

Perchlorate Exposure Induces Hypothyroidism and Affects Thyroid-Responsive Genes in Liver But Not Brain of Quail Chicks

Yu Chen · F. M. Anne McNabb · Jill C. Sible

Received: 23 October 2008 / Accepted: 23 February 2009
© Springer Science+Business Media, LLC 2009

Abstract Ground-dwelling birds in perchlorate-contaminated areas are exposed to perchlorate ion, a known thyroid disruptor, and might be vulnerable to the developmental effects of perchlorate-induced hypothyroidism. We hypothesized that perchlorate-induced hypothyroidism would alter the expression of thyroid-responsive genes involved in thyroid hormone (TH) regulation and in the development of target organ function. Japanese quail chicks were exposed to 2000 mg/L ammonium perchlorate in drinking water for 7.5 weeks beginning on day 5 posthatch. Hypothyroidism was evident after 2 weeks of exposure as lower plasma THs and lower TH content in exposed chicks than in controls. The degree of hypothyroidism was increased at 7.5 weeks, as indicated by significant thyroid gland hypertrophy and sustained changes in thyroid function. After 2 weeks of exposure, hypothyroidism increased type 2 5'-deiodinase (D2) mRNA level and decreased Spot 14 (SP14) mRNA level in the liver, whereas D2 mRNA and RC3 mRNA levels in brain were not affected. After 7.5 weeks of exposure, mRNA levels in the exposed group did not differ from those in controls in either the liver or brain, suggesting the responsiveness of these genes to THs decreased during development. These results suggest that the brain, but not the liver, was protected from the effects of hypothyroidism, probably by changes in D2 activity at the protein level and/or regulation of TH entry and exit from the

brain. We concluded that perchlorate exposure caused hypothyroidism in young Japanese quail and affected the expression of thyroid-responsive genes during early posthatch development.

Perchlorate ion is a thyroid disruptor that inhibits iodine uptake by the thyroid gland and reduces the production of thyroid hormones (THs). The manufacture and use of ammonium perchlorate as an oxidizer in solid rocket fuels has led to the contamination of groundwater, rivers, and lakes in at least 11 states in the United States (Jackson et al. 2006; Mayer et al. 2006). Wildlife species such as ground-dwelling birds in perchlorate-contaminated areas might develop hypothyroidism at young ages. Perchlorate-induced hypothyroidism might alter the expression of thyroid-responsive genes in young animals and thereby interfere with their growth and development. Recently, we have shown that perchlorate deposited in eggs by quail hens can decrease thyroid function and alter gene expression in embryos (Chen et al. 2008); the present article addresses the effects of posthatch perchlorate exposure in quail chicks.

Thyroid hormones play essential roles in vertebrate development. THs control the expression of thyroid-responsive genes during critical developmental windows and are thus required for tissue or organ development. Hypothyroidism during these times might result in irreversible defects (McNabb 2007).

Of the two forms of THs, T_3 is the physiologically active, receptor-binding form. The conversion of T_4 , the most abundant hormone, to T_3 by 5'-deiodination is catalyzed by type 1 and 2 deiodinases (D1 and D2). D2 is the major 5' deiodinase in the central nervous system and D2 activity is influenced by TH concentrations at both mRNA

Y. Chen · F. M. A. McNabb (✉) · J. C. Sible
Department of Biological Sciences, Virginia Polytechnic
Institute and State University, Blacksburg, VA 24061-0406,
USA
e-mail: happy@vt.edu

and protein levels (Burmeister et al. 1997). D2 activity is enhanced by low TH concentrations but inhibited by high TH concentrations. Studies in chickens have demonstrated that brain T_3 concentration is relatively stable despite changes in plasma T_4 concentration, and D2 is one of the key contributors to this stability (Rudas et al. 2005). D2 activity also is found in several other organs such as brown adipose tissue, thyroid gland, skeletal muscle in mammals, and liver in both birds and fish (Bianco et al. 2002; Gereben et al. 1999). Thyroid status regulates brain D2 inversely, a phenomenon that has been most studied in mammals and birds (Bianco et al. 2002).

In mammals, T_3 regulates the expression of many thyroid-responsive genes such as RC3/neurogranin (RC3) in both developing and adult animals. RC3 is a neuron-specific, calmodulin-binding protein that regulates calcium availability in neurons (Gerendasy and Sutcliffe 1997). A significant increase in the RC3 expression during development coincides with the timing of synaptogenesis, which has led to the assumption that RC3 plays an important role during brain development. In mammals, hypothyroidism reduces RC3 mRNA and protein levels in a number of brain regions and such reductions of RC3 expression during brain development are potentially related to some irreversible mental deficits associated with hypothyroidism (Bernal 2002).

Thyroid hormones also are involved in the control of metabolic processes in the liver. The mRNA level of Spot 14 (SP14), a protein involved in the lipogenic pathway in the liver, is directly and rapidly upregulated by T_3 . The control of SP14 in relation to thyroid status has been well studied in both mammals and birds. Hypothyroid animals characteristically have lower SP14 mRNA levels than euthyroid animals and their lipogenic function might be disrupted (Brown et al. 1997; Wang et al. 2004).

In this study, we hypothesized that perchlorate-induced hypothyroidism would alter the expression of thyroid-responsive genes in the brain and liver in birds. We used Japanese quail as a model for ground-dwelling galliform birds, in general, and high levels of perchlorate exposure to produce overt hypothyroidism in the chicks. Hypothyroidism was evaluated by measurements of plasma TH concentrations, thyroid gland weight [to assess activation of the hypothalamic–pituitary–thyroid axis (HPT)] and TH storage in the thyroid gland. The steady-state D2 mRNA levels were measured in the brain and liver, RC3 mRNA levels were measured in the brain, and SP14 mRNA levels were measured in the liver to evaluate tissue-specific responses of thyroid-responsive gene expression. Our results demonstrated that perchlorate-induced hypothyroidism altered the mRNA level of thyroid-responsive genes in the liver but not in the brain.

Materials and Methods

Animal Maintenance, Treatment, and Sampling

Japanese quail (*Coturnix japonica*) eggs were collected from a breeding colony in the animal care facilities in the Department of Biological Sciences at Virginia Tech. Eggs were incubated and hatched at $39 \pm 1^\circ\text{C}$ and $>90\%$ relative humidity in a forced-air incubator (Humidaire Hatchette Incubator; New Madison, OH). Newly hatched chicks were divided into two treatment groups, banded, and kept in separate shelves in a brooder. Game bird feed (Big Spring Mills, Ellison, VA) and drinking solutions were provided ad libitum. Birds were moved to taller cages from 4 weeks of age until the end of the experiment. All maintenance, handling, and sacrifice procedures of the animals were approved by the Virginia Tech Animal Care Committee (IACUC) in accordance with federal guidelines.

