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Original Communication

Melatonin and suppression of the transport response in rat pups (*Rattus norvegicus*)

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ABSTRACT

Elevated levels of either endogenous or exogenous melatonin modulate physiology and behavior in many vertebrates. However, there are no data that address the hormonal action of melatonin on the behavioral transport response in rat pups though there is much evidence suggesting that serotonin, the anabolic precursor to melatonin, modulates this behavior. We conducted two experiments that demonstrate melatonin's effect on the behavioral transport response in the laboratory rat (*Rattus norvegicus*). In our first experiment, we found a significant effect of age. We then controlled for age as a variable in our second experiment to investigate possible dose effects of melatonin. We found all doses of melatonin to significantly suppress the transport response, and briefly discuss the neurochemistry and ontogeny for the effects of melatonin on the transport response in rats. This study is the first to document the suppression of the transport response in rats by melatonin and could serve as a new experimental model for investigating the influence of melatonin on the behavioral physiology of vertebrate systems. More importantly, the evolutionary significance of melatonin's role in physiologically modulating activity and the behavioral transport response may be of interest to future investigators.

KEYWORDS: melatonin, serotonin, transport response, *Rattus norvegicus*, behavior, behavioral physiology, endocrinology

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INTRODUCTION

The transport response, first described by Brewster and Leon [1], is a behavioral response of rat pups between 8 and 28 days of age. A firm grasp of the pup's dorsal surface elicits flexion and adduction of hind limbs, extension and adduction of forelimbs, and adduction of the tail creating a compact body posture or "package" (Figure 1) for transport [2]. This behavioral transport response allows a parental female to efficiently carry and transport pups. Pups which do not demonstrate the transport response are often dragged, stepped on, and possibly abandoned by the parental female. Because this behavior may be critical to a young rat's survival, this behavior is highly conserved and has served as an excellent behavioral model for testing pharmacological agents [3-6].

Melatonin (*N*-acetyl-5-methoxytryptamine), secreted by the pineal gland, is known to influence the physiological and behavioral rhythms of vertebrates [e.g. 7-12]. In the absence of light stimuli, postganglionic fibers innervating the mammalian pineal gland stimulate pinealocytes to produce endogenous melatonin from tryptophan, with serotonin and *N*-acetylserotonin as intermediates in the biochemical pathway [13, 14]. Melatonin is then metabolized in the liver to 6-hydroxymelatonin. Although studies have addressed the effects of serotonin and serotonergic agents on the behavioral transport response of rats [6], there are no data that address the hormonal action of melatonin on this behavior.

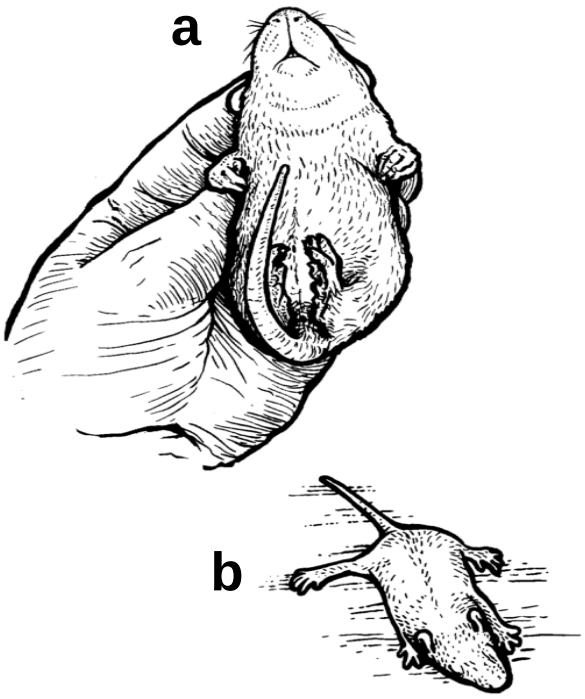

Figure 1. Illustration of the transport response in rat pups first described by Brewster and Leon [1] in which all appendages are adducted to the ventrum (a), giving a transport response score of five. This differs from a typical body position (b), prior to eliciting the transport response.

