculated by taking the sum of all pixels changed between images. Sleep was defined as an activity value of zero between frames. For 2nd instar sleep experiments done across the day, total sleep was summed over 6 h beginning 2 h after the molt to second instar (Figs. 1–4). For sleep experiments performed at certain circadian times, total sleep in the 2nd hour after the molt to second (or third) instar was summed. For all experiments, sleep metrics were normalized to the average value for the control for a given biological replicate.

2.4. Juvenile and adult sleep assays

Ontogeny experiments were conducted as previously described (Chakravarti Dilley et al., 2020). Briefly, day one males and females
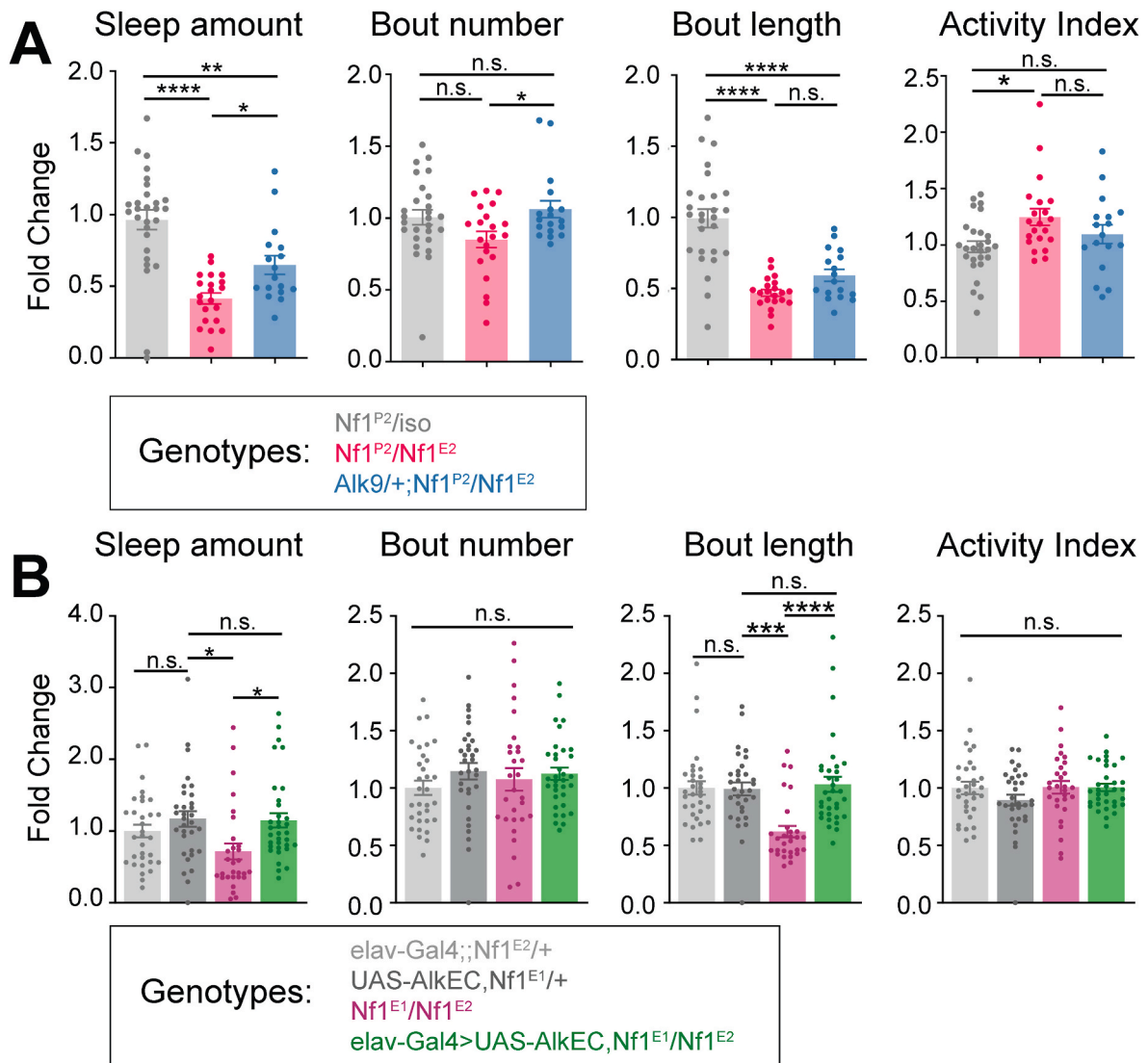
Fig. 4. Nf1-related sleep deficits are caused by disrupted Alk signaling in the second instar larval stage. A) Quantification of sleep amount, bout number, bout length, and waking activity in *Nf1* mutant flies with or without loss of one copy of the receptor tyrosine kinase, *Alk* (*Alk⁹*). From left to right, n = 28, 21, 17 larvae per genotype in A. **B)** Quantification of sleep amount, bout number, bout length, and waking activity in *Nf1* mutant flies with or without neuronal expression of a dominant-negative Alk (AlkEC). From left to right, n = 32, 32, 39, 34 larvae per genotype in B. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by One-Way ANOVA with Tukey's multiple comparisons test.

