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The effects of gestational perchlorate exposure on development and behavior in rats

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THE EFFECTS OF GESTATIONAL PERCHLORATE EXPOSURE ON DEVELOPMENT AND BEHAVIOR IN RATS

by

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ABSTRACT

The Effects of Gestational Perchlorate Exposure on Development and Behavior in Rats

by

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Pregnant Long Evans rats were exposed to ammonioum perchlorate in their drinking water at doses of 0.8 mg/kg-day and 8.7 mg/kg-day (or at concentrations of 5.0 mg/L perchlorate and 50.0 mg/L perchlorate) from GD 7-GD 21. The offspring were observed for developmental landmarks and tested in a battery of behavior trials. Dams exposed at the 5.0 mg/kg-day perchlorate exhibited delayed or deficient responses in the number of pups per dam, pinna unfolding, negative geotaxis, startle response IP on PND 61, figure 8 maze on PND 21, and a more reactive tactile startle response on PND 21. Pups exposed at the higher concentrations showed delayed pinna unfolding, delayed negative geotaxis, and less reactive tactile startle response on PNDs 21 and 61, and a more reactive startle response IP on PND 21. The low dose-dependent effects observed in this experiment indicate that a U-shaped linear dose-response curve may be supported and behavioral responses appear to be compensatory and temporal after exposure to perchlorate during gestation.

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CHAPTER 1

INTRODUCTION

Ammonium perchlorate has been manufactured in large quantities in the United States since the mid 40's (U.S. EPA, 1999). Used as an oxidizing component, the perchlorate ion ($ClO4^-$) has been combined with ammonia to create ammonium perchlorate (U.S. EPA, 2002). Ammonium perchlorate is the primary ingredient in solid propellants for rockets, missiles, and fireworks due to its high thermodynamic energy release (Rogers, 1998). Perchlorate salts are also used in nuclear reactors, electronic tubes, lubricating oils, leather finishing, aluminum refining, paints and enamels (U.S. EPA, 1999). Perchlorate is a stable anion that results from the dissolution of soluble salts such as ammonium perchlorate and potassium perchlorate in water. Perchlorate is highly soluble in water, persistent, and extremely mobile in aqueous systems (U.S. EPA, 1998). One major source of contamination is the improper disposal methods used during manufacture processes (U.S. EPA, 2002). Currently, the EPA reports that eighteen (18) states have sites where environmental contamination exists and has thirty-nine (39) documented perchlorate manufacturers (U.S. EPA, 2004). Heightened concerns about widespread contamination in the United States emerged in 1997 when the California Department of Health Services developed advances in analytical detection capability that reduced the detection limit of perchlorate to 4 ppb (Wirt, Laikhtman, Rohrer, and Jackson, 1998) in drinking water supplies.

The presence of perchlorate in drinking water has forced the EPA to focus research on its potential adverse health effects in humans. Toxicological concerns are at the forefront of proposed and ongoing perchlorate research efforts. Prior to April 1997, research was focused on the repeated oral administration of perchlorate while treating Graves disease, an autoimmune condition that results in hyperthyroidism (Jubiz, 1979).

Thyroid hormones have an effect on almost every tissue of the body. The regulation of metabolism and developmental processes is dependent on the molecular actions of thyroid hormones (Oppenheimer and Schwartz, 1997). The persistence of iodine deficiency in many areas of the developing world still presents an important problem and has raised the possibility that even minimal degrees of undetected hypothyroidism in infants born in such iodine-deficient regions lead to sub-optimal intellectual development (Delange and Ermand, 1979; DeLong, Stanbury, and Fierro-Benitez, 1985), ataxia, spasticity, and deafness (DeLong, 1996). Perchlorate has been associated with a reduction in thyroid hormones in laboratory animals (Wolff, 1998) and is speculated to interfere with this system to alter normal development (Oppenheimer, et. al., 1997). While minimal exposure to $ClO4^-$ may have little significance on adult human thyroid function (Lamm, Braverman, Li, Richman, Pino, and Howearth, 1999) these same exposures during critical windows of development may have permanent adverse consequences to the developing fetus (Brechner, Parkhurst, Humble, Brown, and Herman, 2000). Thus, the present study was designed to investigate the relationship between developmental abnormalities in rodents exposed to perchlorate via their drinking water at two concentrations.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Perchlorate

The perchlorate ion has been combined with most every group in the Periodic Table with exception of the inert gases (Von Burg, 1995). Commercial production of perchlorate is typically achieved by oxidizing sodium chloride electrolytically to sodium chlorate (U.S. EPA, 2002). Ammonium perchlorate (CAS No: 7790-98-9) is prepared by the reaction of ammonia and perchloric acid (Grayson, 1978). Perchlorate is a strong oxidizer, can ignite violently (Von Burg, 1995) and therefore, is used as a primary ingredient in solid propellant for rockets, missiles, and fireworks (U.S. EPA, 2002). Large volumes of the compound have been produced and disposed of in the United States since the mid-1940s (U.S. EPA, 1999).

Perchlorate is exceedingly mobile in aqueous systems and can persist for many decades under typical groundwater and surface water conditions (U.S. EPA, 2002). Because perchlorate is difficult to remove from water, there is no current process to treat perchlorate contaminated water (Logan, 1998). Typical water treatment technologies such as ion exchange, air stripping, and advanced oxidation are not viable options to remediate perchlorate; which is extremely stable in water and does not adsorb well to activated carbon (AWWARF, 1997).

Chemical Properties

The perchlorate anion is a tetrahedral structure with four oxygen atoms at the vertices and the chlorine atom at the center as shown in Figure 1. A low association with cations is responsible for the extremely high solubilities of perchlorate salts in aqueous environments (U.S. EPA, 2002). Due to its structure, the chlorine atom is sterically blocked from the attack of an incoming reducing agent; the oxygen bonds increase in strength in aqueous solutions.

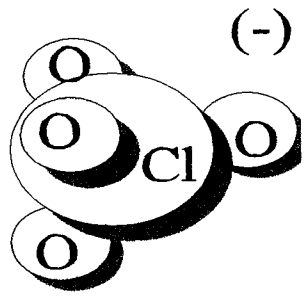

Figure 1. Perchlorate anion (ClO4-) structure.

Biotransformation of Perchlorate

The activation energy to perchlorate reduction is so high that perchlorate cannot be expected to act as an oxidant under human physiological conditions – this idea is supported by limited absorption, distribution, metabolism, and elimination (ADME) studies that show perchlorate is excreted virtually unchanged after absorption in both rats (Eichler and Hackenthal, 1962) and humans (Anbar, Guttmann, and Lewitus, 1959). ClO_4^- is eliminated rapidly in the urine with 95% of the administered doses being accounted for after 5 hrs in humans (Anbar, et. al., 1959) and 60 hrs in rats (Wolff, 1998). Goldman and Stanbury (1973) demonstrated that peak uptake of perchlorate at the thyroid

occurred at 4-6 hours. Chow and Woodbury (1970) used histological measurements, and examined inulin and sulfate spaces, to determine that ClO_4^- is distributed intrathyroidally to the follicular lumen. Perchlorate appears to undergo a two phased urinary elimination process in rats and calves (Selivanova and Arefaeva, 1986). Phase one accounts for approximately 96% of the administered dose with a half life of 1-2 hours. Phase two accounts for approximately 4% of the administered dose with a half life of 72-80 hours (Selivanova and Arefaeva, 1986). In other studies, half-lives for the rat vary from 6 to <8 hrs (Kutzim, Modder, and Bushsieweke, 1980), approximately 8 hours (Eichler and Hackental, 1962), and approximately 20 hours (Goldman and Stanbury, 1973).

Toxicity

The mode-of-action framework model proposed by the Environmental Protection Agency (EPA)(2002) (Figure 2) has served as the conceptual construct for the development of Physiologically Based Pharmacokinetic (PBPK) models. This model presents a schematic of the biomarkers of exposure from ingestion of perchlorate in drinking water, uptake into the blood, and the subsequent effects on thyroid hormonal balance that leads to neurodevelopmental and neoplastic sequelae.

Based on research that contributed to the Toxicology Excellence for Risk Assessment (TERA) group's objectives for developmental toxicity in rats, the EPA (2002) concluded that there are signs of maternal and developmental toxicity at the 30.0 mg/kg-day level suggesting it as a LOAEL with a NOAEL at the 3.0 mg/kg-day. The EPA states a clear caveat that while none of the previous study results were so clear that a definitive assessment can be made, the suggestive results are important to consider in light of the overall database of previous research and mode-of-action for the toxicity of perchlorate.

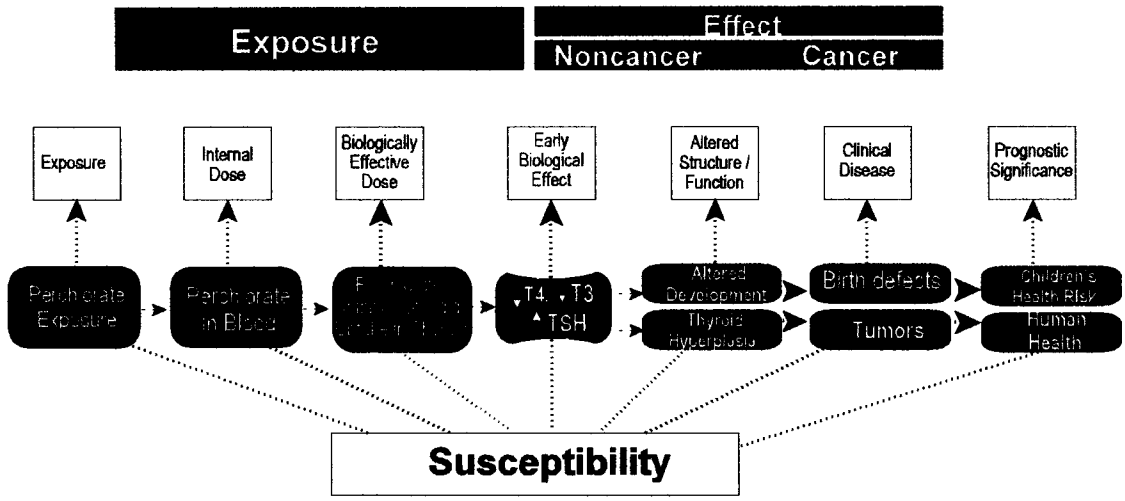

Figure 2. Mode of action diagram reproduced from (US EPA, 2002). Mode-of-action model for perchlorate toxicity proposed by the US EPA (U.S. EPA, 1998). Schematic shows the exposure-dose-response continuum considered in the context of biomarkers (classified as measures of exposure, effect and susceptibility) and level of organization at which toxicity is observed. The model maps the toxicity of perchlorate on this basis by establishing casual linkage or prognostic correlations of precursor lesions.

Thyroid Hormone System

Once perchlorate enters the blood, the first biological effect occurs with the competitive inhibition of iodide at the thyroid. This creates downstream effects that perturbate the thyroid hormone system.

