

Hippocampal Mossy Fibers and Swimming Navigation Learning in Two Vole Species Occupying Different Habitats

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ABSTRACT: We showed previously for mice that size differences of the infrapyramidal hippocampal mossy fiber projection (IIP-MF) correlate with spatial learning abilities. In order to clarify the role of the IIP-MF in a natural environment, we studied the bank vole (*Clethrionomys glareolus*), adapted to a wide range of different habitats, and the root vole (*Microtus oeconomus*), living in homogenous grassland habitats with small home ranges.

Morphometry on Timm-stained horizontal brain sections of six *C. glareolus* and six *M. oeconomus* revealed that the size of the entire mossy fiber projection was 42% larger in *C. glareolus* than *M. oeconomus*. *C. glareolus* had also an IIP-MF projection about 230% larger than that of the root vole. A sample of captured animals was then transferred to the laboratory (*C. glareolus*, *n* = 23; *M. oeconomus*, *n* = 15) and underwent testing for swimming navigation according to a standardized protocol used to assess water maze learning in about 2,000 normal and transgenic mice. Both species learned faster than laboratory mice. Overall escape times showed no differences, but path length was significantly reduced in *C. glareolus*, which also showed superior performance in a variety of scores assessing spatial search patterns. On the other hand, *M. oeconomus* showed faster swimming speed, and strong thigmotaxis combined with circular swimming. *M. oeconomus* also scored at chance levels during the probe trial, about as poorly as mutant knockout mice considered to be deficient in spatial memory.

These differences probably reflect differential styles of water maze learning rather than spatial memory deficits: *C. glareolus* appears to be superior in inhibiting behavior interfering with proper spatial search behavior, while *M. oeconomus* succeeds in escaping by using rapid circular swimming. We assume that size variations of the IIP-MF corre-

spond to a mechanism stabilizing hippocampal processing during spatial learning or complex activities. This corresponds to the ecological lifestyle of the two species and is in line with previous observations on the role of the IIP-MF. *Hippocampus* 2000;10:17–30. © 2000 Wiley-Liss, Inc.

KEY WORDS: ecology; artifactual memory deficits; transgenic mice; *Clethrionomys glareolus*; *Microtus oeconomus*

INTRODUCTION

The distribution of hippocampal mossy fibers in mice and rats shows remarkable genetic variation. A well-investigated hereditary trait is the intra/infrapyramidal mossy fiber projection (IIP-MF) formed by the terminal boutons of the granule cell axons synapsing upon the basal dendrites of pyramidal cells in the CA3 region (Barber et al., 1974). Numerous studies in mice and rats have reported correlations between the extent of the IIP-MF projection and behaviors thought to be mediated by the hippocampal formation, larger IIP-MF projections being frequently associated with superior performance.

Mice and rats with larger IIP-MF projections committed fewer reentry errors during radial maze learning (Crusio et al., 1987, 1993; Schwegler et al., 1990, 1993; Jamot et al., 1994; Schwegler and Lipp, 1995; Prior et

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al., 1997), showed better relearning after dislocation of the target platform in the swimming navigation task (Schöpke et al., 1991; Bernasconi-Guastalla et al., 1994), and exhibited better-controlled search behavior in a complex water maze (Schwegler et al., 1988). Mice selectively bred for high locomotor activity in the open field show larger IIP-MF projections (Hausheer-Zarmakupi et al., 1995). Likewise, mice with more extended IIP-MF projections showed more rearing in an open field (Crusio et al., 1989a,b, 1991), while another study by the same authors found decreased locomotor activity possibly related to stronger habituation (Crusio and Schwegler, 1987). Other correlates with hippocampus-dependent tasks include two-way avoidance learning (usually improved by hippocampal lesions), with poor avoiders showing larger IIP-MF projections (Lipp et al., 1989). Detailed reviews are available (Crusio, 1995; Lipp and Wolfer, 1995; Schwegler and Lipp, 1995; Schwegler and Crusio, 1995).

On the other hand, recent studies have shown that the IIP-MF projection can also covary with behaviors not generally associated with hippocampal function. For example, the IIP-MF correlates positively with the ability of (aversively motivated) discrimination learning in a Y-maze (Schwegler and Lipp, 1995): mice with larger IIP-MF projections showing superior adaptation of choice strategies. Differences in IIP-MF projections were also shown to correlate with paw lateralization. Mice which had larger IIP-MF projections in both hippocampi used a given forepaw more predictably in order to pick up a food reward, and they preferentially used the forepaw on the side with the relatively larger IIP-MF projection (Hausheer-Zarmakupi et al., 1995). Also, mouse strains with shorter attack latencies in the intruder test paradigm showed smaller IIP-MF projections (Guillot et al., 1994; Sluyter et al., 1994). Neither paw preference nor attack behavior involved any learning. A few studies, however, have found behavioral differences in hippocampus-dependent tasks without associated size differences of the IIP-MF projection (Crusio et al., 1990; Wahlsten et al., 1991; Hoffmann et al., 1992; Roullet and Lassalle, 1992).

Taken together, the findings suggest that variations of the IIP-MF, and, possibly, variations of the other mossy fiber subfields in mice are associated with the strength of an intrahippocampal process not identified yet. We have proposed that IIP-MF variations correlate with the stability of hippocampal processing (Lipp and Schwegler, 1989), mice with small IIP-MF projections being more vulnerable to disruption of hippocampal processing by sensory stimuli or motivational changes (Hausheer-Zarmakupi et al., 1995).

