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The effects of exogenous melatonin and melatonin receptor blockade on aggression and estrogen-dependent gene expression in male California mice (*Peromyscus californicus*)

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HIGHLIGHTS

- Mice were given exogenous MT or an MT receptor antagonist (luzindole).
- MT facilitated aggression in long days, which was partially blocked by luzindole.
- MT suppressed non-estrogen-dependent genes in the medial preoptic area (MPOA).
- MT may not be affecting aggression through regulation of estrogen-dependent genes.
- MT may be affecting aggression through receptor dependent and independent pathways.

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ABSTRACT

Photoperiodic regulation of aggression has been well established in several vertebrate species, with rodents demonstrating increased aggression in short day photoperiods as compared to long day photoperiods. Previous work suggests that estrogens regulate aggression via rapid nongenomic pathways in short days and act more slowly in long days, most likely via genomic pathways. The current study therefore examines the role of melatonin in mediating aggression and estrogen-dependent gene transcription. In Experiment 1, male California mice were housed under long day photoperiods and were treated with either 0.3 μg/g of melatonin, 40 mg/kg of the melatonin receptor antagonist luzindole, or vehicle for 10 days. We found that melatonin administration significantly increased aggression as compared to mice receiving vehicle, but this phenotype was not completely ameliorated by luzindole. In Experiment 2, male California mice were injected with either 1 mg/kg of the aromatase inhibitor letrozole or vehicle, and oxytocin receptor (OTR), estrogen receptor alpha (ERα), and c-fos gene expression was examined in the bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA). In the BNST, but not MPOA, OTR mRNA was significantly downregulated following letrozole administration, indicating that OTR is an estrogen-dependent gene in the BNST. In contrast, ERα was not estrogen dependent in either brain region. In the MPOA, OTR mRNA was inhibited by melatonin, and luzindole suppressed this effect. C-fos and ERα did not differ between treatments in any brain region examined. These results suggest that it is unlikely that melatonin facilitates aggression via broad spectrum regulation of estrogen-dependent gene expression. Instead, melatonin may act via regulation of other transcription factors such as extracellular signal regulated kinase.

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1. Introduction

Photoperiod is a seasonal cue which predicts changes in resource availability and environmental conditions, and is widely known to affect aggressive behavior in a variety of vertebrate species [1], including rodents [2–6], birds [7,8], and fish [9]. Aggression can change seasonally as individuals engage in resource defense, whether it be territorial and/or mate defense during the breeding seasons, or limited food resources during the non-breeding seasons. A major pathway through which seasonal changes in day length alter vertebrate physiology is via the effects of melatonin on the central nervous system. Pineal melatonin synthesis is inhibited when light hits retinal ganglion cells [10], causing peak melatonin release during the dark phase [11]. During the winter seasons (short day photoperiods), animals experience longer periods of peak melatonin activity due to shorter photoperiods and demonstrate increased aggressive behavior [4,11–13]. In California mice

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(*Peromyscus californicus*) and Siberian hamsters (*Phodopus sungorus*), aggression in short day photoperiods has been decoupled from reproductive responsiveness [14,15]. In these species, individuals that do not show gonadal regression during exposure to short day photoperiods continue to demonstrate increased aggression. This increased aggression phenotype closely resembles the increased aggression observed in rodents responding to short days with regressed gonads [15–17], suggesting that aggression is most likely not mediated by gonadal hormones [12,15].