The thyroid hormone system involves the thyroid gland, its uptake of iodide via the sodium/iodide symporter, and its secretion of hormones as regulated by the hypothalamic pituitary axis feedback system. The thyroid is an endocrine gland that plays a central role in metabolism. Thyroid hormones are of fundamental importance for the development of the central nervous system in the fetus and the newborn (DeLong, et. al. 1985). Two hormones are biosynthesized in the thyroid, triiodothyronine (T3) and thyroxine (T4 or tetraiodothyronine). Along with T3 reverse (rT3), an inactive isomer of T3, these hormones are the only iodine containing hormones in vertebrates. An adequate supply of I^- iodide is necessary for the thyroid gland to function (Goodman, 1994). Most of the ingested dietary I^- is accumulated in thyroid follicular cells in the gland and made available for T3 and T4 biosynthesis. A negative-feedback mechanism regulated by the concentrations of circulating thyroid hormones signals the thyroid to synthesize T3 and T4. (Goodman, 1994)

There are six main steps in the synthesis, storage and secretion of thyroid hormones. First, iodine is ingested in the diet and circulated as iodide (Anbar, et. al., 1959). Second, iodide is taken up by the follicle cells of the gland by active transport at the sodium/iodide symporter (NIS) (De La Vieja, Dohan, Levy, Carrasco, 2000). Third, oxidation of iodide to hypoiodate (OI^-) by the membrane bound thyroid peroxidase and H_2O_2, and the subsequent iodination of tyrosine residues of the thyroglobulin. Forth, coupling of iodotyrosine residues to generated iodothyronines. Fifth, the proteolysis of thyroglobulin and release of thyroid hormones into the blood. Sixth, the conversion of T4 to T3 in peripheral tissues. (Hadley, 1984)

NIS Symporter

The ability of thyroid follicular cells to concentrate I^- was first reported in 1915 (Jubiz, 1979). The thyroid gland was found to concentrate I^- by a factor 20-40 with respect to its concentration in the plasma (Wolff, 1964)(Figure 3). The Na^+/I^- symporter (NIS) system located in the basolateral membrane actively transports I^- against an electrical gradient (Goodman, 1994) stimulated by thyrotropin (De La Vieja, et. al, 2000) and has a Na^+ dependent secondary active transport process mediated by the NIS (Eskandari, Loo, Dai, Levy, Wright, and Carrasco, 1997). The transport of iodide into the thyroid, catalyzed by the NIS is the initial and rate limiting step in the formation of thyroid hormones (Ajjan, Findlay, Medcalfe, Watson, Crisp, Ludgate, and Weetman, 1998). Efflux of iodide from the lumen to the circulation requires crossing the same two membranes, as in influx, but in the reverse order (Wolff, 1998). The stoichiometry of the co-transport of Na^+/I^- is 2:1 (Eskandari, et. al., 1997). The inhibition of iodide uptake at the NIS results in a transient decrease in serum T4 and T3.

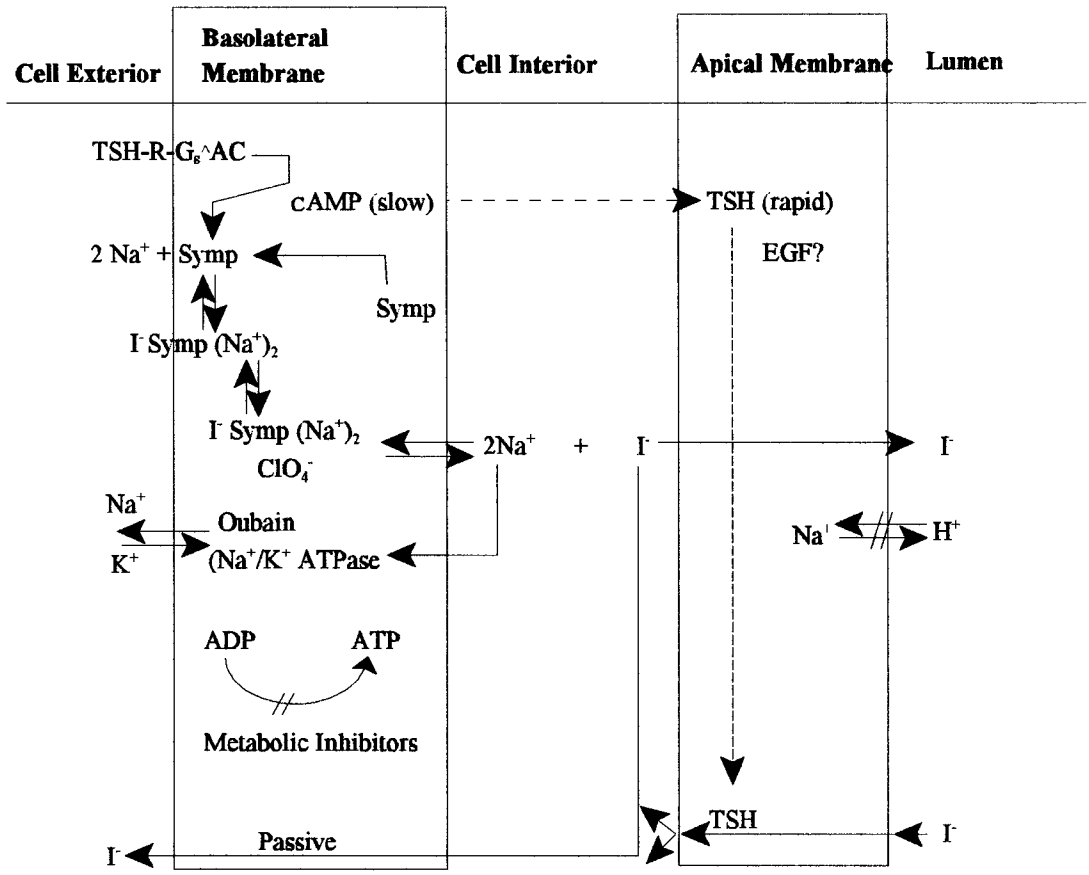

Figure 3. The NIS symporter: the thyroid is at least a three compartment system and much of the iodide in the thyroid is stored in the follicular lumen. Iodide accumulation occurs in two steps: an electrochemically "active" step across the basolateral membrane and an electrically "downhill" step across the apical membrane into the lumen. (Reproduced from Wolff, 1998)

Pituitary Axis Feedback System

The hypothalamic-pituitary axis is an important negative feedback loop that controls thyroid hormone secretion (Figure 4). The basic mechanisms for control in this system are: neurons in the hypothalamus secrete thyroid releasing hormone (TRH), which

stimulates cells in the anterior pituitary to secrete thyroid-stimulating hormone (TSH). TSH binds to receptors at the basolateral membrane in the thyroid gland, stimulating synthesis and secretion of thyroid hormones. When blood concentrations of T4 increase above a certain threshold, TRH-secreting neurons in the hypothalamus are inhibited and stop secreting TRH. Inhibition of TRH secretion leads to shut-off of TSH secretion, which leads to a lack of hormone/receptor complexes being formed, shutting off thyroid hormone secretion. As thyroid hormone levels drop below the threshold, negative feedback is relieved, TRH secretion starts again, leading to TSH secretion. (Hadley, 1984, Wolff, 1998)

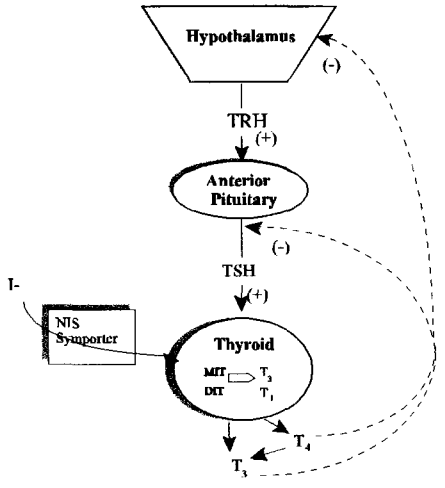

Figure 4. The hypothalamus pituitary feedback loop regulates thyroid hormone production. Iodide enters the thyroid at the NIS symporter.

Thyroid Hormone Synthesis

The functional unit of the thyroid gland is the follicle. The follicular lumen is filled with a thick colloid which contains thyroglobulin. The synthesis of thyroid hormones

begins with the tyrosyl amino-acid residues of thyroglobulin, a highly glycosylated protein of two subunits that contain 115 tyrosine residues (Lissitzky, 1984). Ingested iodine is absorbed in the intestines and taken up by the thyroid via the NIS and converted by a peroxidase to an oxidized species of iodine to make monoiodotyrosine (MIT). MIT is further iodinated to diiodotyrosine (DIT). The oxidative coupling of two DIT molecules forms T4 and the oxidative coupling of a DIT with an MIT molecule forms T3 (Hadley, 1984). The iodotyrosines are deiodinated in the thyroid by deiodinase enzymes leaving free iodide to be re-utilized for thyroid hormone synthesis. In situations of iodine deficiency this reutilization is maximized (Medeiros-Neto, 2001). In humans, iodotyrosines and thyronines are bound to thyroglobulin that have been hydrolyzed by proteolytic enzymes before entering the bloodstream (Goodman, 1994). Most thyroxine circulates bound to one of three major thyroxine-binding proteins in humans: about 70% to 75% is bound to thyroxine binding globulin (TBG), 15% to 20% to thyroxine-binding prealbumin (TBPA), and 5% to 10% to albumin (Hadley, 1984). The free T3 appears to be the most biologically active thyroid hormone under normal serum levels of iodothyronines. Triiodothyronine binds to TBG and albumin, but not TBPA, with less bond strength than that of T4. While both T4 and T3 are secreted by the thyroid, the major source of serum T3 is via conversion from T4. (Goodman, 1994 and Hadley, 1984)

Mechanism of Perchlorate/Thyroid Hormone System Interaction

Perchlorate is a unique chemical when tested for transportation into the thyroid gland. In a study conducted by Eskandari, et. al. (1997), the NIS was capable of transporting a wide variety of anions (I^-, ClO_3^-, SCN^-, $SeCN^-$, NO_3, BR^-, BF_4^-, IO_4^-, BrO_3^-), but perchlorate (ClO_4^-) was not transported. Although no conclusion was drawn about the

molecular commonality between the molecules, Eskandari's team thought that the tetrahedral geometry with an anionic volume similar to that of I^- is the reason ClO_4^- appeared to completely inhibit I^- transport. Wolff and Maury (1963) showed that the spherical halides and tetrahedral ions are concentrated by the thyroid tissue as well as being inhibitors of iodide transport. In the Eskandari's study the perchlorate ion was found to have greater anion selectivity than any other ion tested on the NIS, although he did not show it was transported. In other studies by Wolff, (1964) and Wolff and Maurey (1963), the ability of an anion to be concentrated by thyroid tissue showed that iodide is not the most highly selected in the following potency series for monovalent anion-based inhibition of I^- transport: TcO_4^->ClO_4^-->ReO_4^-->SCN^->BF_4^->I^->NO_3^->Br^->Cl^-. These studies demonstrate that selectivity for iodide or perchlorate in the thyroid gland might be based on an ion exchange mechanism. The larger anion will have the highest affinity for the exchanger because it has the lowest hydration energy. Hydration energy (rather than size) is a prime determining factor for selectivity (Wright and Diamond, 1977).

