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ORIGINAL ARTICLE

Molecular resolution of a behavioral paradox: sleep and arousal are regulated by distinct acetylcholine receptors in different neuronal types in *Drosophila*

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Abstract

Sleep and arousal are both important for animals. The neurotransmitter acetylcholine (ACh) has long been found to promote both sleep and arousal in mammals, an apparent paradox which has also been found to exist in flies, causing much confusion in understanding sleep and arousal. Here, we have systematically studied all 13 ACh receptors (AChRs) in *Drosophila* to understand mechanisms underlying ACh function in sleep and arousal. We found that exogenous stimuli-induced arousal was decreased in *nAChRα3* mutants, whereas sleep was decreased in *nAChRα2* and *nAChRβ2* mutants. *nAChRα3* functions in dopaminergic neurons to promote exogenous stimuli-induced arousal, whereas *nAChRα2* and *β2* function in octopaminergic neurons to promote sleep. Our studies have revealed that a single transmitter can promote endogenous sleep and exogenous stimuli-induced arousal through distinct receptors in different types of downstream neurons.

Statement of Significance

Endogenous sleep and stimuli-induced exogenous arousal are important behaviors, while their regulation mechanisms are still largely unknown. Although being the first neurotransmitter discovered, the role of acetylcholine (ACh) in sleep regulation remains elusive. In this study, we systematically investigated all the 13 ACh receptors in the *Drosophila*, and found that two nicotinic ACh receptors promote endogenous sleep, whereas one promotes stimuli-induced exogenous arousal, and they function in octopaminergic and dopaminergic neurons respectively. These results revealed that endogenous sleep and stimuli-induced exogenous arousal are regulated by distinct pathways, and provided an insight into the molecular and cellular basis for ACh regulation of sleep.

Key words: sleep; arousal; *Drosophila*; nAChR; behavioral genetics

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Introduction

Both sleep and arousal are important in animals ranging from insects, fish, to mammals [1–3]. Sleep deprivation can lead to negative consequences such as impaired memory, disturbed metabolism, and even death [3]. Molecular mechanisms and neural circuits controlling sleep and arousal are under active research. *Drosophila melanogaster* has been used as a model for sleep research for nearly two decades [4, 5] and it shares features of mammalian sleep including behavioral immobility, increased arousal threshold, and homeostatic rebound after sleep deprivation. In mammals, sleep is divided into rapid eye movement (REM) sleep and non-REM (NREM) sleep, which are different in electroencephalogram (EEG), electromyogram (EMG), and arousal levels [6, 7]. Local field potential recordings also revealed distinct sleep stages in flies [8, 9].

Sleep is regulated by two processes: circadian and homeostatic [10, 11]. The circadian clock controls the timing of sleep, while the homeostatic process regulates the sleep drive. Molecular and cellular mechanisms of circadian rhythm have been well studied [12–14], whereas our understanding of homeostatic regulation of sleep is limited [15, 16]. Genes regulating sleep have been identified in both mammals and flies [17–23]. In mammals, sleep is regulated by monoaminergic, cholinergic, glutamatergic, and GABAergic neurons in multiple regions including the brain stem, the preoptic hypothalamus, the lateral hypothalamus and the basal forebrain [24–26]. In flies, sleep is also regulated by multiple brain regions including the mushroom bodies (MBs) [27–30], the dorsal fan-shaped body (dFB) [31–35], the ellipsoid body (EB) [36], the pars intercerebralis (PI) [37–39], and the ventral lateral neurons (LNvs) and dorsal neurons (DN1s) clock neurons [40–45].

In mammals, decades of research have shown that cholinergic neurons participate in sleep regulation. Cholinergic neurons in different brain regions have distinct roles in sleep regulation: Ascending cholinergic projections neurons in the basal forebrain (BF) are important in sleep–wake transitions, whereas descending cholinergic projections in the laterodorsal and the pedunculopontine tegmental nuclei (LDT/PPT) were found to be involved in REM sleep regulation [24, 46, 47]. Moreover, anatomical and functional differences of cholinergic projections across mammalian species have been identified [48], which increased the complexity of the roles of cholinergic neurons in sleep regulation.

Activation of all of the cholinergic neurons in the flies caused arousal [49]. However, recent studies have also found subsets of cholinergic neurons which promote sleep [30, 50]. Thus, in both mammals and flies, cholinergic neurons are paradoxical in being able to promote both sleep and arousal. This complexity could be due to multiple reasons: One is that the synthase of acetylcholine, choline acetyl transferase (ChAT) is ubiquitously expressed in flies, and thus could be coexpressing other sleep regulation transmitters and play dual functions. For example, cholinergic neurons have been found to colocalize with dopaminergic neurons [51] and other neuropeptidergic neurons [52] in flies; Another important reason is that the diversity and wide distribution of AChRs may contribute to this complexity. AChRs are highly conserved from flies to humans [53]. There are two types of ACh receptors: nicotinic AChRs (nAChRs), which are ligand-gated ion channels composed of five subunits, and muscarinic AChRs (mAChRs), which are G-protein coupled receptors. Sixteen nAChR subunits (nAChRα1–α7, α9, α10, β1–β4, δ, γ, ε) and five mAChRs (CHRM1–M5) have been identified in humans and other mammals [54, 55], whereas only 10 nAChR subunits (nAChRα1–α7, β1–β3) and three mAChRs (mAChRA, RB, RC) have been identified in flies [56–65]. In mammals, nAChRs are expressed in both the central nervous systems (CNS) and the neuromuscular junctions (NMJ) [54]. In insects, nAChRs act as excitatory receptors in the CNS, while glutamate receptors function in the NMJ [66–70] and the CNS.

Although ACh has long been known to play important roles in sleep regulation, the molecular basis of cholinergic signaling remains elusive, and little is known about the roles of AChRs in sleep regulation. Dissecting the roles of different AChRs in sleep regulation could help us to understand the molecular basis of ACh circuitry in sleep. We took advantage of the relatively simple brain, smaller number of AChRs, and availability of genetic tools in the flies to study functional roles of AChRs in sleep and arousal. Here, through a systematic generation and screen of 13 AChR fly mutants, we show that nAChRα2 and nAChRβ2 promote sleep, whereas nAChRα3 promotes arousal. And through further immunochemistry, genetic and pharmacological studies, we found that nAChRα2 modulate sleep in octopaminergic neurons probably through octopaminergic signaling, and nAChRα3 modulate arousal in dopaminergic neurons through dopaminergic signaling.

Methods

Fly stocks and rearing conditions

Flies were raised in standard corn meal at 25°C and 60% humidity and kept in a 12 h:12 h light:dark cycle, unless constant darkness assay were used. (1) *UAS-β2RNAi* (THU2877), (2) *UAS-α3RNAi* (THU2756), and (3) *UAS-TβHRNAi* (TH02221) were from TsingHua Fly Center. (4) *UAS-α2RNAi* (v1195), and (5) *UAS-Dicer* were from Vienna *Drosophila* RNAi Center. (6) *UAS-THRNAi* was a gift from Mark N. Wu (Johns Hopkins University) [71]. (7)*UAS-mCD8::GFP*, (8) *UAS-Syt::GFP*, (9) *UAS-DenMark*, (10) *UAS-FRT-STOP-FRT-mCD8::GFP*, (11) *LexAop-myr::GFP* were from the Bloomington Stock Center. (12) *UAS-stinger::GFP* was from Dickson lab (Janelia Research Campus, U.S.). (13) *UAS-LexADBD* was from Chi-Hon Lee (ICOB, Academia Sinica, Taiwan, China). (14) *LexAop-Flp* was from Rosbash lab (Brandeis University, U.S.). (15) *LexAop-Gal4DBD* was constructed by Bowen Deng from Rao Lab's previous study.

nAChRα5KORFP, *nAChRα6KORFP*, *mAChR-AKORFP*, *mAChR-BKORFP*, *mAChR-CKORFP*, *TβHKIGal4*, *THKIGal4*, *TH-p65AD*, *α2p65AD, β2-KILexA, LexAop-Gal4DBD* were from the lab's previous work [51], and the schematic genotypes of the five AChRKORFP lines are shown in Figure S2. *TrhKIGal4* was from the lab's previous work [72].

All the flies used in this study have been backcrossed into a Canton-S background for at least five generations.

Generation of transgenic, knockout and knockin flies

Total RNA of *wt* flies was isolated using TRIzol reagent (Invitrogen), first strand cDNA was then made by the PrimeScript II first strand cDNA synthesis kit (Takara, 6210A). The coding sequences of *nAChRα2* and *nAChRα3* were amplified from the first strand cDNA and inserted into the PACU2 vector and the Addgene plasmid #26224 vector respectively, resulting in the UAS-α2, UAS-α3, and LexAop-α3 DNA constructs. The PACU2

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vector was from the Jan Lab at UCSF [73]. The constructs were inserted into *attP2* site.

The CRISPR/Cas9 system was used to generate knockout and knockin flies. The materials and protocols for designing and generating the guide RNA and Cas9 mRNA were generously provided by Renjie Jiao (Institute of Biophysics, CAS) [74]. Two gRNAs targeting the coding sequence were injected together with Cas9 mRNA to generate deletion and indel lines, including *α2KO*, *α3KO*, *α4KO*, *β2KO*, and *α7-earlystop* (Figure S1A).

