The NMDA Receptor Promotes Sleep in the Fruit Fly, Drosophila melanogaster

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Abstract

Considerable evidence indicates that sleep is essential for learning and memory. Drosophila melanogaster has emerged as a novel model for studying sleep. We previously found a short sleeper mutant, fumin (fmn), and identified its mutation in the dopamine transporter gene. We reported similarities in the molecular basis of sleep and arousal regulation between mammals and Drosophila. In aversive olfactory learning tasks, fmn mutants demonstrate defective memory retention, which suggests an association between sleep and memory. In an attempt to discover additional sleep related genes in Drosophila, we carried out a microarray analysis comparing mRNA expression in heads of fmn and control flies and found that 563 genes are differentially expressed. Next, using the pan-neuronal Gal4 driver elav-Gal4 and UAS-RNA interference (RNAi) to knockdown individual genes, we performed a functional screen. We found that knockdown of the NMDA type glutamate receptor channel gene (Nmdar1) (also known as dNR1) reduced sleep. The NMDA receptor (NMDAR) plays an important role in learning and memory both in Drosophila and mammals. The application of the NMDAR antagonist, MK-801, reduced sleep in control flies, but not in fmn. These results suggest that NMDAR promotes sleep regulation in Drosophila.

Introduction

Sleep is a physiological state with unique characteristics. Behaviorally, sleep constitutes a consolidated period of rest and immobility without bulky movements, accompanied by an apparent reduced responsiveness to outside stimuli. The amount of sleep is regulated by both circadian and homeostatic processes [1]. Scientifically, sleep has been defined by electroencephalogram criteria in humans [2] and thus is mainly described in mammalian and avian species. In insects, sleep-like resting states were described first in the cockroach [3] and in the fruit fly, Drosophila melanogaster [4, 5]. We discovered a mutant with a reduced amount of these sleep-like states, and named it fumin, meaning ‘insomnia’ in Japanese and identified the mutation in the dopamine transporter gene [6]. This was a striking finding, since dopamine is also used to maintain wakefulness in the mammalian brain. Thus, it demonstrated the similarity...
between the molecular mechanism for regulating sleep-like states in *Drosophila* to that of the mammalian system [6]. Since then, cumulative evidence has continued to reveal similarities with mammalian sleep, so the sleep-like state of *Drosophila* is now simply called “*Drosophila* sleep”.

In addition to similar behavioral characteristics and molecular mechanisms, mammalian sleep and *Drosophila* sleep share common physiological traits. Many short sleeping mutants have a reduced life span [7–10], but despite their short sleeper phenotype, *fmn* have an equivalent life span to control flies [6]. In addition, together with other short sleep mutants, *fmn* have a memory retention defect and deprivation of sleep impairs their memory [11]. These findings indicate that sleep plays an important role in lifespan and memory in *Drosophila* and may provide an insight into why sleep evolves in a broad range of species.

In order to further elucidate the molecular mechanisms regulating sleep in *Drosophila*, we attempted to isolate more sleep related genes using the *fmn* mutant. In this report, we describe a gene expression analysis of *fmn*, followed by the successful isolation of the *N*-methyl-d-aspartic acid type glutamate receptor channel (NMDAR) gene, *Nmdar1*, as a novel sleep related gene and its function in promoting sleep.