Perchlorate ions concentrate in the thyroid tissue where they compete with iodide ions at the NIS for the limited capacity of the thyroid gland thereby blocking thyroid hormone production and output (Anbar, et. al., 1959, and Wolff, 1998). The I^- accumulation in the thyroid is an active transport process that occurs against the I^- electrochemical gradient stimulated by thryrotropin (TSH) and blocked by perchlorate which acts as a competitive inhibitor (De La Vieja, et. al., 2000) as a result, ClO_4^- blocks hormone production and output. The work of Chow, Chang, and Yen (1969) and Chow and Woodbury (1970) showed that, as for iodide, perchlorate accumulation occurred in two steps: an electrochemically "active" step across the basolateral membrane and an electrically

"passive" step across the apical membrane into the lumen. However, perchlorate distribution into the lumen was greater than that of chloride (Wolff, 1998). Perchlorate promotes a very rapid and nearly complete discharge of iodide (Gray, Greig, Thomson, and MeLennan, 1974). As a result of the lack of iodide, a transient rise in serum TSH and a fall in serum T3 or T4, or both, occur (Mannisto, Ranta, and Leppaluoto, 1979). This relationship can be seen in the hypothalamic pituitary-axis feedback system (Figure 4).

During pregnancy and development a thyroid hormone deficit is of concern due to the critical role that these hormones play in preventing adverse neurodevelopmental sequelae (Maccia, 2000).

Comparative Thyroid Physiology

The threat of perchlorate is that it is a known competitive inhibitor of I^- at the thyroid gland. Thyroid tissue is present in all vertebrates, although some variations in the gross morphological arrangement occurs, Koibuchi and Chin (2000) note that thyroid hormone receptors are expressed in all cerebellar neuron types. The rat is an altricial species, born with a relatively undeveloped brain and with the thyroid pituitary hypothalamic axis not yet fully matured (Oppenheimer and Schwartz., 1997). Porterfield and Hendrich (1993) point out that the rat brain at birth is equivalent to the human fetus at 5 to 6 months of gestation and the rat at 10 days postnatal age is equivalent to the human brain at birth. Transplacental transport of thyroid hormones from the mother occur before the start of the rat embryos independent secretion which occurs on gestation day 17 (Oppenheimer, et. al., 1997).

The rat pituitary gland binds T3 more strongly than T4 (Oppenheimer, 1979). These binding sites appear to be specific for T3 and exhibit a high affinity but low capacity for this iodothyronine (Oppenheimer, 1979). The inhibitory action of the thyroid hormones

on pituitary TSH secretion in the rat are regulated through T3 (Hadley, 1984).

Both humans and rodents have low affinity serum protein carriers of thyroid hormones, like albumin, that are non-specific. However, in humans, there is a high affinity protein carrier thyroxine binding globulin (TBG) which binds T4. TBG is absent in rodents and therefore T4 is more susceptible to removal from the blood and has a half-life of only one day compared to 5-9 days in humans (Capen, 1997). Serum T3 half life in the rat is approximately 6 hr compared to 24 hr in humans (Capen, 1997). As a result, there is a more frequent need for exogenous T3 and T4 in the rat with a non-functioning thyroid than in the adult human (Capen, 1997). By demand, the follicular cells produce thyroid hormones at an accelerated rate (about 6-60 fold higher than humans) which requires chronic stimulation by TSH. As a result, thyroid-pituitary disruption effects in rats may be more sensitive than in humans (Hadley, 1984).

Hypothyroidism

In 1997, concern about potential adverse human health effects of perchlorate induced hypothyroidism were expressed by an external review panel convened by Toxicology Excellence for Risk Assessment (TERA). Symptoms of this disease include increased synthesis and secretion of iodide-containing hormones into the blood by the thyroid gland, enlarged thyroid gland, increased basal metabolism, and loss of weight when ingested perchlorate competitively inhibits iodide uptake which then depletes the iodine overload (deGroot and Buhler, 1971).

Goitrogenic chemicals are known to adversely affect the development of many organ systems, including the nervous and reproductive systems (Porterfield and Hendrich, 1993 and Jammin, 1995). Since perchlorate can cross the placenta, there is concern for severe developmental hypothyroidism caused by iodine deficiencies which can have devastating

effects on fetal and postnatal development, including mental deficiencies and hearing, speech, and motor deficits.

Pathophysiology of the Thyroid Gland

Thyroid hormones affect many physiological processes and are necessary for the normal development and physiology of an individual. Therefore it is not surprising that abnormalities of thyroid function can lead to gross alterations in thyroid function (Hadley 1984). Hypothyroidism and hyperthryoidism can occur at birth or later in life. Since perchlorate passes the placental barrier, abnormalities of thyroid function may result due to failure in growth or normal function of the thyroid gland at critical stages of fetal development.

Perchlorate antagonizes iodide transport through competitive inhibition (Hadley, 1984). Failure of the thyroid to produce T4 and T3 may result in the excessive secretion of TSH. The increased concentrations of TSH lead to hypertrophy of the thyroid follicular cells which results in the absence of thyroid hormone production (Hadley, 1984). As a result of the absence of any negative feedback to the hypothalamus and pituitary gland, excessive secretion of TSH may result in a goiter (Hadley, 1984).

Physiological Effects of Thyroid Hormones

Thyroid hormones have profound effects on growth, maturation and metabolism, therefore an insult to the homeostasis of the thyroid due to perchlorate exposure could have long-term effects. The skeletal system appears to be affected by a permissive or synergistic relationship with growth hormones and growth factors that promote bone formation. In most animals, the central nervous system develops during the perinatal period where thyroid hormones must be present for normal development of the brain. It is thought that the autonomic nervous system (particularly the sympathetic branch) is

affected by thyroid hormones by increasing the number of receptors for epinephrine and norepinephrine in myocardium and other tissues (Goodman, 1994), resulting in hyperactivity.

Oxidative metabolism, carbohydrate metabolism, lipid metabolism, and nitrogen metabolism are impacted by the cellular levels of thyroid hormones regulating such important systems as basal metabolic rates, glucose absorption, and the synthesis and degradation of body proteins (Goodman, 1994). Barker and Klitgaard (1952) demonstrated that a decrease in oxygen consumption results from a deficiency of thyroid hormones and excessive thyroid hormones increase the basal metabolic rate. Increased glucose absorption from the digestive tract, as well as glycogenolysis and gluconeogenesis in the hepatocytes, occurs with increased T3 concentrations in isolated cultures (Mariash and Oppenheimer, 1982). Since glucose is the major precursor for lipids, it is no surprise that the availability of carbohydrate and insulin are signaled by T3 acting as a control (Goodman, 1994). Both synthesis and degradation of body proteins are slowed when thyroid hormones are absent (Goodman, 1994). With thyroid deficiency, there is accumulation of a mucus-like material in extracellular spaces, particularly in the skin which causes water to accumulate, giving rise to edema (Goodman, 1994).

Objectives

The question of whether ammonium perchlorate is a toxicant is not disputed in the literature (Stanbury and Wyngaarden, 1952, Lampe, Modis and Gehl, 1966, Wolff, 1998, York, 2001, and Thuett, Roots, Mitchell, Geltles, Anderson, and Smith, 2002). Defining the effects of perchlorate in the developing brain after exposure to perchlorate during gestation has yet to be fully understood. It is assumed that developmental effects would

result from the absence and/or reduction of circulating thyroid hormones which have been proven to affect auditory and motor function (Goldley, Kehn, Rehnberg, and Crofton, 1995), and other endpoints identified with iodine deficiency disorders in fetuses, such as abortions, stillbirths, congenital anomalies, increased perinatal and infant mortality, mental deficiencies, and psychomotor defects (Medieiros-Neto, 2001).

Deficiencies of morphogenesis are presumed to be the basis for the failure of normal development of learning and motor skills in the hypothyroid rat (Schwartz, Ross, and Oppenheimer, 1997). These animals have poor development of the neuropil as a result of the dimished axonal and dendritic outgrowth, elongation and branching as well as the reduced number of dendritic spines (Thompson and Potter, 2000). A reduction in myelination, a multilamellar membrane that surrounds and supports axons, in the hypothyroid rat is due to a reduction in the myelin lipids and proteins which may be linked to the influence of thyroid hormones on oligodendrocyte differentiation. (Schwartz, 1983).

Thus, a study was designed to investigate the relationship between perchlorate exposure and possible toxic endpoints during development, using the rat as an animal model. The study tested two low concentrations of perchlorate, 5.0 mg/L and 50.0 mg/L and was based on the following two main hypotheses: (1) after exposure to low doses of perchlorate during gestation, offspring will exhibit altered development such as weight gain, reduced survival index, lower pup weight, delayed eye opening, delayed incisor eruption, delayed or premature pinna unfolding, as well as difficulties in surface righting, reflex suspension, and negative geotaxis; and (2) after exposure to a low dose of perchlorate during gestation, offspring will exhibit changes in behavior effects as measured by footsplay, gripstrength, startle response, and the figure 8 maze.

CHAPTER 3

METHODOLOGY

Approval of this research was given by the University of Nevada, Las Vegas Institutional Animal Care and Use Committee via protocol R993-0697-131.

Animals

Nineteen timed-pregnant Long-Evans hooded rats were obtained on gestational day (GD) 4 from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). The rats were singly housed in plastic shoe box style cages in an AAALAC approved animal facility. Ambient conditions consisted of a light-dark cycle of 12 hours, a temperature rage between 21-23 degrees Celsius and a constant 35% humidity. Rats were fed Purina certified rat chow *ad libitum* and were weighed and handled daily.

Dosing

The nineteen timed-pregnant rat dams were randomly divided into two main groups and a control group. Ammonium perchlorate (CAS No. 7790-98-9), 99.8% purity, was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Test formulations of ammonium perchlorate in deionized water were prepared weekly at two concentrations. The first group received a low concentration supplement of 5.0 mg/l. The second group received a high concentration supplement of 50.0 mg/l. The third group was given deionoized drinking water with no supplements. These concentrations are consistent with proposed research by the EPA.

Water intake in ml was calculated daily for each dam. The actual dose each animal received was calculated by multiplying the concentration of perchlorate by the 24 hr intake and dividing this by the animal's body weight for the same period. At the end of the 14 day dosing period, the actual dosages were 0.82 mg/kg-day and 8.70 mg/kg-day, which were slightly above the target of 0.4 mg/kg-day and 4 mg/kg-day concentration figured when the EPA guideline water consumption rate of 0.30 l/day is used, but still considered fairly accurate due to the minor leakage that occurs after animals drink from this type of bottle. A complete dosing schedule is given in Table 1.

Table 1. Dosing of timed pregnant Long-Evans rats during GD 7-21.

		Dose	
Group	Number of Dams	Concentration (ClO_4-) mg/L	Dose mg/kg-day
5.0 Mg/L Perchlorate	6	5.0	0.82
50.0 Mg/L Perchlorate	6	50.0	8.70
Saline	7	0.0	Pure DI water

Developmental Landmarks

The temporal appearances of developmental landmarks are good indicators of future neurological functioning (Levine, Carey, and Crocker, 1999). The dams were examined daily during the 14 day exposure period and daily during the pre- and post-weaning periods for signs of: clonic/tonic seizures, vocalizations, gait, piloerection, weaning behavior, water consumption, weight gain, and other elements of rat behavior.