On the other hand, the negative findings cited above imply that the functional relevance of the mechanism associated with mossy fiber morphology may be subtle. It could also depend on the experimental context or on the genetic background of the rats and mice studied. In order to assess the functional relevance of the mossy fiber trait one might, in theory, test many samples of animals in many different tests and test schedules. Alternatively, one could try to assess the significance of mossy fiber variations in real-life situations of mice and other species. To study the natural role of hippocampal mossy fiber variations, we adopted two strategies. For one, mouse populations with genetic variation of

the IIP-MF projections are subjected to natural selection in outdoor pens established in a field station in Western Russia (Lipp et al., 1997). In parallel, we investigated the mossy fiber distribution of small European mammals studied extensively in ecological research, as initiated by Slomianka and West (1987, 1989) and Donovan and Slomianka (1996). We tried to evaluate their abilities in hippocampus-dependent tasks, as demonstrated for North American small rodents (Galea et al., 1994b, 1996; Sawrey et al., 1994; Perrot Sinal et al., 1998), and seek for morphobehavioral correlates as shown for the size of the hippocampal formation in relation to home ranges of kangaroo rats (Jacobs and Spencer, 1994) and American voles of the genus *Microtus* (Jacobs et al., 1990), or hippocampal size in relation to memory for caches in food-storing birds (Sherry et al., 1992; Clayton and Krebs, 1995).

In this report, we evaluate whether the correlations between the size of the IIP-MF projection and behavioral scores in water maze learning that have been observed in mice predict abilities for water maze learning in other small rodent species with differential IIP-MF projections. For this purpose, two vole species were chosen for which a qualitative pilot study had revealed substantial differences in mossy fiber morphology.

The bank vole, *Clethrionomys glareolus* Schreber (1789), is widespread over Europe and parts of Asia, mostly within and along all types of forest, but also in tundra and forest-steppe (Bashenina et al., 1981b). Bank voles occur numerously and occupy a variety of habitats, preferring light open parts of forest, glades, and cleared spaces, but they can also be found in the center of Moscow (Sokolov et al., 1995) and have been observed to climb on trees (Bashenina et al., 1981a). *C. glareolus* is herbivorous, and has a varied diets. For example, the diet of Russian *C. glareolus* includes seeds as obligatory high-calory food (Gromov and Poliakov, 1977; Bashenina et al., 1981c), while *C. glareolus* in Spain consumes virtually no seeds (Castien and Gosalbez, 1996). It is reported to show seasonal variation of brain size (Yaskin, 1984).

The root vole, *Microtus oeconomus* Pallas (1776), is found in Europe, Asia, Alaska, and western parts of Canada. It is also known as the tundra vole. It mainly occupies damp open meadow habitats, borders of swamps, and river and lake banks. *M. oeconomus* are good swimmers (Meyer et al., 1996). Their diet consists almost completely of the sappy vegetative parts of plants. No seasonal variation of brain weight has been reported for this species (Yaskin, 1984).

In a first step, the observed differences in mossy fiber distributions were quantified in a sample of animals selected for approximate ages and sex. In order to test functional correlates of these differences, samples of wild voles were collected again and transferred to the laboratory for behavioral testing, using an identical setup and test parameters as used for mice (see below). The test chosen was swimming navigation learning, using the reference version of the Morris test (Morris, 1984), for the following reasons: 1) the test is known to be very sensitive to hippocampal lesions in rats (Morris et al., 1982) and mice (Logue et al., 1997); 2) the same standardized version of this test has been applied to about 2,300 normal and transgenic mice, enabling us

to recognize cognitive and noncognitive factors playing a role in this task (Lipp and Wolfer, 1998; Wolfer et al., 1998); and 3) there were quantitative correlative data from mice with differential IIP-MF projections (Schöpke et al., 1991; Bernasconi-Guastalla et al., 1994). From these data (see Discussion), it was expected that the species with the larger IIP-MF projection would show a behavioral phenotype characterized by slower swimming, more goal-directed search behavior, less swimming along the walls, and superior abilities in finding a shifted platform position.

MATERIALS AND METHODS

Animals

Animals were trapped in standard Sherman live traps baited with dark rye bread soaked in sunflower oil. The traps were placed in the typical habitat of the species, for *C. glareolus* in the forest, for *M. oeconomus* in damp meadows lining the forest. The location of trapping was near a field station located 400 km west of Moscow and 400 km south of St. Petersburg (Biological Station Chicti Lec, Director V.V. Pazhetnov, Bubonizi, Tvierskaja Region). The approximate age of the animals was determined by an experienced field ecologist. In all cases, the animals were diagnosed as having been born in early summer of the same year. Hence, age at neuroanatomical inspection was about 3 months, while animals tested for behavior were on average 4 months old. Approximately half of the voles were caught in summer 1996, the other half in summer 1997.

Animals for behavioral testing were trapped as described above. One batch of animals was caught in August 1996, the other in August 1997. Five to 10 days after trapping, they were transferred to Moscow and kept in standard animal facilities under a natural lighting schedule. They received commercial mouse food supplemented with vegetables, apples, and fir-cones, the latter for *C. glareolus* only. Behavioral testing in September started after an adaptation period of 1 week. A total of 38 animals was available for testing: 23 *C. glareolus* (16 males, 7 females) and 15 *M. oeconomus* (7 males, 8 females). To monitor stress effects, the animals were weighed on the first and the last day of water maze testing. Statistical analysis showed no systematic differences between the years of testing. Thus, the data were pooled for further statistical analysis.

Histology

In the neuroanatomical laboratory of the field station, the voles were euthanized shortly after trapping by an overdose of Nembutal injected intraperitoneally. They were then perfused transcardially with Ringer solution for 2 min, followed by a 1% solution of sodium sulfide dissolved in 0.1 M phosphate buffer (pH 7.4) over 3 min. This was followed by perfusion with a 3% solution of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After removal, the brains were weighed to the nearest 2 mg, and kept in the glutaraldehyde fixative overnight. After a 24-h storage in 20%

sucrose solution in 0.1 M phosphate buffer, they were frozen and stored at -18°C for up to 3 weeks. The brains were sectioned horizontally in a cryostat (40- μm thickness), thaw-mounted in three parallel series, and developed for about 40–60 min in a solution containing Arabic gum, hydroquinone, citric acid, and silver nitrate (Schwegler and Lipp, 1983). Two series were left unstained for image analysis; another was counterstained with neutral red. Sections were then transferred to Zurich for morphometrical analysis.

