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Quinpirole Increases Melatonin-Augmented Pentobarbital Sleep via Cortical ERK, p38 MAPK, and PKC in Mice

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Abstract

Sleep, which is an essential part of human life, is modulated by neurotransmitter systems, including gamma-aminobutyric acid (GABA) and dopamine signaling. However, the mechanisms that initiate and maintain sleep remain obscure. In this study, we investigated the relationship between melatonin (MT) and dopamine D2-like receptor signaling in pentobarbital-induced sleep and the intracellular mechanisms of sleep maintenance in the cerebral cortex. In mice, pentobarbital-induced sleep was augmented by intraperitoneal administration of 30 mg/kg MT. To investigate the relationship between MT and D2-like receptors, we administered quinpirole, a D2-like receptor agonist, to MT- and pentobarbital-treated mice. Quinpirole (1 mg/kg, i.p.) increased the duration of MT-augmented sleep in mice. In addition, locomotor activity analysis showed that neither MT nor quinpirole produced sedative effects when administered alone. In order to understand the mechanisms underlying quinpirole-augmented sleep, we measured protein levels of mitogen-activated protein kinases (MAPKs) and cortical protein kinases related to MT signaling. Treatment with quinpirole or MT activated extracellular-signal-regulated kinase 1 and 2 (ERK1/2), p38 MAPK, and protein kinase C (PKC) in the cerebral cortex, while protein kinase A (PKA) activation was not altered significantly. Taken together, our results show that quinpirole increases the duration of MT-augmented sleep through ERK1/2, p38 MAPK, and PKC signaling. These findings suggest that modulation of D2-like receptors might enhance the effect of MT on sleep.

Key Words: Sleep, Dopamine 2 receptor, Quinpirole, Melatonin, Pentobarbital

INTRODUCTION

Sleep supports human health and brain function. However, modern lifestyles and the advent of artificial lighting have resulted in sleep problems for many individuals. Several groups have reported that sleep disturbances are caused by altered biological rhythms, mood disorders, and neurodegenerative diseases (Parry *et al*., 2006; Costandi, 2013). Many research groups have proposed methods of overcoming sleep problems, including controlling clock transcription factors, modulating neurotransmitters, and administering sleep aids, especially melatonin (MT) (El Helou *et al*., 2013; Proença *et al*., 2014; Wilhelmsen-Langeland *et al*., 2013).

Sleep is modulated by gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system. Activation of $GABA_A$ receptors modulates sleep. Pentobarbital, a major hypnotic drug, binds to $GABA_A$ receptors and increases chloride ion influx (Lees *et al*., 1998; Ma *et al*., 2008; Shah *et al*., 2014). In addition, pentobarbital modulates $GABA_A$ receptor conductance by increasing the duration of inhibitory postsynaptic currents (IPSCs) in the brain and initiates sleep at low and moderate doses (Wan *et al*., 2003). Furthermore, GABA currents are modulated by MT, which stimulates glutamic acid decarboxylase, an enzyme involved in GABA synthesis (Wang *et al*., 2002), and directly binds to $GABA_A$ receptors (Li *et al*., 2001). Recent studies suggest that the relationship between MT and sleep is mediated, at least in part, through modulation of synaptic transmission by GABA.

MT (5-methoxy N-acetyltryptamine) is a neurohormone with a tryptamine structure that binds to G-protein-coupled recep-

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tors MT_1 and MT_2. A recent review of MT receptors established that the MT_1 receptor is distributed in the cerebral cortex, suprachiasmatic nucleus, cerebellum, hippocampus, central dopaminergic pathways (i.e., ventral tegmental area, substantia nigra, and striatum), and peripheral organs, whereas the MT_2 receptor is expressed throughout the brain (Pandi-Perumal *et al*., 2008). The same review described the manner in which functional MT_1 receptors modulate the protein kinase A (PKA) pathway by activating G proteins, including G_i, $G_{\alpha q}$, $G_{\alpha s}$, $G_{\alpha z}$, and $G_{\alpha 16}$, while MT_2 receptor activation modulates PKA and protein kinase C (PKC) signaling pathways. Recent studies show that MT regulates the activity levels of extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (p38 MAPK), although the precise intracellular mechanisms underlying these effects remain unclear (Bondi *et al*., 2008; Vilar *et al*., 2014).