**Materials and Methods**

**Fly strains and culture conditions**

Flies were reared at 25°C in 50–60% relative humidity on standard fly food consisting of corn meal, yeast, glucose, wheat germ and agar. They were kept under 12 h light (zeitgeber time, ZT 0–12) followed by 12 h dark (ZT 12–24) cycles defined as the LD conditions. The transgenic RNA interference (RNAi) lines for *Nmdar1* (VDRC 37333 and 104773), UAS-Dicer-2 flies (60008) and the *w*^1118^ (60000) which is the genetic background of the RNAi line were obtained from the Vienna *Drosophila* RNAi Center (VDRC) [12]. MB247 was provided by Dr. Hiromu Tanimoto. 11Y, 30Y, 201Y, 7Y, 104Y, 121Y, c232 and c767 were a gift from Dr. J. Douglas Armstrong. c747 and c772 were provided by Dr. Toshiro Aigaki. TH- Gal4 was a gift from Dr. Jay Hirsh. GAD-Gal4 was provided by Dr. Takaomi Sakai. OK371- Gal4 was a gift from Dr. Hermann Aberle. *dilp2*-Gal4 was a gift from Dr. Linda Partridge. npf- Gal4 was a gift from Dr. Ping Shen. OK307- Gal4 and c17 were a gift from Dr. Tanja Godenschwege. *tsh*-Gal4 was provided by Dr. Julie H. Simpson. The following stocks were ordered from the Bloomington Stock Center, Indiana University: *elav*-Gal4 (Stock number: 458), c309 (6906), c739 (7362), Ddc-Gal4 (7009), Tdc2-Gal4 (9313), Cha-Gal4 (6793), dimm-Gal4 (25373), per-Gal4 (7127), tim-Gal4 (7126), repo-Gal4 (7415) and D42 (8816). NP3529, NP6510, NP10, NP1004 and NP5103 were obtained from the *Drosophila* Genetics Resource Center, Kyoto Institute of Technology, Japan. The dopamine transporter mutant *fumin* (*fmn*) flies were isolated in our laboratory in a stock of *y* w flies, and backcrossed over five generations to the control strain (*w*^1118^) [6]. The backcrossed *w;fmn* line and the original *y* w; *fmn* line showed similar short sleeping phenotypes. Knockdown and control flies were obtained by crossing the pan-neuronal Gal4 driver *elav*-Gal4 with the UAS-Dicer-2 on the second chromosome to each of the RNAi lines and *w*^1118^, respectively. The 2- to 4-d-old post-eclosion male flies were used in this study.

**Microarray analysis**

Control (*w*^1118^) and *fmn* flies subjected to four LD cycles were harvested every 4 h at seven time points under the following LD conditions (ZT 0, 4, 8, 12, 16, 20 and 24) and immediately frozen in liquid nitrogen. Frozen flies were vigorously shaken and sieved to collect the heads which had separated from the bodies. Total RNA was extracted from approximately 400 male and female fly heads homogenized in TRIzol reagent (Invitrogen) and its quality was assessed.
with an RNA 600 Nano Assay Kit using the Agilent 2100 Bioanalyzer (Agilent Technologies). Double-stranded cDNA was synthesized from 5 μg of total RNA using a One-Cycle cDNA Synthesis Kit (Affymetrix) and served as a template to synthesize biotin-labeled cRNA using a GeneChip IVT Labeling Kit (Affymetrix). Biotin-labeled cRNA was fragmented and hybridized to an Affymetrix GeneChip Drosophila Genome 2.0 Array, which represents the entire Drosophila genome with over 18,500 transcripts, as recommended by the manufacturer. Hybridized arrays were washed and stained using a GeneChip Hybridization, Wash and Stain Kit (Affymetrix) on a Fluidics Station 400, and scanned on an Affymetrix GeneChip Scanner 3000. Expression measures for each probe set were calculated using the MAS5 algorithm. To find differentially expressed genes in control and fmn fly heads, data were normalized, prefiltered and analyzed by the Subio Platform (http://www.subio.jp/). Probe sets that were not called Present by the MAS5 and whose raw signal intensity were less than 100 in at least 7 of 14 samples were excluded from analysis. We identified the 8,740 probe sets above the threshold. Subsequently, a t-test was performed on the prefiltered list. The Benjamini-Hochberg false discovery rate (FDR) was set to 5%. At a cutoff of at least 1.25 \times up or down, 563 genes were determined to be differentially expressed between control and fmn flies. Functional Annotation Chart tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 was used to find enriched gene ontology (GO) terms in the biological process category. Count threshold, minimum number of differentially expressed genes involved in individual term was set to 10. The p-value was set to < 0.05.

