Long-Term Effects of Propylthiouracil-Induced Neonatal Hypothyroidism

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Hypothyroidism was induced in neonatal Sprague-Dawley rats by adding propylthiouracil to the lactating female's food and water. Behavioral evaluation on a 6-item battery occurred from 70 to 114 days of age. Results indicated long-lasting behavioral changes in the neonatal hypothyroid animals characterized by increased activity and decreased performance on avoidance and escape learning. Serum thyroxine levels were reduced in the hypothyroid animals throughout the 120-day period. Experimental animals also had fewer synaptic contacts in the cerebellar cortex when analyzed at 90 days of age.

Experimental neonatal hypothyroidism has been induced either by radiothyroidectomy, surgical thyroidectomy, or by administration of thyroid inhibitors such as tricyanoaminopropene, methylthiouracil, and propylthiouracil (Bakke, Lawrence, Bennett, & Robinson, 1975). Although marked biochemical (Balázs, Kovacs, Teichgräber, Cocks, & Eayrs, 1968), neuropathological (Brown, Akers, & Verity, 1974; Brown, Verity, & Smith, 1976; Nicholson & Altman, 1972; Rosman, Malone, Helfenstein, & Kraft, 1972) and behavioral (Bogdanove, Doody, & Nadackapadam, 1971; Davenport, 1970; Davenport & Dorcey, 1972; Eayrs & Levine, 1963) changes have been demonstrated in experimental neonatal hypothyroid animals, these studies either have not evaluated the long-term effects of the neonatal condition, or have not monitored biochemical parameters so that the subject's endocrine state could be related to the structural-behavioral changes produced (Brown *et al.*, 1976; Rosman *et al.*, 1972).

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The specific purposes of the present study were as follows: (1) to use propylthiouracil ingested orally as the inducing agent in order to produce both uniform growth reduction and thyroid damage in contrast with either surgical or radiothyroidectomy (Brown *et al.*, 1976); (2) to monitor throughout the lifespan studied the degree of hypothyroidism through radioimmunoassay for serum thyroxine (T_4) estimation; (3) to evaluate the long-term (from 70 to 114 days of age) behavioral effects of neonatal hypothyroidism by employing a battery of behavioral assessments sensitive to drug effects and which have previously been used (Schalock, Brown, Copenhaver, & Gunter, 1975) to circumvent motivational differences in animals (Denenberg & Myers, 1958a,b); and (4) to determine long-term neuropathological changes in neonatal hypothyroid rats employing the same techniques used to demonstrate retarded maturation in the cerebellar cortex in young neonatal hypothyroid rats (Brown *et al.*, 1974; Brown *et al.*, 1976).

Method

Subjects

Pregnant Sprague-Dawley rats (*Rattus norvegicus*) were obtained from a commercial distributor and allowed to deliver normally. At parturition, 8 litters (4 Experimental; 4 Control) were randomly reduced to 8 pups each (4 of each gender) and assigned to either the experimental or control condition.

Experimental Treatment

Neonatal hypothyroidism was induced by adding propylthiouracil (6 propyl-2-thiouracil; Nutritional Biochemicals Corp., Cleveland, Ohio) to the mother's food (.3% w/w) and water beginning at parturition and continuing until the pups were weaned at 30 days of age. Pulverized standard rat chow containing the propythiouracil was moistened slightly and reassembled into pellets which were fed ad libitum to the nursing mothers (and pups) until 30 days of age. The drinking water contained .001% propylthiouracil. Control litters were maintained on standard laboratory rat chow and tap water *ad libitum*.

Neuroanatomical Analyses

The subjects included 4 animals (2 Experimental; 2 Control) that were killed at 90 days of age.

Light Microscopy

The rats were decapitated and 2-4-mm sagittal slices of cerebellum were placed in Golgi-Cox fixative for 2 months; blocks were dehydrated and embedded in low-viscosity nitrocellulose. The blocks were hardened with chloroform and terpineol and cut dry at 50-um thickness and lifted into xylol. Sections were mounted with Permount. The preparations were examined and drawings were made with the aid of a

Leitz microscope with a camera lucida attachment. In an attempt to quantify differences in the receptive fields of Purkinje cell dendrites, we used a Zeiss stage micrometer calibrated in .01-mm divisions to standardize an ocular reticule. Two observers then determined the spine density on lengths of dendrite measured in microns.