Recently, a great deal of work in the field of sleep has focused on dopamine; however, little attention has been focused on the effects of D2-like dopamine receptor modulation in sleeping animals. Increasing evidence suggests an important role for D2-like dopamine receptors in various aspects of sleep (Dimpfel, 2008; Volkow *et al*., 2012). Recent studies have demonstrated that interaction between D2-like dopamine receptors and MT is involved in the antidepressant-like effect of MT (Zawilska and Iuvone, 1990; Binfaré *et al*., 2010). However, the effects of D2-like dopamine receptor activation on MT-related sleep and cell signaling in the cerebral cortex of mice during pentobarbital-induced sleep have not been studied.

In the present study, we hypothesized that MT augments pentobarbital-induced sleep in mice. Moreover, we hypothesized that D2-like receptor agonist quinpirole modulates the sleep-inducing effect of MT in mice. In addition, western blotting analyses were used to study the mechanism by which quinpirole modulates intracellular signal transduction pathways related to ERK1/2, p38 MAPK, PKA, and PKC in the cerebral cortex. Our findings provide the first evidence that D2-like receptor activation affects MT-augmented sleep in mice.

MATERIALS AND METHODS

Animals

Male CD-1 mice (3-weeks-old, 15-16 g) were purchased from Koatech (Pyeongtaek, Korea). For 1 week prior to the experiments, 10 animals were housed in each cage and allowed access to water and food *ad libitum*. During the acclimation period, the animals were kept in a room maintained at constant temperature (23 ± 1°C) and humidity (55 ± 5%) under a 12-h light/dark cycle (lights on from 07:00-19:00 h). After the acclimation period, the mice were divided randomly into groups. All experiments were conducted in accordance with the NIH Guide for Laboratory Animals. The study protocol was approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University.

Drugs and chemicals

MT and (-)-quinpirole were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). MT was dissolved in 10% dimethyl sulfoxide (DMSO) and 5% Tween-80 in normal saline. Quinpirole was dissolved in 0.9% physiological saline. All drugs were administered intraperitoneally (i.p.). Mouse anti-β-actin antibodies for western blotting were purchased from Sigma Chemical Co. Rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) and anti-p38 MAPK antibodies for western blotting were purchased from Epitomics (Burlingame, CA, USA). Rabbit anti-phospho-ERK1/2 (Thr202/Thr204), anti-ERK1/2, anti-phospho-PKA (Thr197), anti-PKA, anti-phospho-PKC (gamma Thr514), and anti-PKC antibodies for western blotting were purchased from Cell Signaling Technology (Boston, MA, USA). All other chemicals were of analytical grade.

Pentobarbital-induced sleep test

The pentobarbital-induced sleep test was carried out according to the method described by Ma *et al*. (2009) with some modification. Briefly, mice were treated with quinpirole or saline for 30 min prior to administration of MT or vehicle, followed by administration of pentobarbital sodium (40 mg/kg) 30 min after the administration of MT. Following administration of pentobarbital, mice were placed in individual cages and the time to sleep onset and sleep duration were measured. The observers were blinded to the treatments. Mice that stayed immobile for more than 3 min were considered asleep. The time to sleep onset was measured from the time of pentobarbital administration to the time of sleep onset. The sleep duration was defined as the difference in time between loss and recovery of the righting reflex. Animals that failed to fall asleep within 15 min after pentobarbital treatment were excluded from the study (Ma *et al*., 2009).

Locomotor activity test

To test the effects of MT and quinpirole on locomotor activity, each mouse was placed in an activity cage (locomotor box: 30×30×30 cm) and habituated for 40 min. Mice were administered quinpirole (1 mg/kg, i.p.) 20 min before MT (30 mg/kg, i.p.), and the subsequent horizontal activity was recorded using a video tracking system (Neurovision, Pusan, Republic of Korea). Locomotor activity was measured for 70 min, and the distance travelled over the final 30 min in the chamber was expressed using bar graphs and tracking patterns.

