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Melatonin potentiates the anticonvulsant action of phenobarbital in neonatal rats

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Abstract

Phenobarbital is the most commonly utilized drug for neonatal seizures. However, questions regarding safety and efficacy of this drug make it particularly compelling to identify adjunct therapies that could boost therapeutic benefit. One potential adjunct therapy is melatonin. Melatonin is used clinically in neonatal and pediatric populations, and moreover, it exerts anticonvulsant actions in adult rats. However, it has not been previously evaluated for anticonvulsant effects in neonatal rats. Here, we tested the hypothesis that melatonin would exert anticonvulsant effects, either alone, or in combination with phenobarbital, the most commonly utilized anticonvulsant in neonatal medicine. Postnatal day (P)7 rats were treated with phenobarbital (0–40 mg/kg) and/or melatonin (0–80 mg/kg) prior to chemoconvulsant challenge with pentylenetetrazole (100 mg/kg). We found that melatonin significantly potentiated the anticonvulsant efficacy of phenobarbital, but did not exert anticonvulsant effects on its own. These data provide additional evidence for the further examination of melatonin as an adjunct therapy in neonatal/pediatric epilepsy.

Keywords

melatonin; phenobarbital; seizure; neonatal; pentylenetetrazole; GABA; neurprotection; apoptosis; adjunct therapy

Introduction

Identification of adjunct treatments for neonatal seizures is a high priority, in large part due to the adverse side effect profile and questionable efficacy of current first-line treatments (Sankar and Painter, 2005). Presently, the most commonly used drug for the treatment of neonatal seizures is phenobarbital (Bartha et al., 2007; Blume et al., 2009). Identification of compounds that would allow lower doses of phenobarbital to be utilized, reducing side effects associated with this treatment and/or ameliorate other safety concerns associated with phenobarbital would be ideal.

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One compound with an established safety record in humans, and promise as an adjunct therapy is melatonin. Melatonin has been examined in neonates with respiratory distress, sepsis, or hypoxia-ischemia. In these patient populations the high doses (e.g., 10 mg/kg) appears to be well tolerated (see Aversa et al., 2012 or Gitto et al., 2011 for a review). While no studies of melatonin have been conducted for neonatal seizures, data from children and adults suggest that melatonin is not only well-tolerated, but may have clinical benefits. For example treatment with 10 mg/day of melatonin decreased diurnal seizures in a study of ten patients with intractable epilepsy (Goldberg-Stern et al., 2012). Treatment with 3 mg of melatonin before bed reduced seizures in five of six patients in another small trial (Peled et al., 2001). A single case has been reported using higher doses of melatonin (>5 mg/kg/day) in a child with severe myoclonic epilepsy, which also indicated an anti-seizure effect of melatonin treatment (Molina-Carballo et al., 1997). Above and beyond anticonvulsant effects of melatonin, a small clinical trial of melatonin as an adjunct therapy with valproate showed an increase in quality of life in children with epilepsy (M. Gupta et al., 2004).

In mouse seizure models, melatonin has been documented to potentiate the anticonvulsant action of phenobarbital and carbamazepine against electroshock seizures in adult animals (Borowicz et al., 1999). Melatonin has also been reported to exert an anticonvulsant action when given alone to adult rats, mice, hamsters, and guinea pigs (Albertson et al., 1981; Bikjdaouene et al., 2003; Costa-Lotufo et al., 2002; Golombek et al., 1992; Solmaz et al., 2009; Yahyavi-Firouz-Abadi et al., 2007) and to postnatal day (P)21 rats (Costa-Lotufo et al., 2002). However, the effects of melatonin alone or in combination with phenobarbital have not been examined in neonatal animals.

