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Infrapyramidal Mossy Fibers and Two-Way Avoidance Learning: Developmental Modification of Hippocampal Circuitry and Adult Behavior of Rats and Mice

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The extent of the intra- and infrapyramidal mossy fiber projection (IIP-MF) in the hippocampus of mice and rats is strain-specific, and correlates negatively with the strain-specific capacity of avoidance learning. If variations of the IIP-MF influence the capacity for 2-way avoidance learning, then developmental modification of the IIP-MF projection in an individual member of a strain should remain correlated with its adult behavior.

Pups of strains with high avoidance capacity and small IIP-MF projections (RHA rats, DBA/2 and BALB/c mice) were injected with varying doses of thyroxine during the postnatal period. This transient hyperthyroidism resulted in a strong, yet largely unpredictable, variability of the IIP-MF projection in the adult animals. Furthermore, postnatal saline injections also increased the variability of the IIP-MF projection; however, this was to a lesser degree than when using thyroxine. The animals were tested for 2-way avoidance learning at the age of 90–120 d. Many showed strain-atypical avoidance scores. These deviations from the inherited level of 2-way avoidance learning were strongly correlated with the magnitude of the IIP-MF projection, regardless of whether the structural changes resulted from thyroxine or saline injections. A multivariate analysis showed that the observed correlations could neither be explained by thyroxine-induced changes in brain weight nor by individual differences of other terminal fields in the hippocampal region CA3.

These results suggest that the extent of the IIP-MF projection is influenced by several genetic and epigenetic factors. Irrespective of the underlying causes, the magnitude of the IIP-MF (or of an unknown but well-correlated variable) appears to bias the adult capacity for 2-way avoidance learning predictably.

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The ability of mice and rats to learn and perform a 2-way avoidance task often varies from one animal to another. The expression of this *task-specific* behavior is strongly mediated by genetic factors (Bignami and Bovet, 1965; Oliverio et al., 1973; Brush et al., 1979; Buselmaier et al., 1981). Furthermore, hippocampal morphology shows genetic variability (Barber et al., 1974; Fredens, 1981). We could show that a natural and hereditary variation of intrahippocampal circuitry was closely correlated with 2-way avoidance performance: the more mossy fibers (the efferents of the dentate granule cells) were synapsing on the basal dendrites of pyramidal cells in CA3, the poorer this so-called "shuttlebox" learning (Schwegler and Lipp, 1981, 1983; Schwegler et al., 1981; Lipp and Schwegler, 1982). The correlation coefficients ranged from −0.80 to −0.90, and a multivariate analysis suggested that the extent of the intra- and infrapyramidal mossy fiber projection (IIP-MF) alone might be a strong predictor of the individual ability for shuttlebox learning. Given the critical role of the hippocampal formation in mediating 2-way avoidance (O'Keefe and Nadel, 1978; Olton et al., 1979; Gray, 1982) and the commanding position of the mossy fibers in the hippocampal circuitry (Fig. 1*A*), one might postulate that variations of this trait, or of its associated hippocampal circuitry, could underlie the variations in behavior.

To validate such a hypothesis, we have employed a double strategy. The first seeks covariations of hippocampal circuitry with other behavioral tasks known to be sensitive to hippocampal lesions. So far, we have found that the IIP-MF projection covaries positively with behavioral scores in classical hippocampal tasks (Crusio et al., 1987; Wolfer et al., 1987), as well as with a variety of other behaviors (Lipp et al., 1983, 1986a, b, c, 1987; Schwegler et al., 1988). The second strategy for obtaining evidence for our basic hypothesis is to modify the IIP-MF distribution during its development, in order to test whether an experimentally altered neuroanatomical trait would remain correlated with the individual level of behavioral performance in adulthood. This, obviously, is the *sine qua non* for the hypothesis of a functional role of hippocampal circuitry variations. One must note that the studies reported here were started, and had yielded the first positive results, before we undertook a search for further morphobehavioral correlates (Lipp et al., 1984). Since then, both strategies were pursued simultaneously.

The behavioral task chosen for investigation was 2-way avoidance. Its correlation with the IIP-MF was the first to be discovered, and the genetic aspects of this covariation have been

studied extensively (Heimrich et al., 1985; Crusio et al., 1986). Given the difficulties in interpreting 2-way avoidance (Anisman, 1978; Lipp and Schwegler, 1982; Bignami et al., 1985), we refrained from a specific behavioral analysis, and accepted the behavioral scores at a phenomenological level, planning further analysis in case of a positive outcome of the experiment. For testing, we selected strains with superior capacities for 2-way avoidance, since it is known that a transient hyperthyroidism produces an irreversible hyperplasia of the IIP-MF fibers (Lauder and Mugnaini, 1977, 1980). If our hypothesis were correct, the degree of hyperplasia would predict the impairment in 2-way avoidance of the adult animals. Since 2-way avoidance is generally improved after hippocampal lesions, potential behavioral consequences resulting from nonspecific damage of septohippocampal circuitry should be discernible from the behavioral impairment associated with a larger IIP-MF projection.

Parts of the results of these studies have been published as short or preliminary communications.

Materials and Methods

This report consists of 2 studies, one with rats, which was performed first, the other with mice, designed on the basis of the results with rats.

Experimental design and statistical procedures. The basic aim of the studies was to increase the variability of the IIP-MF projection in order to analyze its covariation with adult behavior, with no attempt being made to control the development of the IIP-MF. Thus, a morphological trait (which is normally under genetic control) is "randomized" experimentally during development. Since the strains used are either inbred or genetically selected, other genetic factors that might affect the IIP-MF or avoidance are being held constant. In genetic terms, the treatment increases the phenotypical variability of a given genotype, i.e., the within-strain variability. Given the anticipated multiple effects of thyroxine treatment (see Discussion), the randomization of the IIP-MF is likely to be accompanied by unpredictable developmental "noise." We believe that correlations that can be observed under such (adverse) conditions are likely to reflect robust and biologically meaningful relationships (the reader may note that similar principles are used in digital image analysis and enhancement).

The first step was to determine whether the IIP-MF projection of adult rats and mice would show the thyroxine-dependent hyperplasia described by Lauder and Mugnaini (1977, 1980), or at least an increased variability of that projection.

The second step was analyzing the relations between adult 2-way avoidance and developmentally varied IIP-MF projections, including developmental changes of other terminal fields, using multiple linear regression (MLR) and assuming a random-effect model (Zar, 1974; Edwards, 1979) with no explicit control over the degree of structural variation. The tabulated values of the partial regression and correlation coefficients describe the correlation between a particular structural variable and the behavior score, which is independent of the common variance of the remaining set of structural variables. The squared multiple-regression coefficient (R^2) indicates (in percentages) the amount of behavioral variability in 2-way avoidance that can be explained (statistically) by the sum of the linear regressions of all structural variables.

To facilitate comparisons of variables with different units of measurement (i.e., brain weight and volumes of terminal fields), the partial-regression coefficients in this report are given in standardized form (Edwards, 1979), with a sample mean of 0 and individual values scaled in units of 1 SD.

Histology and morphometry. Timm's stain was administered by perfusing the animals with a 1.17% solution of phosphate-buffered Na_2S (pH 7.4) for 1 min, followed sequentially by a phosphate-buffered solution of 3% glutaraldehyde for 5 min and a 5 min rinse with Na_2S (Schwegler and Lipp, 1983). The brain was postfixed in a buffered sucrose solution containing 3% glutaraldehyde and cut horizontally in sections of 40 μm thickness. The sections were mounted on glass slides and immersed for about 60 min in a solution containing a protective colloid, hydroquinone, citric acid, and silver nitrate. The resulting stain visualizes the hippocampal mossy fiber system with particular clarity, but also the lamination of the other intrinsic hippocampal connections that terminate in separate layers on the apical and basal dendrites of pyramidal cells.

For morphometry, 30 sections per animal were sampled. Sampling started immediately below the ventralmost extension of the septal pole of the fascia dentata. Five sections were analyzed per animal, either from a left or a right hippocampus. In rats, every fourth section was taken, in mice every second, starting at the beginning of the sampling level. These sampling planes correspond to the middle (midseptotemporal) portion of the long axis of the hippocampus. All sections were analyzed without knowledge of the behavior scores.

The hippocampal subregions of CA3 and CA4 (the regio inferior and the hilus of the fascia dentata) were outlined from a projected image (magnification: rats, 100×; mice, 150×). Boundaries and subdivisions are given in Figure 1*B*. The IIP-MF distribution was drawn according to the individual patches and dots (for an example, see Schwegler and Lipp, 1983). The cross-sectional areas of the hippocampal layers were determined planimetrically with the aid of a graphic tablet and a stylus (CA4, the strata lacunosum moleculare, radiatum, pyramidale, oriens, and the suprapyramidal mossy fiber layer). The area covered by the (scattered) IIP-MF projection was obtained by a stereological procedure (overlaying the drawings with a point grid and counting the number of points coincident with dark Timm reaction product; Barber et al., 1974). For the reliability of these measurements, see Schwegler and Lipp (1983). The area measures were used to calculate the total volume of a given terminal field within the midseptotemporal sampling region, and these volumes were used for regression analysis. The volumes of stratum radiatum are given after subtraction of the suprapyramidal mossy fiber layer; the volumes of stratum oriens include the IIP-MF projection, as in Schwegler and Lipp (1983). In this report, the term hippocampal terminal field means a layer or cluster made evident by Timm's stain.

Experiment 1: Postnatal circuitry modification and behavioral outcome in RHA/Verh rats

Methods

Animals and treatments. The total number of rats (RHA/Verh) used for correlative analysis was 75. Fifty-one rats were subjected to postnatal hyperthyroidism of variable degree, 24 were treated with saline.

The thyroxine-treated rats received subcutaneous or intraperitoneal injections for 17 d postnatally, starting at the day of birth. Dosage was varied by injecting 7.5 μg of L-thyroxine, which was dissolved in 0.05 ml saline made alkaline by the addition of NaOH, either daily (total dose, 135 μg/rat; $n = 17$) every second day (total, 67.5 μg/rat; $n = 22$), or every third day (total, 37.5 μg/rat; $n = 12$). The saline-treated animals received the same amount of 0.9% saline (see Lauder and Mugnaini, 1980), the numbers of animals with differential injection schedules being approximately proportional to the thyroxine groups. Members of a given litter received the same treatment (7 litters used for thyroxine injections, 2 for saline), since littermates cannot be treated differently in studies of postnatal hyperthyroidism (the thyroxine-treated animals develop much faster, the control siblings suffer from starvation). Sex ratios are given in Table 1. Pooling of the litters is not problematic, since this rat strain is genetically homogeneous with regard to 2-way avoidance and IIP-MF (Schwegler and Lipp, 1983). The treatment of the rats mostly follows the procedures and dosages employed by Lauder and Mugnaini (1980) to obtain a differential hyperplasia of the IIP-MF.