Two phases of testing were observed in pups (F1-generation). Phase one included records being kept on litters only from post natal day (PND) 1 through PND 20. While phase two included the recording of data on individual rats from PND 21 through 60.

Pregnancy outcome measures were evaluated at birth and included the number of pups per dam, live birth index, sex ratios of pups, and viability. All offspring were observed for selected developmental landmarks including eye opening, incisor eruption, pinna unfolding, surface righting, and negative geotaxis as described in Norton (1986).

On the day of birth, the live birth index was determined for each litter and the pups were sexed. The survival index was determined for the pups at 24 hrs and at 4, 7, 14, and 21 days of age. Litters were culled to 8 pups per dam on PND 4. Litters were cross-fostered within dose groups as necessary to achieve 4 females and 4 males per dam. The percent of pups with bilateral pinna unfolded per liter was observed on PNDs 3-7 and recorded. Incisor eruption observations were recorded as a percentage of the litter on PNDs 9, 10, and 11. The percentage of pups with both eyes opened per litter was recorded on PNDs 15, 16 and 17.

Time in secs to right during surface righting, the ability of a rat pup to roll to an upright position after being placed on its back, was observed and recorded for 1 pup picked randomly from each litter on PND 1 and 6, and for 1 pup of each sex per litter on PND 7 and 13. One female and 1 male randomly selected from each litter were tested for negative geotaxis, the ability of a rat pup to orient themselves in an upward position after being pointed downward on a sloped surface, on PNDs 6-12 by placing them on a 45 degree sloped screen with their head pointing down then recording the time (in secs) it took for each rat to orient itself uphill. Reflex suspension times, the duration of the ability of the pup to hold a grip using forpaws and suspend themselves in the air on a wire rod (1.85 mm in diameter) with no other support, were determined for one randomly selected pup of each sex in all litters on PNDs 7, 13 and 17.

Behavior Tests

A modified Functional Observational Battery (FOB) similar to that followed by Moser, McCormick, Creason, and McPhail (1988) was used to monitor offspring for home cage observations, ease of handling, grip strength, startle response, foot splay, and a Figure 8 maze on PNDs 17 and 21. Observations of home cages included posture, clonic and tonic convulsions, vocalizations, palpebral closure, lacrimation, piloerection, and salivation. Startle response evaluates auditory development, which is linked to fetal thyroid hormones.

As a measure of motor ability, foot splay was recorded by painting the rats' hind limbs with tempera paint. While being held parallel to the landing surface, rats were released from a height of 30 cm onto a sheet of paper. The hind limb placement was marked and the distance between marks were measured for two trials. Grip strength (designed by Dr. J. Mattson, Dow Chemical) is a measurement of the rats ability to resist a backward pull when placed squarely on wire mesh screen (22 cm x 14 cm) attached to a gauge support mounted on a clear plexiglass base plate (60 x 26 cm). The fore- or hind-limbs of the rats were placed on a bench (7.6 cm high x 25 cm long) allowing the rat to grip the screen. The resistance was measured in kilograms with three trials recorded.

Startle Response

Startle response (SR) system (San Diego Instruments, San Diego, CA) chambers were used to provoke a stereotypic motor response to an unpredictable intense stimulation for 4 males and 4 female pups randomly selected from each dose group on PND 21 and PND 61. SR equipment measures response to both acoustic (118 dB for 300 msec) and tactile (air puff at 15 psi for 20 msec). The SR chambers are an isolated cabinet with an adjustable transparent acrylic cylindrical rodent enclosure inside.

Figure 8 Maze

One of the most commonly used tests to assess activity levels is the Figure 8 maze (F8M). The F8M was used to determine motor activity for rats in this study (F8M, San Diego Instruments). Eight infrared beams positioned around a plexiglass maze with a wire mesh floor recorded rat movement by beam breaks. During a 30 minute period, beam breaks were counted and summed every 5 min. Four pups of each sex from each group were randomly selected and tested in the F8M on PNDs 12, 17, and 21.

Statistical Analysis

Standardization of litter size and sex distribution accommodates a smaller 'n' as uniform data can be analyzed with more robust statistical tests (Cox, 1994). In order to increase statistical power and decrease error, members of the same litter that were combined and treated as an 'n' of one were averaged within litters (Holson and Pearce, 1992). Laboratory book data and behavioral system software program data were entered into Excel (Microsoft 2000 - SR1) spreadsheets. After the accuracy was checked by another person, the group means with standard deviations were defined for each parameter. Excel spreadsheet files were then converted into SPSS (Version 11) software for advanced statistical analysis. The excel graphs were marked with the statistical test used, as well as any overall test or individual group significance ($p=.05$ or less) obtained using SPSS.

Differences in dam weight gain were analyzed as the percent of body weight gain for both gestational and postnatal periods using a one way analysis of variance (ANOVA). The ANOVA was also used to analyze differences in the number of pups per litter. The Kilmogorov-Smirnow test indicated that most of the data were not normally distributed in the pinna unfolding, eye opening and incisor eruption observation data with a $p=<0.05$ cut off. Further statistical analyses were conducted after the data were normalized using the

arcsin square root transformation (Zar, 1999) using a p=0.05 cut off. The significance of effects on developmental landmarks and behavior were analyzed statistically using a general linear model (GLM) for repeated measures to reveal group by day effects. Tukey's post hoc tests were used when appropriate.

A GLM repeated measures model was used to compare the means of all observed developmental events (survival index, pinna unfolding, eye opening, incisor eruption, surface righting, reflex suspension, and negative geotaxis). Foot splay data group variance were analyzed using both the average splay measured and average trial 1 to trial 2 differences.

The Startle Response and Figure 8 Maze compared behavioral responses over time. Startle response system data examined in two startle response tests were compared against each other across dose groups using a GLM repeated measures analysis for the maximum response time after audio, tactile, and IP stimulation. Activity over time (trend in total beam break number) in each F8M test was averaged and graphed for each group, the statistical test (GLM repeated measures) was performed for total beam breaks per test (or day) averaged per group.

Morphometric data including body weight, liver weight, ovary/testes weight, and adrenal weight were compared as ratios to body weight and differences in groups were obtained using one-way ANOVAs. Weight was used as a covariate in subsequent tests.

CHAPTER 4

RESULTS

Developmental Observations

No treatment-related effects on dam weight gain during gestation or postnatal periods were detected using a one-way ANOVA test to compare the differences among treated group means (Figure 5, Table 2). However, both perchlorate treated groups had a smaller number of pups per dam compared to the control with the 5.0 perchlorate concentration group having statistical significance (Figure 6). Since no mortalities occurred in either treatment group, the survival index was unaffected by perchlorate concentrations for the groups tested (data not shown). Eye opening and incisor eruption data showed no significant delays in either dose group, but there was a significant difference in that premature pinna unfolding (i.e. the low dose group opened quicker) which occurred in the low dose group on PNDs 5 and 6 and the high dose group on PND 5 when they were compared to the saline group using a GLM for repeated measures (Figure 7). Using covariate analysis, the variability is highest on PND4 for the low dose group, this variability decreases by the next developmental day. The percent of pinna unfolded per dam results in a non-linear trajectory of the low dose group between PNDs 4 and 6 and between PNDs 4 and 5 for the high dose group (Figure 8).

There were no statistically significant developmental difference in surface righting or reflex suspension in either dose group on PND 21 and 60 (Table 2) compared to the control group. There was a slower dose response in negative geotaxis for both perchlorate exposed

groups on PND6 and for the higher 50.0 perchlorate concentration group on PND 10 using the same test for repeated measures (Figure 9).

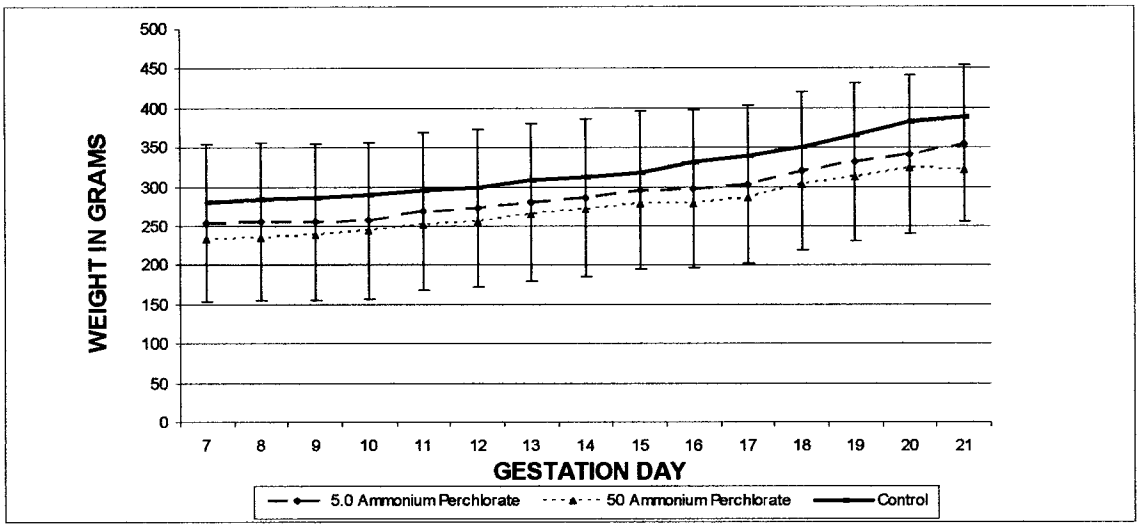

Figure 5. Average dam weight by dose group for mothers exposed to perchlorate.

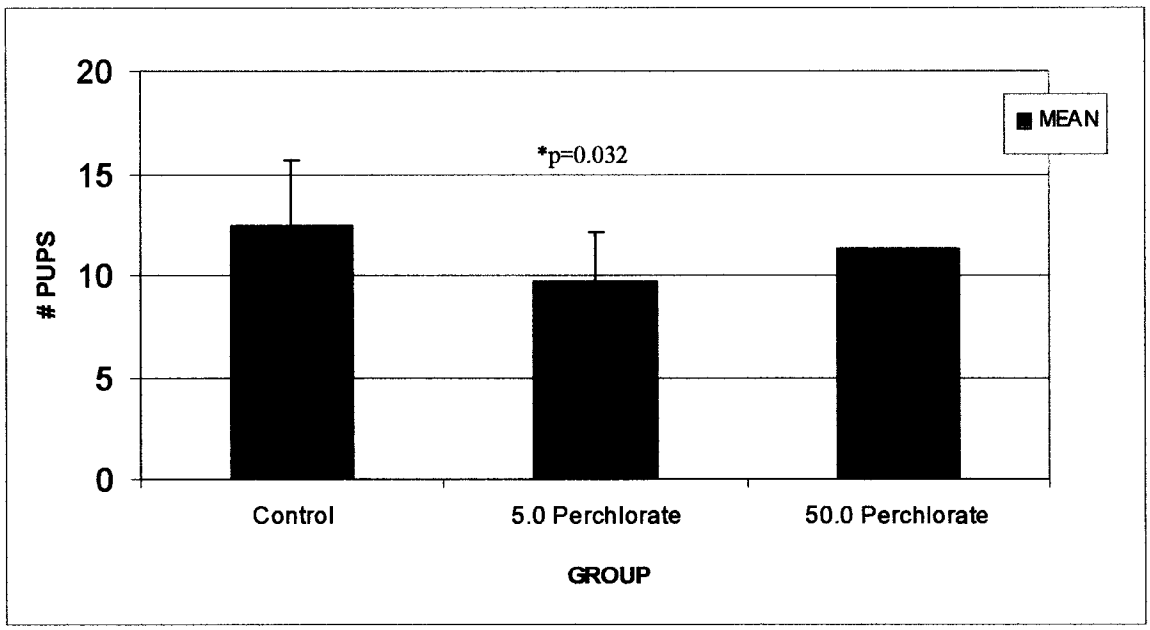

Figure 6. Average pups per dam by dose group after gestational exposure to perchlorate. See Table 1 for exact doses. P values for ANOVA in Table 2.

