Melatonin-mediated attenuation of fluphenazine-induced hypokinesia in C57BL/6 mice is dependent on the light/dark phase

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\textbf{Keywords:}
- Dopamine antagonist
- Catalepsy
- Circadian
- Basal ganglia
- Pineal gland

\textbf{ABSTRACT}

Our aims were to assess the effect of melatonin on fluphenazine-induced hypokinesia during the light (ZT 9.5–10.5) and dark (ZT 17.5–18.5) phases in mice lacking endogenous pineal melatonin (C57BL/6 mouse), and to investigate the effects of the manipulation of environmental lighting in mice with a targeted deletion of the MT1 melatonin receptor. In both knockout (C57KO MT1) and wild type (C57WT) mice, fluphenazine (1 mg/kg) induced hypokinesia during the light phase (C57WT: M=105, SEM=31.2, n = 31; C57 MT1KO: M=118, SEM = 32.6 s, n = 29). During the light phase melatonin (10 mg/kg, sc) significantly reduced hypokinesia in both genotypes (C57WT: M=33.1, SEM=8.4 s; C57 MT1KO: M=33.3, SEM=13.0 s). In the dark, fluphenazine did not induce a substantial hypokinesia in either C57WT or C57 MT1KO mice. Manipulating the lighting environment during testing, experiments conducted during the light phase in a dark environment served to abolish the hypokinetic effect of fluphenazine in all groups regardless of melatonin treatment. Conversely, experiments conducted during the dark phase in a light environment showed mice to have hypokinetic effects by fluphenazine treatment in both C57WT (M=98.4, SEM=20.2 s) and C57 MT1KO (M=40.4 SEM=9.5 s) groups. These data suggest that fluphenazine-induced hypokinesia is more pronounced under light than dark conditions, and that melatonin is only able to counteract hypokinesia during the light phase. Importantly, our data suggest that the effect of melatonin on hypokinesia was not solely mediated by the MT1 melatonin receptor in the C57BL/6 mouse, leaving the possible activation of MT2 receptor as the mechanism of action which is regulated by the light/dark environment.

\section{1. Introduction}

Patients treated with typical antipsychotics show amelioration of the positive symptoms of the schizophrenia, however, debilitating extra-pyramidal side effects are very common \cite{20,24,26,30}. These side effects include parkinsonism-like symptoms that consist of tremor, muscle rigidity, hypokinesia, and symptoms related to tardive dyskinesia manifested in oral-facial repetitive movement mainly of the mouth and tongue. The motor side effects are thought to occur via D\textsubscript{2} antagonism in the nigro-striatal system \cite{33,44}. Indeed, D\textsubscript{2} occupancy has been highly correlated with clinical efficacy, and increased risk of extra-pyramidal side effects with chronic typical antipsychotic use \cite{8,12,18,19,39}. As a result, neuroleptics used in the clinic provide transient animal models of parkinsonism and tardive dyskinesia.

Fluphenazine, a typical antipsychotic belonging to the phenothiazine family, induces differential hypokinetic effects in the light and the dark phases when used as an animal model of parkinsonism \cite{43}. In rats, fluphenazine induced substantial hypokinesia only in the light phase; however, it did not induce hypokinesia in the dark phase \cite{43}. Others have reported a similar differential effect with the classic neuroleptic, haloperidol, exhibiting maximal hypokinetic inducing-effects in rats during mid-light with minimal effects at mid-dark \cite{6,16,31,50}. Interestingly, in humans, similar patterns of extrapyramidal symptoms have been reported after neuroleptic treatment. Torner et al., \cite{46} reported parkinsonian type symptoms after haloperidol treatment to be more severe during the morning evaluations than during the evening tests.