Although gonadal hormones do not appear to facilitate increased aggression during short days, steroid hormones have important effects on aggression. Evidence from song sparrows demonstrated that estrogens can be synthesized *de novo* in the brain, and that neuroestrogen synthesis is increased during aggressive interactions in the non-breeding season [18]. Although the enzymes necessary to produce estrogens *de novo* are present in the brain of many mammalian species, a direct link between neuroestrogens and aggressive behavior has not yet been established in mammals. The mechanism through which estrogens act, however, depends on the photoperiodic conditions of the surrounding environment. Estrogens rapidly affect aggression under short day photoperiods, but not long day photoperiods, in California mice [14]. This rapid action occurs independently of protein synthesis, suggesting nongenomic regulation of aggression in short days [19]. Utilizing microarrays, eleven estrogen-dependent transcripts in the BNST were upregulated during long day photoperiods in oldfield mice (*Peromyscus polionotus*), suggesting that estrogen-dependent gene transcription is downregulated under short days [20]. The transcriptional effects of estrogens can be mediated by nuclear estrogen receptors alpha (ERα) and beta (ERß) interacting with different response elements such as estrogen response elements (ERE) and AP-1 [reviewed in 21]. Both ERα and ERß have been observed to regulate aggressive behaviors [22–24], but are not themselves estrogen-dependent genes [25]. ERα$^{-/-}$ mice show decreased inter-male aggression as compared to wild type mice, whereas ERß$^{-/-}$ mice show comparative or increased levels of inter-male aggression as compared to wild type mice [23,24]. Although these results suggest that the two receptor types have opposing effects on aggressive behavior, this is mostly likely due to developmental effects. Studies using ERα and ERβ selective agonists in adult oldfield mice suggest that the two receptor types may have similar effects on aggression in the adult [20]. Oxytocin receptor (OTR), an estrogen-dependent gene in the rat brain [26], has also been observed to affect aggressive behavior, where mice lacking OTR demonstrate enhanced aggression [27,28]. These receptors are of particular interest because their actions could mediate the effects of short day photoperiod on aggressive behavior.

Interestingly, melatonin can also affect estrogen signaling, and may exert its effects through at least two mechanisms. Melatonin can act through its associated receptors MT_1 and MT_2 to inhibit cyclic adenosine monophosphate (cAMP) [29]. Increased expression of cAMP facilitates ERα expression [30]. Conversely, melatonin has been shown to inhibit estrogen-dependent gene expression by binding to calmodulin, thereby reducing transport of ERα dimers to the nucleus [31]. Intriguingly, this pathway is independent of melatonin receptor activity and ER expression. Injections of melatonin have been shown to facilitate aggressive behavior [4,12,13], but the mechanism of action remains unclear. Here we tested whether exogenous melatonin increases aggression in male California mice housed under long days, and whether any behavioral effects are mediated by melatonin receptors. We also investigated the effects of melatonin and its receptors on estrogen-dependent gene expression (OTR) and non-estrogen-dependent gene expression (ER expression) [25]. These genes were specifically chosen for investigation because of their previous association with aggressive behavior. We hypothesized that melatonin treatment would induce a behavioral profile akin to short day California mice. If melatonin acts via the MT receptor pathway, then a melatonin receptor antagonist (luzindole) should reverse any effects of melatonin. If melatonin acts independently of MT receptors, then the effects of melatonin should persist in the presence of luzindole.

2. Methods

2.1. Animals

Male California mice were bred in our colony or purchased from the Peromyscus Stock Center (Columbia, SC) and housed on long day photoperiods (16 h light:8 h dark). All mice were housed in polypropylene cages on either Carefresh (Absorption Corp, Ferndale, WA) or sanichip (Harlan Teklad, Indianapolis, IN) bedding with up to two same-sex cage mates before behavioral testing. Food (2016 Harlan Teklad, Madison, WI) was provided ad libitum. Mice were tested at approximately six months of age. Housing temperatures were maintained at between 20 and 26 °C, and humidity ranged from 30 to 70% in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the Association of Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee.

2.2. Experiment 1: the effects of melatonin on aggression

Male mice housed on Carefresh bedding were randomly assigned to receive subcutaneous (s.c.) injections of either 150 μL vehicle (10% DMSO (Sigma, St. Louis, MO) in saline) or 0.3 μg/g of melatonin (Sigma, St. Louis, MO) in vehicle [4,12]. To test the extent to which melatonin receptors mediated the effects of melatonin, an additional set of mice were assigned to receive injections of melatonin with 40 mg/kg of luzindole (Sigma Aldrich, St. Louis, MO) [32,33]. Animals received one injection per day three hours before the dark phase (1500 h) over the course of ten days. Three hours following the last injection, mice were tested in a resident-intruder aggression test, in which a novel male mouse was introduced into the home cage of the focal mouse [2,3,19]. Each test lasted seven minutes, and was conducted during the dark phase (1500 h). Tests were monitored and video recorded for subsequent scoring of aggressive behavior. Aggression was characterized by the number of bites the focal mouse directed toward the intruder and latency for the focal mouse to bite the intruder mouse.