mRNA analysis by quantitative RT-PCR

The efficiency and specificity of Nmdar1 RNAi were examined by quantitative RT-PCR (qPCR). For biological replicates, the knocked down and control male flies were collected from each of three culture vials at ZT 6. Total RNA was prepared from 35–40 heads by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesized from the total RNA using oligo (dT)\text{20} primer and ReverTra Ace reverse transcriptase (Toyobo) was used for qPCR using THUNDERBIRD SYBR qPCR Mix (Toyobo). GAPDH2 expression levels were quantified and used as an internal control. The primers were 5'-AGGAAGGAAAA AGCG GAAAGG-3' and 5'-GGGGAGGATA AACGAGGTGT-3' for Nmdar1, 5'-TCGGTTTCGGTT TTGGATGAG-3' and 5'-TTGTCCTTTC CGCCTGTATG T-3' for GluRIB and 5'-GCCCTGTATG T-3' for CanA-14F, 5'-TGCTGGTCTA-3' and 5'-TGGCAACTGC TTCGTGTCTA-3' for CanB, 5'-GGGGAGGATA AACGAGGTGT-3' and 5'-GGGGAGGATA AACGAGGTGT-3' for Rpl32 (rp49) and 5'-TGGTACGACA ACGAGTTTG-3' and 5'-TGGTACGACA ACGAGTTTG-3' for GAPDH2. First, each mRNA expression level was normalized to GAPDH2. Then, the normalized values were normalized to the average of three independent control samples, which was set at 100%.

Locomotor activity and sleep analysis

Flies were individually housed in glass tubes (length, 65 mm; inside diameter, 3 mm) containing either standard fly food or sucrose-agar (1% agar supplemented with 5% sucrose) at one end and were entrained for at least 3 d under LD cycle conditions before being transferred to constant dark (DD) conditions. Locomotor activity was monitored by recording the number of infrared beam crossings for individual flies in 1 min bins using the Drosophila activity monitoring (DAM) system (Trikinetics). Data were continuously collected for 3 d under either LD or DD conditions. Under LD, ZT was used and under DD, circadian time (CT, with CT 0 as 12 h after lights-off of the last LD conditions) was used to indicate daily time, respectively. Based on previous reports [4–6, 13], sleep was defined as periods of inactivity lasting 5 min or longer.
Total activity, total sleep, sleep bout length and sleep bout number in LD and DD conditions were analyzed by a Microsoft Excel-based software as previously described [6] and averaged over 3 d for each condition. This software was used to scan activity data over 3 d in 1 min bins, i.e., a total of 4,320 bins. Five or more continuous bins with the value of zero (lack of activity) are regarded as one sleep bout and the bins composing sleep bouts were assigned to sleep. To quantify sleep, the number of bins assigned to sleep were counted and expressed as a percentage of the total bins. The number and the length of each sleep bout were also recorded and the mean bout length of sleep was calculated. For the pharmacological experiments, sucrose-agar food was used as a control. (+)-MK-801 hydrogen maleate (MK-801) and 3-iodo-L-tyrosine (3IY) were purchased from Sigma-Aldrich. MK-801 or/and 3IY was directly mixed with sucrose-agar food at a concentration of 0.1 mg/ml and 3 mM, respectively.

Statistical analysis
Data were analyzed as described in the figure legends using Microsoft Excel and the freely available statistical software package R 3.0.0. (http://www.r-project.org/).

Results
Microarray analysis
We took RNA samples from the heads of both the control and fmn mutant flies which had been backcrossed for 5 generations to the control background flies [6]. Under LD conditions, 7 RNA sample sets were collected every 4 hours around the clock (ZT 0, 4, 8, 12, 16, 20 and 24). The samples at ZT 0 and ZT 24 were overlapped and used as a measure for the experimental variations. The RNAs were analyzed as described in Materials and Methods and the results were uploaded to the Gene Expression Omnibus (GEO) as GSE56149. As shown in Fig 1,

Fig 1. Clock gene cycling is not altered in fmn. Temporal expression patterns of circadian clock genes (per, tim, Clk and cyc) in the heads of control (w1118) and fmn flies under LD conditions. Data were normalized to the average signal intensity from each time point, which were set to 1.

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circadian clock genes (per, tim, Clk) cycled similarly in both flies and there was no apparent difference between them. The expression level of three dopamine receptor genes (DopR, DopR2, D2R) did not differ in fmnn. Tyrosine hydroxylase and other dopamine related genes including, Dopamine N acetyltransferase (Dat), L-3,4-dihydroxyphenylalanine (DOPA) decarboxylase (Ddc) and β-alanyl-dopamine synthase (ebony) also did not show any difference, suggesting that no compensatory changes in the expression level occurred in fmnn (data not shown).

