Tacrolimus is not Neuroprotective Against Bilirubin Induced Auditory Impairment

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TACROLIMUS IS NOT NEUROPROTECTIVE AGAINST BILIRUBIN INDUCED AUDITORY IMPAIRMENT

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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ABSTRACT

TACROLIMUS IS NOT NEUROPROTECTIVE AGAINST BILIRUBIN INDUCED AUDITORY IMPAIRMENT

By Lori Elizabeth Walker, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

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In newborns, unconjugated bilirubin (UCB) is not readily excreted, and when bilirubin levels exceed the serum albumin binding capacity, pathological levels of UCB exist. Hyperbilirubinemia may lead to auditory damage and ultimately cause a hearing disorder called auditory neuropathy/dys-synchrony, characterized by absent or abnormal brainstem auditory evoked potentials (BAEPs) with evidence of normal inner ear function assessed by either otoacoustic emissions or cochlear microphonic responses. Phototherapy and double volume exchange transfusion are used as treatment methods for neonatal hyperbilirubinemia. Spontaneously jaundiced Gunn rat pups given
sulfadimethoxine to displace bilirubin from serum albumin develop bilirubin encephalopathy and have abnormal BAEPs comparable to human neonates. BAEPs are a noninvasive electrophysiological measure of neural function of the auditory system. High levels of calcineurin activity are believed to be involved in the mechanism of this bilirubin induced auditory neuropathy. FK506, a calcineurin inhibitor, was administered 3 hours prior to sulfa in concentrations of 0.1mg/kg, 1.0mg/kg, and 10.0mg/kg body weight. Due to the observation that all animals had abnormal BAEPs after treatment with FK506 and sulfa, it can be concluded that none of the treatment doses protected against bilirubin induced auditory impairment.
INTRODUCTION

Physiological Jaundice

About 60% of newborn babies develop visible jaundice within their first week of life due to an elevation of unconjugated bilirubin (UCB) levels. This is a common condition called physiological jaundice. Physiological jaundice normally develops as fetal hemoglobin is replaced with adult hemoglobin as there are increases in bilirubin production due to the breakdown of red blood cells, as well as due to a relative immaturity of the hepatic enzyme UDP-glucuronosyl transferase (UGT), which converts unconjugated (indirect) bilirubin to conjugated (direct) bilirubin (Blackburn 1995). Plasma bilirubin levels normally peak in the neonate at about 5 days of age. This peak in bilirubin tends to be self-correcting as the hepatic enzyme, UGT, becomes expressed and is therefore able to conjugate and excrete bilirubin at a faster rate.

Hyperbilirubinemia

Neonatal hyperbilirubinemia results from a predisposition to the production of bilirubin in newborn infants and their limited ability to excrete it. In newborns, unconjugated bilirubin (UCB) is not readily excreted, and when levels exceed the serum albumin binding capacity or when other substances such as sulfonamides compete for binding sites, pathological levels of unbound bilirubin exist (Diamond and Schmid 1966). This unconjugated bilirubin not bound to albumin (free bilirubin) exits the circulation and
can freely enter the brain, interstitial fluid, and cerebrospinal fluid, and is responsible for neurotoxicity (Shapiro 2003). Extreme hyperbilirubinemia may result in kernicterus. Kernicterus is characterized by choreathetoid cerebral palsy, high frequency central neural hearing loss (deafness), palsy of vertical gaze, and dental enamel hypoplasia (Perlstein 1960). Kernicterus is still relatively uncommon but has been on the rise with the institution of early postnatal discharge policies (Bhutani and Johnson 2004; Johnson et al. 2002). Average full term newborn infants have a peak serum bilirubin concentration of 5-6mg/dl. Serum bilirubin concentrations higher than 17mg/dl in full term infants are considered pathological (Diamond et al 1951). Phototherapy is the standard care for treatment of hyperbilirubinemia in infants as this rapidly reduces serum bilirubin concentrations. Double volume exchange transfusion was the first successful therapy for severe neonatal jaundice as this eliminates bilirubin from circulation (Diamond et al. 1951).

Less severe cases of hyperbilirubinemia may result in auditory damage. This auditory damage causes an auditory processing disorder known as auditory neuropathy/auditory dys-synchrony (AN/AD), which is defined as absent or abnormal brainstem auditory evoked potentials with evidence of normal inner ear function assessed by either otoacoustic emissions or cochlear microphonic responses (Shapiro 2003). The structure of the inner ear is intact and functional, however, transmission of auditory input is impaired due to impairment of the auditory nerve or other brainstem auditory pathways (Berlin et al. 2003). Children with AN/AD have a severe disruption in the temporal
coding of speech and an inability to cope with the dynamics of speech (Rance et al. 2002).

