Different Effects of Hypnotics and Stimulants on Sleep Between Wild Type and Sleep-less Flies

【Article】

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Summary

We compared the effects of hypnotics (triazolam and pentobarbital) and stimulants (caffeine and methylphenidate) on sleep between wild-type and sleep-less mutant, *fumin* (*fmn*) flies, in which a mutation of dopamine transporter (DAT) gene was found (Kume et al. 2005). Triazolam at small doses had no effect on sleep and at large dose significantly decreased nighttime sleep in wild-type flies, compared with vehicle treatment, while in *fmn* flies triazolam significantly increased sleep in early periods at night. Pentobarbital increased sleep in both wild-type and *fmn* flies. Nighttime sleep was more enhanced by pentobarbital in *fmn* flies than in wild-type flies. Thus, the results by hypnotics obtained in *fmn* flies were more consistent with the results in insomnia patients than those

obtained in wild-type flies. Caffeine decreased sleep in both wild-type and *fmn* flies. *Fmn* flies were more sensitive to caffeine than wild-type flies. Methylphenidate significantly decreased daytime sleep in wild-type flies, whereas in *fmn* flies methylphenidate dose-dependently and significantly increased sleep. Since both triazolam and pentobarbital exhibit their hypnotic actions through GABA receptors, the present study suggests that GABAergic mechanisms are altered in *fmn* flies and contribute more effectively to wake-sleep cycles in *fmn* flies than in wild-type flies. Methylphenidate exhibited sleep-promoting effects on *fmn* flies, presumably by other actions than inhibiting DAT, because *fmn* fly has a mutation of DAT gene. Thus, using flies provide researching tool for effects of hypnotics and stimulants on rest and wake in animals with sleep problems.

I. Introduction

Drugs are usually used in humans who suffer from diseases. Since patients stand at pathophysiological background different from healthy conditions, effects of drugs on the patients may be different from those on the healthy individuals. It is, therefore, very important to know whether there are any differences in the effects of drugs between healthy individuals and subjects with diseases.

Drosophila melanogaster, with its accessibility to genetic analysis, has been used as model systems for studying molecular and cellular mechanisms of human diseases, and for discovering therapeutic drugs of human diseases (O'Kane, 2003 ; Pandey and Nichols, 2011). Since pathophysiological states associated with human diseases were caused in *Drosophila melanogaster* by genetic manipulations, *Drosophila melanogaster* is an attractive model system to compare pharmacological effects of drugs on animals between normal and pathophysiological states (Tabuchi et al., 2018).

Two groups of researchers provided the conclusive proof that *Drosophila* sleep shares all the fundamental features of mammalian sleep (Hendricks et al. 2000 ; Shaw et al. 2000). Recently, Kume et al. (2005) found the *Drosophila* sleep-less mutant called *fumin* (*fmn*) and demonstrated that the behavioral phenotype was caused by a genetic lesion in the dopamine transporter (DAT) gene (Porzgen et al., 2001). The analysis of *fmn* suggests a role for dopamine in the modulation of insect arousal and highlights a similarity between insects and mammals regarding the molecular basis of arousal.

In the present study, we compared the effects of hypnotics and stimulants on sleep between wild-type and sleep-less mutant, *fmn*, flies. We found that two hypnotics, triazolam (Pakes et al., 1981) and pentobarbital (Lopez-Muoz et al., 2005) exhibited more potent hypnotic effects on *fmn* flies than on wild-type flies. Caffeine decreased sleep in both wild-type and *fmn* flies. In contrast, methyl-

phenidate decreased sleep in wild-type flies, while it increased sleep in *fmn* flies. Although caffeine and methylphenidate showed opposite effects on sleep in *fmn* flies, the effects of two drugs on sleep in *fmn* flies were consistent with those in DAT-knockout mice (Wisor, 2001). Thus, the present study validates the use of Drosophila as a model system for studies into pharmacological therapeutics of human insomnia.

II. Materials and methods

2.1. Fly stock

Wild-type, Canton-S and sleepless mutant, *fmn* (Kume et al., 2005) flies were reared at 25 C and 50-60% humidity, on a 12 h light/12 h dark cycle, and on a conventional corn meal, yeast meal. The light was put on at 8 in the morning (Zeitgeber time ; ZT 0) and off at 20 in the evening (ZT 12) on every day. *fmn* flies were kindly provided by K. Kume (Kumamoto University, Kumamoto, Japan).