(young adults) were compared to day 4–5 males and females (mature adults) of the same genotype. For day 4–5 flies, newly eclosed males and females were collected and aged in group housing on standard food at 25° on a 12 h:12 h LD cycle, and flipped onto new food every 2–3 days. For day 1 flies, newly eclosed males and females were housed until loading into the sleep experiment. Flies were anesthetized on CO_2 pads (Genesee Scientific Cat #59–114) and loaded into individual glass tubes (containing 5% sucrose and 2% agar food) for monitoring locomotor activity in the *Drosophila* Activity Monitoring (DAM) system (Trikinetics, Waltham, MA). Data collection began at ZT0 the following day, at least 16 h after CO_2 exposure. Activity was measured in 1 min bins and sleep was identified as 5 min of consolidated inactivity (Shaw et al., 2000; Hendricks et al., 2000). Data was processed using custom MATLAB script.

2.5. Statistical analysis

All statistical analyses were performed in GraphPad (Prism). Individual tests and significance are detailed in the figure legends.

3. Results

A number of available *Drosophila* mutants of neurodevelopmental disorders with adult sleep phenotypes were assessed for early developmental sleep phenotypes as 2nd instar larvae. Genes of interest included Neurofibromin 1[38] (*Nf1*), *neurexin* (Larkin et al., 2015; Zeng et al., 2007) (*Nrx-1*), and *Fmr1* (Dockendorff et al., 2002). We also assessed sleep in mutants of *gigas* (Ito and Rubin, 1999) (*gig*), the fly homolog of *TSC2*, since sleep disturbances are one of the most common behavioral features of tuberous sclerosis (Hunt and Stores, 1994; van Eeghen et al., 2011; Zambrelli et al., 2021), and in the adult sleep mutant *insomniac* (Stavropoulos and Young, 2011) (*inc*), as inc serves as an adaptor for Cul3, a high confidence Autism Spectrum Disorder (ASD) risk gene (Kong et al., 2012; Li et al., 2017; O'Roak et al., 2012). Mutant larvae and controls were loaded into the LarvaLodge (Szuperak et al., 2018; Churgin et al., 2019) and sleep features were measured for 6 h. Despite prominent sleep phenotypes in adulthood, most mutants showed minimal to no change in sleep measurements as larvae. However, transheterozygous *neurofibromin 1* (*Nf1*) mutant flies exhibited a reduction in sleep duration,
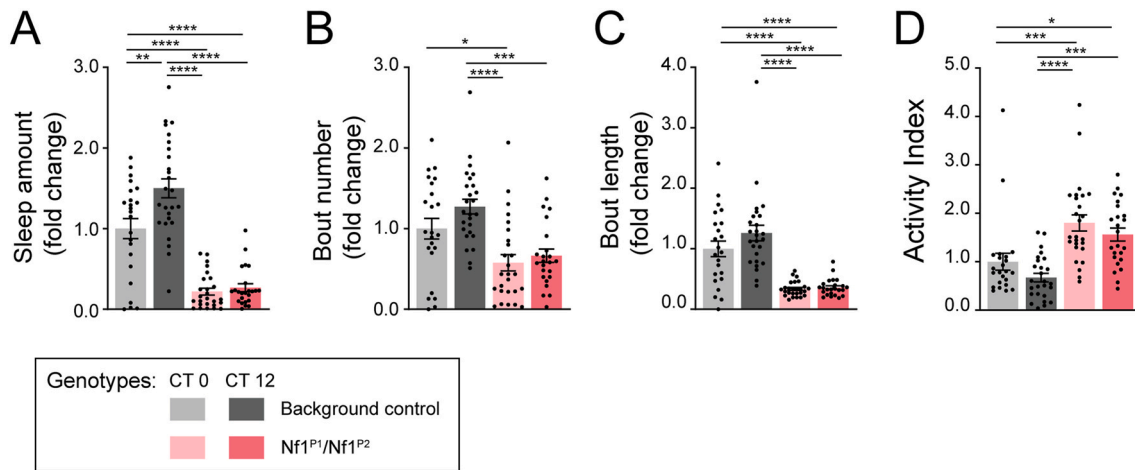
Fig. 5. *Nf1* **mutant larvae exhibit deficits in 3**rd **instar sleep amount. A)** Quantification of total sleep amount. There were significant main effects of genotype (p < 0.0001), circadian time (p = 0.0032) and a significant interaction between genotype and circadian time (p = 0.0144). **B)** Quantification of bout number, with a significant main effect of genotype (p < 0.0001). **C)** Quantification of bout duration, with a significant main effect of genotype (p < 0.0001). **D)** Quantification of waking activity, with significant main effects of genotype (p < 0.0001) and circadian time (p = 0.0499). From left to right, n = 23, 26, 26, 24 larvae (A–D). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Two Way ANOVA with Sidak's multiple comparisons test. All sleep and activity metrics were normalized to background controls at CT0.