Chicks, 4–5 days old, were divided into two groups and housed in separate cages. One group of 17 chicks was given 2000 mg/L ammonium perchlorate (AP; Fluka Chimka, Steinheim, Germany) solution in tap water as drinking water. Previous work in our laboratory on Bobwhite quail chicks showed that this concentration of AP was sufficient to cause decreased thyroid function after a 2-week exposure period (McNabb et al. 2004a). The control group of 13 chicks was given tap water. After 2 weeks, five chicks, randomly selected from each group, were sacrificed to evaluate the short-term effects of perchlorate exposure. The remaining chicks were continued in the experiment and blood samples were drawn from the brachial vein at 6 weeks of exposure to determine their plasma T_4 concentrations. After it was shown that at 6 weeks the mean plasma T_4 concentration was significantly lower in the perchlorate-exposed group than the control group, these chicks were sacrificed at 7.5 weeks of exposure. Chicks were sacrificed by decapitation; trunk blood was collected in heparinized capillary tubes and plasma was stored at -20°C until analysis. Brains and livers were dissected immediately after sacrifice, flash-frozen in liquid nitrogen, and stored at -80°C until RNA isolation. Thyroid glands were removed, weighed, frozen, and stored at -20°C in snap-cap tubes.

Thyroid Assays

Plasma TH concentrations were determined by a double antibody radioimmunoassay (RIA) described by Wilson and McNabb (1997). Hormone standards were prepared in charcoal-stripped chicken plasma. Duplicate aliquots for each plasma sample were used and sample volumes were 12.5 μL for T_4 and 25 μL for T_3 . Primary antibodies were purchased from Fitzgerald (Fitzgerald Industries

International, Inc, Concord, MA). ^{125}I -labeled hormones (high specific activity; 1200 $\mu\text{Ci}/\mu\text{g}$) were purchased from Perkin-Elmer Life Sci (Boston, MA). Three levels of Randox Immunoassay Control serum (Randox Laboratories, San Diego, CA) were included in each assay to evaluate assay performance. The lowest sensitivity of the RIA is 0.125 ng/mL for T_3 and 1.25 ng/mL for T_4 . The intra-assay precision of RIA, ± 2 SE, was 3.1% of the mean for T_4 ($n = 6$) and 2.6% of the mean for T_3 ($n = 6$; McNabb et al. 2004b).

The plasma of quail at 6 weeks contained high lipid content that interfered with T_4 antibody binding in the RIAs. We addressed this by extracting plasma by mixing equal volumes of plasma with 100% ethanol for the 6-week samples for the T_4 assay only. After centrifugation at 12,000g to remove insoluble material, hormones in the supernatant were measured by RIA using standards prepared in 50% ethanol. This method was validated by demonstrating that dilutions of plasma extract paralleled the standard curve. This strategy did not allow us to measure T_4 in the 7.5-week samples. The lipid content had much less interference with T_3 antibody binding and plasma T_3 assays were performed without ethanol extraction on all samples.

Activation of the HPT axis was evaluated by comparing mean thyroid gland weights from the control and perchlorate-exposed groups. The pair of thyroid glands from each bird was weighed to the nearest 0.01 mg.

Thyroidal hormone content of the glands was measured using the method described by McNabb and Cheng (1985). Thyroid gland tissue (10 mg or less) was digested in 350 μL of digestion medium containing 25 mg of Pronase (Sigma-Aldrich, St. Louis, MO) at 37°C for 24 h. When the combined weight of the gland pair exceeded 10 mg, each gland was digested separately. The digestion was stopped by the addition of 1.0 mL of absolute ethanol and the tubes were stored at -20°C for 24 h to extract the THs, tubes were centrifuged at 13,500g for 5 min, and the supernatant was stored at -20°C until analysis. Dilutions of the supernatant in 75% ethanol were analyzed for T_4 and T_3 by RIA as described earlier, using standards prepared in 75% ethanol.

Thyroid hormone concentrations in samples within each dataset (e.g., plasma T_4 at 2 weeks) were measured in a single assay. Therefore, for each thyroid variable, comparisons among the different treatment groups were made with data determined in the same assay.

Total RNA Isolation

Total RNA was isolated from brain and liver tissues using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) following the protocol provided by the manufacturer. Total RNA from each brain or liver tissue was isolated individually. Tissues

were homogenized in Tri-Reagent with a Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Inc., Westbury, NY). For each 50 mg of tissue, 1 mL of the Tri-Reagent was added. Insoluble material in the homogenate was eliminated by centrifuging at 12,000g for 10 min. The supernatant was mixed with chloroform (0.2 mL for each milliliter of Tri-Reagent) and centrifuged at 12,000g for 15 min for phase separation. The top aqueous phase containing RNA was transferred to a new tube and mixed with isopropanol (0.5 mL for each milliliter of Tri-Reagent) to precipitate the RNA for 15 min at room temperature. After centrifugation, RNA pellets were washed in 75% ethanol, dried, and resuspended in RNase-free water. The RNA samples were then stored at -80°C for further analysis.

Northern Blotting

The mRNA levels of thyroid-responsive genes were determined by Northern blot following the protocol described by Sible et al. (1997). Before Northern blotting, total RNA samples within the same group were pooled. For electrophoresis, 20 μg from each pooled total RNA sample was loaded on a 1% denaturing agarose gel containing formaldehyde. After electrophoresis, the RNA was transferred to a nylon membrane and cross-linked by ultraviolet (UV) irradiation. ^{32}P -labeled probes were prepared using the Random Primed DNA labeling kit (Roche Applied Science, Indianapolis, IN) and ^{32}P -labeled dCTP (Amersham Life Sci, Pittsburgh, PA). The cDNA probes of Japanese quail D2, RC3, SP14, and β -actin were obtained from young Japanese quail brain and liver total RNA by reverse transcription–polymerase chain reaction (RT-PCR). Sequence information of the cDNAs is available in the NCBI nucleotide database (NCBI accession Nos: RC3, EU558133; DII, EU558134; and SP14, EU558135). Membranes were hybridized with 20×10^6 cpm/mL denatured probes in QuikHyb solution (Stratagene, La Jolla, CA) and washed following the manufacturer's instruction. Brain total RNA was hybridized with D2 and RC3 probes and liver total RNA was hybridized with D2 and SP14 probes. The experiments were repeated three times for the same samples to confirm the results. In addition, β -actin, which is constitutionally expressed in most tissues, was used as the housekeeping gene to test the quantity and integrity of RNA.

