

Evidence of hypothyroidism in the genetically epilepsy-prone rat

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A number of neurochemical and behavioral similarities exist between the genetically epilepsy-prone (GEPR) rat and rats made hypothyroid at birth. These similarities include lower brain monoamine levels, audiogenic seizure susceptibility and lowered electroconvulsive shock seizure threshold. Given these similarities, thyroid hormone status was examined in GEPR rats. Serum samples were collected from GEPR-9 and non-epileptic control rats at 5, 9, 13, 16, 22, 31, 45, 60, 90, 150 and 350 days of age. Serum thyroxine (T_4) levels were significantly lower in GEPR-9 rats compared to control until day 22 of age. GEPR-9 thyrotropin (TSH) levels were significantly elevated during the period of diminished serum T_4 . GEPR-9 triiodothyronine (T_3) levels were lower than control throughout the first year of life. The data indicate that the GEPR-9 rat is hypothyroid from at least the second week of life up to 1 year of age. The critical impact of neonatal hypothyroidism on brain function coupled with the development of the audiogenic seizure susceptible trait by the GEPR-9 rat during the third week after birth suggests that neonatal hypothyroidism could be one etiological factor in the development of the seizure-prone state of GEPR-9 rats.

INTRODUCTION

Evidence is accumulating that hormones may play a role in the pathogenesis of some forms of epilepsy. For example catamenial epilepsy, or seizures occurring during menses, has been described in patients³⁰ and in animal models^{14,30}. Thyroid hormone imbalances may also be an etiological factor in epilepsy. Thyroid hormone deficiencies have been suggested to be one contributing factor in the epilepsy of some adult patients^{18,54}. Another consideration is the putative role of neonatal hypothyroidism in the development of seizure susceptibility. Experimentally induced neonatal hy-

pothyroidism in rats results in a persistent heightened sensitivity to acoustic stimulus-induced (audiogenic) seizures^{56,57} and a lower electroconvulsive shock seizure threshold²⁷.

The genetically epilepsy-prone (GEPR) rat has been developed by selective inbreeding of Sprague–Dawley rats susceptible to audiogenic seizures¹⁹. GEPR rats exhibit abnormally low thresholds for a variety of other seizure provoking stimuli^{7,23–25,41,47} including electroconvulsive shock⁷. Given the similarities in seizure susceptibility and expression between GEPR rats and rats made hypothyroid at birth, thyroid hormone status was examined in the developing GEPR rats. Serum levels of thyroxine (T_4), triiodothyronine (T_3) and thyroid-stimulating hormone (TSH, thyrotropin) were examined in GEPR-9 rats and age matched non-epileptic Sprague–Dawley controls. In addi-

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tion, serum thyroid binding protein capacity was measured to obtain indices of free (unbound) serum T_4 and T_3 .

MATERIALS AND METHODS

Breeding colony

Breeding pairs of genetically epilepsy-prone rats (GEPR-9 rats) and non-epileptic Sprague-Dawley control rats were received from breeding colonies housed at the University of Illinois College of Medicine at Peoria. GEPR-9 rats characteristically exhibit full tonic extensor seizures in response to an acoustic stimulus, whereas non-epileptic control rats display no seizure responses. Rats were housed in an isolated wing of the animal resource facility, maintained on a 07.00–17.00 h light cycle, and given Purina Breeder Blocks and water ad libitum. Litters used for the thyroid hormone determinations were obtained by brother-sister inbreeding, following the breeding protocol described by Reigel et al.⁴². Pups used prior to weaning age were taken from their mothers immediately before sacrifice. Otherwise, pups were weaned at 28 days of age and maintained in gang cages of 4 until sacrifice. In each of the 3 experiments conducted, the serum hormone data collected at each time point represent 1–4 animals each from 2 to 4 litters of each of the respective breeding colonies.

Serum collection

GEPR-9 and control rats were sacrificed by decapitation between 11.00 and 14.00 h on days 5, 9, 13, 16, 22, 31, 45, 60, 90, 150 or 350 \pm 10 (hereafter referred to as 350) days of age. Trunk blood was collected and immediately centrifuged at $13,000 \times g$ for 12 min. Serum was stored at -70°C until the hormone assays were performed. Serum samples were assayed in duplicate.