*Bilirubin Metabolism*

Unconjugated bilirubin (UCB), a breakdown product of the porphyrin ring of red blood cell hemoglobin, is lipid soluble, water insoluble, and neurotoxic. UCB is conjugated in the liver by UGT to a water-soluble, non-toxic glucuronide, known clinically as conjugated bilirubin or direct bilirubin (Shapiro 2003). The water-soluble, conjugated bilirubin, is excreted into bile and then into the gut for elimination (Gourley 2004). Conjugated bilirubin can however be deconjugated back to UCB in the gut so that the bilirubin is reabsorbed into circulation (Dennery et al. 2001).  \(\beta\)-Glucuronidase, an enzyme responsible for the deconjugation of UCB, is found in considerable amounts in breast milk (Gourley 2002), so exclusively breast fed newborns are at risk for high levels of UCB (Figure 1). Free bilirubin, not bound to albumin, seems to be the best parameter to estimate bilirubin neurotoxicity, but measurements of free bilirubin are not available in clinical practice. Treatment is therefore based on total serum bilirubin (TSB), which includes a combination of conjugated and unconjugated bilirubin, but treatment thresholds are not evidence based (Maisels and Watchko 2003). In newborns, due to low levels of UGT expressed, the bilirubin detected in TSB is primarily unconjugated.

Abnormalities of bilirubin metabolism cause elevated levels of UCB and place infants at risk for the development of bilirubin encephalopathy or kernicterus. Factors known to be associated with higher risk of bilirubin injury include prematurity,
dehydration, infection, genetic deficiencies, or breast feeding with inadequate intake (Shapiro 2003). A common genetic predisposition to deficient hepatic uptake of bilirubin is caused by mutations in the promoter region of UGT, as seen in Crigler-Najjar or Gilbert syndromes (Kadakol et al. 2000; Stevenson et al. 2001). In the first few days of life, breast-fed infants tend to have a lower fluid and calorie intake than formula-fed infants. They become relatively dehydrated and underfed (Gartner 2001). This minimizes the amount of conjugated bilirubin excreted in the feces and therefore allows it to be reabsorbed into blood through the enterohepatic circulation. Blood-group incompatibilities between mother and fetus may also result in increased production of bilirubin (Dennery et al. 2001) as this causes antigens in the mother’s blood to destroy the blood cells of the fetus.

Animal Model of Hyperbilirubinemia

The classic Gunn rat model was first described in 1938 by C.H. Gunn as a mutant jaundiced rat of the Wistar rat strain (Gunn 1938). The Gunn rat model of neonatal jaundice has been used to study the effects of neonatal hyperbilirubinemia on the developing central nervous system (Johnson et al. 1959; Johnson et al. 1961; Conlee and Shapiro 1997). Neonatal hyperbilirubinemia in homozygous j/j Gunn rat pups results from a deficiency of the bilirubin conjugating enzyme UGT (Roy-Chowdhury et al. 1991) homologous to human patients with Crigler-Najjar type 1 syndrome. The heterozygous N/j Gunn rat pups have about 50% UGT enzyme activity and do not develop hyperbilirubinemia. The homozygous jaundiced animal has many of the same
neurological symptoms and histopathological lesions that are exhibited by hyperbilirubinemic human newborns. Bilirubin levels peak around 15-17 days of age and slowly drop over the lifetime of the animal (Johnson et al. 1961; Sawasaki et al. 1976). These j/j pups can be made acutely symptomatic by injection of sulfadimethoxine, a long acting sulfonamide which competes with bilirubin for blood albumin binding sites promoting its net transfer out of blood into hydrophobic tissues including the brain (Diamond and Schmid 1966).

**Brainstem Auditory Evoked Potentials**

Brainstem auditory evoked potentials (BAEPs) are a noninvasive electrophysiological measure of neural function of the auditory system. They record surface electrical potentials in response to auditory stimuli (Buchwald and Huang 1975; Huang et al. 1977). BAEPs detect the efficacy of neural transmission between the brainstem auditory nuclei, one of the brain areas damages by high bilirubin levels (Shapiro 1988; Shapiro and Conlee 1991; Shapiro 2003; Rice and Shapiro 2006). BAEPs are highly sensitive to abnormalities of neuronal function such as conduction delay, desynchronization, and loss of cells (Shapiro 2003). Waves I, II, and III in the rat correspond to waves I, III, and V in humans and reflect neural transmission between the auditory nerve (wave I) and the cochlear nucleus (wave I in rat, III in human) and midbrain structures (wave III in rat, V in human) (Møller et al. 1981; Huang and Buchwald 1977; Huang 1980; Shapiro and Conlee 1991; Conlee and Shapiro 1991). Sulfa treated jaundiced jj Gunn rat pups show dramatic BAEP abnormalities just hours
after treatment. BAEP recordings typically show a range of abnormalities from absent responses at worst to delayed latencies and reduced amplitudes or absence of waves (Spencer et al. 2002). These BAEP wave amplitude abnormalities could be a result of reduced synchrony at which the auditory neurons are firing and the wave latency abnormalities could result from increased conduction time.

**Mechanism of bilirubin toxicity**

The exact mechanism of bilirubin induced neuronal cell injury and dysfunction in specific brain regions is not completely understood at this time, however, the pathogenesis of kernicterus is known to be quite complex. The molecular pathogenesis likely reflects the problematic effects of hazardous unconjugated bilirubin concentrations on plasma, mitochondrial, and/or endoplasmic reticulum membranes. It is suggested that bilirubin induced neuronal injury is initiated at the levels of these membranes with resultant perturbations of membrane permeability and function (Brito et al. 2004).