An additional donor plasmid was co-injected with two gRNAs and the Cas9 mRNA to generate *KOGal4*, *KIGal4*, and *KILexA* flies. The 5′ homologous arm (~2.5 kb) and 3′ homologous arm (~2.5 kb) were inserted into the pBSKII vector to generate the donor plasmids, with the target sequence lying between the two homologous arms. After the target sites were cut by gRNAs, homology-directed repair introduced target sequence (Gal4 or LexA) from the donor into a specific site in the genome, replacing the original genome sequence between two homologous arms. The 3′ end of the 5′ homologous arm was designed to locate right behind the start codon in *KOGal4* (Figure S1B), so that the translation was ended by the stop codon in the target sequence. The 3′ end of the 5′ homologous arm was designed to locate right before the stop codon in *KIGal4* and *KILexA* (Figure S1C), so that the translation was not disrupted, and hence the *KIGal4* and *KILexA* could represent the expression pattern of the target gene as natural as possible. *nAChR-KOGal4*, *KIGal4*, and *KILexA* flies were generated by the above strategies.

Identification of transgenic flies

The targeted regions of generated transgenic lines and the primer sequences for genotyping are listed in Tables S1 and S2.

For indel/deletion lines, a pair of primers flanking the targeted region was used for PCR and sequencing, flies were then backcrossed for at least five generations to exclude possible off-target editing.

For Gal4/LexA lines, a LoxP-flanked 3xP3-RFP target sequence is fused behind Gal4/LexA sequence as a selective marker, which would express RFP in flies' eyes. All the transgenic knockin flies have been picked for RFP-expressing eyes and then genotyped to check in-target gene editing. Two pairs of primers were used for each line: one 5′ pair with the forward primer in the genomic region upstream the 5′ arm and the reverse primer in the Gal4/LexA target sequence; and one 3′ pair with the forward primer in the RFP target sequence and the reverse primer in the genomic region downstream the 3′ arm, to make sure that the whole target sequence was correctly incorporated into the target site. Flies were then backcrossed for at least five generations to exclude possible off-target editing.

Behavioral assays

Sleep, sleep deprivation and circadian analyses were carried out as previously described [75]. Male and female flies were separated in 5 h after eclosion, and flies aged 5–8 days were used in behavioral assays. Standard corn meal was used in behavioral assays except for L-DOPA feeding assay. Each experiment was repeated for more than three times. Periods of uninterrupted behavioral immobility lasting for more than 5 min were defined as sleep [76].

Briefly, a single fly was transferred into the monitor tube (5 mm × 65 mm) containing fly food, 48 monitor tubes were fixed on a recording plate, and the flies were recorded for 3–5 days. Then the positions of the flies were tracked, sleep duration, and speed were analyzed using Matlab (Mathworks), flies that died during experiment were excluded in analyses. Background of the frames was calculated after obtaining the video data, and the contours of flies of each frame were obtained by subtracting the background from the picture. A threshold was applied to turn the contour of flies into a binary picture, subtle information such as mouth and legs was lost in this procedure. The geometric center of each fly contour was then extracted, the distance between the geometric centers of two continuous frames was calculated to indicate the status of flies. Flies were defined as moving when the distance was more than one pixel.

Sleep was deprived by random shaking in the whole night. The recording tubes were fixed to a silica gel holder, and then placed horizontally into a holding box. The box was rotated clockwise or counter-clockwise and bumped to plastic stoppers under the control of a servo motor (TowerPro MG995) to shake the flies. The flies were shaken with random intervals of 2–5 min. Each shaking lasts 18 s, including nine times of continuous rotation of the box. Rebound rate for every 30 min was calculated as (sleep duration after the deprivation—sleep duration for the equivalent time before the deprivation)/(sleep loss). Accumulated rebound rate was calculated as the sum of rebound rate since the deprivation stopped.

For circadian analyses, flies were entrained to 12h:12h LD cycles for 3 days and then entrained to constant darkness for 9 days. Locomotor activity was measured and analyzed by chi-square analysis in the Actogram J plugin [77]. Relative rhythm power was calculated by dividing the detected peak amplitude by the significance threshold value at the same period length in rhythmic flies ($p < 0.05$).

In arousal assay, flies were stimulated three times at night (ZT16, ZT18, and ZT20) by eccentric vibration motors (1.0 g). Eccentric vibration motors were fixed underneath the recording plates to stimulate the flies, and the strength of the stimuli was controlled by modulating the voltage output. The stimulation strength was measured by an acceleration sensor (model CJMCU_ADXL345, read by an Arduino plate) attached in the surface of the plate. The stimulation strength was set to 1.0 g (1.0 g equals the gravitational force at the surface of the earth, 9.8 m/s^2). Each stimulation contains three times of a vibration lasting 200 ms, with an interval of 800 ms. Sleep state of the flies was analyzed using our video-based fly tracing system, and flies that were sleeping before the stimuli were counted and further analyzed to measure their state right after the stimuli. For each experiment, ~48 flies were loaded, and arousal rate was calculated as the ratio of (the total number of flies awakened by the stimuli at ZT16, ZT18, and ZT20) to (the total number of flies slept before the stimuli at ZT16, ZT18, and ZT20).

Immunohistochemistry and confocal imaging

Adult female brains were dissected, fixed, and stained as previously described [75]. The following primary antibodies were used: chicken anti-GFP (1:1,000) (Invitrogen), mouse nc82 (1:40) (DSHB). The following secondary antibodies were used: AlexaFluor488 antichicken (Life technologies), AlexaFluor633 antimouse (Life technologies). Brains were imaged using a Zeiss LSM710 confocal microscope, and images were processed with Imaris (Bitplane AG, Zurich, Switzerland) and ImageJ (National Institutes of Health, USA) softwares.

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Drug treatment

L-DOPA (Sigma, D-9628) was dissolved in DMSO firstly and then added to fly food containing 5% sucrose and 2% agar to make the L-DOPA food, with the ultimate concentration being 8 mg/mL. Control food was made by adding equivalent DMSO to the food containing 5% sucrose and 2% agar. *wt* and *α3KO* flies were transferred to monitor tubes containing L-DOPA food or control food for sleep and arousal analyses.

Statistics

Statistical analyses were performed with Prism 5 (GraphPad). Mann–Whitney *U*-test was used to compare two columns of data. Kruskal–Wallis test with Dunn's posttest was used to compare multiple columns of data from mutants, rescue and RNAi. Fisher's exact test was used to compare arousal rates. Statistical significance was denoted by asterisks: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, n.s., $p > 0.05$.

Results

A systematic construction of AChR mutants in flies

To systematically study the role of AChRs in sleep regulation, we used the CRISPR–Cas9 system to generate knockout (KO) mutants and in-frame fused knockin (KI) lines for genes encoding 10 nAChRs and 3 mAChRs (Figures S1 and S2, Table S1). Out of the 13 AChR mutants, KO of *nAChRβ1* was lethal, and most of *nAChRα4*$^{-/-}$ flies died in three days after eclosion, thus, *nAChRβ1*$^{+/-}$, *nAChRα4*$^{+/-}$, and *nAChRα4*$^{-/-}$ flies, along with other AChR mutants, were used in the following screen.

The switch from sleep to wakefulness could be endogenous (daily wake promoting) or exogenous (induced by stimuli), previous studies have suggested that these two forms of arousal were regulated differentially [78, 79]. To investigate the role of AChRs in these two aspects, we measured the sleep durations and stimuli-induced arousal rates of wild type (*wt*) and *AChR* mutant lines using a video-based fly recording system as described previously [51, 72, 75].

nAChRα2 and *nAChRβ2* promote sleep

Among the 13 *AChR* mutant lines, sleep was most drastically reduced in *nAChRα2* knockout (*nAChRα2KO*, *α2*$^{-/-}$) and *nAChRβ2* knockout (*nAChRβ2KO*, *β2*$^{-/-}$) mutant flies, with the ratio to wt being 0.39 and 0.51, respectively (Figure S3A). While *nAChRα4* was found to promote sleep in a previous study [21], we could not replicate this phenotype with *nAChRα4* knockout (*nAChRα4KO*) flies (Figure S3D). This could be due to the different genotypes of *nAChRα4* mutants: point mutation was introduced to flies used in the previous study [21], whereas 10,296 base pairs (bp) in the coding region of the *nAChRα4* gene have been deleted in our *nAChRα4KO* flies (Figure S2A, Table S1). Besides *α2*$^{-/-}$ and *β2*$^{-/-}$ flies, *α1*$^{-/-}$, and *β1*$^{+/-}$ flies also displayed statistically significantly decreased sleep, and *α5*$^{-/-}$ flies displayed increased sleep. However, the effect of *α1*$^{-/-}$, *β1*$^{+/-}$, and *α5*$^{-/-}$ flies on sleep was moderate, with the ratio to wt being 0.82, 0.84, and 1.17. Thus, we then focused on *nAChRα2* and *nAChRβ2* to investigate their roles in sleep promoting.