Since the fmnn mutation does not affect circadian rhythm, we statistically compared 7 samples as a whole using a t-test with an FDR of 5%, and found that 563 genes showed a significantly different expression level between fmnn and the control, as shown in S1 Table. Of these, expression levels in 272 genes were up-regulated and 291 genes were down-regulated in fmnn. Using gene ontology enrichment analysis, we found that the differentially expressed genes in fmnn are involved in many different biological processes including metabolism, transport, development, protein modification and more (S2 Table). Dopamine transporter (DAT) gene expression was barely detectable in fmnn, which validated the experiment (S1 Table). We first noticed the scab (scb) gene, that codes for α-integrin, and its mutant, called Volado, which shows abnormal synaptic transmission and memory deficit [14, 15] and is dramatically down regulated in fmnn. Thus, we confirmed its expression in the control and fmnn using RT-PCR. To our disappointment, there was no difference in scb mRNA levels between the control and fmnn when we used primers for the reading frame. By sequencing the scb gene of fmnn, we identified single-base substitutions in the 3' untranslated region, which is on position 1629803_a_at of the Affymetrix DNA array probe set (data not shown), resulting in a very low signal on a microarray. Since the scb gene is located on the right arm of the second chromosome (2R) at 51E10-51E11 (Sequence location, 2R: 11,136,290..11,146,003) and fmnn (DAT) at 51E10-51E11 (12,446,062..12,452,763), the distance between the two is 1.3 M bases. Our backcross was apparently insufficient to isogenize this region.

Functional screening for a sleep related phenotype

Next, we performed a loss-of-function screen to isolate sleep-related genes. As a first trial round of screening, we selected 26 candidate genes with different properties, i.e. genes whose expression levels are largely different but whose functions are unknown and genes which functions in the nervous system, such as receptors for neurotransmitters and molecules in the signal transduction. We knocked these 26 genes and fmnn RNAi (VDRC 37333)-expressing flies in LD and DD conditions. In contrast, the total daily sleep of the Nmdar1 RNAi flies was significantly decreased in both LD and DD conditions relative to the control (Fig 2D). We confirmed the efficiency and specificity of the Nmdar1 knockdown by quantification of mRNA. As shown in Fig 2E, the Nmdar1 mRNA levels were significantly decreased to
approximately 50%. Although the glutamate receptor IB (GluRIB) gene is reported as a potential off-target gene for the Nmdar1 RNAi construct by target predictions of the VDRC [12], the GluRIB mRNA levels were comparable in the Nmdar1 RNAi and control flies. We also
examined the mRNA levels of CanA-14F and CanB for neuronal genes and RpL32 for ubiquitously expressed genes and found there were no differences in their mRNA levels in the Nmdar1 knockdown flies. Another Nmdar1 RNAi line (104773) gave a significant increase in locomotor activity and reduction in sleep only under DD conditions (S1A and S1B Fig). The Nmdar1 mRNA levels tended to be reduced in the RNAi flies, but did not reach statistical significance (S1C Fig).

Since Nmdar1 knockdown flies were more active than control flies, especially during the subjective night period under DD conditions, we calculated the parameters of day sleep and night sleep separately. In Nmdar1 RNAi (37333)-expressing flies, there was no significant difference in the amount of sleep during daytime under LD conditions (Fig 3A). Night sleep under LD conditions and both subjective day and subjective night sleep under DD conditions were significantly shorter in the Nmdar1 knockdown flies. There were no significant differences in the bout length of sleep in the Nmdar1 RNAi and control flies under both LD and DD conditions (Fig 3B). As shown in Fig 3C, the decrease in sleep in the Nmdar1 RNAi flies was mainly due to the reduction in sleep bout number. Similarly, another Nmdar1 RNAi line (104773) gave a significant decrease not in sleep bout length but in sleep bout number (S2B and S2C Fig).

