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Abstract

Background: Pesticides have been extensively used around the word but concerns over their influence on environment and health is growing. Synthetic pyrethroids are potent insecticides considered to be neurotoxicant.
Hypothesis: We hypothesize that perinatal exposure to type I (Cypermethrin) and type II (d-Allehtrin) pyrethroids would produce behavioral effects on offspring during development.
Methods: Pregnant Wistar rats were exposed during gestation and lactation periods, and their pups were evaluated for somatic and sensory motor changes after weaning, and for locomotor activity, motor coordination, and anxiety-like behavior at post natal day 23 and 75 (PND23 and PND25).
Results: Cyp and All provoked somatic and sensory-motor reflex alterations in weaned pups. In PND23 pups both Cyp and All decreased locomotor activity and motor coordination, and provoke anxiogenic-like effect. In PND75 pups both Cyp and All decreased locomotor activity and motor coordination. Cyp but not All provoked anxiogenic-like effect.
Conclusion: Perinatal exposure to selected type I (d-allethrin) and type II (cypermethrin) pyrethroids provoked physical and sensory-motor alterations in weaned pups and persistent behavioral effects during offspring development, suggesting Cyp with a major power to cause neurotoxicity through time.

Key words: pyrethroids, development, behavior, neurotoxicity, rat

Introduction

Throughout the time pesticides have largely benefited the human life through enhancement of agricultural products and controlling infectious diseases, but their extensive use, in turn, has caused continuous problems of toxicity in nontarget organisms including humans, due to continued low level exposure[1].

In addition, occupational exposition occurs at higher levels compared to the population in general, is more significant, and toxicity is enhanced in this special situation[2].

Along with the wide use of pesticides in the world, the concerns over their health impacts are rapidly growing. There is a huge body of evidence on the relation between exposure to pesticides and elevated rate of chronic diseases such as different types of cancers, diabetes, neurodegenerative disorders like Parkinson, Alzheimer, and amyotrophic lateral sclerosis, birth defects, and reproductive disorders[3,4].

Scientific reports in the last years have shown that there is a high potential for pesticides toxicity to humans and the association of the indiscriminate use of these substances with neurodevelopmental disorders in children has drawn attention to the various problems arising from its use and exposures occurring during the pre and perinatal periods[5,6].

For many years now, there has been public concern raised about the potential health effects of pesticides on the developing fetus and in childhood. Depending on stage of development, the fetus is selectively sensitive to particular chemical toxicants[7,8]. Currently, there is much epidemiological evidence linking pre- and post-natal exposures to pesticides with congenital disorders[9].

With further regard to maternal exposure to pesticides in early pregnancy, epidemiological studies suggest that maternal employment in agriculture or gardening may be a risk factor for birth defects[10].

Because studies evaluating possible neurological and neurobehavioral effects of pesticides in children are indeed rare, authors identified the need to examine neuro-behavioral and neurodevelopmental status of children exposed to these toxicants[11, 12].

Synthetic pyrethroids are a very potent insecticides class that is widely used in forestry, public health, household products and in agriculture throughout the world and generally considered to be the safest class of insecticides available[13].

Pyrethroid neurotoxicity to adult animals has been well characterized and literature data offers several comprehensive reviews of pyrethroid toxicity, metabolism, and actions[14]. However, information regarding the potential developmental neuro-toxicity of this class of pesticides is still scarce.

Historically, pyrethroids were grouped into two subclasses (Types I and II) based on chemical structure and the production of either the T (tremor) or CS (choreoathetosis with salivation) intoxication syndrome following intravenous or intracerebral administration to rodents[15].

Pesticides of pyrethroids group are know by provoke nervous paralysis for drawing out the opening sodium channels of the neuronal membrane delaying the repolarization and by increase nervous excitability due to inhibition of the chlorine flow regulated by γ-aminobutyric acid (GABA). Pyrethroids also may act in voltage-sensitive calcium channels. All effects together may contribute to the neurotoxicity of these chemical compounds[15.16].

Very few studies have reported specific functional consequences linked with perinatal exposure to two prototypical pyrethroids studied, d-allethrin (type I) and/or cypermethrin (type II), neither investigated persistent behavioral toxicity occurrence thought weaned to adult animals.