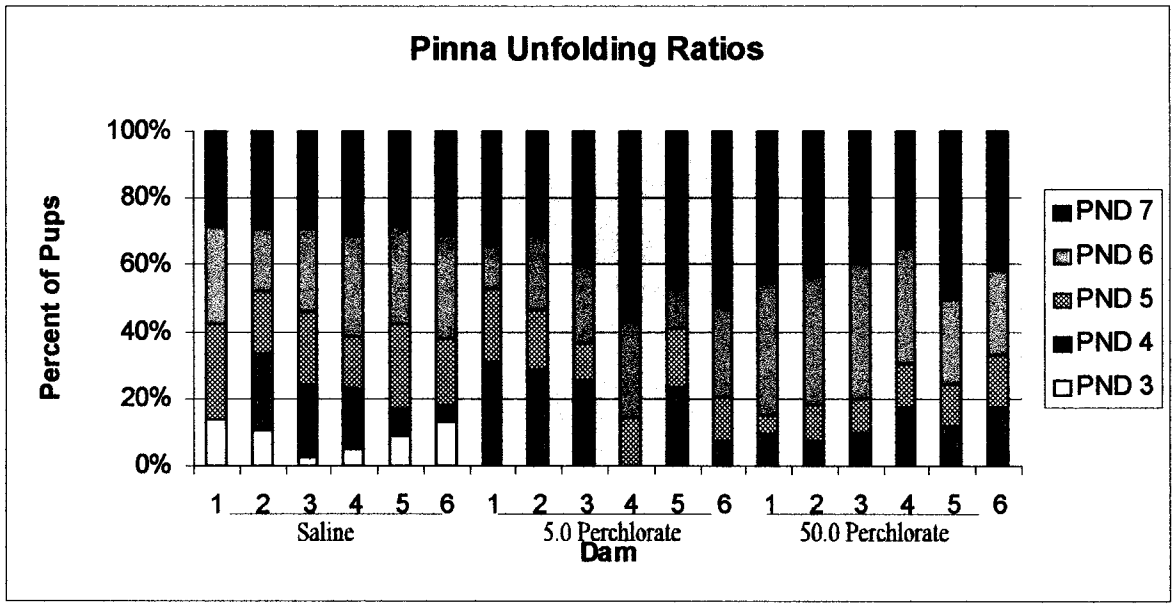

Figure 7. Pinna unfolding ratios of pups after gestational exposure to perchlorate. Exact doses given in Table 1. P values for ANOVA in Table 2.

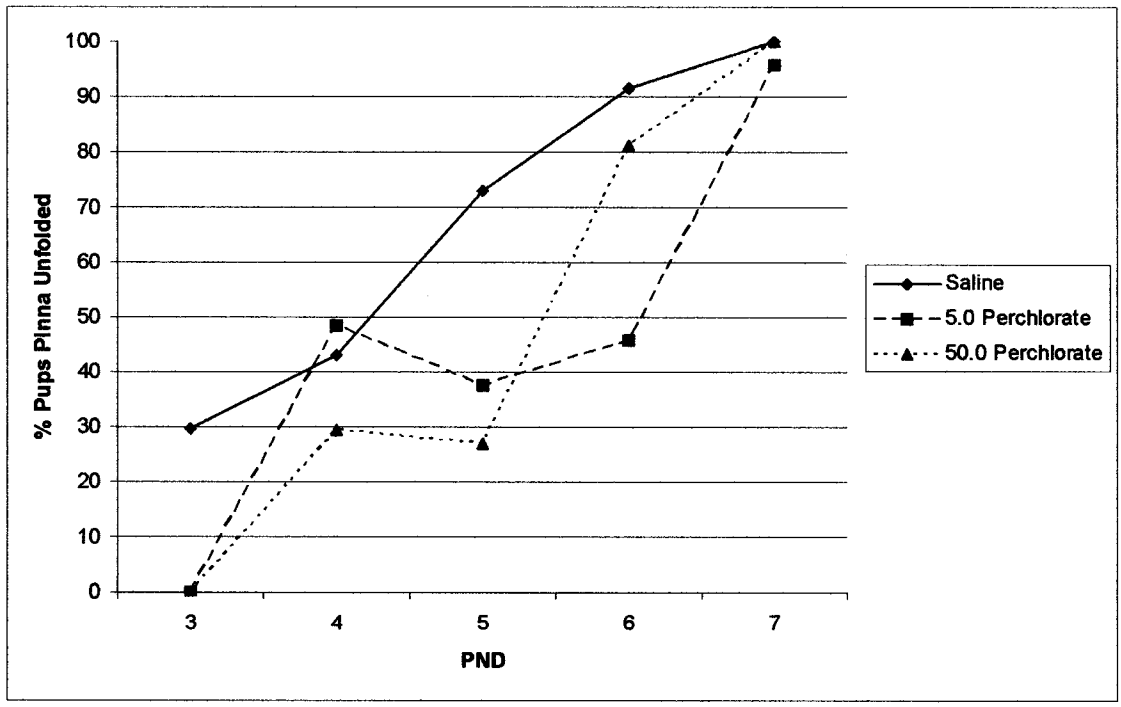

Figure 8. Percent of pinna unfolding from PND 3 through 7 after gestational exposure to perchlorate. P values for ANOVA in Table 2.

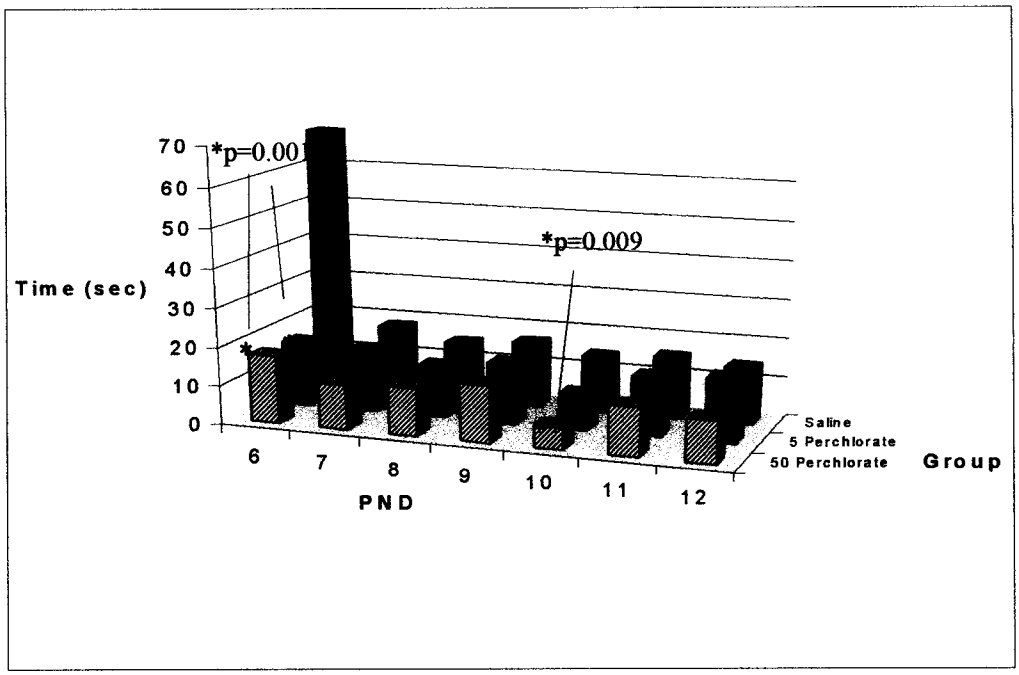

Figure 9. Negative Geotaxis measures from PND 6 through 12. Exact doses given in Table 1. P values for Repeated Measures ANOVA in Table 2.

Table 2. Summary of Statistical Analyses for Developmental Observations

Developmental Observations

Test	Statistical Test	n	F	P	Post Hocs
Dam % wt. gain (gestation)	1-way ANOVA	6	0.094	0.911	N/A
Dam % wt gain (postnatal)	1-way ANOVA	6	0.053	0.949	N/A
# Pups per dam	1-way ANOVA Group Male Female	6	 4.072 1.349 2.988	 0.039 0.289 0.081	 5.0 Perchlorate v. Control mean diff = +4.667, p=0.032
Pup wts.	GLM RM	6	0.572	0.567	N/A
Pinna unfolding	GLM RM	6	3.801	0.001	5.0 Perchlorate v. Control for PND 5; mean diff = +0.468, p=0.002
					50.0 Perchlorate v. Control for PND 5; mean diff = +0.531, p=0.001
					5.0 Perchlorate v. Control for PND 6; mean diff = +0.659, p= 0.001
Eye opening	GLM RM	6	2.356	0.129	N/A
Incisor eruption	GLM RM	6	0.266	0.770	N/A
Surface Righting	GLM RM	6	2.918	0.069	N/A
Reflex Suspension	GLM RM	6	1.704	0.202	N/A
Negative geotaxis	GLM RM	6	11.608	0.001	5.0 Perchlorate v. Control for PND 6; mean diff = -53.167, p=0.001
					50.0 Perchlorate v. Control for PND 6; mean diff = -52.250, p=0.001
					50.0 Perchlorate v. Control for PND 10; mean diff = -9.000, p=0.009

Behavioral Observations

Behavior tests of F1 footsplay distance and fore- and hind-limb gripstrength indicate no significant differences (Table 3). There were no treatment-related effects on audio measures for either dose group in the Startle Response Audio test (Figures 10 and 13).