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https://doi.org/10.1016/j.bbr.2022.113827

Received 28 May 2021; Received in revised form 11 February 2022; Accepted 2 March 2022

Available online 3 March 2022

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Additionally, during fluphenazine-induced hypokinesia, treatment with the indolamine, melatonin, dramatically attenuated hypokinesia in rats only during the light phase [43]. Recently, Hussain et al. [16] reported similar effects of melatonin lessenings hypokinesia after injections of haloperidol in the light phase in adolescent rats. In humans, similar effects have been shown where melatonin treatment during the daytime and not the nighttime reduced tardive dyskinesia induced by chronic neuroleptic treatment [41]. However, the clinical significance of these findings has been questioned [34].

Rodent studies have corroborated these findings in humans, whereby melatonin was used to attenuate extrapyramidal side-effect induced by antipsychotics. For example, haloperidol and reserpine orofacial induced dyskinesias, characterized by vacuous chewing movement, tongue protrusions [32,36] and oral-facial movements [1], have been significantly repressed by melatonin treatment during the light phase. Further, continuous exposure to light served to increase reserpine-induced oral movements [1], suggesting that disruption in the endogenous rhythm of melatonin may play a role in exaggerating motor dysfunction. Important to our research is the recent finding that during the dark phase, when haloperidol did not induce hypokinesia, testing in light conditions revised the lack of effect of the D2 antagonist [16]. These data portend that the effect of melatonin on extrapyramidal dysfunction via D2 receptor modulation may depend on the response of the dopaminergic and melatonergic systems to light and dark. Taken together, differences in the diurnal pattern of typical neuroleptic-induced side-effects may represent receptor-related mechanisms exhibiting varying diurnal patterns contributing to the induction of extrapyramidal symptoms. In fact, diurnal expression of D2 receptor mRNA and D2 protein levels in mouse striatal tissue has been shown to exhibit a rhythm similar to that of the cataleptic response with maximal mRNA and protein at mid-light and minimal mRNA and protein at mid-dark [2,50].

Given the differential findings in the light versus dark in both rodents and humans, the distinct circadian rhythm of melatonin (acrophase during the dark and nadir during the light in both diurnal and nocturnal species) [3] is of utmost relevance. Melatonin thus serves as an exquisite endogenous biochemical marker conveying the status of the external environment to the internal environment. It has been well established that melatonin plays a central role in circadian and seasonal rhythms most notably in the sleep-wake cycle [5,9,23,42] and seasonal breeding in nonhuman animals [14,25]. In mammals, melatonin exerts its effects via activation of at least two molecular and pharmacologically distinct G-protein coupled membrane bound receptors, the MT1 and MT2 (for review see [10,23]). Transgenic mice with genetic deletion of these receptors have been generated allowing the investigation of the mechanism of action and functional role that melatonin exerts via receptor mediated function.

1.1. Purpose

Mice with a genetic deletion of the MT1 melatonin receptor can be used to identify receptor type specific melatonin responses and function in mammals [17,22,23]. Here, our purpose was to study the effect of melatonin on fluphenazine-induced hypokinesia in mice lacking endogenous pineal melatonin ([13,38]) and to investigate the effects of the manipulation of environmental lighting in mice with a targeted deletion of the MT1 melatonin receptor, the C57BL/6 MT1KO mouse. Here we report melatonin to ameliorate hypokinesia induced by the D2 dopamine receptor antagonist, fluphenazine, in both the C57WT and C57 MT1KO only during the light phase. In the dark, the hypokinetic effect of fluphenazine was absent in all groups. Manipulation of the environmental lighting during the dark served to restore the hypokinetic effects of the D2 antagonist.

2. Method

2.1. Animals

Wild type(C57 WT) homozygous and MT1 receptor mutant mice (C57 MT1 KO) were bred at the Northwestern University Center for Comparative Medicine from 129/Sv/C57BL/6 MT1KO mice donated by Dr. Steven M. Reppert (Massachusetts General Hospital, Boston, MA) by back-crossing C57BL/6 mice (Harlan, USA) for one generation, referred here as C57WT and C57 MT1KO, respectively. Genotypes were confirmed by PCR analysis of DNA samples prepared from tail tips at the time the colony was established, and periodically confirmed during the tenure of the colony.