Statistical Analyses

Multivariate analysis of variance (MANOVA) was used to analyze the thyroid variables measured. For chicks sacrificed at 2 weeks, plasma T_4 and T_3 concentration, thyroid gland weight, and thyroidal T_4 and T_3 storages were included as responses. For chicks sacrificed at 7.5 weeks, thyroid

gland weight and thyroidal T_4 and T_3 storage were included as responses. For post hoc analysis, univariate analysis of variance (ANOVA) was used to investigate the data for each individual response. Body weight at 2 and 7.5 weeks and plasma T_4 and T_3 concentrations at 6 weeks were analyzed using the Student's *t*-test. Statistically significant differences were defined as probabilities of $p \leq 0.05$. Statistical analyses were performed using Minitab 15 (Minitab, Inc., State College, PA). Birds sacrificed at 2 weeks of exposure were randomly selected, and the sex of the birds could not be distinguished by appearance at that age. As a result, all five chicks in the control group at 2 weeks turned out to be females and three out of the five chicks in the perchlorate-exposed group were females. Of the birds sacrificed at 7.5 weeks, 3 out of 8 birds in the control group were females, whereas 6 of 12 birds in the exposed group were females. Therefore, the effects of perchlorate exposure on difference sexes were not analyzed statistically.

Results

Growth and Development

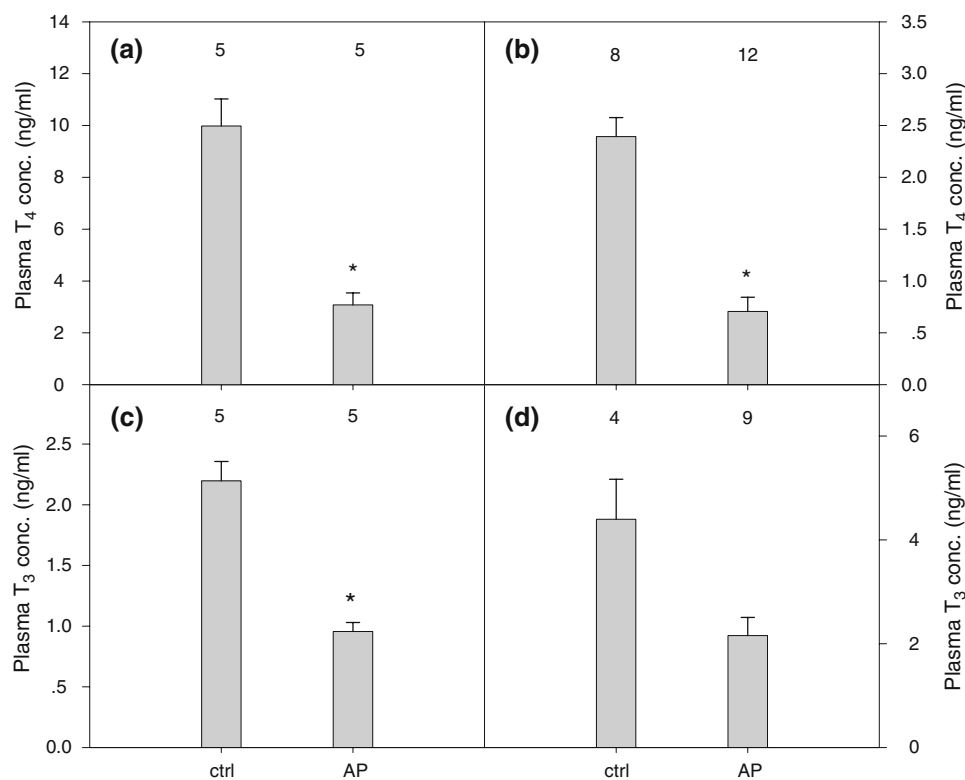
The body weights of the control group and perchlorate-exposed groups were not significantly different at either 2 weeks ($p = 0.168$, $\text{mean}_{\text{ctrl}} = 33.49 \pm 3.7$, $\text{mean}_{\text{AP}} = 27.07 \pm 1.0$) or 7.5 weeks ($p = 0.657$, $\text{mean}_{\text{ctrl}} =$

105.25 ± 2.3 , $\text{mean}_{\text{AP}} = 103.3 \pm 3.6$) of treatment. In males of the perchlorate-exposed group, the change of their plumage from the dull immature color to a brighter adultlike color was delayed for about 1 week compared with those of the control group. When sacrificed at 7.5 weeks, four of the six males in the perchlorate-exposed group were sexually mature and the other two had underdeveloped testes; all control males had testicular development characteristic of sexually mature adults. Thus, perchlorate exposure did not affect the overall growth of the birds, but there was evidence that the development of the male reproductive system might have been delayed.

Thyroid Function

Perchlorate exposure significantly affected overall thyroid function at both 2 weeks (MANOVA: $F_{5,4} = 12.258$; $p = 0.015$) and 7.5 weeks (MANOVA: $F_{3,16} = 81.228$; $p < 0.001$). The development of organismal hypothyroidism was assessed by measuring THs in blood samples collected at 2 weeks ($N = 5/\text{group}$) and 6 weeks (8–12 chicks/group) of perchlorate exposure. At 2 weeks of treatment, plasma T_4 concentrations in the perchlorate-exposed group were significantly lower than those in the control group (ANOVA: $F_{1,8} = 36.55$; $p < 0.001$; Fig. 1a). At 6 weeks of treatment, the plasma T_4 concentrations after ethanol extraction also were significantly lower in the perchlorate-exposed group than those in the

Fig. 1 Plasma T_4 concentrations after 2 weeks (a) and 6 weeks (b) of ammonium perchlorate (AP) treatment and plasma T_3 concentrations after 2 weeks (c) and 7.5 weeks (d) of AP treatment in young Japanese quail. AP was administered, beginning at 4–5 days of age, at 2000 mg AP/L in drinking water. Values are means \pm S.E. Asterisks indicate significant differences at $p < 0.05$ between control (C) and perchlorate-exposed groups. *N* values are above each bar. Note that the lower plasma T_4 concentration at 6 weeks is due to the loss of hormone during ethanol extraction of T_4 from plasma before RIA. This extraction was necessary because of lipid interference with the T_4 antibody in the 6-week samples