Specimens of vermis from formalin-perfused brains were washed and embedded in paraffin; sections were stained with cresyl violet for views of the cortical cytology. The width of the cerebellar molecular layer was then measured by enlarging photomicrographs of the selected region and the distance from the superficial edge of the pialglial membrane to the shoulder of the Purkinje cell at the apical dendrite determined, thereby including both the external granular layer and the molecular layer. Measurements were made of the molecular layer from straight portions of the cortex of lobules IX and X, avoiding the depths of sulci and the convexities of folia. These determinations were done in age-matched experimental and normal control cerebella.

Electron Microscopy

Parasagittal slices were made of the cerebellar vermis from which thin (1 mm) tissue slabs were cut from the nodulus and other lobules. Blocks were washed for 12 hr at 4°C in a .12*M* phosphate buffer-dextrose solution, postfixed for 1 hr in osmium tetroxide (2% v/v in double strength phosphate buffer-dextrose solution) before dehydrating and embedding in Epon 812 resin. Sections were stained with lead citrate and uranyl acetate, and examined in a Siemens 1A electron microscope.

Serum Thyroxine (T_4) Levels

Blood samples were obtained from 3 Experimental and 3 Control animals by intracardiac puncture at 8, 14, 21, 30, 59, 73, 101, and 122 days of age. Separate rats were used for each age period for the 8- to 30-day determinations; the same rats (n = 6) were reused for the 59-122-day data points. The T_4 levels were determined by the radioimmunoassay technique of Chopra (1972). Animals used for blood samples were not assessed behaviorally.

Apparatus and Behavioral Testing Procedure

Behavioral evaluation was conducted on 20 animals (10 Experimental; 10 Control) and began at 70 days of age. Five females and 5 males were within each group. The sequence of testing was random. All animals were evaluated on a differential reinforcement of low rate of responding 12-sec schedule (DRL:12), an instrumental escape task, and an active avoidance task. The DRL:12 learning is insensitive to motivational differences (Kramer & Rilling, 1970) and was included to assess both simple acquisition and complex learning in an appetitive task. Escape and avoidance learning were included to circumvent possible motivational differences due to the reduced value of food reinforcement in animals of different sizes (Denenberg & Myers, 1958b).

(1) *DRL:12 Learning.* The DRL:12 learning was evaluated in a Gerbrands Operant Conditioning Chamber (standard model) using 23-hr food deprivation; reinforcements were 45-mg Noyes reward pellets. The original evaluation involved 2 stages: (a) an initial acquisition period on a continuous reinforcement schedule until a 110

responses/30 min learning criterion was reached; and (b) 6 consecutive days of DRL:12 learning in which only those responses that occurred after a 12-sec period of not responding were reinforced (25 reinforcements per session). Scores were the number of responses emitted to reach the original 110/30 learning criterion (acquisition) and the mean number of responses and responses per minute for each of the 6 DRL:12 sessions (DRL:12 Learning).

(2) *Escape Learning.* This behavior was evaluated in an electrified T-maze, 106.7-cm wide and 76.2-cm long, with 15.2-cm wide alleys. A 15.2-cm square raised goal box with a nonelectrified floor and removable gate was at either end of the T. Each animal's preference was determined by running 20 trials without shock. Thereafter, 3 successive reversals (to a criterion of 18 out of 20 correct responses) were run: first, against the original preference and, thereafter, alternating for Reversals 2 and 3. The shock source was scrambled via a Lafayette A-615C Master Shocker-Scrambler Interrupter, with shock amplitude .6 to .8 mA. The intertrial interval was 15 sec. The test data included 2 reversals of the originally correct response (total = 3 discriminations); the scores were the number of responses and the time required to make an 18/20 correct response criterion for each of the 3 discriminations.