Part of the ligand-binding domain (LBD) and most of the transmembrane domain (TMD) were eliminated in *α2*$^{-/-}$ flies (Figure 1, A), and most of LBD and TMD were eliminated in *β2*$^{-/-}$ flies (Figure 1, B). Durations of both daytime and nighttime sleep were significantly reduced in *α2*$^{-/-}$ female (Figures 1, C and D) and male (Figures 1, G and H) flies. Daytime and nighttime sleep were significantly reduced in *β2*$^{-/-}$ male flies (Figure 1, I and J) and nighttime sleep was significantly reduced in *β2*$^{-/-}$ female flies (Figure 1, E and F). Because nighttime sleep was consistently reduced in both genders of *α2*$^{-/-}$ and *β2*$^{-/-}$ flies, and flies are diurnal animals, thereafter we focused on nighttime sleep. The sleep duration of *α2*$^{+/-}$ and *β2*$^{+/-}$ were comparable with that of *wt*, suggesting that both *nAChRα2KO* and *nAChRβ2KO* are recessive. *α2*$^{-/-}$ and *β2*$^{-/-}$ flies were phenotypically similar to the *wt* in sleep recovery after deprivation (Figure S4, A and B), in circadian rhythm (Figure S4, D and E) and arousal with normal strength stimuli (Figure S5, C and I) or with weak stimuli (Figure S5, N).

nAChRα3 promotes arousal

For the stimuli-induced exogenous arousal screen, we stimulated the flies with mechanical stimuli (strength: 1.0 g) at ZT16, ZT18, ZT20 in the night. To distinguish arousal from startle response, only flies that were asleep right before the stimuli were selected for further analysis, and the number of them was counted as "number of asleep flies before stimuli ($Number_{pre}$)." Among these flies, the number of flies that were awakened by the stimuli was counted as $Number_{wake}$, and the number of flies that did not awaken by the stimuli was counted as $Number_{sleep}$. Arousal rate is defined as $Number_{wake}/Number_{pre}$, standing for the rate of flies awakened from sleep by the stimuli. For each experiment, $Number_{pre}$, $Number_{wake}$, and $Number_{sleep}$ were the total number under three times of stimuli at ZT16, ZT18, and ZT20.

We measured the arousal rate of 13 *AChR* mutant lines induced by mechanical stimuli (Figure S5), and found that the arousal rate was most drastically reduced in *nAChRα3* knockout (*nAChRα3KO*, *α3*$^{-/-}$) flies (Figure S5, A): while the arousal rate of wt flies was ~0.8, the arousal rate of *α3*$^{-/-}$ flies was only ~0.2. $Number_{wake}$ was plotted as a solid bar and $Number_{sleep}$ plotted as an empty bar for statistical analyses, the ratio of the solid bar to the whole bar represented for arousal rate, which was denoted under each bar. Fisher's exact test was used to compare the arousal rate between the two groups. Through statistical analyses, we found that besides *α3*$^{-/-}$ flies, which showed most drastically reduced arousal rate, *α5*$^{-/-}$, *β1*$^{+/-}$, *β3*$^{-/-}$, *mAChRA*$^{-/-}$, *mAChRB*$^{-/-}$ flies also showed moderately reduced arousal rate (Figures S5, B–M).

Because the arousal rate of *α3*$^{-/-}$ flies was reduced most drastically, with only ~20% of *α3*$^{-/-}$ flies could be awakened by the stimuli, we next went on to investigate *nAChRα3*, which probably plays a key role in the regulation of exogenous stimuli-induced arousal. Our *nAChRα3KO* mutants were created by deleting a large part of LBD and TMD (Figure 2, A). The arousal rate was significantly reduced in both female *α3*$^{-/-}$ (Figure 2, B) and male *α3*$^{-/Y}$ (Figure 2, F) mutant flies. When extremely strong mechanical stimuli (strength: 3.0 g) were applied, almost all the *nAChRα3KO* mutant flies and wt flies were aroused from sleep (Figures 2, C and G), suggesting that the mechanosensation of *nAChRα3KO* mutants is normal. The speed during locomotion in *α3*$^{-/-}$ females was not different from that in the *wt* (Figure 2, D), and the speed of *α3*$^{-/Y}$ males was even slightly increased (Figure

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2, H), indicating that the low responsiveness of *nAChRα3KO* was not due to defective locomotion.

Nighttime sleep in *nAChRα3KO* was not different from that in the *wt* (Figure 2, E and I), suggesting that *nAChRα3* specifically regulates exogenous stimuli-induced arousal but not endogenous sleep. The arousal rate of $\alpha3^{+/-}$ flies lies between those of $\alpha3^{-/-}$ and $\alpha3^{+/+}$ (Figure 2, B), suggesting that *nAChRα3* is haploinsufficient. Sleep recovery after sleep deprivation was slightly reduced in $\alpha3^{-/-}$ flies (Figure S4, C), suggesting a possible role of *nAChRα3* in sleep homeostasis. The circadian periods were not different between $\alpha3^{-/-}$ and $\alpha3^{+/+}$ (Figure S4, D), and the relative rhythm power of $\alpha3^{-/-}$ is higher than $\alpha3^{+/+}$ flies (Figure S4, E).

nAChRα2 and *nAChRβ2* are widely expressed in the brain

Through a mini-screen of sleep duration and arousal rate in 13 AChR mutants, we found that the sleep duration of $\alpha2^{-/-}$ and $\beta2^{-/-}$ were drastically reduced to nearly half of that of wildtype flies, and only ~20% of $\alpha3^{-/-}$ flies could be awakened from sleep by exogenous stimuli, comparing with the arousal rate of wildtype flies being ~80%. These results suggested that *nAChRα2* and β2 participate in sleep promoting, while *nAChRα3* participate in arousal promoting.

Next, we sought to understand in which types of neurons do these three receptors function in, to gain insights into the possible downstream circuits.

nAChRα2 encodes one protein while *nAChRβ2* encodes two isoforms differing in the carboxyl (C) terminal. *2A-Gal4* was fused in-frame to the C terminal precisely before the stop codon, to produce *α2KIGal4*, *β2KIGal4* (for the long isoform of *nAChRβ2*), and *β2-PB-KIGal4* (for the short isoform of *nAChRβ2*) (Figure S2, B).

mCD8::GFP, stinger::GFP, Syt::GFP, and DenMark were driven by *α2KIGal4*, *β2KIGal4*, and *β2-PB-KIGal4* to label the membrane, the nuclei, the axons and the dendrites of neurons expressing nAChRα2 or nAChRβ2, respectively. *α2KIGal4* and *β2KIGal4* labeled the membrane of neurons in multiple brain regions, including the antenna lobe (AL), the subesophageal ganglion (SOG), and the sleep-regulating regions MBs and PI, and in the mesothoracic, metathoracic neuromere (MN, MtN) and the abdominal center (AC) of the ventral nerve cord (VNC) (Figure 3, A–F). The nuclei and axons of neurons were labeled by *α2KIGal4* and *β2KIGal4* in multiple brain regions similar to that of membrane, and dendrites in the PI, the SOG, and the optical lobe were labeled by *α2KIGal4* and *β2KIGal4* (Figure S6, A–F). *β2-PB-KIGal4* showed no membrane expression in the brain and the VNC (Figure S6, M–O), no nuclei expression in the brain (Figure S6, G), rare synapse expression in the SOG (Figure S6, H), and dendrites expression in the optic lobe (Figures S6, I). Because membrane and nuclei expression were not labeled in the brain by *β2-PB-KIGal4*, the long isoform of *nAChRβ2* probably plays a more important role in sleep regulation than the short isoform, and we thereafter label β2-expressing neurons with *β2KIGal4*.

nAChRα2 and *nAChRβ2* may function together to promote sleep

RNAi knockdown of *nAChRα2* driven by *α2KIGal4* and RNAi knockdown of *nAChRβ2* driven by *β2KIGal4* both significantly

