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SHORT COMMUNICATION

Age-Dependency of Blood-Brain Barrier Penetration by *cis*- and *trans*-Permethrin in the Rat

Tanzir B. Mortuza, Gaylen L. Edwards, Catherine A. White, Vandan Patel,
Brian S. Cummings, and James V. Bruckner

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy
(T.B.M., C.A.W., B.S.C., J.V.B.)

Interdisciplinary Toxicology Program
(G.L.E., C.A.W., B.S.C., J.V.B.)

Department of Physiology and Pharmacology, College of Veterinary Medicine (G.L.E.)

University of Georgia

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Running Title: Blood-Brain Barrier Penetration by Pyrethroids in Rats

Corresponding Author: James V. Bruckner
Department of Pharmaceutical and Biomedical Sciences
College of Pharmacy
University of Georgia
(706) 542-5405
(706) 542-5358 FAX
bruckner@uga.edu

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ABSTRACT

Permethrin (PER), a Type I pyrethroid, is the most widely used insecticide in domestic settings in the U.S. The overall objective of this study was to assess the efficiency of the blood-brain barrier (BBB) as an obstacle to the *cis* (CIS) and *trans* (TRANS) isomers of PER, and to determine whether its barrier function changes during maturation of the rat. Experiments were conducted to quantify brain uptake of CIS and TRANS in postnatal day (PND) 14, 21 and 90 Sprague-Dawley rats. The common carotid artery of anesthetized rats was perfused for 2 or 4 min with 1, 10 or 50 µM ^{14}C-CIS or ^{14}C-TRANS in 4% albumin. Brain deposition of each isomer was inversely related to age, with levels in the youngest animals >5 times those in adults. Brain uptake was linear over the 50-fold range of pyrethroid concentrations, indicative of passive, non-saturable BBB permeation. The extent of uptake of toxicologically-relevant concentrations of CIS and TRANS was quite similar. Thus, dissimilar BBB permeation does not contribute to the greater acute neurotoxic potency of CIS, but greater permeability of the immature BBB to CIS and TRANS may contribute to the increased susceptibility of pre-weanling rodents to the insecticides.

INTRODUCTION

Sales of pyrethroid insecticides have increased substantially worldwide due to their effectiveness and restrictions on the use of organophosphates (Williams, 2008). A variety of pyrethroids are widely used agriculturally and in households for pest control. Studies of general populations reveal that a large proportion of those surveyed have been exposed to permethrin (PER) and several other pyrethroids (Morgan et al., 2012; Saillenfait et al., 2015). Children often exhibit higher urinary pyrethroid metabolite levels than adults, apparently due largely to ingestion from hand-to-mouth activities involving contaminated surfaces. PER, commonly sold as a mixture of its *cis* (CIS) and *trans* (TRANS) isomers, is the most widely utilized insecticide in the U.S. for residential use. PER is also often the treatment of choice for infestation of pets and children with lice and other parasites (Anadon et al., 2009; Frankouski and Bocchini, 2010).

High doses of pyrethroids can be acutely neurotoxic, although their potency differs substantially (Wolansky et al., 2006). Sufficient doses of Type I pyrethroids elicit tremors and sensitivity to external stimuli. Type II pyrethroids can produce salivation, hyperexcitability and choreoathetosis. The parent compounds are the proximate neurotoxic entities. They produce stimulus-dependent depolarization block, primarily by interference with voltage–sensitive sodium channels (VSSC) (Cao et al., 2011; Soderlund, 2012). Disturbance of VSSC may result in residual impairment of the maturing nervous system (Shafer et al., 2005). Several recent epidemiology studies have found an association between long-term pyrethroid exposure and neurobehavioral disorders in children (Richardson et al., 2015; van Wendel de Joode et al., 2016; Viel et al., 2015; Wagner-Schumann et al., 2015). Other researchers failed to find an

association between prenatal or postnatal PER exposure and neurodevelopmental problems (Horton et al., 2011; Quiros-Alcala et al., 2014).