driven by truncated sleep bout length (Fig. 1; Supplemental Fig. 1). Waking activity was not significantly different between mutants and controls, indicating the sleep loss phenotype was not due to hyperactivity in mutant flies.

To confirm the early developmental sleep phenotype in *Nf1* mutants, we assessed sleep in other *Nf1* mutant alleles. *Nf1*[E1] and *Nf1*[E2], the alleles tested as a transheterozygote in Fig. 1, were generated by ethyl methanesulfonate (EMS)-induced mutation (Walker et al., 2006), and prematurely truncate the *Nf1* protein. *Nf1*[P1] and *Nf1*[P2] alleles are null alleles generated by a P-element insertion (The et al., 1997). *Nf1*[P1]/*Nf1*[P2] transheterozygotes exhibited severely fragmented sleep, with an increase in sleep bout number and reduction of bout length (Fig. 2A; Supplemental Fig. 2). Transheterozygous combinations of P-element and EMS generated alleles all showed loss of sleep duration associated with dramatic reduction in sleep bout length (Fig. 2B–D; Supplemental Fig. 2). The E and P mutants are complete loss of function, suggesting that background differences most likely account for the inconsistent effects on sleep duration. However, a CRISPR-generated *Nf1* mutant (*Nf1*[C1]) also showed sleep loss and truncated sleep bout duration, providing further confirmation of the severe sleep disruption with loss of *Nf1*. (Fig. 2E; Supplemental Fig. 2). In many of the *Nf1* mutants examined, waking activity was actually decreased, indicating that sleep loss was not due to increased overall activity. Sleep in heterozygous *Nf1* mutants was unaffected (Supplemental Fig. 3). Together, these results demonstrate that loss of *Nf1* causes deficits in early developmental sleep.

In mature adult flies, *Nf1* functions in neurons to regulate sleep (Bai and Sehgal, 2015; Machado Almeida et al., 2021). To determine if the same is true during early developmental periods, we knocked down *Nf1* specifically in neurons using the elav-Gal4 driver. Knockdown of *Nf1* in neurons with two different RNAi lines led to significantly decreased sleep in 2nd instar larvae without changes to wake activity (Fig. 3A and B). In both RNAi lines, sleep loss was associated with decreased sleep bout length, and one line (KK) showed a significant decrease in bout number as well (Fig. 3A and B). To test whether *Nf1* re-expression in neurons is sufficient to rescue the mutant sleep phenotype, we used elav-Gal4 to drive UAS-*Nf1* in an *Nf1*[E1]/*Nf1*[E2] mutant background. Expression of *Nf1* under the elav-Gal4 driver rescued deficits in total sleep and bout length (Fig. 3C). Thus, neuronal *Nf1* is both necessary and sufficient for normal sleep in *Drosophila* larvae.