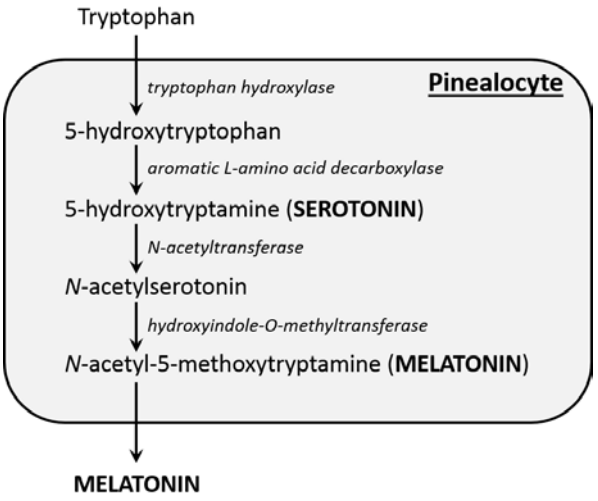

Figure 2. Illustration modified from Lutterschmidt *et al.* [14] showing a simplified biosynthetic pathway for the synthesis of melatonin by the pinealocyte. Enzymes involved in the anabolism of melatonin are shown in italics.

Elevated levels of both endogenous and exogenous melatonin have been shown to modulate physiology and behavior in many vertebrates. For example, exogenous melatonin significantly lowers temperature preferences in several ectothermic species [15-19] and reduces thermal tolerance in both adult *Necturus maculosus* [20] and in *Mus musculus* [21]. Pharmacological doses of melatonin during the day induce sedation and hypothermia, reduction in food intake, and suppression of the hypothalamic-pituitary-adrenal (HPA) axis in rodents [22-26]. Melatonin can also influence stress and stress responses [e.g. 27-29]. Antigonadotropic influences of melatonin occur in various species including hamsters [30] and the lizards, *Calotes versicolor* [31] and *Anolis carolinensis* [32]. Similarly, chronic administration of melatonin inhibits sexual activity in rats [e.g. 33-35]. More recent literature has reviewed the role that melatonin may play in the adaptive behavior of animals to their environment, including entrainment of daily and annual physiological rhythms, behavior and performance [e.g. 36]. Recent studies using rats as experimental models [e.g. 37-39] have also investigated the significance of melatonin as a hormone that modulates physiology and behavior. For example, melatonin is shown to reduce reward seeking, aggression and activity in the diurnal fat sand rat [37] and melatonin's suppression of the perceptual component of nociception in male Wistar rats resulted in increased perception of noxious thermal stimuli and improved memory retrieval for environmental signals [40].

Tactile stimulation by the parental female rat induces the secretion of serotonin in rat pups and intensifies their transport response [2, 6]. Because serotonin is a precursor in the anabolism of melatonin (Figure 2), we were interested in evaluating the effects of melatonin on the transport response in rats. Elevated levels of endogenous serotonin may later increase subsequent levels of melatonin thus suppressing the transport response. We conducted two experiments to investigate the following questions: (1) Does melatonin suppress the transport response in rat pups?; (2) Does melatonin differentially affect the transport response with respect to age and sex?; (3) Is there a dose effect of melatonin on the transport response?, and (4) Is there a time course for the catabolism of melatonin indicated by transport response recovery?

MATERIALS AND METHODS

Animals, acclimation, and captive care

Sprague-Dawley albino rats 12, 18, and 22 days of age were used in this study. All individuals were obtained from an established breeding colony housed in the Sam Houston State University animal facility. Rats were housed in plexiglass breeding cages within an environmentally-controlled room maintained at 25 °C. All rats were acclimated to a 12L:12D photoperiod with the photophase centered on 1300 h CST and beginning at 0700 h. Food and water were available *ad libitum*. All captive care regimes followed the guidelines of the National Institute of Health and the National Science Foundation for the ethical care and treatment of animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Sam Houston State University [41].