The present study aimed to study perinatal exposure by d-allethrin (type I) and cypermethrin (type II) pyrethroids in rats, by evaluate social behavior (anxiety) and motor (locomotor activity and motor coordination) changes during offspring development.

Material and Methods

Animals and treatments

All procedures for animal experimentation were approved by the institutional Committee of Ethics for Use of Animals, Biosciences Institute, São Paulo State University (UNESP), in accordance with international guidelines for animal use and care.

Wistar female and male rats, acquired with 22 day-old from our institutional colony were kept under standard conditions (up to four rats per cage, controlled temperature of 22±2°C and 70% humidity, 12-h light/dark cycle starting at 6a.m. with light, continuous exhaustion, receiving water and food ad libitum) until they reached 75 days of age. Animals were mated by keeping overnight two females and one male per cage. On the pregnancy day 0 (determined by the presence of sperm in vaginal smears) the pregnant dams were divided in d-allethrin (All), cypermethrin (Cyp) and control (Ct) groups (N=15), housed singly and were orally administered, by gavage, d-allethrin (Sigma Aldrich), cypermethrin (Sigma Aldrich), as a single daily dose dissolved in corn oil or only corn oil as control vehicle. The treatment lasted throughout pregnancy and lactation.

Choose of dose was made based in LD50 to no produce clear mother's alterations (e.g. food and water intake reduction and weakness, fine to whole-body tremor, decreased motor activity, neuromuscular weakness, weight loss, impaired viability, or some other behavioral manifestation), which could affect pups normal development[17]. In accordance to this paradigm the 1/20 LD_{50} dosage was selected to use in experiments, respectively 43 mg/kg for All and 12,5 mg/kg for Cyp.

At birth, all litters were culled to six male pups per mother to avoid interference of litter size on nutritional state of the pups and, consequently, on their development. Whenever possible only male rats were kept within the litter and females were kept just to maintain equal litter sizes. Pups were maintained with their mothers and weaned at PND21.

Toxicity Evaluations

Mothers

Maternal toxicity during exposition period was evaluated through observations on weight gain, food and water consumption measured on a weekly basis, and also weakness, fine to whole-body tremor, decreased motor activity, neuromuscular weakness, mood swings or some other behavioral manifestation or impaired viability. The level of mother's exposition was confirmed through insecticides dosage at 20th gestational day[18]. Additionally number of pups per litter was scored.

Pups

Pup weights at birth and during developing period were recorded on a weekly basis. In order to assess the level of offspring pyrethroid exposure, for each litter, pups (N=8) were euthanized by decapitation at PND-1, 23 and 75, and blood was collected for pyrethroids quantification[18].

PND1 to PND15: There were evaluated the somatic (weight gain, incisors eruption, eyes opening, first appearance of hair, unfolding of ears, and testes descent) and sensory-motor reflex (postural, negative geotaxis, palmar grasp, and acoustic startle reflex) development[19]. In rats, palmar grasp reflex is present at birth, and disappears during growth and development, contrary to other animals in which it appears in the first days of growth.

PND23 and PND75: At these selected ages rats of the control, allethrin, and cypermethrin groups were submitted to open-field (OF), elevated plus-maze (EPM), and hole-board (HB) behavioral tests.

Open-Field tasks

The OF behaviour was assessed using a wood box measuring 97 x 32.5 cm (diameter x height), similar to that described previously[20]. This was divided into three concentric circles, which were subdivided by painted black lines into 18 similar spaces. For OF observations, each rat was individually placed in the centre of the arena and was observed during 3 minutes. Locomotor activity was assessed by the number of floor units entered with the paws. Additionally, was recorded the number of entries into the central area which can be considered an unprotected area for rats. Entries into the central circle of the arena provided a measure of anxiety-like behavior[21]. Rats that spend less time in the center and takes more time to first cross through it, are regarded as more "anxious".

For the social interaction test, rats exposed to the same treatment were housed in pairs for 7 days prior to the test. The test, which is sensitive to anxiogenic drugs[22], consisted of familiarizing each pair (cagemates) of rats with the arena for a period of 8 min on 2 consecutive days. On the third day, each rat was randomly assigned to an unfamiliar partner according to weight. These animals were placed in the arena to observe social interaction behavior for 5 min. Social interaction time (in seconds) per pair of rats was measured as the time spent sniffing the partner, climbing over and crawling under the partner, mutual grooming, genital investigation, and following and walking around the partner[22]. Aggressive behavior was not considered to be a social interaction behavior.