Total serum T_4 and T_3 assays

Serum total thyroxine (T_4) and triiodothyronine (T_3) assays were performed using radioimmunoassay (RIA) kits (Kit nos. KC4D1 and TKC31 respectively, Diagnostic Products Corporation, Los Angeles, CA). Since T_4 and T_3 are not species specific, canine T_4 and T_3 RIA kits were used because

the kit standard curve ranges were optimal for determining serum T_4 and T_3 levels in neonatal rats. In the T_4 assay, $10\ \mu\text{l}$ of each serum sample were incubated at room temperature for 2 h with $200\ \mu\text{l}$ of canine buffer, $50\ \mu\text{l}$ ^{125}I - T_4 and $50\ \mu\text{l}$ of canine T_4 antiserum. Following the incubation period, $2\ \text{ml}$ of ice-cold polyethylene glycol-saline were added to each tube and the tubes centrifuged at $3000 \times g$ for 15 min. In the T_3 assay, $100\ \mu\text{l}$ of each sample were added to tubes coated with T_3 antibody. One ml of ^{125}I - T_3 was added to each tube and the tubes were incubated at 37°C for 2 h and then decanted. In both assays, the RIA tubes were counted for 1 min using an LKB gamma counter. Standard curves were established by logit-log plots and the amount of thyroid hormone in serum samples determined by regression analysis. The standard curve range for T_4 ranged from 0.15 to $9\ \mu\text{g/dl}$. The T_3 standard curve ranged from 20 to $600\ \text{ng/dl}$.

Serum TSH assay

Serum TSH levels were assayed using materials supplied by the National Hormone and Pituitary Program (NIH-NIADDK). Rat TSH was iodinated using the chloramine-T method. Ten micrograms of rat TSH (NIADDK-rTSH-1–8) were solubilized in $20\ \mu\text{l}$ of 0.05 M phosphate-buffered saline (PBS) and added to a mixture of $25\ \mu\text{l}$ of 0.5 M phosphate buffer and $1.0\ \mu\text{Ci}$ of ^{125}I -NaI (Amersham-Searle; spec. act. = $100\ \text{mCi/mg}$). Fifty microliters of 0.06% chloramine-T in 0.05 M phosphate buffer was added and the reaction vessel gently agitated for 60 sec. The reaction was stopped by the addition of $50\ \mu\text{l}$ of 0.09% sodium metabisulfite in 0.05 M phosphate buffer. The reaction mixture was applied to a $10 \times 1\ \text{cm}$ diameter Sephadex G-50 column, and 0.5 ml fractions were collected by elution with 0.05 M phosphate-buffered saline. The radioactivity of each fraction tube was monitored with a Geiger counter. Iodinated rat TSH was eluted in the seventh through eleventh fractions. Each fraction was tested for the presence of iodinated protein by perchloric acid (PCA) precipitation. Ten microliters of each fraction were mixed with 1.0 ml of 23% PCA, centrifuged for 10 min, decanted and the pellet counted and compared to the total radioactivity in $10\ \mu\text{l}$ to determine amount of iodine in precipitated

protein. Radioactivity of PCA precipitated pellets averaged 88% of the total radioactivity.

Serum TSH levels were determined using a double antibody procedure with a standard curve of TSH reference preparation (NIADDK-rTSH-RP-2) ranging from 0.01 to 50 ng rat TSH/100 μ l. One hundred and fifty microliters of standard or serum sample were added to 100 μ l of 1% bovine serum albumin (BSA) in 0.05 M PBS. A 100 μ l volume of 125 I-labeled rat TSH containing approximately 20,000 counts/min was added to each tube, followed by 200 μ l of primary antibody (rabbit anti-rat TSH antiserum: NIADDK-anti-rTSH-S-5; 1/5000 dilution). After a 24 h incubation at room temperature, 200 μ l of second antibody (goat anti-rabbit gamma-globulin: 1/5 dilution) were added to each tube. Following another 24 h incubation at room temperature, the assay tubes were centrifuged at $1000 \times g$ for 30 min. The supernatant was decanted and the precipitate counted for 1 min using a gamma counter. Under these assay conditions, 30% of the 125 I-TSH bound to antibody in the absence of unlabeled TSH. About 4% of the iodine bound non-specifically. A logit-log plot of percent of 125 I-TSH bound versus concentration of unlabeled TSH was used to construct the TSH standard curve. Serum TSH values were determined by regression analysis.