The breeding regimes described here follow that of Wilson and Pulido [6]. Prior to parturition, pregnant female rats were placed in breeding cages with pine-shaving bedding. Cages were checked daily at 0900 and 1600 h for the presence of recently born litters. The day a new litter was observed, the day was deemed postnatal (PN) day zero (PN-0). On PN-1, the litters were culled to eight to ten animals and were subjected to experiments on either PN-12, 18, or 21. On the day that pups were tested, each pup was assigned randomly to an experimental treatment (i.e. control or melatonin) based upon a split-litter design and placed in a cage containing fresh bedding.

Reagents

We purchased melatonin (*N*-acetyl-5-methoxytryptamine) from Sigma Chemical Company (St. Louis, MO). Doses of melatonin (25 mg kg^{-1}, 5 mg kg^{-1}, and 1 mg kg^{-1}) were prepared by dissolving 25 mg, 5 mg, or 1 mg of melatonin in 1.0 ml of 95% ethanol and 19 ml of mammalian ringers solution. Control injections also consisted of a 1:19 ratio of 95% ethanol and mammalian ringers. Injection volumes for the control and melatonin treatments were 2% body mass (M_b).

Transport response

The transport response (Figure 1) is a behavioral response displayed by rat pups between 8 and 28 days of age. The transport response was elicited by grasping and squeezing firmly (using our thumb and first two fingers) the nape of each rat pup [5]. A subject received a point for each appendage (i.e. forelimbs, hind limbs, and tail) that was adducted to the ventrum, giving a minimum score of zero and a maximum score of five. Thus, we scored qualitatively the transport response intensity using a relative scale ranging from 0 to 5. A mean transport response for each pup was calculated from three independent trials separated by 2 min intervals. This mean transport response now provided a continuous measure (range = 0 to 5) for statistical analyses.

Experimental procedure

Randomly selected rat pups were sexed, weighed, and assigned to one of two treatment groups (i.e. control or melatonin). Double-blind experiments were performed in which the experimenter administering the injections and the experimenter testing the transport response were unable to identify treatment groups. This was accomplished by labeling the injection solutions and the treatment groups as “red” or “green”, in which only the experimenter preparing the injection solutions knew which was the control and which was the melatonin injection solution.

Prior to a pup receiving an intraperitoneal injection, we tested its “pre-injection” transport response. All experiments were performed in the middle of the photophase with injections being administered between 1200 h and 1300 h. After injection, the pup was placed in a cage and tested for the transport response every 30 min over a 2 h period. This testing period was determined *a priori* because the half-life of melatonin in endotherms is 1 h or less [42].

We conducted two separate experiments in this study. Experiment-I first identified if a pharmacological dose (25 mg kg^{-1}) of melatonin could suppress the transport response in rat pups. After we established that melatonin suppressed the transport response in rat pups 30 min after injection, we investigated the possible time-course by testing for this suppressed transport response at 120 min. In Experiment-II, we used only PN-18 pups to test for a possible dose response and determine the time-course of melatonin. These data from only PN-18 rat pups were used also to investigate possible differences in the time-course of the suppressed transport response among all three melatonin doses.

Statistical analyses

In Experiment-I, we used a general linear model, 3x2x2 factorial, model one, three-way analysis of variance to investigate possible differences among age (PN-12, 18, and 22), between sex (male and female), and between treatments (control and melatonin injections). In Experiment-II, we used a general linear model, 3x5 factorial repeated measures two-way analysis of variance with dose (25 mg kg^{-1}, 5 mg kg^{-1}, 1 mg kg^{-1} M_b) and time (pre-injection, 30 min, 60 min, 90 min, and 120 min) as the repeated factor.

