# Thyroid Hormone Influence on the Susceptibility of Mice to Audiogenic Seizures

Abstract. *Serum thyroxine levels peak earlier and are significantly higher in audiogenic seizure-susceptible DBA/2J mice than in seizure-resistant C57BL/6J mice during early postnatal life. The seizure susceptibility of DBA/2J mice is suppressed by administration of an antithyroid drug or by radiothyroidectomy, while the seizure susceptibility of C57BL/6J mice is enchanced by treatment with excess thyroxine.*

Audiogenic seizures (AS) are one of the most spectacular behavioral abnormalities in the mouse. When mice of the DBA strain are exposed to loud, high-frequency sound, they experience violent generalized seizures and frequently die. Susceptibility to these seizures also follows a developmental program. They are most intense around the third postnatal week, but gradually subside with age (*1*). Mice of the C57 strain, on the other hand, are generally resistant to AS at all ages. Although this strain difference for seizure susceptibility has been recognized since 1947 (*2*), the underlying mechanism (or mechanisms) responsible for these seizures has not been conclusively elucidated.

We recently found that the levels of cerebrosides and $G_{M1}$ ganglioside, which are generally enriched in myelin, were significantly higher in the brains of AS-susceptible DBA/2J (D2) mice than in the brains of AS-resistant C57BL/6J (B6) mice at 21 days of age (*3*). These results indicated that D2 mice possessed a more heavily myelinated central nervous system (CNS) than B6 mice. An elevated level of myelin could increase the excitability of the CNS by lowering the threshold for electrical conductivity (*4*). In addition, we found that the cerebellum of D2 mice was less developed than the cerebellum of B6 mice at 21 days of age (*3*). The cerebellum, because of its intense inhibitory efferent function, is thought to play an important role in the control of seizure activity (*5*). Hence, we proposed that the AS susceptibility of D2 mice could result from accelerated myelinogenesis coupled with an underdevelopment of the cerebellum. It occurred to us that these disparate features in D2 mice may both be consequences of an elevation in the level of thyroid hormone.

Thyroid hormone is known to have profound effects on several aspects of early postnatal brain development (*6*). It is well documented that thyroid hormone can significantly stimulate myelinogenesis in the mammalian CNS (*7*). Cerebellum growth is also noticeably affected by slight alterations in the level of thyroid hormone (*8*). Furthermore, Salas *et al.* (*9*) reported that the presence of an excess level of thyroid hormone during early stages of brain development could significantly advance and exaggerate responsiveness to various types of environmental stimuli. Hence, the inherent susceptibility of D2 mice to sound-induced seizures might be related to an elevated level of thyroid hormone during early periods of brain development. This report demonstrates that the levels of serum thyroxine ($T_4$) peak earlier and are significantly higher in D2 mice than in B6 mice during critical stages of brain development. Furthermore, the susceptibility of these mouse strains to AS can be reversed by artificial manipulation of their thyroid hormone levels.

Audiogenic seizure-resistant B6 and susceptible D2 mouse strains were initially purchased from Jackson Laboratory, Bar Harbor, Maine, and then propagated in our laboratory by sibling matings. The husbandry conditions were as previously described (*3*). Blood samples were taken from the mice at several ages. An animal was lightly anesthetized with ether, decapitated, and the trunk blood was then collected within 1 minute. Blood samples were collected between noon and 5 p.m. The total level of $T_4$ and the $T_4$ binding capacity (TBC) were determined from the serum of each blood sample. These determinations were conducted by the Clinical Chemistry Department, Yale University School of Medicine, with a radioimmunoassay procedure (*10*). The estimated free $T_4$ levels were determined by a procedure based on the equilibrium between the level of $T_4$ and TBC (*10*). Hypothyroidism was induced in D2 mice by either 6-*n*-propyl-2-thiouracil (PTU) (*11*) or by an $^{131}I$ radiothyroidectomy procedure (*12*). The mice received PTU (0.2 percent in the food) from 2 days before birth until they were tested for AS susceptibility at 18 ± 1 days of age. Control animals received untreated food. Mice treated with $^{131}I$ received one intraperitoneal injection (7.5 μCi in 10 μl of physiological saline) 2 days after birth and a second injection (7.0 μCi in the same volume) 5 days after birth. Control animals received injections of saline. Hyperthyroidism was induced in B6 mice by injecting intraperitoneally 20 μg of $T_4$ (in 10 μl of saline) per day on postnatal days 5 to 8. Control animals received saline injections. The PTU and $T_4$ (L-thyroxine, sodium salt) were obtained from Sigma Chemical Co., and the $^{131}I$ from New England Nuclear.