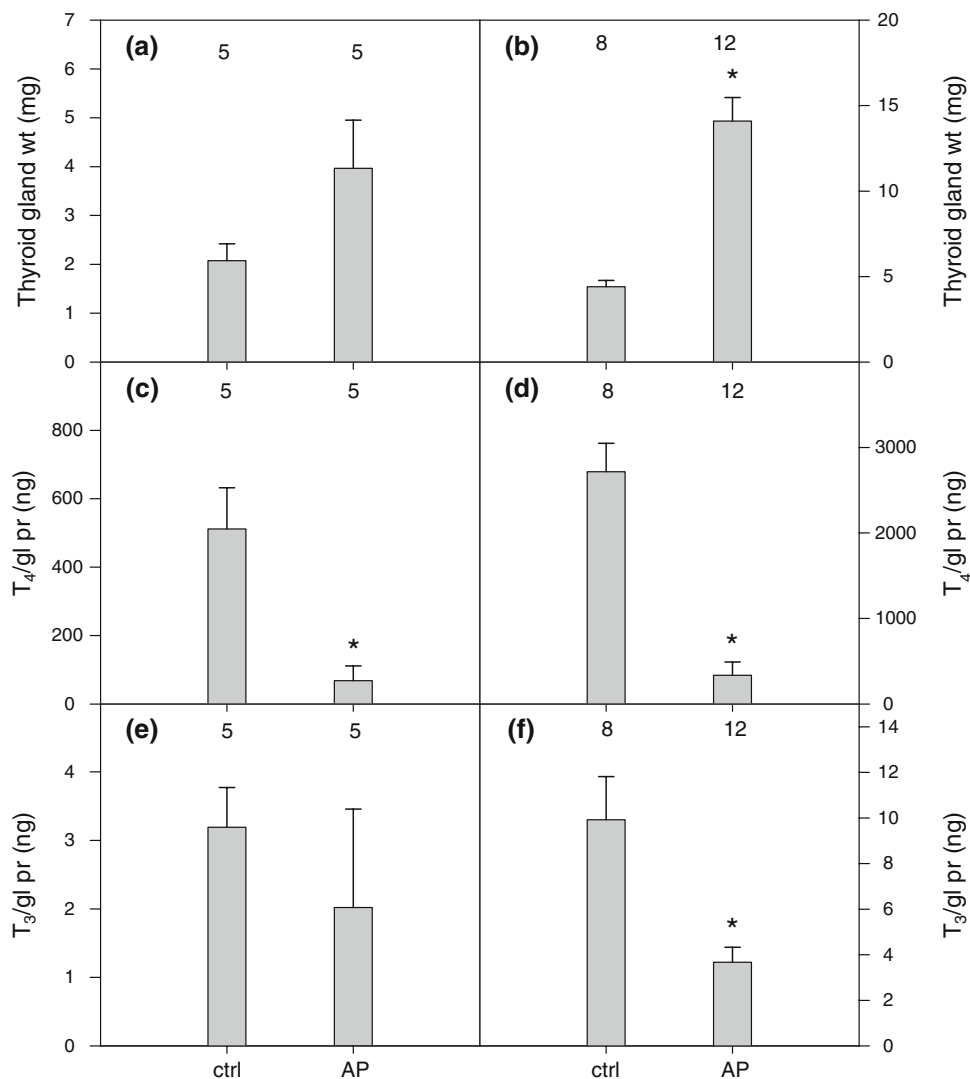
control group (t -test: $p < 0.0001$; Fig. 1b). The proportional difference in plasma T_4 concentration (perchlorate group/control group) was very similar at 2 weeks (~ 0.31) and 6 weeks (~ 0.29) of treatment. Plasma T_3 concentrations were significantly lower in the perchlorate-exposed group than in the control group at 2 weeks (ANOVA: $F_{1,8} = 49.31$; $p < 0.001$; Fig. 1c), but the groups did not differ significantly at 7.5 weeks (t -test: $p = 0.057$; Fig. 1d) due to greater variability at the later time. Note that T_3 concentrations in a few samples in both groups were not measurable due to the lipid interference. Most of the birds were sexually mature at the time of sacrifice and the lipid content in the plasma increased dramatically between 6 and 7.5 weeks of perchlorate exposure. Ethanol extraction was not effective in eliminating lipid interference with antibody binding in either T_4 or T_3 RIAs at this age.

The involvement of the HPT axis in developing hypothyroidism was assessed by thyroid gland weight. At 2 weeks, gland weight in the perchlorate-exposed group

tended to be significantly different from the control group (ANOVA: $F_{1,8} = 3.29$; $p = 0.107$; Fig. 2a). Mean thyroid weights of the perchlorate-exposed controls were about twofold those of the controls at 2 weeks, but the high variability in the exposed group accounted for the lack of significant differences in groups at this stage. At 7.5 weeks, the gland weight was significantly higher in the perchlorate-exposed group (about a threefold weight difference) than in the control group (ANOVA: $F_{1,18} = 31.44$; $p < 0.001$; Fig. 2b). The results suggest the activation of the HPT axis was more consistent and somewhat greater at 7.5 weeks than at 2 weeks of perchlorate exposure.

Thyroid gland storage of T_4 per bird (ng T_4 /pair thyroid glands) was significantly lower in the perchlorate-exposed group at both 2 weeks (ANOVA: $F_{1,8} = 11.98$; $p = 0.009$; Fig. 2c) and 7.5 weeks of treatment (ANOVA: $F_{1,18} = 52.50$; $p < 0.0001$; Fig. 2d). The perchlorate-exposed group showed a 7.5-fold decrease in gland T_4 after 2 weeks of exposure compared with the control group and a slightly

Fig. 2 Thyroid gland weight and thyroidal TH storage of perchlorate-exposed young Japanese quails: thyroid gland weight after 2 weeks (a) and 7.5 weeks (b) of perchlorate exposure; thyroidal T_4 storage after 2 weeks (c) and 7.5 weeks (d) of perchlorate exposure; thyroidal T_3 storage after 2 weeks (e) and 7.5 weeks (f) of perchlorate exposure. AP was administered, beginning at 4–5 days of age, at 2000 mg AP/L in drinking water. Values are means \pm S.E. Asterisks indicate significant differences at $p < 0.05$ between control (C) and perchlorate-exposed groups. N values are above each bar



greater 8.1-fold decrease after 7.5 weeks. Thyroidal T_3 content, which accounts for a very small proportion of gland hormones, was not significantly different in the perchlorate-exposed group from controls at 2 weeks of exposure (ANOVA: $F_{1,8} = 0.57$; $p = 0.472$; Fig. 2e) but was significantly lower at 7.5 weeks of exposure (ANOVA: $F_{1,18} = 175.37$; $p < 0.001$; Fig. 2f). Thyroidal hormones decreased quickly after perchlorate exposure started (by 2 weeks of exposure) and remained low through the exposure period.