In vitro studies have shown that unbound UCB is extremely toxic and causes damage to mitochondria, impairing energy metabolism (for review see Ostrow et al. 2004). In developing rat brain neurons, UCB permeabilizes mitochondria membranes (Rodrigues et al. 2002), leading to mitochondrial swelling and the release of cytochrome c into the cytosol (Rodrigues et al. 2000). This then triggers activation of caspase 3 and translocation of Bax (Rodrigues et al. 2002), an apoptosis promoter, resulting in cell death by apoptosis via the mitochondrial pathway. UCB concentration exposures in the lower neurotoxic range were associated with apoptosis, whereas moderate to high
neurotoxic UCB concentrations were associated with necrosis (Hanko et al. 2005). UCB is also toxic to plasma membranes, causing oxidative damage and disrupting transport of neurotransmitters.

Also seen in vitro is a decrease in phosphorylation of synapsin I, a protein associated with vesicle storage, following bilirubin exposure (Hansen et al. 1988). Due to a decrease in phosphorylation of synapsin I, there is less neurotransmitter available for release when a neuron is stimulated. In support of decreased neurotransmitter release, it has been shown that there is a decrease in synaptic activation following exposure to bilirubin (Hansen et al 1988).

Numerous kinases known to regulate neuronal function, including calcium calmodulin dependent protein kinase II (CaM kinase II), cAMP dependent kinase (PKA), calcium dependent protein kinase C (PKC), have been shown by in vitro studies to be inactivated by the removal of the phosphate group or inhibited by bilirubin (Hansen et al. 1988). Loss of function of these kinases or decreased phosphorylation of their substrates could be producing the neuronal dysfunction seen in humans as well as Gunn rats.

Our lab has performed in vivo studies using minocycline and has found this drug to be neuroprotective in jaundiced Gunn rat pups (Geiger et al. 2007). Minocycline has anti-inflammatory, anti-apoptotic, and antibiotic properties. Thus, although it is neuroprotective, minocycline is not useful for determining the mechanism of bilirubin neurotoxicity.
**Calcium/Calcineurin**

Along with the probable mechanisms mentioned above, calcium is believed to be a key factor in the neuropathology of bilirubin toxicity. Unpublished studies from the lab have shown that exposure to bilirubin causes a dramatic increase in levels of intracellular calcium as well as an increase in the activity of a calcium dependent enzyme, calcineurin. Calcium binding proteins have an important role in maintaining intracellular calcium homeostasis, and in the sulfadimethoxine injected jj Gunn rat, the density of immunoreactive staining for these proteins was reduced substantially in comparison to the littermate Nj control rats (Spencer et al. 2002). Loss of calcium homeostasis is a principal mechanism that results in neuronal cell death in models of global ischemia and increased neuronal excitability (Churn 1995). Calcium can enter the cytoplasm through many different channels, can be released from internal stores, and can be maintained in the cytoplasm due to loss of function of extrusion mechanisms. Exposure of astrocytes to UCB decreases uptake of glutamate, the main excitatory neurotransmitter in the brain, thus prolonging the time glutamate is in the synaptic cleft (Silva et al 1999). This causes overstimulation of N-methyl-D-aspartate (NMDA) receptors leading to excitotoxicity both in vitro (developing rat brain neurons) (Grojean et al. 2000; Grojean et al. 2001) and in vivo (jaundiced Gunn rat) (McDonald et al. 1998). Excitotoxicity induces neuronal cell swelling due to excessive influx of sodium, calcium, chloride, and water, triggering cell death by both apoptosis and necrosis (Mattson 2003). However, we have demonstrated that calcium entering through NMDA channels is not responsible for neurological dysfunction due to bilirubin (Shapiro et al. 2007).
Calcineurin is a calcium calmodulin dependent phosphatase found throughout neural tissue. It is associated with many neuronal functions. Calcineurin plays a role in many neuronal processes. It is known to regulate gene transcription through the nuclear factor of activated T-cells (NFAT) family of molecules, it modulates the function of NMDA receptors, it regulates neurotransmitter release, and it may be involved in the initiation of apoptosis (Hansen et al. 1988). Calcineurin can dephosphorylate, and in turn deactivate, many key enzymes including neurotransmitter receptors and voltage gated channels thereby inhibiting neural transmission. Calcineurin is abundantly expressed in areas of the brain that are vulnerable to stroke, epilepsy and neurodegenerative diseases. Calcineurin is important in inflammation (Parry and June 2003). Unpublished studies from the lab have shown that calcineurin activity increases after exposure to bilirubin, and our lab hypothesizes that this activity accounts for the neuronal dysfunction observed in bilirubin toxicity.

Whatever the mechanism may be, the amount and duration of bilirubin exposure determine the extent of neuronal damage.