Mutation of the receptor tyrosine kinase Anaplastic lymphoma

kinase (*Alk*) rescues some behavioral phenotypes in adult *Nf1* mutant flies, at least in part by decreasing Ras signaling (Bai and Sehgal, 2015; Gouzi et al., 2011; Walker et al., 2013). To determine if Alk signaling plays a role in early developmental sleep in *Nf1* mutants, we expressed a loss of function mutant allele of *Alk* (*Alk*[9]) in an *Nf1* mutant background. We found that the heterozygous *Alk*[9] mutation ameliorated the sleep phenotype in *Nf1*[E1/P2] mutants, leading to an increase in sleep duration, though not to control levels (Fig. 4A). The *Alk*[9] mutation alone had no effect on larval sleep measures (Supplemental Fig. 4A). We next sought to determine if suppression of Alk signaling specifically in neurons could rescue the developmentally-timed sleep deficits. To do so, we used elav-Gal4 to drive expression of a dominant negative Alk construct, UAS-AlkEC (Bazigou et al., 2007). Neuronal expression of AlkEC rescued the sleep duration and sleep bout length deficits normally observed in *Nf1* transheterozygous mutants (Fig. 4B; Supplemental Fig. 4B). These results suggest that, as in adults, developmental *Nf1*-dependent sleep impairments involve Alk signaling pathways.

Drosophila development is characterized by three larval stages prior to pupation. Initial work (Szuperak et al., 2018) characterized sleep during the 2nd instar stage, but more recent efforts have studied sleep in 3rd instar larvae (Poe et al., 2022). We therefore assessed whether *Nf1* loss of function has a persistent effect on sleep across developmental periods. During the 3rd instar stage, *Nf1*[P1/P2] mutants exhibited a dramatic reduction in sleep duration compared to controls (Fig. 5A). This loss of sleep was caused by fewer sleep bouts but also severe curtailment of sleep bout length (Fig. 5B–C). Notably, *Nf1* mutants at this stage, in contrast to 2nd instars, were also more active during periods of wake (Fig. 5D), perhaps indicative of increased feeding related to known metabolic abnormalities with loss of *Nf1* (Botero et al., 2021).