Elevated Plus-Maze tasks

The elevated plus maze apparatus consisted of two open arms, 50 x 10 cm (length x width) and two closed arms, 50 x 10 x 50 cm (length x width x height) with an open roof arranged such that the two arms of each type were opposite to each other. The maze was elevated from the floor. For the test, each animal was placed in the center of the maze, facing one of the closed arms and the number of entries into, and the time spent in the open and the closed arms were registered for 5 min[23].

Hole-Board tasks

Motor coordination in hole-board was evaluated according Godinho et al. (2014)[24]. The apparatus consists of a square box (28x28x20cm), taking the floor painted white and containing 36 holes of 2 cm diameter by 1 cm deep (arrangement of 6x6). An acrylic lid covers the box to allow viewing and filming inside the box. For each animal, motor coordination was evaluated during 5 min, according to the number of times the animal's leg disappeared into a hole (paw-dip number).

All apparatus (OF, EPM, and HB) used in behavioral tests were carefully cleaned with 5% ethanol before each rat was introduced, to remove traces of the previous animal. All tasks assessed in OF, EPM, and HB were filmed for further evaluation.

Blood levels quantification

At the ages of 1, 23 and 75 days old and after behavioral assessment (PND 23 and 75) animals (N=8) of all treatment groups were euthanized by decapitation for blood collection and pyrethroids determination[18].

Statistical analysis

The results were statistically analyzed using GraphPad Instat Software (San Diego, California, USA). Data were compared using the One-way analysis of variance (ANOVA). A Tukey–Kramer post hoc test was used for comparisons between means when ANOVA

was significant at the $P < 0.05$ level[25]. Values were expressed as mean ± standard error mean (S.E.M.).

Results

Administrations of Cyp or All to mothers not modified their food and water consumption, weight gain, nor do modified litters size or pups weight at birth, compared to Ct group (data not shown). Neonates born from pyrethroid-treated dams (both groups) were phenotypically normal at gross level, regardless presence of drug parent in blood. In addition, none signal of maternal toxicity was observed during Cyp or All exposure period (data not shown). The non occurrence of maternal toxicity seen in present experiment indicates that any change occurring with offspring's was therefore exclusively due to exposure to fipronil.

Somatic and sensory motor development of pups

We observed no significant differences ($p > 0.05$) in the weight gain between animals of the Ct, Cyp and All groups (data not shown).

Figure 1 shows somatic (A) and sensory motor reflex (B) development in pups after weaning. As observed in Fig. 1A, the time for incisor eruption, first appearing of hair and ear unfolder were significantly decreased ($P<0.05$) in animals of Cyp and All groups compared to control, but eye opening and testes descent were normal ($p>0.05$) in Cyp and All groups compared to Ct.

Figure 1B shows that puppies of mothers receiving both Cyp or All, had significant increase ($P<0.05$) in palmar grasp disappearance and negative geotaxis and postural reflex appearance compared to Ct pups nevertheless, the acoustic startle reflex was significantly lower ($P<0.05$) in Cyp and All animals compared to Ct pups.