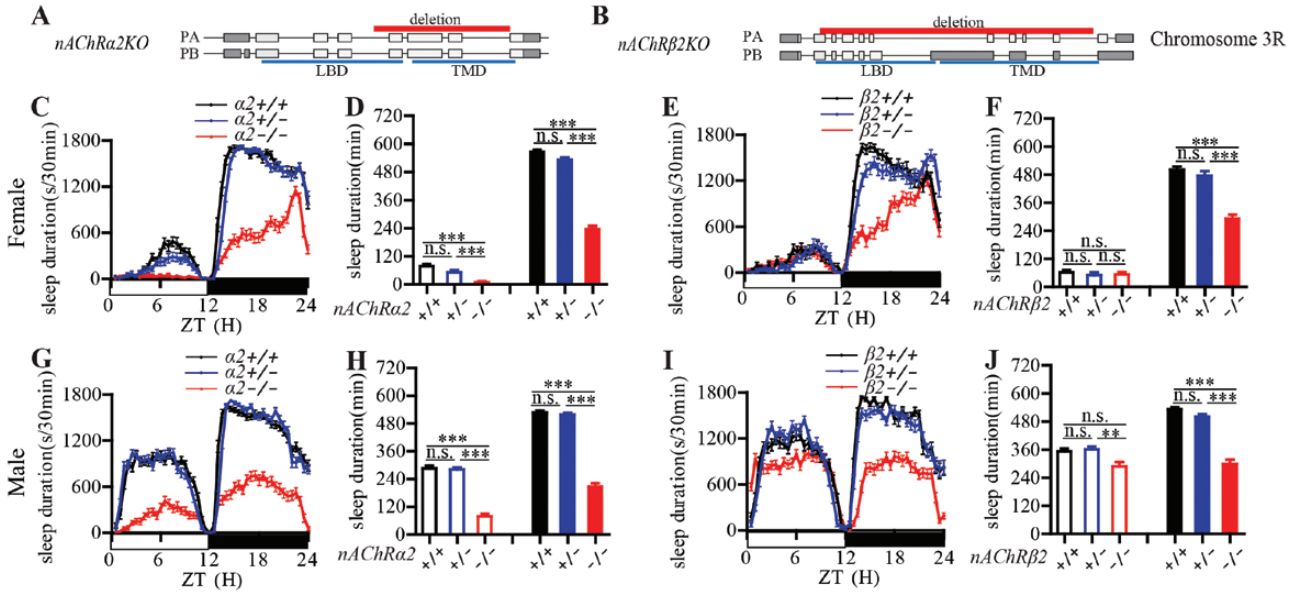

Figure 1. Sleep phenotypes of *nAChRα2* and β2 mutants. (A, B) Schematic representations of *nAChRα2* (A) and *nAChRβ2* (B) genes with red bars indicating the deleted regions in *nAChRα2KO* and *nAChRβ2KO*. Predicted transmembrane domain (TMD) and part of ligand-binding domain (LBD) were deleted in *nAChRα2KO*, most part of LBD and TMD were deleted in *nAChRβ2KO*. Blue lines denote LBD and TMD, white boxes denote CDS, grey boxes denote UTR. Deleted part: *nAChRα2KO*, NP_524482.1, 197aa–512aa. *nAChRβ2KO*, NP_524483.1, 57aa–459aa. (C–J) Sleep profiles and statistical analysis of α2 (C, D, G, H) and β2 (E, F, I, J) female (C–F) and male (G–J) mutant flies. (C) Sleep profiles of $\alpha2^{-/-}$ (red), $\alpha2^{+/-}$ (blue), and *wt* ($\alpha2^{+/+}$, black) female flies, plotted in 30 min bins. Open bar under the *x*-axis denotes light phase, filled bar denotes dark phase. (D) Statistical analysis. Daytime (open bars) and nighttime (filled bars) sleep durations of $\alpha2^{-/-}$ (red) ($n = 83$) were significantly less than $\alpha2^{+/-}$ (blue) ($n = 46$), and $\alpha2^{+/+}$ female flies (black) ($n = 47$). (E) Sleep profiles of $\beta2^{-/-}$, $\beta2^{+/-}$, and $\beta2^{+/+}$ female flies, plotted in 30 min bins. (F) Statistical analysis. Daytime and nighttime sleep durations of $\beta2^{-/-}$ ($n = 47$) were significantly reduced than $\beta2^{+/-}$ ($n = 48$) and $\beta2^{+/+}$ female flies ($n = 45$). (G) Sleep profiles of $\alpha2^{-/-}$, $\alpha2^{+/-}$, and $\alpha2^{+/+}$ (male flies, plotted in 30 min bins). (H) Statistical analysis. Daytime (open bars) and nighttime (filled bars) sleep durations of $\alpha2^{-/-}$ ($n = 46$) were significantly less than $\alpha2^{+/-}$ ($n = 45$) and $\alpha2^{+/+}$ male flies ($n = 47$). (I) Sleep profiles of $\beta2^{-/-}$, $\beta2^{+/-}$, and $\beta2^{+/+}$ male flies, plotted in 30 min bins. (J) Statistical analysis. Nighttime sleep durations of $\beta2^{-/-}$ ($n = 48$) were significantly less than $\beta2^{+/-}$ ($n = 48$) and $\beta2^{+/+}$ male flies ($n = 44$). $^{***}p < 0.001$, $^{**}p < 0.01$, n.s., $p > 0.05$. Kruskal–Wallis test with Dunn's posttest. Error bars represent SEM.

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reduced nighttime sleep (Figure 3, J and K), further supporting the sleep-promoting roles of *nAChRα2* and *nAChRβ2*, and their function in neurons labeled by *α2KIGal4* and *β2KIGal4*.

When *nAChRα2* and *nAChRβ2* genes were knocked out, sleep was both reduced to about half of *wt* level (Figure 1, C–J), and these two genes are tightly linked: only ~18 kb apart from each other in the *Drosophila* genome. Because a functional nAChR is composed of five subunits *in vivo*, and studies have shown that nAChRα2 can form homo-pentamer by itself [60], or form hetero-pentamer with nAChRα1 and nAChRβ2 in *Drosophila* [80], it is plausible that nAChRα2 and nAChRβ2 form a functional nAChR pentamer to promote sleep.

To explore this possibility, we intersected *nAChRα2* and *nAChRβ2* by simultaneously expressing *UAS-FRT-STOP-FRT-GFP* in β2-expressing neurons driven by *β2KIGal4*, and *LexAop-Flp* in α2-expressing neurons driven by *α2KILexA*. In *nAChRα2*$^{+}$ *nAChRβ2*$^{+}$ neurons, the STOP cassette between the UAS and GFP was removed by the Flp recombinase, thus labeling the neurons with GFP. Intersection of *nAChRα2* and *nAChRβ2* was identified in multiple brain regions, including known sleep-regulating regions such as the MB Kenyon cells and the PI (Figure 3, G–I).

Also, RNAi knockdown of *nAChRβ2* in *nAChRα2*-expressing cells and knockdown of *nAChRα2* in *nAChRβ2*-expressing cells