An NMDAR antagonist reduces sleep

In order to pharmacologically examine NMDAR function on sleep, we assessed the effect of the NMDAR antagonist, MK-801. MK-801 is a non-competitive antagonist, which binds to the phencyclidine binding site of NMDAR and prevents its channel activities. We mixed MK-801 into the food and fed the flies during the sleep assay. At concentrations of 0.1 mg/ml, amount of sleep was significantly decreased compared to untreated controls, whereas lower doses (0.01 and 0.03 mg/ml) had no effect on sleep (S3 Fig). In 16 flies with 0.1 mg/ml MK-801, 3 ones died during the sleep assay. Thus, we used 0.1 mg/ml MK-801 in following experiments. As shown in Fig 4, MK-801 reduced sleep significantly in w1118 flies but not in fmn flies, which suggested that the effect of the Nmdar1 knockdown was due to a nondevelopmental effect. Although the Nmdar1 RNAi flies showed the reduction in sleep bout number, administration of MK-801 shortened sleep bout length (Fig 5B). We also examined the effects of the tyrosine hydroxylase inhibitor, 3-iodo-L-tyrosine (3IY), which inhibits dopamine biosynthesis. 3IY increased the amount of sleep in fmn almost to the control level (Fig 4C). Simultaneous addition of 3IY with MK-801, partially hindered the effects of MK-801, suggesting the additive effects of MK-801 and dopamine signaling in reducing sleep. These data indicated that NMDAR functions to promote sleep in Drosophila.

Nmdar1 knockdown in various regions of the fly brain

To identify neurons important for the function of NMDAR in sleep regulation, we screened 36 neural Gal4 drivers for their potency to induce Nmdar1 knockdown-induced decreased sleep (Fig 6A). Nmdar1 knockdown in the mushroom body or central complex did not show the reduced sleep comparable to that in the pan-neuronal knockdown flies. Nmdar1 knockdown in the fan-shaped body (104Y), which receives projection of sleep-regulating dopaminergic neurons [17, 18] also did not decrease sleep. On the other hand, Nmdar1 knockdown by at least two Gal4 drivers (121Y and c17) significantly decreased sleep (Fig 6B), suggesting that NMDAR expressed in several brain regions were involved in sleep regulation.

Discussion

This study provides evidence that NMDAR is involved in the regulation of sleep in flies.
To identify molecular candidates involved in sleep and arousal, we performed a genome-wide screen of genes that were differentially expressed in heads of control and \textit{fmn} flies. Since DAT functions as part of the clearance mechanism for released dopamine, extracellular dopamine levels are persistently elevated and postsynaptic dopamine signaling is increased in the DAT mutant \textit{fmn}. Although we predicted that expression of dopamine-related genes including NMDAR1 would be affected, our results showed that NMDAR1 RNAi flies exhibited a decreased sleep bout number.

**Fig 3.** Nmdar1 knockdown flies show a decreased sleep bout number. Total sleep (A), sleep bout length (B) and sleep bout number (C) for control (elav-Gal4;UAS-Dicer-2 \times \textit{w^{1118}}, white bars) and Nmdar1 RNAi (VDRC 37333)-expressing flies using the elav-Gal4;UAS-Dicer-2 (black bars) during day (ZT 0–12), night (ZT 12–24), subjective day (CT 0–12) and subjective night (CT 12–24). A total of 48 flies were tested for both genotypes in (A) and (C). Flies with no sleep during 12 h were excluded from the calculations of sleep bout length in (B) (n = 24–48). Data are presented as mean ± SEM. Asterisks indicate statistically significant differences from control determined by a t-test (p < 0.05).
dopamine receptors and genes of the dopamine biosynthesis pathway would be altered in fmnh flies, such adaptive changes were not observed at the mRNA level. In DAT knockout mice, the levels of both D1 and D2 dopamine receptors are significantly reduced [19]. We ourselves found that D1 dopamine receptor protein level was reduced in the mushroom body area of fmnh mutant [20]. Therefore, it is possible that expression levels of these genes are posttranscriptionally regulated in fmnh flies. Oscillation of circadian clock genes, per, tim and Clk under LD conditions were barely affected by the fmnh mutation (Fig 1) and fmnh flies showed a substantial circadian rhythmicity of locomotor activity [6], suggesting that fmnh flies have intact molecular clocks. We identified 563 genes with a significant difference in expression levels over a day between control and fmnh flies using microarray analysis (at 5% FDR). Of the 563 genes, 272 genes were up-regulated and the remaining 291 genes including DAT were down-regulated.
in fmn flies. Although the scb gene was identified as a markedly down-regulated gene in fmn flies in the microarray data, the false difference in expression was due to a single-base substitution in the 3' untranslated region, for which probe set was designed. This is a problem of gene expression analysis using this type of DNA microarray. Furthermore, it should be noted that our backcross was apparently insufficient to isogenize a neighboring scb locus. Gene ontology enrichment analysis using the 563 differentially expressed genes suggested a number of altered
biological processes in \textit{fmn} flies, listed in S2 Table. The changes in expression detected in many genes involved in metabolism likely reflect hyperactivity in locomotion in \textit{fmn} flies \cite{6} and dopamine function in modulating metabolic rate \cite{21}. Interestingly, genes involved in response to stress are also identified as wakefulness-related genes in \textit{Drosophila} and rats \cite{22, 23}. We found that differentially expressed genes were involved in nervous system development. These