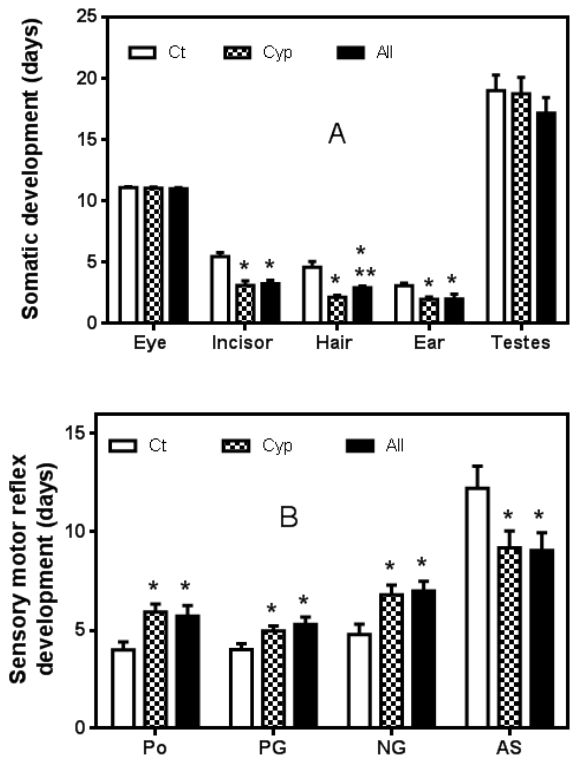

Figure 1- Somatic development (A) and Sensory motor reflex development (B) in pups of mothers exposed to treatments (Ct, Cyp, and All) during the perinatal period (N = 8-10 litters). Ct=control; Cyp=cypermethrin; All=d-allethrin; Po=postural reflex; PG=palmar grasp reflex†; NG=negative geotaxis reflex; AS=Acoustic Startle reflex. Values are the mean of time ± S.E.M. (ANOVA). *p<0.05 vs. Ct. **P<0.05 vs. Cyp.

Blood pyrethroids levels

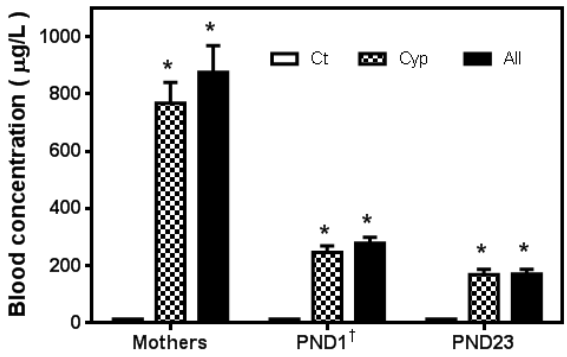

Figure 2- Blood pyrethroids concentration in mothers (21th day gestation), PND1 pups†, and PND23 pups (N = 8-10). Ct=control; Cyp=cypermethrin; All=d-allethrin. †Pool of 3 animals. Values are the mean ± S.E.M. (ANOVA). *p<0.05 vs. Ct.

Figure 2 shows that mothers exposed to pyrethroids during gestation and lactation period have high blood level (P<0.05) of Cyp or All at 20th gestational day and that your pups had high blood levels (P<0.05) of Cyp or All at birth and in PND23. Nevertheless, while the level of Cyp or All was smaller in PND23 pups compared to PND1 (respectively 31%for Cyp and 38% for All), in PND75 Cyp or All were not detected in blood (data not shown). These results on pyrethroid dosage can reflect a predominant fetal more than milk transference of pyrethroids for the pups and lack of bioaccumulative effect.

Tasks	Ct	Cyp	All
PND 23			
Ambulation (count)	61.88±6.59	45.33±4.28 *	44.4±4.96 *
Crossings (count)	4.6±0.51	3.2±0.19 *	3.5±0.21 *
Latency to cross (s.)	105.3±10.8	140.1±13.5 *	145.3±14.0 *
Interaction time (s.)	55.30±5.67	34.42±3.94 *	36.41±3.71 *
PND 75			
Ambulation (count)	79.00±7.12	56.50±5.22 *	59.80±5.74 *
Crossings (count)	3.9±0.31	2.8±0.22 *	3.2±0.29
Latency to cross (s.)	130.5±13.7	170.7±16.8 *	160.3±15.6
Interaction time (s.)	47.94±4.74	32.44±3.91*	39.42±4,11

Table 1- Effects of perinatal Cyp and All exposure on open field tasks in PND23 and PND75 pups (N = 13-15). Ct=control; Cyp=cypermethrin; All=d-allethrin. Values represents the mean ± S.E.M. (ANOVA). *p<0.05 vs. Ct.

Behavioral assessment

Table 1 shows the effects of Cyp and All perinatal exposition on tasks assessed in animals in OF. We observed that pups exposed to Cyp or All had a significant decrease (P<0.05) in locomotor activity in both PND23 and PND75. The number of crossings through the center of arena was significantly decreased (P<0.05) by Cyp or All treatments in PND23 pups and only by Cyp treatment in PND75 pups, compared to Ct group. The latency time to first cross was significantly increased (P<0.05) by Cyp or All treatments in PND23 pups and only by Cyp treatment in PND75 pups compared to Ct group. We observed that the interaction time was significantly decreased (P<0.05) by Cyp or All treatments in PND23 pups and only by Cyp treatment in PND75 pups, compared to Ct group.