**FK506**

FK506 (tacrolimus) is a calcineurin inhibitor that belongs to a group of chemicals referred to as the macrolides. Tacrolimus is an immunosuppressive drug used mainly after allogenic organ transplant to prevent attack of the transplanted organ by the patient’s immune system. It is highly lipophilic in nature, and is very soluble in methanol, chloroform, acetone, ethyl acetate, ethanol, and propylene glycol (Venkataramanan et al.
The immunosuppressive agent FK506 has neuroprotective properties not only in rodents (Sharkey and Butcher 1994; Butcher et al. 1997; Maeda et al. 2002) but also in nonhuman primates (Takamatsu et al. 2001). Studies using FK506 as a neuroprotectant have been conducted in many neurological conditions such as epilepsy (Kurz et al. 2008), cerebral ischemia (Sharkey and Butcher 1994), and traumatic brain injury (Marmarou and Povlishock 2006). FK506 has shown promise as a neuroprotective agent in models of traumatic brain injury (Kurz et al. 2005). The mechanisms underlying the neuroprotective effects of the immunosuppressant tacrolimus, observed in vivo, remain unclear (Labrande et al. 2006). Calcineurin, however, appears to be a major target for neuroprotection in damaged axons demonstrating impaired axonal transport (Marmarou and Povlishock 2006). FK506 initially binds to its specific binding protein, FKBP-12, forming a complex that then interacts with calcineurin preventing its activation.

**Objective**

Minocycline, with its anti-inflammatory, anti-apoptotic, and antibiotic properties, is neuroprotective in our animal model, however, the specific mechanism of action in bilirubin neurotoxicity has yet to be determined. Because calcineurin is involved in inflammation and minocycline, which is neuroprotective, has anti-inflammatory properties, we wanted to test whether inhibiting calcineurin activity would prevent bilirubin neurotoxicity. Calcineurin is a calcium regulated phosphatase and unpublished in vitro studies have shown an increase in intracellular calcium levels as well as increased activity of calcineurin after exposure to bilirubin, therefore, the calcineurin data fits the
minocycline data. We hypothesized that if we could block calcineurin activity, we could prevent bilirubin neurotoxicity.

The objective of this study is to determine if FK506 (tacrolimus) can protect the brain against hyperbilirubinemia and prevent neurotoxicity and its devastating consequences. This study will provide a better insight into the cellular mechanism of bilirubin induced neurotoxicity and hopefully lead to a therapeutic window for modulating calcineurin activity. FK506 is unlikely to be used in neonates, however, I am using it to help understand the mechanism involved in bilirubin induced neuronal damage. This will hopefully help lead to the development of compounds that interfere with this mechanism and are safe for neonates.
Bilirubin is a product of hemoglobin catabolism. (A) Hemoglobin is converted to biliverdin by heme oxygenase and produces equimolar concentrations of bilirubin and carbon monoxide (CO). (B) Nontoxic biliverdin is catalyzed by biliverdin reductase to unconjugated bilirubin (UCB), a natural antioxidant at low levels, but neurotoxic at high levels. (C) UCB is water insoluble and is bound to serum albumin. Thus little UCB exists in the form of unbound or free unconjugated bilirubin (Bf). It is Bf and not albumin-bound UCB that enters brain (D), interstitial fluid, and cerebrospinal fluid, and is responsible for neurotoxicity. UCB is taken up by liver cells (E), conjugated with glucuronide by UDP-glucuronosyl transferase (UGT) to nontoxic, water-soluble conjugated bilirubin and excreted in bile. Conjugated bilirubin is eliminated in stool (F) but can also be deconjugated in the gut to UCB, which is then reabsorbed back into the bloodstream, through the enterohepatic circulation. β-Glucuronidase, an enzyme responsible for the deconjugation of UCB, is found in considerable amounts in breast milk (Gourley 2002) so exclusively breast fed newborns are at risk for high levels of UCB. Taken from Shapiro (2003).
METHODS

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University, and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Gunn rats were obtained from the breeding colony maintained at Virginia Commonwealth University in which phenotypically normal non-jaundiced (Nj) females are bred with homozygous, autosomal recessive jaundiced (jj) males to produce litters with approximately equal numbers of Nj and jj pups. The rat pups were genotyped based on their phenotypic skin color at 5 days of age.

Experimental Method

At 16 days of age, healthy pups were weighed, blood was obtained by cheek puncture to analyze hematocrit (Hct) and total plasma bilirubin (TB), and they were randomly assigned to a treatment group. Jj pups were assigned to one of two control groups or one of three treatment groups while Nj pups were assigned to one of two groups. The jj animals were first given an intraperitoneal (i.p.) injection of either the treatment drug (0.1 mg/kg, 1 mg/kg, or 10 mg/kg FK506) or vehicle, which consisted of 20% EtOH, 40% glycerol, and 40% water. The Nj animals were given an i.p. injection of either vehicle or 10 mg/kg FK506. Three hours following the treatment injection, pups were injected (i.p.) with 200 mg/kg sulfadimethoxine (sulfa) or saline. The time of the
second injection was recorded as zero hours. 6 hours after sulfa treatment, animals were weighed again and anesthetized with an intramuscular injection of acepromazine (6mg/kg) and ketamine (60mg/kg) and a brainstem auditory evoked potential (BAEP) was recorded (Figure2).