The lower dose group showed significantly more reaction on PND 21, although not as extreme as the higher dose group in the Tactile test on PND 21 and showed significantly less reaction in the IP test on PND 61 (Figures 11 and 15). Pups of both sexes receiving the higher dose of perchlorate were more reactive in the Startle Response IP test than controls on PND 21 and significantly less active on PND 61 and in the Tactile test on PND 21 they were more active (Figures 11, 12 and 115). Figure 8 Maze data show that compared to the control group on PND 21, the higher dose perchlorate group was not different, but the lower dose group was significantly less active. (Table 3)

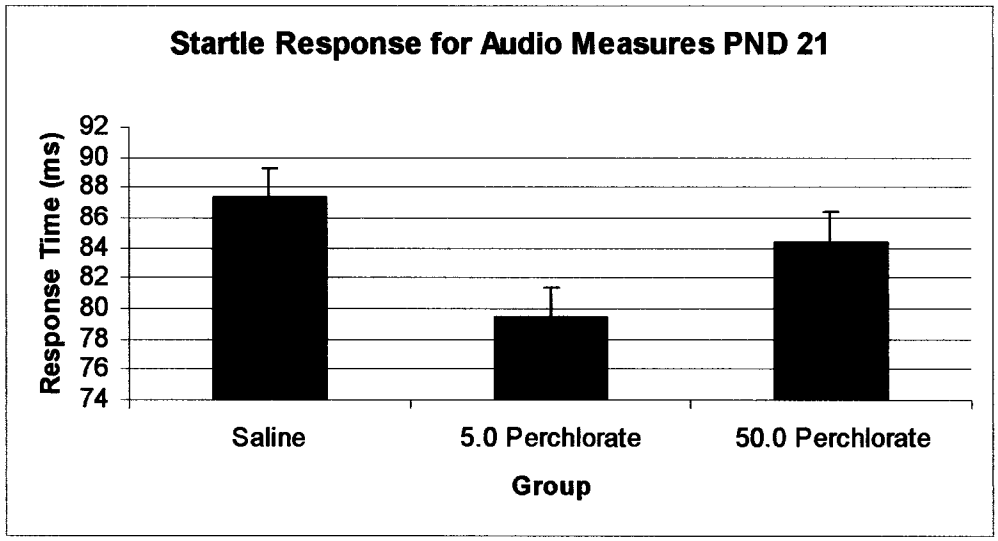

Figure 10. Startle response for audio measures at PND 21 after maternal exposure to perchlorate.

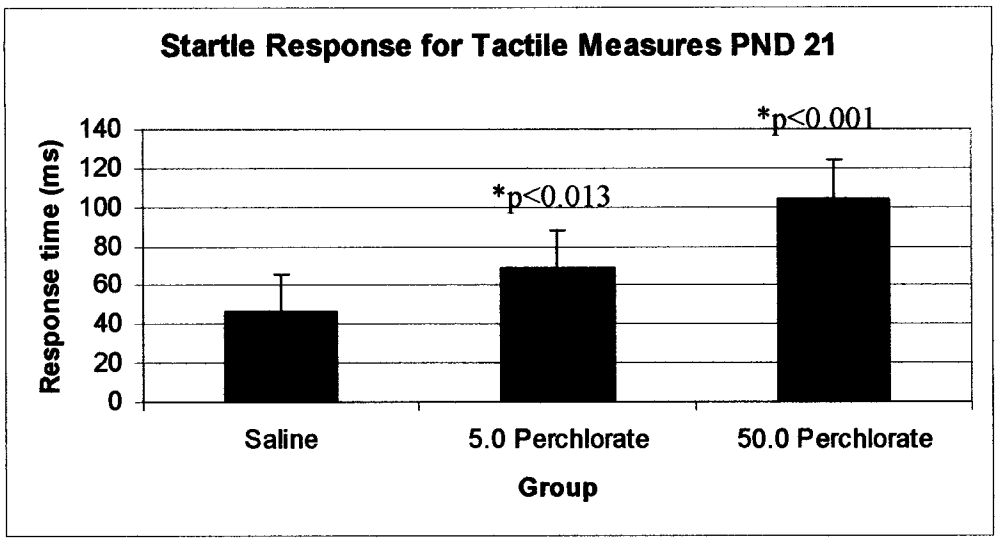

Figure 11. Startle response for tactile measures at PND 21 after maternal exposure to perchlorate. P values from ANOVA in Table 3.

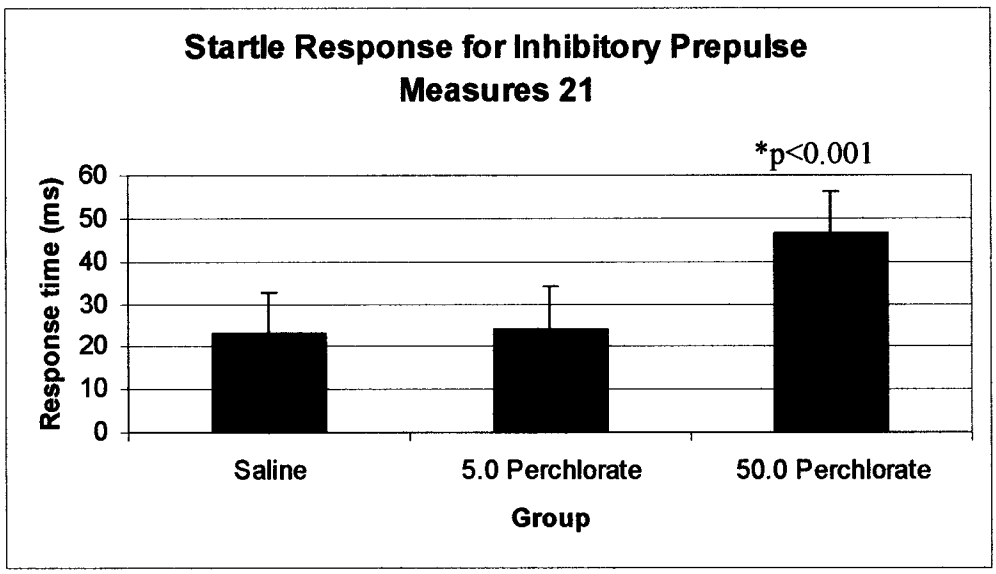

Figure 12. Startle response for inhibitory prepulse measures at PND 21 after maternal exposure to perchlorate. P values from ANOVA in Table 3.

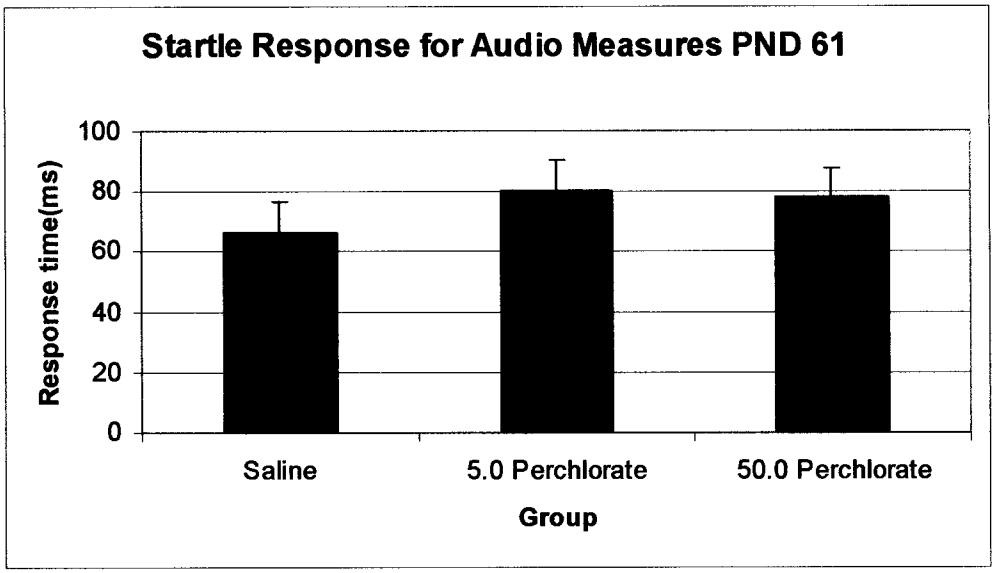

Figure 13. Startle response for audio measures at PND 61 after maternal exposure to perchlorate.

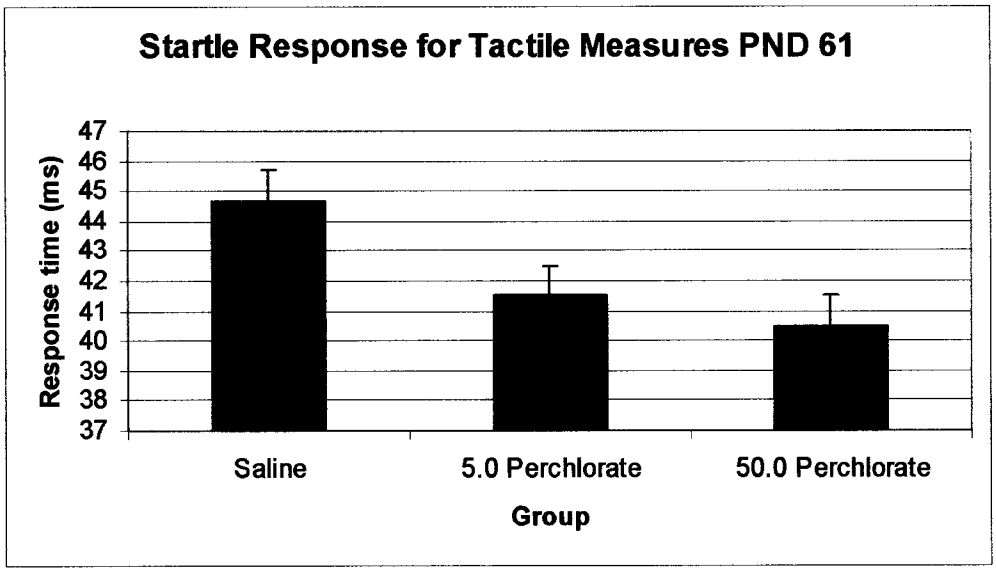

Figure 14. Startle response for tactile measures at PND 61 after maternal exposure to perchlorate.

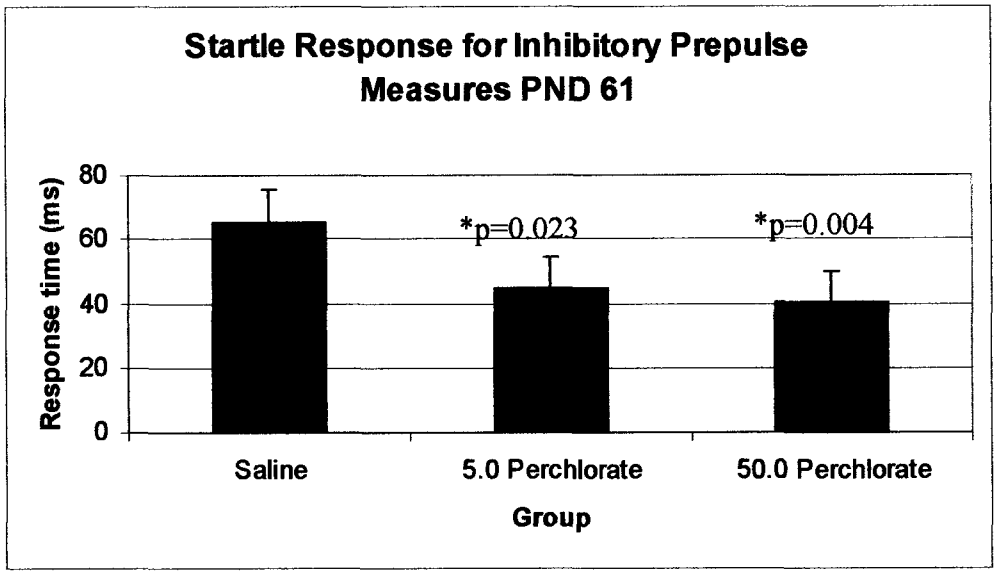

Figure 15. Startle response for inhibitory prepulse measures at PND 61 after maternal exposure to perchlorate. P values from ANOVA in Table 3.