Western blot analysis

Each mouse was decapitated, after which the cerebral cortex was quickly dissected on an ice-cold metal surface. The dissected brain tissue samples were homogenized in ice-cold lysis T-per tissue protein extraction buffer (Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Basel, Switzerland) and incubated on ice for 30 min. After centrifugation at 13,000×rpm for 15 min at 4°C, the supernatant was separated and stored at -70°C. Protein concentrations were determined using a protein assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples (30 μg protein) were separated on 10% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride transfer membranes (Pall Corporation, Pensacola, FL, USA), and blocked with 5% skim milk containing 0.5 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. The membranes were subsequently incubated with primary antibodies overnight at 4°C (1:1,000 dilution for anti-phospho-p38 MAPK [Thr180/Tyr182], anti-p38 MAPK, anti-phospho-PKA [Thr197], and anti-PKA; 1:2,000 dilution for anti-ERK1/2 [Thr202/Thr204], anti-ERK1/2, anti-phospho-PKC [gamma Thr514], and anti-PKC; 1:20,000 dilution for β-actin). After three washes with Tris-buffered saline containing 0.1%

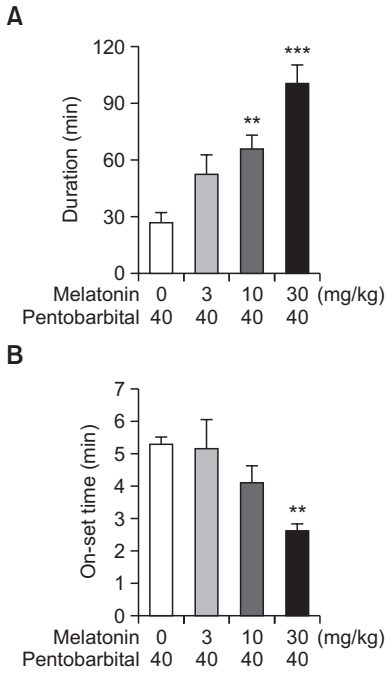

Fig. 1. Effect of MT on pentobarbital-induced sleep. (A) MT increased the duration of pentobarbital-induced sleep. (B) MT decreased the time to onset of pentobarbital-induced sleep. Each column represents the mean ± S.E.M (n=6). **p<0.01 and ***p<0.001 were considered to be statistically significant in comparison with the control group (one-way ANOVA followed by the Fisher's LSD post-hoc test).

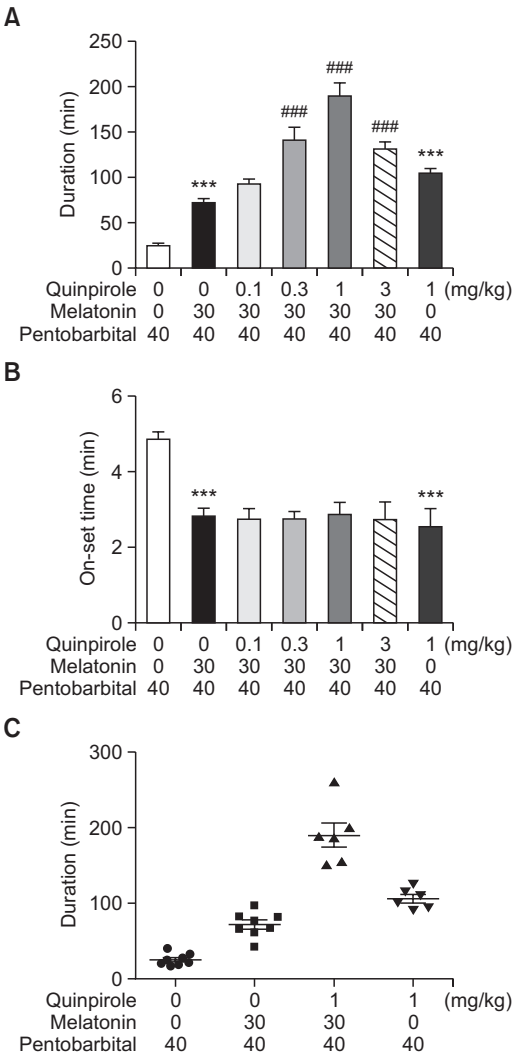

Fig. 2. Effect of quinpirole on MT-augmented pentobarbital-induced sleeping behavior in mice. (A) Quinpirole increased the duration of MT-augmented sleep. (B) Quinpirole had no effect on the time to onset of MT-induced sleep. (C) Distribution of sleep duration in each drug treatment group. Each column represents the mean ± S.E.M (n=6-8). ***p<0.001 vs. the pentobarbital-treated group; ###p<0.001 vs. the group treated with MT and pentobarbital (one-way ANOVA followed by Fisher's LSD post-hoc test).

Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in TBST with 5% skim milk (1:5,000) for 1 h at room temperature. The blots were washed three times in TBST, immersed in an enhanced chemiluminescence (ECL) mixture for 5 min (Perkin Elmer, Boston, MA, USA) (reagents A and B at a 1:1 ratio), and exposed to photographic film. Each western blot was quantified by densitometry using ImageJ 1.44 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were analyzed with Prism 6.0 software (Graphpad Software, Inc., San Diego, CA, USA). The results are expressed as the mean ± S.E.M. of each group. For the assays of sleeping behavior and locomotor activity, data were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test. Western blot data were analyzed using two-way ANOVA followed by Fisher's LSD post-hoc test to detect intergroup differences. Results of p<0.05 were considered statistically significant.

RESULTS

MT prolonged the duration of pentobarbital-induced sleep in mice

A previous study reported that the ED_{50} values for MT-potentiated sleeping time was 6.1 mg/kg (i.p.) in pentobarbital-induced sleep in mice (Sugden, 1983). Therefore, we estimated the dose of MT that prolonged the duration of pentobarbital-induced sleep in our system. Administration of MT dose-dependently decreased sleep latency and dose-dependently increased the duration of pentobarbital-induced sleep (Fig. 1). A single injection of MT (10 and 30 mg/kg) markedly increased the duration of pentobarbital-induced sleep, consistent with a previous study (Sugden, 1983). Furthermore, the group treated with 30 mg/kg MT showed a decreased time to sleep onset in comparison with that of the pentobarbital-treated group (Fig. 1A; $F_{(3,20)}$=12.59, p<0.001; 10 mg/kg MT: t=3.17, p<0.01; 30 mg/kg MT: t=6.03, p<0.001; Fig. 1B; $F_{(3,20)}$=5.00; 30 mg/kg MT: t=3.42, p<0.01). The most significant effects on sleep duration and onset time were produced by 30 mg/kg MT; therefore, this dose was utilized for future experiments and investigation of

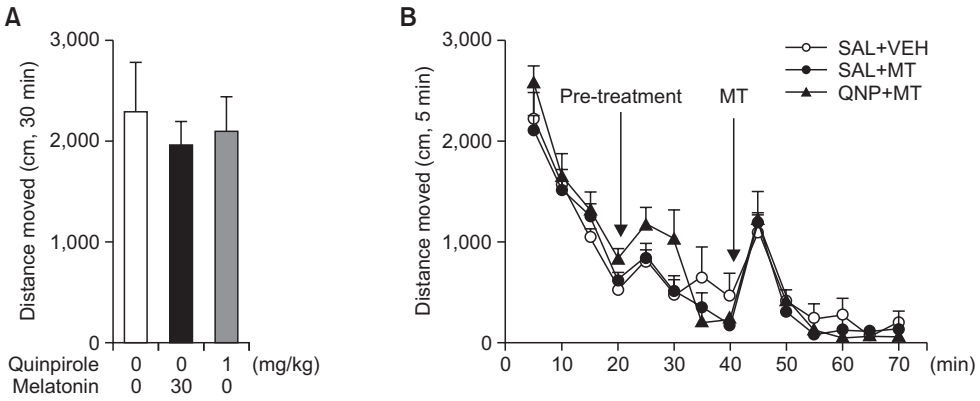

Fig. 3. Effects of MT and quinpirole on locomotor activity in mice. (A) Locomotor activity was assessed by measuring the distance travelled by each mouse for 30 min after MT injection (A), as well as the distance travelled every 5 min for 70 min (B). Each column represents the mean ± S.E.M (n=8-9) (one-way ANOVA followed by Fisher's LSD post-hoc test). SAL, saline; VEH, vehicle; QNP, quinpirole.