Behavioral testing. Treated rats were left undisturbed in group cages up to the age of 90 d. They were then tested for the acquisition of 2-way avoidance in a shuttlebox. A technical description of the apparatus is given by Driscoll and Baettig (1979). The conditioning stimulus was a buzzer (duration, 5 sec), the punishment a continuous electric shock (1 mA) of 10 sec duration that could be terminated by escaping to the other compartment. Between trials (30 sec), animals could move with

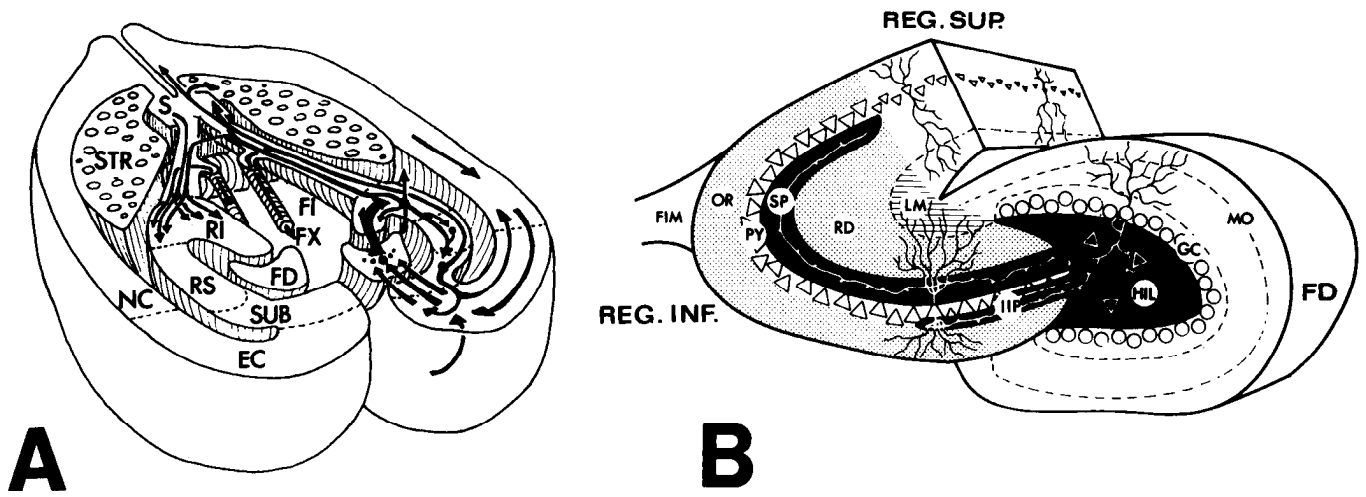

Figure 1. Hippocampal circuitry diagrams. *A,* Schematic view of a horizontal section through a rodent brain (anterior pole toward *upper left*) showing the relation of the hippocampal formation to the other parts of the brain. *Arrows* indicate the (simplified) organization of hippocampal connections. *B,* Schematized cross section through the hippocampal formation. *Hatched, shaded,* and *black* areas delineate the hippocampal subfields examined morphometrically. Abbreviations: *EC,* entorhinal cortex; *FD,* fascia dentata; *FI* and *FIM,* fimbria fornicis; *FX,* columna fornicis; *GC,* granule cell layer; *HIL,* Hilus of fascia dentata (CA4); *IIP,* intra/infrapyramidal mossy fibers; *LM,* stratum lacunosum moleculare; *MO,* molecular layers of the fascia dentata; *NC,* neocortex; *PY,* stratum pyramidale; *OR,* stratum oriens; *RD,* stratum radiatum; *RI* and *REG. INF.,* regio inferior (CA3/CA4); *RS* and *REG. SUP.,* regio superior (CA1); *S,* septum; *SP,* suprapyramidal mossy fibers (stratum lucidum); *STR,* striatum; *SUB,* subiculum.

impunity across the compartments. The shuttlebox contained no hurdles. The main behavioral variable was the number of trials required to reach a criterion of 4 consecutive avoidance responses. In addition, we measured the escape latency and noted the occurrence of freezing responses (a behavioral pattern characterized by crouching, ruffled hairs, closed or half-closed eyes, and retracted ears). Testing was terminated after 35 trials. Rats failing to reach criterion during these trials were given a behavior score of 35. This behavioral procedure was chosen because it corresponds to the routine test used to select RHA/Verh rats for breeding (Driscoll and Baettig, 1979, 1982). Hence, the results from the study could be compared with a large data base available from the ongoing genetic selection program.

Statistics. Predicted differences between means or variances of the IIP-MF projection were tested by means of a t test and a variance ratio test, respectively (Zar, 1974). Treatment-dependent differences between other terminal fields were characterized by t values *post hoc,* for no predictions were made.

The prediction for the regression analysis was that the extent of the IIP-MF would show a significant partial regression with avoidance behavior, so that the more IIP-MF, the more trials to criterion the rats would need. As in all other MLR analyses, all independent variables were entered simultaneously into the equation.

The behavioral data from the rats contradict some of the assumptions underlying a multivariate parametric analysis, pri-

Table 1. Means and SD of morphological variables in thyroxine- and saline-treated RHA/Verh rats

			Volumes of midseptotemporal terminal fields in CA3/CA4 ($mm^3 \times 10^{-2}$)							
Treatment	n	Brain weight (mg)	Str. oriens	Str. pyramidale	Str. radiatum	Str. lac. mol.	CA4–MF	Supra-pyramid. MF	IIP-MF	"Entorhinal input"[a]
Saline, 10 ♂, 14 ♀	24	1438 ±97	34.96 ±3.21	10.67 ±1.34	24.74 ±2.90	6.94 ±1.02	15.39 ±1.17	11.25 ±1.45	3.05 ±0.80	33.59 ±2.93
Thyroxine, 31 ♂, 20 ♀	51	1458 ±110	33.66 ±2.87	11.44 ±1.21	25.19 ±2.13	7.78 ±0.83	16.36 ±1.96	11.87 ±1.19	5.06 ±1.54[b]	36.02 ±3.32
Variance ratio (thyroxine vs saline), $F(24,51)$		1.29	1.25	1.23	1.85	1.51	2.81**	1.48	**2.93****	1.28
t Test (saline vs thyroxine) $df = 73$		−0.76	−1.76	2.50*	0.76	3.79**	2.24*[c]	1.97	**6.02***[c]	3.06**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; 2-tailed. Bold print: predicted changes.

[a] Str. lac. mol., suprapyramidale, and CA4; IIP-MF *not* included.

[b] Dosage effects on IIP-MF: 37.5 μg thyroxine ($n = 12$), 4.48 ± 0.33; 67.5 μg thyroxine ($n = 22$), 4.79 ± 1.77; 135.0 μg thyroxine ($n = 17$), 5.83 ± 1.49.

[c] Verified by t test for unequal variances.

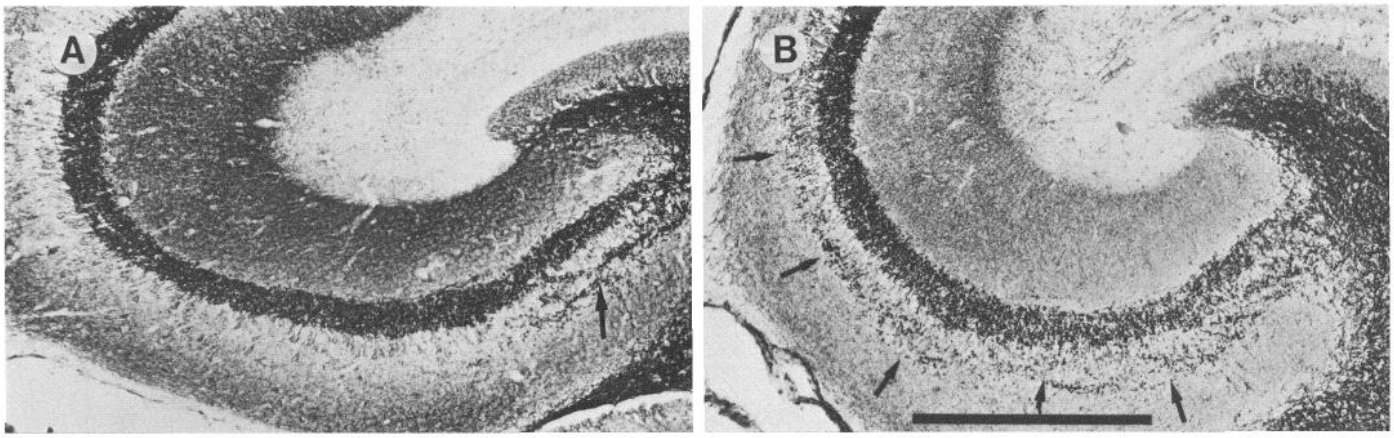

Figure 2. Thyroxine effects on the infrapyramidal mossy fiber distribution of RHA rats at the midseptotemporal level. *Dense black patches* indicate the distribution of the mossy fiber boutons, and *differentially colored bands* indicate the laminar synaptic fields of intrinsic and extrinsic projections. *A,* Horizontal section through the midseptotemporal hippocampus of an adult RHA/Verh rat that received saline. Intra- and infrapyramidal mossy fibers boutons (IIP-MF) are seen in the hilar region only (*arrow*). *B,* Same plane of an adult RHA/Verh rat that received the highest dose of thyroxine (135 μg). *Arrows* indicate the hyperplastic IIP-MF along the basal dendrites of the entire CA3 region. Bar, 0.5 mm.

marily because of the cutoff point at a score of 35. Thus, bivariate correlation coefficients (Pearson's product–moment) that were important for our conclusions were always checked by a nonparametric measure (Spearman's rho, corrected for multiple ties; Siegel, 1956) and by visual inspections of the scatterplots. We recommend, however, treating the coefficients of the data for rats as approximations only. They are presented here in this form for comparison with the mouse data.

Results

Treatment-induced changes of hippocampal circuitry

IIP-MF. The thyroxine treatment induced a massive hyperplasia of the IIP-MF, as described by Lauder and Mugnaini (1977, 1980). The most distinct increase in IIP-MF was found in the distal parts of CA3, but there were more IIP-MF terminals in the normal zone of termination as well (close to the hilus; see Fig. 2).

The mean volume of the IIP-MF in the treated rats showed a significant enlargement of about 65%, as compared to the saline controls (Table 1). The enlargement was weakly dose-dependent (correlation dosage/IIP-MF: $r = 0.37, p < 0.01$), but dosages of either 67.5 or 135 μg resulted in highly variable IIP-MFs.

It must be noted here that infrapyramidal mossy fibers in the distal parts of CA3 do not represent a pathological trait, since they can be seen regularly in the more septal (dorsoanterior) portions of the hippocampus (West et al., 1981a; West, 1983).