Table 3. Summary of Statistical Analysis for Behavioral Tests.

Behavioral Tests

Test	Statistical Test	n	F	P	Post Hocs
Footsplay	GLM RM	12	1.408	0.260	N/A
Grip strength	GLM RM				
Forelimb	GLM RM	12	0.429	0.655	N/A
Hindlimb	GLM RM	12	0.856	0.435	N/A
Startle Response					
PND 21 Audio	ANOVA	perchl =42 saline= 48	0.858	0.425	N/A
Tactile	ANOVA	perchl =42 saline= 48	27.706	<0.001	5.0 Perchlorate v. Control mean diff= +22.278, p<0.013
					50.0 Perchlorate v. Control mean diff= +57.611, p<0.001
IP	ANOVA	perchl =42 saline= 48	15.621	<0.001	50.0 Perchlorate v. Control mean diff= +23.597, p<0.001
PND 61 Audio	ANOVA	perchl =42 saline= 48	2.844	0.062	N/A
Tactile	ANOVA	perchl =42 saline= 48	0.437	0.647	N/A
IP	ANOVA	perchl =42 saline= 48	6.195	0.003	5.0 Perchlorate v. Control mean diff= -20.667, p=0.023
					50.0 Perchlorate v. Control mean diff= -25.261 p=0.004
Figure 8 Maze					
PND*Group	GLM RM	perchl =48 saline= 72	9.481	<0.001	N/A
PND 17	GLM RM	perchl =48 saline= 72	3.827	0.024	only sig differences between treated groups p=0.017
PND 21	GLM RM	perchl =48 saline= 72	4.095	0.019	5.0 Perchlorate v. Control mean diff= -5.510, p=0.019

"perchl" in n column indicates both 5.0 perchlorate and 50.0 perchlorate dose groups

Morphometric Observations

Analysis of morphometric data suggests a significant difference in dam weight between groups, but when compared to the difference in weight gain using one-way ANOVA, it was determined that this significance was consistent with the group mean at intake of dams for each group (Table 4 and Figure 14). ANOVA tests revealed no other morphometric data significance in the dam cull, pup cull or final cull for dam weight, pup weight, liver weight, ovary/testes weight, or adrenal weight between groups using bodyweight as a covariate (Table 4).

Table 4. Summary of Statistical Analysis for Morphometric Observations.

Cull Measures

	Covariate	Parameter	Statistical Test	n	F	P
Dams	Weight	Dam Wt	1-way ANOVA	6	5.860	0.013
		Liver Wt	1-way ANOVA	6	3.080	0.076
		Ovary Wt	1-way ANOVA	6	0.574	0.575
		Adrenal Wt	1-way ANOVA	6	2.789	0.094
Culls Pups	Weight	Dam Wt	1-way ANOVA	6	0.566	0.579
		Liver Wt	1-way ANOVA	6	0.416	0.667
		Ovary/Testes Wt	1-way ANOVA	6	0.100	0.906
		Adrenal Wt	1-way ANOVA	6	0.400	0.677
Culls Final	Weight	Dam Wt	1-way ANOVA	6	0.049	0.952
		Liver Wt	1-way ANOVA	6	0.136	0.874
		Ovary/Testes Wt (Group/Sex)	1-way ANOVA	6	0.002	0.998
		Adrenal Wt	1-way ANOVA	6	1.254	0.314

Table 5. Summary of significant test results for both groups treated with perchlorate.

		Dose	
End Point	**Test**	**5.0 Perchlorate**	**50.0 Perchlorate**
Developmental	Dam % wt. gain (gestation)	N/A	N/A
	Dam % wt. gain (postnatal)	N/A	N/A
	# Pups per dam	FEWER PUPS	N/A
	Pup weights	N/A	N/A
	Pinna unfolding	SLOWER PND 5 & 6	SLOWER PND 5
	Eye opening	N/A	N/A
	Incisor eruption	N/A	N/A
	Surface righting	N/A	N/A
	Reflex suspension	N/A	N/A
	Negative geotaxis	SLOWER PND 6	SLOWER PND 6 & 10
Behavioral	Footsplay	N/A	N/A
	Grip strength	N/A	N/A
	Startle Response Audio PND 21	N/A	N/A
	Startle Response Audio PND 61	N/A	N/A
	Startle Response Tactile PND 21	MORE REACTIVE	MORE REACTIVE
	Startle Response Tactile PND 61	N/A	N/A
	Startle Response IP PND 21	N/A	MORE REACTIVE
	Startle Response IP PND 61	LESS REACTIVE	LESS REACTIVE
	Figure 8 maze PND 17	N/A	N/A
	Figure 8 maze PND 21	LESS ACTIVE	N/A
Cull weight measures	Dams - Dam, liver, ovary, & adrenal	N/A	N/A
	Final - Dam, liver, ovary/testes, & adrenal	N/A	N/A
	Pups - Dam, liver, ovary/testes, & adrenal	N/A	N/A

CHAPTER 5

DISCUSSION

The lower dosed, 5.0 perchlorate treated group, demonstrated delayed or deficient trends in development and behavior in 5 of the 23 endpoints evaluated including: the number of pups per dam, pinna unfolding, negative geotaxis, startle response IP on PND 61, and figure 8 maze on PND 21, as wells as, an upward, more reactive tactile startle response was seen at PND 21. The 50.0 perchlorate dosed group showed similar trends to the lower dosed group with delayed pinna unfolding, delayed negative geotaxis, and less reactive tactile startle response on PND 21 and on PND 61. In addition to these commonalities, the startle response for the 50.0 perchlorate group was also more reactive than controls in the startle response IP on PND 21. These results are surprising because, in comparison to the body of knowledge now available on perchlorate exposure, it would be expected that a linear dose response curve that supports the suggested NOAEL and LOAEL of 0.3 mg/kg-day and 30.0 mg/kg-day respectively would have eliminated the possibility of significant results at such low doses. But, the low level exposure to perchlorate, did elicit a response, one that eliminates the notion of sampling fluctuation and one that may mimic a U-shaped dose response curve. With 23 different measures used, we were bound to see some effect by mere chance, but when significance is demonstrated in 5 of the endpoints, the results are likely a

real biological effect. This was clearly demonstrated with respect to the developmental landmarks.

Development

The assessment of early developmental landmarks are often the first indicators of specific neurological disorders (Levine, Carey and Crocker, 1999). Research conducted by Goldey, et.al. (1995) found that gestational exposure to a known iodine-uptake inhibitor, propylthiouracil, demonstrated a delay in eye opening, reduced body weights, decreased and/or delayed pre-weaning motor activity and persistent, post-weaning hyperactivity. The differences in time of administration and chemical structure may account for the different effects in our study. Goldey, et. al. (1995) exposed their rats during GD 18 to PND 21. More developmental delays could have been witnessed in our study with a longer exposure period, although, the perinatal window of exposure used in our study on GD 7 to GD 21 appears to elicit delayed developmental landmarks not seen in the Goldey study. Delayed developmental landmarks may be indicative of maternal thyroid hormone deficiencies in neonatal rats.

Figures 7 and 8 show that the percentage of pinna unfolding in pups following gestational exposure to perchlorate is erratic, when compared to the controls. On PND 6 each dam in the control had approximately 70% of pups with pinna unfolded, where both perchlorate treated groups exhibited unpredictable unfolding patterns. At PND 4 the variability is the greatest for the low dose group. This variability shows that not all dams react the same to the perchlorate exposure and genetic alterations might lead to different

reactions that can be perterbated through perchlorate exposure. If we look at Figure 8 we see a non-linear trajectory for the 5.0 perchlorate group that extends from PND 4 through 6, where over 50% of the pups demonstrated pinna unfolding that converges by PND 7. The achievement of pre-weaning landmarks of development is highly correlated with body weight, but, covariant analysis eliminated this potential influence. Since 100% of pinna unfolding occurred by PND 7, which is considered within a normal range, we can infer that exposure to low levels of perchlorate during early gestation is associated with delayed and erratic appearance of landmarks and the activation of a mechanism that compensates for the perterbation.

Since the developing fetus is dependent upon maternal thyroid hormones until GD17, neurological abnormalities increase when a deficiency of these hormones exists (Oppenheimer, 1979). Although, due to the lack of equipment and available quantities of serum after the sacrifice, we did not measure thyroid hormone levels directly, enough evidence exists (Table 5) to infer that developmental effects after exposure to perchlorate, known to adversely affect TSH, T4 and T3 levels (York, et. al., 2001), are the result of the reduction of thyroid hormones during neonatal development which correlates with delayed developmental feats (Koibuchi and Chin, 2000). The actual thyroid hormone genes that are responsible for normal development are not well characterized, but, since hypothyroidism is associated with reduced growth and branching of Perkinje cells, reduced synapotgenesis between Perkinje cells and granule cell axons, delayed proliferation and migration of granule cells, delayed myelination, and changes in synaptic connections among cerebella neurons and afferent neuronal fibers (Koibuchi and Chin, 2000), an insult

to the developing fetus' thyroid hormone homeostasis could inevitabley result in a cascade of effects. This leaves multiple possibilities of how thyroid hormone action affects cerebellar development. While we are uncertain as to whether proteins, factors, or gene regulation is the actual molecular effect that is the result of perchlorate exposure, our data suggest that low doses of perchlorate disrupts the regulation of some transcription or translational activities that ultimately results in high variability and compensation toward developmental feats.

Number of Pups per Dam

Previous studies have observed conflicting results between litter size and exposure to perchlorate. Thuett, et. al. (2001) observed smaller litter sizes following gestation and postnatal perchlorate treatment in deer mice. While York et. al. (2001) found no differences in litter size in a 2-generation reproductive study that administered perchlorate in drinking water at doses between 0.3 up to 30.0 mg/kg-day. Our study contradicts York's findings and hints to a gestation related dose-response interpretation of the Thuett study. Both 5.0 and 50.0 perchlorate groups exhibited smaller litter sizes (Figure 5) with the lowest dose group being most affected with litter sizes below 10 pups per dam, supporting a U-shaped dose response curve.

Since perchlorate passes the placental barrier, a change in the fetus' hormonal balance could affect the environment necessary for healthy development. The York study exposed their rats for two generations from weaning through sacrifice which may have allowed the their dams' endocrine system (thyroid) an opportunity to compensate for exposure to

perchlorate at low doses and maintain homeostasis during pregnancy. In our study, the insult began at GD 7 which may have altered the maternal homeostasis, resulting in lower numbers of offspring.

Behavior

Delayed development of the acoustic startle response may be a particularly sensitive indicator of perinatal hypothyroidism (Goldey, et. al, 1995). In the Goldey study, delays in acoustic startle response occurred in hypothyroid rats at PND 25. No existing data was found for the tactile or IP response measures in hypothyroid rats. In our study, no significant results occurred in the auditory startle response, but mixed reactions in the tactile and IP measures suggests other effects of perchlorate exposure. It is possible that perchorate delayed the development of sensory and/or motor processes necessary for responding to tactile and IP stimuli. Previous tests using methimazole, thoiurea (Schneider & Golden, 1986), and propylthiouracil (Goldey, et. al, 1995) to inhibit the formation of thyroid hormones resulted in acoustic startle response delays. Methimazole and thiourea react at the thyroid gland only and propylthiouracil reacts at both the thyroid and prevents the deiodination of T4 to T3 (Geffner, et. al., 1975).