(3) *Avoidance Learning.* Avoidance learning was evaluated in a Lehigh Valley Shuttle Cage (Model 146-04) with a Gerbrands Operant Manipulandum (Model G 6312) mounted in one end. Floor sections were wired alternately with current presented randomly via a Lafayette A-615C Master Shocker-Scrambler Interrupter. Each rat was adapted to the apparatus for one 15-min session on the day preceding regular testing. Neither shock nor noise was present during the adaptation period. Thereafter, up to 80 (20 per day) conditioning trials were conducted. Each trial consisted of a 15-sec adaptation period, a 15-sec conditioned stimulus period (CS, white noise), and a 30-sec simultaneous CS and shock (UCS) presentation. The shock amplitude was .6-.8 mA and shock duration alternating .5 sec. The avoidance response was depressing the manipulandum during the 15-sec condition stimulus period. The animal's score was the total number of trials required to reach an 18/20 correct avoidance response criterion.

(4) *Activity.* The activity apparatus was a cylindrical enclosure (31-cm radius, 44-cm high walls) whose floor sections rested on quadrant microswitches that permitted automated data recording. Activity scores were obtained during two 15-min daily test sessions and consisted of the number of floor quadrants depressed.

(5) *Exploration.* The exploratory apparatus, which has been described in detail elsewhere (Schalock & Copenhaver, 1973), was a 50-cm high rectangular plywood box containing 3 alley and 3 room sections. Following a 5-min adaptation period in the entry section, the rat was allowed 2 (24 hr apart) 15-min periods to explore the entire maze. Exploration scores were the total number of entries into the 3 room sections.

(6) *Emotionality.* The number of fecal boli was recorded for each testing session in the activity and exploratory apparatuses. The scores were the total boli for each animal.

Results

Neuroanatomical Findings

Light microscopy of the cerebellar cortex revealed some thinning of the molecular layer. Cells such as the Purkinje, Golgi II, granule, and basket neurons all appeared

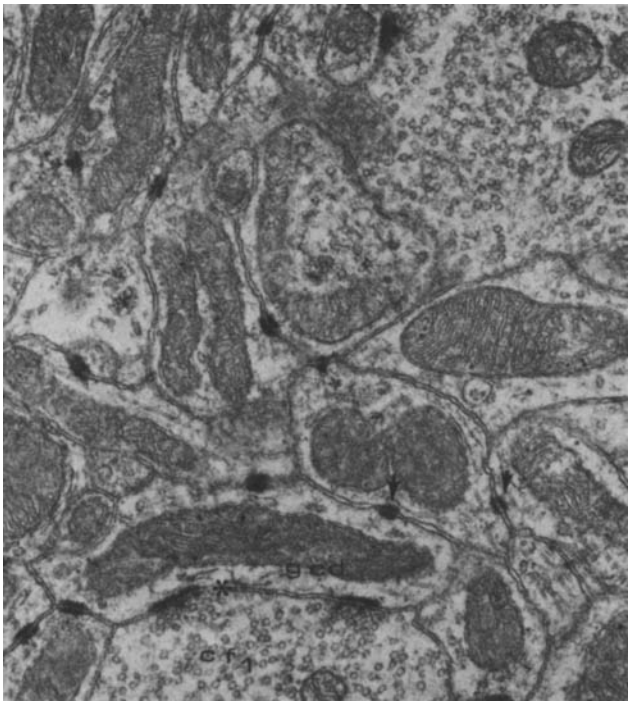

Fig. 1. Two segments of climbing fiber terminals (cf) in the normal upper cerebellar granule cell cortex of the 90-day-old rat. Present and synapsing (*) with the lower segment (cf) is a dendrite (gcd) of a granule cell. Note that some electron dense features (↓) are symmetrically disposed on each side of the intracellular membranes. These (↓) are puncta adherens and not synapses. Compare this micrograph with that of Figure 2. (x 31,000.)

normal by 90 days of age (see Figs. 1 & 2). Electron microscopy showed the general conformation of these cells to be indistinguishable from each other when comparing the normal (Fig. 3) with the previously thyroid deficient rat (Fig. 4). The synaptic bars, vesicles, mitochondria, and general outline of the basket axosomatic junctions were the same in experimental and control animals.