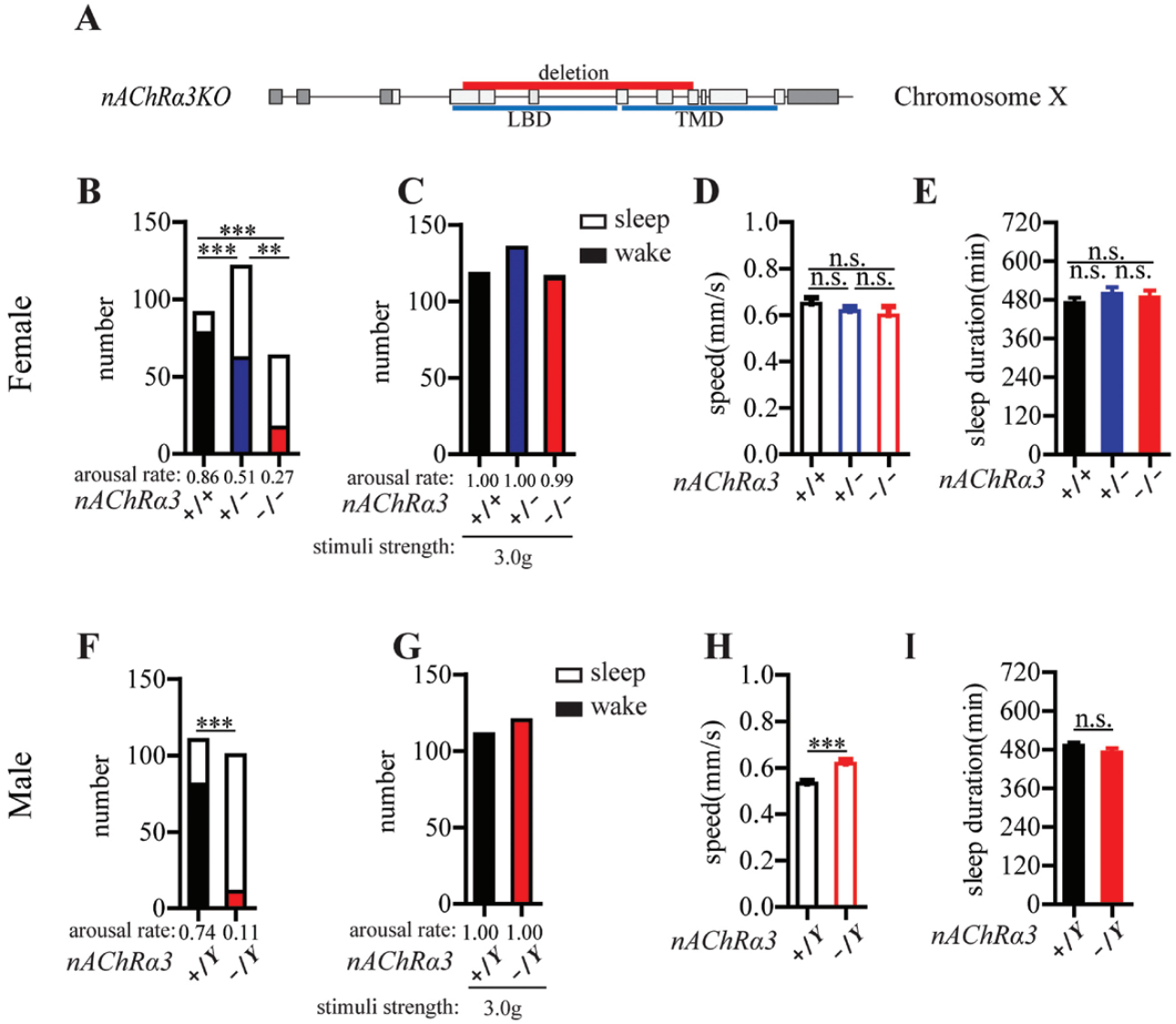

Figure 2. Sleep and arousal phenotypes of *nAChRα3* mutants. (A) Schematic representations of *nAChRα3* with a red bar indicating the deleted regions in *nAChRα3KO*. Deleted part: NP_525079.3, 94aa–335aa. (B) Arousal rate of $\alpha3^{+/+}$ (black) (n = 91), $\alpha3^{+/-}$ (blue) (n = 121), and $\alpha3^{-/-}$ (red) (n = 63) female flies under mechanical stimuli. Open bars denote the number of flies stayed asleep after the stimuli ($Number_{sleep}$), filled bars denote the number of flies awakened by the stimuli from sleep ($Number_{wake}$). Arousal rate was denoted under each bar. (C) Almost all the $\alpha3^{-/-}$ (n = 116), $\alpha3^{+/-}$ (n = 135), and wt (n = 118) female flies were awakened from sleep by extremely strong stimuli (3.0 g). (D) The speed of $\alpha3^{-/-}$ (n = 30) and $\alpha3^{+/-}$ (n = 48) flies during locomotion was normal compared to $\alpha3^{+/+}$ flies (n = 48). (E) $\alpha3^{-/-}$ (n = 48) and $\alpha3^{+/-}$ (n = 48) flies had normal nighttime sleep duration compared to $\alpha3^{+/+}$ (n = 45) flies. (F) Arousal rate of $\alpha3^{-/Y}$ (n = 100) was significantly less than $\alpha3^{+/Y}$ (n = 110) male flies. (G) All the $\alpha3^{-/Y}$ (n = 120) (red) and wt (n = 111) (black) male flies were awakened from sleep by extremely strong stimuli (~3.0 g). (H) Speed of $\alpha3^{-/Y}$ (n = 47) was higher than $\alpha3^{+/Y}$ (n = 48) flies. (I) Nighttime sleep durations of $\alpha3^{-/Y}$ (n = 47) had no significant difference with $\alpha3^{+/Y}$ flies (n = 47). Daytime sleep durations of $\alpha3^{-/Y}$ were less than $\alpha3^{+/Y}$ flies. $^{***}p < 0.001$, $^{**}p < 0.01$, n.s. $p > 0.05$, Fisher's exact test was used in (B), (C), (F), and (G), Kruskal–Wallis test with Dunn's posttest was used in (D) and (E). Mann–Whitney test was used in (H) and (I). Error bars represent SEM.

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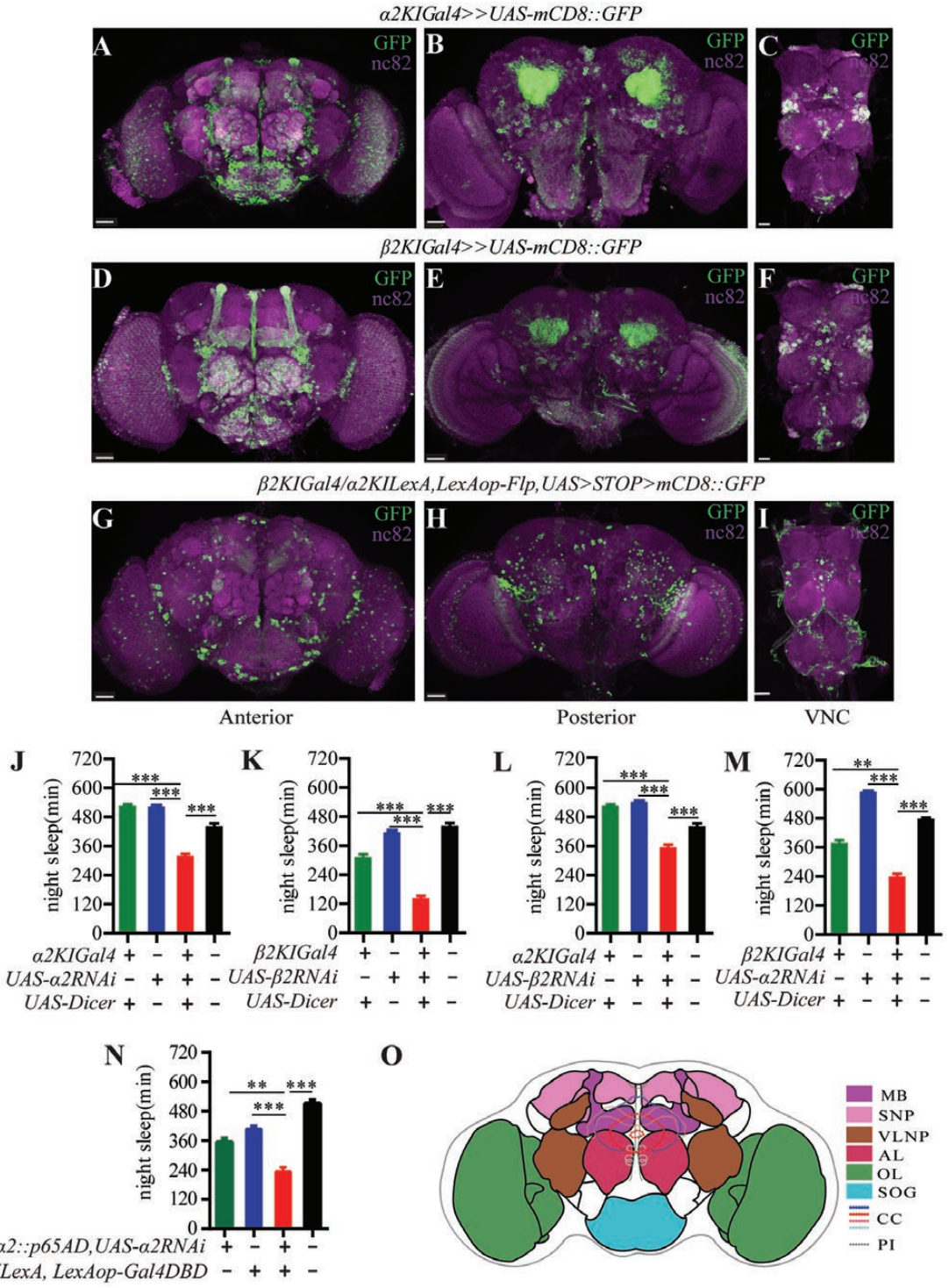

Figure 3. *nAChRα2* and β2 function together to promote sleep. (A–C) Expression patterns of *α2KIGal4* labeled by mCD8::GFP in the brain (A, anterior view; B, posterior view) and the VNC (C). (D–F) Expression patterns of *β2KIGal4* labeled by mCD8::GFP in the brain (D, anterior view; E, posterior view) and the VNC (F). (G–I) Expression patterns of *β2KIGal4/α2KILexA, LexAop-Flp, UAS-FRT-STOP-FRT-mCD8::GFP*. Colocalized neurons expressing *nAChRα2* and *nAChRβ2* were labeled with GFP in the brain (G, anterior view; H, posterior view) and the VNC (I). The tissues were stained with anti-GFP (green) and nc82 (magenta). Scale bars are 30 μm. (J) Knockdown of *nAChRα2* in *nAChRα2*-expressing cells (red) ($n = 48$) significantly reduced nighttime sleep duration compared to *α2KIGal4/+* (green) ($n = 48$), *UAS-α2RNAi/+* (blue) ($n = 46$) and *wt* (black) ($n = 48$). (K) Knockdown of *nAChRβ2* in *nAChRβ2*-expressing cells (red) ($n = 47$) significantly reduced nighttime sleep duration compared to Gal4 control (green) ($n = 48$), RNAi control (blue) ($n = 48$), and *wt* (black) ($n = 48$). (L) Knockdown of *nAChRβ2* in *nAChRα2*-expressing cells (red) ($n = 46$) significantly reduced nighttime sleep duration compared to *α2KIGal4/+* (green) ($n = 48$), *UAS-β2RNAi/+* (blue) ($n = 42$), and *wt* (black) ($n = 48$) flies. (M) Knockdown of *nAChRα2* in *nAChRβ2*-expressing cells (red) ($n = 48$) significantly reduced nighttime sleep duration compared to *β2KIGal4/+* (green) ($n = 48$), *UAS-α2RNAi/+* (blue) ($n = 47$) and *wt* (black) ($n = 44$) flies. (N) Knockdown of *nAChRα2* in α2–β2-coexpressing cells (red) ($n = 43$) significantly reduced nighttime sleep duration compared with *α2::p65AD,UAS-α2RNAi/+* (green) ($n = 46$), *β2KILexA, LexAop-DBD/+* (blue) ($n = 46$), and *wt* (black) ($n = 47$). $^{***}p < 0.001$, $^{**}p < 0.01$, Kruskal–Wallis test with Dunn's posttest. Error bars represent SEM. Female flies were used. (O) Schematic representations of fly brain regions. MB, mushroom body; SNP, superior neuropils; VLNP, ventrolateral neuropils; AL, antennal lobe; OL, optic lobe; SOG, subesophageal ganglion; CC, central complex; PI, pars intercerebralis.

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both significantly reduced nighttime sleep durations (Figure 3, L and M). We also used split Gal4 strategy to express *nAChRα2* RNAi in *nAChRα2*-β2-coexpressing cells, and found that nighttime sleep duration was significantly decreased when α2 is knocked-down in *nAChRα2*-β2-coexpressing cells (Figure 3, N). Taken together, these data showed that *nAChRα2* and *nAChRβ2* in the same neurons regulate sleep. The simplest explanation for the previous finding of pentameric nAChR composition and our results of expression, intersection, and RNAi experiments is that *nAChRα2* functions with *nAChRβ2* to promote sleep.

nAChRα2 and *β2* function in octopaminergic neurons to promote sleep

To further investigate the sleep-promoting mechanisms of *nAChRα2*, we intersected *nAChRα2* with a group of neurotransmitter synthases encoding genes, which were found to affect sleep when mutated. Among the immunochemistry and confocal imaging results, synthases of serotonin (tryptophan hydroxylase, or *Trh*) and octopamine (tyramine β hydroxylase, or *TβH*), which were known to participate in sleep regulation [37, 51, 72, 81, 82], were found to have intersections with *nAChRα2*. *Trh* and *nAChRα2* were found to have an overlapping expression in the SOG (Figure S7, A). *TβH* and *nAChRα2* were found to be overlapped widely in the brain and the VNC (Figure 4, A and B), including the AL, the ventral unpaired median (VUM) cells, the anterior superior medial protocerebrum (ASM) cells, the protocerebral bridge (PB) cells, and the ventrolateral protocerebrum (VL) cells.