To examine the effects of melatonin on the anticonvulsant action of phenobarbital in neonatal rats, we selected seizures evoked by pentylenetetrazole (PTZ, 100 mg/kg) in P7 rat pups. This model, as described extensively by Mares and colleagues (Kubová and Mares, 1993; Kubova and Mares, 1991; Stankova et al., 1992), and previously employed by our group (Forcelli et al., 2012c), has been widely used to evaluate the anticonvulsant efficacy of compounds (including phenobarbital) in developing rats. Pups were pretreated with doses of phenobarbital (10–40 mg/kg) consistent with the anticonvulsant range previously described in rat pups (Kubova and Mares, 1991). Doses of melatonin (20–80 mg/kg) were selected based on the anticonvulsant range previously described in adult animals (Bikjdaouene et al., 2003). It is worth noting that even the highest dose utilized in our study (i.e., 80 mg/kg) is similar to those used in human neonates when allometric scaling is applied.

Methods

Animals

Female Sprague-Dawley (Harlan, Indianapolis, IN, U.S.A.) rats were used to generate the 121 pups used for these experiments. Pups were maintained with their dam until testing on postnatal day (P)7. This age is approximately equivalent to term infants in humans (Dobbing and Sands, 1979). Animals were housed in the Georgetown University Division of Comparative Medicine in temperature (21°C) controlled rooms with a standard 12h light cycle (lights on 0600h). All experiments were approved by the Georgetown University Animal Care and Use Committee and conducted in accordance with AALAC recommendations and the *Guide for Care and Use of Laboratory Animals* (National Research Council (U.S.) et al., 2011).

Drugs

Phenobarbital (Sigma-Aldrich) was dissolved in saline at a concentration of 1, 2, and 4 mg/ml to allow a standard volume of injection (0.01ml/g body weight). Melatonin (Sigma-Aldrich) was dissolved in 1% ethanol in saline with 1% tween immediately before use. Respective vehicle controls (saline for phenobarbital; 1% ethanol, 1% tween in saline for melatonin) were used for comparison. Drug doses were randomized within groups of animals, such that each group was represented during a given test session.

Seizure testing

PTZ (100 mg/kg, s.c.) was dissolved in saline. Animals were removed from their home cage, weighed, numbered, and treated with phenobarbital 90 min before PTZ injection. This time was selected on the basis of the previously described time-course of phenobarbital action in neonatal animals (Kubova and Mares, 1991). Melatonin was administered 30 min before PTZ injection. This time was selected on the basis of the previously described time-course of melatonin action against PTZ seizures in adult rats using a dose range equivalent to that in the present study (Bikjdaouene et al., 2003). Animals were returned to their dam to maintain body temperature until immediately prior to PTZ testing. PTZ was injected, and animals were placed in clear plexiglass boxes for observation of seizure activity. Latency to seizure onset, as well as the incidence of seizures, were recorded by treatment-blind observers (P.A.F., C.S., and/or A.D.). Animals were observed for 25 min following PTZ injection.

Seizure scoring

Both incidence of seizures of different severities and latency to seizure onset were recorded. Seizure duration was not recorded because using this dose of PTZ in rat pups of this age, seizures typically last the entire observation period. To assess seizure severity we employed the rating system of Kubova and Mares (Kubová and Mares, 1993) to allow consistency across studies from our lab (Forcelli et al., 2012c) and theirs (Kubová and Mares, 1993; Kubova and Mares, 1991; Mares et al., 1989; Stankova et al., 1992) that have assessed anticonvulsant action against PTZ in neonatal rats. The rating system was: 0 = no change in behavior, 1 = myoclonic jerks, 2 = unilateral clonus, chewing/shuffling, Straub tail, 3 = facial and forelimb clonus, 4 = running/bouncing clonus with loss of righting, 5 = running/bouncing clonus with loss of righting and tonic extension (this is equivalent to the “complete major seizure” described by (Kubová and Mares, 1993)). We report mean latencies only for groups with a seizure incidence of at least 50%

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, LaJolla, CA). Seizure latencies were analyzed using a one-way analysis of variance with Holm-Sidak post hoc tests (one-tailed). Seizure scores were analyzed in using two methods 1. Because these data are non-parametric in nature, a Kruskal-Wallis tests with Dunn’s post-hoc (one-tailed) was used 2. Contingency tables for proportions of animals displaying minimal vs. running bouncing clonic and tonic-clonic seizures were evaluated using Fisher’s exact test (Kirkman, n.d.), as has been previously employed for these types of data (Forcelli et al., 2012c; Kubova and Mares, 1991; Stankova et al., 1992). Adjusted standardized residuals were calculated for tables that had significant Fisher’s Exact Test results and used as post-hoc measures of cell-by-cell contributions to the overall effect. Values for residuals greater than 1.96 are statistically significant at the $P < 0.05$ level.