Climbing fiber terminals appeared to be normally constructed in the previously deficient rat, but dendritic fields of the Purkinje cells were not. The granule cell connections of both the normal rat and that which developed after early hypothyroidism appeared similar (compare Fig. 1 with Fig. 2). The synapses between the Purkinje cell dendritic spines and parallel fibers, and those between mossy fiber terminals and Golgi II cells and/or granule cell dendrites, all appeared normal. The major deficiency in the hypothyroid rats was apparent only when the actual synaptic apparatus was selectively stained with phosphotungstic acid and actual counts made of the stained bars. In this regard, hypothyroid animals had only 60% of the synapses seen in age-matched controls.

Weight and Serum Thyroxine Determinations

Hypothyroid rats were significantly lighter (p's $< .05$ in all comparisons) until 126 days of age, at which time the weights were not significantly different. Representative

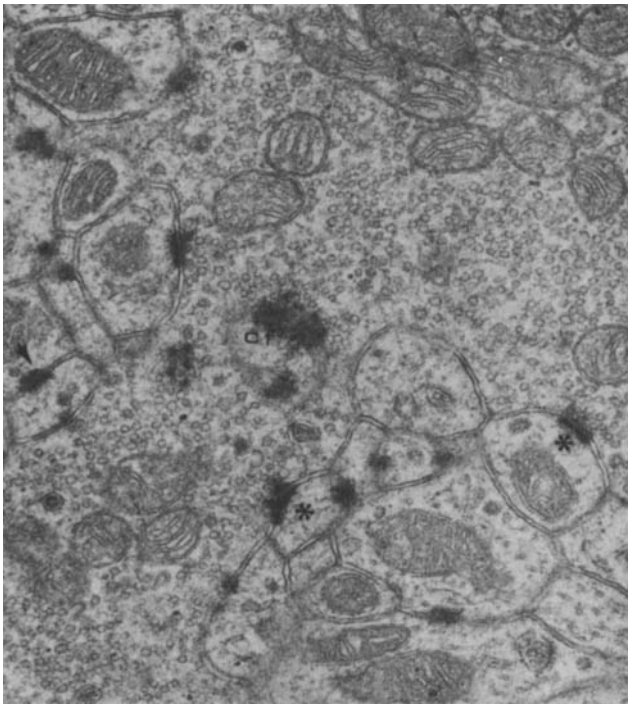

Fig. 2. A climbing fiber terminus (cf) from the cerebellar cortex of a 90-day-old rat. The animal was raised to the point of weaning under the influence of thyroid deficiency. Aside from the variation in contour which is related to sectioning, note that this is essentially the same as Figure 1. Note again the presence of granule cell synapses (*) and puncta (↓). (x 31,000.)

weight differences were as follows (Hypothyroid vs Control, respectively): Day 8: 14 ± .9 g vs 18 ± .8 g; Day 30: 46 ± 1.4 g vs 100 ± 2.5 g; Day 56: 110 ± 5 g vs 232 ± 16 g; Day 77: 193 ± 12 g vs 300 ± 24 g; Day 98: 188 ± 13 g vs 265 ± 19 g; Day 119: 249 ± 17 g vs 309 ± 20 g; Day 126: 268 ± 20 g vs 325 ± 24 g. Throughout the study, females averaged 18.7% less than males.

In general, serum thyroxine (T_4) levels (μg T_4/100-ml serum) remained depressed in the hypothyroid rats until approximately 120 days of age (Fig. 5).

Behavioral Results

Because no gender differences were obtained in initial analyses, data for females and males were combined in all subsequent analyses. Scores for any one behavioral measure were examined initially by either simple or 2-way analyses of variance (ANOVA). If significant, subsequent mean comparisons were made using Fisher's Least Significant Difference (LSD) multiple-comparison procedure (Fryer, 1966).