Thyroid-Responsive Gene mRNA Levels

The mRNA levels of thyroid-responsive genes were compared using the β -actin mRNA levels as references. The D2 mRNA level in the brain was not affected by perchlorate exposure at either 2 weeks or 7.5 weeks (Fig. 3a). In the liver, the D2 mRNA level was higher in the perchlorate-exposed group than the control group at 2 weeks. At 7.5 weeks, however, no difference in D2 mRNA levels could be observed between the control and perchlorate-exposed groups. In addition, D2 mRNA levels in the liver at 7.5 weeks of perchlorate exposure in both control and treated groups appeared to be slightly lower than those at 2 weeks of perchlorate exposure (Fig. 3a). The SP14 mRNA level in the liver was lower in the perchlorate-

exposed group compared with the control group at 2 weeks but was about the same in both groups at 7.5 weeks. Moreover, the SP14 mRNA of both the perchlorate-exposed group and the control group at 7.5 weeks was expressed at a higher level than the control group at 2 weeks (Fig. 3b).

Two bands were detected after Northern blotting using RC3 probe. This result was consistent with previous findings in Japanese embryos by our laboratory. Both bands were believed to be RC3 mRNA products and they share the same pattern in their levels during development (Chen et al. 2008). Therefore, both bands were included for comparison of the RC3 mRNA level in this study. There was no difference in the RC3 mRNA levels in the brain between the control and perchlorate-exposed groups at either 2 or 7.5 weeks of perchlorate exposure (Fig. 3c).

Discussion

Ammonium perchlorate disrupted thyroid function in Japanese quail in this study, as has been observed in a limited number of other studies of perchlorate effects in birds (for review, see McNabb et al. 2006). Hypothyroidism was evident in perchlorate-exposed chicks after 2 weeks, as indicated by decreased plasma THs and largely depleted thyroidal TH content compared to the control group. However, although there was a trend toward a HPT axis response (as indicated by thyroid gland weight), the response was not significantly different in the perchlorate-treated birds from the controls after 2 weeks. This was surprising because the depleted TH content in the perchlorate-exposed birds indicates that a large proportion of the stored THs had been released into the circulation to sustain plasma TH concentrations (note that the thyroid is unique among endocrine glands in having large stores of hormone). The release of stored hormones occurs because decreases in plasma THs (as seen in these quail at 2 weeks) have a negative feedback effect on the HPT axis, which increases the release of thyrotropin from the pituitary. Increased thyrotropin, in addition to stimulating TH release, also stimulates thyroid gland growth. The thyroid weights were highly variable at 2 weeks of perchlorate exposure, probably due to individual differences in chick responses (i.e., not all birds had developed hypothyroidism after only 2 weeks of perchlorate exposure).

In the Japanese quail in this study, the degree of hypothyroidism was increased with continued duration of perchlorate exposure. By 7.5 weeks of exposure, the most notable change was seen in the HPT axis response; that is, thyroid gland weights were significantly greater by 3.2-fold in the perchlorate-exposed group than the control group. Plasma T_4 in the perchlorate-exposed birds remained

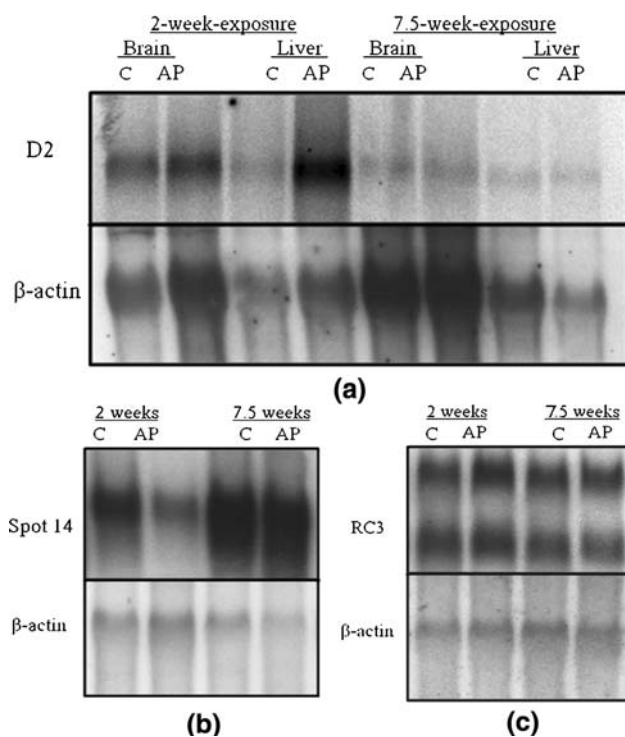


Fig. 3 Effect of ammonium perchlorate exposure on the mRNA level of (a) type 2,5' deiodinase (D2), (b) Spot 14, and (c) RC3. All RNA samples also were probed with β -actin to test the quality and consistency of the RNA

significantly lower than in control birds (ratio perchlorate group/control group = 0.31 at 2 weeks and 0.29 at 6 weeks). Thyroidal T_4 stores also continued to decrease slightly from 2 weeks to 7.5 weeks of treatment (perchlorate group/control group; 0.13 vs. 0.12, respectively).

The thyroid-responsive gene for D2 is important in the central nervous system enzymatic regulation of T_3 , the most active TH. We predicted that the brain mRNA level of D2 would increase in hypothyroid chicks, reflecting a compensatory D2 response that would protect brain T_3 concentrations. However, the mRNA level of D2 was not affected in the brain of Japanese quail chicks after 2 or 7.5 weeks of perchlorate exposure. The brain is known to be protected from short-term hypothyroidism by several mechanisms: (1) increases in D2 activity, which increase T_3 production, (2) increases in the uptake of THs into the brain, and (3) decreases in the loss of THs from the brain [rats (Bernal 2002); young chickens (Gereben et al. 1999; Rudas et al. 2005)]. The third of these mechanisms results from decreased T_3 deactivation by inner-ring deiodination (Type 3, a 5-deiodinase) and consequent decreased loss of T_3 to the circulation (Rudas et al. 2005). Our measurements focused only on whether decreases in circulating TH affected D2 expression at the level of mRNA, as a mechanism for increasing T_3 generation. However, if the second and third mechanisms listed protected brain T_3 concentrations in these quail chicks, then increases in D2 gene expression or enzyme activity would not be “needed.” Thus, the lack of D2 mRNA increase in hypothyroid chicks in this study argues that brain T_3 concentrations were maintained by the second and third mechanisms listed within the exposure times and perchlorate concentrations we used. In addition, levels of mRNA might not be a direct reflection of the D2 activity in specific tissues. D2 activity is regulated by both T_3 and T_4 through different mechanisms. Studies in mammals have revealed that T_4 alters D2 activity at a posttranslational level; that is, high T_4 concentrations facilitate the degradation of the enzyme (Kim et al. 1998; Steinsapir et al. 1998). In contrast, T_3 regulates at the level of D2 mRNA, which also ultimately changes the activity of the enzyme. High T_3 concentrations decrease and low T_3 concentrations increase steady-state D2 mRNA levels. The effect of T_4 is usually fast, whereas that of T_3 is slow (Burmeister et al. 1997). Moreover, studies have shown that D2 protein stability and activity were also regulated by complex mechanisms at the posttranscriptional level (Gereben et al. 2002; Zeold et al. 2006). Therefore, Northern blotting might not detect the effects of all these mechanisms. Furthermore, D2 might not be affected by hypothyroidism to the same degree in all brain areas (Gereben et al. 2004; Verhoelst et al. 2004). Subtle changes in regions more sensitive to hypothyroidism might not be detected by measuring mRNA levels in brain total RNA.