Results

PTZ seizure response in vehicle controls

As shown in Figure 1a, vehicle pretreated animals displayed a mean seizure score of 4.5, corresponding to running/bouncing behavior, but no tonic extensor component. As shown in Figure 1b, vehicle pretreated animals displayed a mean latency to seizure onset of 157sec.

PTZ seizure response in animals pretreated with melatonin and/or phenobarbital

Treatment with melatonin (20–80 mg/kg), in the absence of phenobarbital, did not significantly impact seizure score or seizure latency. Moreover, when animals were treated with the lowest dose of phenobarbital (10 mg/kg), either alone or in combination with melatonin (20, 40 or 80 mg/kg), neither seizure severity, nor latency differed from controls.

When animals were treated with a dose of 20 mg/kg of phenobarbital, no significant decrease in seizure severity was obtained. When this dose of phenobarbital was combined with a high dose of melatonin (80 mg/kg), animals displayed a significant reduction in seizure severity as compared to controls ($P < 0.001$), and as compared to 20 mg/kg phenobarbital without subsequent melatonin (Mann Whitney U, $U = 3.0$, d.f., 12, $P < 0.05$).

Latency to seizure onset was significantly ($p < 0.05$) increased relative to controls by 20 mg/kg of phenobarbital, alone, or in combination with 20 mg/kg of melatonin ($P < 0.05$, Figure 1B). While the combination of 20 mg/kg of phenobarbital and 40 mg/kg of melatonin did not induce a statistically significant increase in latency to seizure onset as compared to control, it is worth noting that the latency for this group does not significantly differ from the phenobarbital alone condition ($P > 0.05$).

The highest dose of phenobarbital tested (40 mg/kg) significantly attenuated seizure severity. Melatonin did not significantly modify this effect at any dose tested (Dunn's test, $P_s < 0.001$ as compared to control).

The above effects were revealed by omnibus analyses showed a main effect of group ($H = 83.88$, d.f. 121, $P < 0.0001$) on seizure score (Kruskal-Wallis test) and on latency to seizure onset (ANOVA, $F_{10,64} = 3.28$, $P < 0.005$).

To provide another quantification of drug action we examined the incidence of minimal vs. Stage 4 vs. Stage 5 seizures as a function of treatment. These data, expressed as both the count and as percent are shown in Table 1. Fisher's exact test was used to assess the contingency tables for these data. The data from controls (animals that received both vehicles, i.e., 0 mg/kg phenobarbital, 0 mg/kg melatonin) were included in the analysis of each of the four following contingency tables: each dose of melatonin without phenobarbital, 10 mg/kg phenobarbital and each dose of melatonin, 20 mg/kg phenobarbital and each dose of melatonin, and 40 mg/kg phenobarbital and each dose of melatonin.

These tests revealed that the 10 mg/kg ($P < 0.05$), 20 mg/kg and 40 mg/kg tables ($P < 0.001$) differed from expected distributions. Post-hoc analysis of adjusted standard residuals identified significant contributors to the overall effect as indicated in Table 1 by an asterisk.

Discussion

Our present findings demonstrate a dose-dependent potentiation of the anticonvulsant effects of phenobarbital by melatonin in P7 rat pups. Melatonin, when administered in the absence of phenobarbital failed to alter the latency to seizure onset, seizure score, or the relative percentages of animals displaying minimal vs. Score 4 or 5 seizures. However, melatonin

significantly potentiated the effect of phenobarbital as evidenced by increases in latency to seizure onset or a decrease in seizure severity; in particular, we found that the combination of 80 mg/kg melatonin and 20 mg/kg of phenobarbital completely abolished Score 4 and 5 seizures, an effect that was equivalent to that seen after treatment with 40 mg/kg phenobarbital alone.