Pilot Studies

A series of pilot studies were done prior to the experimental method that was eventually used. In the first pilot study 16 day old animals were first used to do a 24 hour study. 16 day old jj pups were weighed and blood was obtained for analysis of Hct and TB. They were randomly assigned to the treatment groups. 24 hours after sulfa treatment, BAEPs were obtained. This experimental design proved to be rather difficult. One problem was dissolving FK506. Litters were treated with 0.5mg/ml FK506 in 50% EtOH and 50% saline. Once injected, the rats became very intoxicated. This caused them to remain sleeping for several hours, which in turn did not allow them to nurse from their mother, they become very dehydrated, and were not able to urinate. Most jaundiced animals died within 24 hours.

In the second pilot study 16 day old rat pups were again used, although this time FK506 was dissolved in 12ml saline and 3ml EtOH for a 20% EtOH solution. All the animals were once again very intoxicated and severely dystonic after this injection. They remained asleep for hours and all died within 24 hours.

In the third pilot study FK506 was dissolved in EtOH and glycerol for a 1mg/ml solution consisting of 20% EtOH. Animals injected with this solution were much less
intoxicated. They were much more awake and moving around within 2 hours after the injection. Because most of the pups were still quite dystonic after the injections and dying because of dehydration, I tested to see whether younger animals would show auditory impairments without the confounding effects of motor impairments. 13 day old jj pups were injected with sulfa. All the animals survived with no sign of dystonia and BAEP recordings done at 16 days of age showed auditory impairment. The pups were then injected on day 13 and BAEPs were obtained on day 16. However, some pups still showed signs of dystonia and died before day 16, so the experimental design was changed to the final method, outlined in figure 2.

**Plasma measurements**

The blood sample obtained by cheek puncture was collected in an 85-µl heparinized hematocrit tube, centrifuged, and hematocrit (% red blood cells) was obtained. To remove any plasma turbidity due to milk fat, the plasma was transferred to a 0.6 ml microcentrifuge tube and re-centrifuged for 10 minutes at 3,400 rpm. 23.5 µl of the plasma sample was then pipetted into a cuvette for measurement of TB by a bilirubinometer.

**Brainstem auditory evoked potentials**

Rats were anesthetized with an intramuscular injection of 6mg/kg acepromazine and 60mg/kg ketamine. The left ear was occluded with petrolatum to block any auditory stimuli from interfering with the BAEP recording. Temperature was measured by a rectal
probe and was maintained at $37.0 \pm 0.1^\circ C$ by a servo-controlled heating lamp with a red bulb to avoid the possibility of converting bilirubin to its water soluble isomer, photobilirubin, by blue wavelengths of light. BAEP recordings were begun after $\geq 5$ minutes of temperature stabilization. BAEPs were recorded using a Nicolet Spirit 2000 evoked potential system (Biosys, Inc.). They were obtained to monaural 100 $\mu$s duration rarefaction clicks given at a rate of 31.7/s to the right ear through a Sony walkman 4LIS headphone speaker. The sound intensity was set at 70 dB which corresponds to a level of about 62 dB above a normal Gunn rat pup BAEP threshold (Rice and Shapiro 2006). Surface electrical potentials were recorded from 13 mm long subcutaneous platinum needle electrodes inserted behind the left and right mastoid and on the scalp over the vertex with a ground electrode in the flank. Two channel BAEP recordings were obtained from the contralateral to the ipsilateral mastoid (horizontal) and the vertex to the ipsilateral mastoid (vertical). The horizontal data is reported and the vertical is used only to help identify the waves. Each BAEP is a summed response of three replicated responses, each to at least 2000 stimuli (Figure 3). If the responses were judged to be reproducible then the three responses were summed and the peak latencies and peak to trough amplitudes for waves I, II, and III were scored (Figure 3). Interwave intervals were obtained by subtracting latencies and correspond to the time of neural transmission between the auditory nerve, the cochlear nucleus, and the midbrain structures respectively.
**Statistical Analysis**

A-priori variables of interest were amplitude of waves I, II, and III, with decreases indicating abnormality, and I-II and I-III IWIs as well as latency of wave I, with increases indicating abnormality. BAEP values were compared by ANOVA and if significant, with post-hoc Tukey test. A p-value of 0.05 was corrected for the 6 independent variables to give a criterion p-value of 0.00833 for each comparison. Weights, TBs, and Hct values were also compared between groups by one-way ANOVAs.
Figure 2. Experimental Design