Table 1. Developmental profile of serum $T_4$ levels and TBC in B6 and D2 mice.

| Age (days) | Strain | *N** | Concentration (μg per 100 ml of serum) | |
|---|---|---|---|---|
| | | | $T_4$ | TBC |
| 7 | B6 | 1 | 2.2 | 9.6 |
| | D2 | 1 | 2.9 | 9.4 |
| 9 | B6 | 3 | 2.9 ± 0.1† | 10.2 ± 0.8 |
| | D2 | 5 | 4.8 ± 0.4 | 10.0 ± 0.8 |
| 14 | B6 | 3 | 6.8 ± 0.8† | 16.5 ± 1.6 |
| | D2 | 3 | 12.0 ± 0.3 | 17.5 ± 1.1 |
| 16 | B6 | 3 | 9.1 ± 0.9 | 18.2 ± 1.3 |
| | D2 | 4 | 10.0 ± 0.6 | 15.8 ± 0.7 |
| 21 | B6 | 3 | 6.6 ± 0.2 | 23.3 ± 1.7 |
| | D2 | 4 | 5.9 ± 0.4 | 20.7 ± 1.4 |
| 30 | B6 | 3 | 6.3 ± 0.2 | 23.3 ± 1.0 |
| | D2 | 3 | 5.2 ± 0.5 | 22.9 ± 0.8 |
| 42 | B6 | 3 | 6.2 ± 0.2 | 22.7 ± 0.6 |
| | D2 | 3 | 6.0 ± 0.1 | 24.3 ± 0.4 |
| 80 | B6 | 3 | 4.7 ± 0.2 | 25.6 ± 1.0 |
| | D2 | 3 | 5.8 ± 0.6 | 25.8 ± 2.4 |

*N, number of samples. Each sample consisted of blood from two to six animals (including both males and females) except the samples at 7 days of age, each of which consisted of nine animals. †The B6 mice were significantly different from the D2 mice at $P < .01$ (*t*-test).

Mice were tested for AS susceptibility at 18 ± 1 days of age between noon and 5 p.m. Each mouse received only one 90-second exposure to a pure tone sound (120 dB at 11 kHz). The apparatus used for sound treatment consisted of a circular glass chromatography jar (30 by 30 cm), a high-frequency speaker (model T350, Electro-Voice, Buchanan, Michigan), and a Heathkit amplifier. The onset of each phase of the AS response, as described by Collins (*13*), was recorded for each mouse. Each mouse was classified as either a nonseizer (no response or wild running only) or a seizer (clonic or tonic seizure). Mice used for the developmental analysis of serum $T_4$ levels were not tested for AS susceptibility.

Serum $T_4$ levels were significantly higher in D2 mice than in B6 mice during the earlier ages (Table 1). Maximum $T_4$ levels in D2 mice were reached at 14 days of age, when the values were almost double those found in B6 mice. Maximum $T_4$ levels in B6 mice were not reached until 16 days of age. The $T_4$ levels in both mouse strains fell sharply after 16 days of age. No striking changes in $T_4$ levels were seen in either strain after 21 days of age. The TBC rapidly increased from 7 to 14 days of age and then