2.2. Behavioral assays

Sleep behaviors were measured using *Drosophila* Activity Monitoring System (DAMS) (Trikinetics, Waltham, MA, USA) in glass tubes (6.5 cm X 0.4 cm) maintained in a well humidified incubator (Yamato, IN61, Japan) at 25C. To exclude unhealthy flies, we chose only flies which climbed at 9 cm high in response to negative geotaxis. Male flies were selected under carbon dioxide anesthesia and maintained for a period of 5 days. Each glass tube was connected with a short silicon tube (1.5 cm long and 0.5 cm in diameter), which contained 5% sucrose/2% agar. Sleep was identified as a minimum of 5 min of locomotor inactivity as described previously (Hendricks., et al. 2000 ; Shaw et al. 2000). Unless otherwise specified, flies used in behavioral experiments were pre-entrained for 2 d in a 5% sucrose/2% agar and then treated with drugs for additional 2 days in 12/12 h light/dark (L/D) cycle. *fmn* flies, which showed more than 50% sleep during nighttime at pre-entrained period, were excluded. Male flies were 5-8 d-old at the start of the behavioral experiments. Data was collected using TriKinetics software and analyzed in Excel (Microsoft, Redmond, WA, USA). The light was put on at 8 in the morning (ZT 0) and off at 20 in the evening (ZT 12). Total sleep (%), nighttime sleep (%) and daytime sleep (%) in wild-type or *fmn* flies are the fraction of 5 min intervals in 24 h time period (between ZT 12-12) with no activity, 12 h time period (between ZT 12 and 0) with no activity and 12 h time period (between ZT 0 and 12) with no activity, respectively. Percentage change from baseline sleep at total time, nighttime and daytime in experiment was calculated within

the same individual flies as [(sleep (%) in experiment – sleep (%) in baseline)/sleep (%) in baseline] x 100 and effects of drugs on the sleep were evaluated by comparing the percentage changes after drug treatment with those after vehicle treatment in total, nighttime and daytime sleep (Fig. 1A).

2.3. Stimulant treatment

We could control access to stimulant substrate by changing the silicon tube connected with glass tubes (Fig. 1B). Stimulants were mixed in 5% sucrose/2% agar solution. For pentobarbital, caffeine and methylphenidate treatment, after pre-entrainment, the silicon tubes were changed in daytime and flies were fed in 5% sucrose/2% agar containing either solvent only or stimulants. For triazolam treatment, flies were treated as for above stimulants, except that they were fed triazolam in doses ranging from 0.5 to 2 mg/ml in 1% ethanol, and corresponding control flies were fed 1% ethanol.

2.4. Stimulants

Stimulants used were triazolam (Wako, Osaka, Japan), methylphenidate hydrochloride (Sigma), pentobarbital sodium salt (Tokyo Kasei Kogyo, Tokyo, Japan) and caffeine (Wako, Osaka, Japan). Triazolam was solved in ethanol. Methylphenidate hydrochloride, pentobarbital sodium salt and caffeine were solved in H_2O.

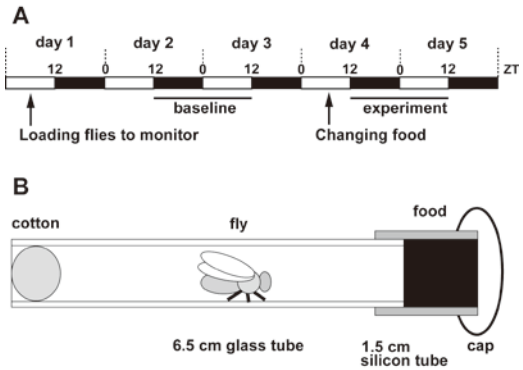

Fig. 1. Simple method quantifying drug-effects on sleep. (A) Paradigm for measuring the effects of drugs on sleep. Experiments took place over 5-day period after the loading flies to standard DAM tubes. Baseline (sleep before treatment) : all groups were kept on standard food (sucrose 5%/ 2% agar). Experiment (sleep after treatment) : the food was switched at daytime on 4 days to either vehicle food (contained the same concentration of solvent used for the drugs) or standard food containing drug, and the flies were fed for additional 2 days. (B) An individual fly was housed in each glass tube (6.5 cm × 0.4 cm) connected with short silicon tube (1.5 cm × 0.5 cm) containing food, sealed with dome cap for PCR. The opposing end of glass tube was sealed with cotton wool plug to allow for air transfer.