Tasks	Ct	Cyp	All
PND 23			
Open arms entries (%)	35.3±3.3	22.4±2.3 *	21.7±2.2 *
Time spent on open arms (%)	41.1±5.2	28.2±3.1 *	27.8±3.1 *
PND 75			
Open arms entries (%)	30.5±3.2	22.8±2.3 *	25.4±2.9
Time spent on open arms (%)	40.2±4.9	29.0±3.2 *	35.2±4.1

Table 2- Effects of perinatal Cyp and All exposure on elevated plus maze tasks in PND23 and PND75 pups (N = 13-15). Ct=control; Cyp=cypermethrin; All=d-allethrin. Values represents the mean ± S.E.M. (ANOVA). *p<0.05 vs. Ct.

In Table 2 we observed that Cyp or All exposure significantly reduced (P<0.05) the percentage of open arms entries and time spent on open arms compared to Ct group, in PND23 pups. In PND75 pups Cyp exposure significantly reduced (P<0.05) the percentage of open arms entries and time spent on open arms compared to Ct group, while All exposure not affected significantly (P>0.05) open arms entries and time spent on open arms, compared to Ct group.

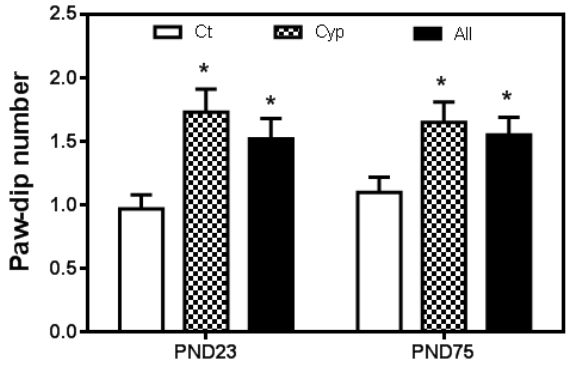

Figure 3- Effects of perinatal Cyp and All exposure on paw-dip number in PND23 and PND75 pups (N = 13-15). Ct=control; Cyp=cypermethrin; All=d-allethrin. Values are the mean ± S.E.M. (ANOVA). *p<0.05 vs. Ct.

Figure 3 shows that Cyp or All exposure significantly increased (P<0.05) the paw-dip number compared to Ct group in both, PND23 and PND75 animals.

Discussion

Findings obtained in animals indicate toxicity by pyrethroids exposure[26,27]. Repeated low-level rat exposure during gestation and lactation may provoke contamination of offspring and once pyrethroids reaching the nervous system in enough concentration, they could cause adverse neurotoxic effects in developing rats in absence of maternal toxicity[15,28,29]. In accordance with these previous data, his work report toxic effects during offspring development related to perinatal exposure to two prototypic pyrethroids, Cyp and All.

Ahlbom et al. (1994)[30] related dose-response changes in brain muscarinic receptors and behavior in neonatal and adult mice, after neonatal exposure to the type-I pyrethroid bio-allethrin. Data reported by Biernacki et al. (1991)[31] and Santoni et al. (1998)[32] showed that, during gestation, cypermethrin exposure induces severe and persistent alterations in pups neural and physical development and behavior. Our results shows clearly changes in physical and sensory motor development in weaned pups and are in according these previous findings.

Present results showed that the blood levels of Cyp and All dosed in pups were lowers in PND23 than PND1 rats. This fact may suggest the prevalence of fetal transference more than the pyrethroid absorption via milk process. Both, fetal transference and transference via milk had been documented from pyrethroids by other authors[32,33]. This is worrisome due to the possibility of toxic pyrethroids effects could be enhanced in risks groups as pregnant women, infants, and children[34].

Recent epidemiological data has raised concerns about human's exposition to pyrethroids once exposure to pyrethroids has been shown to occur in humans, including exposure to pregnant women, infants, and children[34]. Recently, Shelton et al. (2014)[5] observed that children of mothers residing near pyrethroid insecticide applications just prior to conception or during 3rd trimester were at greater risk for both autism spectrum disorders (ASD) and developmental delay.

In the present work was observed that pyrethroids perinatal exposure induced changes in the onset time of many physical features and reflexes in developing pups. Undernutrition, a common confounding variable in developmental research, could be ruled out as a causative agent for the changes observed, but here, mother's and pups had normal weight gain in the course of the study.