It has been recognized for 25 years that immature rodents are more susceptible to high-dose pyrethroid neurotoxicity than adults. PER, a Type I pyrethroid, as well as cypermethrin and deltamethrin (DLM), Type II pyrethroids, were found to be much more acutely toxic to neonatal than to adult rats (Cantalamessa, 1993; Sheets et al., 1994). Kim et al. (2010) subsequently observed that DLM levels in plasma, brain and other tissues were inversely proportional to rats' stage of development. Deficient metabolic detoxification and systemic clearance by liver cytochrome P450s (CYPs) and carboxylesterases (CaEs) and plasma CaEs in the immature animals were reported to be a cause of this phenomenon (Anand et al., 2006). Low adipose tissue content in neonatal and preweanling rats was observed to promote enhanced deposition of the highly lipophilic pyrethroids in the CNS (Amaraneni et al., 2017a). Postnatal day (PND) 15 and 21 rats with plasma DLM levels comparable to adults have recently been observed to exhibit significantly higher brain levels, suggesting that the immature blood-brain barrier (BBB) is relatively permeable to the chemical (Mortuza et al., 2018). An *in situ* experiment revealed that brain uptake of DLM was inversely related to the age of rats (Amaraneni et al., 2017b). A goal of the current investigation was to determine whether this was also true for PER, the insecticide to which children are most frequently exposed in the U.S.

The pharmacokinetics (PK), as well as the acute neurotoxic potency of CIS and TRANS, are quite different. Pilot PK experiments revealed that rats of different ages can tolerate substantially higher single oral doses of TRANS. TRANS is eliminated more

rapidly from the blood and brain of rats than CIS (Tornero-Velez et al., 2012; Willemin et al., 2016). CIS is metabolized solely by CYPs, while TRANS is oxidized by CYPs and extensively hydrolyzed by CaEs in rats (Scollon et al., 2009). The ester linkage of the TRANS configuration of PER is quite labile to CaE-catalyzed hydrolysis (Hosokawa, 2008). It is also possible that CIS is more potent than TRANS, because CIS penetrates the immature BBB more readily.

MATERIALS AND METHODS

^{14}C-*cis*-permethrin (CIS) and ^{14}C-*trans*-permethrin (TRANS) (each of 99.9% radiochemical purity) were provided by Moravic Inc. (Brea, CA). Hanks' balanced salt solution (HBSS) was obtained from Mediatech (Manassas, VA). Human serum albumin (HSA) was purchased from Golden West Biologicals (Temecula, CA). Ecolite scintillation cocktail was obtained from MP Biochemicals (Solon, OH), while glycerol formal (GF) was purchased from Sigma Aldrich (St. Louis, MO).

Adult males (~300g), dams with ~10 pups, and pregnant female rats (gestation day 17-19) were purchased from Charles River Labs (Raleigh, NC). The Sprague-Dawley rats were delivered to and housed at the AAALAC-accredited University of Georgia (UGA) Life Sciences Animal Care Facility. The animals were acclimated to a 12-h light/dark cycle (light 0700 -1900 h) in a temperature (25°C)- and humidity (40%)-controlled room for at least 1 week prior to use. Tap water and Purina Rat Chow # 5001 were provided *ad libitum.* Adult male rats were housed 4 per solid–bottom polycarbonate cage. Dams with pups and pregnant females were housed singly in the cages with corncob bedding. The pregnant rats' delivery date was defined as postnatal day (PND) 0 for their pups. Pups were maintained with their mother until PND 15 or 21.

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Groups of 4 adults and 3-5 unsexed pups were used for each experiment according to the research protocol approved by the UGA Animal Care and Use Committee. The study was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The initial activity of both ^{14}C-CIS and ^{14}C-TRANS was 61 mCi/mmol. A stock solution of each was prepared by dissolving 6.4 mg in 2 ml of GF. Aliquots of stock were used to make working solutions of 10, 100 and 500 µM of each isomer in GF. 200 µl of working solution were added to 1800 µl of 4% HSA in HBSS to obtain final concentrations of 1, 10 and 50 µM for infusion.