Bonferroni multiple comparisons procedures [43, 44] were used *a posteriori* in Experiment-I because the investigation of treatment differences were between a "control" injection and the melatonin injection. These same multiple comparison procedures were used in Experiment-II because transport response time-0 (pre-injection) served as a single "control" or pre-group that was compared to the repeated "treatment" or testing of the transport response (i.e. at time-30, time-60, time-90, and time-120). A Student-Newman-Keuls multiple comparison procedure [44] was used to compare mean transport response among different melatonin doses.

We used SPSS7 8.0 and SigmaStat 11.0 statistical software for all analyses and graphical representation of data. Results were considered significant at $P < 0.05$ and all assumptions of normality and equal variance were tested and met prior to analyses.

RESULTS

Experiment-I: Age, sex, and treatment effects with a pharmacological dose of melatonin

We found a significant effect of age in the transport response (Table 1). Bonferroni pairwise multiple comparison procedures [44] indicated that only PN-18 and PN-22 rat pups differed in the transport response. The least square means of transport response among age showed both PN-12 pups (mean = 2.25, SE = 0.414, n = 8) and PN-22 pups (mean = 1.35, SE = 0.359, n = 11) to have the lowest transport response and that they did not differ statistically (t = 1.65, P = 0.35) from each other. The highest mean transport response was by PN-18 pups

Table 1. Results of a 3x2x2 factorial, model one, three-way analysis of variance investigating differences in transport response (at 30 min post-injection) indicate significant effects of age (PN-12, 18, and 22) and treatment (control and melatonin) on the transport response. Bonferroni pairwise multiple comparison procedures [44] indicated that only PN-18 and PN-22 rat pups differed in transport response and that the transport response was reduced significantly by melatonin injections in comparison to control injections. Sample sizes of rat pups for PN-12, 18, and 22 were 8, 11, and 11, respectively with a 50:50 sex ratio between control and melatonin injection treatments; for n = 11, a sex ratio of 6 males to 5 females was used.

Source	SS	df	MS	F	P
Age	19.039	2	9.520	6.940	0.006
Sex	0.296	1	0.296	0.216	0.648
Treatment	54.683	1	54.683	39.865	<0.001
Age x Sex	1.335	2	0.668	0.487	0.622
Age x Treatment	3.853	2	1.926	1.404	0.271
Sex x Treatment	4.289	1	4.289	3.127	0.094
Age x Sex x Treatment	0.349	2	0.175	0.127	0.881
Residual	24.691	18	1.372		
Total	109.038	29	3.760		

(mean = 3.26, SE = 0.368, n = 11). Although PN-12 pups did not differ from PN-18 ($t = 1.83$, $P = 0.25$), the lowest transport response by PN-22 pups did differ significantly from this highest transport response by PN-18 ($t = 3.72$, $P = 0.005$). This significant effect of age with PN-12 and PN-22 pups demonstrating a greater suppression in transport response may indicate that these ages have a greater initial (time-30 min) sensitivity to melatonin than PN-18 pups.

We found no differences in transport response between sex and no significant interactions among age, sex, and treatment (Table 1). However, we did find a significant treatment effect (Table 1) indicating that a pharmacological dose (25 mg kg^{-1} M_b) of melatonin significantly suppressed the transport response in rat pups (at 30 min post-injection) compared to control injections.

We ran an identical and independent analysis to investigate if these results (at 30 min post-injection) were conserved at the end of the 120 min testing and observation period for transport response. At 120 min post-injection, there was no longer an age effect ($F = 1.461$; $df = 2, 18$; $P = 0.258$) and only treatment differences between control injections and melatonin injections were significant ($F = 63.795$; $df = 1, 18$; $P < 0.001$). This may indicate that age differences in transport response may be a significant factor only for the initial increase in exogenous melatonin. These results were further investigated in a second experiment to address the potential time-course of melatonin.

Experiment-II: Dose response and time-course of melatonin

Because we observed an effect of age on the 30 min post-injection transport response (Table 1), we controlled for age in this experiment by using only PN-18 rat pups to test for a potential dose effect and the time-course of melatonin. We used PN-18 to be conservative as this age group showed the highest transport response (i.e. lowest reduction in transport response) to the pharmacological dose of melatonin.