Hippocampal Morphometry and Statistics

For morphometry, histological series from the brains of six *C. glareolus* and six *M. oeconomus* were selected according to sex (three males and females for each species) and same approximate age (young adult animals), as judged by dentition and body weight.

Quantitative analysis of the mossy fiber system was done on five horizontal sections spaced by 80 μm , plus a sixth one located 320 μm more ventrally. This level was included to assess better septotemporal gradients of the IIP-MF projection. Sampling started immediately below the most ventral extension of the septal pole of the hippocampus (Schwegler and Lipp, 1983), the last section matching approximately the horizontal level of the anterior commissure. Both left and right hippocampi were analyzed. The levels for analysis were determined separately for each hemisphere in order to correct for tilted cutting planes.

Images of the hippocampi (Fig. 1) were digitized at a resolution of $1,280 \times 1,024$ pixels, using a Videk[™] Megaplus CCD camera mounted on a Zeiss Axioplan. In Adobe[™] Photoshop, images were optimized for contrast and analyzed using NIH Image 1.61 public-domain software. The mossy fiber distribution was then transformed into a binary image. In previous studies, this had been done by adjusting the threshold values manually until the binary image matched the camera image of the mossy fiber distribution. However, in 40- μm sections, Timm-staining produces an undefined shadow zone along the mossy fibers which, for small IIP-MF projections, can cause interobserver errors by a factor of 2 (Moos, 1997). Here, the gray level threshold for binary transformation was determined in four pairs of small circular masks along the suprapyramidal mossy fiber layer (SP-MF) and the hilar mossy fiber field (CA4-MF). For each pair of masks, one was placed within and the other outside the darkly stained mossy fiber zone. The average of the eight gray levels was then used to define the threshold level for obtaining the binary image of the mossy fiber distribution. Boundaries between the three mossy fiber fields (IIP-MF, SP-MF, and CA4-MF) were then drawn on the computer screen in order to measure the areas of each subfield. This procedure permitted area measurements with much higher interobserver reliability than subjective visual matching of the mossy fiber distribution (data not shown). Note that these subfields refer only to the areas as covered by mossy fiber boutons. Thus, the hilar mossy fiber field referred to as CA4-MF does not include the full polymorphic layer of the dentate gyrus as defined by Amaral and Witter (1995).

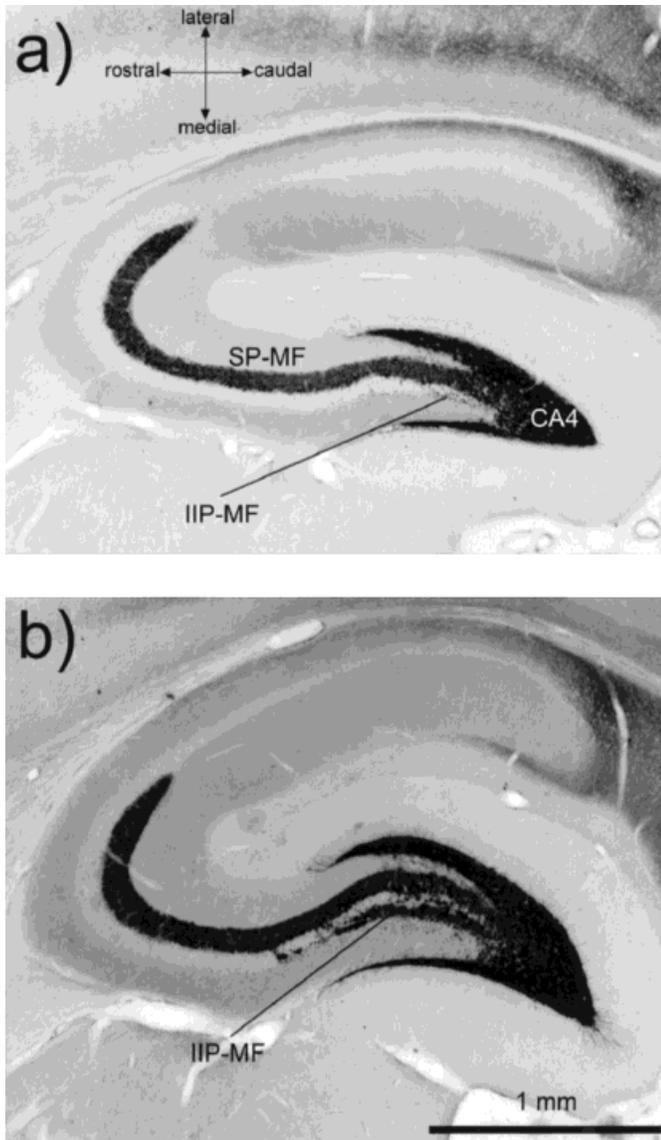


FIGURE 1. Mossy fiber distribution in *Microtus oeconomus* (a) and *Clethrionomys glareolus* (b). Note the scarce infrapyramidal mossy fiber projection (IIP-MF) in *M. oeconomus*. Timm-stained horizontal sections from the mid-septotemporal level.

The morphometric scores for a given individual were the mean cross-sectional areas of the left and right mossy fiber fields, averaged across the six sections (CA4-MF left, CA4-MF right, SP-MF left, SP-MF right, IIP-MF left, and IIP-MF right). For data presentation, the areas from left and right sections were then averaged again. To quantify possible left-right differences, asymmetry scores were computed according to the formula $(R - L) / 0.5(R + L)$ (Schöpke et al., 1991; Bernasconi-Guastalla et al., 1994). A percentage ratio (IIP-MF/SP-MF) was computed also, as this index has been found useful in computing correlations between the relative size of the IIP-MF projection and behavioral scores in mice.