0036-8075/79/0810-0598$00.50/0 

gradually increased to adult values (Table 1). The absence of a significant strain difference in TBC at all ages suggests that the $T_4$ binding globulin is functionally similar in both strains. Hence, the increased levels of $T_4$ seen in D2 mice should reflect an increase in the free or unbound $T_4$ levels. This is readily apparent in Fig. 1. The D2 mice not only have significantly higher levels of free $T_4$ during earlier periods of brain development, but also reach their maximum level about 2 days before B6 mice. Since the free or unbound $T_4$ is the fraction available for physiological action (*11*), the differences observed between the strains for the levels of free $T_4$ assume particular importance. The increased level of CNS myelin and the early underdevelopment of the cerebellum observed in D2 mice (*1, 3*) may both be consequences of the elevated $T_4$ levels in these mice. An early increase in $T_4$ levels could also disturb the normal balance between excitatory and inhibitory actions and thus exaggerate responsiveness to auditory stimulation at later ages (*9*). Hence, the AS susceptibility of D2 mice, which is maximal between 16 to 25 days of age (*1*), may be related to the early elevation of $T_4$ levels.

To test this hypothesis, we induced hypothyroidism in D2 mice with two different antithyroid agents: PTU and $^{131}I$. It is clear from Table 2 that the exposure of young D2 mice to these agents significantly reduced their susceptibility to AS at 18 ± 1 days of age (*14*). The $T_4$, TBC, and estimated free $T_4$ levels in a group of 14-day-old D2 mice, which were treated with PTU from 2 days before birth until 14 days of age, were 0.8 μg, 21.4 μg, and 0.2 ng per 100 ml of serum, respectively. Thus this treatment effectively induces hypothyroidism in these mice. The D2 controls for the $^{131}I$ experiment are not shown, but their AS susceptibility was identical to that of the untreated D2 mice. The hypothyroid D2 mice differed in physical appearance from the control D2 mice. They had delayed eye opening, rounder and shorter snouts, smaller and less pointed ears, slightly shorter tails, and were generally smaller in overall body size than the untreated D2 mice (*15*). Woodbury *et al.* (*16*) suggested that PTU may inhibit seizure activity by acting as an anticonvulsant drug. We therefore treated D2 mice with 10 μg of PTU per gram of body weight 6 hours or 3 days (one injection per day) before sound exposure. This treatment had no influence on the AS susceptibility of these mice (Table 2). Thus PTU does not appear to inhibit AS by acting as an anticonvulsant drug under these circumstances. Furthermore, there were a few mice that did have AS even though they were exposed to PTU from 2 days before birth until 18 days of age. This further argues against the possible anticonvulsant action of PTU toward AS.

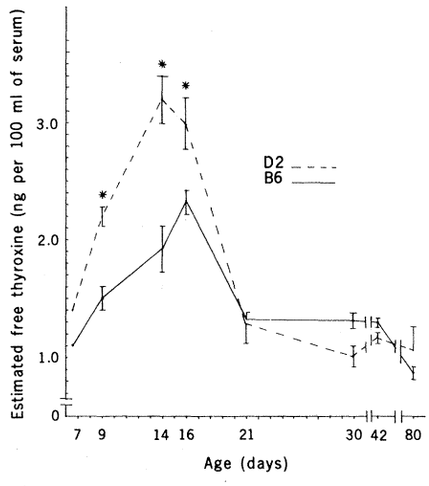


Fig. 1. Developmental profile of the estimated free thyroxine levels in serum from B6 and D2 mice. Values are expressed as means ± standard errors, and the number of samples analyzed at each age are as shown in Table 1. Asterisks indicate $P < .01$ ($t$-test).

Vicari (*17*) also reported that PTU could inhibit AS in D2 mice. However, the developmental pattern of seizure susceptibility, the classification of the seizure phenotype, the age at which the mice were treated, and the form and intensity of the sound were all different between our study and that of Vicari. We use some caution, therefore, in making a direct comparison of the influence of PTU on AS between the two studies.