Thyroxine effects on other terminal fields. The thyroxine treatment resulted in an enlargement of the stratum lacunosum moleculare, of the CA4 mossy fiber field, and of the stratum pyramidale. With the exception of the slight changes in stratum pyramidale, the remaining enlarged fields (CA4, stratum lacunosum moleculare, and the IIP-MF) correspond to the zone of entorhinal input, an indirect one via the granule cells and their mossy fibers, and a direct one to stratum lacunosum moleculare (see Fig. 1). Quantitatively, the most massive effect of the thyroxine treatment was the enlargement of this combined "entorhinal input" field. Obviously, part of this difference was due to the inclusion of the IIP-MF. Yet, even after subtracting the IIP-MF, the difference between the remaining "entorhinal input" of saline- and thyroxine-treated rats would be highly significant (see Table 1).

Body and brain weight. Neither body nor brain weight showed significant differences between saline- and thyroxine-treated rats, such as are reported in the literature (Chen and Fuller, 1975; Balázs et al., 1977; Bass et al., 1977; Pascual-Leone et al., 1985). Yet, from the inspection of the individual data, it was apparent that some of the rats that had received the highest doses of thyroxine were distinctly small and that the individual variability of brain weight was rather large in the animals treated with higher doses.

Adult behavior of the treated animals

General observations. During the treatment period, the postnatal hyperthyroidism induced the classical signs, such as shivering, hyperactivity, and a general acceleration of brain and body maturation (earlier eye-opening, earlier development of fur), as reported by Eayrs (1964), Schapiro and Norman (1967), and Sokoloff (1977). The thyroxine treatment was apparently not very toxic to the RHA/Verh rats, since only 2 pups died. During adulthood, the treated rats appeared to the observer to be nervous and hyperactive, as compared to normal RHA/Verh rats (Sjöden and Söderberg, 1976; Brunjes and Alberts, 1981; McCarty et al., 1983).

Thyroxine effects on adult 2-way avoidance. During behavioral testing in the shuttlebox, the thyroxine-treated rats showed neither freezing responses nor any signs of motor impairment. They needed significantly more trials to reach criterion than did the saline-treated animals (21.2 vs 16.4), with 13 rats not reaching criterion. This difference, though significant, appears to be rather subtle, chiefly because of the highly variable scores in both groups (4 saline-treated rats did not reach criterion). The 2 groups did not differ in escape latencies (thyroxine: 6.0 ± 2.4 sec; saline: 5.5 ± 1.8 sec). All failing rats that had received a shock (5 sec after the onset of the warning signal) were quick to change the compartment. There were no sex differences in escape and avoidance behavior.

The (unexpected) variability of the avoidance scores among the saline-treated rats prompted us to analyze the results in more detail. Since a normal RHA/Verh rat reaches criterion after

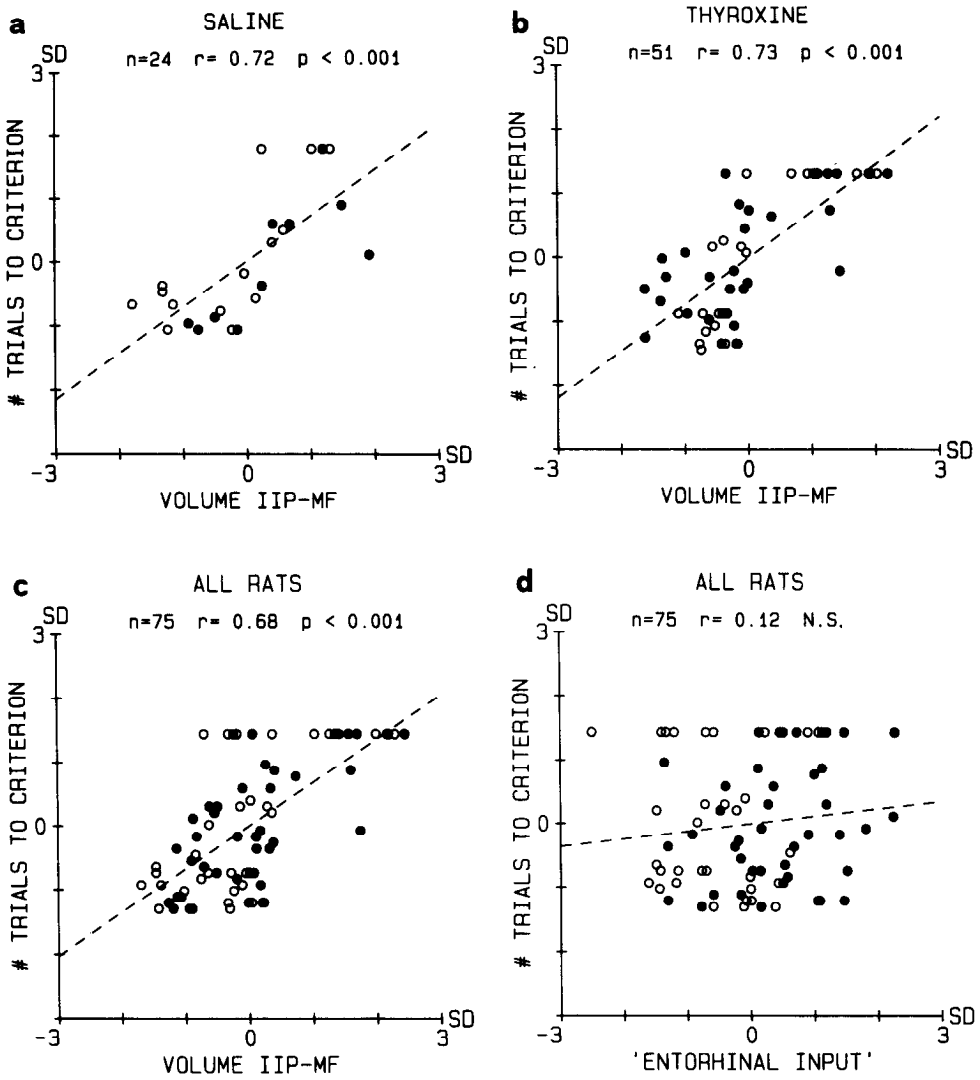

Figure 3. Standardized scatterplots of bivariate regressions in thyroxine and saline-treated RHA/Verh rats. *a,* Regression of avoidance scores (trials to criterion) on the IIP-MF varied by saline. *b,* Same regression in thyroxine-treated rats. *c,* Same regression on the IIP-MF of all rats. *d,* Regression of the avoidance scores of all rats on the remaining "entorhinal input" (suprapyramidal mossy fibers, CA4, stratum lacunosum moleculare). Note that this combined terminal field showed strong variation after the thyroxine treatment as well (quantitatively as much as the IIP-MF), but that the correlation is almost 0. This indicates that the IIP-MF variation is behaviorally relevant, while the remaining variation of other terminal fields is not. *Abscissae and ordinates,* deviation from the sample mean (SDs). *Open circles,* female rats; *filled circles,* male rats.

about only 10 trials, the scores were compared *post hoc* with routine data obtained during the ongoing genetic selection program. Indeed, the difference between saline controls and naive and unhandled RHA/Verh rats was highly significant (saline: 16.3 ± 9.3, $n = 24$; normal RHA/Verh: 10.4 ± 4.5, $n = 80$, $p < 0.0001$; 2-tailed *post hoc t* test). Data plots (Fig. 3) showed that the saline controls were not a homogenous population. Apparently, the postnatal saline injections resulted in some rats obtaining unusually poor avoidance scores. These animals included about one-third of the sample; the others were not different from normal RHA/Verh rats. The relatively slow acquisition of 2-way avoidance by these saline controls was not caused by freezing behavior nor due to slower escape latencies.

Morphobehavioral correlations

Since the inspection of the morphometrical and behavioral data indicated that the saline treatment might have had some effects of its own, separate regression analyses were performed for thyroxine- and saline-treated animals to see whether the saline "deviants" would show corresponding changes in their IIP-MF projection.

Thyroxine group. The extent of the hyperplastic IIP-MF remained well correlated with the shuttlebox score: the more IIP-MF, the more trials were needed to achieve criterion ($r = 0.73$, $p < 0.0001$; Spearman's rho = 0.71; corrected for ties, $p < 0.0001$; see Fig. 3). The ensemble of the morphometric variables (as listed in Table 2) could explain as much as 64.7% of the variation in the avoidance score. Apart from the predicted significant partial regression of avoidance on IIP-MF, there was a weak but significant partial regression of avoidance on CA4: the larger this field, the faster was avoidance learning. However, when the IIP-MF variable was eliminated from the multiple-regression equation, the multiple-regression coefficient was no longer significant, and no significant partial regressions were evident. The thyroxine-induced variability of the volume of the "entorhinal input" was not correlated with the behavior score ($r = 0.12$, n.s.; see Fig. 3), nor was this the case with brain weight ($r = 0.14$, n.s.). The correlation IIP-MF avoidance was not dependent on the postnatal dosage of thyroxine, since largely similar correlations were found among rats that had received equal doses (37.5 μg: $n = 12$, $r = 0.57$, $p = 0.053$; 67.5 μg: $n = 22$, $r = 0.75$, $p < 0.001$; 135 μg: $n = 17$, $r = 0.74$, $p < 0.01$).

Saline group. The variability of these behavior scores was strongly correlated with the IIP-MF distribution ($r = 0.72$, $p < 0.01$; Table 2, Fig. 3). The behavioral "deviants" had, indeed, the largest IIP-MF of this group. The combined morphometric

Table 2. Multiple-regression analysis of RHA/Verh rats: 2-way avoidance scores on hippocampal terminal fields in CA3/CA4

Terminal field (SD)	Dependent variable: trials to criterion (SD)		
	All rats ($n = 75$)	Saline ($n = 24$)	Thyroxine ($n = 51$)
	Standardized partial regression coefficients (b_i) ± SE[a]		
Str. oriens	−0.06 ± 0.10 (−0.07)	−0.02 ± 0.23 (−0.02)	−0.13 ± 0.10 (−0.18)
Str. pyramidale	0.38 ± 0.14 (0.31)**	0.63 ± 0.32 (0.43)	0.26 ± 0.15 (0.25)
Str. radiatum	0.01 ± 0.12 (0.01)	0.02 ± 0.23 (0.03)	0.07 ± 0.14 (0.07)
Str. lac. mol.	−0.28 ± 0.11 (−0.30)*	−0.42 ± 0.24 (−0.40)	−0.07 ± 0.13 (−0.09)
CA4–MF	−0.28 ± 0.12 (−0.28)*	−0.43 ± 0.18 (−0.52)*	−0.32 ± 0.15 (−0.31)*
Suprapyramid. MF	−0.08 ± 0.10 (−0.07)	−0.31 ± 0.21 (−0.36)	−0.02 ± 0.17 (−0.02)
IIP-MF	**0.77 ± 0.10 (0.70)*** **	**0.80 ± 0.17 (0.76)*** **	**0.83 ± 0.12 (0.74)*** **
df	67	16	43
% of Behavioral variation explained by MLR ($R^2 \times 100$)	58.9***	77.8*	64.7**
After removal of variable IIP-MF	19.0 (n.s.)	43.9 (n.s.)	23.3 (n.s.)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (2-tailed). Bold print: predicted partial regressions.