Similar to the pharmacological dose of melatonin (Figure 3a), we found the transport response to be significantly reduced by the melatonin dose of 5 mg kg^{-1} (Figure 3b) and 1 mg kg^{-1} (Figure 3c). The two-way repeated measures analysis of variance indicated that there was no significant effect among the three different melatonin doses ($F = 0.284$; $df = 2, 64$; $P = 0.756$). All transport

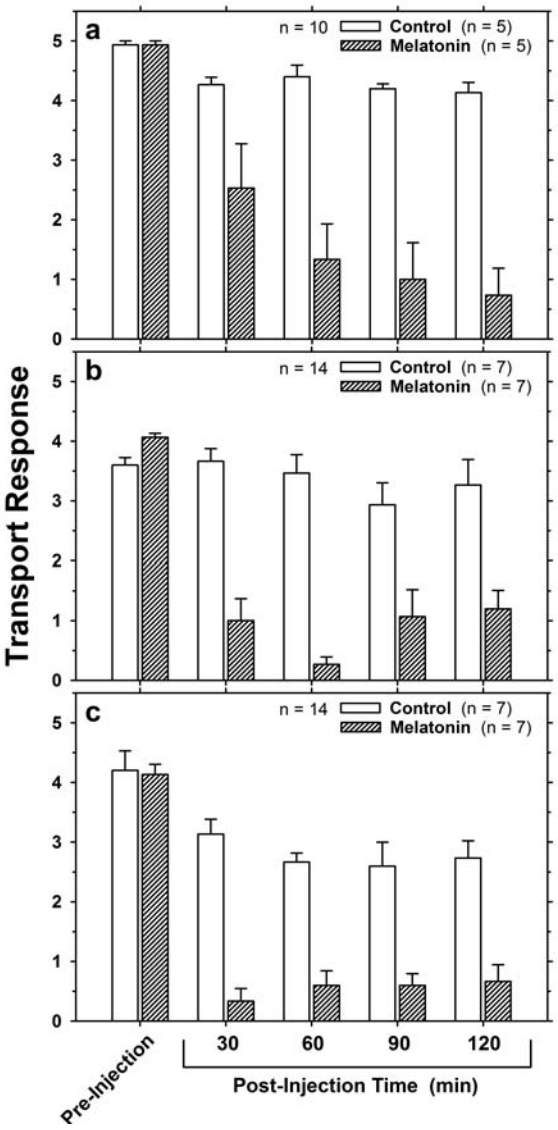

Figure 3. Comparison of mean transport response of PN-18 rat pups between control injections (white bars) and melatonin injections (hashed bars) for the pre-injection and all post-injection times over the entire time-course of 120 min. Melatonin doses 25 mg kg^{-1} M_b (a), 5 mg kg^{-1} M_b (b), and 1.0 mg kg^{-1} M_b (c) are shown. Caps above each bar indicate the standard error (SE) of the mean transport response. A total of 38 PN-18 rat pups were used in this experiment and sample sizes among melatonin doses and between treatments (control and melatonin) are indicated in each graph panel (a, b, and c).

responses in rat pups among melatonin doses were similar over the entire time-course (Figure 4). The repeated measure of time (pre-injection and post-injection 30 min, 60 min, 90 min, and 120 min) was significant ($F = 79.396$; $df = 4, 64$; $P < 0.001$). Multiple comparison procedures indicated that only the pre-injection differed from all other post-injection transport responses over the entire time-course of 120 min (Figure 4).

A gradual (but non-significant) increase in the transport response between 60 min and 120 min for PN-18 pups was observed in the 5 mg kg^{-1} melatonin treatment group (Figure 3b). In these experiments, we observed the transport response over a 120-minute time-course in an attempt to observe transport response recovery. Because our results showed that rat pups do not regain their transport response after 120 minutes post-injection for any of the melatonin doses (Figures 3 and 4), rat pups may not demonstrate a typical dose-response curve or catabolize melatonin in the expected endothermic half-life [42].