T₃ uptake assay

Serum thyroid hormone binding protein capacity and free T₄ and T₃ indices were determined by the T₃ uptake method as described by Chopra and Solomon³ using a liquid phase T₃ uptake kit (Kit no. KTUD1, Diagnostics Products Corporation, Los Angeles, CA). T₃ uptake provides an index of circulating thyroid hormone carrier protein levels by measuring the residual capacity of the serum sample to bind 125 I-T₃. An index of free (unbound) T₄ and T₃ can be obtained when total T₄ and T₃ are assayed along with T₃ uptake. One hundred microliter aliquots of each serum sample were incubated with 100 μ l of 125 I-T₃ and 100 μ l of charcoal dextran slurry. Samples were incubated at room temperature for 15 min and then centrifuged at $2000 \times g$ for 10 min. The supernatant was decanted and the pellets counted for 1 min in a gamma counter. The percent T₃ uptake of each sample was determined

by dividing the sample counts/min data by the counts/min data of the kit serum calibrator and multiplying the dividend by the lot specific T₃ percent uptake calibrator. Free T₃ and T₄ indices were determined by multiplying the sample T₃ percent uptake value by the sample total T₃ and T₄ values and dividing by 100.

Statistics

Statistical comparisons of control and GEPR-9 data for each hormone assay were performed using a Student's 2-tailed *t* test⁶⁰.

RESULTS

Three different experiments were performed in this study. In the first experiment, total serum T₄, T₃ and TSH from control and GEPR-9 rats were analyzed at ages ranging from 5 to 45 days of age. The results of the first experiment are presented in Fig. 1. Total serum TSH, T₄ and T₃ values in non-epileptic Sprague-Dawley control rats were similar to values reported previously by other laboratories^{9,12,58}. Total serum T₄ values in GEPR-9 rats were significantly lower than control from day 5 through 16 but not different from control from day 22 to 45. Total serum T₃ values in GEPR-9 rats were decreased relative to control levels at all time points examined. Serum TSH levels were significantly elevated in GEPR-9 rats compared to control from day 5 to 22 and returned to control values by day 31.

A second experiment was conducted to obtain a measure of serum thyroid binding proteins by the T₃ uptake method, enabling calculation of free serum T₄ and T₃ indices. Serum was collected from control and GEPR-9 rats at days 16, 31 and 45. These time points were chosen because these were the times at which there was the greatest difference in either total serum T₄ or T₃ between control and GEPR-9 rats (see Fig. 1). The results of this experiment are presented in Fig. 2. In confirmation of the first experiment, total serum T₄ levels were decreased at day 16 but not days 31 or 45 of age while total serum T₃ levels were significantly decreased at all 3 time points. At 16 days of age, both the free T₄ and T₃ indices were reduced significantly confirming the impression that the