Blood samples were obtained by cheek puncture from each animal (jj and nj) before treatment to measure TB and Hct levels. Pups were weighed and randomly assigned to a treatment group. FK506 or vehicle was given by i.p. injection 3 hours before sulfa or saline was administered by i.p. injection. The sulfa injection was marked as time 0 hours. 6 hours after sulfa injection, pups were anesthetized by an intramuscular injection of 6 mg/kg acepromazine and 60 mg/kg ketamine and BAEPs were recorded.
Figure 3. BAEP Wave Summation
BAEP recording. Three replicated responses, each to 2000 stimuli, (A) are summed to give a BAEP with waves I, II, and III (B). Latencies (peak to peak) and amplitudes (peak to trough) are scored for each wave.
RESULTS

The mean baseline TB for all jj animals (n=37) before treatment injection was 12.0 ± 1.0 (mean ± SD) mg/dL. There were no significant differences in baseline TB levels between groups or within groups, except for the normally low TB Nj pups, as expected. The mean hematocrit level for all animals was 35.3 ± 1.8% and was not significantly different between groups or within groups. The mean weight for all animals did not differ significantly and was 28.0 ± 3.7g (Table 1). The two Nj treatment groups showed no significant differences so they were pooled together for statistical analysis.

BAEP Latencies and IWIs

Latency of wave I was not significantly different between any group with an overall ANOVA $p=0.664$. The latency of IWI I-II for the vehicle/saline treated animals as well as the Nj animals differed significantly from the vehicle/sulfa treated and FK506 treated animals with an overall ANOVA $p<0.001$. ANOVA could not be performed for the latency of IWI I-III due to the fact that there were too few groups that actually had a wave III. BAEPs for jj groups are illustrated in Figure 4.

Latency of wave I for all treated animals in all groups was 1.26 ± 0.06 ms. The latency of wave I for all jj pups was 1.27 ± 0.06 ms and was 1.25 ± 0.04 for all Nj pups. Post-hoc Tukey analysis $p\leq0.0083$ showed no statistically significant differences between (Figure 5).
By post-hoc Tukey analysis $p \leq 0.0083$, the latency of IWI I-II for all jj FK506 treatment groups differed significantly from the vehicle/saline treated jj control animals. The average latency of IWI I-II for FK506 treated jj animals was $1.66 \pm 0.19$ ms and that for the jj vehicle/saline control animals was $1.22 \pm 0.12$ ms. The overall average latency of IWI I-II for vehicle/sulfa treated control animals was $1.63 \pm 0.15$ ms and did not differ significantly from the FK506 treated animals. The latency of IWI I-II for Nj pups did not differ significantly from the vehicle/saline treated animals, with the average latency for Nj pups being $1.12 \pm 0.10$ ms (Figure 6).

**BAEP Amplitudes**

Wave I amplitude was significantly different (ANOVA $p=0.007$) between all groups but not significantly different between jj groups (ANOVA $p=0.066$). The amplitude of waves II and III for the vehicle/saline treated animals as well as the Nj pups differed significantly from the amplitude of waves II and III for the vehicle/sulfa treated and the FK506 treated animals. Overall ANOVA for amplitude of both waves II and III was $p<0.001$. Shifts in BAEP wave amplitudes are depicted between all treatment groups (Figure 4).

The amplitude of wave I for all treated animals in all groups was $0.90 \pm 0.39 \mu$V. The amplitude of wave I for all jj pups was $0.82 \pm 0.35 \mu$V and was $1.21 \pm 0.41 \mu$V for all Nj pups. Post-hoc Tukey analysis $p \leq 0.0083$ showed no significant differences between groups (Figure 7).
By post-hoc Tukey analysis $p \leq 0.0083$, the amplitude of wave II for all jj FK506 treated animals differed significantly from all vehicle/saline treated jj control animals. The average amplitude of wave II for all FK506 treated jj pups was $0.25 \pm 0.20 \mu V$ and that for all vehicle/saline treated jj animals was $1.26 \pm 0.32 \mu V$. The average amplitude of wave II for all vehicle/sulfa treated jj control animals was $0.22 \pm 0.17 \mu V$ and did not differ significantly from the jj FK506 treated animals. The amplitude of wave II for Nj pups did not differ significantly from the vehicle/saline jj control animals, with the average amplitude for Nj pups being $1.42 \pm 0.55 \mu V$ (Figure 8).

By post-hoc Tukey analysis $p \leq 0.0083$, the amplitude of wave III for all jj FK506 treated animals differed significantly from all vehicle/saline treated jj control animals. The average amplitude of wave III for all FK506 treated jj pups was $0.02 \pm 0.06 \mu V$ and that for all vehicle/saline treated jj animals was $1.00 \pm 0.55 \mu V$. The average amplitude of wave III for all vehicle/sulfa treated jj control animals was $0.04 \pm 0.08 \mu V$ and did not differ significantly from the jj FK506 treated animals. The amplitude of wave III for Nj pups did not differ significantly from the vehicle/saline jj control animals, with the average amplitude for Nj pups being $1.33 \pm 0.47 \mu V$ (Figure 9).