[a] In parentheses: partial correlation coefficients.

variables accounted for 77.8% of the variation in 2-way avoidance (Table 2), with the IIP-MF distribution showing a strong and significant partial regression. As in the thyroxine-treated rats, the volume of CA4 had a significant partial regression with avoidance (the more CA4, the better avoidance), which, however, disappeared after the elimination of the IIP-MF variable from the MLR equation. Apparently, the control treatment had a similar but smaller effect on the IIP-MF projection than did the thyroxine injections, the smaller morphological changes remaining closely associated with minor behavioral changes.

Discussion

These data show that the thyroxine treatment had induced considerable variation among the volumes of hippocampal terminal fields. The most prominent effect was the thyroxine-dependent increase in the combined volumes of those synaptic fields whose axons grow in during the treatment period. This greatly increasing postnatal input is made up of the 3 mossy fiber fields representing granule cell projections, and by the stratum lacunosum moleculare receiving the axons from the entorhinal cortex. This result was to be expected, since it has been shown that postnatal hyperthyroidism stimulates fiber growth in differentiating projection systems, particularly in the hippocampus and the cerebellum (Lauder, 1977; Lauder and Mugnaini, 1980). Yet, certain subfields of this entorhinal projection system did not expand equally in all individuals. For example, a large increase of the IIP-MF projection was found in one rat, and in another the stratum lacunosum moleculare appeared disproportionately enlarged. Thus, it seems that the thyroxine treatment had a general growth effect on the synaptic fields formed by the entorhinal input. However, unknown factors (perhaps the microenvironment in the target zones, or timing disturbances) made it impossible to predict which subfield would expand most. Yet the individual differences in behavior could be related *only to the treatment-induced variability of the IIP-MF*. This suggests that the variations in 2-way avoidance do not simply reflect a trophic, i.e., systemic effect of hyperthyroidism, for in that case one would expect the total entorhinal input to covary most closely with the behavior score. However, this was not the case. Therefore, the individually varying avoidance performances must be attributed to a factor that is *specifically* linked to the extent of the IIP-MF.

The results obtained with the saline-injected rats support this conclusion. The treatment produced an increase of the IIP-MF in a few animals. Those were the only rats to display a deficit in 2-way avoidance. The individual differences in the IIP-MF were possibly a consequence of handling stress (repeated injections), which may have hormonal consequences (Smith and Mills, 1983) interfering with the ongoing differentiation of hippocampal circuitry. More enigmatic is the observation that only a few animals showed strain-atypical IIP-MF. The treatment protocols gave no clue to this riddle.

There is no evidence that the variations in the IIP-MF of both thyroxine- and saline-treated rats were associated with a motor impairment or altered sensitivity to electrical footshock. Poor learners escaped from shock as quickly as did good learners. It has also been shown that postnatal hyperthyroidism in rats does not affect adult sensitivity towards electrical footshock (McCarty et al., 1983), and selective breeding for differential thresholds of neuromuscular excitability has not resulted in a concomitant differentiation of IIP-MF distribution (Dimitrieva et al., 1984). This suggests that the variations in avoidance learning, as observed here, were related to some unknown form of central processing.

The main conclusion from the experiments with rats is that the null hypothesis of no functional relation between the extent of the IIP-MF and the capacity for avoidance learning must be rejected: much of the variation in adult 2-way avoidance can be exclusively regressed by an artificially induced variation of the IIP-MF, despite considerable treatment-induced variability of other terminal fields. Furthermore, this regression can be found even after *different* treatments.

Experiment 2: Postnatal modification of hippocampal circuitry and behavioral outcome in mice

Methods

Experimental design. The aim of these studies was to replicate the findings of the rat study in another rodent species, and to test whether a systemic side effect of the thyroxine treatment might result in a spurious relation between 2-way avoidance and the IIP-MF. The main study concentrated on the strain DBA/2; a smaller sample from the strain BALB/c was used to rule out any strain-specificity of the main thyroxine effect (IIP-MF and avoidance learning), and to observe whether the thyroxine treatment might alter a genetic anomaly, the lamination of the mossy fibers, typical for this strain (Nowakowsky, 1984).

The analysis of systemic side effects of the thyroxine treatment is based on the assumption that a systemic effect of a drug ought to be *dose-dependent.* Hence, should any of the structural variables show significant dose-dependency, and if behavior could be regressed significantly by a dose-dependent structural alteration, then a common systemic effect underlying the regression cannot be rejected. Conversely, the hypothesis of a systemic effect is untenable if dose-dependent structural changes cannot be correlated with behavior.

Animals and treatments

DBA/2 mice. Seventy-five mice (locally bred from a Jackson line) were studied. They were divided into 5 treatment groups. One group received no treatment and handling, one group was given saline injections (0.9% NaCl), and 3 groups were treated with thyroxine. For 12 d, subcutaneous injections (volume, 0.05 ml of 0.9% NaCl, with or without thyroxine) were given daily to all mice. Thyroxine dosage was varied by adding a standard dose (2 μg) of sodium levothyroxine to the saline at different intervals (1, 2, or 3 d), the saline made alkaline (pH 9) by adding a few drops of NaOH. Thus, 3 groups received a total dose of 8, 12, or 24 μg of thyroxine per animal. Sex ratios are given in Table 5. One treatment group consisted of animals from 4–7 litters. Such pooling was necessary because DBA/2 pups had a rather high mortality rate and litters were usually small (3–6 animals). Pooling is not a critical factor because the strain is inbred. Prior to testing, group size was equalized to 15 mice per group. This facilitated the assessment of dosage effects in a multiple-regression analysis. The untreated animals served to measure the genuine variability of the traits under investigation (any variability found in an inbred strain reflects environmental variance and measurement errors), the saline-treated animals to replicate the rat findings of a possible saline effect, and the 3 thyroxine groups to assess dosage effects.

BALB/c mice. Three litters of 6–8 pups were treated with daily subcutaneous injections, starting at postnatal day 0 and ending at postnatal day 12. Thyroxine dosage was varied by adding either 1 or 3 μg sodium levothyroxine to the daily injection. Two pups died. Since the sample size was small, data from treated and control mice were pooled for the analysis of correlations.

Behavioral testing

At approximately 3 months of age, all treated mice were tested in an automated shuttlebox, described in detail by Buselmaier et al. (1981). The conditioning stimulus was light (10 sec duration), the punishment was an electric shock of 50 μA (10 sec duration), which could be terminated by escaping to the other compartment. Note that the punishment was rather weak, since the mice showed no jumping or squeaking, but only locomotor responses. During the intertrial phase (10 sec), the mice could move across compartments with impunity. The shuttlebox contained no hurdles. The animals were tested for 5 consecutive days. A daily training session included 80 trials.

The behavior score used for correlative analysis was the percentage of correct avoidance responses on day 5 of training. This variable had shown the best correlations with the IIP-MF distribution in earlier studies (Schwegler et al., 1981; Schwegler and Lipp, 1983), and was thus chosen as the main dependent variable. In addition, the number of escapes (leaving the compartment after the onset of the shock) was measured, as well as the number of failures to escape. Failure to escape is defined as a trial in which the animal remains in the shocked compartment for more than 10 sec, regardless of its activity, such as continuous jumping or freezing.

Statistical analysis

To assess the results of the rat study, the following predictions were tested:

1. For both mouse strains, the thyroxine treatment will result in enlarged IIP-MFs compared to those of nontreated or saline-treated animals.
2. The saline-treated DBA/2 mice will differ from the untreated mice with respect to the IIP-MF, either with regard to the mean volume or to the variance of the IIP-MF.
3. In the treated animals, their 2-way avoidance behavior at day 5 will show a significant partial regression in the volume of the IIP-MF.

Results

Treatment-induced changes of hippocampal circuitry

Thyroxine effects on the IIP-MF. A hyperplasia of the IIP-MF was seen in many thyroxine-treated mice. Yet it was more subtle than in the rats, most animals lacking IIP-MF in the far distal portions of CA3 (Fig. 4). In the BALB/c mice, the overall enlargement of the thyroxine-treated mice was about 22%, in comparison with the saline group (Table 3). In the DBA/2 mice, however, the thyroxine-dependent enlargement of the IIP-MF was approximately 75%, compared to the untreated and saline-treated mice (Table 4). Note that such treatment differences were also apparent upon visual inspection of the brains of animals that were not exposed to avoidance training (Fig. 4). As analyzed in the DBA/2 mice, the thyroxine-induced changes of the IIP-MF were, at least statistically, *not* dose-dependent (see Fig. 7). Generally, mice that had received maximal doses of the hormone had the largest projections. However, mixed thyroxine effects were the rule, with individual mice of any dosage group reacting with vastly different enlargements of the IIP-MF.

Saline effects. As predicted from the rat results, the volume of the IIP-MF showed a significantly larger variance in the saline-treated DBA/2 mice than in the untreated mice, but the means of these 2 groups were almost identical.

Thyroxine effects on other terminal fields. Among the other terminal fields, only the volume of the suprapyramidal mossy fiber layer showed a weak increase in the thyroxine-treated DBA/2 mice (+12%; Table 4). As with the IIP-MF, this enlargement was not dose-dependent.