2.3. Experiment 2: the effects of letrozole on OTR and ERα gene expression

Male mice housed on either Carefresh or sanichip bedding were randomly assigned to receive intraperitoneal (i.p.) injections of either 1 mg/kg letrozole (an aromatase inhibitor; Sigma Aldrich, St. Louis, MO) in 1% DMSO in saline, or 100 μL of vehicle [34]. Mice were injected once per day for 9 days. Twenty-four hours following the final injection, mice were anesthesized under isoflurane gas and rapidly decapitated. Brains were cut in 2 mm thick sections to prepare tissue for punch sampling [3]. Brain sections were frozen on a freezing plate, and 1 mm punch samples were taken from the bed nucleus of the stria terminalis (BNST) and the medial preoptic area (MPOA), two regions where OTR, ERα, and aggression-induced c-fos are expressed [19,35–37, Steinman et al. unpublished]. OTR gene expression has been shown to be estrogen-dependent in the rat brain [26], whereas ERα gene expression is not [25]. Here we tested whether these effects generalized to California mice using real-time quantitative PCR (qPCR).

2.4. Experiment 3: the effects of melatonin on OTR and ERα gene expression

Immediately after behavior testing, focal mice from experiment 1 were anesthesized under isoflurane gas and rapidly decapitated. Brains were cut in 2 mm thick sections to prepare tissue for punch sampling [3]. Brain sections were flash frozen on a freeze plate, and 1 mm punch samples were taken from the BNST and the MPOA for qPCR analysis.

2.4.1. Quantitative real-time polymerase chain reaction

RNA was extracted using an Ambion® RNAqueous Micro-kit (AM1931, Life Technologies, Grand Island, NY, USA). An iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA, USA) was used to reverse transcribe 1 μg of RNA. *Peromyscus* specific primers and probes were used to amplify OTR (Genbank accession: HQ651236), ERα (DQ357060.1), and c-fos (JN601063.1) in California mice.

OTR Forward: 5′-GCCCTTGACGCCTTTCTTCT-3′
OTR Reverse: 5′-TTCCTTGGGCGCATTGAC-3′
OTR Probe: 5′-CGTGCAGATGTGGAGCGTCTGGG-3′
ERα Forward: 5′-GAACAGCCCCGCCTTGT-3′
ERα Reverse: 5′-GCATCCAGCAAGGCACTGA-3′
ERα Probe: 5′-TGACAGCTGACCAGATG-3′
c-fos Forward: 5′-TGTTTCACGCACAGATAAGGT-3′
c-fos Reverse: 5′-TGGTGCATTTCAGAGAGGAGAA-3′
c-fos Probe: 5′-CTCCCTAGGTCTACGGGAACCCTCGAG-3′

TaqMan® 18s ribosomal RNA primers and a probe labeled with a VIC dye were used to amplify the housekeeping gene (Applied Biosystems, Foster City, CA, USA). The OTR probe was labeled with a NED dye and the ERα probe was labeled with a FAM dye. All reactions were run on a ViiA 7 Fast Real-Time System with a TaqMan® system (Life Technologies). Reaction cycles were as follows: one cycle of 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sections, and 60 °C for 1 min [22,38,39]. All samples were run in triplicates. Expression data for OTR and ERα were then normalized to 18s CT values. The standard curve was generated from pooled paraventricular nucleus samples taken from male and female California mice (1:2, 1:4, 1:5, and 1:10).

2.5. Data analysis

Mice were classified as non-aggressive (demonstrating 0 bites during the aggression test) or aggressive (demonstrating 1 or more bites during the aggression test). The percentage of aggressive mice was analyzed for each treatment group utilizing Fisher's Exact Test. Attack latency, number of bites per test, and gene expression data were all analyzed using Mann–Whitney *U* tests due to non-homogeneity of variance in our data sets. Gene expression for mice receiving saline and luzindole was analyzed as a percentage of the average saline value for each respective brain region. A p-value of <0.05 was considered statistically significant.