To find in which type of neurons does *nAChRα2* function, *UAS-nAChRα2* was reintroduced into different types of neurons in the $\alpha2^{-/-}$ background. The nighttime sleep duration of $\alpha2^{-/-}$ was rescued by reintroduction of *UAS-nAChRα2* into α2-expressing cells (Figure 4, C) labeled by *α2KOGal4* in which *2A-Gal4-STOP* was fused to the start codon of *nAChRα2* (Figure S2, B). Expression of *nAChRα2* in the octopaminergic cells labeled by *TβHKIGal4* rescued the nighttime sleep duration (Figure 4, D), whereas expression in the serotonergic cells labeled by *TrhKIGal4* failed to rescue (Figure S7, B), indicating that *nAChRα2* functions in octopaminergic neurons, but not serotonergic neurons, to promote sleep. RNAi knockdown of *nAChRα2* in octopaminergic cells driven by *TβHKIGal4* also reduced nighttime sleep duration (Figure 4, E), suggesting that nAChRα2 in octopaminergic cells was necessary for proper sleep duration. Furthermore, RNAi knockdown of *TβH* in nAChRα2-expressing cells reduced nighttime sleep duration (Figure 4, F), indicating that octopamine in α2-expressing cells was also necessary for sleep.

Because *nAChRα2* probably functions with *nAChRβ2* to promote sleep, we then tested whether *nAChRβ2* also functions in octopaminergic neurons. We labeled the colocalization neurons of *TβH* and *nAChRβ2* using split-LexA strategy [83], and found that *TβH* and *nAChRβ2* colocalized in multiple brain regions and the VNC (Figure 4, G and H). Knockdown of *nAChRβ2* in octopaminergic cells also significantly reduced nighttime sleep duration (Figure 4, I), indicating that *nAChRβ2* also functions in octopaminergic neurons to promote sleep. Also, knockdown of TβH in nAChRβ2-expressing cells significantly reduced nighttime sleep duration (Figure 4, J), suggesting that octopamine in β2-expressing cells was necessary for sleep.

Taken together, these data suggest that *nAChRα2* and β2 function in octopaminergic neurons to promote sleep, most likely through octopaminergic signaling.

nAChRα3 functions in dopaminergic neurons to promote arousal

Our mini-screen revealed that *nAChRα3KO* flies are hard to be awakened from sleep by exogenous stimuli (Figure 2), indicating an arousal-promoting role of *nAChRα3*. To examine the expression of arousal-promoting *nAChRα3*, we fused *2A-Gal4* in-frame to *nAChRα3*, making the *α3KIGal4* line (Figure S2, B). It was then used to drive UAS lines expressing mCD8::GFP, stinger::GFP, Syt::GFP, and DenMark to label the membrane, the nuclei, the axons and the dendrites of *nAChRα3*-expressing neurons, respectively. *α3KIGal4* was expressed in the membrane of neurons in the SOG, the MBs, the superior neuropils (SNP), and the ventrolateral neuropils (VLNP), and in the MN, the MtN and the AC of the VNC (Figure 5, A–C). Nuclei and axons were labeled in brain regions similar to that of the membrane by *α3KIGal4* (Figure S6, J and K). Dendrites in the SOG, the VLNP, and the accessory medulla (AME) were labeled by *α3KIGal4* (Figure S6, L).

The arousal rate of $\alpha3^{-/-}$ was fully rescued by reintroduction of *nAChRα3* into *nAChRα3*-expressing cells (Figure 5, G) with *UAS-nAChRα3* driven by *α3KOGal4* in which the translation of *nAChRα3* was interrupted by *2A-Gal4-STOP* (Figure S2, B), proving that the arousal phenotype of $\alpha3^{-/-}$ was caused by the deletion of *nAChRα3*.

Dopamine, dopamine receptor, and dopaminergic neurons are known to promote endogenous wakefulness and exogenous stimuli-induced arousal [32, 33, 78, 79, 84, 85]. To test the possibility of nAChRα3 promoting exogenous stimuli-induced arousal in dopaminergic neurons, we first checked whether nAChRα3 is expressed in the dopaminergic neurons labeled by the synthase of dopamine, tyrosine hydroxylase (TH). We used the split-LexA strategy [83] to detect overlaps in *nAChRα3* and *TH* expression, and found that nAChRα3 and TH were colocalized in multiple regions in the CNS (Figure 5, D–F), including the optical lobe, the MBs, the PB, the central complex (CC), the SOG, and the VNC. The colocalization of nAChRα3 and TH allowed nAChRα3 to signal through dopamine.

nAChRα3 was reintroduced into the *nAChRα3* and *TH* intersectional cells in $\alpha3^{-/-}$ background, and the arousal rate was partially rescued (Figure 5, H), suggesting that nAChRα3 in the α3-expressing dopaminergic neurons is sufficient to promote arousal. RNAi knockdown of *nAChRα3* in dopaminergic neurons driven by *THKIGal4* significantly reduced the arousal rate (Figure 5, I), indicating that nAChRα3 in the dopaminergic neurons is necessary for arousal promoting. Taken together, these data suggest that *nAChRα3* functions in dopaminergic neurons to promote arousal.

RNAi knockdown of *TH* in *nAChRα3*-expressing neurons also reduced the arousal rate significantly (Figure 5, J), indicating that dopamine in the *nAChRα3*-expressing neurons is necessary for arousal. We fed flies with the dopamine precursor, L-DOPA, to test whether *nAChRα3* functions through dopamine signals. L-DOPA rescued the arousal rate of $\alpha3^{-/-}$ to the level of $\alpha3^{+/+}$ flies fed with mock (Figure 5, K). Taken together, our results indicate that *nAChRα3* functions in dopaminergic neurons to promote arousal, by positively regulating the dopamine signaling.

Discussion

Sleep and arousal are two important processes regulated by multiple molecules. Although the cholinergic neurons have long been identified as a key modulator in sleep regulation in both mammals [49, 86–91] and flies [30, 50], the specific function of cholinergic neurons in sleep and arousal remains

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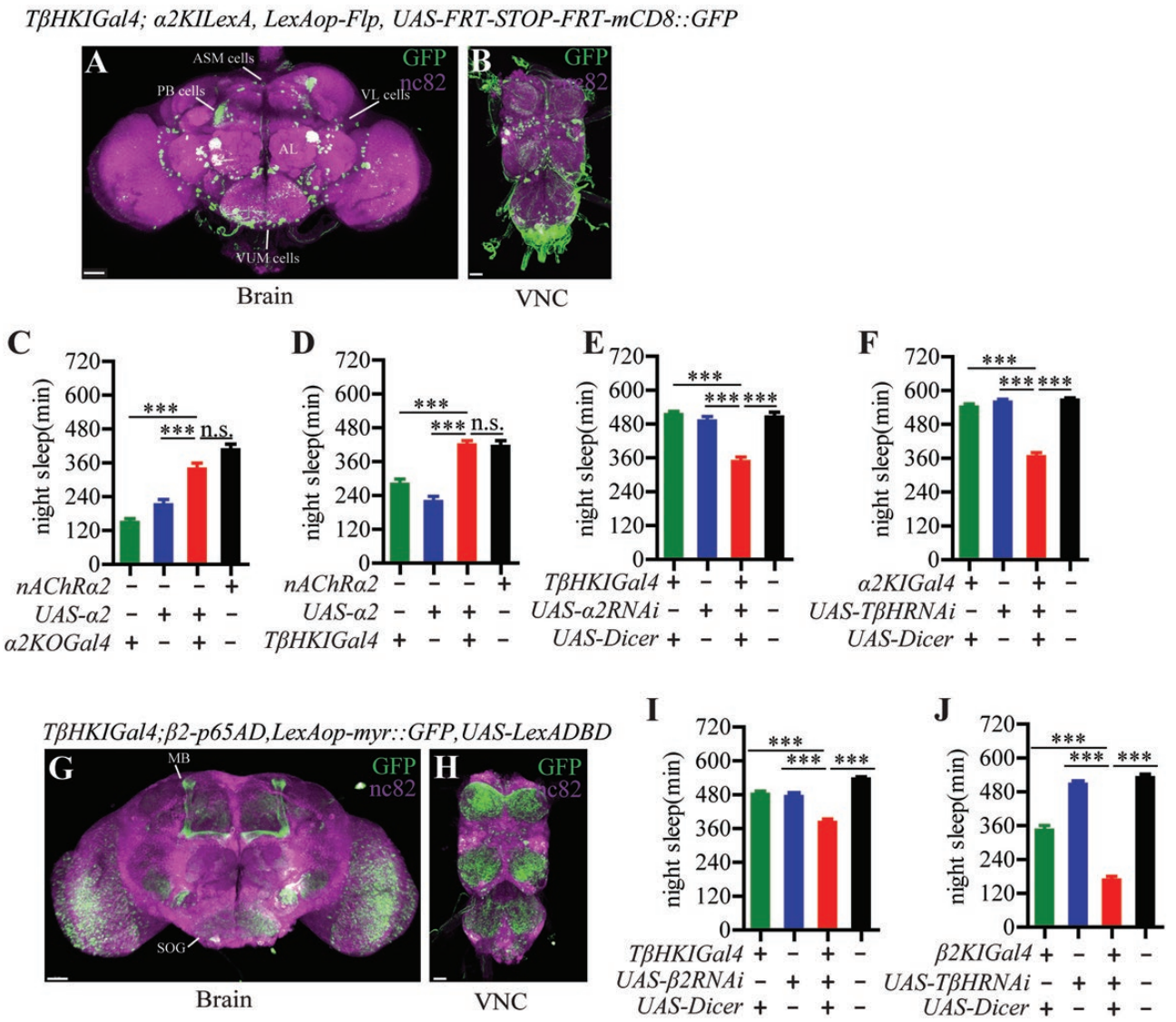