Since the statistical comparisons of correlated traits between species using analysis of variance (ANOVA) of percentage ratios has been discouraged (Packard and Boardman, 1990), analysis of

covariance (ANCOVA) was employed for assessing differences between the averaged values of the mossy fiber fields, using at least one other variable as covariate (Table 1). In addition, results were checked by means of a simple discriminant analysis, using multiple regression with the three mossy fiber fields as independent and species as dependent dummy variable (Krebs et al., 1989).

Water Maze Testing

The design of the water maze followed the description given by Morris (1984) and was almost identical with the system used in Zurich to study mouse water maze learning. It consisted of a white Plexiglas circular pool of 143 cm diameter and 50 cm height, filled with water (16 cm deep, 24–26°C), made opaque by the addition of milk. Distant visual cues for navigation were provided by the environment of the laboratory. A wire mesh platform (16 × 16 cm) was placed 0.5–0.8 cm below the water surface, with a pool perimeter distance of 35 cm. To avoid visual orientation prior to release, voles were transferred from their cages to the pool in a small opaque cup. They were released from eight symmetrically placed positions on the pool perimeter in a predetermined but not sequential order. They were allowed to swim until they found the platform or until 120 s had elapsed. Between trials, the animals were placed under 60 W bulbs and allowed to warm up and dry off for a few minutes. Intertrial times varied from 30–60 min. During this time, the animals were kept in cages containing dry hygroscopic sphagnum moss to permit complete drying of the fur.

The entire procedure took 5 days; each vole did a total of 30 trials, six per day. The position of the hidden platform remained fixed for the first 3 days (18 trials, acquisition phase). Afterwards, it was placed in the opposite quadrant for 2 days (12 trials, reversal phase). The first 60 s of trial 19 (the first reversal trial) were defined as probe trial to analyze the search behavior of the animals. For testing, *C. glareolus* and *M. oeconomus* were released alternately during a test session.

Water Maze Swim Path Analysis

Swim paths were recorded by means of a video camera suspended above the center of the pool and fed to an electronic imaging system (ASBA Wild & Leitz[®]) which extracted and stored X-Y coordinates at a frequency of 4.2 Hz and with 256 × 256 pixels of spatial resolution. These coordinates were then analyzed off-line, using custom software (WINTRACK by D.P. Wolfer) as based on an earlier DOS version (Wolfer and Lipp, 1992). In order to characterize species-specific peculiarities of water maze learning, the program extracted from the swim paths a large set of variables (see also Table 2). *Learning performance* was quantified by escape time (s), length of swim path (m), a cumulative search index according to Gallagher et al. (1993) (average distance between subject and goal multiplied by escape time, time needed to reach the goal on a straight path being excluded from the calculation), and the number of trials in which the animals failed to find the platform. *Orientalional behavior* was quantified by the percentage of swimming time spent within 50 cm of the start point (a measure which is generally high in well-orienting animals), by the percentage of time spent in the

TABLE 1.

ANCOVA of Mossy Fiber Morphometry in *Clethrionomys glareolus* and *Microtus oeconomus*, mean \pm S.E.M.*

Variables	C. <i>glareolus</i> (n = 6)	M. <i>oeconomus</i> (n = 6)	P (Scheffé)	Covariate
Body weight (g)	18.0 \pm 1.6	37.8 \pm 5.3	0.005	None
Brain weight (g)	0.58 \pm 0.01	0.67 \pm 0.01	<0.0001	Body wgt
CA4-MF (μ^2)	222,342 \pm 11,877	158,063 \pm 9,530	0.002	Brain wgt
SP-MF (μ^2)	186,794 \pm 6,359	148,061 \pm 8,155	0.006	Brain wgt
IIP-MF (μ^2)	25,723 \pm 2,880	5,317 \pm 622	0.0002	SP-MF
IIP-MF/suprapyramidal MF (%)	13.81 \pm 1.49	3.71 \pm 0.56	0.0004	Brain wgt
Asym-Coeff CA4-MF (%)	-1.64 \pm 5.37	-3.92 \pm 2.6	n.s.	Brain wgt
Asym-Coeff SP-MF (%)	-2.44 \pm 1.87	-10.33 \pm 10.25	n.s.	Brain wgt
Asym-Coeff IIP-MF (%)	7.90 \pm 15.31	-13.21 \pm 9.87	n.s.	Brain wgt
AbsAsym-Coeff CA4-MF (%)	9.20 \pm 3.53	5.50 \pm 1.75	n.s.	Brain wgt
AbsAsym-Coeff SP-MF (%)	4.10 \pm 1.15	13.41 \pm 9.35	n.s.	Brain wgt
AbsAsym-Coeff IIP-MF (%)	22.03 \pm 12.24	15.15 \pm 9.15	n.s.	Brain wgt

*wgt, weight; n.s., not significant; Asym-Coeff, asymmetry coefficient; AbsAsym-Coeff, absolute asymmetry coefficient.

actual target quadrant, by the average distance to the target, by the absolute initial directional error within 30 cm from the release point, and by an airline corridor index, defined as percentage of swimming time in a corridor of 16 cm width connecting release and target sites. The *organization of the swim path* was assessed by a walltime index measuring thigmotaxis (defined as percentage of swim time within 22 cm to the wall; equals 50% of the surface area of the pool), by the average number of wall contacts, by an index measuring path efficiency or goal-directedness (defined as percent of the swim path during which the trigonometric component of swimming towards the goal is 75% or more), and by an index quantifying swimming in parallel to the walls (percentage of path which does not meet the criterion of goal-directedness as defined above and in which the component of swimming perpendicularly to and from the walls is 25% or less), and by the tortuosity of the swim path. This measure was obtained by subdividing the path into periods of floating (see below), straight segments, and curves with consistent change of swim direction. Absolute direction changes of all curves in a trial were then summed and divided by the total swim path length. *Motor peculiarities* were measured by assessing swim speed (m/s) while moving faster than 6 cm/sec, and by the percentage of time spent floating (defined as speed below 6 cm/s). *Spatial retention and memory* were defined by probe trial measures, namely by the percentage of time spent in former target quadrants and other quadrants, by the number of annulus crossings over the former target position and over annuli in control quadrants, and by the average distance to the previous goal location.