DRL:12 Learning. The 2 groups did not differ significantly on any of the 4 DRL:12 acquisition measures (see Table 1 and Fig. 6). Both groups demonstrated learning ability by emitting fewer responses across the 6 DRL:12 sessions ($F = 2.35$, $df = 5/108$, $p < .05$);

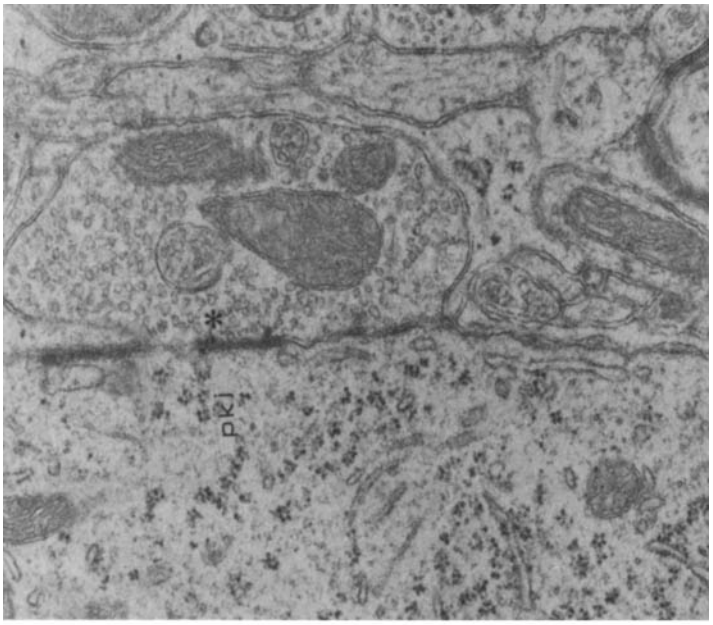

Fig. 4. A portion of a Purkinje cell (pkj) and an axosomatic synapse (*) of the 90-day-old animal which sustained until 30 days a significantly reduced (< 1 μg) T_4 level. (x 31,000.)

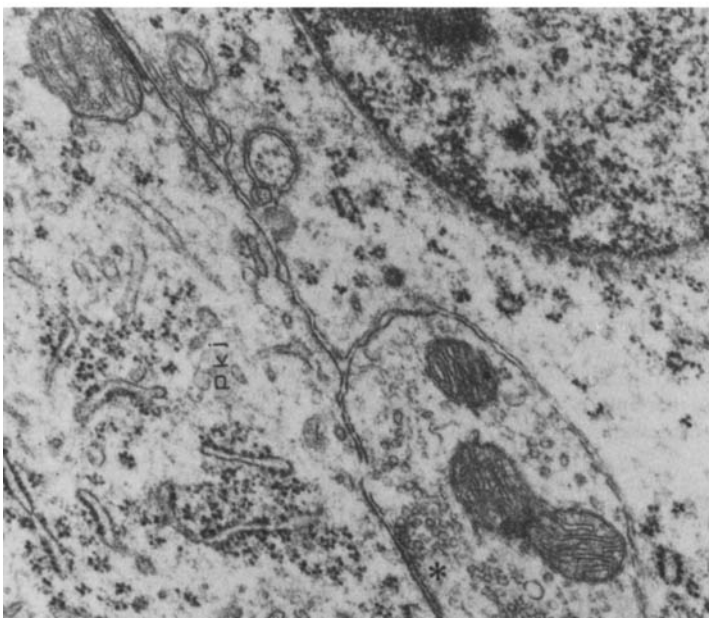

Fig. 3. An axosomatic synapse (*) of a Purkinje cell soma (pkj). The cellular segment in the lower right includes cytoplasm and *nucleus of a Bergmann Glial cell*. This preparation is from the normal 90-day-old rat cerebellum. (x 35,000.)