Figure 4. *nAChRα2* and β2 function in octopaminergic neurons to promote sleep. (A, B) Expression patterns of *TβHKIGal4/ α2KILexA, LexAop-Flp, UAS-FRT-STOP-FRT-mCD8::GFP* in the brain (A) and VNC (B). Colocalized neurons expressing *nAChRα2* and *TβH* were labeled with GFP, clusters are labeled according to Crocker et al. [37]. ASM, anterior superior medial protocerebrum; PB, protocerebral bridge; VL, ventrolateral protocerebrum; VUM, ventral unpaired median (VUM). (C) Reintroduction of *nAChRα2* into *nAChRα2*-expressing cells rescued nighttime sleep loss of *nAChRα2* mutants. Nighttime sleep durations of *α2KO/α2KOGal4* (green) (n = 48), *α2KO/α2KO, UAS-α2* (blue) (n = 46), *α2KOGal4/ α2KO, UAS-α2* (red) (n = 35), and *wt* (black) (n = 48) flies were plotted. (D) Reintroduction of *nAChRα2* in octopaminergic cells rescued the sleep defect of *nAChRα2* mutants. Nighttime sleep durations of *TβHKIGal4/+; α2KO/α2KO* (green) (n = 47), *α2KO/α2KO, UAS-α2* (blue) (n = 47), *TβHKIGal4/+; α2KO/α2KO, UAS-α2* (red) (n = 46), and *wt* (black) (n = 47) flies were plotted. (E) Knockdown of *nAChRα2* in octopaminergic cells (red) (n = 48) significantly reduced nighttime sleep duration compared to *TβHKIGal4/+* (green) (n = 36), *UAS-α2RNAi/+* (blue) (n = 46), and *wt* (n = 47). (F) Knockdown of *TβH* in *nAChRα2*-expressing cells (red) (n = 46) significantly reduced nighttime sleep duration compared to *α2KIGal4/+* (green) (n = 39), *UAS-TβHRNAi/+* (blue) (n = 44), and *wt* (n = 48). (G, H) Expression patterns of *TβHKIGal4; β2-p65AD, LexAop-myr::GFP, UAS-LexADBD*. Neurons coexpressing nAChRβ2 and TβH were labeled with GFP in the brain (G) and VNC (H). (I) Knockdown of *nAChRβ2* in octopaminergic cells (red) (n = 44) significantly reduced nighttime sleep duration compared to *TβHKIGal4/+* (green) (n = 47), *UAS-β2RNAi/+* (blue) (n = 46), and *wt* (n = 47). (J) Knockdown of *TβH* in *nAChRβ2*-expressing cells (red) (n = 48) significantly reduced nighttime sleep duration compared to *β2KIGal4/+* (green) (n = 48), *UAS-TβHRNAi/+* (blue) (n = 47), and *wt* (n = 48). ***$p < 0.001$, n.s., $p > 0.05$. Kruskal–Wallis test with Dunn's posttest. Error bars represent SEM. The tissues were stained with the anti-GFP (green) and nc82 (magenta). Scale bars are 30 μm. Female flies were used.

controversial [92–94], and little is known about the AChRs' role in sleep regulation. Cholinergic neurons in different brain regions have been found to promote wakefulness, REM sleep, or NREM sleep. Genome-wide association studies (GWAS) in humans have found associations of nAChRs with sleep disorders [95, 96]. Muscrinic AChRs have recently been found to be essential for REM sleep in mice [97]. A recent chemical genetic screen in flies has implicated *nAChRα4* in sleep [21], and another study studying mechanisms downstream of the *sleepless* (*sss*) gene has found that *sss* promotes sleep through inhibition of nAChRα3 in flies [98]. But the specific roles of AChRs in sleep regulation and their mechanisms are still largely unknown.

Here, through a systematic genetic investigation and further intersection and genetic studies of 13 ACh receptors, we have found that *nAChRα3* promotes arousal in dopaminergic neurons and *nAChRα2* and β2 promote sleep in octopaminergic neurons in *D. melanogaster*, providing an insight into the molecular mechanisms of sleep regulation by ACh (Figure 5, L).

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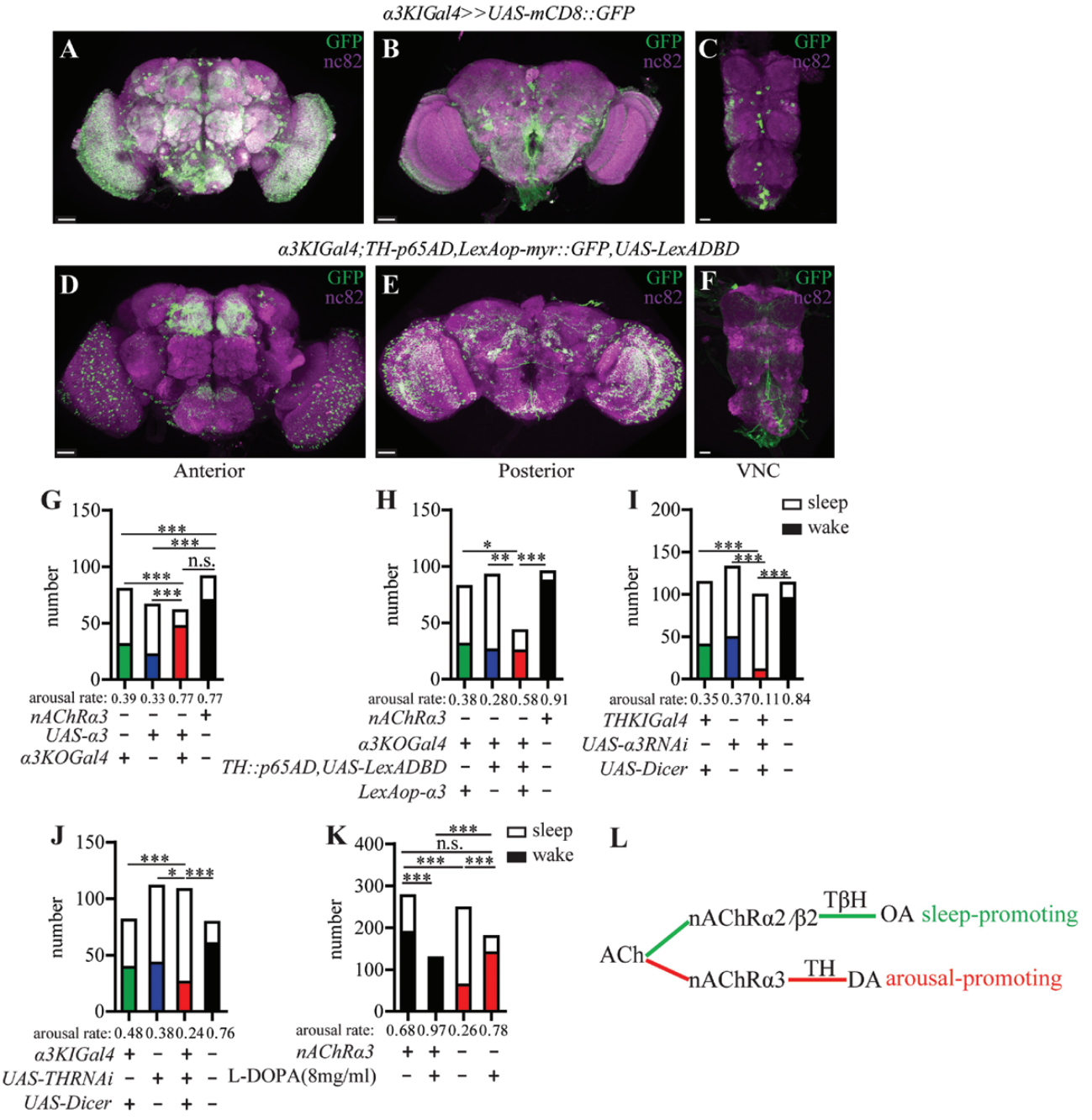