The effects of phenobarbital we described are similar to those reported by Kubova and Mares (Kubova and Mares, 1991), who found that phenobarbital was most effective at reducing the tonic component of seizures in P7 animals (i.e., reducing Score 5 seizures). The right-shifted dose-response in our study as compared to that of Kubova and Mares may reflect the fact that we used Sprague-Dawley rats, whereas they used Wistar rats; differences in seizure responses between these strains have been previously reported (e.g., (Statler et al., 2008)).

Here, we found that melatonin given alone did not significantly attenuate seizures. In adult animals, melatonin has previously been reported to attenuate seizures induced by electroshock (Borowicz et al., 1999; Y. K. Gupta et al., 2004), kindling (Albertson et al., 1981), or certain chemoconvulsants (Golombek et al., 1992; Lapin et al., 1998). However, results regarding anticonvulsant effects against PTZ, with melatonin given to rodents using a protocol comparable to ours (10–30 min pretreatment) have been inconsistent (Bikjdaouene et al., 2003; Moezi et al., 2011; Xu and Stringer, 2008; Yahyavi-Firouz-Abadi et al., 2007, 2006). It has been suggested that melatonin may attenuate seizures via enhancement of GABAergic inhibition (Borowicz et al., 1999; Ray et al., 2004); this may explain the inconsistent findings. Furthermore, this could explain both the melatonin enhancement of phenobarbital and the relative insensitivity of PTZ seizures to melatonin alone in our present study. This also raises the possibility that melatonin may be more effective (either alone, or in combination with phenobarbital) in other models of neonatal seizures that do not directly manipulate GABAergic signaling (e.g., kainate, electroshock (Kim et al., 2010; Velíšek et al., 1992)), a question that merits further investigation.

In addition to the potential contribution of GABAergic signaling to melatonin's phenobarbital-potentiating effect, there are other possible mechanisms by which it may exert anticonvulsant action. These may include a nitric oxidergic pathway with the nitric oxide synthetase inhibitor L-NAME attenuating the anticonvulsant effect of melatonin in mice (Yahyavi-Firouz-Abadi et al., 2006). The effects of melatonin appear to be receptor-dependent, as co-administration of melatonin receptor antagonists have been shown to block the anticonvulsant effect of melatonin in several seizure models ((Moezi et al., 2011; Ray et al., 2004). Moreover, several melatonin receptor agonists have been shown to exert anticonvulsant effects in animal models (Aguiar et al., 2012; Fenoglio-Simeone et al., 2009). The degree to which specific receptors or signaling cascades contribute to the potentiation of phenobarbital's anticonvulsant effect by melatonin in the present manuscript remains to be determined.

Differences in the maturational state of the limbic seizure network in neonatal rats as compared to adults make it difficult to compare effects across different ages. In young animals (e.g., P7–P10), seizures are poorly organized, and the seizure semiology is completely different than that manifest at later ages (P17 and older; (Kim et al., 2010)). It should be noted that little is known about the maturational time course of the melatonin receptor system, which may vary across brain regions (Zitouni et al., 1996). Interestingly, Costa-Lotufo and colleagues found that while melatonin exerted anticonvulsant effects against pilocarpine-evoked seizures in P21 rats, it was significantly less efficacious than it was in adult animals (Costa-Lotufo et al., 2002).

The ability to protect the majority of animals from behavioral seizures by treatment with the combination of 20 mg/kg phenobarbital and 80 mg/kg of melatonin provides for an opportunity to explore electrographic seizure manifestations in the protected animals. This will be of particular interest because of the electroclinical decoupling (Painter et al., 1999; Sankar and Painter, 2005; Scher et al., 2003) reported in neonates after treatment with phenobarbital: this phenomenon is manifest by the presence of electrographic seizures even with doses of phenobarbital that fully suppress behavioral seizures. It would be interesting to determine if decoupling still occurs in the presence of melatonin.