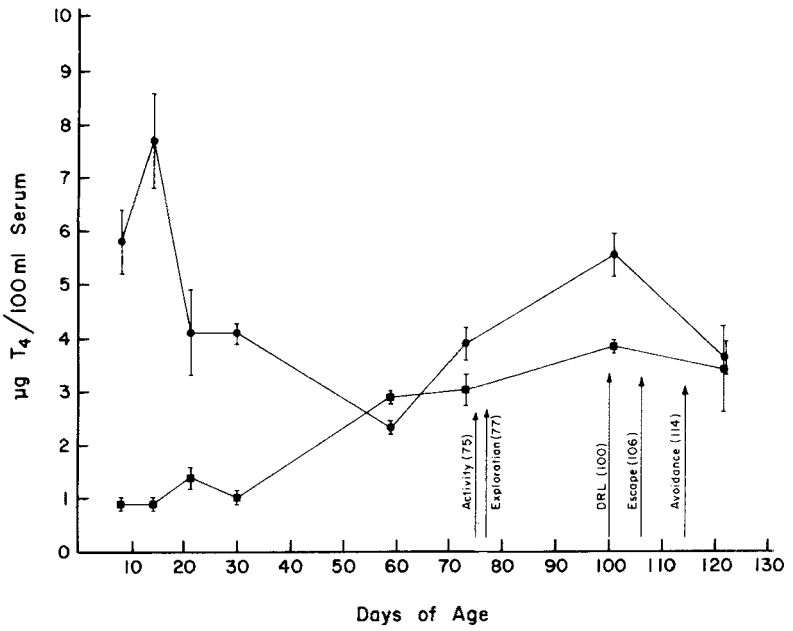

Fig. 5. Serum thyroxine (T_4) levels and behavior evaluation periods. ● = Normal, ■ = hypothyroid.

TABLE 1. Summary of Behavioral Data.

Measure	Group	
	Hypothyroid (n = 10)	Control (n = 10)
Learning		
DRL:12-Acquisition		
No. of days to 110/30	3.1 ± .6[a]	2.8 ± .4
Correct responses/day	64 ± 5.9	71 ± 5.7
Total number of responses	250 ± 33	229 ± 23
Total time (min)	85 ± 16	74 ± 12
Avoidance		
Responses to criterion	42.2 ± 3.0	31.2 ± 2.5
Activity (floor depressions)		
Day 1	239 ± 44	245 ± 32
Day 2	219 ± 61	132 ± 32
Exploration (room sections)		
Day 1	127 ± 25.2	107 ± 11.8
Day 2	138 ± 25.4	84 ± 9.3
Emotionality (total boli)		
Day 1	4.2 ± 1.1	2.2 ± .8
Day 2	3.4 ± 1.2	1.6 ± .6

[a]Standard error of mean.