Thyroxine treatment and genetic mossy fiber anomaly. In the BALB/c mice, the thyroxine treatment did not alter the ap-

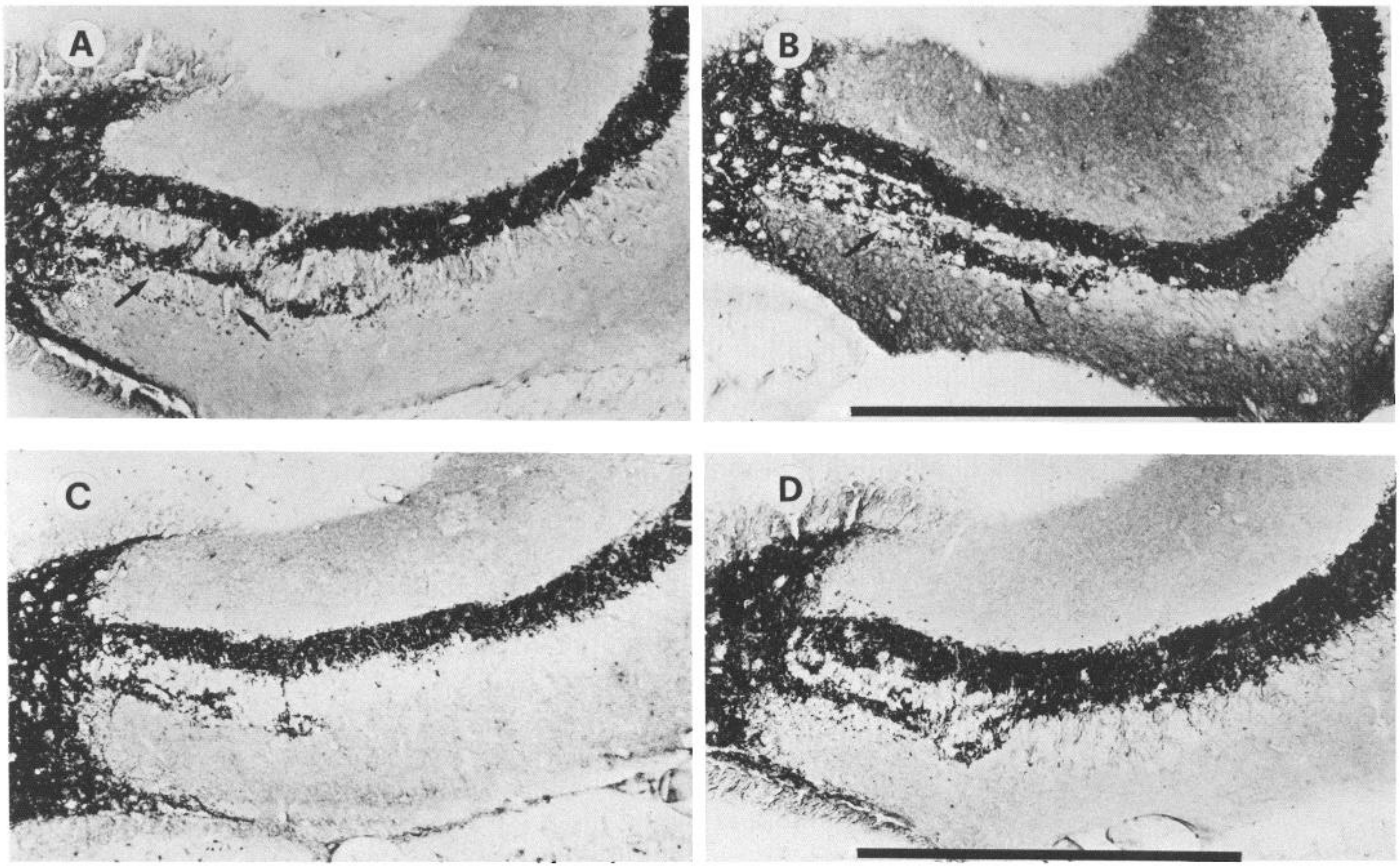

Figure 4. Thyroxine effects on the IIP-MF in mice. *A,* Infrapyramidal mossy fiber projection of an untreated adult BALB/c mouse. *B,* Same region of an adult BALB/c mouse with postnatal hyperthyroidism (total dose, 36 μg). *C,* Untreated DBA/2 mouse. *D,* Thyroxine-treated DBA/2 mouse (total dose, 24 μg). Note the unaltered intrapyramidal termination of the IIP-MF typical for BALB/c mice (*arrows* point at ectopic pyramidal cells). Note also that these photographs are of mice never tested in the shuttlebox; the visible expansion of the IIP-MF is thus not a consequence of exposure to shock. Bars, 0.5 mm.

pearance of the genetic lamination defect characteristic of this strain. In these mice, most IIP-MF actually terminate in-between the pyramidal cells (Barber et al., 1974; Nowakowsky, 1984). The projection was enlarged but retained its anomalous position.

Thyroxine effects on brain weight

The thyroxine injections reduced body weight and, similarly, the brain weight of the adult DBA/2 mice. This effect appeared to be strongly dose-dependent (Table 4, Fig. 7). Except for the usual differences in body size and (correlated) brain weight, no male/female differences were found.

Behavioral consequences of the treatments

Postnatal hyperthyroidism. As analyzed in the DBA/2 mice, the most distinct effect of the postnatal hyperthyroidism was an impairment of the avoidance performance at day 5 of training, the treated animals having an average of about 65% correct responses (Fig. 5). On day 1 of training, group differences were detectable but small. Animals from all groups rapidly improved their avoidance scores during days 1 and 2. Afterwards, untreated and saline-treated mice showed a less pronounced, yet distinct, improvement until day 5. The performance of the thyroxine-treated mice stagnated from day 3 to 5, showing only weak improvement in 2 groups (8 and 24 μg), or a performance drop at day 5 (12 μg). No dose-dependency of the avoidance scores was observed on day 5, nor any difference between males and females.

Apparently, the poorer performance of the thyroxine-treated mice was caused by a persisting tendency to commit errors despite the fact that they had learned the task. Two-thirds of these persistent errors were escapes (20% of all responses), one-third (10%) was a failure to escape. Observation of the actual behavior of DBA/2 mice in the late stages of conditioning showed that errors were chiefly caused by the unresponsiveness of the mouse to the conditioning stimulus. The most common situation was that the animals were grooming themselves and continued to do so until delivery of shock. They then escaped to the other compartment.

Saline injections. A comparison between untreated and saline-treated DBA/2 mice revealed a behavioral consequence of the saline injections. As in rats, some, but not all, mice responded to the treatment with an impairment of avoidance performance. This did not yield significant group differences, but resulted in *increased variability* in 2-way avoidance performance.

Another difference was found upon analysis of the error scores. After postnatal saline injections, adult DBA/2 mice showed significantly fewer escapes and more failures to escape (i.e., jumping or freezing) during the first day of conditioning compared to the untreated mice. On later days, this difference disappeared.

Table 3. Means and SD of morphological variables of thyroxine- and saline-treated BALB/c mice

		Volumes of midseptotemporal terminal fields ($mm^3 \times 10^{-2}$)						
Treatment	*n*	Str. oriens	Str. pyram-idale	Str. radiatum	Str. lac. mol.	CA4–MF	Supra-pyramid. MF	IIP-MF
Saline	6	8.55 ± 0.74	3.78 ± 0.31	6.38 ± 0.33	1.86 ± 0.29	2.61 ± 0.18	3.02 ± 0.31	0.69 ± 0.10
Thyroxine	12	8.74 ± 0.78	3.82 ± 0.34	6.32 ± 0.49	1.83 ± 0.16	2.54 ± 0.29	2.99 ± 0.32	0.84 ± 0.14
Variance ratio (thyroxine vs saline)								
$F(6,12)$		1.11	1.20	2.20	3.29	2.60	1.07	2.22
t-Test (saline vs thyroxine)								
$df = 16$		0.49	0.28	−0.27	−0.34	−0.56	−0.24	**2.33***

* $p < 0.05$; 2-tailed. Bold print: predicted changes.

Morphobehavioral correlations

Correlation of IIP-MF with avoidance. As in the rats, the thyroxine-induced variability of the IIP-MF remained significantly and strongly correlated with 2-way avoidance. In the DBA/2 mice, the correlation was −0.80, both bivariate and partial correlations being equal (Fig. 6, Table 6). In the BALB/c mice, the partial correlation coefficient was −0.88 (Table 5), the bivariate correlation −0.77. The correlation between IIP-MF and avoidance was greatest at day 5. Significant correlations between IIP-MF and avoidance were observed for the other days as well. Yet, as in several previous studies with nonexperimental mice, the correlations became stronger with each day of training.

Correlations of other terminal fields with avoidance. In the sample of BALB/c mice, a significant partial correlation was found between avoidance and the volume of stratum oriens: the larger the field, the poorer avoidance ($r = -0.63$, $p < 0.05$; Table 5). This relation was not seen in the DBA/2 mice, but weak positive partial correlations were seen for the volumes of stratum pyramidale ($r = 0.30$) and CA4 ($r = 0.37$; Table 6). However, all these partial correlations *disappeared* when the volume of the IIP-MF was eliminated from the regression equation, the corresponding multiple correlation coefficients no longer being significant either (bottom of Tables 5 and 6). Thus, these correlations are secondary to the IIP-MF/avoidance relation, i.e., they correlate only with residuals of this very strong correlation. When the IIP-MF variable remained in the regression equation, about 88% of the behavioral variability of the BALB/c mice could be linearly regressed by the combination of hippocampal variables, and about 75% in the DBA/2 mice.

Saline-treatment and untreated mice. The regression analysis in untreated and saline-treated DBA/2 mice showed no significant correlation in the untreated animals (Table 6, Fig. 6). On the other hand, the variability of the IIP-MF produced by saline

Table 4. Means and SD of morphological variables of treatment groups of DBA/2 mice

			Volumes of midseptotemporal terminal fields in CA3/CA4 ($mm^3 \times 10^{-2}$)						
Treatment	*n*	Brain weight (mg)	Str. oriens	Str. pyramidale	Str. radiatum	Str. lac. mol.	CA4–MF	Supra-pyramid. MF	IIP-MF
a. Treatments									
Untreated									
(6 ♂, 9 ♀)	15	376 ± 16	8.80 ± 0.79	3.10 ± 0.26	6.39 ± 0.71	1.22 ± 0.21	2.04 ± 0.25	2.72 ± 0.38	0.30 ± 0.05
Saline									
(9 ♂, 6 ♀)	15	390 ± 16	8.13 ± 0.91	3.26 ± 0.26	6.19 ± 0.63	1.24 ± 0.18	2.00 ± 0.21	2.60 ± 0.35	0.29 ± 0.09*[b]
Thyroxine[a]									
(14 ♂, 31 ♀)	45	353 ± 22	8.01 ± 1.32	3.19 ± 0.34	6.10 ± 0.79	1.26 ± 0.17	2.00 ± 0.21	2.96 ± 0.37	0.51 ± 0.12***[b]
$F(2,72)$		21.59***	2.55	0.96	0.82	0.28	0.19	6.12**	**50.47***[c]
b. Dosage levels of thyroxine									
8 μg									
(7 ♂, 8 ♀)	15	363 ± 17	8.60 ± 1.04	3.24 ± 0.31	6.30 ± 0.81	1.25 ± 0.19	2.10 ± 0.18	2.98 ± 0.38	0.49 ± 0.13
12 μg									
(1 ♂, 14 ♀)	15	363 ± 11	7.87 ± 1.42	3.20 ± 0.37	6.04 ± 0.80	1.27 ± 0.20	1.95 ± 0.23	2.91 ± 0.28	0.49 ± 0.12
24 μg									
(6 ♂, 9 ♀)	15	332 ± 21	7.55 ± 1.34	3.12 ± 0.35	5.95 ± 0.78	1.26 ± 0.13	1.94 ± 0.20	3.01 ± 0.45	0.54 ± 0.11
$F(2,42)$		15.81***	2.49	0.44	0.73	0.05	2.69	0.26	0.81

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; 2-tailed. Bold print: predicted changes.