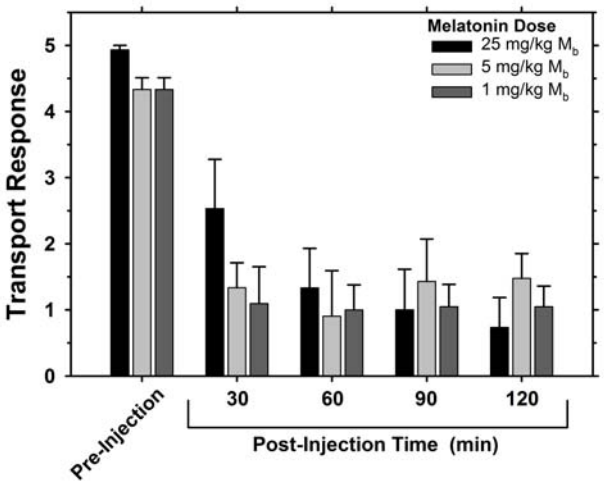

Figure 4. Illustration of mean transport response of PN-18 rat pups taken from figure 3 without control data allowing for direct comparison among all melatonin doses over the entire time-course of 120 min. Melatonin doses are presented in the same order as presented in figure 3 where 25 mg kg^{-1} M_b, 5 mg kg^{-1} M_b, and 1.0 mg kg^{-1} M_b are represented by black, light grey, and dark grey bars, respectively. Caps above each bar indicate the standard error (SE) of the mean transport response.

DISCUSSION

Transport response suppressed by a pharmacological dose of melatonin

Our results in Experiment-I clearly demonstrate that elevated levels of exogenous melatonin significantly disrupt the behavioral transport response in rat pups. We also found this suppression of the transport response by melatonin to be dependent on age (Table 1). Our data indicate that PN-12 and PN-22 rat pups are more sensitive than the PN-18 rats. Ristine and Spear [45] asserted that precocial portions of serotonergic (5-HT) systems may mediate some behaviors critical to survival in young, altricial animals, while the later-developing portions of these systems, which mediate adult behaviors, may suppress these early systems. Based upon this assumption, Wilson and Pulido [6] investigated age-related differences in transport response intensity with serotonergic agents in rats. They reported that administration of the 5-HT antagonists ketanserin and cinanserin resulted in a significant suppression of the transport response in PN-23 rats. In addition, administration of the 5-HT agonist quipazine reinstated the transport response in 40- and 50-day-old rats, animals typically too old to show the response [6]. Given that quipazine has an affinity for 5-HT3 receptors [46], Wilson and Pulido [6] speculated that giving quipazine may induce enough activity in the 5-HT3 receptors to overcome more later-developing aspects of these systems, thus causing a reinstatement of the transport response.

With respect to melatonin, it may be that this hormone interacts with the precocial portions of these systems or with aspects of the later-developing portions of these systems, resulting in a suppression of the response. The nature of how melatonin causes this suppression is unknown, but given that melatonin reduces arousal, it is possible that developing rats, when under the influence of melatonin, lack the ability to actively adduct their tail and limbs, a necessary condition for demonstrating the response [5]. An alternative explanation for the effects of melatonin reported here is that the drug may be interacting with other neurotransmitter systems (i.e. dopamine, acetylcholine, and norepinephrine) that have been related to the elicitation and/or suppression of the transport response [3, 4, 47]. Overall, our results suggest that elevated melatonin levels disrupt the transport response, suggesting

that melatonin modulates mechanisms mediating transport behavior.