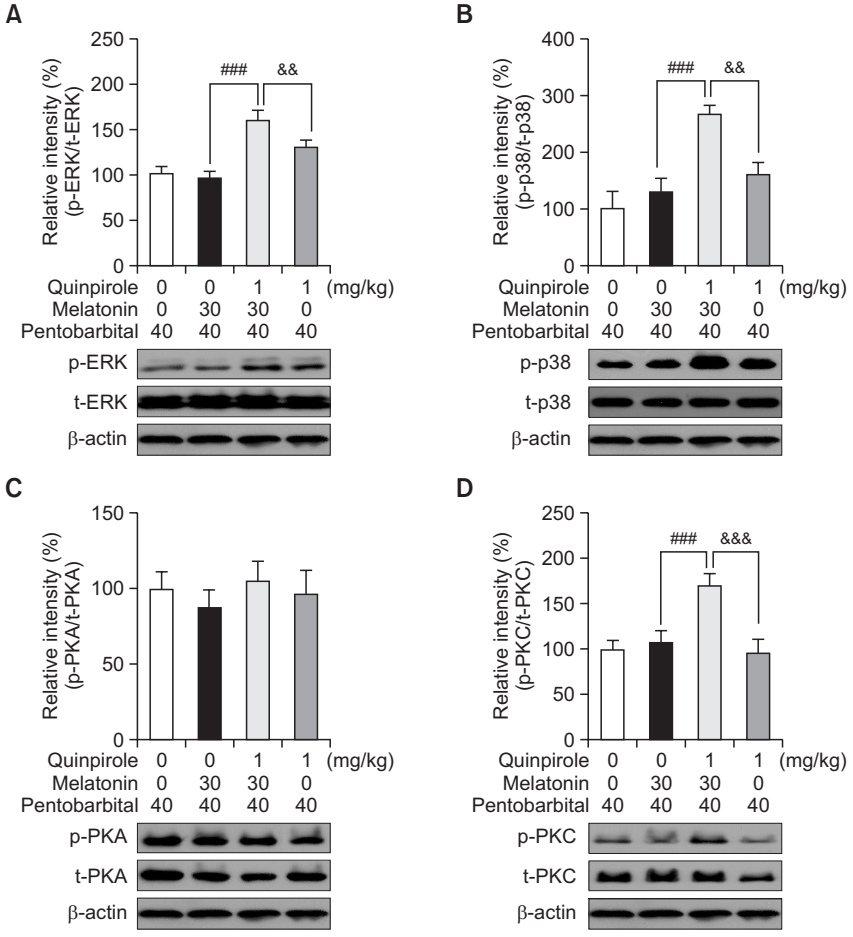

Fig. 4. Effects of quinpirole on ERK1/2, p38 MAPK, PKC, and PKA activation in the cerebral cortex. Quinpirole increased phosphorylation of ERK1/2 (A), p38 MAPK (B), and PKC (D), but not that of PKA (C), in the cerebral cortex. Each column represents the mean ± S.E.M (n=3-5). $^{###}p<0.001$ vs. the group treated with MT and pentobarbital $^{&&}p<0.01$ and $^{&&&}p<0.001$ vs. the group treated with quinpirole and pentobarbital (two-way ANOVA followed by Fisher's LSD post-hoc test).

the hypnotic effect of MT.

Quinpirole increased the duration of MT-augmented pentobarbital-induced sleep in mice

In order to determine whether D2-like receptor activation

altered MT-augmented sleeping behavior, mice were pretreated with selective D2-like receptor agonist quinpirole 30 min before MT treatment. Quinpirole had no significant effect on sleep onset time (Fig. 2B; $F_{(6,39)}$=7.66, p<0.001); however, quinpirole at doses of 0.3, 1, and 3 mg/kg increased the duration of MT-augmented sleep in a dose-dependent manner (Fig. 2A; $F_{(6, 39)}$=37.38, p<0.001; 0.3 mg/kg quinpirole, t=5.73, p<0.001; 1 mg/kg quinpirole, t=9.61, p<0.001; 3 mg/kg quinpirole, t=4.95, p<0.001, compared to the group treated with MT+pentobarbital). Interestingly, in the absence of MT, quinpirole also significantly affected sleep duration (t=6.55, p<0.001, compared to the control group). Furthermore, treatment with the combination of quinpirole, MT, and pentobarbital also prolonged the duration of sleep in comparison with quinpirole treatment alone (t=6.39, p<0.001). The sleeping time distribution of each mouse is shown in Fig. 2C.