[a] Pooled values of all thyroxine-treated mice.

[b] Predicted larger variance of mice treated with saline or thyroxine (variance ratio test).

[c] Predicted enlargement of the IIP-MF projection; corrected for unequal variances ($F(2,35)$).

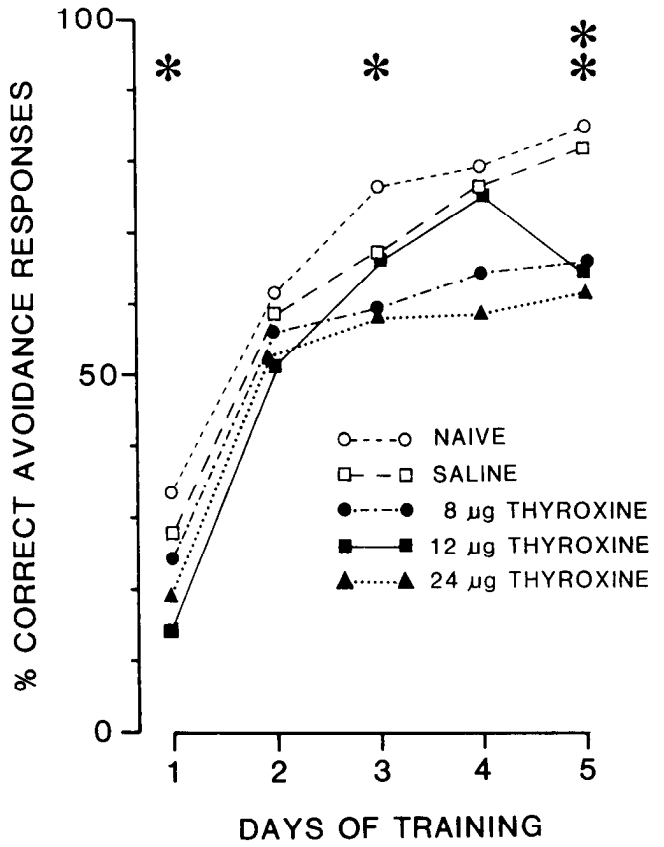

Figure 5. Development of 2-way avoidance performance in the 5 treatment groups of DBA/2 mice. Values for each day correspond to the mean percentage of correct responses (80 trials/d per animal). Significant treatment differences were seen on days 1, 3, and 5, as indicated by analysis of variance. Note the progressive development of differences between thyroxine-treated mice and the naive and saline controls, respectively, due to a plateauing of performance levels in the thyroxine-treated mice. $*p < 0.05$; $**p < 0.01$.

injections remained extremely well correlated with behavior (bivariate $r = -0.94$; partial $r = -0.95$).

Analysis of systemic side effects in the DBA/2 mice

Brain weight, and with it body weight, was strongly reduced by the treatment. This reduction, undoubtedly a systemic effect of the treatment, showed a significant linear regression with thyroxine dosage (Fig. 7*c*). Yet, if this systemic effect were a determinant of the avoidance scores, and the IIP-MF hyperplasia a by-product (a covariate), one would expect, first, that both variables would be dose-dependent. Furthermore, there should be a *positive* correlation between brain weight and avoidance, and a *negative* one between the IIP-MF and avoidance.

Figure 7 and Table 6 show that the correlation between IIP-MF and avoidance *cannot be a covariate of a systemic side effect.* First, there was a dose-dependency of brain weight, but not of the IIP-MF, indicating that the variables were not well correlated among themselves. Second, the positive correlation between brain weight and avoidance expected under the conditions of a systemic side effect did not occur: it was virtually 0 in the subgroup of the thyroxine-treated mice ($n = 45$; Fig. 7*d*), and was slightly positive ($r = 0.28$) for the entire sample of mice. Also, there was a strong correlation between the IIP-MF and avoidance within a given subgroup of thyroxine-treated mice,

Table 5. Multiple-regression analysis of BALB/c mice: 2-way avoidance scores on hippocampal terminal fields in CA3/CA4

Terminal field (SD)	Dependent variable: % correct responses day 5 (SD) $b_i \pm$ SE	Partial correlation
Standardized partial regression coefficients and corresponding partial correlations		
Str. oriens	−0.77 ± 0.30	−0.63*
Str. pyramidale	0.13 ± 0.21	0.19
Str. radiatum	0.65 ± 0.37	0.49
Str. lac. mol.	−0.33 ± 0.25	−0.38
CA4–MF	0.30 ± 0.15	0.53
Suprapyramid. MF	0.09 ± 0.19	0.15
IIP-MF	**−0.87 ± 0.15**	**−0.88*** **
df	10	
% of Behavioral variation explained by MLR ($R^2 \times 100$)	87.6**	
After removal of variable IIP-MF	44.8 (n.s.)	

$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; 2-tailed. Bold print: predicted partial regressions.

but none with brain weight. More important, the multiple-regression analysis showed that the weakly positive correlation for the entire sample was *spurious*: there was a significant partial regression of avoidance on brain weight, but it was negative (Table 6): the larger the brain, the poorer avoidance.

This implies that the treatment had 2 effects that were statistically independent: it induced a strong (but not dose-dependent) variability of the IIP-MF, and a dose-dependent decrease of brain weight. The MLR analysis shows that these 2 effects had opposite behavioral consequences. An increase of the IIP-MF decreased 2-way avoidance, but the dose-dependent decrease of brain weight was associated with better avoidance scores. Hence, the dose-dependent loosening of the IIP-MF/avoidance correlation, as seen in Figure 7, was a consequence of a systemic side effect that, at high doses, reduced brain weight and induced a relative improvement of avoidance learning. In statistical terms, the residuals of the IIP-MF/avoidance regression increased with thyroxine dosage, and this increase was significantly correlated with reduction of brain weight. Because of this, the hypothesis of a systemic side effect as a determinant of the avoidance scores, with the changes of the IIP-MF projection being a behaviorally irrelevant covariate, must be rejected.

Discussion

The mouse data confirmed all predictions made from the rat study. The postnatal thyroxine treatment led to a variable hyperplasia of the IIP-MF, which, as in rats, was barely dose-dependent. Most mice reacted to the treatment, but the extent of the IIP-MF was highly variable in every treatment group, with the (expected) exception of the untreated animals. The variable growth of the IIP-MF cannot be attributed to a specific thyroxine effect, because the variability of the IIP-MF in the saline-treated DBA/2 mice was significantly increased as well. As in the rats, the differential IIP-MFs appeared to result from

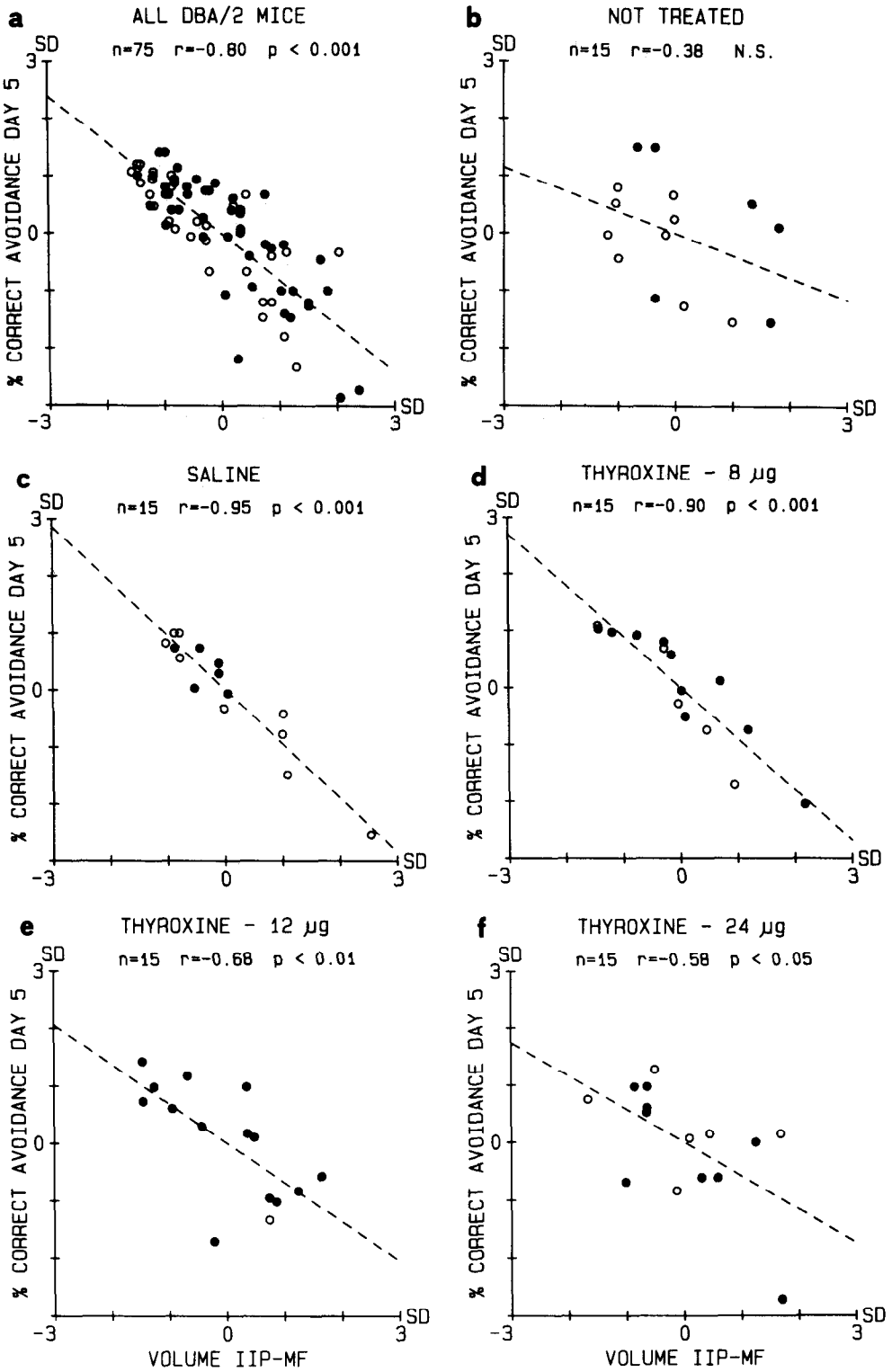

Figure 6. Standardized scatterplots of avoidance performance at day 5 on the volume of the IIP-MF in treatment groups of DBA/2 mice. *a,* All mice (treated and untreated). *b,* Untreated (isogenic) mice. *c,* Mice that received saline. *d,* Mice with mild postnatal hyperthyroidism (8 µg total/animal). *e,* Mice with moderate postnatal hyperthyroidism (12 µg total/animal). *f,* Mice with strong postnatal hyperthyroidism (24 µg total/animal; $n = 15$). *Open circles,* female mice; *filled circles,* male mice.

a developmental disturbance caused by hormonal imbalances.