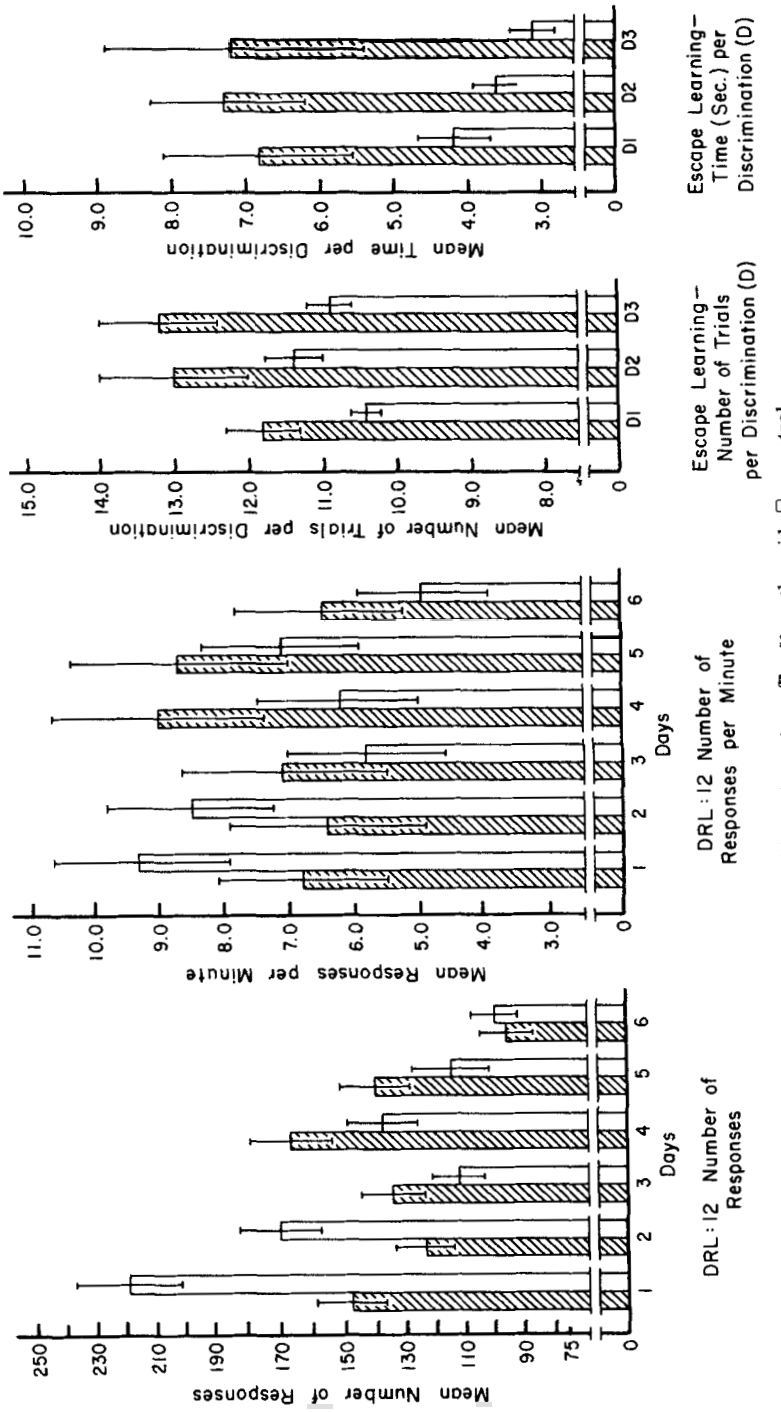

Fig. 6 Summary of behavioral data. ▨ = Hypothyroid; □ = control.

however, neither the differences between groups ($F < 1$) nor the Groups x Session interaction ($F = 1.12$, $df = 5/108$, $p > .05$) was significant. Despite the insignificant interaction, the trend was different for the 2 groups: Controls improved more than Hypothyroids over the 6 DRL:12 sessions (see Fig. 6). Both groups reduced significantly the time required during the 6 sessions to emit 50 correct responses ($F = 2.73$, $df = 5/108$, $p < .05$), with controls requiring less time per 50 reinforcements than hypothyroid animals ($F = 6.22$, $df = 1/108$, $p < .01$).

Escape and Avoidance Learning. In escape learning, the groups differed significantly on both number of trials per discrimination ($F = 13.38$, $df = 2/54$, $p < .01$) and time per discrimination ($F = 16.54$, $df = 2/54$, $p < .01$). For all 3 discriminations, control rats were both faster ($LSD_{.01} = 2.51$) and required significantly fewer trials ($LSD_{.01} = 1.36$) to reach the discrimination learning criterion. The interaction was insignificant in both analyses. Control animals also learned the avoidance response significantly faster than hypothyroid animals ($t = 2.83$, $df = 18$, $p < .02$).

Activity and Exploration. The analysis of the activity data resulted in significant differences between Days ($F = 6.12$, $df = 1/30$, $p < .05$), Groups ($F = 5.23$, $df = 1/36$, $p < .05$), and for the interaction ($F = 6.83$, $df = 1/36$, $p < .05$). Subsequent mean comparisons indicated that hypothyroid animals were significantly ($LSD_{.05} = 2.21$) more active on Day 2 than controls. Similarly, although not differing on Day 1 exploration scores, hypothyroid rats approached a significant increase in exploration on Day 2 ($LSD_{.05} = 56$).

Emotionality. Neither main effect nor the interaction was significant.

Discussion

The present data indicate that experimental neonatal hypothyroidism results in long-lasting behavioral changes characterized by increased activity and decreased performance on avoidance and escape learning. Most of the behavioral data can be explained by the hypothyroid animal's tendency to maintain a fairly consistent response rate over testing sessions. This tendency, which is generally counterproductive and associated with maladaptive behavior (Eayrs, 1964; Eayrs & Levine, 1963; Schalock *et al.*, 1975), is exemplified best in the activity, exploratory, DRL:12 (response/min), and escape (sec/discrimination) data. In reference to activity and exploration, differences appeared only on Day 2, during which hypothyroid rats maintained their high response levels, rather than decreasing them, which is normally the case (Schalock *et al.*, 1975). The same perseverative tendency was evident in the DRL:12 and escape time data.