Amplitude of BAEP waves is more variable from animal to animal than latency. In order to decrease animal to animal variability in wave amplitude, a ratio of wave II:I amplitude was compared, however, the results were the same (Figure 10).
<table>
<thead>
<tr>
<th>Genotype</th>
<th>FK506</th>
<th>Sulfa</th>
<th>N</th>
<th>Weight ± SD (g)</th>
<th>TB ± SD (mg/dL)</th>
<th>Hct ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>jj</td>
<td>0mg/kg</td>
<td>0mg/kg</td>
<td>6</td>
<td>28.6 ± 4.1</td>
<td>12.7 ± 1.4</td>
<td>35.5 ± 2.2</td>
</tr>
<tr>
<td>jj</td>
<td>0.1mg/kg</td>
<td>200mg/kg</td>
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<td>11.9 ± 1.2</td>
<td>35.1 ± 1.8</td>
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<tr>
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<td>200mg/kg</td>
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<td>27.3 ± 5.0</td>
<td>12.1 ± 0.6</td>
<td>35.4 ± 1.6</td>
</tr>
<tr>
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<td>200mg/kg</td>
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<td>26.6 ± 3.1</td>
<td>11.8 ± 0.8</td>
<td>36.0 ± 1.5</td>
</tr>
<tr>
<td>jj</td>
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<td>200mg/kg</td>
<td>8</td>
<td>28.5 ± 3.6</td>
<td>11.9 ± 1.1</td>
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<td>0mg/kg</td>
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<td>29.9 ± 4.2</td>
<td>0.02 ± 0.04*</td>
<td>34.0 ± 1.7</td>
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<tr>
<td>Nj</td>
<td>10mg/kg</td>
<td>200mg/kg</td>
<td>5</td>
<td>30.3 ± 2.3</td>
<td>0.2 ± 0.4*</td>
<td>35.4 ± 2.5</td>
</tr>
</tbody>
</table>

Table 1. Baseline Weight, TB, and Hematocrit Measurements
Baseline weight, TB, and hematocrit measurements were taken before treatment injection. There were no significant differences in either variable between groups or within groups, except for TB levels between jjs and Njs as expected. p≤0.001. compared to jj vehicle/saline group is indicated by an asterisk.
Figure 4. Waveforms for Treatment Groups
BAEP recordings 6 hours after sulfa. The vehicle/saline jj group (A.) shows typical waveforms with normal amplitudes and latencies. The vehicle/sulfa jj group (E.) shows decreased amplitudes for all waves and prolonged latencies when compared with vehicle/saline controls. The 0.1 mg/kg, 1.0 mg/kg, and 10 mg/kg FK506/sulfa jj groups (B, C, D, respectively) appear similar to the vehicle/sulfa jj group with decreased amplitudes for all waves and prolonged latencies. Often some of the waves are diminished. Amplitudes are represented on the vertical axis with each mark denoting 1.24μV, and latency (time) is represented on the horizontal axis with each mark denoting 1.0 ms.
The first 5 groups are jj animals. There were no statistically significant differences in Wave I latency between groups or within groups. $p=0.664$
Figure 6. Interwave Interval I-II

The first 5 groups are ji animals. Vehicle/saline ji animals and FK506-treated ji animals showed prolonged latencies for IW I-II compared to vehicle/saline ji controls and NJ controls. ANOVA, *p* < 0.001. There were no differences in latency of IW I-II between the vehicle/saline treated ji animals and the NJ controls. *p* = NS. *p* ≤ 0.0083 compared to ji vehicle/saline groups is indicated by an asterisk.
Figure 7. Amplitude of wave I
The first 5 groups are jj animals. Wave I amplitude was statistically significant (ANOVA $p=0.007$) between all groups but not statistically significant between jj groups (ANOVA $p=0.066$).
Figure 8. Amplitude of wave II
The first 5 groups are jj animals. Vehicle/sulfa jj animals and FK506 treated jj animals showed decreased amplitudes for wave II compared to vehicle/saline jj controls and Nj controls. ANOVA  $p<0.001$. There were no differences in amplitude of wave II between the vehicle/saline treated jj animals and the Nj controls. $p=NS$. $p \leq 0.0083$ compared to jj vehicle/saline group is indicated by an asterisk.
Figure 9. Amplitude of wave III
The first 5 groups are jj animals. Vehicle/sulfa jj animals and FK506 treated jj animals showed decreased amplitudes for wave III compared to vehicle/saline jj controls and Nj controls. ANOVA $p<0.001$. There were no differences in amplitude of wave III between the vehicle/saline treated jj animals and the Nj controls. $p=\text{NS}$. $p\leq 0.0083$ compared to jj vehicle/saline group is indicated by an asterisk.
Figure 10. Amplitude Ratio of Wave II:I
The first 5 groups are jj animals. Ratio of wave II:I amplitude was taken to reduce animal to animal variability of wave amplitude, however, the results were the same as before waves were compared. $p \leq 0.0083$ compared to jj vehicle/saline group is indicated by an asterisk.
DISCUSSION