Adult rats were anesthetized by i.m. injection of 0.1 ml/100 g bw of a “cocktail” consisting of ketamine HCI (100 mg/ml), acepromazine maleate (10 mg/ml) and xylazine (20 mg/ml) (3:2:1, v/v/v). PND 15 and 21 rats received the same dose i.p. The common carotid artery of adults was exposed and cannulated with a 23G needle attached to PE-50 tubing. It was necessary to use a 30G needle affixed to PE-10 tubing to cannulate PND 15 and 21 pups. Each animal’s cardiac ventricles were then cut to stop the flow of blood to the brain before infusion was begun, as well as to allow drainage of the perfusate. Two ml (for adults) or 0.5 ml (for pups) of saline were then slowly injected into the cannula to flush as much blood as possible from the brain’s vasculature. Dosing solutions were incubated at 37°C in an orbital shaker for 15 min prior to injection. A Harvard syringe pump was used to infuse dosing solutions into the left brain of each rat at a rate of 500 µl/min for 4 min in adults and 250 µl/min for 2 min in pups. After the infusions were completed, 2 ml (for adults) or 0.5 ml (for pups) of saline were slowly perfused through the cannula to flush unabsorbed test compound

from the vasculature. *In situ* brain perfusion is reported to be one of the most sensitive and reliable techniques for estimating CNS uptake of xenobiotics (Bickel, 2005).

The left half of the brain was removed after perfusion and processed for measurement of the two isomers. Portions of brain were homogenized in 2 volumes of ice-cold distilled water with a Tekmar Tissuemizer. One ml of brain homogenate was added to 4 ml of Ecolite scintillation cocktail, and each isomer quantified by liquid scintillation counting with a Beckman Coulter LS 6500 and normalized for tissue weight.

Data were analyzed and expressed as mean ± S.D. by use of Microsoft Excel (Microsoft Co., Redmond, WA.). The statistical significance of differences between groups was assessed by one-way ANOVA, followed by Tukey's multiple comparison test, with a significance level of $p< 0.05$, using GraphPad Prism 5.01 (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Maturation of the BBB had a pronounced effect on penetration by both CIS and TRANS *in situ*. Brain uptake of each of the three infused concentrations of CIS and TRANS was significantly greater in the PND 15 than in the PND 21 or PND 90 rats (Table 1). The extent of CNS deposition of 1 μM of each isomer, a toxicologically-relevant concentration, was significantly different in all three age-groups. Brain uptake of 10 and 50 μM CIS and TRANS consistently appeared to be slightly higher in PND 21 weanlings than adults, but the apparent differences were not sufficient to be statistically significant. The magnitude of difference in pyrethroid permeation, as a function of stage

of BBB maturation, is included in parentheses in the table. Interestingly, uptake of 1 µM of each isomer decreases by ~50% with each stage of maturation.

Relatively little information was found in the literature on changes in BBB permeability to xenobiotics during development of rodents or humans. The BBB of PND 10 – 11 rats was reported to be more permeable than that of adults to the lipophilic corticosteroid triamcinolone (Arya et al., 2006). Cerebral deposition of inulin progressively decreased from PND 4 – 26 in rats (Ferguson and Woodbury, 1969). Structural integrity of the BBB was reported to be achieved by PND 21, the time of weaning of rats (Schulze and Firth, 1992). Tightening of endothelial junctions occurred in conjunction with increasing thickness of the basement membrane and envelopment by pericytes. Pericyte, astrocyte and basement membrane signaling were reported to maintain integrity of the tight cell junctions (Liebner et al., 2011). Pediatric studies involving iv drug injection and cerebrospinal fluid (CSF) monitoring have not provided definitive information on the progression of permeability changes in the BBB or chorid plexus of humans. Most investigations have been limited to neonatal and adult subjects. The most definitive data have been obtained from measurements of CSF protein levels and dye penetration. CSF protein levels decrease rapidly during the first 6 months of life (Wong et al., 2005), with the highest levels measured 2 – 4 weeks after birth (Shah et al., 2011). Misra et al. (1987) reported an inverse relationship between entry of Fluorescein into the CSF and age up to 6 months, with the largest drop in CSF fluorescein levels during the first 2 weeks. It might be assumed from the foregoing that reasonably effective BBB function is achieved within 2 to 4 weeks of parturition in both

rodents and humans. The immature rat thus appears to be a reasonable animal model with which to forecast CNS dosimetry in infants and children (Semple et al., 2013).

