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Developmental neurotoxicity of Propylthiouracil (PTU) in rats: Relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes

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ABSTRACT

Markedly lowered thyroid hormone levels during development may influence a child's behaviour, intellect, and auditory function. Recent studies, indicating that even small changes in the mother's thyroid hormone status early in pregnancy may cause adverse effects on her child, have lead to increased concern for thyroid hormone disrupting chemicals in the environment.

The overall aim of the study was therefore to provide a detailed knowledge on the relationship between thyroid hormone levels during development and long-lasting effects on behaviour and hearing. Groups of 16–17 pregnant rats (HanTac:WH) were dosed with PTU (0, 0.8, 1.6 or 2.4 mg/kg/day) from gestation day (GD) 7 to postnatal day (PND) 17, and the physiological and behavioural development of rat offspring was assessed. Both dams and pups in the higher dose groups had markedly decreased thyroxine (T_4) levels during the dosing period, and the weight and histology of the thyroid glands were severely affected. PTU exposure caused motor activity levels to decrease on PND 14, and to increase on PND 23 and in adulthood. In the adult offspring, learning and memory was impaired in the two highest dose groups when tested in the radial arm maze, and auditory function was impaired in the highest dose group. Generally, the results showed that PTU-induced hypothyroxinemia influenced the developing rat brain, and that all effects on behaviour and loss of hearing in the adult offspring were significantly correlated to reductions in T_4 during development.

This supports the hypothesis that decreased T_4 may be a relevant predictor for long-lasting developmental neurotoxicity.

Introduction

Normal thyroid hormone status is essential for a child's neurological development, and even small disruptions in the mother's thyroid status early in gestation, may cause intellectual and behavioural abnormalities in her children. Early in gestation, thyroid hormones are necessary for correct neural development, but during the first few months of pregnancy the human foetus does not produce thyroid hormones itself and therefore depends totally on the supply of thyroid hormones from the mother (Morreale de Escobar et al., 2000). Recent studies, using cohorts of healthy pregnant women not suffering from hypothyroidism and without dietary iodine deficiency, have shown that both low maternal thyroxine (T_4) levels and high levels of thyroid-stimulating hormone (TSH) early in pregnancy correlate significantly with impaired psychomotor development of the children (Pop et al., 1999; Haddow et al., 1999). These findings have contributed to increased concern as to the presence of thyroid hormone disrupting chemicals (TDC) in the environment.

The relationship between adverse neurobehavioral development and low thyroid hormone levels during development has been studied in laboratory animals for many years. In most cases Propylthiouracil (PTU) has been used as an anti-thyroid agent, because of its specific and well-characterized mode of action. PTU is an anti-thyroid drug which inhibits both the synthesis of thyroid hormones in the thyroid gland, and the conversion of thyroxine (T_4) to its active form, triiodothyronine (T_3), in peripheral tissues. For a detailed description of the many modes of action of PTU and the observed neurological abnormalities resulting from PTU exposure, see the review by Zoeller and Crofton (2005). In early rat studies developmental hypothyroidism was often induced by dosing pregnant dams with high doses of PTU in the drinking water. The observed effects included delayed development and reduced growth in the young offspring and persistent neurobehavioral effects, e.g. impaired learning and memory abilities and increased spontaneous activity (Davenport et al., 1976a,b, Davenport and Dorcey 1972; Shalock et al., 1979). In more recent PTU

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studies, in which more moderate changes in thyroid hormone homeostasis have been investigated, similar developmental and behavioural effects have been seen (Akaike et al., 1991; Gilbert and Sui 2006; Goldey et al., 1995b; Kobayashi et al., 2005; Noda et al., 2005). Hearing loss has also been demonstrated after both pre- and postnatal PTU treatment (Uziel et al., 1985; Goldey et al., 1995b; Henley and Rybak 1995). Crofton and co-workers have shown that hypothyroidism during the early postnatal period in the rat leads to impaired hearing and permanent damage in the cochlea (Crofton et al., 2000a,b). Furthermore, the hearing loss correlates significantly with decreases in early postnatal T_4 levels, seen after e.g. prenatal exposure to polychlorinated biphenyls (PCBs) (Crofton, 2004; Crofton and Zoeller, 2005).