The animals were kept in a 14 L/10D cycle. The light period was from ZT0 to ZT14 and the dark period was from ZT14 to ZT24 (Zeitgeber Time (ZT) 0 is defined as onset of light). Mice were housed under controlled conditions at 22 °C and food and water were provided ad libitum. Mice were group housed until day of testing where they were temporarily placed in individual housing for ease of administration of drug just prior to treatments. The mice were used and handled in accordance with institutional guidelines set forth by the National Institutes of Health standards and approved by the Institutional Animal Care and Use Committee at Northwestern University.

2.2. Primary behavioral screening – phenotyping of C57WT and C57MT1 KO mice

To rule out basic behavioral differences at baseline that might serve to adversely affect performance in the bar test, we tested gross motor and muscle function. Motor strength (Hanging Wire Test), and motor coordination (Vertical Pole Test) were assessed in C57 MT1KO mice as compared to the C57WT controls (male, 25–30 g: C57WT, n = 22 and C57 MT1KO, n = 22). The mice were tested between ZT4 and ZT6. No significant differences were found in the various motor and muscle domains (see Fig. 1). Further, in order to rule of basic differences in locomotor behavior in the light and dark that might affect bar testing, we placed mice on the bar test without fluphenazine treatment and found mice, regardless of phase, to immediately jump off the bar test showing no hypokinesia (data not shown here).

2.3. Drugs & Lighting

Male mice (25–30 g) were pretreated with fluphenazine (FLU, 1 mg/kg, I.P, Fluphenazine Hydrochloride, Sigma, St. Louis) 2.5 h prior to a bar test either in the light or the dark. The mice were then randomly assigned to receive either vehicle (VEH: saline for FLU/ 10% ETH for melatonin) or melatonin (MLT, 10 mg/kg, sc) treatment. The light level
in the testing room was maintained at 250–300 lux during the light phase and at < 3 lux during the dark phase (15 W GE soft light with Kodak red filter 1 A).

### 2.4. Measurement of hypokinesia

The effects of melatonin on fluphenazine-induced hypokinesia was assessed via the bar test \([29]\). Originally developed for the rat and adapted here for the mouse, the animals were placed on a Plexiglas apparatus with the front paws placed on a horizontal bar (6 mm thick, 15 cm wide, 3 cm in height) and the hind paws placed on the base. The horizontal bar was connected to a microswitch whereby the weight of the animal triggered a timer. The total time the mouse remained on the bar was recorded in seconds. The degree of hypokinesia was quantified by how long it took the animal to remove itself from the bar. A maximum of 300 s was allowed.

#### 2.5. Testing during the light (ZT9.5-10.5) or the dark phase (ZT17.5-18.5)

Once pretreated with fluphenazine, mice were injected with either vehicle or melatonin 30 min prior to a bar test in both the light (VEH: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 8\) and MLT: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 8\)) and the dark (VEH: C57WT, \(n = 7\); C57MT\(_1\) KO, \(n = 6\) and MLT: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 8\)). Mice were tested between ZT9.5–10.5 during the light phase and between ZT17.5–18.5 during the dark phase (see Fig. 2A for lighting schedule).

### 2.6. Testing during the light phase (ZT9.5-10.5) in the dark or testing during the dark phase (ZT17.5-18.5) in the light

Once pretreated with fluphenazine, mice were injected with either vehicle or melatonin 30 min prior to a bar test in both the light (VEH: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 6\) and MLT: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 7\)) and the dark (VEH: C57WT, \(n = 8\); C57MT\(_1\) KO, \(n = 7\) and MLT: C57WT, \(n = 7\); C57MT\(_1\) KO, \(n = 6\)). Mice underwent the same procedure as followed in the testing paradigm above with the exception of manipulation of environmental lighting during drug treatment and bar testing. During the light phase in a dark environment, the lights were turned off at ZT6. The animals were placed in the dark one hour prior to the pre-treatment with fluphenazine. Bar testing was then conducted between ZT9.5–10.5 (see Fig. 2B for lighting schedule). During the dark phase with testing in a light environment, the lights were turned on at ZT14. The animals were placed in the light one hour prior to pre-treatment with fluphenazine. Bar tests were then conducted between ZT17.5–18.5 (see Fig. 2C for lighting schedule).