The magnitude of age-dependent differences in pyrethroid permeation, as a function of the stage of BBB maturation, is included in parentheses in Table 1, which includes measured brain concentrations. BBB permeability varied with the concentration of infused CIS and TRANS in rats in the current investigation. Uptake of 1 μM CIS and TRANS is 5.4- to 5.5-fold greater in the brain of the least mature pups than in the adult brain (Table 1). The disparity in brain levels between pups and adults was less pronounced at the two highest CIS and TRANS concentrations. Saturation of an active influx process with limited capacity in immature rats may be responsible for this phenomenon. A low affinity influx transporter was described for DLM, CIS and TRANS uptake by caco-2 cells, as well as DLM uptake by hCMEC/D3 (human brain microvascular endothelial) cells (Zastre et al., 2013; Amaraneni et al., 2016).

The isomer appeared to have little effect on the extent of CNS deposition of CIS and TRANS in any age group in the present study. Very similar levels of the two isomers were measured in the brain of animals infused with 1 μM (Table 1). CIS concentrations consistently appeared to slightly exceed TRANS concentrations for the 10 and 50 μM infusions. The apparent prominence of CIS increased with infused concentration and animal age, although CIS deposition was significantly higher only in adults perfused with 50 μM.

Brain uptake of both CIS and TRANS was linear in all three age-groups of rats over the 50-fold range of concentrations (Fig. 1). R^2 values for all ages of animals exceeded 0.999. Amaraneni et al. (2017b) also found brain uptake of DLM to be linear.

In vitro experiments with CMEC/D3 cells revealed uptake of DLM to be a passive, non-saturable process (Amaraneni et al., 2016). BBB penetration by xenobiotics has been observed to increase with lipophilicity up to a point, beyond which higher Log P values result in diminishing permeation (Banks, 2009). DLM's Log P and molecular weight are 6.1 and 505.2 (ATSDR, 2003). The similarly high Log P (6.5) and molecular mass (391.3) of permethrin satisfy two of Lipinski et al's. (2001) criteria for poor membrane permeability. Lipid partitioning, coupled with hydrophobic bonding, serve to reduce the flux of large, highly lipophilic chemicals such as pyrethroids through the BBB, virtually trapping them there in membranes (Liu et al., 2011; Waterhouse, 2003). The magnitude and the age-dependency of brain uptake of DLM in rats *in situ* (Amaraneni et al., 2017b) were strikingly similar to CIS and TRANS in the current study. Comparable brain uptake of CIS and TRANS indicate that dissimilar BBB permeation does not contribute to the greater acute neurotoxic potency of CIS in rats. Although the permeability of the BBB to pyrethroids in limited, it appears likely that increased permeability of the immature BBB contributes to the elevated target organ dosimetry manifest in this age-group (Kim, et al., 2010; Mortuza, et al., 2018), possibly resulting in enhanced neurological effects of the insecticides during early development.

AUTHORSHIP CONTRIBUTIONS

Participated in the research design: Mortuza, Edwards, White, Cummings and Bruckner

Conducted experiments: Mortuza, Edwards and Patel

Contributed new reagents or analytical tools: NA

Performed data analysis: Mortuza, White, Patel and Cummings

Wrote or contributed to writing the manuscript: Mortuza and Bruckner

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FOOTNOTES

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Figure Legends

Figure 1: Concentration-dependent brain uptake of CIS (A) and TRANS (B) by PND 15, 21, and 90 rats infused with 1, 10, or 50 μM CIS or TRANS. Linear regression was conducted by GraphPad Prism. R^2 values > 0.999 were obtained for each group. Each point represents mean ± S.D. (n = 3-5).

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TABLE 1

Age-dependency of brain uptake of CIS and TRANS

Uptake is represented as pmol CIS or TRANS/g brain. Data are expressed as mean ± S.D. (n=3-5).

Isomer	µM	PND 15	PND 21	PND 90
CIS	1	53 ± 6*# (5.4)	23 ± 5* (2.4)	10 ± 1
CIS	10	498 ± 70*# (3.7)	188 ± 24 (1.4)	136 ± 22
CIS	50	2044 ± 415*# (3.1)	900 ± 218 (1.4)	666 ± 55
TRANS	1	52 ± 15*# (5.5)	24 ± 5 (2.5)	10 ± 1
TRANS	10	405 ± 65*# (3.7)	146 ± 16 (1.3)	110 ± 9
TRANS	50	1918 ± 632*# (3.8)	754 ± 146 (1.5)	510 ± 80

*Denotes significant difference from adults (P<0.05).