MT and quinpirole did not affect locomotor activity

Sugden (1983) suggested that high doses of MT affect locomotor activity in rodents. In our study, we investigated the effect of 30 mg/kg MT on locomotor activity in mice. Locomotor activity was assessed by measuring the distance travelled every 5 min for 70 min, as well as the distance travelled for 30 min after MT injection. As shown in Fig. 3A, administration of 30 mg/kg MT did not significantly affect locomotor activity. In addition, the locomotor activity level of mice treated with quinpirole or MT was similar to that of the control mice (Fig. 3A; $F_{(2,23)}$=0.2151, p>0.05).

Quinpirole increased expression of phospho-ERK1/2, phospho-p38 MAPK, and phospho-PKC in the cerebral cortex

To examine the intracellular mechanisms underlying quinpirole-induced prolongation of sleep, we isolated the brain 3 hours after pentobarbital injection. At this time point, mice treated with quinpirole showed augmented activity levels of ERK1/2 (Fig. 4A; $F_{(3,12)}$=24.71, t=7.648, p<0.001), p38 MAPK (Fig. 4B; $F_{(3,6)}$=117.4, t=14.52, p<0.001), and PKC (Fig. 4D; $F_{(3,6)}$=213.1, t=18.57, p<0.001) in the cerebral cortex in comparison with those of mice treated with MT and pentobarbital. Further, mice treated with quinpirole, MT, and pentobarbital showed up-regulated expression of p-ERK1/2 (Fig. 4A; t=3.584, p<0.01), p-p38 MAPK (Fig. 4B; t=11.32, p<0.001), and p-PKC (Fig. 4D; t=21.97, p<0.001) in comparison with that of mice treated with quinpirole and pentobarbital. Mice treated with quinpirole, MT, and pentobarbital showed PKA activity similar to that of mice treated with either MT or quinpirole and pentobarbital (Fig. 4C; $F_{(3,9)}$=1.306; p>0.05).

DISCUSSION

This study indicates that quinpirole modulates MT-augmented sleeping behavior in mice and elucidates several intracellular mechanisms underlying this action in the brain. We showed that MT, which alone has no hypnotic or sedative effects, enhances pentobarbital-induced sleeping behavior. The duration of MT-augmented sleep was further increased by a single administration of quinpirole. Interestingly, quinpirole affected pentobarbital-induced sleep even in the absence of MT. In addition, quinpirole, alone or in combination with MT, did not affect locomotor activity, suggesting that quinpirole potentiates the effects of MT on sleep duration without affecting the motor system. Western blot analyses revealed increased phosphorylation and activation of ERK1/2, p38 MAPK, and PKC in the cerebral cortex following treatment with quinpirole, MT, and pentobarbital. However, PKA activation was not involved in the effect of quinpirole on the duration of MT-augmented sleep. Although the precise molecular mechanisms by which quinpirole controls ERK1/2, p38 MAPK, and PKC remain unknown, these findings suggest a unique role for MAPKs and PKC in the action of quinpirole.

Consistent with a previous report, systemic administration of MT prolonged the duration of pentobarbital-induced sleep and decreased the time to sleep onset (Wang *et al*., 2002). These findings suggest that MT prolongs the duration of pentobarbital-induced sleep via the GABAergic system. Furthermore, the additional effects of MT, such as locomotor depression, anti-convulsive effects, and analgesic effects, may be due to interaction with benzodiazepine receptors in the brain (Sugden, 1983). Indeed, MT binds to the $GABA_A$ receptor and inhibits [^{3}H]-diazepam binding in the brain (Holmes and Sugden, 1982). Thus, we investigated the effect of 30 mg/kg MT alone on locomotor activity and determined whether this dose produced hypnotic effects. In our mouse model, MT had no significant effect on locomotor activity and did not produce hypnotic effects, suggesting that 30 mg/kg MT augmented pentobarbital-induced sleep without modulating the motor system or producing sedative effects.