Toxicity, ranging from induction of neuronal apoptosis (Bittigau et al., 2003, 2002; Forcelli et al., 2011; Katz et al., 2007; Kim et al., 2007a, 2007b) and impaired synaptic maturation (Forcelli et al., 2012a) in animal models to longlasting cognitive effects in both rodents (Bhardwaj et al., 2012; Forcelli et al., 2012a, 2012b, 2010) and humans (Farwell et al., 1990; Sulzbacher et al., 1999) has been described after early-life exposure to phenobarbital. The fact that melatonin can enhance the anticonvulsant action of phenobarbital in neonates may have a particular advantage for addressing these concerns by allowing a reduction in the dose of phenobarbital used. Moreover, antioxidant doses of melatonin (Reiter et al., 2013) have previously been demonstrated to ameliorate toxicity in response to neonatal drug exposure (Forcelli et al., 2012a; Yon et al., 2006), perhaps through the same antioxidant effects that attenuate damage caused by prolonged seizures (Lima et al., 2011; Manev et al., 1996; Tan et al., 1998; Tchekalarova et al., 2013). These prior data showing neuroprotective effects of melatonin, combined with our present findings may provide a rationale for the examination of melatonin as an adjunct therapy for the treatment of seizures in neonates.

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Highlights

- We tested melatonin with phenobarbital for anticonvulsant effects in neonatal rats
- Melatonin potentiated the anticonvulsant effect of phenobarbital
- Melatonin did not exert an anticonvulsant effect on its own
- This supports further examination of melatonin as an adjunct therapy.

Figure 1. Pentylentetrazole seizures in animals treated with melatonin and/or phenobarbital
 A) Mean seizure score (as described in methods) as a function of dose of phenobarbital and melatonin. Median seizure scores: Vehicle control (4.5); 20 mg/kg melatonin (4.0); 40 mg/kg melatonin (4.0), 80 mg/kg melatonin (5.0); 10 mg/kg phenobarbital + 0 mg/kg melatonin (4.0); +20 mg/kg melatonin (4.0); +40 mg/kg melatonin (4.0); +80 mg/kg melatonin (3.0); 20 mg/kg phenobarbital + 0 mg/kg melatonin (3.0); +20 mg/kg melatonin (3.0); +40 mg/kg melatonin (2.5); +80 mg/kg melatonin (0.0); 40 mg/kg phenobarbital + 0 mg/kg melatonin (0.0); +20 mg/kg melatonin (0.0); +40 mg/kg melatonin (0.0); +80 mg/kg melatonin (0.0);
 (B) Mean latency to seizure onset as a function of dose of phenobarbital and melatonin.

Asterisks indicate a significant differences from control (vehicle), * = $p < 0.05$; n.a. = not analyzed

Table 1

Effect of treatments on the distribution of animals in each seizure category. Numbers in parentheses are the number of animals with a given response. Minimal seizure (less than a Score of 4 on the rating scale as described in methods), Generalized running bouncing clonus with loss of righting reflex; Score of 4; running bouncing clonus with tonic extension; Score of 5.

PB (mg/kg)	MEL (mg/kg)	No Seizure	Minimal Seizure (Score 1-3)	Score 5	Score 4	Total n=
00	0	0% (0)	0% (0)	50% (5)	50% (5)	10
	20	0% (0)	22% (2)	56% (5)	22% (2)	9
	40	0% (0)	17% (1)	50% (3)	33% (2)	6
	80	0% (0)	0% (0)	20% (1)	80% (4)	5
1010	0	0% (0)	0% (0)	83% (5)	17% (1)	6
	20	0% (0)	20% (2)	60% (6)	20% (2)	10
	40	10% (1)	20% (2)	60% (6)	10% (1)	10
	80	0% (0)	75% (3) **	0% (0) **	25% (1)	4
2020	0	0% (0)	83% (5) **	0% (0) **	17% (1)	6
	20	20% (1)	60% (3)	0% (0) **	20% (1)	5
	40	20% (1)	60% (3)	0% (0) **	20% (1)	5
	80	57% (4) **	43% (3) **	0% (0)	0% (0)	7
4040	0	80% (8) **	20% (2)	0% (0) **	0% (0)	10
	20	71% (5) **	29% (2)	0% (0) **	0% (0)	7
	40	50% (5) **	40% (4)	0% (0) **	10% (1)	10
	80	91% (10) **	9% (1)	0% (0)	0% (0)	11

** = significant contribution to the overall effect in Fisher's Exact Test.