In addition to its role in adult sleep regulation, *Nf1* also has a known function in rest:activity circadian rhythms (Williams et al., 2001; Bai et al., 2018; Machado Almeida et al., 2021). Adult *Nf1* mutants have arrhythmic daily locomotor patterns and loss of *Nf1* leads to dampened cycling of calcium in *Drosophila* circadian neurons and in mammalian astrocytes (Williams et al., 2001; Bai et al., 2018). While sleep in 2nd instar larvae is not under circadian control (Szuperak et al., 2018), recent work has established that clock control of sleep patterns emerges during the early 3rd instar period (Poe et al., 2022); as in adulthood, 3rd instar larvae sleep more during the dark (night) phase (CT12) than the light (day) phase (CT0). Consistent with these observations, we found that control flies showed an increase in sleep duration at CT12 relative to
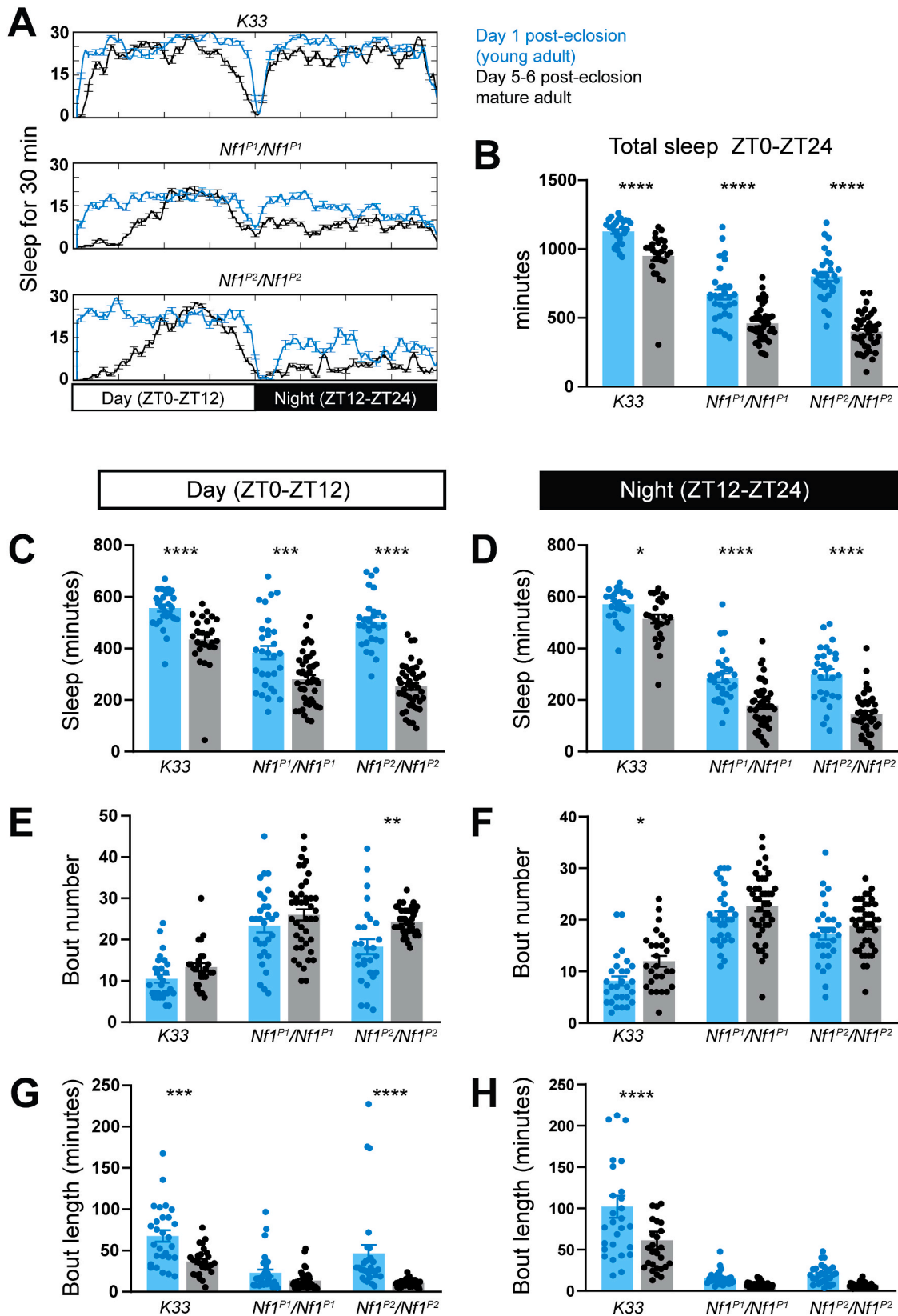
Fig. 6. Ontogenetic changes to adult sleep are preserved in *Nf1* mutants. A) Representative traces of sleep behavior in juvenile (blue) and mature (black) adult *Nf1* P allele mutants and control male flies. **B)** Quantification of total sleep time over 24 h in juvenile and mature adult *Nf1* and control flies. **C-D)** Quantification of total sleep time during day (ZT0-ZT12, C) and night (ZT12-ZT24, D) in juvenile and mature adult *Nf1* and control flies. **E-F)** Quantification of sleep bout number during day (E) and night (F) in juvenile and mature adult *Nf1* and control flies. **G-H)** Quantification of sleep bout length during day (G) and night (H) in juvenile and mature adult NF1 and control flies. From left to right, n = 28, 27, 30, 42, 28, 43 flies (B–H). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by One Way ANOVA with Sidak's multiple comparisons test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

CT0 (Fig. 5A). This pattern was not apparent in *Nf1* mutants, suggesting a potential early developmental origin of circadian abnormalities (Fig. 5A). However, interpretation of these data is potentially confounded by the dramatic suppression of sleep in these mutants that might prevent detection of a circadian pattern.