Statistical Analysis

Morphometrical variables and behavioral scores representing averaged values were analyzed by factorial ANOVA (species and

sex) or *t*-tests where appropriate. For analysis of learning curves, trials were grouped in blocks of two trials and analyzed by a two-way ANOVA, with trials as repeated factors. All variables were similarly checked for sex effects using a two-way ANOVA (species by sex) on the averaged values from the variables.

RESULTS

C. glareolus Has Larger Infrapyramidal Mossy Fiber Projections Than *M. oeconomus*

A morphological comparison of *C. glareolus* and *M. oeconomus* is listed in Table 1. Physically, *M. oeconomus* was about twice as large as *C. glareolus* ($P = 0.0005$). The male *M. oeconomus* of this sample were significantly larger than the females (males, 49 \pm 3 g; females, 27 \pm 2 g). The average brain weight of both sexes, however, was only about 15% larger in *M. oeconomus* ($P < 0.0001$). Correspondingly, the brain-to-body weight ratio was about 70% higher in *C. glareolus* than *M. oeconomus*. Both *C. glareolus* and *M. oeconomus* had larger brains than laboratory mice, even after correcting for body weight (data not shown).

A comparison of hippocampal morphology (Fig. 1) revealed that *M. oeconomus* had a slender and more elongated hippocampus, which appeared to be somewhat smaller than the hippocampus of *C. glareolus*. The latter showed a well-developed dentate gyrus and CA3 region, whereas CA1 and the subiculum appeared to be approximately the same size in the two species. This impression was corroborated by the quantitative analysis of the mossy fiber distribution, which revealed that all three mossy fiber fields were significantly larger in *C. glareolus* than in *M. oeconomus*.

TABLE 2.

*Behavioral Data in Water Maze Learning of Clethrionomys glareolus and Microtus oeconomus, means ± S.E.M. of 30 Trials**

Variables	<i>C. glareolus</i> (n = 23)	<i>M. oeconomus</i> (n = 15)	P (t-test)	Gender effects, Scheffé post hoc
Body weight (g)	18.08 ± 0.50	27.07 ± 1.52	<0.0001	n.s.
Performance				
Escape time (s)	19.88 ± 1.66	19.57 ± 1.31	n.s.	n.s.
Path length (m)	3.89 ± 0.30	5.08 ± 0.32	0.012	n.s.
Cumulative search (Gallagher) index (m * s)	8.50 ± 0.82	9.88 ± 0.83	n.s.	n.s.
Number of failed trials	3.96 ± 1.12	2.82 ± 0.69	n.s.	n.s.
Orientalional behavior				
Time in start zone (%)	54.82 ± 1.58	43.63 ± 1.31	<0.0001	n.s.
Average distance to release point (m)	0.47 ± 0.12	0.56 ± 0.14	<0.0001	n.s.
Directional error (absolute degrees)	37.65 ± 1.79	56.28 ± 2.41	<0.0001	n.s.
Airline corridor index (% time)	29.86 ± 1.60	18.29 ± 1.19	<0.0001	
Organization of swim path				
Wall time index (% time within 22 cm to wall)	24.62 ± 2.09	41.50 ± 2.83	<0.0001	n.s.
Average number of wall contacts	0.99 ± 0.12	1.63 ± 0.23	0.011	n.s.
Path efficiency (% time heading to goal)	44.07 ± 1.81	30.72 ± 1.54	<0.0001	n.s.
Parallel to border swimming (% time)	13.11 ± 1.04	26.23 ± 1.67	<0.0001	P = 0.02, F > M
Path tortuosity (absolute degrees/m)	223.67 ± 10.53	192.62 ± 7.58	0.038	n.s.
Search behavior				
Time spent in current goal quadrant (%)	38.74 ± 1.58	36.62 ± 1.05	n.s.	n.s.
Average distance to current goal (m)	0.43 ± 0.01	0.48 ± 0.01	0.011	n.s.
Probe trial first 60 s				
Time in old goal quadrant (%)	40.29 ± 4.14	24.99 ± 3.89	0.017	n.s.
No. of crossings over old platform position	2.74 ± 0.65	1.79 ± 0.47	n.s.	n.s.
Average distance to old goal position (m)	0.42 ± 0.02	0.54 ± 0.03	0.001	n.s.
Motor behavior				
Swimming speed (m/s)	0.21 ± 0.01	0.27 ± 0.01	<0.0001	n.s.
Time spent floating (%)	1.82 ± 0.68	0.26 ± 0.13	0.07	n.s.

*n.s., no significance.

The SP-MF projection was larger by 28%, the hilar projection (CA4-MF) by 45%, and the IIP-MF by 339%. Expressed as percentage of the SP-MF, the IIP-MF was still 234% larger in *C. glareolus* than in *M. oeconomus* (13.8 ± 1.5 SEM vs. 4.12 ± 0.45 SEM). In fact, in many *M. oeconomus*, the IIP-MF projection at the midseptotemporal level was almost missing. A detailed analysis at all midsepto-temporal levels of the hippocampus showed that the larger IIP-MF projections of *C. glareolus* were not an artifact due to differential levels of sectioning. (Fig. 2). For comparison, data from 200 mice (*Mus musculus*) with mixed genetic backgrounds showed an IIP-MF/SP-MF index of about 15%. The two vole species did not show differences in left-right asymmetries of the mossy fiber fields, nor were there significant differences in the degree of asymmetries (Table 1). None of the hippocampal measures showed any sex differences.