Our study also demonstrated that D2 mRNA is present in Japanese quail liver, a peripheral tissue exposed to circulating TH concentrations; that is, the liver does not have “protective” mechanisms for controlling tissue hormones. This is in contrast to the central nervous system, where the entry and exit of THs is regulated (see section above). In avian species, the presence of hepatic D2 has been confirmed by both detection of PTU insensitive 5'-deiodinase activity in Japanese quail liver (Hughes and McNabb 1986; McNabb et al. 1986) and the presence of hepatic D2 mRNA in chickens (Gereben et al. 1999). Liver is one of the tissues responsible for the production of a major fraction of circulating T_3 from T_4 , and, historically, D1 was believed to be the sole source of T_3 from peripheral deiodination in mammals (Chopra 1991). Nonetheless, some studies in rats indicate that D2 in brown adipose tissue (BAT), and perhaps in other tissues, might account for as much as 50% of the T_3 production for release to the circulation. The ratio might be even higher in hypothyroid rats, where D1 activity is reduced by low circulating T_4 (Nguyen et al. 1998; Silva and Larsen 1985). In birds, although D1 predominates in the liver, enzymatic assays have indicated that D2 activity accounts for some T_3 production (probably < 10% of the total) during posthatch development (Hughes and McNabb 1986). In adult chickens, the level of D2 mRNA in the liver is comparable to that in the brain (Gereben et al. 1999). However, D2 activity is much lower in the liver than in the brain. This might be a result of alternative splicing of D2 mRNA in the liver, which can result in higher proportions of inactive D2 (Gereben et al. 2002).

The presence of D2 in avian liver raises the question of whether there is a hepatic D2 response to changes in circulating THs and whether D2 might be elevated in the liver of hypothyroid birds, thus contributing to circulating T_3 . In the present study, hypothyroidism increased D2 mRNA level in the liver of Japanese quail chicks after 2 weeks of perchlorate exposure (although this was not the case at 7.5 weeks of exposure). Similar results were reported by our laboratory in an earlier study on perchlorate-exposed Japanese embryos, in which D2mRNA was detected in perchlorate-exposed embryos but not in the control embryos (Chen et al. 2008). Unlike the brain, for which hormone entry and exit are regulated, liver tissue is not protected against hypothyroid conditions. In the present study, decreased plasma THs increased the expression of D2 in the livers of perchlorate-exposed quail chicks, which suggests that the liver initially responded to hypothyroidism by increasing T_3 production. In contrast, at 7.5 weeks, although the perchlorate-exposed birds remained hypothyroid, the liver D2 mRNA level was not different from that of the control group. There is evidence that D2 expression changes during the course of development. In rats, the D2/

D1 activity ratio decreases substantially from the neonatal period to that in adults in tissues such as thyroid gland and pituitary (Bates et al. 1999). Hence, developmentally, D2 function and regulation might be different in quail chicks than adults. Furthermore, D2 might be regulated differently in the liver than in the brain in adult chickens (Gereben et al. 2002). Our results in both Japanese embryos and young chicks might indicate a more active role of D2 in TH regulation during early development.

In this study, RC3 was investigated as an example of a thyroid-responsive gene with developmental effects on a target tissue (brain). We predicted that perchlorate-induced hypothyroidism would result in decreased brain RC3 mRNA levels. However, the mRNA level of RC3 in the brain was not affected by hypothyroidism (in the body) at either 2 or 7.5 weeks of perchlorate exposure. Because the brain D2 mRNA level also was not affected in the perchlorate-exposed group, these two congruent results argue that T₃ concentrations in the brain did not change appreciably (see discussion above about other hormone regulatory mechanisms that protect the brain). However, it is possible that there were changes in RC3 mRNA in some brain regions that were not detectable in our studies of total brain mRNA. Many of the previous studies on RC3 were done by *in situ* hybridization of RC3 mRNA in brain tissues and they have found that RC3 mRNA level did not change the same way in all brain areas (Ishitobi et al. 2007; Lein et al. 2007; Piosik et al. 1995, 1996; Wilcoxon et al. 2007; Zoeller et al. 2000).

In the liver, hypothyroidism lowered the SP14 mRNA level (another thyroid-responsive target tissue gene) at 2 weeks of perchlorate exposure. SP14 codes for a protein that regulates enzymes in the lipogenic pathway in the liver and its mRNA level is rapidly upregulated by THs (Jump et al. 1984; Narayan et al. 1984). As predicted, this result indicates that some liver functions might have been affected by perchlorate-induced hypothyroidism. At 7.5 weeks, no significant difference was seen between the perchlorate-exposed and control groups. With increasing maturity, SP14 mRNA levels were increased considerably at 7.5 weeks compared to those at 2 weeks in both control and treated birds. SP14 is known to be responsive to factors other than plasma TH concentrations (e.g., circulating insulin concentrations as well as carbohydrate content of the diet; Jump et al. 2001; LaFave et al. 2006). At 7.5 weeks of treatment, quail are entering breeding age and we observed plasma lipids increased markedly, indicating that lipogenesis was highly active at that time. In such conditions, SP14 expression and lipogenesis might be regulated by multiple mechanisms and the influence of THs might not be as significant as at 2 weeks of perchlorate exposure.