Fig 6. \textit{Nmdar1} knockdown in various regions of the fly brain. (A) Total sleep for progeny collected from between 36 \textit{Gal4} drivers and \textit{UAS-Nmdar1 RNAi} (VDRC 37333) with the \textit{UAS-Dicer-2} on the 2nd chromosome in DD conditions (black bars). For controls, each \textit{Gal4} driver was crossed to \textit{w^{1118}} flies (white bars). Data are presented as mean ± SEM (\(n = 6–8\) for each group). Asterisks indicate statistically significant differences from control determined by a \(t\)-test (\(p < 0.05\)). (B) Total sleep for flies expressing \textit{Nmdar1} RNAi and \textit{Dicer-2} transgenes under indicated \textit{Gal4} drivers (black bars) in DD conditions. For controls, each \textit{Gal4} driver was crossed to \textit{w^{1118}} (white bars) or \textit{UAS-Dicer-2} flies (gray bars). Data are presented as mean ± SEM (\(n = 7–16\) for each group). Groups with asterisks indicate statistically significant differences (Tukey-Kramer HSD test for normally distributed data, \(p < 0.05\)).

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findings may be associated with the physiological function of sleep and regulation of neuronal plasticity in Drosophila [24, 25]. Using transgenic RNAi screening, we showed that a pan-neuronal knockdown of Nmdar1 resulted in locomotor hyperactivity and sleep reduction (Fig 2 and S1 Fig). Moreover, the application of an NMDAR blocker, MK-801 also reduced sleep in control flies (Fig 4). These results indicate a sleep-promoting role for NMDAR in Drosophila. We observed that Nmdar1 knockdown decreased sleep bout number, while MK-801 shortened sleep bout length (Fig 3, S2 Fig and Fig 5). These different effects may be due to the difference in the degree of inhibition of NMDAR function, or it is possible the pharmacological interference by NMDAR antagonist may not mimic NMDAR knockdown. Sleep was unaffected in fmn flies that were treated with MK-801 (Fig 4). This can be explained by the decrease in Nmdar1 gene expression in fmn. As previously reported, tyrosine hydroxylase inhibitor 3IY-treated fmn flies demonstrated significantly increased sleep levels, almost similar to that of control flies [26]. Simultaneous administration of 3IY and MK-801 partially suppressed the effects of MK-801 in controls and those of 3IY in fmn flies, suggesting an additive effect for MK-801 and dopamine signaling in reducing sleep. Although we have no data on the physiological relationship between NMDAR and dopamine signaling in sleep regulation, calcineurin, a heterodimeric Ca2+/calmodulin-dependent serine/threonine protein phosphatase, may link these two pathways. We found that CanA-14F and CanB genes were down-regulated in fmn flies (S1 Table). Calcineurin is activated by Ca2+ influx through the NMDAR. We recently reported that pan-neuronal CanA-14F and CanB RNAi flies significantly decrease sleep in a similar way to Nmdar1 RNAi flies [16]. In the mammalian brain, calcineurin dephosphorylates dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and thus modulates neuronal functions [27–29]. The Drosophila NMDAR subunits, Nmdar1 (dNR1) and Nmdar2 (dNR2), are widely expressed in the adult brain [30–33]. NMDAR expressed in the mushroom body and central complex is required for memory formation [31, 32]. The mushroom body has been shown to function not only as a memory center but also as a sleep center in Drosophila [34–36]. However, Nmdar1 knockdown in these brain regions hardly decreased sleep (Fig 6). The physiological association between sleep and memory in Drosophila has recently been described in various reports. Sleep deprivation causes impairment in aversive olfactory learning and memory [37, 38]. The short sleeper fmn mutants have poor memory retention [11]. Another short sleep mutant, Hyperkinetic [39] and CanA-14F RNAi flies [16] also have an impaired memory. Taken together, our results indicate that NMDAR-calcineurin signaling plays an important role not only in memory function but also in sleep regulation. Understanding NMDAR-calcineurin signaling in Drosophila may provide novel insights into the molecular relationship between sleep and memory.