In addition to sleep changes that occur during *Drosophila* larval stages, sleep maturation is also evident across the first days of adult life (Shaw et al., 2000; Kayser et al., 2014; Chakravarti Dilley et al., 2020). While sleep deficits with loss of *Nf1* are well-characterized in mature adulthood (Bai and Sehgal, 2015; Machado Almeida et al., 2021), the developmental trajectory of the sleep phenotype, from juvenile to mature adult flies, has not been investigated. To determine if *Nf1* mutants display normal sleep ontogenetic changes across juvenile adulthood, we compared sleep characteristics at day 1 post-eclosion (juvenile) to day 5–6 post-eclosion (mature) in adult *Nf1* mutant or control male flies. Consistent with previous work (Bai and Sehgal, 2015), mature adult *Nf1* mutant males slept less overall than controls (Fig. 6A–D); however, the typical pattern of excess sleep in juvenile compared to mature flies was preserved (Fig. 6A–D). Ontogenetic changes in sleep were driven primarily by a decrease in sleep bout length from juvenile to mature adulthood (Fig. 6G–H) in both control and *Nf1* mutants. *Nf1$^{E1/E2}$* mutants similarly exhibited preservation of juvenile to mature adult ontogenetic sleep changes (Supplemental Fig. 5), although their sleep patterns appeared arrhythmic even in LD conditions, suggestive of more severe circadian disturbances than P-allele mutants (Williams et al., 2001). These findings support the idea that *Nf1* plays a role in determining sleep duration across the lifespan, but not sleep maturation itself.

4. Discussion

Accumulating evidence indicates that genetic regulators of sleep change across the lifespan. In humans, there appears to be no genetic correlation between the GWAS of adults and children with regard to sleep duration (Dashti et al., 2019; Marinelli et al., 2016). Distinct mechanisms regulating early developmental versus adult sleep have likewise been described in worms (Trojanowski and Raizen, 2016) and flies (Dilley et al., 2018). Temporally-specific genetic manipulations in *Drosophila* and other systems are poised to help pinpoint sleep-relevant genes that are active during particular life stages. Here, we report that *Nf1*, in contrast to most other genes examined thus far, regulates sleep duration and continuity across numerous developmental periods and in mature adulthood.

As in adults (Morris et al., 2016; Licis et al., 2013), *Nf1* appears to be acting in neurons and through Alk-dependent signaling cascades to affect sleep. While these molecular features are shared, the larval brain is far simpler than the adult, so it will be of interest to understand whether *Nf1* functions in the same cells/circuits to control sleep or if the cellular mechanisms diverge. Indeed, other behavioral studies in *Drosophila* have demonstrated developmental roles for *Nf1* (King et al., 2020; Dyson et al., 2022), raising the possibility of shared mechanisms and highlighting the utility of this model to understand how early developmental events impinge upon behaviors in a NDD model. For example, does *Nf1* act acutely during different phases of life to regulate sleep at each point in time, or is there a critical period of *Nf1* action during larval stages, with long-lasting effects into maturity? Prior evidence support an ongoing acute role, as adult-specific *Nf1* manipulations are associated with circadian and locomotor phenotypes (Bai et al., 2018). Nonetheless, these questions will be of interest to explore with regard to sleep and other phenotypes.

Recent work has implicated two other NDD-associated genes in both early developmental and adult sleep. Studies of *kismet* in flies (*CHD8/CHD7* ortholog) have revealed sleep fragmentation with loss of function in both larvae and adults (Coll-Tané et al., 2021). Additionally, *Shank3* mutant mice exhibit sleep deficits across numerous developmental periods, and the specific sleep features affected depend upon age of the

animal (Medina et al., 2022). Together with our results, these findings support a model in which certain genes act across the lifespan to regulate sleep while others function in a more constrained manner in the adult. We suspect another class of genes might mediate sleep features *only* during developmental periods, though no such example has yet been described. These temporally-specific subclasses will be important to define, as they suggest critical windows of action for intervention.

CRediT authorship contribution statement

Jaclyn Durkin: Conceptualization, Investigation, Formal analysis, Writing – original draft, Supervision, Funding acquisition. **Amy R. Poe:** Investigation, Formal analysis, Supervision, Funding acquisition. **Samuel J. Belfer:** Investigation, Formal analysis, Supervision, Funding acquisition. **Anyara Rodriguez:** Investigation, Formal analysis, Supervision, Funding acquisition. **Si Hao Tang:** Investigation, Formal analysis, Supervision, Funding acquisition. **James A. Walker:** Investigation, Formal analysis, Supervision, Funding acquisition. **Matthew S. Kayser:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbscr.2023.100101.

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