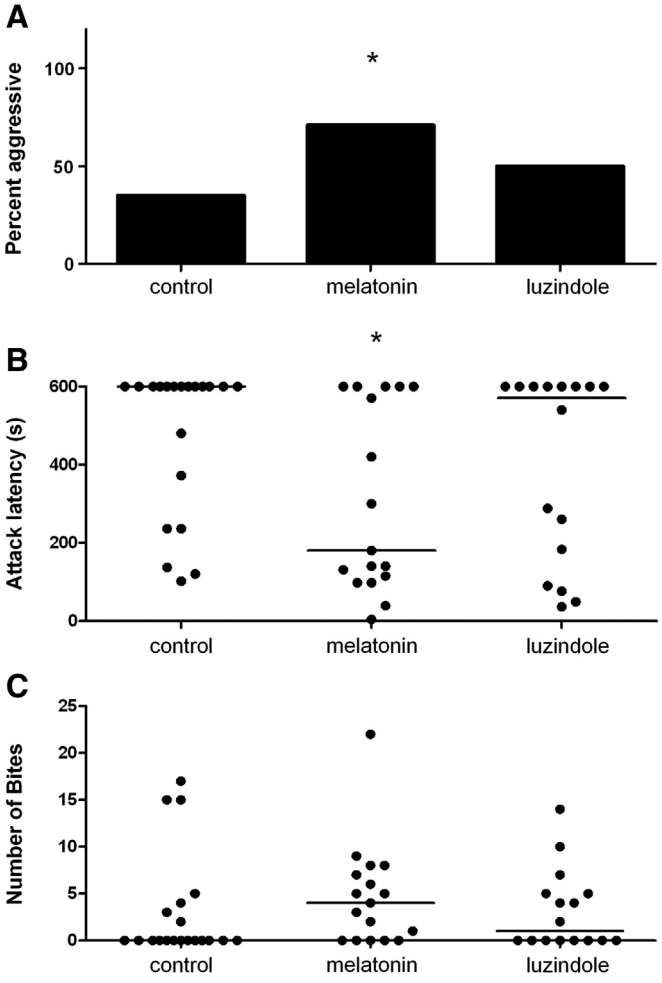

Fig. 1. (A) Bar graphs indicating the percent of mice in each treatment group (control, melatonin or luzindole) who demonstrated aggression. Those mice receiving melatonin injections demonstrated significantly more aggression than controls, but not mice receiving luzindole. (B) and (C) indicate scatterplots of attack latency and number of bites, respectively. Mice receiving melatonin demonstrated significantly lower latencies to attack as compared to control mice, but not mice receiving luzindole. Number of bites did not differ between treatment groups. Horizontal lines indicate the median for each group. *signifies difference from control, $p < 0.05$.

3. Results

3.1. Experiment 1: the effects of melatonin on aggression

The percentage of mice that were aggressive was significantly higher following melatonin treatment (12 aggressive mice out of 17 total mice) compared to mice receiving vehicle control (7 aggressive mice out of 20 total mice) ($p < 0.05$; Fisher's exact test), but were not significantly more aggressive than mice receiving luzindole (8 aggressive mice out of 16 total mice) ($p > 0.05$; Fisher's exact test) (Fig. 1). The percentage of mice that were aggressive did not significantly differ between vehicle and luzindole treatment groups ($p > 0.05$; Fisher's exact test). Mice receiving melatonin had a significantly shorter attack latency than mice receiving vehicle ($U = 97.50$, $p < 0.05$), but not luzindole. Mice receiving luzindole did not have a significantly different attack latency from mice receiving vehicle ($U = 128.00$, $p > 0.05$). There were no significant differences between any treatment groups for number of bites, but there was a non-significant trend towards an increased number of bites in mice that received melatonin as compared to mice receiving vehicle ($U = 227.00$, $p = 0.085$).

3.2. Experiment 2: the effects of letrozole on OTR and ERα gene expression

Mice receiving letrozole showed significantly less OTR gene expression in the BNST as compared to mice receiving saline ($U = 3.00$; $p < 0.05$), but there were no differences between letrozole and saline mice in the MPOA ($U = 7.00$; $p > 0.05$) (Fig. 2). Mice treated with letrozole or saline showed comparable ERα expression in both the BNST ($U = 14.00$; $p > 0.05$) and MPOA ($U = 14.00$; $p > 0.05$).

3.3. Experiment 3: the effects of melatonin on OTR and ERα gene expression

OTR gene expression was significantly reduced in the MPOA of mice receiving melatonin as compared to both vehicle ($U = 26.00$, $p < 0.05$) and luzindole ($U = 79.00$, $p < 0.05$) mice (Fig. 3). There were no differences between treatment groups for OTR expression in the BNST (all p's > 0.05), or for ERα gene expression in the MPOA or BNST (all p's > 0.05). There were also no differences between treatment groups for c-fos expression in the BNST or MPOA (all p's > 0.05).