In contrast to the RHA/Verh rats, the thyroxine-treated mice did not show much growth of other terminal fields. However, we have found that the reactions of mice and rats to postnatal hyperthyroidism are very different across strains. In some strains, we found that doses similar to those employed here invariably killed the pups—for instance, in the (normally fairly robust) C57BL/6 strain. Hence, thyroxine sensitivity depends strongly on the genetic background of the animals used. Perhaps it is associated with the genetic variation in endogenous thyroxine levels found across rodent strains (Amin et al., 1957; Chai, 1958).

The results seen in the mice also show that the behavioral mechanism associated with the IIP-MF cannot be a sole determinant of 2-way avoidance performance. First, the statistical analysis identified a second factor that had an independent and

Table 6. Multiple-regression analysis of DBA/2 mice: 2-way avoidance scores on hippocampal terminal fields in CA3/CA4

Independent variables (SD)	Dependent variable: % correct avoidance at day 5 (SD)					
	All mice ($n = 75$)	Not treated ($n = 15$)	Saline ($n = 15$)	Thyoxine, 8 μg ($n = 15$)	Thyroxine, 12 μg ($n = 15$)	Thyroxine, 24 μg ($n = 15$)
Brain weight	−0.30 ± 0.08 (−0.41)***	0.63 ± 0.34 (0.60)	0.09 ± 0.12 (0.27)	−0.12 ± 0.11 (−0.39)	−0.31 ± 0.15 (−0.66)	−0.12 ± 0.39 (−0.12)
Str. oriens	0.02 ± 0.12 (0.02)	1.05 ± 0.50 (0.65)	−0.02 ± 0.21 (−0.04)	0.48 ± 0.24 (0.63)	−0.09 ± 0.28 (−0.13)	−1.33 ± 0.72 (−0.60)
Str. pyramidale	0.24 ± 0.10 (0.30)*	−1.22 ± 0.64 (−0.62)	0.04 ± 0.16 (0.10)	−0.31 ± 0.15 (−0.64)	1.10 ± 0.27 (0.86)**	0.93 ± 0.62 (0.52)
Str. radiatum	−0.22 ± 0.16 (−0.17)	−0.72 ± 0.55 (−0.47)	−0.17 ± 0.21 (−0.31)	−0.20 ± 0.39 (−0.20)	−0.38 ± 0.30 (−0.46)	0.60 ± 0.88 (0.27)
Str. lac. mol.	−0.08 ± 0.09 (−0.11)	−0.08 ± 0.34 (−0.10)	0.09 ± 0.13 (0.29)	0.12 ± 0.21 (0.23)	−0.35 ± 0.22 (−0.54)	−0.33 ± 0.49 (−0.27)
CA4–MF	0.25 ± 0.08 (0.37)**	0.84 ± 0.35 (0.70)	−0.10 ± 0.16 (−0.24)	0.21 ± 0.14 (0.53)	0.24 ± 0.16 (0.53)	0.77 ± 0.42 (0.60)
Suprapyramid. MF	−0.05 ± 0.11 (−0.08)	−0.32 ± 0.75 (−0.43)	0.26 ± 0.23 (0.42)	−0.17 ± 0.19 (−0.33)	−0.29 ± 0.22 (−0.47)	−0.80 ± 0.66 (−0.44)
IIP-MF	**−0.95 ± 0.09 (−0.80)*****	0.33 ± 0.39 (0.33)	**−1.02 ± 0.13 (−0.95)*****	−0.79 ± 0.11 (−0.95)***	−0.73 ± 0.14 (−0.91)**	−0.70 ± 0.35 (−0.64)
df	66	6	6	6	6	6
% of Behavioral variation explained by MLR ($R^2 \times 100$)	74.3***	79.9 (n.s.)	94.7*	96.3**	89.7*	86.7 (n.s.)
After removal of variable IIP-MF	29.1 (n.s.)	77.3 (n.s.)	41.5 (n.s.)	63.6 (n.s.)	42.6 (n.s.)	58.3 (n.s.)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; 2-tailed. Bold print: predicted partial regressions.

[a] Standardized partial regression coefficients (b_i) ± SE. In brackets: partial correlation coefficients.

facilitating influence. Second, and more important, the impairment associated with the IIP-MF was not severe, the average performance decreasing from about 85 to 65%. However, in the same apparatus, poorly performing strains had shown an average performance of about 20% (Buselmaier et al., 1981; Schwegler and Lipp, 1983; Heimrich et al., 1985). It would seem that the still relatively good performance of DBA/2 mice with epigenetically modified IIP-MFs was caused by other genetic factors responsible for good 2-way avoidance, but which are not altered by the treatment. If that is true, then the behavioral mechanisms associated with the IIP-MF would be responsible for determining about one-third of the *total possible* range of genetically determined avoidance performance in mice.

The behavioral process(es) associated with variations of the IIP-MF cannot be analyzed by means of measurements obtained in the shuttlebox. Efficient 2-way avoidance learning requires that the animal learn to maintain a high degree of locomotor activity. However, any factor that interferes with this (rather unnatural) response will produce an impairment, be this factor cognitive (e.g., choice conflicts), mnemonic (e.g., forgetting), or peripheral (e.g., foot-shock sensitivity). On the other hand, 2-way avoidance is very sensitive to a variety of cerebral changes, because there is only the choice between running or not running. Since this choice is repeated several hundred times, every cerebral bias affecting it is likely to be unmasked. Thus, shuttlebox learning has the advantage of being a sensitive indicator, but the mechanism underlying behavioral changes must be analyzed, finally, by using more specific tasks (see the introduction).

Yet some of the possible mechanisms underlying the changes in 2-way avoidance that have been correlated with the IIP-MF can be ruled out or appear unlikely. The behavioral changes in 2-way avoidance performance on day 5 were probably not caused by an altered sensitivity towards electrical foot-shock, as a recent analysis showed no correlation between avoidance performance and electrical shock thresholds inducing locomotion in DBA/2 mice (unpublished data; see also Dimitrieva et al., 1984). The variations of the IIP-MF cannot be related to a general debilitation or sensory impairment either, since all mice showed rapid acquisition of the task. Moreover, individual differences in performance during the first test session were only poorly correlated with the IIP-MF. The reason that some mice committed errors in the late stages of conditioning is unclear. From observation, it appears that this performance decrement was caused by inattentiveness towards the conditioned stimulus, or by competing behavioral tendencies, such as avoidance response versus ongoing activities, such as grooming. This suggests that variations of the IIP-MF correlate with the probability of a behavioral change (Kimble, 1975).

General Discussion

The hypothesis underlying these studies is that variations in circuitry in the hippocampus can influence its function, and thus be reflected in individual differences in hippocampus-dependent behavior. A critical prediction stemming from this hypothesis is that developmentally induced changes of the IIP-MF should remain correlated with adult 2-way avoidance capacity. The results confirmed this prediction. If certain conditions were met, such as a uniform genetic background and mild developmental

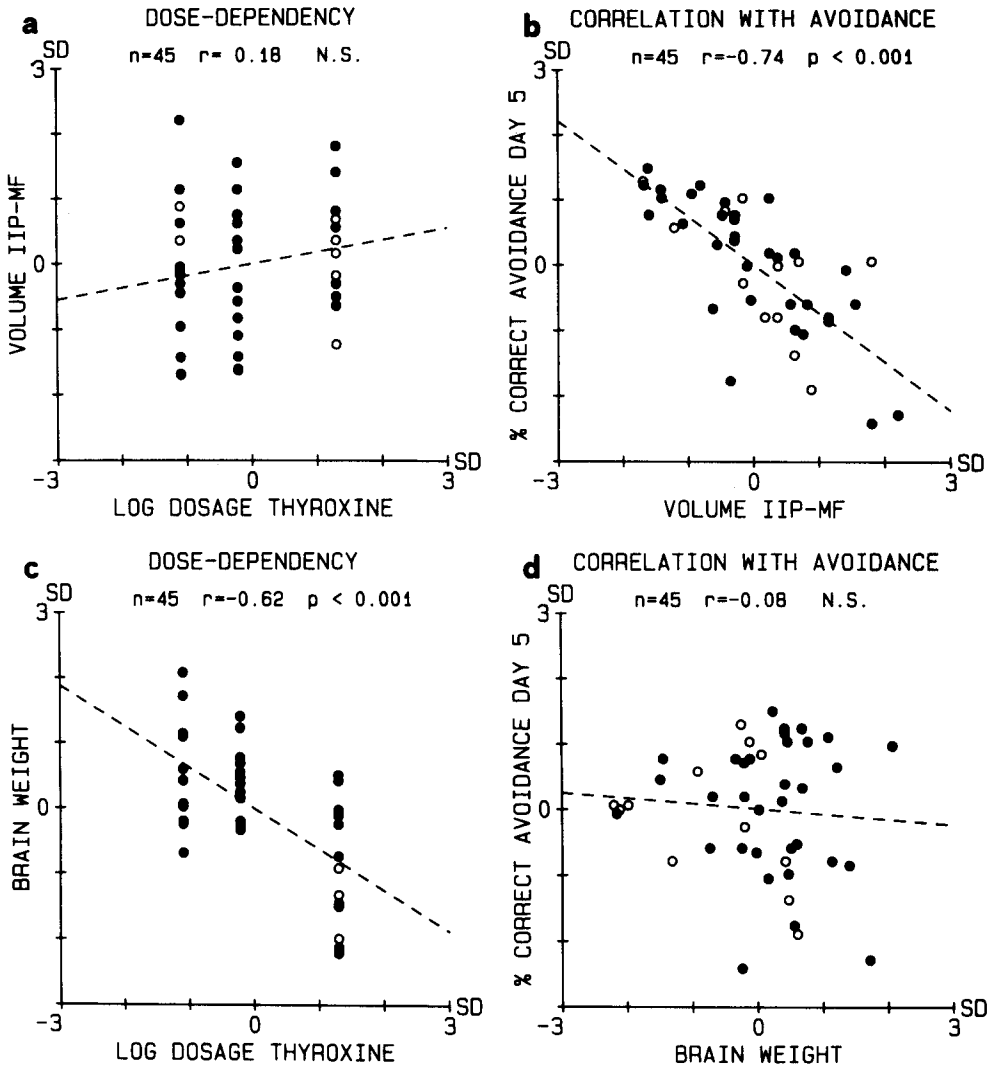

Figure 7. Analysis of systemic side effects in the thyroxine-treated DBA/2 mice. Comparison of dose-dependency of the IIP-MF and brain weight, and the regression of behavior with these 2 structural variables. *a,* Standardized scatterplot of the volumes of adult IIP-MFs on the thyroxine doses received postnatally ($n = 45$; logarithmic scaling of dosages). Note the lack of dose-dependency. *b,* Regression of avoidance with dose-independent variation of the IIP-MF. *c,* Regression of brain weight on thyroxine doses (same mice). Note the strong dose-dependency. *d,* Regression of avoidance scores on the dose-dependent variation of brain weights. Note the lack of any correlation. Together with the analysis in Table 6, these plots show that the differences in avoidance behavior cannot be a function of a systemic side effect (which is evident as a dose-dependent decrease of brain weight). If they were, there should be a strong positive correlation between brain weight and avoidance. *Open circles,* female mice; *filled circles,* male mice.

interference, individual differences in adult 2-way avoidance learning could be regressed almost linearly with individual differences in the extent of the IIP-MF caused by developmental manipulation. Statistically, the regression cannot be explained by observable systemic side effects of the treatment. Since the approach chosen here is probabilistic, the results do not prove, in a mechanistic sense, that the behavioral differences among the mice and rats were caused by hippocampal circuitry variations. Nevertheless, confirmed predictions and correlations approaching unity certainly permit the assumption that variations in hippocampal circuitry might be of behavioral relevance.