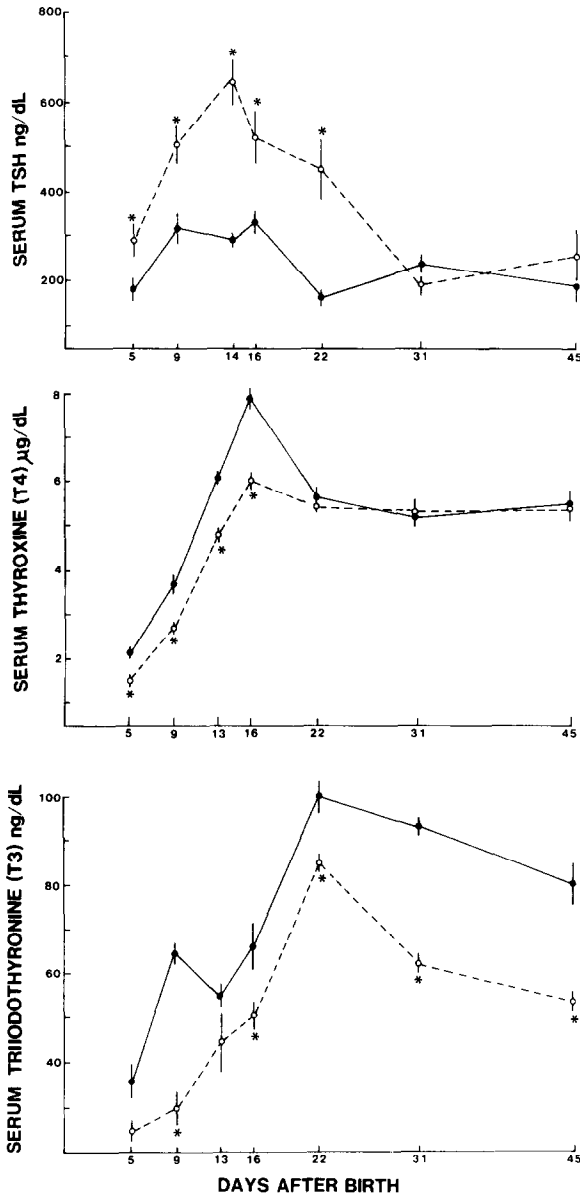


Fig. 1. Serum thyroid hormone levels in developing non-epileptic Sprague-Dawley control (filled circles) and GEPR-9 rats (open circles). Each data point represents the mean \pm the standard error of the mean of 5–9 rats. Asterisks denote data significantly different than control ($P < 0.05$; Student's 2-tailed t test).

GEPR-9 rat is hypothyroid at this age. At 31 and 45 days of age, there was a statistically significant elevation in the free T₄ index in GEPR-9 rats. However, the free T₃ index remained significantly lower than control.

The question of whether the GEPR-9 rat is hy-

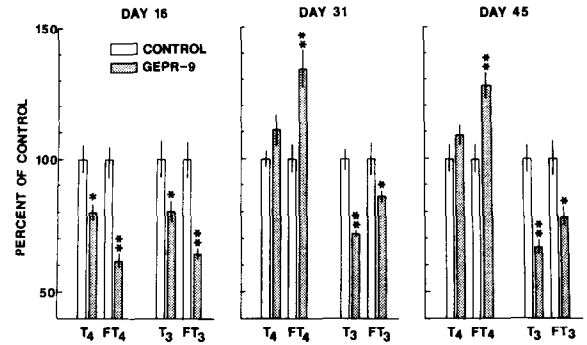


Fig. 2. Total serum thyroxine (T₄) and triiodothyronine (T₃) and free serum T₄ (FT₄) and free T₃ (FT₃) indices in developing non-epileptic Sprague-Dawley control and GEPR-9 rats. Each bar represents the mean \pm the standard error of the mean of 10–13 rats expressed as percent of control. Asterisks denote data significantly different than control: * $P < 0.05$ and ** $P < 0.005$, Student's 2-tailed t test.

pothyroid beyond the growth and maturation phase was examined in a third experiment by measuring serum T₄ and T₃ levels and T₃ uptake in older rats. Serum was collected from rats aged 60, 150 and 350 days of age. The results of this experiment are presented in Fig. 3. At 60 and 150 days of age, GEPR-9 total T₄ was normal. In addition, the free T₄ index was not different than control, whereas total and free T₃ remained at 61–65% of control values. At 350 days of age, GEPR-9 serum total T₄ and the free T₄ index were reduced significantly to 72–75% of control values while total T₃ and the

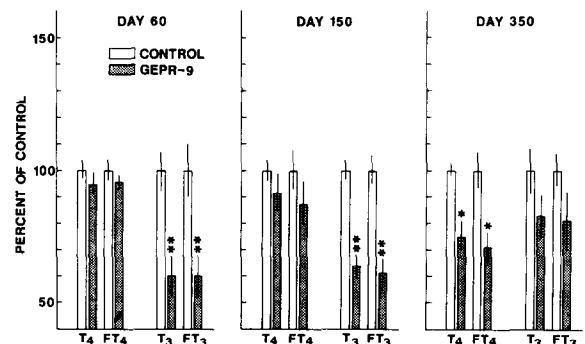


Fig. 3. Total serum thyroxine (T₄) and triiodothyronine (T₃) and free serum T₄ (FT₄) and free T₃ (FT₃) indices in mature non-epileptic Sprague-Dawley control and GEPR-9 rats. Each bar represents the mean \pm the standard error of the mean of 3–5 rats expressed as percent of control. Asterisks denote data significantly different than control: * $P < 0.05$ and ** $P < 0.005$, Student's 2-tailed t test.

free T_3 index were decreased, though not significantly, to about 80% of control.