Although many drugs and hormones are known to affect AS susceptibility in mice (*18*), no hormone treatment has been shown to significantly enhance AS susceptibility in 18-day-old inbred mice that are normally resistant to AS at this age. If thyroid hormone truly influences the susceptibility of mice to AS, then an elevation of $T_4$ levels in normally AS-resistant mice should enhance their AS susceptibility. Hamburgh and Vicari (*19*) reported that the treatment of AS-resistant mouse strains with excess thyroid hormone did not enhance their seizure susceptibility. We also found that the daily injection of 0.6 μg of $T_4$ into B6 mice from birth until 18 days did not influence their AS susceptibility. The injection of a single large dose (1 mg) of $T_4$ into 16-day-old B6 mice was also unable to enhance their AS susceptibility at 21 days of age even though this treatment effectively induced hyperthyroidism. Hence, hyperthyroidism at the time of testing does not necessarily enhance AS susceptibility.

In view of the developmental differences in serum $T_4$ levels between the two strains (Fig. 1), we decided to elevate the $T_4$ levels of B6 mice only at earlier ages. It is clear from Table 2 that a series of high $T_4$ doses (20 μg per mouse per day) administered to B6 mice from 5 to 8 days of age significantly enhances their AS susceptibility at a later age. Thus, timing and dosage appear to be critical factors that can affect the influence of thyroid hormone on the AS susceptibility of mice. In contrast to the untreated B6 mice, the $T_4$-treated mice had earlier eye opening, longer and more pointed snouts, more pointed ears, and were smaller in overall body size. Although each phase of the AS phenotype appeared identical in both the $T_4$-treated B6 mice and the D2 mice, the onset of wild running in the treated B6 mice did

Table 2. Influence of thyroid hormone manipulation on the AS susceptibility of B6 and D2 mice at 18 ± 1 days of age.

| Strain | Treatment | Number tested | Response (%) | | Chi-square* |
|---|---|---|---|---|---|
| | | | No seizure | Clonic-tonic seizure | |
| D2 | Controls† | 30 | 0 | 100 | |
| D2 | PTU in diet from 2 days before birth until sound treatment | 24 | 83 | 17 | 36.21 ($P < .01$) |
| D2 | Iodine-131 | 10 | 40 | 60 | 9.26 ($P < .01$) |
| D2 | PTU (10 μg/g) 6 hours before sound treatment | 6 | 0 | 100 | |
| D2 | PTU (10 μg/g-day) for 3 days before sound treatment | 7 | 0 | 100 | |
| B6 | Controls | 24 | 96 | 4 | |
| B6 | $T_4$ (20 μg/day per mouse) at 5 to 8 days of age | 44 | 70 | 30 | 4.66 ($P < .05$) |

*The chi-square values were obtained from a 2 by 2 contingency table, using Yates' correction for continuity. Compared with $\chi^2$ (1 d.f.), the treated mice were significantly different from the control mice at the $P$ values shown in parentheses. †Since no differences in AS susceptibility were observed between 15 mice given untreated food and 15 mice receiving saline injection ($^{131}I$ controls), these groups were pooled.

not occur until about 6 to 9 seconds after the commencement of sound stimulation. The D2 mice usually experience wild running within 1 second after sound stimulation. It appears, therefore, that the $T_4$-treated B6 mice still possess a factor that temporarily offers resistance to AS.

In conclusion, we have demonstrated a close relationship between $T_4$ levels and the susceptibility of mice to AS. The functional state of the thyroid gland is also believed to exert an important influence on the manifestation of AS in rats (*20*). Furthermore, the strain difference for $T_4$ levels may serve as a useful natural model for studying the influence of thyroid hormone on various aspects of brain maturation. It would be important to determine whether thyroid hormone acts alone or in conjunction with other hormones to influence AS susceptibility. Further studies are needed to elucidate the role of thyroid hormone on the manifestation of AS in mice.