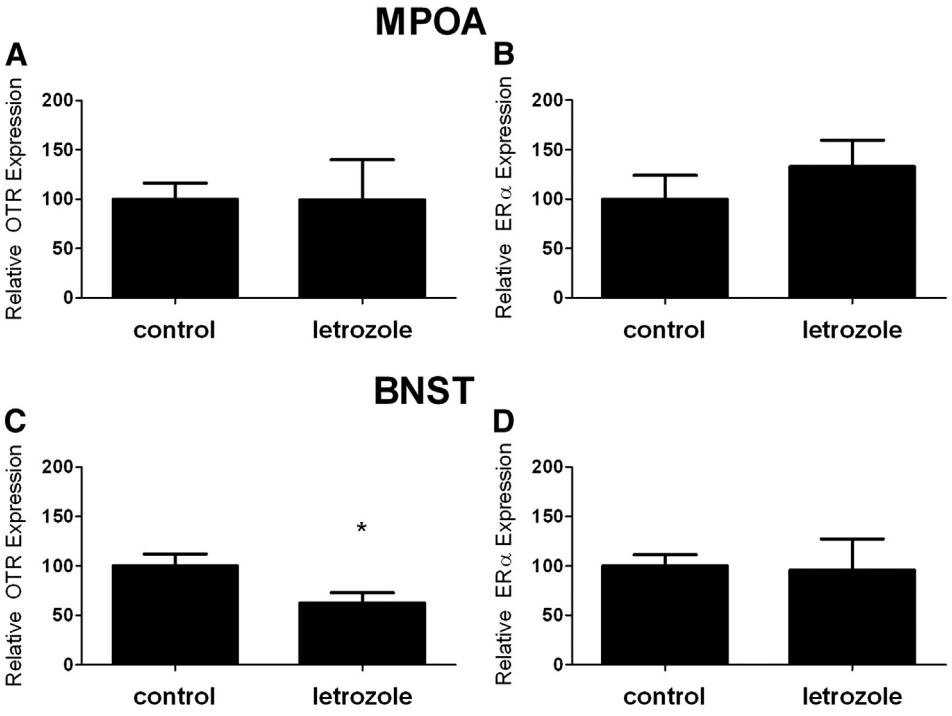

Fig. 2. Bar graphs of mice receiving the aromatase inhibitor letrozole or vehicle injections. Graphs indicate that letrozole had no effect on OTR (A) or ERα (B) in the MPOA, nor did it affect ERα in the BNST (D). OTR expression in the BNST (C), however, was significantly reduced by letrozole injection. *$p < 0.05$.

4. Discussion

Here we have shown that male California mice treated with exogenous melatonin demonstrate higher levels of aggression and a lower latency to attack than mice receiving vehicle. Behaviors observed in mice receiving melatonin parallel those observed in California mice housed in short days [14,20]. In accordance with the current findings, previous studies utilizing exogenous melatonin have shown that melatonin injections increase aggressive behavior in mice (*Mus musculus*) [13], Syrian hamsters (*Mesocricetus auratus*) [4,40] and Siberian hamsters [12]. This increase in aggression under short days can be blocked by adrenalectomy [12], indicating that adrenal hormones play a significant role in mediating the relationship between melatonin action and aggression [41].