2.5. Data analysis and Statistics

Values shown are the mean±S.E.M. The Student's *t* –test was used for statistical analysis of two groups (directional evaluation was used) and for comparisons of more than two doses, one-way ANOVAs with dose as a between-subject factor were used, and if there was a significant effect, *post hoc* comparison with Tukey honestly significant differences were performed. The statistical software used is the Origin 8 (OriginLab Corporation, Northampton, MA 01060, USA).

III. Results

3.1. Characteristics of sleep in wild-type and sleep-less mutant, fmn, flies, and methods quantifying stimulant effects on sleep

When sleep patterns of flies were compared between wild-type and *fmn* flies, sleep in *fmn* flies was markedly reduced (Figs. 2 and 4, compare sleep profiles before Stimulant treatment between wild-type and *fmn* flies), as reported by Kume et al (2005). Especially, nighttime sleep (%) in *fmn* flies

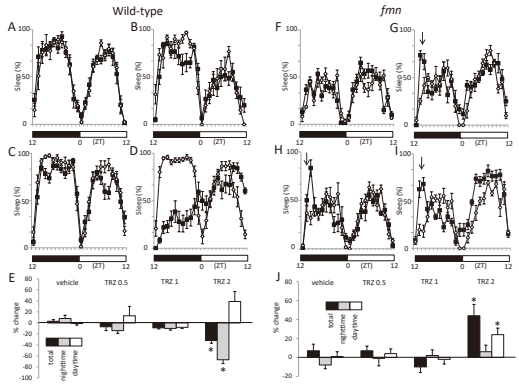

Fig. 2. Effects of triazolam (TRZ) on sleep in wild-type (A-E) and *fmn* (F-J) flies. Conventional sleep plot of wild-type (A-D) and *fmn* (F-I) flies showing sleep in a 12-h dark (nighttime)-light (daytime) cycle before (at nighttime on 2 day and at daytime on 3 day after loading flies to monitor, open diamonds) and after either vehicle or triazolam treatment (at nighttime on 4 day and at daytime on 5 day after loading flies to monitor, closed squares). Dark and white bars below each graph indicate nighttime and daytime, respectively, and time shows Zeitgeber time (ZT). Values (mean ±S.E.M) indicate sleep (%) per h of groups of flies fed with either vehicle or various doses of triazolam. A ; vehicle, n=11, wild-type flies, B ; 0.5 mg/mL triazolam, n=10, wild-type flies, C ; 1 mg/mL triazolam, n=14, wild-type flies, D ; 2 mg/mL triazolam, n=16, wild-type flies, F ; vehicle, n=10, *fmn* flies, G ; 0.5 mg/mL triazolam, n=10, *fmn* flies, H ; 1 mg/mL triazolam, n=10, *fmn* flies, I ; 2 mg/mL triazolam, n=12, *fmn* flies). Percentage change of total sleep (black columns), nighttime sleep (gray columns) and daytime sleep (white columns) from the corresponding baseline sleep show effects of vehicle and triazolam on total, nighttime and daytime sleep in wild-type (E) and *fmn* (J) flies. Values represent mean±S.E.M. * indicates $P<0.05$ compared to the corresponding vehicle treatment using one-way ANOVA factor 'triazolam dose' and Tukey *post hoc test*. Vehicle ; 0 mg/mL triazolam,, n=11 for wild-type flies and n=10 for *fmn* flies, TRZ 0.5 ; 0.5 mg/mL triazolam, n=10 for wild-type flies and n=10 for *fmn* flies, TRZ 1 ; 1 mg/mL triazolam, n=14 for wild-type flies and n=10 for *fmn* flies, TRZ 2 ; 2 mg/mL triazolam, n=16 for wild-type flies and n=12 for *fmn* flies.

was significantly decreased, compared with that in wild type flies (69.6±4.5%, n=16 in wild-type flies *vs* 31.5±2.8%, n=16 in *fmn* flies, Student's *t*-test, P<0.01). Daytime sleep (%) in *fmn* flies was also significantly decreased, compared with that in wild type flies (53.7±2.3%, n=16 in wild-type flies *vs* 35.5±2.6%, n=16 in *fmn* flies, Student's *t*-test, P<0.01,). In both wild-type and *fmn* flies, sleep at nighttime and daytime, and the timing of the major sleep phase vary significantly among individuals of the same fly population, even when age and housing conditions were kept constant. The same parameter in the same fly of both wild-type and *fmn* flies, however, were extremely consistent from one day to another. When we calculated the ratio of nighttime sleep on 4 days to that on 2 days within the same flies (Fig. 1A), the ratio value showed almost 1.00 (1.03±0.05, n=16 in wild-type flies and 0.94±0.04, n=16 in *fmn* flies). The ratio of daytime sleep on 5 days to that on 3 day (Fig. 1A) was also about 1.00 in the same flies (1.01±0.27, n=16 in wild-type flies and 1.13±0.06, n=16 in *fmn* flies). These results are consistent with those reported by Cirelli (Cirelli 2003) and require that stimulant effects on sleep should be evaluated by using the same flies.