In our experiment, quinpirole affected the pentobarbital-sleep onset time, but not the MT-sleep onset time. Because quinpirole as a D2-like receptor agonist produces a sedative effect related to GABA receptor modulation (Canales and Iversen, 2000; Joung *et al*., 2015), quinpirole may indirectly affect pentobarbital-induced sleep. However, in this study, quinpirole did not modulate the sleep onset time in MT-administered mice. There may be two reasons why quinpirole did not change the sleep onset time in MT-administered mice. First, as you can see in Fig. 2, 1 mg/kg quinpirole, and not 3 mg/kg quinpirole, which was the highest dose used in our study, showed the highest effect on MT-pentobarbital sleep duration. However, 0.1 to 3 mg/kg quinpirole did not affect MT-induced sleep onset time. This result indicates that a shortened pentobarbital-induced onset time by MT has the maximum effects. Second, it is well known that sleep onset time may be related to the GABA receptor channel opening time (Kim *et al*., 2012). In our experiment, quinpirole did not change the MT-induced sleep onset time. This result indicates that quinpirole may not change the channel opening time shortened by melatonin because the channel opening time is already shortened by melatonin. Therefore, quinpirole may affect MT-induced pentobarbital sleep behavior by prolonging channel opening without a change in onset time.

Interestingly, we found that mice treated with the combination of quinpirole, MT, and pentobarbital were consistently asleep more than 3 hours after the last injection, while the mice in the other groups were fully awake at that time. These results suggest that the combination of quinpirole, MT, and pentobarbital prolongs sleep duration by affecting a specific signaling pathway. To identify the intracellular mechanisms underlying the effect of the combination of quinpirole, MT, and pentobarbital on sleep duration, we measured the abundance of proteins involved in cell signaling in the brain 3 hours after the last injection.

We investigated phosphorylation of ERK1/2, p38 MAPK, PKA, and PKC in mice treated with the combination of quinpirole, MT, and pentobarbital. Although few studies have reported that activation of MAPKs and protein kinases directly induces sleeping behavior, several studies suggest that activation of ERK signaling enhances sleeping behavior in *Drosophila* (Foltenyi *et al*., 2007), whereas PKA activation inhibits sleeping behavior induced by GABA receptor agonist baclofen (Datta, 2007). However, the relationship between p38 MAPK activation, PKC activation, and sleeping behavior has not been studied comprehensively. Our study is the first demonstration of the effects of protein kinase activation on pentobarbital-induced sleeping behavior in mice.

The ERK pathway is one of several signal transduction pathways that propagate and amplify cellular signaling (Lim *et al*., 2012). Several studies have reported modulation of sleeping behavior by ERK activation. Cortical ERK is involved in sleep consolidation in cats (Dumoulin *et al*., 2015). When the sleep-wake cycle was altered in cats, the number of hippocampal dendritic spines was altered via an ERK-dependent mechanism (Ikeda *et al*., 2015). In this study, quinpirole significantly increased cortical ERK activation. Quinpirole binds to D2-like receptors coupled to Gq proteins, thus activating PLCβ and inducing phosphorylation of ERK1/2 via the PKC/Ras/Raf/MEK pathway in neurons (Yan *et al*., 1999). Furthermore, activated ERK1/2 signaling increases GABA release in the brain (Cui *et al*., 2008), suggesting that phosphorylation of ERK1/2 in response to quinpirole may increase GABA currents by increasing GABA levels in the synaptic cleft, thereby potentiating sleeping behavior.

Increased p38 MAPK expression is associated with sleep apnea and disease-induced alterations in sleep patterns (Wood *et al*., 2006; Dyugovskaya *et al*., 2012). SB203580, a selective p38 MAPK inhibitor, inhibits hydrogen peroxide-induced GABAergic miniature IPSCs (Takahashi *et al*., 2007), whereas quinpirole increases p38 MAPK phosphorylation (Lee *et al*., 2006). Consistent with these reports, we found that expression of phospho-p38 MAPK was increased in the quinpirole-treated group, suggesting that quinpirole potentiates the effects of MT on sleep duration through GABAergic activity linked to the p38 MAPK pathway.