In summary, perchlorate exposure causes organismal hypothyroidism in young Japanese quail and thyroid

function indicators suggest the degree of hypothyroidism increased as the time of exposure lengthened. The effects of perchlorate exposure on the expression of thyroid-responsive genes were more complicated. Genes in different tissues responded differently. The brain showed no effects of organismal hypothyroidism on the mRNA level of the hormone regulatory gene, D2, or the developmentally relevant gene, RC3, suggesting that other mechanisms provided protection of the brain from the decreased TH concentrations in the body. In the liver, a hormone regulatory gene, D2, and a developmentally relevant gene, SP14, were influenced by hypothyroidism early in perchlorate exposure (2 weeks) but not after sustained exposure (7.5 weeks). This study suggests that wild birds in perchlorate-contaminated areas might develop hypothyroidism from perchlorate exposure during their posthatch life. The effects of hypothyroidism on thyroid-responsive genes might differ in different target tissues and with exposure time and age. Thus, thyroid disruption by perchlorate exposure might affect development and reduce the overall fitness of both young and adult birds living in contaminated habitats. However, it should be noted that we used higher perchlorate exposures than commonly occur environmentally because our purpose was to address the effects of hypothyroidism on thyroid-responsive genes in different target tissues.

Acknowledgments The study was supported by the Strategic Environmental Research and Development Program (SERDP), grants from Graduate Research and Development Program (GRDP), grants from Sigma-Xi grants-in-aids, and a Waste Policy Institute (WPI) summer fellowship. We thank Dr. Michael Denbow, Dr. Bradley Klein, and Dr. Ignacio Moore for useful advice during the study. Laila Queral-Kirkpatrick, Catherine Webb, and Eric Weigel participated in animal sampling. Bambi Jarrett helped with animal care and maintenance.

References

- Bates JM, St. Germain DL, Galton VA (1999) Expression profiles of the three iodothyronine deiodinases, D1, D2, and D3, in the developing rat. *Endocrinology* 140:844–851. doi:10.1210/en.140.2.844
- Bernal J (2002) Thyroid hormones and brain development. In: Pfaff D, Arnold A, Etgen A, Fahrbach S, Rubin R (eds) *Hormones, brain and behavior*. Academic Press, Burlington, pp 543–587
- Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 23:38–89. doi:10.1210/er.23.1.38
- Brown S, Maloney M, Kinlaw W (1997) “Spot 14” protein functions at the pretranslational level in the regulation of hepatic metabolism by TH and glucose. *J Biol Chem* 272:2163–2166. doi:10.1074/jbc.272.4.2163
- Burmeister LA, Pachucki J, St. Germain DL (1997) Thyroid hormones inhibit type 2 iodothyronine deiodinase in the rat cerebral cortex by both pre- and post-translational mechanisms. *Endocrinology* 138:5231–5237. doi:10.1210/en.138.12.5231