#Denotes significant difference from PND 21 animals (P< 0.05).

Values in parentheses are multiples of PND 90 values.

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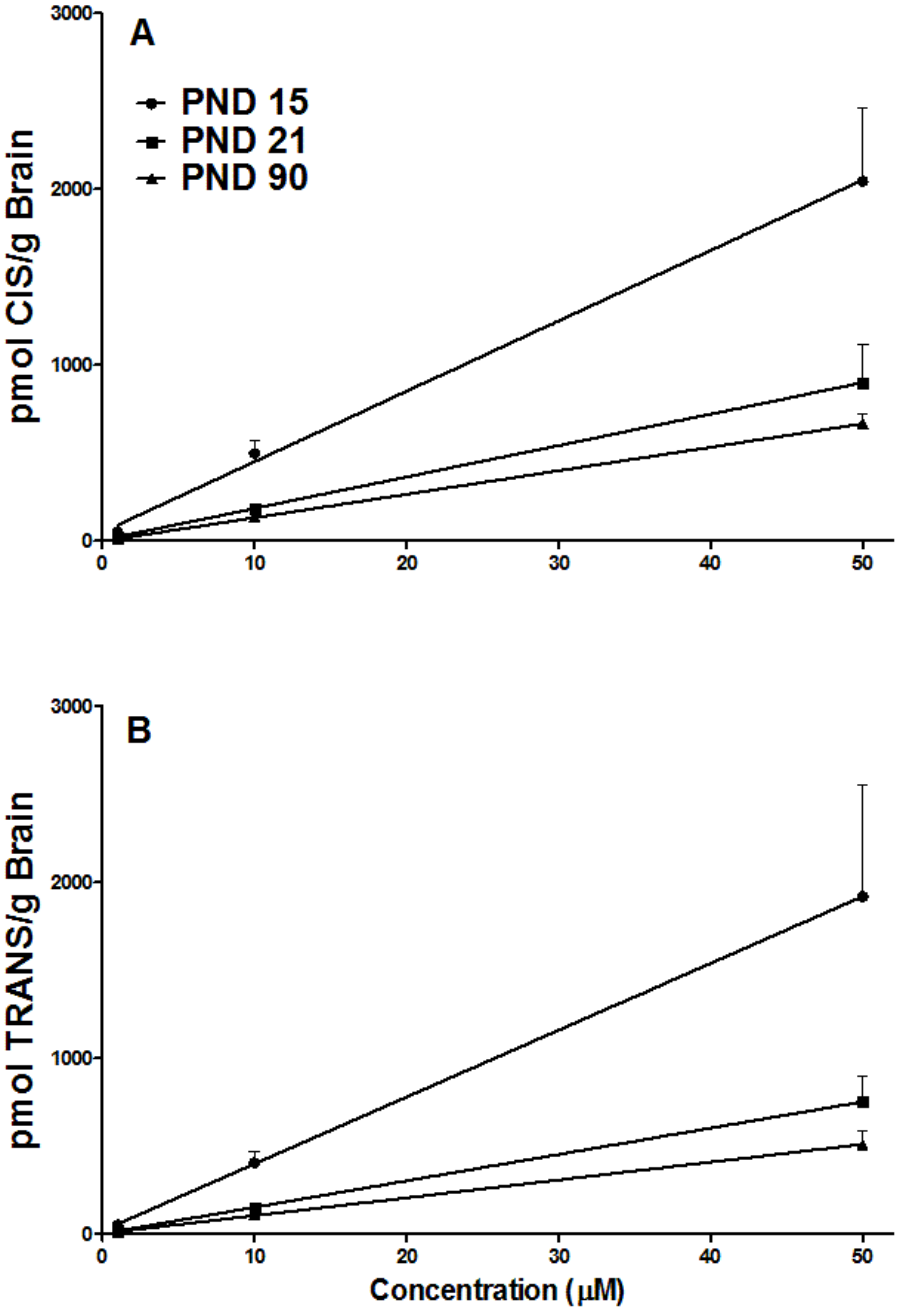

Figure 1:

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