Significance levels of these differences as assessed by ANCOVA are given in Table 1. For the size of the IIP-MF, we additionally computed a multiple regression using the three mossy fiber fields

as independent variables, and the species as dependent dummy variable. This revealed a highly significant multiple regression on species ($R = 0.93$; $F_{3,8} = 17.35$; $P = 0.0007$), reflecting the overall size differences between the two species, but also an independent partial regression of the IIP-MF variable on species (standardized partial regression coefficient = 0.876, $P = 0.009$), indicating an independent size difference after partialing out a common size difference between *C. glareolus* and *M. microtus*.

Both Species Learn a Spatial Task About Equally Fast

A preliminary data analysis revealed no significant sex effects, nor effects due to testing in 1996 vs. 1997. Swimming navigation data are shown in Table 2, and Figures 3 and 4. Both species tolerated the handling and the swim tests well, judging by their behavior and by the lack of body weight loss between begin and end of the training week. *C. glareolus* could be handled easily,

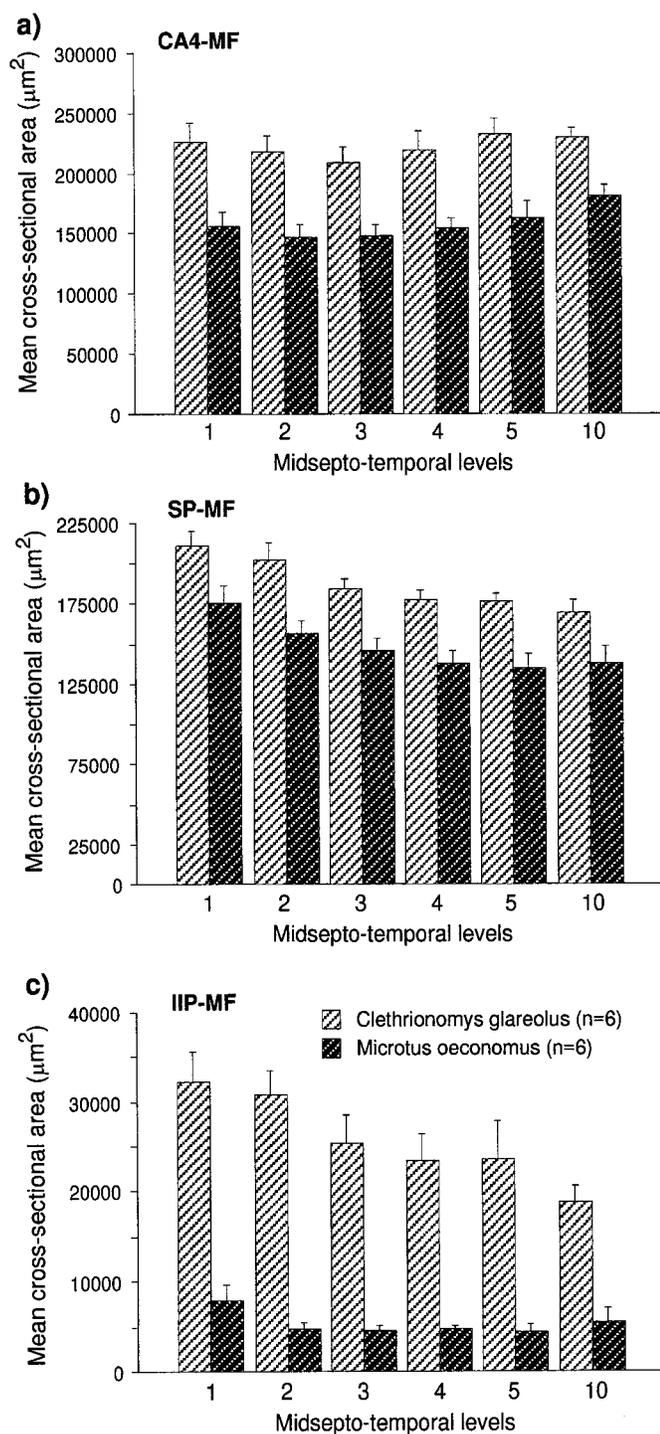


FIGURE 2. Cross-sectional areas (mean areas and SEM, averaged from left and right hippocampus) of the hilar (a), suprapyramidal (b), and intra/infrapyramidal (IIP-MF) mossy fiber fields (c) at different horizontal levels in *M. oeconomus* and *C. glareolus*. Level 1 corresponds to a plane immediately below the ventralmost extension of the septal pole of the hippocampus, level 10 corresponds to a horizontal plane including the anterior commissure.

while *M. oeconomus* sometimes tried to bite the experimenters. Both species were peaceful in comparison to wild wood mice (*Apodemus flavicollis*) which, for that reason, could not be tested systematically for water maze learning.

Escape times to the platform were not different between the two species (Fig. 3a, Table 2). In fact, both of them learned the task during the first day, improved during the second day, and showed asymptotic escape times between 10–15 s during the third day. Platform reversal caused a steep increase in escape times, followed by rapid relearning of the platform position. Thus, both species had developed a spatial reference memory. The number of trials in which the voles failed to find the platform was slightly yet not significantly higher in *C. glareolus*, and no species difference was found for Gallagher's cumulative search error. Comparison of swim paths, however, revealed that these were significantly longer by about 30% in *M. oeconomus* ($F_{1,14,35} = 5.34$, $P = 0.039$), chiefly because *C. glareolus* reduced swim paths more quickly after during days 2 and 3 of acquisition and after platform reversal.

Swimming speed was about 30% faster in *M. oeconomus* ($P < 0.0001$). Neither species showed much passive floating, but a few *C. glareolus* individuals showed floating during the first training sessions.