THOMAS N. SEYFRIED
GILBERT H. GLASER
ROBERT K. YU

*Department of Neurology,*
*Yale University School of Medicine,*
*New Haven, Connecticut 06510*

### References and Notes

1. T. N. Seyfried, G. H. Glaser, R. K. Yu, *Genetics* **88**, S90 (1978).
2. C. S. Hall, *J. Hered.* **38**, 2 (1947).
3. T. N. Seyfried, G. H. Glaser, R. K. Yu, *Trans. Am. Soc. Neurochem.* **9**, 133 (1978); *J. Neurochem.* **31**, 21 (1978).
4. A. Vernadakis and D. M. Woodbury, in *Basic Mechanisms of the Epilepsies*, H. H. Jasper, A. A. Ward, Jr., A. Pope, Eds. (Little, Brown, Boston, 1969), pp. 535–541; P. S. Timiras, *ibid.*, pp. 727–736.
5. I. S. Cooper and R. S. Snider, in *The Cerebellum, Epilepsy and Behavior*, I. S. Cooper, M. Riklan, R. S. Snider, Eds. (Plenum, New York, 1974), pp. 245–256.
6. G. D. Grave, *Thyroid Hormones and Brain Development* (Raven, New York, 1977).
7. M. Hamburgh, *Dev. Biol.* **13**, 15 (1966); K. B. Dalal, T. Valancana, P. S. Timiras, R. R. Einstein, *Neurobiology* **1**, 211 (1971); S. J. Wysocki and W. Segal, *Eur. J. Biochem.* **28**, 183 (1972); S. Murad, G. D. Strycharz, Y. Kishimoto, *J. Biol. Chem.* **251**, 5237 (1976); T. J. Flynn, D. S. Deshmukh, R. A. Pieringer, *ibid.* **252**, 5864 (1977).
8. R. Balazs, S. Kovacs, N. A. Cocks, A. L. Johnson, J. T. Eayrs, *Brain Res.* **25**, 555 (1971); J. L. Nicholson and J. Altman, *ibid.* **44**, 13 (1972); A. Rabie, C. Favre, M. C. Clavel, J. Legrand, *ibid.* **120**, 521 (1977); J. Lauder, *ibid.* **126**, 31 (1977).
9. M. Salas, S. Diaz, L. Cintra, *Physiol. Behav.* **17**, 239 (1976).
10. H. Seligson and D. Seligson, *Clin. Chim. Acta* **38**, 199 (1972); D. Seligson and H. Seligson, *ibid.* **89**, 47 (1978). Free $T_4$ was estimated from total $T_4$ and free $T_4$ binding sites by the formula (T/TBC) × $k$, where $k$ was determined from a group of serums with measured free $T_4$ by a dialysis procedure. Estimated free $T_4$ was well correlated with directly measured free $T_4$ over a substantial range (D. Seligson and H. Seligson, in preparation).
11. A. G. Gilman and F. Murad, in *The Pharmacological Basis of Therapeutics*, L. S. Goodman and A. G. Gilman, Eds. (Macmillan, New York, 1975), pp. 1398–1422.
12. R. C. Goldberg and I. L. Chaikoff, *Endocrinology* **45**, 64 (1949).
13. R. L. Collins, in *Experimental Models of Epilepsy—A Manual for the Laboratory Worker*, D. P. Purpura, J. K. Penry, D. M. Woodbury, D. B. Tower, R. D. Walter, Eds. (Raven, New York, 1972), pp. 347–372.
14. The seizure susceptibility of D2 mice could also be suppressed by adding 0.1 percent PTU in the drinking water from 3 days before birth until testing.
15. In general, the PTU-treated mice were quite healthy in appearance, but some of the $^{131}$I-treated mice appeared somewhat runtish.
16. D. M. Woodbury, R. E. Hurley, N. G. Lewis, M. W. McArthur, W. W. Copeland, J. F. Kirschvink, L. S. Goodman, *J. Pharmacol. Exp. Ther.* **106**, 331 (1952).
17. E. M. Vicari, *Proc. Soc. Exp. Biol. Med.* **78**, 744 (1951).
18. ———, A. Tracy, A. Jongbloeb, *ibid.* **80**, 47 (1952); J. L. Fuller and R. E. Wimer, in *Biology of the Laboratory Mouse*, E. L. Green, Ed. (McGraw-Hill, New York, 1966), pp. 609–678; K. Schlessinger and B. J. Greik, in *Contributions to Behavior—Genetic Analysis*, G. Lindzey and D. D. Thiessen, Eds. (Appleton-Century-Crofts, New York, 1972), pp. 219–257; C. Kellogg, *Naunyn-Schmiederberg's Arch. Pharmacol.* **293**, 145 (1976).
19. M. Hamburgh and E. Vicari, *J. Neuropathol. Exp. Neurol.* **19**, 461 (1960).
20. L. V. Krushinsky, G. N. Molodkina, D. A. Fless, L. P. Dobrokhotova, A. F. Steshenko, A. F. Semiokhina, Z. A. Zorina, L. G. Romanova, in *Physiological Effects of Noise*, B. L. Welch and A. S. Welch, Eds. (Plenum, New York, 1970), pp. 159–183.
21. We thank R. DeLorenzo, D. Seligson, and R. Donabedian for their many helpful comments and suggestions. Supported by USPHS grant NS 11853 and grants from the Esther and Joseph Klingenstein Fund and the Kroc Foundation. T.N.S. is a recipient of USPHS postdoctoral fellowship 1F32NSO5443.