PKC activation has been observed in the hippocampus and prefrontal cortex of sleep-deprived rats (Abrial *et al*., 2015). Stimulation of D2-like receptors via activation of G-protein-coupled receptors modulates $GABA_A$ receptors via PKC-dependent signaling pathways (Di Marzo *et al*., 1993; Brandon *et al*., 2002). In cortical neurons, a PKC-dependent pathway that does not involve changes in $GABA_A$ receptor expression modulates $GABA_A$ receptor phosphorylation and function (Brandon *et al*., 2000). Our findings suggest that PKC activation may influence the effect of quinpirole on sleep duration by potentiating the function of $GABA_A$ receptors in the cerebral cortex.

Rapid eye movement (REM) sleep duration and non-rapid eye movement (NREM) fragmentation are increased in PKA knockout mice, suggesting that PKA may regulate sleep quantity (Hellman *et al*., 2010). In our study, ERK, p38 MAPK, and PKC were markedly activated in the cerebral cortex of mice treated with the combination of quinpirole, MT, and pentobarbital in comparison with mice treated with the combination of MT and pentobarbital or the combination of quinpirole and pentobarbital, suggesting that ERK, p38 MAPK, and PKC may be crucial mediators of the effect of quinpirole on sleeping behavior. However, PKA was not activated in the cerebral cortex of mice treated with quinpirole or MT; this discrepancy between previous reports and the present study may be due to differences in animals and protocols.

The effect of the combination of quinpirole, MT, and pentobarbital on sleep duration was similar to those of the combinations of MT and pentobarbital and quinpirole and pentobarbital, suggesting that quinpirole and MT may have additive effects on pentobarbital-induced sleeping behavior. The effect of a drug combination is classified as synergistic, additive, or antagonistic, respectively, when the effect is greater than, equal to, or less than the sum of the individual effects of each drug (Jia *et al*., 2009). The additive effects of drug combinations on particular targets are classified into several subtypes. In this study, the combination of quinpirole, MT, and pentobarbital activated ERK1/2, p38 MAPK, and PKC in the cerebral cortex, suggesting that quinpirole and MT may act via similar pathways, such as those associated with ERK1/2, p38 MAPK, and PKC; however, further studies are required to illuminate the mechanisms underlying the effects of quinpirole and MT on the brain.

The specified signaling pathways investigated by us may be indirectly involved in quinpirole-augmented modulation of sleeping behavior, because signaling pathways seem synergistically affected by the combination of quinpirole, MT, and pentobarbital. Although the mechanism between sleep maintenance and intracellular signaling pathway needs to be further explored, it is possible that phosphorylation of ERK, p38 MAPK, and PKC may be more active when sleep duration is maintained than when sleep duration decreases. Signaling modulation not only affects sleep duration but also sleeping behavior. Sleep increases the activation of ERK signaling; moreover, a molecular or behavioral feedback mechanism causes persistent intracellular signaling in *Drosophila* (Foltenyi *et al*., 2007). Thus, signaling modulation examined by us may be expressed longer and higher than sleep behavior change.

A majority of sedative hypnotics produce major side effects, including psychomotor impairment; therefore, many researchers have developed drugs aimed at maintaining sleep-induction and reducing side effects, such as motor dysfunction (Miyamoto, 2009). In the present study, we investigated the modulatory effects of quinpirole and MT on locomotor activity. Pretreatment with MT, with or without quinpirole, had no effect on locomotor activity in mice. In addition, pretreatment with quinpirole had no significant effect on the total distance moved in comparison with that of saline. A study reported that a single systemic injection of quinpirole (5 or 10 mg/kg) increased locomotor activity in mice (Jung and Shim, 2011), whereas lower doses (0.1 mg/kg) of quinpirole decreased locomotor activity (Schindler and Carmona, 2002). In contrast, our analysis of the effects of quinpirole on locomotor activity suggests that quinpirole has no effect on the motor system at a dose of 1 mg/kg.

Taken together, our results suggest that the combination of quinpirole and MT increased the duration of pentobarbital-induced sleep by activating signaling pathways associated with MAPKs and PKC in the cerebral cortex. These findings enhance our understanding of the manner in which D2-like receptor activation enhances sleeping behavior in the presence of MT.

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