DISCUSSION

The observation of a state of persistent hypothyroidism in the GEPR-9 rat is the principal finding of this study. The data demonstrate a decrease in serum T_4 in the GEPR-9 rat during the first 3 weeks of life. The ' T_4 surge,' which normally occurs between days 9 and 16 in rat⁹, is diminished in GEPR-9 rats (Fig. 1). This surge has been suggested to play an important role in the maturation of the central nervous system^{6,51}. The T_4 surge is thought to occur in response to a surge in TSH^{9,12}. One reason for the diminished T_4 surge in GEPR-9 rat might have been lower levels of TSH released from the pituitary gland during the first 3 weeks of life. As monoamine deficits are thought to diminish TSH levels^{5,21,22}, lower brain monoamines in GEPR-9 rats⁴ might have caused a decrease in serum T_4 and T_3 . However, the data in Fig. 1 indicate that TSH levels were elevated in the GEPR-9 rat during the time of lowered serum T_4 levels, an effect opposite to what would have been expected if serum thyroid hormone deficits were due to lowered brain monoamines. Comparing the TSH and T_4 curves in Fig. 1, it would appear that TSH levels in the GEPR-9 rat returned to normal after T_4 levels returned to normal, suggesting that the pituitary-thyroid axis was intact and that TSH was elevated in response to lower T_4 levels.

One possible explanation for this pattern of response may be that the thyroid gland in GEPR-9 rats was not fully responsive to the stimulatory effects of TSH, especially between days 9 and 16 of age. A light microscopic examination of hematoxylin and eosin stained sections of thyroid glands from 16 day control and GEPR-9 rats revealed no gross anatomical alterations in thyroid gland histology in GEPR-9 rat (data not shown). No differences in the gross size or shape of the glands, the epithelial cells, follicles or the presence of colloid in the lumen were observed. However, gross histological examination does not rule out the possibility that a more subtle deficit in TSH-stimulated synthesis, storage or release of thyroxine might exist in the neonatal GEPR-9 rat.

From 22 to 150 days of age total T_4 remained normal in the GEPR-9 rat. A transient elevation in the free T_4 index occurred at 31 and 45 days of age. The cause of this elevation is not known. The elevation may have been an 'overshoot' phenomenon in response to the T_4 deficit present in the previous 2–4 weeks of life. Interestingly, the only time points at which the deficit in the free T_3 index was not equal to or greater than the deficit in total T_3 was when the free T_4 index was elevated.

Although GEPR-9 T_4 levels were normal by day 22, serum total T_3 and the free T_3 index remained lower than control at all time points measured (Figs. 1–3). The T_3 decrease was most striking after T_4 levels had returned to control T_4 values. Thus, decreases in T_3 after 22 days of age cannot be explained by parallel deficits in T_4 . As the major source of circulating T_3 is contributed by peripheral 5'-monodeiodination of T_4 (refs. 16, 17), the persistent decrease in T_3 levels compared to control suggests either: (1) a defect in the conversion of T_4 to T_3 by 5'-monodeiodinase, (2) an increase in the conversion of T_4 to reverse T_3 , or (3) an increase in the subsequent metabolism of T_3 to di- and moniodothyronine. At present, there is no direct evidence to implicate any of these 3 possibilities. There is indirect evidence to support the possibility of a functional defect in 5'-monodeiodinase activity in the GEPR-9 rat. An increase in the amount of TSH or a decrease in T_4 increases 5'-monodeiodinase activity in the central nervous system of hypothyroid rats^{26,52,53}. TSH also stimulates 5'-monodeiodinase activity in perfused liver of euthyroid rats¹⁶. As elevations in TSH and decreases in serum T_4 occur in the GEPR-9 rat during the first 3 weeks of life, this regulatory effect may explain why serum T_3 levels are not as consistently low in the first 3 weeks of life as they are in the fourth to sixth week when TSH and T_4 have returned to normal in the GEPR-9 rat (Fig. 1).