- Chen Y, Sible JC, McNabb FMA (2008) Effects of maternal exposure to ammonium perchlorate on thyroid function and the expression of thyroid-responsive genes in Japanese quail embryos. *Gen Comp Endocrinol* 159:196–207. doi:[10.1016/j.ygcen.2008.08.014](https://doi.org/10.1016/j.ygcen.2008.08.014)
- Chopra IJ (1991) Nature, sources, and relative biologic significance of circulating THs. In: Braverman LE, Utiger RD (eds) *Werner and Ingbar's the thyroid: a fundamental and clinical text*, 6th edn. J. B. Lippincott, Philadelphia, pp 126–143
- Gereben B, Bartha T, Tu HM, Harney JW, Rudas P, Larsen PR (1999) Cloning and expression of the chicken type 2 iodothyronine 5'-deiodinase. *J Biol Chem* 274:13,768–13,776. doi:[10.1074/jbc.274.20.13768](https://doi.org/10.1074/jbc.274.20.13768)
- Gereben B, Kollar A, Harney JW, Larsen PR (2002) The mRNA structure has potent regulatory effects on type 2 iodothyronine deiodinase expression. *Mol Endocrinol* 16:1667–1679. doi:[10.1210/me.16.7.1667](https://doi.org/10.1210/me.16.7.1667)
- Gereben B, Pachucki J, Kollar A, Liposits Z, Fekete C (2004) Ontogenic redistribution of type 2 deiodinase messenger ribonucleic acid in the brain of chicken. *Endocrinology* 145:3619–3625. doi:[10.1210/en.2004-0229](https://doi.org/10.1210/en.2004-0229)
- Gerendasy DD, Sutcliffe JG (1997) RC3/neurogranin, a postsynaptic calpacitin for setting the response threshold to calcium influxes. *Mol Neurobiol* 15:131–163. doi:[10.1007/BF02740632](https://doi.org/10.1007/BF02740632)
- Hughes TE, McNabb FMA (1986) Avian hepatic T-3 production by two pathways of 5'-monodeiodination: effects of fasting and patterns during development. *J Exp Zool* 238:393–399. doi:[10.1002/jez.1402380312](https://doi.org/10.1002/jez.1402380312)
- Ishitobi H, Mori K, Yoshida K, Watanabe C (2007) Effects of perinatal exposure to low-dose cadmium on thyroid hormone-related and sex hormone receptor gene expressions in brain of offspring. *Neurotoxicology* 28:790–797. doi:[10.1016/j.neuro.2007.02.007](https://doi.org/10.1016/j.neuro.2007.02.007)
- Jackson WA, Anderson TA, Canas JE, Snyder SA, Tan K (2006) Environmental fate of perchlorate. In: Kendall RJ, Smith PN (eds) *Perchlorate ecotoxicology*. SETAC Press, Pensacola, pp 21–45
- Jump DB, Narayan P, Towle H, Oppenheimer JH (1984) Rapid effects of triiodothyronine on hepatic gene expression. Hybridization analysis of tissue-specific triiodothyronine regulation of mRNAs14. *J Biol Chem* 259:2789–2797
- Jump DB, Thelen AP, Mater MK (2001) Functional interaction between sterol regulatory element-binding protein-1c, nuclear factor Y, and 3,5,3'-triiodothyronine nuclear receptors. *J Biol Chem* 276:34,419–34,427. doi:[10.1074/jbc.M105471200](https://doi.org/10.1074/jbc.M105471200)
- Kim SW, Harney JW, Larsen PR (1998) Studies of the hormonal regulation of type 2 5'-iodothyronine deiodinase messenger ribonucleic acid in pituitary tumor cells using semiquantitative reverse transcription-polymerase chain reaction. *Endocrinology* 139:4895–4905. doi:[10.1210/en.139.12.4895](https://doi.org/10.1210/en.139.12.4895)
- LaFave LT, Augustin LB, Mariash CN (2006) S14: insights from knockout mice. *Endocrinology* 147:4044–4047. doi:[10.1210/en.2006-0473](https://doi.org/10.1210/en.2006-0473)
- Lein PJ, Yang D, Bachstetter AD, Tilson HA, Harry GJ, Mervis RF, Kodavanti PRS (2007) Ontogenetic alterations in molecular and structural correlates of dendritic growth after developmental exposure to polychlorinated biphenyls. *Environ Health Perspect* 115:556–558
- Mayer KP, Jackson WA, Snyder SA, Smith PN, Anderson TA (2006) State of the science: background, history, and occurrence. In: Kendall RJ, Smith PN (eds) *Perchlorate ecotoxicology*. SETAC Press, Pensacola, pp 1–21
- McNabb FMA (2007) The hypothalamic-pituitary-thyroid (HPT) axis in birds and its role in bird development and reproduction. *Crit Rev Toxicol* 37:163–193. doi:[10.1080/10408440601123552](https://doi.org/10.1080/10408440601123552)
- McNabb FMA, Cheng M-F (1985) Thyroid development in ring doves, *Streptopelia risoria*. *Gen Comp Endocrinol* 58:243–251. doi:[10.1016/0016-6480\(85\)90340-5](https://doi.org/10.1016/0016-6480(85)90340-5)
- McNabb FMA, Lyons LJ, Hughes TE (1986) Avian hepatic T3 generation by 5'-monodeiodination: characterization of two enzymatic pathways and the effects of goitrogens. *Comp Biochem Physiol A* 85:249–255. doi:[10.1016/0300-9629\(86\)90247-1](https://doi.org/10.1016/0300-9629(86)90247-1)
- McNabb FMA, Larsen CT, Pooler PS (2004a) Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. *Environ Toxicol Chem* 23:997–1003. doi:[10.1897/03-362](https://doi.org/10.1897/03-362)
- McNabb FMA, Jang DA, Larsen CT (2004b) Does thyroid function in developing birds adapt to sustained ammonium perchlorate exposure? *Toxicol Sci* 82:106–113. doi:[10.1093/toxsci/kfh247](https://doi.org/10.1093/toxsci/kfh247)
- McNabb FMA, Hooper MJ, Smith EE, Scott M, Gentles BA (2006) Perchlorate effects on birds. In: Kendall RJ, Smith PN (eds) *Perchlorate ecotoxicology*. SETAC Press, Pensacola, FL, pp 99–127
- Narayan P, Liaw CW, Towle HC (1984) Rapid induction of a specific nuclear mRNA precursor by thyroid hormone. *Proc Natl Acad Sci USA* 81:4687–4691. doi:[10.1073/pnas.81.15.4687](https://doi.org/10.1073/pnas.81.15.4687)
- Nguyen TT, Chapa F, DiStefano JJ III (1998) Direct measurement of the contributions of type I and type II 5'-deiodinases to whole body steady state 3,5,3'-triiodothyronine production from thyroxine in the rat. *Endocrinology* 139:4626–4633. doi:[10.1210/en.139.11.4626](https://doi.org/10.1210/en.139.11.4626)
- Piosik PA, van Groenigen M, Ponne NJ, Bolhuis PA, Baas F (1995) RC3/neurogranin structure and expression in the caprine brain in relation to congenital hypothyroidism. *Mol Brain Res* 29:119–130. doi:[10.1016/0169-328X\(94\)00237-9](https://doi.org/10.1016/0169-328X(94)00237-9)
- Piosik PA, van Groenigen M, Baas F (1996) Effect of thyroid hormone deficiency on RC3/neurogranin mRNA expression in the prenatal and adult caprine brain. *Mol Brain Res* 42:227–235. doi:[10.1016/S0169-328X\(96\)00126-X](https://doi.org/10.1016/S0169-328X(96)00126-X)
- Rudas P, Ronai Z, Bartha T (2005) Thyroid hormone metabolism in the brain of domestic animals. *Domest Anim Endocrinol* 29:88–96. doi:[10.1016/j.domaniend.2005.02.032](https://doi.org/10.1016/j.domaniend.2005.02.032)
- Sible JC, Anderson JA, Lewellyn AL, Maller JL (1997) Zygotic transcription is required to block a maternal program of apoptosis in *Xenopus* embryos. *Dev Biol* 189:335–346. doi:[10.1006/dbio.1997.8683](https://doi.org/10.1006/dbio.1997.8683)
- Silva JE, Larsen PR (1985) Potential of brown adipose tissue Type II thyroxine 5'-deiodinase as a local and systemic source of triiodothyronine in rats. *J Clin Invest* 76:2296–2305. doi:[10.1172/JCI112239](https://doi.org/10.1172/JCI112239)
- Steinsapir J, Harney J, Larsen PR (1998) Type 2 iodothyronine deiodinase in rat pituitary tumor cells is inactivated in proteasomes. *J Clin Invest* 102:1895–1899. doi:[10.1172/JCI4672](https://doi.org/10.1172/JCI4672)
- Verhoelst CHJ, Darras VM, Roelens SA, Artykbaeva GM, Van der Geyten S (2004) Type II iodothyronine deiodinase protein in chicken choroid plexus: additional perspectives on T3 supply in the avian brain. *J Endocrinol* 183:235–241. doi:[10.1677/joe.1.05743](https://doi.org/10.1677/joe.1.05743)
- Wang X, Carre W, Zhou H, Lamont SJ, Cogburn LA (2004) Duplicated Spot 14 genes in the chicken: characterization and identification of polymorphisms associated with abdominal fat traits. *Gene* 332:79–88. doi:[10.1016/j.gene.2004.02.021](https://doi.org/10.1016/j.gene.2004.02.021)
- Wilcoxon JS, Nadolski GJ, Samarut J, Chassande O, Redei EE (2007) Behavioral inhibition and impaired spatial learning and memory in hypothyroid mice lacking TH receptor [alpha]. *Behav Brain Res* 177:109–116. doi:[10.1016/j.bbr.2006.10.030](https://doi.org/10.1016/j.bbr.2006.10.030)
- Wilson CM, McNabb FMA (1997) Maternal thyroid hormones in Japanese quail egg and their influence on embryonic development. *Gen Comp Endocrinol* 107:153–165. doi:[10.1006/gcen.1997.6906](https://doi.org/10.1006/gcen.1997.6906)

- Zeold A, Pormuller L, Dentice M, Harney JW, Curcio-Morelli C, Tente SM, Bianco AC, Gereben B (2006) Metabolic instability of type 2 deiodinase is transferable to stable proteins independently of subcellular localization. *J Biol Chem* 281:31,538–31,543. doi:[10.1074/jbc.M604728200](https://doi.org/10.1074/jbc.M604728200)
- Zoeller RT, Dowling ALS, Vas AA (2000) Developmental exposure to polychlorinated biphenyls exerts thyroid hormone-like effects on the expression of RC3/Neurogranin and myelin basic protein messenger ribonucleic acids in the developing rat brain. *Endocrinology* 141:181–189. doi:[10.1210/en.141.1.181](https://doi.org/10.1210/en.141.1.181)