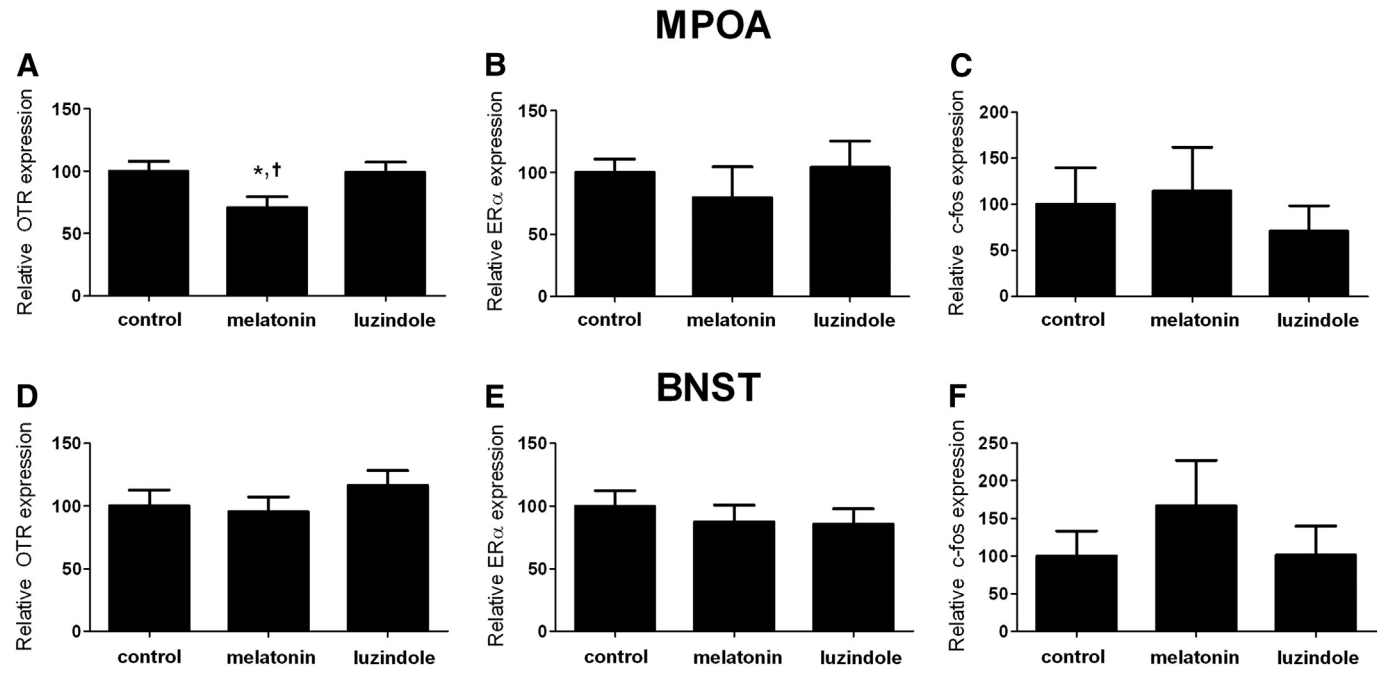

Fig. 3. Bar graphs indicating OTR, ERα, and c-fos relative gene expression in the MPOA (A–C) and BNST (D–F). Mice receiving melatonin injections had significantly less OTR expression in the MPOA, but not the BNST, as compared to control mice and mice receiving luzindole. ERα and c-fos relative gene expression did not differ between treatment groups in the MPOA or BNST. *signifies difference from control, $p < 0.05$. †signifies difference from luzindole treatment, $p < 0.05$.

4.1. Melatonin, aggression, and the BNST

Previous studies have shown that under short day photoperiods, aggression in male California mice and oldfield mice is rapidly modulated by estradiol administration [19] and is correlated with reduced estrogen-dependent gene expression [20]. Since melatonin can modulate both estrogen-dependent gene expression [31] and ERα expression [30], we hypothesized that melatonin might affect aggression by modulating estrogen dependent gene expression. Overall, this hypothesis was not supported. In rats, OTR is regulated by an ERE [26], and we demonstrated that letrozole, an aromatase inhibitor, significantly reduced OTR gene expression in the BNST of male California mice. Our lab has also previously found that c-fos expression in the BNST of California mice was estrogen dependent [19]. However, neither melatonin nor luzindole treatment affected the expression of OTR or c-fos in the BNST. It is possible that testosterone may play an important role in photoperiod dependent changes in gene expression in the BNST. Reduced estrogen dependent gene expression in the BNST was observed in oldfield mice, a species in which short days reduce testosterone levels [42]. Short days do not reduce testosterone levels in California mice [14], which may explain the differences in gene expression patterns between the two species. Although short days clearly have important effects on estrogen-dependent signaling, it appears that the behavioral effects of melatonin are not directly mediated by changes in estrogen-dependent gene expression in the BNST.

One alternative mechanism for melatonin regulation of BNST activity is via extracellular signal-regulated kinase (ERK). When slices of the suprachiasmatic nucleus (SCN) of Wistar rats (*Rattus norvegicus*) and Syrian hamsters were prepared for immunoblotting following melatonin administration, it was found that pERK was upregulated in slices that had received melatonin treatment [43]. Interestingly, pERK is part of a signaling pathway that is upregulated in the BNST and medial amygdala (MEA) of male California mice housed in short day photoperiods following a resident-intruder test [3]. In the BNST, the number of pERK positive cells is also correlated with the number of bites demonstrated during a resident-intruder test [3]. It is possible, therefore, that melatonin facilitates aggression through upregulation of pERK in male mice. Female California mice also demonstrate greater levels of territorial aggression in short day photoperiods [2]. pERK, however, is upregulated in the BNST following aggressive encounters under both short and long day photoperiods [2]. This suggests that there are important differences in the mechanisms by which short days increase aggression in males and females. Future studies are needed to more directly examine the role of melatonin and pERK signaling.