Like PCB, several other industrial chemicals and pesticides have been shown to affect thyroid function and/or thyroid hormone levels (Brucker-Davis, 1998). Independently, some of these substances have also been shown to cause developmental neurotoxicity (DNT) in experimental animals (Schantz and Widholm, 2001). However, correlations between the degree of hypothyroidism and the severity of behavioural changes have not previously been established. Consequently, a major uncertainty in assessing the risk of developmental exposure to TDCs, is the lack of a clear characterization of the relationship between disruption of thyroid hormones and adverse effects on the brain. Thus, the main aim of the present study was to investigate how developmental thyroid hormone reduction is associated with adverse effects on the developing nervous system.

Furthermore, studies like this may in the future reduce the need for large DNT studies. If a clear and consistent connection can be established between thyroid hormone disruption and adverse neurobehavioral effects, then chemicals known to disrupt the thyroid hormone axis may in the future be evaluated and classified for developmental neurotoxicity based solely on the effect on thyroid hormones. This will decrease the need for costly DNT studies and also reduce the use of experimental animals.

The study was designed based on the newly approved OECD Test Guideline for Developmental Neurotoxicity testing TG426 (OECD, 2007) and the dams were dosed with PTU during both pregnancy and lactation. Since the rat brain and auditory system undergo substantial development postnatally, induction of hypothyroidism during the pre- and postnatal period in the rat corresponds to the thyroid sensitive stages of the nervous and auditory system development during prenatal life in humans (Goldey et al., 1995b). The study included measurements of thyroid hormone levels in dams and offspring during the gestation and lactation period, assessment of postnatal growth and physical development in the offspring, and histology of the thyroid gland. To test the effects of hypothyroxinemia on the development of the brain and the organ of hearing, the animals were tested in a battery of behavioural and physiological tests, including tests of activity, learning and memory, and auditory function.

Materials and methods

Test compound

The test compound was 6-propyl-2-thiouracil, PTU. CAS no. 51-52-5, product number P3755, purity >99.0% (Sigma-Aldrich, Brøndby, Denmark). Corn oil (Bie & Berntsen, Herlev, Denmark) was used as vehicle.

Animals and treatment

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the Inhouse Animal Welfare Committee. Eighty eight (88) time-mated, nulliparous, young adult Wistar rats (HanTac: WH, Taconic Europe, Ejby, Denmark) were supplied at day 3 of pregnancy. Upon arrival, the females were randomly distributed in pairs and housed under standard conditions: semitransparent plastic cages (15×27×43 cm) with Aspen bedding (Tapvei, Gentofte, Denmark) situated in an animal room with controlled environmental conditions (12h reverse light–dark cycles with light starting at 9 p.m., light intensity 500lx, temperature 22±1 °C, humidity 55±5%, ventilation 10 air changes per hour). Food (Altromin Standard Diet 1314) and acidified tap water were provided ad libitum.

The day after arrival, i.e. gestation day (GD) 4, the animals were weighed and assigned to four groups of 22 animals, with similar weight distributions. They were given 4 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. The study was run in three blocks, with 2 weeks in between each block and an equal representation of each dose group in each block. Dams in the four experimental groups were gavaged once a day at approximately the same time, from GD 7 to postnatal day (PND) 17 with 0, 0.8, 1.6, or 2.4 mg/kg PTU. The vehicle control and the PTU solutions were continuously stirred during the dosing period, and prepared anew for each of the three study blocks. The dams were treated at a constant volume of 2ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing. The dams were pair-housed until GD 17 and individually hereafter. They were observed daily for signs of toxicity, and body weights were recorded on GD 4 and during the entire dosing period.