To measure sleep in the same flies before and after drug treatment, we developed a simple method (Fig. 1B). We measured sleep (%) at nighttime and daytime in flies fed on 5% sucrose plus 2% agar at 12 h dark/light cycle for 3 days (Fig. 1A, baseline) and then fed on either sucrose 5%/2% agar or sucrose 5%/2% agar containing drugs for additional 2 days (Fig. 1A, experiment). The change of food was easily achieved by changing the short silicon tube, which contained either vehicle or drug-containing food, attached with monitor glass tube. To quantify drug effects on sleep, percentage change from baseline sleep at total time, nighttime and daytime in experiment was calculated within the same individual flies as [(sleep (%) in experiment - sleep (%) in baseline)/sleep (%) in baseline] x 100 (Fig. 1A). Effects of drugs on total, nighttime and daytime sleep were statistically evaluated by comparing percentage changes after drug treatment with those after vehicle treatment in corresponding sleep.

3.2. Effects of triazolam on sleep in wild-type and fmn flies

Triazolam is a short-acting hypnotic of benzodiazepine derivatives (Pakes et al., 1981). In wild-type flies, sleep profiles did not change after treatment with triazolam at 0.5 and 1 mg/mL (Figs. 2B and C). Percentage change from baseline sleep indicated that 0.5 and 1 mg/mL triazolam treatment had no effect on total sleep, compared with vehicle treatment (Fig. 2E, black columns with TRZ 0.5 and TRZ 1). After treatment with triazolam at 2 mg/mL, sleep decreased in wild-type flies (Fig. 2D). Percentage change from baseline sleep showed that triazolam treatment at 2 mg/mL had signif-

icant decreasing effect on total sleep, compared with vehicle treatment. ANOVA was used for comparison of more than two groups. If the ANOVA was significant ($P_{\text{degree of freedom}}$ and $P<0.05$), Tukey's test was used as a *post-hoc test* (ANOVA, $F_3=11.43$, $P=1.3\times10^{-6}$, Tuky's test, $P<0.05$). When total sleep was divided into nighttime sleep and daytime sleep, nighttime sleep was significantly decreased by 2 mg/mL triazolam treatment (Fig. 2E, grey column with TRZ 2 ; ANOVA, $F_3=34.82$, $P=1.0\times10^{-9}$, Tukeys test, $P<0.05$) but not daytime sleep, compared with vehicle treatment in the corresponding sleep.

In contrast to wild-type flies, sleep in *fmn* was enhanced by triazolam. Although percentage change from baseline sleep showed that 0.5 and 1 mg/mL triazolam treatment had no effects on total sleep (Figs. 3G and H), compared with vehicle treatment (Fig. 3J, black columns with TRZ 0.5 and TRZ 1), sleep in early period after dark was significantly enhanced by treatment with triazolam at 0.5 and 1 mg/mL (Figs. 3G and H, arrows). Vehicle treatment increased sleep during 2 hours after dark by 5.5±1.1 min/2 h (n=10) from baseline sleep. When compared with vehicle treatment, 0.5 mg/mL and 1 mg/mL triazolam treatment significantly increased sleep during 2 hours after dark by 22±1.7 min/2 h (n=10) and by 19 ± 1.7 min/2h (n=10) from baseline sleep, respectively (ANOVA, $F_3=11.21$, $P=0.026$, Tukey's test, $P<0.05$ for both treatment with triazolam at 0.5 and 1 mg/mL). At dose of 2 mg/mL, triazolam treatment significantly increased sleep during 2 hours after dark by 27.5±4.4 min/2 h from baseline sleep (Fig. 3I, n=12, Tukey's test, $P<0.05$), compared with vehicle treatment. Per-