C. glareolus Searches Efficiently, While *M. oeconomus* Shows Thigmotaxis

A detailed analysis of the swim paths showed the reasons for the discrepancy between escape times and path length. As evident from the inspection of all plotted swim paths (for an example, see Fig. 4), *C. glareolus* appeared to learn the task more efficiently than *M. oeconomus*, which often swam along the walls and/or traversed the pool straightforwardly, bouncing back once or twice. The resulting longer swim paths were compensated by the higher swimming speed, however. A quantitative analysis of spatial search behavior confirmed the visual analysis. *M. oeconomus* spent a significantly larger fraction of their swimming time in proximity in parallel along the walls ($P < 0.0001$, Fig 3c, Table 2) than *C. glareolus*. They also showed an increased number of wall contacts ($P = 0.011$), which appeared not as escape attempts but rather as bouncing (Fig. 4).

On the other hand, *C. glareolus* showed a much more efficient swimming strategy. After release, they swam rather slowly, as indicated by a significantly longer fraction of swim time spent near the release site ($P < 0.0001$), were better oriented towards the target when leaving the release zone ($P < 0.0001$), spent more time in the direct airline corridor connecting release site and target ($P < 0.0001$), and showed significantly higher scores for goal-directedness ($P < 0.0001$) than *M. oeconomus*. The more precise spatial orientational behavior of *C. glareolus* was partially reflected in scores of spatial selectivity. There was no difference in the time the animals spent in the actual goal quadrant (both species spending significantly more time there than the expected 25%; see Table 2), but the average distance to the current goal (a measure which is less dependent of peculiarities of the swimming path) was significantly shorter in *C. glareolus* ($P = 0.011$). One may note that bank voles are able to learn the Morris task in a single trial (Fig. 4b).

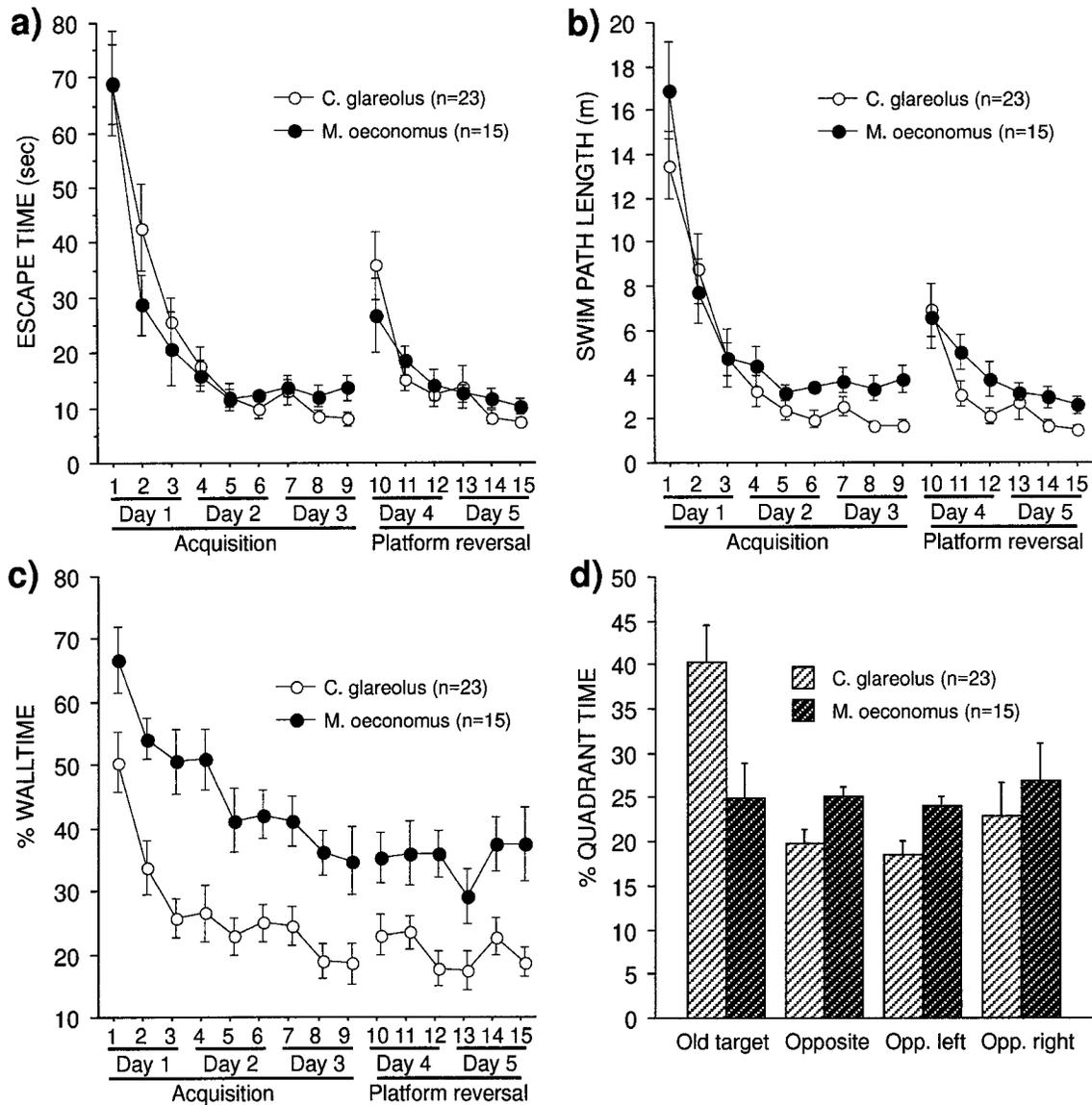


FIGURE 3. Escape times (a), swim path length (b), thigmotactic swimming along the walls (c), and spatial retention scores from probe trial at the begin of reversal learning (d) in *M. oeconomus* and *C.*

glareolus (means and S.E.M.). Blocks of trials in a–c represent averages from two trials. Note the massive thigmotaxis in *M. oeconomus* and the probe trial scores suggesting deficient spatial memory.