### 3. Design and analysis

The experiments in the light and the dark phases each represent a 2 × 2 between subjects factorial design whereby the independent variables were treatment (VEH/MLT) and genotype (C57WT/C57MT\(_1\)KO) in each phase. A 2 × 2 between subjects analysis of variance (ANOVA) was used to analyze the effect of treatment and genotype on hypokinesia (bar test in sec) with p values < 0.05 considered statistically significant. Independent samples t-tests were used to assess differences between the genotypes in motor strength and muscle function. All statistical analyses were conducted using SPSS (11.0 for Windows - SPSS, Inc., Chicago, IL).

### 4. Results

In C57 WT mice, fluphenazine (1 mg/kg, ip) induced hypokinesia during the light phase (Fig. 3A) but not in the dark phase (Fig. 3B). The effects of melatonin on fluphenazine-induced hypokinesia in both C57WT and C57 MT\(_1\) KO mice during the light phase are shown in Fig. 3A. The between subjects two-way ANOVA showed a significant main effect for treatment, \(F (1, 28) = 10.87, p < 0.005\). Regardless of genotype, melatonin treatment significantly decreased hypokinesia, decreasing time on the bar test (VEH: 105.3 \(±\) 31.2 s vs MLT: 33.1 \(±\) 8.4 s in C57WT; VEH:118 \(±\) 32.6 s vs MLT: 33.3 \(±\) 13.0 s in C57 MT\(_1\)KO) . During the dark phase, there was also a significant main effect for treatment, \(F (1, 25) = 6.63, p = 0.01\), however, paradoxically melatonin potentiated rather than attenuated the fluphenazine-induced hypokinesia in the C57 MT\(_1\)KO group (VEH: 8.9 \(±\) 1.1 s vs MLT:27.4 \(±\) 6.2 s).

To test the effects of manipulation of environmental light (light or dark) on fluphenazine-induced hypokinesia, light phase bar testing was conducted in a dark environment. Under these circumstances testing in the dark served to dampen fluphenazine-induced hypokinesia in all mice. Fig. 4A shows that melatonin did not have an effect on the dampened fluphenazine-induced hypokinesia in either the C57WT or C57 MT\(_1\)KO groups. For dark phase testing done in a light environment, a main effect for genotype was shown, \(F (1, 24) = 4.39, p < 0.05\). The C57WT group showed a greater degree of hypokinesia as compared to their KO counterparts (C57WT: 98.4 \(±\) 20.2 vs C57 MT\(_1\)KO: 40.4 \(±\) 9.5 s; Fig. 4B) regardless of melatonin treatment. Additionally, in order to assess the effects of manipulation of the testing environment between the VEH groups (fluphenazine only groups) from Figs. 3B and 4B, we conducted a 2 × 2 between subjects ANOVA (Type of Testing: During
the Dark Phase Under Dark Conditions or During the Dark Phase Under Lit Conditions X Genotype: WT or KO). The analysis revealed a main effect for Type of Testing, $F(1,25) = 11.56, p = 0.002$ (Figs. 3B and 4B) showing that light restored the hypokinetic effect of the D2 antagonist in both C57WT and C57 MT$_1$KO groups during the dark phase.

5. Discussion

These results show that in the C57 mouse, pharmacological treatment with melatonin served to ameliorate hypokinesia induced by the D$_2$ dopamine receptor antagonist, fluphenazine. Melatonin blocked the effect of the classic neuroleptic by nearly 70%. These data corroborate previous findings in the rat showing melatonin to reduce hypokinesia by over 75% [43]. As reported in the rat, this effect was found only during the light phase. Further, during the dark phase fluphenazine’s ability to induce hypokinesia was reduced by 87% and 92% in the C57WT and C57 MT$_1$KO, respectively, as compared to light phase levels. These data support previous reports on typical neuroleptics producing maximal cataleptic inducing-effects in rats during mid-light with minimal effects at mid-dark [6,31,50]. In the case of haloperidol, dark phase conditions served to dampen cataleptic responses by nearly 50% [50]. Comparatively, it does appear that fluphenazine is more susceptible to the dampening effects of the dark environment as compared to haloperidol.