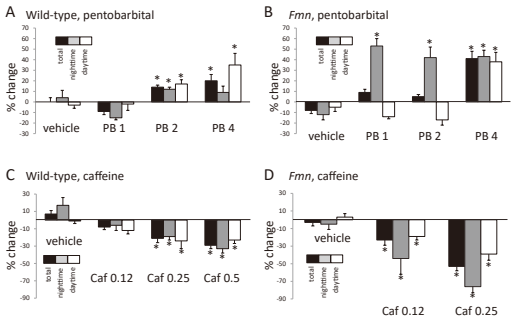

Fig. 3. Effects of pentobarbital (PB) and caffeine (Caf) on sleep in wild-type and *fmn* flies.
Percentage change of total sleep (black columns), nighttime sleep (gray columns) and daytime sleep (white columns) from the corresponding baseline sleep show effects of vehicle and pentobarbital on total, nighttime and daytime sleep in wild-type (A) and *fmn* (B) flies. Values represent mean±S.E.M. * indicates $P<0.05$ compared to the corresponding vehicle treatment using one-way ANOVA factor 'pentobarbital dose' and Tukey *post hoc test*. Effect of pentobarbital on sleep in wild-type flies (A). Vehicle ; 0 mg/mL pentobarbital, n=10, PB 1 ; 1 mg/mL pentobarbital, n=10, PB 2 ; 2 mg/mL pentobarbital, n=10, PB 4 ; 4 mg/mL pentobarbital, n=10. Effects of pentobarbital on sleep in *fmn* flies (B). Vehicle ; 0 mg/mL pentobarbital, n=10, PB 1 ; 1 mg/mL pentobarbital, n=10, PB 2 ; 2 mg/mL pentobarbital, n=10, PB 4 ; 4 mg/mL pentobarbital, n=10. Effects of caffeine on sleep in wild-type flies (C). Vehicle ; 0 mg/mL caffeine, n=5, Caf 0.12 ; 0.12 mg/mL caffeine, n=6, Caf 0.25 ; 0.25 mg/mL caffeine, n=11, Caf 0.5 ; 0.5 mg/mL caffeine, n=10. Effects of caffeine on sleep in *fmn* flies (D). Vehicle ; 0 mg/mL caffeine, n=6, Caf 0.12 ; 0.12 mg/mL caffeine, n=7, Caf 0.25 ; 0.25 mg/mL caffeine, n=12.

centage change from baseline sleep showed that treatment with triazolam at 2 mg/mL had significant increasing effects on total (Fig. 2J, black column with TRZ 2 ; ANOVA, F_3 =4.19, P=0.01, Tukey's test, P<0.05) and daytime (Fig. 2J, white column with TRZ 2 ; ANOVA, F_3 =7.22, P=7.87×10^{-4}, Tukey's test, P<0.05) sleep, compared with vehicle treatment in the corresponding sleep.

3.3. Effects of pentobarbital on sleep in wild-type and fmn flies

Pentobarbital has been used as both hypnotic and anesthetic drug (Lopez-Munoz et al., 2005). Drug effects on sleep were evaluated by percentage change from baseline sleep. In wild-type flies, percentage change from baseline sleep indicated that 1 mg/mL pentobarbital treatment had no effect on total, nighttime and daytime sleep, compared with vehicle treatment (Fig. 3A, black, grey and while columns with PB 1), but 2 and 4 mg/mL pentobarbital treatment had significant increasing effects on total sleep, compared with vehicle treatment (Fig. 3A, black columns with PB 2 and PB 4 ; ANOVA, F_3=13.18, P=8.41 × 10^{-5} and Tukey's test, P<0.05 for both pentobarbital at 2 and 4 mg/mL). Nighttime sleep was significantly increased by 2 mg/mL pentobarbital treatment (Fig. 3A, grey column with PB 2 ; ANOVA, F_3=8.30, P=0.037 and Tukey's test, P<0.05), but not 4 mg/mL pentobarbital treatment (Fig. 3A, grey column with PB 4), compared with vehicle treatment. Daytime sleep was significantly increased by 2 and 4 mg/mL pentobarbital treatment, compared with vehicle treatment (Fig. 3A, white columns with PB 2 and 4 ; ANOVA, F_3=6.88, P=2.26 × 10^{-4} and Tukey's test, P<0.05 for both pentobarbital at 2 and 4 mg/mL).

In *fmn* flies, percentage change from baseline sleep showed that 4 mg/mL pentobarbital treatment increased total sleep, compared with vehicle treatment (Fig. 3B, black column with PB 4 ; ANOVA, F_3=24.20, P=1.75 × 10^{-9} and Tukey's test, P<0.05). Nighttime sleep was significantly increased by 1, 2 and 4 mg/mL pentobarbital treatment in *fmn* flies, compared with vehicle treatment (Fig. 3B, grey columns with PB 1, PB 2 and PB 4 ; ANOVA, F_3=18.04, P=1.35 × 10^{-3} and Tukey's test, P<0.05 for pentobarbital at 1, 2 and 4 mg/mL). Daytime sleep was significantly increased by 4 mg/mL pentobarbital treatment in *fmn* flies, compared with vehicle treatment (Fig. 3B, white column with PB 4 ; ANOVA, F_3=6.88, P=2.26 × 10^{-4} and Tukey's test, P<0.05).