On other hand, the in uterus exposition to pyrethroids might affect the general development, including the neural development of pups[29]. As dams continued exposed during lactation period and their pups receive the pyrethroids through the milk, maturation of the central nervous system in offspring probably also was prejudiced. The fact that we observed behavioral alterations in PND23 and PND75 pups exposed to Cyp and All, through their mother's, strength this

hypothesis. These results are in according with findings of literature suggesting that exposure to drugs and environmental agents during pre- and postnatal periods can affect behavior during childhood and adult life of individuals[35,36].

Behavior has been recognized as a biological indicator of function for neurotoxicity provoked by chemicals agents[37]. Neurobehavioral screening is still a recommended and sensitive approach to differentiate between Type I and Type II pyrethroids for hazard identification[38], and it is part of multidisciplinary approaches to assess adult and developmental neurotoxicity due to the complex and diverse functions of nervous system[39].

Previous studies with pyrethroids in animals observed decreases in motor activity[40, 41,42]. Our results are in agreement with these previous findings although the profile of our experiment is different because, while these authors performed experiments to observe acute effects and sometimes with high doses, we used rat perinatal exposure, at low doses, and studied offspring's to obtain data on the neurotoxic effect during development. Recently, work by Mozer et al. (2016)[43] with the pyrethroid cyhalothrin in rats suggested differences between isomers (λ and γ) in the effect on locomotor activity.

Nasuti et al. (2007)[44] studying the effects of neonatal exposure of rats to permethrin, observed an increase in the spontaneous locomotor activity in OF arena. Contrary to these results, in our experiments we found decrease in spontaneous locomotor activity in OF arena. Factors such as dose, pyrethroids type, age of animals, exposure time and quantitative relation between isomers of compound used, may be responsible for these discrepancies between results.

Here, the results obtained in OF arena and in EPM, for anxiety assessment in PND23 pups, are very consistent with reduced social interaction and together, indicate an anxiogenic-like effect provoked in pups by Cyp and All perinatal exposure which must be related to pyrethroids presence in animal's body. Already in PND75 pups was observed that Cyp effect on anxiety persisted despite the disappearance of pyrethroids from the animal blood. This fact is consistent with the hypothesis sustained by others authors that environmental toxicants can induce fetal reprogramming due to effects in uterus and provoke diseases that persists during development until adulthood[5,45]. The effect of All on locomotor activity persisted in PND75 pups but the anxiogenic-like effect no. As the molecule of All not contain an alfacyano group, perhaps this radical is important to the persistence of pyrethroid effect on anxiety but not on motor effect.

Unfortunately, there is limited evidence available indicating that pyrethroids exposure may disrupt some aspects of social behavior, including anxiety. Our result it's opposite from those obtained by Spinosa et al. (1999)[46] which not observed anxiety alterations by fenvalerate administration, but are similar to those performed by Righi and Palermo-Neto (2003)[47] which found a anxiogenic-like response due to cyalothrin administration in rats.

An important aspect to be commented on is the fact that although the animals receiving Cyp or All presented less amount of ambulation, they still had a decrease in the motor coordination assessed in HB. The importance of this is the fact that increased locomotor activity could influence the paw-dip number increasing it. Other important aspect is the fact that the effect of All on locomotor activity persisted in PND75 pups but the anxiogenic-like effect was not observed. As the molecule of Cyp contain a CN^- radical but All no, and it provides a greater half-life to pyrethroid molecule[48], perhaps this radical is important to persistence of pyrethroid effect on motor activity but not

on anxiety status, showing specificity of action related to molecular type.

Some mechanisms for neurotoxicity by pyrethroids had been suggested including neurotransmitter modulation and apoptosis [30,49,50], calcium channels modulation[24], and cell damage by oxidative stress[44]. Results obtained in the present work not discriminates about mechanistic actions of the two types of pyrethroids studied, however strengths the need of more studies on pyrethroids actions to understand better their toxicity at CNS level.

Overall, in attention to the present data seems prudent that pregnant mothers should avoid exposure to chemicals as pyrethroids insecticides during periods of pregnancy and lactation to minimize offspring exposure in uterus and through breast milk wherever possible.

Concluding, perinatal exposure to selected type I (d-allethrin) and type II (cypermethrin) pyrethroids provoked physical and sensory-motor alterations in weaned pups and persistent persistent behavioral effects during offspring development, suggesting Cyp with a major power to cause neurotoxicity through time.

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