Figure 5. *nAChRα3* functions in dopaminergic neurons to promote arousal. (A–C) Expression patterns of *α3KIGal4* labeled by mCD8::GFP in the brain (A, anterior view; B, posterior view) and the VNC (C). (D–F) Expression patterns of *α3KIGal4; TH-p65AD, LexAop-myr::GFP, UAS-LexADBD*. Neurons co-expressing *nAChRα3* and *TH* were labeled with GFP in the brain (D, anterior view; E, posterior view) and the VNC (F). (G) Reintroduction of *nAChRα3* in *nAChRα3*-expressing cells rescued the arousal rate of $\alpha3^{-/-}$. $Number_{wake}$ (filled bars) and $Number_{sleep}$ (open bars) were plotted for *α3KO/α3KOGal4* ($n = 80$) (green), *α3KO/α3KO; UAS-α3/+* ($n = 66$) (blue), *α3KO/α3KOGal4;UAS-α3/+* ($n = 61$) (red), and *wt* ($n = 91$) (black) flies. Arousal rate was denoted under each bar. (H) Reintroduction of *nAChRα3* in nAChRα3$^+$ TH$^+$ cells rescued the arousal rate of *nAChRα3* mutants. $Number_{wake}$ and $Number_{sleep}$ were plotted for *α3KO/α3KOGal4; LexAop-α3/+* ($n = 82$) (green), *α3KO/α3KOGal4; TH-p65AD, UAS-LexADBD/+* ($n = 92$) (blue), *α3KO/α3KOGal4; TH-p65AD, UAS-LexADBD/ LexAop-α3* ($n = 43$) (red), and *wt* ($n = 96$) (black) flies. (I) Knockdown of *nAChRα3* in dopaminergic cells resulted in reduced arousal rate. $Number_{wake}$ and $Number_{sleep}$ were plotted for *UAS-Dicer/+; THKIGal4/+* ($n = 114$) (green), *UAS-α3RNAi/+* ($n = 132$) (blue), *UAS-Dicer/+; THKIGal4/ UAS-α3RNAi* ($n = 99$) (red), and *wt* ($n = 113$) (black) flies. (J) Knockdown of *TH* in *nAChRα3*-expressing cells resulted in reduced arousal rate. $Number_{wake}$ and $Number_{sleep}$ were plotted for *UAS-Dicer/+;α3KIGal4/+* ($n = 81$) (green), *UAS-THRNAi/+* ($n = 111$) (blue), *UAS-Dicer/+;α3KIGal4/ UAS-THRNAi* ($n = 108$) (red), and *wt* ($n = 79$) (black) flies. (K) Oral administration of L-DOPA rescued the arousal rate of $\alpha3^{-/-}$. $Number_{wake}$ and $Number_{sleep}$ were plotted for $\alpha3^{+/+}$ flies fed with DMSO (276), $\alpha3^{+/+}$ flies fed with L-DOPA ($n = 128$), $\alpha3^{-/-}$ flies fed with DMSO ($n = 247$) and $\alpha3^{-/-}$ flies fed with L-DOPA ($n = 179$) (from left to right bars). (L) Summary of nAChRs' roles in sleep regulation. *nAChRα2/β2* promote sleep in octopaminergic (OA) neurons, probably through octopaminergic signaling (green), *nAChRα3* promotes exogenous stimuli-induced arousal in dopaminergic (DA) neurons, probably through dopaminergic signaling (red). The tissues were stained with anti-GFP (green) and nc82 (magenta). Scale bars are 30 μm. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, n.s. $p > 0.05$, Fisher's exact test. Female flies were used.

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In mammals, nAChRs have been found to modulate norepinephrine (NE) release [99, 100]. Norepinephrinergic neurons in the locus coeruleus (LC) are part of the ascending arousal system [25]. LC neurons are most active during wakefulness, less active during REM sleep, and inactive during NREM sleep [101]. NE levels in multiple brain regions are high in wakefulness and low in sleep in mice, rats, and cats [102–105]. Administration of antagonists of Gq-coupled NE receptors and agonists of Gi-coupled NE receptors had the sedative effect in rat [106, 107], and blocked the sleep–wake switch induced by activation of LC neurons in mice [108]. Mice deficient in dopamine β-hydroxylase ($D\beta h^{-/-}$), the synthase of NE, showed conflicting sleep phenotypes: one group found reduced latency to sleep, but no significant change in sleep in $D\beta h^{-/-}$ mice [109], but another group found reduced wakefulness and REM sleep duration in $D\beta h^{-/-}$ mice [110]. Because NE is synthesized from dopamine by Dβh in mammals, $D\beta h^{-/-}$ mice not only had reduced NE, but also elevated dopamine level [111]. Given that dopamine is also an important sleep regulator, the sleep phenotypes of $D\beta h^{-/-}$ mice could result from changes in both NE and dopamine.

Octopamine is insect equivalent of norepinephrine in mammals. Octopamine is synthesized from tyramine by *TβH* in flies, thus the function of octopamine and dopamine on sleep could be separated. The role of octopamine in sleep regulation in flies is elusive. Currently, five octopamine receptors have been identified in flies: OAMB, Oa2(Octβ1R), Octβ2R, Octβ3R, and Oct-TyrR (which could bind both octopamine and tyramine) [112]. Previous studies found that sleep duration was increased in *TβH* mutant flies [81] and *OAMB* receptor mutants [37] using the *Drosophila* Activity Monitor (DAM)-based method, suggesting a wake-promoting role of octopamine. However, a recent study from our lab has found that sleep durations were decreased to approximately half of that of wt flies in *TβH* mutant flies and *Octβ2R* receptor mutant flies [51] using the video-based method, indicating a sleep-promoting role of octopamine. As demonstrated in our recent study, the conflict results are probably due to different methods of sleep measurement: when video-based data were reanalyzed using the DAM-based method, *TβH* mutant and *Octβ2R* mutant showed increased sleep as previous studies [51]. Because DAM-based method measures the fly activity by counting the times fly crossing the infrared beam set at the center of the tube, flies that are moving without crossing the beam would be lost, resulting in an increased sleep duration compared with our video-based method. Here, we found that *nAChRα2* functions in octopaminergic neurons to promote sleep, probably through octopaminergic signaling, added evidence to octopamine promoting sleep and provided a candidate upstream circuit for it. It was previously reported that nicotine stimulation-induced octopamine release through nAChRs in the fly brain [113], providing a basis for *nAChRα2* to promote sleep through octopamine.

nAChRα3 was found to act downstream of *sss* [98]. Knockdown of *nAChRα3* could restore the sleep duration of *sss* mutants, but no effect was found when *nAChRα3* was knocked down in *wt* flies, making the role of *nAChRα3* unclear [98]. Here, we found that the environment-induced arousal of *nAChRα3KO* flies was reduced, but the sleep durations were not different from the wt, suggesting that *nAChRα3* mediates exogenous stimuli-induced arousal. Groups of cholinergic neurons have been found to promote sleep homeostasis [49]. After sleep deprivation, *nAChRα3KO* flies showed reduced sleep rebound rate, suggesting that *nAChRα3* may mediate ACh's regulation of sleep homeostasis.

We found that *nAChRα3* promotes the exogenous stimuli-induced arousal in dopaminergic neurons, probably through dopaminergic signaling. Previous studies have suggested distinct mechanisms between endogenous awake and exogenous stimuli-induced arousal [78, 79]. In flies, genetic and pharmacological studies have implicated dopamine in wake promotion [84, 85]. Specific dopaminergic neurons have been identified to promote wakefulness [32, 33, 114], and the dopamine receptor Dop1R1 is important in both endogenous awake and exogenous arousal [78, 79], indicating that dopamine could regulate both endogenous and exogenous arousal. Dopaminergic neurons have been found to be activated by nicotine perfusion in the MBs, the EB, and the FB of the fly brain [115], and dopamine release has also been found to be modulated by nAChRs in mammals [100, 116, 117], providing a basis for *nAChRα3* to function through dopamine.

Single point mutation of *nAChRα4* at the junction between the LBD and the TMD was found to cause decreased sleep [21], but $nAChR\alpha4^{-/-}$ flies generated in our study with LBD/TMD deleted in the coding region showed normal sleep duration (Figure S3, D). $nAChR\alpha4^{-/-}$ flies in our study have increased mortality, and most of them died in 3 days after eclosion, which might affect sleep. Also, this conflict with the previous study could be due to different genotypes of our mutant and previous study, point mutation and CDS deletion probably have different effects on nAChRα4 activity. In this case, the discrepancy of sleep phenotypes between these different nAChRα4 mutants suggests a delicate regulation of sleep by AChR. Besides nAChRα2, β2, and α3, we also identified other ACh receptor mutants that have moderate effects on sleep duration (Figure S3) and exogenous stimuli-induced arousal (Figure S5), future studies investigating the roles of these receptors would be interesting.

A recent study has investigated the sleep phenotypes of AChR mutant mice generated by triple-target CRISPR method [97]. They found that nAChRs play relatively minor roles in sleep regulation, while mAChRs are key to REM sleep: *Chrnb2KO* and *Chrnb3KO* mice showed moderately decreased sleep duration, and *Chrm1/Chrm3* doubleKO mice showed reduced NREM duration and completely REM loss. In contrast, our study in flies revealed a major role of nAChR but not mAChR in sleep regulation, with the mAChR mutants showing normal sleep durations (Figure S3, J–L). One possibility is that the sleep defect in nAChR mutant mice might be compensated. Although NREM- and REM-like sleep haven't been identified in flies, local field potential recording studies have found that sleep in flies also consists of various stages [8]. Whether mAChRs in flies also participate in the regulation of specific sleep stages would be of further interest.

Through a systematic investigation followed with further genetic dissection and pharmacological studies, we found that acetylcholine, octopamine, and dopamine are connected in sleep regulation, different nAChRs function in different types of neurons to promote sleep and exogenous stimuli-induced arousal respectively, indicated that sleep and exogenous stimuli-induced arousal are regulated by distinct molecules and circuits. Our results provide insights into the complicated roles of ACh in endogenous sleep and exogenous stimuli-induced arousal, and shed light on the sleep regulation networks.

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