3.4. Effects of methylphenidate on sleep in wild-type and fmn flies

Methylphenidate is used for treatment of narcolepsy (Leonard et al., 2004). Methylphenidate at 0.5 and 1 mg/mL had no effect on sleep in wild-type flies (Figs. 4B and C). Methylphenidate at 2 mg/mL decreased sleep in wild-type flies (Fig. 4D). Percentage change from baseline sleep

showed that 2 mg/mL methylphenidate treatment had significant decreasing effect on total and on daytime sleep but not on nighttime sleep, compared with vehicle treatment (Figs. 4 E, black column with Ritalin 2 ; ANOVA, F_3 =7.50, P=1.53 × 10^{-3} and Tukey's test, $P<0.05$ and white column with Ritalin 2 ; ANOVA, F_3 =12.84, P=0.006 and Tukey's test, $P<0.05$).

In contrast to wild-type flies, methylphenidate at 0.5, 1 and 2 mg/mL increased sleep in *fmn* flies (Figs. 4G, H and I). Percentage change from baseline sleep showed that 1 and 2 mg/mL methylphenidate treatment had significant enhancing effect on total sleep in *fmn* flies, compared with vehicle treatment (Figs. 4J, black columns with Ritalin 1 and 2 ; ANOVA, F_3 =13.71, P=2.31 × 10^{-4} and Tukey's test, $P<0.05$ for both methtylphenidate at 1 and 2 mg/mL). Nighttime sleep was significantly increased by treatment with methylphenidate at 1 mg/mL, but not at 2 mg/mL, compared with vehicle treatment (Fig. 4J, grey column with Ritalin 1 ; ANOVA, F_3 =3.69, P=2.18 × 10^{-2} and Tukey's test, $P<0.05$). Daytime sleep was significantly increased by treatment with methylphenidate at 1 and 2 mg/mL, compared with vehicle treatment (Fig. 4J, white column with Ritalin 1 and 2 ; ANOVA, F_3 =17.36, P=2.61 × 10^{-4} and Tukey's test, $P<0.05$ for both methylphenidate at 1 and 2 mg/mL).

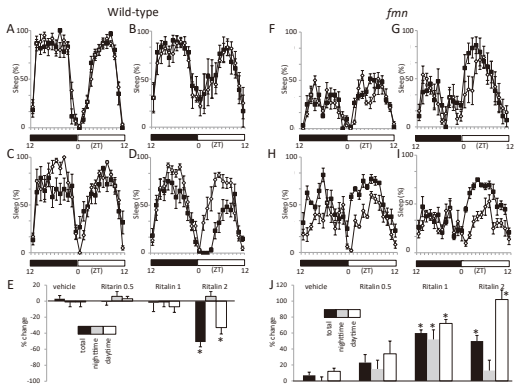

Fig. 4. Effects of methylphenidate (Ritalin) on sleep in wild-type (A-E) and *fmn* (F-J) flies. Conventional sleep plot of wild-type (A-D) and *fmn* (F-I) flies showing sleep in a 12-h dark (nighttime)-light (daytime) cycle before (at nighttime on 2 day and at daytime on 3 day after loading flies to monitor, open diamonds) and after either vehicle or methylphenidate treatment (at nighttime on 4 day and at daytime on 5 day after loading flies to monitor, closed squares). Dark and white bars below each graph indicate nighttime and daytime, respectively, and time shows Zeitgeber time (ZT). Values (mean±S.E.M) indicate sleep (%) per h of groups of flies fed with either vehicle or various doses of methylphenidate. A ; vehicle, n=10, wild-type flies, B ; 0.5 mg/mL methylphenidate, n=6, wild-type flies, C ; 1 mg/mL methylphenidate, n=10, wild-type flies, D ; 2 mg/mL methylphenidate, n=6, F ; vehicle, n=11, *fmn* flies, G ; 0.5 mg/mL methylphenidate, n=7, *fmn* flies, H ; 1 mg/mL methylphenidate, n=9, *fmn* flies, I ; 2 mg/mL methylphenidate, n=13, *fmn* flies. Percentage change of total sleep (black columns), nighttime sleep (gray columns) and daytime sleep (white columns) from the corresponding baseline sleep show effects of vehicle and methylphenidate on total, nighttime and daytime sleep in wild-type (E) and *fmn* (J) flies. Values represent mean±S.E.M. * indicates P<0.05 compared to the corresponding vehicle treatment using one-way ANOVA factor 'methylphenidate dose' and Tukey *post hoc test*. Vehicle ; 0 mg/mL methylphenidate, n=10 for wild-type flies and n=11 for *fmn* flies, Ritalin 0.5 ; 0.5 mg/mL methylphenidate, n=6 for wild-type flies and n=7 for *fmn* flies, Ritalin 1 ; 1 mg/mL methylphenidate, n=10 for wild-type flies and n=9 for *fmn* flies, Ritalin 2 ; 2 mg/mL methlphenidate, n=6 for wild-type flies and n=13 for *fmn* flies.