Our findings are of clinical relevance because the classic antipsychotic, fluphenazine, is used in the treatment of the positive symptoms of schizophrenia, mainly psychosis [21,35,45]. Fluphenazine did not induce extrapyramidal side-effects in this animal model of parkinsonism during dark phase. Albeit, the antipsychotic effects of neuroleptics hinge on the blockade of dopaminergic activity in mesocortical and...
mesolimbic areas of the brain and is distinct from the extrapyramidal inducing effects occurring in nigro-striatal, the question arises whether a similar lack of efficacy may exist in its ability to diminish the positive symptoms of schizophrenia. To date, this question remains unanswered as there is no empirical evidence reporting number of psychotic episodes as a function of the light/dark cycle. However, Torner et al. [46], have reported differential effects with haloperidol-induced extrapyramidal side effects based on the light/dark cycle. Extrapyramidal side effects were found to lessen during the dark phase in humans. Additionally, sleep disturbances have been reported in schizophrenic populations [4, 27,28,54] which may not only represent disruption in the sleep/wake cycle, but also may represent the lack of efficacy of drug treatment during the nighttime. That is to say, lessered efficacy of a typical anti- psychotic during the dark may lead to more psychotic disturbances during the dark phase which may then disrupt sleep parameters. This notion is supported by studies showing less subjective sleep quality in patients taking typical antipsychotics as compared to patients taking the new generation of drugs in the treatment of schizophrenia [52].

Melatonin substantially decreased the effects of the D₂ antagonist, fluphenazine, in C57WT and C57MT KO mice suggesting involvement of the MT₂ melatonin receptor. If in fact this effect is mediated by melatonin receptor activation, the likely candidate is the MT₂ receptor. Future studies would do well to further elucidate the involvement of the MT₂ receptor by using MT₂ receptor antagonist to block this effect (e.g. 4 P-PDOT). Additionally, the ameliorating effect of melatonin may not be contingent on the endogenous circadian rhythm of melatonin as the C57BL/6 mouse is considered a melatonin-deficient strain producing low levels of melatonin in the pineal gland (~ 10 pg/pineal) [51]. However, we cannot rule out that very low levels of melatonin may still be contributing to these effects.

In the dark phase, exogenous melatonin served to potentiate the effect of the D₂ antagonist but only in C57 MT, KO mice. However, the potentiating effect of melatonin on fluphenazine-induced hypokinesia should be interpreted in light of the fact that fluphenazine did not induce a substantial hypokinesia during the dark phase as shown in previous studies with haloperidol [6,31,50]. That is to say, melatonin served to counteract the lack of effect of the D₂ antagonist in the C57 MT, KO group. The potentiating effect of melatonin during the dark phase is difficult to interpret given the degree of hypokinesia in the melatonin treated group in the dark phase was similar to that experienced during the light phase with melatonin treatment.

Opposite behavioral effects were shown in the C57WT group with manipulation of the lighting condition of the testing environment. By placing the C57WT group in the light during the dark phase, light served to restore levels of hypokinesia as experienced during daytime. In the C57 MT, KO group, the same paradoxical effect was found however not as pronounced; testing in the light during the animal’s nocturnal phase restored hypokinetic levels to one third its original level as compared to mice tested in the light phase in a light environment. Using the same manipulation of the lighting condition of the testing environment, we corroborate Hussain et.al’s (2020) recent report in rats investigating haloperidol induced hypokinesia. Taken together, these data show that the hypokinetic effects of classic neuroleptics are more pronounced under light than dark conditions regardless of circadian phase of the animal.