Hypothyroidism causes many profound biological alterations¹⁰. Neonatal hypothyroidism has a significant impact on the growth and maturation of many organ systems. For example, neonatal hypothyroidism decreases the synthesis and release of growth hormone^{20,34,44} resulting in deficits in growth and weight gain¹¹. Other morphological abnormalities include hair cell damage in the or-

gan of Corti⁵⁷ and a shortened and wider skull shape^{10,15}. Thyroid hormone deficits also have a significant impact on the development of the central nervous system^{11,13}. The effects of experimentally induced neonatal hypothyroidism include impaired synaptogenesis, delayed myelination, and decreased dendritic branching^{2,31-33,45}. Alterations in neurotransmitter systems, most notably a deficit of brain monoamine levels, have been observed in animals made hypothyroid at birth^{8,36,40,50}. Neonatal hypothyroidism affects the development of the auditory and cerebellar cortices and the hippocampal formation^{32,33,37-39,46}. A variety of behavioral abnormalities including diminished performance on learning tasks^{51,55} and reproductive function¹ are associated with neonatal hypothyroidism.

In the studies of neonatal hypothyroidism cited above, the level of thyroid hormones remaining after propylthiouracil (PTU) or ¹³¹I treatment usually ranged from 10% to 40% of control values. Serum thyroid hormone levels were never less than 60% of control in the GEPR-9 rat. However, the GEPR-9 rat exhibits many of the same abnormalities present in more severely hypothyroid neonatal rats. Total body weight gain is reduced and deficits in serum growth hormone have been observed in developing GEPR-9 rats²⁹. Damage to organ of Corti hair cells has been reported in GEPR rats³⁵. GEPR-9 rats tend to have shorter and wider skulls than control rats. Monoamine levels are reduced significantly in many brain regions of GEPR-9 rats⁴. The number of similarities between GEPR-9 rats and PTU or ¹³¹I-treated neonates suggests that the GEPR-9 rat is functionally hypothyroid and that more moderate levels of hypothyroidism may be sufficient to cause many of the abnormalities attributable to neonatal hypothyroidism. Furthermore, the similarities suggest that the neonatal period of hypothyroidism in GEPR-9 rat may be the period of time responsible for most of the abnormalities that occur in the GEPR-9 rat. At present, however, the contribution of fetal or postneonatal hypothyroidism cannot be ruled out as contributing factors in the development or persistence of some of the abnormalities present in GEPR-9 rats.

Induction of neonatal hypothyroidism by PTU

treatment leads to development of audiogenic seizure susceptibility⁵⁷ and a lowered threshold for electroconvulsive shock²⁷. As the audiogenic seizure trait does not begin to appear in GEPR-9 rat until 16 days of age⁴³, neonatal hypothyroidism may be a contributing factor in the development of the seizure-prone state in the GEPR-9 rat. One manner in which to test this hypothesis would be to supplement neonatal GEPR-9 rats with thyroid hormones. If deficits in thyroid hormones are responsible for the development of the seizure-prone state, supplemental therapy should prevent the development or diminish the severity of seizure responses in the GEPR-9 rat.

If neonatal hypothyroidism is a contributing factor in the development of epilepsy, another question of interest is what brain regions are altered by neonatal hypothyroidism in such a way as to become hyperexcitable and serve as anatomical foci in the development of epilepsy in the GEPR-9 rat. Brain regions that are particularly susceptible to the effects of neonatal hypothyroidism include the cerebellum, striatum, olfactory bulbs, auditory neocortex and hippocampal formation^{37-39,46}. A number of observations would suggest that some of these areas may be hyperexcitable in the GEPR-9 rat. Monoamine levels are reduced in striatum, cerebellum, neocortex and hippocampal formation of GEPR-9 rat^{4,19}. Noradrenergic enhancement of GABA-mediated inhibition of cerebellar Purkinje cells is reduced in GEPR-9 rat⁵⁹. Glutamate- and naloxone-sensitive dihydromorphine receptor binding sites are elevated in the hippocampal formation of GEPR-9 rat^{28,48,49}. These alterations, which suggest a net increase in excitability, indicate that brain regions most affected by neonatal hypothyroidism should be one focus of continued research on the etiology of epilepsy in the GEPR-9 rat.

Hypothyroidism is the first endocrine imbalance observed in the GEPR-9 rat. The etiology of GEPR-9 hypothyroidism is not known. The recent observation of growth hormone deficits in GEPR-9 rats²⁹ coupled with the influence of thyroid and growth hormones on other endocrine systems suggest that additional endocrine imbalances are likely to be present in the GEPR-9 rat. Hypothyroidism may be one part of a complex matrix of neu-

roendocrine disturbances leading to the development of the seizure prone state of the GEPR-9 rat.

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