The question exists of whether the action of perchlorate at the receptor site of the NIS, the gestational exposure, or the days tested in our study may be responsible for the lack of acoustic response and the presence of tactile and IP response. If inhibition of iodine uptake occurred at the NIS, then similar effects to methimazole, thoiurea, and propylthiouracil would be anticipated. While we did not observe acoustic startle

responses, we did witness significant delays in tests of startle response that rely on similar mechanisms. In normal animals, the acoustic startle response appears by PND 13 (Sheets, et. al., 1988), therefore, significant delays in development may have been missed in the perchlorate groups because the endpoint was not evaluated until PND 21. Testing the rats at an earlier developmental age from PND 13 through adulthood would provide valuable information on the relative sensitivity of the otogeny of the multiple startle response measures over a broader time spectrum and help determine the effects of gestation only exposure.

Tamasay, Meisami, Vallerga, and Timiras, (1986) demonstrated that neonatal hypothyroidism is reversible in an experiment that reversibly inhibited both maternal and infant thyroid function after the administration of propylthiouracil. Their results concluded that the rehabilitation of hyperactive behavioral responses is rapid and restoration to normal or near normal occurs by PND 50, although some residual effects seem to remain until PND 90. Our study demonstrates a recovery or a compensation for earlier perterbation caused by perchlorate exposure after ceasing administration. When testing both perchlorate treated groups in tactile startle response at PND 21 and the 50.0 perchlorate group in IP startle response, the treatment groups showed elevated response amplitudes compared to the controls (Figures 10 and 11). This finding is particularly interesting because it suggests that the cause of hyper-reactivity may occur in early development, but by PND 61 each hyper-reactive response had slowed to a normal, less reactive, or a delayed response (Figures 12, 13 and 14). This trend supports a temporal effect of gestational perchlorate exposure which is not uncommon for neonatal hypothyroid rats, therefore comparisons of normal and an entire dose response continuum

below and above the LOAEL for perchorate at a number of ages should be useful in mapping the development of this pathway and identifying which responses may be dependent on thyroid hormones for normal development.

In contrast to the overall increase in activity seen on PND 21, a decrease in activity was found in the figure 8 maze for the 5.0 perchlorate group. It would have been interesting to follow this behavioral test to PND 50 and beyond. In the Tamasay, et. al. (1986) study, Long Evans rats were exposed to propylthiouracil from birth to PND 25. Hyperactivity was documented followin propylthiouracil exposure as measured by the figure 8 at PND 50 and PND 100, but this hyperactivity lessened in magnitude with age. The opposite effect was seen in the Goldey (1998) paper where the hyperactivity increased with age when dosed with propylthiouracil.

Our study evaluated the figure 8 maze to measure motor activity on PND 17 and 21. Admittedly, these early dates are a very narrow window to demonstrate long term effects, but, since the rats nervous system is particularly vulnerable to neurotoxic effects due to the rapid development of the brain, this window can provide clues for future research. Pups develop the motor conditioning required for ambulation by PND 10 (Bolles Woods, 1964), and follow a linear increase in locomotor activity with age. In our study, pups exposed in the 5.0 perchlorate group at an early stage exhibited decreased activity, an indicator of a possible reduction of thyroid hormones occurred at this low dose, and current research supports that this would affect the cerebellum. Since healthy neuronal development of the rodent cerebellum is dependent on thyroid hormones, and perchlorate is known to reduce TSH, T4, and T3 production (Koibuchi and Chin, 2000), more testing

of cerebellum related tasks would be needed to verify this effect. In addition, the evaluation of this motor activity should be completed at multiple developmental stages to determine whether effects are attributable to perchlorate exposure during gestation and if they are recoverable after the cessation of dosing.

Morphometric

The existing body of knowledge is inconsistent with respect to morphometric changes following perchlorate exposure. Thuett's (2001) perchlorate mouse study showed that all dose groups had significant increases and/or decreases for specific organ weights of the gonads, kidneys, adrenals, liver and heart on PND 21. In the York (2001) Sprague-Dawley rat reproductive toxicity study, differences in thyroid weight and spleen weight were found in F1 generation pups and F1 generation adults with exposure of 30 mg/kg-day of perchlorate. In the York (2001) rabbit developmental toxicity study there were no organ weight differences after similar concentrations of perchlorate were administered. These inconsistencies make it difficult to predict morphometric changes as a result of exposure to perchlorate.

In our study, the data from low concentrations of perchlorate exposure show no statistically significant or discernable morphometric change following oral administration. Although, it appears that some perturbation of oxidative metabolism, carbohydrate metabolism, lipid metabolism, and/or nitrogen metabolism may have influenced the liver weight (although not statistically significant, data still demonstrate a borderline significance) due to exposure.

Weakness of Approach

Using the rat as a model for human risk assessment has inherent problems for all scientific studies that hope to extrapolate the information, but there are specific complications that exist when we are considering interspecies thyroid studies. Humans have the ability to maintain blood thyroid hormone levels due to the presence of TBG, the main thyroxine circulating hormone, and would not be expected to produce similar disruptions as might be seen in rats which have no TBG. The human thyroid is also more able to adapt to changes than rats. Therefore, we find that the existing body of human health studies show no-effect levels at 5 to 6 μg/kg/day which is equivalent to approximately 200 ppb perchlorate--one or two orders of magnitude greater than the existing contamination that is reported in drinking water supplies in the United States.

Since perchlorate is known to decrease thyroid hormone concentrations, it is assumed that the effects seen in rat pups, in our study, would be due to altered maternal/fetal thyroid status. Typically when the term hypothyroidism is applied to rats it refers to the most severe situation where rats are treated with a potent goitrogen such as methimazole or propylthiouracil and then exhibit undetectable T4 and TSH levels (Macchia, 2002). It is difficult, however, to actually diagnose hypothyroidism since we did not measure T4 and TSH directly, but rather, evaluated only some of the potential symptoms associated with hypothyroidism. The only actual documented behavioral effect, is auditory startle habituation (Goldey, et. al., 1995). Our research showed no changes in auditory startle response.

The diet fed to the rats in this study presents a potential confounding variable because it had the ingredient soy. Soy contains isoflavones which are known to inactivate both rat

and human thyroid peroxidase. Ikeda, Nishikawa, Imazawa, Kimura, and Hirose (2000) found a dramatic synergism between soybean intake and iodine deficiency that caused an increase in serum TSH levels. But, even though this dietary confound is present, it is apparent in this study, that a difference does exist between the control and those dosed at low levels of perchlorate. Therefore, this research needs to be considered valuable in support of low level exposure and used to expand the screening assays when considering endpoints not previously tested for thyroid disrupting chemicals.

Conclusion

Gestational exposure to perchlorate appears to result in a biological effect that alters the developmental trajectories of pups exposed at doses lower than the existing NOAEL of 3.0 mg/kg-day, established by the EPA for risk assessment purposes. We have demonstrated that with a dosing of 0.82 mg/kg-day (5.0 perchlorate group) during GD7-21, there are fewer pups per dam, erratic pinna unfolding, slower negative geotaxis, more reactive tactile startle response, less reactive IP startle response and reduced figure 8 maze activity. We also observed consistent and similar responses for those rats dosed 8.7 mg/kg-day (50.0 perchlorate group) with erratic pinna unfolding, slower negative geotaxis, more reactive tactile startle response, and less reactive startle response on PND 61, but in addition this group experienced more reactive startle response on PND 21. (See Table 5)

The developing fetus is dependent upon maternal thyroid hormones until GD17, therefore maternal exposure to perchlorate can result in a thyroid hormone deficiency effect on neonatal rats. Our results may support a U-shaped dose response curve with

significantly fewer pups per dam following perchlorate exposure. Other studies on reproduction at similar to higher concentrations do not suggest that perchlorate is a reproductive toxicant. It is possible that fewer pups per dam in our low dose group could be a result of other factors such as fertility, although a similar response curve is noted for other effects. For instance, pinna unfolding was significantly slower on both PND 5 and 6 for the 5.0 perchlorate concentration group whereas for the 50.0 perchlorate group results indicate only delays on PND 5. These consistencies also eliminate the possibility of sampling fluctuations that naturally occur in all populations. Our results show effects that are consistent with the chemical insult of perchlorate and the hormonal relationship between the pituitary axis feeback system.

Perinatal hypothyroidism can be measured by acoustic startle response. Auditory response is first recordable by PND 12 in rat development and, in normal animals, acoustic startle response appears by PND 13. Unfortunately, we were not able to test our animals until PND 21, therefore earlier data might have helped elucidate the effects from perchlorate. But PND 21 coincides with the timing of reflex and automatic motor functions which are mature by PND 20. Our results show significant effects in the tactile startle response on PND 21 for both 5.0 and 50.0 perchlorate groups as well as the IP startle response for PND 21 (50.0 perchlorate group only) and PND 61 (both groups), that would be consistent with a possible effect to the cerebellum due to insufficient thyroid hormones.

While motor skills are not exclusively due to cerebellar function, the cerebellum is a major brain component that contributes to them (Altman, Anderson, Strop, 1971). Exploratory behavior in the rat is highest between PND 20-30. Again, here we see where

the lower dosed group is most affected with significant test results with a decrease in activity in the Figure 8 maze.

It is known that thyroid hormones can affect development and behavior and much of this is attributed to their effect on the cerebellum. Thyroid hormones regulate neuronal proliferation, migration, process outgrowth, synaptic development, and myelin formation in specific brain regions (Koibuchi and Chin, 2000). Thyroid hormones are also known to effect cerebral development leading to morphological and functional deficits. The information with respect to mechanisms for these effects and deficits is limited to thyroid hormone receptors exerting effects by the regulation of expression of specific genes (Oppenheimer, et. al., 1997). Because brain development occurs during discrete windows of time, low levels of thyroid hormones in definitive periods can produce permanent damage, the nature of which depends upon the timing and magnitude of the insult. In our study, the effects did not appear to be permanent, but rather temporary. The question is then posed as to whether this is due to the low doses, the discrete period of exposure, the molecular complexity of the affected area enable full functioning after exposure ceases, or if the achievement is actually a genetically altered compensatory response. There are a multitude of potential factors, including DNA binding proteins, coactivator and corepressors which need to be coordinated for the governance of gene expression. To date, few direct response genes for thyroid hormones have been identified in the mammalian brain (Thompson and Potter, 2000). The potential for perchlorate to interfere with any of these processes which results in delays and deficits in development or motor behaviors exists.

In conclusion, we have demonstrated that perchlorate has the potential to affect landmarks that are indicators of brain and myelination development (such as causing delays in specific developmental feats like pinna unfolding), therefore, future research may be able to use this chemical to alter developmental pathways and learn about these molecular mechanisms. We have also demonstrated that sensitive markers exist at doses that are several orders of magnitude below the level that the existing regulatory agencies consider to be the NOAEL. It would be prudent for the U.S. EPA to reevaluate the risk assessment of this wide-spread pollutant based on the newly identified sensitive endpoints indicated herein.

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