Although a possible candidate mediating the effects of melatonin’s amelioration of dopamine antagonist-induced hypokinesia appears to be MT₂ receptor action, the mechanism linking the paradoxical effects of fluphenazine during the light and dark phase remains unclear. One possible mechanism of action includes circadian variation within the dopamine (DA) receptor system. For instance, D₂ receptor mRNA levels in mouse striatum oscillated with strongest expression around mid-light and lesser expression during mid-dark [50]. Differences in the diurnal pattern of typical neuroleptic-induced side-effects may represent receptor-related mechanisms exhibiting different circadian profiles contributing to the induction of extrapyramidal symptoms.

Additionally, it could be that melatonin is indirectly modulating D₂ receptor function. Support for this notion is that melatonin was shown to increase the affinity of D₂ striatal neurons after in vitro application of a D₂ antagonist [15]. Further, melatonin increased the expression of MT₁ melatonin receptor mRNA in rodent dopaminergic neurons and MT₁ melatonin receptor protein in post-mortem human striatal tissue [47]. However, the functional relevance of these findings is unknown. It is not out of the realm of possibility that melatonin receptors may modulate dopaminergic motor function in humans.

Another possible mechanism of action could lie at the molecular level of circadian rhythmicity namely the clock genes that are thought to provide the molecular basis of circadian rhythms. First located in the master biological oscillator, the suprachiasmatic nucleus (SCN), the clock genes are regulatory proteins that express persistent circadian rhythms under constant conditions [11,37]. The clock gene, PERIOD1 (Per1), has been identified in rodent striatum. Relevant to our work, peak striatal Per1 protein was found highest during the day and lowest during the night [48]. Additionally, a diurnal rhythm-dependent variation in striatal D₂ protein levels in mice has been shown to have the greatest activity during the day (Abreu-Neto et al. [21]). It is possible that levels of Per1 in the striatum are low during the night, and therefore, the D₂ receptor antagonist, fluphenazine, lacks the ability to induce extrapyramidal side-effects. There may indeed be a relationship between the regulatory clock genes and the D₂ system also via SCN modulation. When mice were treated with haloperidol an increase in SCN Per1 expression occurred with the greatest effect during mid-light [49]. We also cannot rule out the role that photic processing at the retinal level may play. D₂ receptor deficient mice had reduced oscillatory levels of retinal mPer1 expression with a dampened light response in the induction of mPer1 [53].

We have demonstrated that acute pharmacological treatment with melatonin ameliorated hypokinesia in melatonin-deficient mice as induced by the D₂ antagonist, fluphenazine in both C57WT and C57 MT, KO groups highlighting a possible role for MT₂ melatonin receptor mediation. However, melatonin blocked the effect of the classic neuroleptic only during the light phase. Of equal importance, fluphenazine did not induce hypokinesia during the dark phase in either C57WT or C57 MT, KO mice. Since light restored the hypokinetic effect during dark phase testing, the present results demonstrate the importance of the light/dark status in time of dosing of the classic neuroleptic, fluphenazine, and the time of dosing of melatonin in the amelioration of extrapyramidal side effects. These findings provide important insight in determining the optimal time of drug administration to garner a greater clinical effect of typical neuroleptics in the treatment of psychosis. However, clinical trials manipulating drug administration in the light and dark are still needed to provide direct evidence to institute more effective dosing regimens for individuals treated with neuroleptics. Our findings also corroborate others [41] reporting melatonin to ameliorate extrapyramidal side-effects as a result of neuroleptic use in human. However, these effects appear to be dose dependent as others report no effect of melatonin on extrapyramidal side-effects at smaller doses [7, 40]. More studies are needed to determine the genuine efficacy of melatonin in the treatment of extrapyramidal side-effect keeping in the mind the light/dark cycle.

Funding

This work was supported by U.S. Public Health Service Grants MH-42922 and MH-5265 (M.L.D.), and National Institutes of Health T32 NS41234 (ICS).

CRediT authorship contribution statement

Isabel Sumaya: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Project administration, Visualization,
Investigation. Margarita Dubocovich: Resources, Writing – review & editing, Supervision, Funding acquisition.

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