31 July 1978; revised 28 February 1979

## Combination Tones at Frequencies Greater Than the Primary Tones

Abstract. *The existence of audible combination tones at frequencies greater than the primary tones that generate them has long been problematic. With primary tones at frequencies* $f_1$ *and* $f_2$, *combination tones at* $f_1 + f_2$, $2f_2 - f_1$, *and other frequencies can be demonstrated and measured by using a contralateral probe tone to establish a binaural interaction with a given combination tone. The estimated amplitudes of these higher-frequency combination tones are generally 20 to 40 decibels below the amplitude of the primary tones.*

The auditory sensations produced by an acoustic stimulus composed of two sinusoidal primary tones are augmented with audible tones at frequencies other than those of the primaries. With the primary frequencies labeled $f_1$ and $f_2$ ($f_1 < f_2$), the frequencies at which combination tones may be generated are given by $(nf_1 \pm mf_2)$ ($n$ and $m$ are integers). Helmholtz (*1*) proposed that the eardrum and ossicles of the middle ear displaced nonlinearly, thereby distorting the signal and introducing combination tones to the signal transmitted to the inner ear. Recent experimental evidence (*2, 3*) shows that the generation of combination tones, at least those of the particular form $(n + 1) f_1 - nf_2$, involves a high degree of frequency selectivity. Thus the cochlea, wherein the initial auditory frequency analysis is performed, is now implicated as the locus of the distortion mechanism.

Those combination tones thus far noted and measured, with $2f_1 - f_2$ and $f_2 - f_1$ receiving the greatest attention, have all been lower in frequency than the primary tones that produce them. Noteworthy exceptions to this rule have been provided by Helmholtz (*1*), who claimed to hear a pitch at $f_1 + f_2$, and one of Plomp's (*4*) listeners, who was able to identify a tone at $2f_2 - f_1$. Others who have attempted to hear these higher-frequency combination tones have been unsuccessful (*2, 5*), although some evidence suggests that the tones exist, but at very low relative levels (*6, 7*).

The most frequently advanced reason for the inaudibility of combination tones above the primary frequencies is that, owing to the asymmetry of masking—low-frequency tones mask high tones more than the reverse—the higher-frequency combination tones may be masked by the primary tones (*4, 6, 8, 9*). However, determining whether higher-frequency combination tones are not present or are present but inaudible is difficult. Another possible factor contributing to the inaudibility of higher-frequency combination tones is that, if they are introduced by nonlinear motion of the basilar membrane in the cochlear region characteristic of the primary tones, the higher-frequency tones would be at a disadvantage as a result of a directional-coupling effect that favors wave transmission to places of lower characteristic frequency in the cochlea (*10*).

We have found that, with a suitable psychophysical measurement procedure, higher-frequency combination tones can be not only demonstrated to exist (*11*) but also measured. The measurement procedure exploits the fact that a binaural tone seems to be centered intracranially when it is equal both in amplitude and in phase at the ears. To measure the amplitude and phase of a given combination tone generated by low-frequency primary tones presented to the right ear, we present to the left ear a probe tone at the frequency of the combination tone. The subject's task was to adjust the amplitude and phase of this


0036-8075/79/0810-0600$00.50/0 