Based on wave latencies and amplitudes from BAEP recordings, FK506 is not neuroprotective under the conditions addressed in this study against bilirubin induced auditory impairment in the Gunn rat animal model. We found that sulfa treated jj Gunn rat pups had reduced amplitudes of waves II and III when compared to the saline control animals, and pups treated with FK506 had wave amplitudes similar to those of the sulfa treated jj pups. Reduced wave amplitudes could indicate a loss of synchronization with which the auditory neurons are activated and/or a loss of functional neurons that are able to transmit the auditory signal, however this is unlikely to occur as soon as 6 hours after sulfa injection. In more severely affected animals, all waves, including wave I from the auditory nerve, are lost (Shapiro et al., 2006). Sulfa treated jj Gunn rat pups also have longer wave II and III latencies as well as increased latencies of I-II and I-III IWIs. Latencies of waves II and III and I-II and I-III IWIs from FK506 treated pups are affected similarly. Longer wave latencies indicate longer conduction time for neural transmission due to events such as loss of myelination, problems at the synapse, or problems with neurotransmitter release.

Properties of FK506

Intraperitoneal administration is the most feasible injection method in our Gunn rat model because it is difficult to administer an intravenous (i.v.) injection into a 25-30 gram rat, however, no published studies using i.p. administration were found. Most
published studies using FK506 as a neuroprotectant are by intravenous (i.v.) or oral administration. In the rat, FK506 has a mean half-life of approximately 7.4 hours after i.v. administration (Iwasaki et al., 1998) and has a mean half-life in rats after oral administration of less than 3 hours (Venkataramanan et al. 1990). We chose a 3 hour time point between treatment injection and injection of sulfa based on a published study showing neuroprotection 3 hours after i.v. injection of FK506 in an animal model of epilepsy (Kurz et al. 2008). Because FK506 is not neuroprotective in our model, a different time or route of administration might have prevented bilirubin neurotoxicity.

In experiments done with rats, brain concentrations of FK506 are maintained for more than 72 hours after i.v. administration (Butcher et al. 1997). Studies using FK506 in monkey models of stroke conclude that a brain concentration of 20 ng/g of FK506 is sufficient to show efficacy against ischemia and that brain concentrations peak after about 7 minutes following i.v. administration (Murakami et al. 2004). Future studies should explore the effects of different administration routes.

At a drug level of 50ng/mL about 80% of FK506 binds to rat serum albumin (Iwasaki et al. 1991). In rats, FK506 has a high metabolic clearance rate from the body, goes through extensive metabolization, preferentially distributes to the blood cells at a low concentration, and has high biliary excretion (Iwasaki et al. 1991).

There are some adverse reactions when FK506 is used as an immunosuppressant in organ transplant patients. Some of these symptoms are tremor, headache, diarrhea, hypertension, nausea, and abnormal renal function. Pediatric patients tend to need higher doses than adults to achieve similar FK506 concentrations.
Pitfalls

Although FK506 is unlikely to ever be used in neonates, we have used the drug in this study to help identify the mechanisms involved in bilirubin neurotoxicity. Because this drug shows no neuroprotection in the Gunn rat model, calcineurin may not be the mechanism of bilirubin toxicity in the auditory brainstem structures. It could also be that neonatal rat pups like those used in this study have a much faster metabolism than adult rats used in other FK506 studies, thereby eliminating the drug from their body before injection of sulfa to displace bilirubin into the tissues, allowing no protection. Because the primary auditory neurons, represented by wave I, are typically not as affected by bilirubin as are the brainstem structures (wave II and III), the decrease in amplitude of wave I seen in the vehicle/sulfa treated animals and the FK506 treated animals could be a result of these animals being very sick.

Future Studies

Supplemental studies could be done in order to get a better idea of the pharmacokinetics of FK506 in the rat after i.p. administration. Blood samples should be taken at different time points after injection to measure the concentration of FK506 in the blood and better determine the half-life of the drug. Because this study was performed in neonates, higher doses of the drug should be used in supplemental experiments to determine if the doses used were too low to exhibit neuroprotection, especially because neonates tend to have a much faster metabolism than adults. Because neonates have a faster metabolism than do adults, future studies could include shortening the length of
time between the administration of FK506 and the sulfa injection to determine if the drug shows any neuroprotection. Studies using different routes of administration of the drug could also be done to determine if absorption rates have any affect on the level of neuroprotection. Because FK506 binds to rat serum albumin, studies could be conducted to determine if this drug competes with bilirubin for binding sites therefore displacing bilirubin into the neural tissues. Bilirubin levels should be measured before injection of FK506 as well as after. If the levels do not change, FK506 probably does not displace bilirubin.

Conclusion

FK506, at doses of 0.1 mg/kg, 1.0 mg/kg, and 10.0 mg/kg, does not show neuroprotective effects against bilirubin induced auditory impairment in the Gunn rat model when administered 3 hours prior to sulfa.
REFERENCES


Lori Walker was born on June 4, 1984 in Germantown, Maryland and is an American citizen. She graduated from Northwest High School in Germantown, Maryland in 2002. Lori earned her Bachelor of Science degree in psychology from Frostburg State University in 2006. She began her graduate studies at Virginia Commonwealth University in 2006, and received a post-baccalaureate certificate in physiology in 2008.