Delivery and postnatal development

After delivery, weights of dams and individual pups were recorded. The pups were counted, sexed, and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD 22, was designated PND 0 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age as the animals gave birth on GD 22–23. Body weight of offspring was recorded on PND 6, 14, 17, 23, 27, after weaning on PND 66 and at the age of 4, 5 and 7 months.

At PND 16, litter size was standardized to 3 males and 3 females, when possible. From these offspring, 1–3 males and 1–2 females from each litter were weaned on PND 27, and kept for later behavioural testing. The weaned offspring was housed in pairs of the same sex and exposure status. After weaning each of the four dose groups consisted of two subgroups of animals. One had 18–20 male and 18–20 female pups from 16–17 different litters per group. These animals were used for motor activity tests, Morris water maze test and assessment of hearing. The other subgroup consisted of 18–20 male rats per dose group (from 17 litters per group), and these animals were tested in the radial arm maze.

Thyroid weights and histopathology PND 16 and 27

On PND 16, 1–9 pups in each litter were sacrificed depending on the original size of the litter. All sacrificed pups were weighed and decapitated, and trunk blood was collected for T_4 analysis (pooled for all males and all females within each litter). Thyroid glands from two males and two females per litter were used for histopathological investigations.

Dams and offspring (one male and one or two females in each litter) that were not kept for behavioural and functional studies, were sacrificed on PND 27. All animals were weighed and decapitated after CO_2/O_2 anesthesia, and trunk blood was collected for measurement of thyroxine in serum. The uteri of the dams were excised, and the number of implantation scars was registered. The thyroid glands were dissected, weighed, and used for histopathological investigation. In the offspring, the thyroid gland from one male and one female per litter were excised, weighed, and used for histopathology. The glands were fixed in formalin, embedded in paraffin, and examined by light microscopy after staining with haematoxylin and eosin.

The histological evaluation included scoring of thyroids as being "normal" or having "moderate" or "marked" effects. In PND 16 pups, thyroids were classified as having "marked" effects, when hyperplasia and hypertrophy of epithelial cells were observed along with papillary projections into the follicle lumen. A score for "moderate" changes required one of the following: slightly irregular follicular lumen, increased cellularity, and few papillary structures in the follicle lumen. In PND 27 pups, histological changes were generally milder, and a score for "moderate" changes required the presence of enlarged colloid-filled follicles, an increased cellularity and/or pseudostratified epithelium projecting into the lumen. In dams, thyroid effects at PND 27 were generally more marked than in the PND 27 pups, and a score for "marked" changes required the presence of irregular follicles, pseudostratified epithelium, and papillary projections into the follicle lumen. In adulthood, effects were mild, and a score for "moderate" effects was given when an increased number of large follicles with flattened epithelium were observed.

Thyroid hormone analysis

On GD 15 dams were anesthesized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam) and blood was drawn from the tail vein. From PND 16 pups, PND 27 pups, dams on day 27 and from 7 months old offspring, trunk blood was used for the hormone analysis. The plasma level of the thyroid hormone thyroxine (T_4) was analyzed using a modified Delfia T_4 (cat. no. 1244-030) time-resolved fluoroimmunoassay from Perkin Elmer (Wallac Oy, Turku, Finland). Instead of the T_4 standards and the T_4 antibody supplied in the Delfia kits, T_4 standards in T_4-free rat serum (cat. no.30042 and 30041, respectively), as well as biotinylated T_4 (30039) antibody from Biovian Ltd., Finland were used. The assay was run as outlined in the protocol supplied by Biovian Ltd. using streptavidin microtitration strips 8×12 wells (4009–0010) and T_4 assay buffer (1244-029 or 1244-111) from Perkin Elmer. The measurements were performed by use of a Wallac Victor 1420 multilabel counter (PerkinElmer Life Sciences, Turku, Finland).

Behavioral testing

The investigations were performed between 9 a.m. and 4 p.m. during the animals' dark cycle, i.e. their active period. The experimenter was kept unaware as to which group an individual rat belonged. Exposed and control animals were tested alternately and so were females and males.