Supporting Information

S1 Fig. Pan-neuronal Nmdar1 knockdown decreases sleep. Total daily activity (A) and total sleep (B) for control (elav-Gal4;UAS-Dicer-2 × w1118, white bars, n = 19) and Nmdar1 RNAi (VDRC 104773)-expressing flies using the elav-Gal4;UAS-Dicer-2 driver (black bars, n = 20) in LD and DD conditions. Data are presented as mean ± SEM. Asterisks indicate statistically significant differences compared to control according to a t-test (p < 0.05). (C) Efficiency of Nmdar1 gene knockdown. The expression levels of Nmdar1 and RpL32 genes in the head of male flies expressing Nmdar1 RNAi transgenes in all neurons are expressed as relative values to the control flies. Each mRNA level was quantified by qPCR and first normalized to GAPDH2. Then, the values were normalized to the average of independent control samples, which were set at 100%. Data are presented as mean ± SEM. n = 3 for each group. (PDF)
S2 Fig. Nmdar1 knockdown flies show a decreased sleep bout number. Total sleep (A), sleep bout length (B), and sleep bout number (C) for control (elav-Gal4;UAS-Dicer-2 × w^{1118}, white bars, n = 57) and Nmdar1 RNAi (VDRC 104773)-expressing flies using the elav-Gal4;UAS-Dicer-2 (black bars, n = 60) during day (ZT 0–12), night (ZT 12–24), subjective day (CT 0–12) and subjective night (CT 12–24). Data are presented as mean ± SEM. Asterisks indicate statistically significant differences from control determined by a t-test (p < 0.05).

S3 Fig. Addition of the NMDAR antagonist MK-801 to the food at a concentration of 0.1 mg/ml decreases sleep. Total sleep for untreated control (white bars) and MK-801-fed w^{1118} flies (colored bars) in DD conditions. MK-801 was directly mixed with sucrose-agar food at indicated concentration. Data are presented as mean ± SEM (n = 13–16 for each group). Groups with asterisks indicate statistically significant differences (Tukey-Kramer HSD test for normally distributed data, p < 0.05).

S1 Table. List of all genes that were significantly differentially expressed between control (w^{1118}) and fmn flies (t-test, 5% FDR; fold change > 1.25 × up or down). DAT, sch, CanA-14F, Nmdar1 and CanB genes were indicated in bold. The list was arranged by fold change and also included an Affymetrix DNA array probe set, gene symbol, FlyBase ID and Q-Value.

S2 Table. Gene ontology (GO) terms in the biological process category present in differentially expressed genes between control (w^{1118}) and fmn flies (p < 0.05). Count represents the number of differentially expressed genes belonging to indicated categories, respectively. Nmdar1 gene was included in following 10 categories, localization, establishment of localization, transport, response to stimulus, neurological system process, system process, behavior, response to chemical stimulus, cell-cell signaling and transmission of nerve impulse, which were indicated in bold. The list was arranged by Count.

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Author Contributions
Conceived and designed the experiments: JT TU MM SK KK. Performed the experiments: JT TU MM KK. Analyzed the data: JT TU MM KK. Contributed reagents/materials/analysis tools: JT TU MM KK. Wrote the paper: JT TU SK KK.

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