4.2. The MPOA: important for male aggression in short days?

An unexpected result was that melatonin reduced OTR expression in the MPOA, an effect that was reversed by luzindole treatment. In an influential review on the neural control of male rodent social behaviors, Sara Newman summarized evidence for a social behavior network controlling sexual and aggressive behaviors [44]. At that time most evidence suggested that while the MPOA was a critical nucleus for the regulation of male sexual behavior, its role in male aggressive behavior was more limited. However, these studies were mostly conducted under long day photoperiods (i.e. [45,46]). There is emerging evidence that the relationship between the MPOA and male aggression may be context-dependent. Under short day photoperiods, male California mice show an increase in c-fos protein in the MPOA [14]. Male California mice that have had pups also have elevated c-fos in the MPOA following aggressive encounters [14] as do female *M. musculus* following a maternal aggression test [47,48]. Increased c-fos expression in the MPOA has also been associated with higher levels of aggressive behavior in greater long-tailed hamsters (*Tscheskia triton*) [49], indicating that the MPOA may play a more important role in modulating aggression under certain contexts. Although we did not observe an increase in c-fos mRNA in the MPOA of mice treated with melatonin, the timeline for collecting brains (immediately after behavior testing) may not have been optimal for detecting an increase in c-fos gene expression. These results suggest that there may be complementary neural pathways mediating aggressive behavior: one pathway through the MPOA that is independent of estrogen-dependent gene expression, and one pathway through the BNST that is dependent on estradiol acting rapidly through estrogen-dependent gene expression [20].

It was unexpected that letrozole did not affect OTR expression in the MPOA, given previous work in female rats showing that OTR expression in the MPOA is estrogen dependent [50]. Regulation of the OTR promoter can be facilitated by transcription factors other than ERE's [51], including the transcription factor activator protein 1 (AP-1) and nuclear factor kappa B (NF-κB). These alternate promoters may control OTR expression in brain regions where nuclear estrogen receptors are not expressed, such as the nucleus accumbens [52–55]. Interestingly, both endogenous and exogenous melatonin can suppress NF-κB binding [56], consistent with our findings that melatonin reduces OTR gene expression. Our results also demonstrate that the melatonin receptor antagonist luzindole suppressed melatonin's inhibition of OTR mRNA expression, indicating that melatonin is mediating OTR gene expression via MT_1 and MT_2. Indeed, it has been shown that the MT_2 antagonist 4P-PDOT suppressed the inhibition of OTR by melatonin administration [57] providing evidence that MT_2 is responsible for mediating OTR gene expression. This is in contrast to our findings that luzindole did not ameliorate the effects of melatonin on aggressive behavior. Although luzindole did not have a significant effect on aggression, our results show that luzindole partially blocked the effects of melatonin on behavior. It may also be the case that the 40 mg/kg dose of luzindole used in these studies only lead to a partial blockade of MT receptors which would provide an explanation for why luzindole did not fully block the effects of melatonin on behavior. This explanation is unlikely, however, since a 30 mg/kg dose of luzindole used on a related species of *Peromyscus*, the white-footed mouse (*Peromyscus leocopus*), caused a significant increase in plasma melatonin [58], suggesting the efficacy of a lower dose of luzindole than used in the current studies. Taken together, our data suggest that melatonin may act via receptor dependent and receptor independent mechanisms to affect aggression. It is worth noting, however, that MT_2 is not functional in all species, such as Siberian hamsters [59]. The functionality of this receptor has not yet been tested in California mice, and requires further investigation.

4.3. Conclusions

In the current studies, we have shown that administration of exogenous melatonin increases aggressive behavior in long day housed California mice, but luzindole only partially ameliorated this phenotype. Our findings that melatonin suppressed OTR in the MPOA is consistent with recent evidence suggesting a possible context-dependent role for MPOA modulation of male aggression. This effect was reversed by luzindole. The fact that luzindole affected behavior and OTR gene expression in opposing manners suggests that melatonin may be acting via receptor dependent and independent mechanisms to mediate aggression. The inability of melatonin to alter estrogen-dependent gene expression in the BNST does not support the hypothesis that the behavioral effects of melatonin require a broad suppression of estrogen-dependent genes.

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