If so, 3 questions are of interest. (1) Is there additional evidence that different treatments can result in similar correlations between IIP-MF and behavior? (2) What evidence exists of hippocampal involvement? And (3) how might a comparatively subtle change in circuitry express itself in adult behavior, in spite of multiple side effects of the treatment and developmental plasticity?

A common target for drugs and genes

The data from these studies suggest that developmental changes in the IIP-MF may be caused by different processes, but that the behavioral consequences are the same, irrespective of the factors producing morphological changes. This interpretation is supported by studies in which adult rats born to alcoholic mothers, or having consumed alcohol during development themselves, have a hyperplastic IIP-MF resembling the one seen after postnatal hyperthyroidism (West et al., 1981b; West and Hamre, 1985). Other studies have shown that such maternal alcoholism in rats leads to an impairment of 2-way avoidance in the adult offspring (Bond and DiGiusto, 1978), as well as to increased open-field activity (Bond and DiGiusto, 1976). This appears to be very similar to the syndrome induced by postnatal thyroxine administration.

Further support for this interpretation comes from a genetic study in mice (Heimrich et al., 1985). By Mendelian crossing of strains with extremes of IIP-MF distribution and avoidance performance, and then analyzing the morphobehavioral correlation in filial generations (F_1 and F_2), it was found that inheritance of the extent of the IIP-MF was compatible with a *polygenic,* but not a monogenic, model. Yet 2-way avoidance was strongly correlated ($r = -0.80$) with the extent of the IIP-MF in individual mice. Thus, whether that variation in circuitry is caused by genes, epigenetic interference, or environmental factors, seems not to be important—behavior appears primarily to follow morphological changes.

Arguments for hippocampal involvement

Although we make no attempt here to identify specific neural mechanisms underlying the changes in 2-way avoidance behavior, the arguments below strongly suggest that variations in intrahippocampal circuitry influence processes that modulate 2-way avoidance behavior. These processes may be influenced by the IIP-MF distribution itself, or by a spatially associated structural (or biochemical) alteration within the hippocampal circuitry, such as of the number and density of granule cells (Gaarskjaer, 1978; Wimer and Wimer, 1982), of ingrowing noradrenergic fibers that have a predilection for mossy fiber territory (Davis and Martin, 1982), or by the number of recurrent mossy fiber collaterals terminating within the fascia dentata, as is currently being investigated by us.

The following are the arguments for hippocampal involvement:

1. A majority of lesion studies localize the hippocampus (together with other limbic structures) as a critical structure for the control of 2-way avoidance (Isaacson and Kimble, 1972; Jarrard, 1976; O'Keefe and Nadel, 1978; Olton et al., 1979; Gray, 1982).

2. Mossy fibers form a critical 1-way connection in the circuitry of Ammon's horn (Swanson, 1982) that is difficult to bypass (Fig. 1). Variations of their distribution in CA3 might influence the way in which granule cells can drive pyramidal neurons in CA3 (Gozzo and Ammassari-Teule, 1983; Schwegler and Lipp, 1983), or the degree of "cross-talk" between neighboring and remote trisynaptic circuits (lamellae) along the septotemporal axis (Lipp et al., 1987).

3. The IIP-MF/avoidance correlation coefficient is very high. In addition, there is statistical evidence that variations in the volume of *other* terminal fields account for the residuals of the behavioral variation that cannot be regressed on the IIP-MF. It would seem likely that such extremely strong correlations can only be seen close to a site of direct action.

4. Multiple genetic factors and treatments that appear biochemically different seem to influence the IIP-MF distribution and 2-way avoidance in a similar way. This is difficult to explain if we assume that the IIP-MF is a marker, but easy if one assumes that hippocampal circuitry is the common developmental target for genetic factors and developmental interference. We realize that a hidden variable *outside* the hippocampal formation may ultimately be responsible for the behavioral differences observed. However, that variable must fulfill 2 conditions: it must consistently yield higher correlation coefficients than the IIP-MF, for a marker cannot correlate better with behavior than an ultimate determinant, and it must react quantitatively similarly to all the manipulations to which the IIP-MFs were responsive, that is, to thyroxine and saline injections, and to cross-breeding. This is not impossible but would appear unlikely.

5. There are genetic and ontogenetic variations in hippocampal circuitry in mice and rats that are associated with performance in behavioral tasks known to depend on hippocampal functions as well (Lipp et al., 1986a, b, c, 1987; Wolfer et al., 1987; Crusio et al., 1987). Furthermore, Wimer et al. (1983). have reported a significant correlation between 2-way avoidance in mice and the density of granule cells (whose axons are the mossy fibers) in the rostral part of the hippocampus.

6. Once established, the IIP-MF distribution is permanent (Moyer et al., 1983), being modifiable only during the first 3 weeks of life. Afterwards, any of the manipulations known to produce changes in the IIP-MF distribution postnatally have been found to be ineffective (Lauder and Mugnaini, 1980; Laurberg and Zimmer, 1980, 1981). This is in contrast to neighboring synaptic fields, whose afferents maintain adult plasticity (Zimmer, 1974; Lynch et al., 1976). Thus, every natural or extraneously imposed process that influences the development of the mossy fiber distribution in CA3 may have a good chance of producing a structural bias that permanently affects hippocampal functions.

Thyroxine pleiotropy versus developmental last-word effects

The final argument in favor of hippocampal involvement is that the IIP-MF is one of the late-differentiating connections in the rodent brain (Zimmer and Haug, 1978; Amaral and Dent, 1981). Although not evident at first, this would seem to be a necessary condition, permitting a relatively minor variable, such as the IIP-MF, to have recognizable and predictable effects on adult behavior. The problem is that the consequences of a thyroxine-dependent change in the IIP-MF must be expressed in spite of masking side effects and developmental plasticity. We propose that the surprising persistence of an IIP-MF-related factor causing impaired 2-way avoidance may be due to a developmental "last-word effect" affecting the hippocampal formation.

Postnatally administered thyroxine has wide-spread effects on the developing brain. The hormone primarily stimulates RNA synthesis (Blecher and Bar, 1981), and possibly the production of nerve growth factor (Sadiq et al., 1985). Neurons respond to thyroxine with increased metabolism and accelerated growth and proliferation (Sokoloff, 1977), which results in shifts in local cell numbers (Balázs, 1977; Bass et al., 1977), altered neuronal size (Moskovkin and Marshak, 1978), different lengths of axonal projections (Lauder, 1977), and altered balances between afferent projections (this study), not to mention more subtle biochemical changes (Rastogi and Singhal, 1974; Balázs et al., 1977; Patel et al., 1980; Rastogi et al., 1981; Davis and Martin, 1982; Legrand, 1983; McCarty et al., 1983; Pascual-Leone et al., 1985).

Yet most behavioral changes after neonatal hyperthyroidism appear to be transient, as is suggested by studies of sensory and motor systems (Brunjes and Alberts, 1981; Nagy and Forster, 1982). Thus far, the classical long-term effect appears to be open-field hyperactivity, associated with more errors in maze tasks, the changes in underlying brain systems being unknown (Eayrs, 1964; Davenport and Gonzalez, 1973; Davenport et al., 1975; Stone and Greenough, 1975; Sjöden and Söderberg, 1976; McCarty et al., 1983).

It would seem likely that such subtle changes in adult behavior after a rather massive postnatal intervention would be due to developmental reorganization (plasticity). Any structural and biochemical anomaly that occurs early in development may mask its consequences on behavior with a combination of homeostatic mechanisms and developmental reorganization—the developmental buffers (Katz and Lasek, 1978; Lipp, 1979, 1988; Lipp and Schwegler, 1982; Katz, 1983). These may prevent the expression of many early thyroxine effects, or make them, at least, highly unpredictable. However, this would seem less likely for behavioral sequelae based on changes in the mossy fiber circuitry. Behavioral changes caused by late-differentiating structures are most likely compensated by system homeostasis or behavioral adjustment, because there is no more time for structural reorganization. Such compensation may mask the consequences of a structural bias under normal conditions. Yet that bias might be perceptible in situations of stress and re-

stricted choice, such as in 2-way avoidance. Given the adult invariance of the CA3–mossy fiber system, this projection could well preserve the traces of a developmental disturbance as a behavioral trait for life.

Conclusions

The genetically determined extent of the IIP-MF of RHA/Verh rats and BALB/c and DBA/2 mice is modified by developmental processes that are sensitive to postnatal thyroxine administration, and, to a lesser degree, to saline injections. In adulthood, individual differences in 2-way avoidance performance can be related rather precisely to individual deviations from the strain-typical mossy fiber pattern. Systemic side effects of the treatment exist but have opposite behavioral consequences.

Correlations can be observed across rodent species and strains, but the experimental design was to use strains in which the predicted changes were most likely to be observed, that is, good 2-way avoidance learners with scanty IIP-MFs. The same treatment may yield different results when applied to strains with poor 2-way avoidance and differential IIP-MFs.

The IIP-MF/avoidance correlation is seen under *natural conditions,* as well as after *developmental modification* of the morphological trait. The behaviorally relevant factor associated with the IIP-MF distribution is not the sole determinant of 2-way avoidance learning, but appears to be a comediator of appreciable functional strength. Its nature is unknown, but from several natural correlations with hippocampus-dependent behavior it seems likely that variations of the IIP-MF are related to corticolimbic processing.

There is no formal proof that the treatments influenced adult 2-way avoidance by means of interfering with circuitry differentiation in the hippocampal formation, but several arguments support this view: the morphological trait holds a commanding position in a structure mediating 2-way avoidance, is extremely well correlated with behavior, shows plasticity only during a critical postnatal period, and is established so late that its structural variations might dominate, as a developmental "last-word effect," even the multiple consequences of postnatal hyperthyroidism.

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