The long-term effects on learning ability were equivocal and depended upon the type of learning. As previously mentioned, avoidance and escape learning were impaired. Although the hypothyroid rats were not observed to be differentially sensitive to the shock, avoidance learning was related positively to plasma thyroxine concentration (Dupont, 1973). In addition, hypothyroidism desensitizes receptors to the catecholamines (Waldstein, 1966) which play a significant role in both avoidance and escape learning (Latané & Schacter, 1972). Although DRL:12 (appetitive) learning was not impaired significantly in the hypothyroid animals, controls improved more than hypothyroids over the 6 testing sessions, primarily because of the perseverative tendency mentioned above.

The data do not distinguish between the effects of early hypothyroidism and early malnutrition. Neonatal undernourished rats do show irreversible effects related to body size, brain DNA, catecholamine accumulations, and behavior (Bakke *et al.*, 1975). Moreover, Shambaugh and Wilber (1974) have shown that neonatal undernutrition results in hypothyroidism during the period of the nutritional deprivation, suggesting that the persistent hypothyroidism following neonatal insult may be secondary to caloric deprivation. More recently, however, Bakke *et al.* (1975) have reported that the persistent reduction in body weight following early (0-21 day of age) malnutrition does not appear to be due to any abnormality of pituitary-thyroid function in the adult and, more importantly, that the abnormalities in the neonatal hypothyroid syndrome are not similar to those following neonatal food deprivation. In the current study, only exploration scores were significantly ($r = -.53$) related to weight. Additionally, gross examination revealed that the hypothyroid animals were not physiologically debilitated or malnourished: their development was simply delayed. Weights did not differ between hypothyroid and control animals by 126 days of age.

Thyroid inhibition was induced by the use of propylthiouracil because of numerous unsuccessful attempts with other techniques (Brown *et al.*, 1976). The control serum thyroxine (T_4) levels were consistently greater than the values given by Samel (1968) for animals of this age. The probable basis for these higher values is the greater sensitivity of the radioimmunoassay technique. The pronounced peak in T_4 levels at 14 days of age in control animals may be related to the brain growth spurt (Dobbing, 1970). The large increase from 30 to 60 days of age probably corresponds to a delayed growth spurt in the hypothyroid animals. The point in the curves at 60 days where crossing occurred may have been due to the overresponse of the previously deficient thyroid as its T_4 production was activated by the recovery phase and then gradually became less active.

Neuroanatomical changes in younger (8-42 days of age) hypothyroid rats have been reported by the investigators elsewhere (Brown *et al.*, 1974, 1976). In general, the cerebellar cortex and caudate nucleus of younger animals are characterized by nonmigrated granule cells, malformed Purkinje cell dendrites (as shown by Golgi impregnation studies), impaired synaptogenesis, and narrower molecular layers. In the current study, 90-day-old hypothyroid animals continued to show deficient synaptic development in the cerebellar cortex.

The present study permits a number of generalizations regarding experimental neonatal hypothyroidism: first, uncorrected early thyroid deficiency leads to long-lasting neuroanatomical and behavioral deficits. Although not evaluated in this study, other studies have indicated that the learning deficits associated with the condition can be lessened through either early replacement therapy (Eayrs & Levine, 1963), or environmental enrichment (Davenport, Gonzalez, Carey, Bishop, & Hagguist, 1976). Second, despite reference in the literature to "intellectual retardation" associated with hypothyroidism (Bogdanove *et al.*, 1971; Davenport & Dorcey, 1972), the long-term effects are dependent upon the specific behavior measured and the frequency with which that behavior is recorded. Finally, many behavioral domains should be evaluated in that the behavioral effects of neonatal hypothyroidism seem to be more specific than generalized.

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