In our experiments, we tested for the transport response every 30 min over a 2 h period (i.e. 120 min post-injection) to ensure mainly that we did not miss the potential observation of a suppressed transport response by melatonin. The 120 min testing period was determined *a priori* because the half-life of melatonin in endotherms is documented and considered to be an hour or less [42]. It is interesting that our results show that rat pups never regained their transport response after 120 minutes post-injection and that all melatonin doses (Figures 3 and 4) showed the same response over the time-course. Lutterschmidt *et al.* [19] showed the time-course for an ectotherm to return to its set temperature within 3 to 9 h of a first melatonin injection. After a second injection this time-course increased significantly with some animals requiring as much as 24 h to return to their set temperature. Regardless of dose, the potential difficulty in evaluating the time-course of melatonin's action is that the actual elevated plasma levels following injection are unknown. Although we were not able to show the time-course for a regained transport response in rat pups nor demonstrate a typical dose response [48], we showed that a 1 mg kg^{-1} M_b dose has a similar effect on suppressing the transport response as does a pharmacological dose.

Neurochemistry and the transport response

The transport response is not displayed reliably until the second postnatal week [1], which corresponds to maturation of several neurochemical systems. Neurotransmitters shown to be important in controlling transport responses include dopamine [49, 50], norepinephrine [5, 50], serotonin [6], and acetylcholine [4]. This suggests that the behavior is mediated by several neurotransmitter systems, which above all act in concert to ensure that pups display appropriate postures to facilitate effective maternal transportation. Indeed, coordination of the transport response with isolation calling (ultrasonic vocalizations) appears to be an important learning process within the nest [51].

Ontogeny of the melatonin system

Pineal melatonin is detectable within 3 to 4 days after birth [52]. The light entrainment of melatonin levels occurs during the second postnatal week, with patterns of adult control emerging by the third week [52-56]. Rats as young as five days old can metabolize exogenously-injected melatonin, though plasma melatonin levels were significantly elevated after 4 hours following the injection; PN-10 rat pups had a much higher clearance rate [57]. The influence of age on a rat's ability to metabolize melatonin may offer a plausible explanation for the effects of age on the transport response demonstrated in Experiment-1.

Melatonin and its effects on behavior and neurocircuitry

Melatonin has inhibitory effects on a variety of adult rat behaviors and physiologies, including motor movements, heart rate responses, startle reflexes, and lordosis reflexes [33, 58], while facilitating sexual behaviors [59, 60]. Though melatonin effects on rat pup behavior remains unexplored, exogenous melatonin reduces the number of isolation distress calls in young chickens [61] and in lactating female rats, it has been shown to inhibit suckling-induced oxytocin release [62]. Several studies suggest that melatonin may inhibit serotonergic-mediated behaviors by serving as a serotonergic receptor antagonist [23, 63, 64]. Also, melatonin has been shown to inhibit the release of dopamine [65]. Given the importance of serotonin and dopamine in mediating the transport response [5, 49], antagonism of serotonin and dopamine by melatonin offers a plausible mechanism for melatonin's suppression of the transport response. More importantly, both serotonin's and melatonin's roles in physiologically modulating activity and behavior may have greater evolutionary significance. The tactile stimulation by parental female rats increases endogenous levels of serotonin thus intensifying the transport response. This subsequent increase in serotonin (a precursor to the anabolism of melatonin) may increase endogenous levels of melatonin thus suppressing exploratory activity and behavior after initiation of the transport response and physical transport of the rat pups to a new nest [41] and may be evolutionarily adaptive.

CONCLUSION

We conducted two experiments which show that (1) melatonin suppresses the transport response in

rat pups, (2) melatonin differentially affects transport response with respect to age but not sex; (3) melatonin has no detectable dose effect as all doses of melatonin suppressed the transport response significantly; and (4) melatonin suppresses the transport response over the entire 120 min time-course. This study is the first to document the suppression of the transport response in rats by melatonin and could serve as a new experimental model for investigating the influence of melatonin on the behavioral physiology of vertebrate systems. More importantly, the evolutionary significance for melatonin's role in physiologically modulating activity and the behavioral transport response may be of interest to future investigators.

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CONFLICT OF INTEREST STATEMENT

The authors acknowledge no conflict of interest regarding the data, analyses, and interpretations presented in this manuscript.

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