3.5. Effects of caffeine on sleep in wild-type and fmn flies

As previously reported (Hendricks et al., 2000, Shaw et al., 2000, Wu et al., 2009), caffeine decreased sleep in wild-type flies. Percentage change from baseline sleep showed that treatment with caffeine at 0.25 and 0.5 mg/mL had significant decreasing effects on total sleep (Fig. 3C, black columns with Caf 0.25 and Caf 0.5 ; ANOVA, F_3 =11.08, P=1.23 × 10^{-3} and Tukey's test, P<0.05 for both caffeine at 0.25 and 0.5 mg/mL), nighttime sleep (Fig. 3C, grey columns with Caf 0.25 and Caf 0.5; ANOVA, F_3 =12.26, P=7.52 × 10^{-3} and Tukey's test, P<0.05 for both caffeine at 0.25 and 0.5 mg/mL) and daytime sleep (white columns with Caf 0.25 and Caf 0.5 ; ANOVA, F_3 =19.44, P=0.01 and Tukey's test, P<0.05 for both caffeine at 0.25 and 0.5 mg/mL), compared to vehicle treatment.

In *fmn* flies, percentage change from baseline sleep showed that treatment with caffeine at 0.12 and 0.25 mg/mL significantly increased total sleep (Fig. 3D, black columns with Caf 0.12 and Caf 0.25 ; ANOVA, F_3 =87.28, P=4.5 × 10^{-2} and Tukey's test, P<0.05 for both caffeine at 0.12 and 0.25 mg/mL), nighttime sleep (Fig. 3D, grey columns with Caf 0.12 and 0.25 ; ANOVA, F_3 =41.40, P=1.59 × 10^{-5} and Tukey's test, P<0.05 for both caffeine at 0.12 and 0.25 mg/mL) and daytime sleep (Fig. 3D, white columns with Caf 0.12 and Ca 0.25 ; ANOVA, F_3 =12.05, P=1.43 × 10^{-2} and Tukey's test, P<0.05 for 0.12 and 0.25 mg/mL), compared to vehicle treatment. 6 flies of 8 *fmn* flies died within 3 days after treatment with caffeine at 0.5 mg/mL. These data were excluded in the present analysis for sleep.

IV. Discussion

The effects of triazolam and pentobarbital on sleep in sleep-less flies, *fmn*, were more consistent with the results in insomnia patients than those in wild-type flies. Triazolam at small doses in the present study had significant increasing effects on sleep in early periods after dark in *fmn* flies, while in wild-type flies triazolam at small doses had no effects on sleep, compared with vehicle treatment. The effects of triazolam at small doses observed in *fmn* flies is consistent with that small dose of triazolam hastens sleep onset in insomnia patients and its hypnotic effect is short at doses clinically used (Pakes et al., 1981 ; Jonas et al, 1992 ; Mendelson and Jain, 1995). At large dose, triazolam increased total sleep in *fmn* flies, but decreased it in wild-type flies. The effect of triazolam observed in wild-type flies may be unexpected one because benzodiazepine derivatives are widely used in the treatments of multiple neurological disorders, such as sleep disturbance and epilepsy, and in the inducing sedation. However, there were clinical reports that triazolam induced hallucinations and delirium

in healthy humans (Van der Kroef, 1979 ; Goodchild and Donaldson, 2005). Pentobarbital at small dose increased nighttime sleep in *fmn* flies, whereas in wild-type flies it increased both nighttime sleep and daytime sleep. Although nighttime sleep in flies remains identical with sleep at night in humans, this result suggests that pentobarbital had significant enhancing effects on sleep during dark (at night) in *fmn* flies. This result is compatible with that low-dose of pentobarbital is used as hypnotics, drugs which increase sleep during night, but not as anesthetics in humans (Lopez-Munoz et al., 2005 ; Van der Kroef C, 1979 ; Pakes et al., 1981).

Why triazolam and pentobarbital exhibited more sleep-promoting effects in *fmn* flies than in wild-type flies? Dopamine is implicated in the arousal system of *Drosophila* as mammals (Isaac and Berridge, 2003 ; Nishino et al., 1998 ; Wisor et al., 2001 ; Lu et al, 2006 ; Andretic R et al. 2005 ; Kume et al., 2005). A mutation of DAT gene was found in *fmn* flies and resulted in high level dopamine and reduced sleep (Kume et al., 2005). On the other hand, GABA systems are involved in promotion of sleep-in flies as in mammals (Harrison, 2007 ; Agosto et al, 2008). Triazolam and pentobarbital exhibit their sleep-promoting actions through increasing GABA receptor efficacy by changing allosteric structures (Burt, 2003 ; Sieghart, 2006). Above evidence suggest that GABA receptor functions are altered in *fmn* flies. If so, these alterations may be induced through dopamine-GABA interactions, resulted in more effective contribution of GABA systems to sleep-wake behavior in *fmn* flies than in wild flies. GABAergic systems were affected by changes of dopamine signaling during development. When zebrafishes were treated with dopamine receptor stimulants such as dopamine, quinpirole and MPTP during development, significant changes in the GABAergic neuron population were observed throughout the brain (Souza et al., 2011). When both dopaminergic D1 and D3 receptor levels were reduced in the knockout mice, GABA binding was significantly increased (Wong et al., 2003). It is likely that dopamine receptors would be downregulated in *fmn* flies by high concentration of dopamine at synapse due to DAT mutation.

Both caffeine and methylphenidate decreased sleep in wild-type flies as in mammals (Yanik et al., 1987 ; Wu et al., 2009). Caffeine dose-dependently decreased sleep in *fmn* flies as in wild-type flies. *fmn* flies were more sensitive to caffeine and died when fed at 0.5 mg/mL, while wild-type flies were still alive. This result is consistent with the results obtained in *Drosophila* (Andretic et al., 2008) and mammals (Wisor et.al. 2001). *fmn* flies and DAT-knockout mice were grossly hypersensitive to the effects of caffeine (Andretic et al., 2008 ; Wisor et.al. 2001). In contrast, the response of *fmn* flies to methylphenidate, a DAT inhibitor (Makos et al., 2010), was distinct from the response to caffeine. Although methylphenidate at 2 mg/mL significantly decreased total sleep in wild-type flies

as in mammals (Leonard et al., 2004), methylphenidate at 1 and 2 mg/mL significantly increased total sleep in *fmn* flies, compared with vehicle treatment. The results of methylphenidate on sleep in *fmn* flies were unexpected. However, as observed in *fmn* flies in the present study, a decrease in wake state was observed in DAT-knockout mice after treatment with GBR12909 and modafinil, which are considered as DAT inhibitors, when changes in wake state were compared with the base line (Wisor et al., 2001). The clearance of exogenously applied dopamine was inhibited following treatment with methylphenidate, cocaine, amphetamine and methamphetamine in wild-type flies, but these drug-induced inhibition was not observed in *fmn* flies (Makos et al., 2010), indicating that the action of methylphenidate inhibiting DAT was lack in *fmn* flies. The enhancing effects on sleep by methylphenidate observed in *fmn* flies are, therefore, considered as being induced by the other action than inhibiting DAT. It is possible that methylphenidate or modafinil would have unknown sleep-promoting actions in addition to a wake-promoting actions by blockade of DAT. Methylphenidate and modafinil may be reconsidered for treatment of insomnia for which dysfunction of DAT is responsible.

Thus, the present study validates the use of Drosophila as a model system for studies into comparison of effects of drugs on normal and pathophysiological animals and into pharmacological therapeutics of humans with sleep diseases.

V. Conclusions

The effects of two hypnotics, triazolam and pentobarbital, on sleep in sleep-less flies, *fmn*, were more consistent with the results in insomnia patients than those in wild-type flies. Caffeine decreased sleep in both *fmn* and wild-type flies. In contrast, another stimulant, methylphenidate decreased sleep in wild-type flies, but it increased sleep in *fmn* flies. Methylphenidate's result obtained in *fmn* flies is consistent with the result obtained by DAT inhibitors in DAT knockout mice providing new aspect for pharmacological therapeutics of a sort of insomnia.

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