M. oeconomus Shows Poor Spatial Memory Scores

Analysis of the probe trials revealed that many *C. glareolus* showed the typical searching over the previous platform position, at least for a short period, while *M. oeconomus* failed in most cases to do so and continued to follow an unpredictable swim path after having missed the platform in the expected position. Since most *M. oeconomus* actually crossed the platform position at least once, they certainly had acquired a spatial reference memory (as indicated also by the longer swim paths after platform reversal). However, the random swim paths of *M. oeconomus* resulted in a low preference for the previous target quadrant, and they spent, on average, the same fraction of swimming time (about at chance level of 25%) in any of the four quadrants (Fig. 3d). This resulted

also in a significant species difference ($P = 0.011$), because *C. glareolus* spent on average 40% of their time in the former target quadrant. The average distance to the former target position during the 60 s of the probe trial was also significantly shorter in *C. glareolus* ($P = 0.011$). The number of annulus crossings over the old target position was not different in the two species, however, because many *C. glareolus* soon started to search elsewhere in the pool. Their more efficient search behavior for the new platform position entailed then a significant shortening of their swim paths during reversal learning in comparison to *M. oeconomus* ($F_{1,5,35} = 4.58$, $P = 0.039$).

All variables assessed were also checked for sex effects. Except for a slightly but significantly increased tendency of female voles

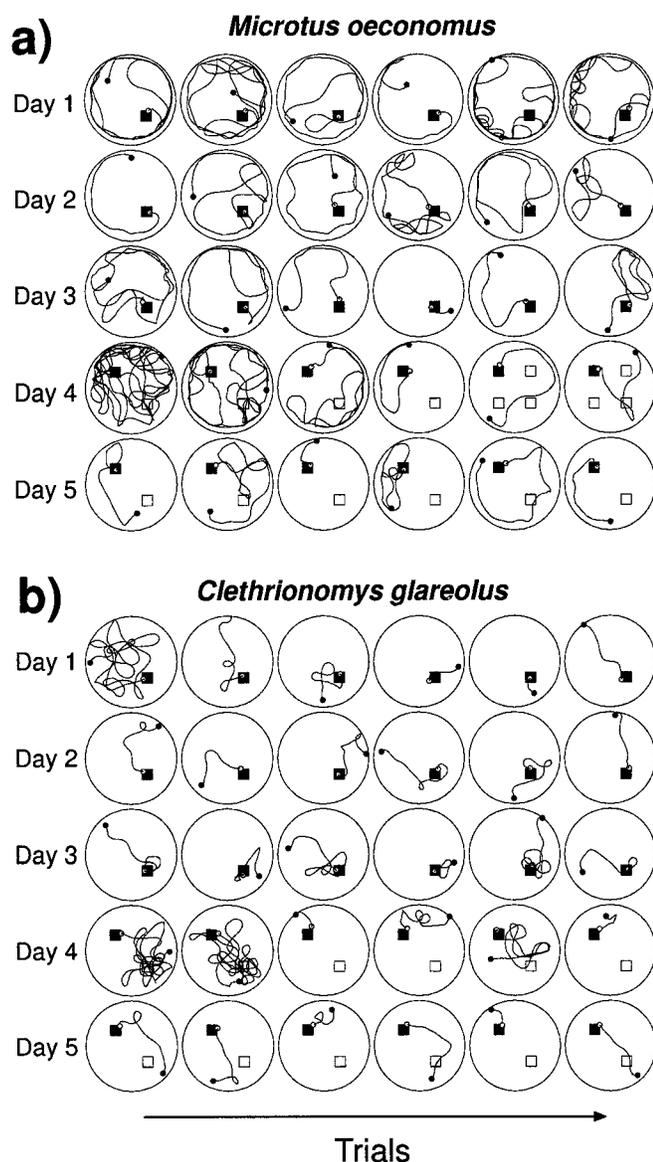


FIGURE 4. Typical swim path patterns over all 36 trials in *M. oeconomus* (a) and *C. glareolus* (b). Black dots indicate release points, open circles the end points of recorded swim path. Black squares, target positions; open squares, previous target positions after platform reversal. All paths analyzed and plotted off-line by WINTRACK. Note that *M. oeconomus* shows strong thigmotaxis (i.e., swimming within a rim of 22-cm width along the walls), but compensates the resulting longer swim paths by faster swimming. Relocating the platform causes increased thigmotaxis and a poorly organized search pattern without quadrant preference. This indicates that this *M. oeconomus* had developed some form of spatial memory based on some other form of spatial mapping. Conversely, *C. glareolus* swims more slowly and shows a more controlled search behavior. Note that learning the platform location takes not more than one trial. Afterwards, performance remains stable, the animal not showing thigmotaxis. After platform reversal, the animal searches during two trials in the former target quadrant and then reliably finds the new platform position. This type of learning is commonly interpreted as “cognitive.”

from either species to swim in parallel to the wall, there were no differences in the variables listed in Table 2.

DISCUSSION

The data show that the two species show remarkable differences in the extent of the hippocampal mossy fiber projection, particularly of the IIP-MF projection. Likewise, the species with the larger IIP-MF projection, *C. glareolus*, showed much better spatial search behavior in the invisible platform version of the water maze than *M. oeconomus*. The latter, however, compensated the poorly developed search behavior by faster swimming and alternative search strategies. Thus, as a net result, the two species had similar escape times from the water. There is at least comparative evidence that the species differences in both the neuroanatomical and behavioral traits during water maze learning are functionally linked.

Differential Morris Maze Learning in Voles and Laboratory Mice Is Caused by the Same Factors

Our data show that the wild voles tested here learned the hidden platform version of the Morris task very rapidly. In comparison to laboratory-bred North American voles (*Microtus ochrogaster*, *M. montanus*, and *M. pennsylvanicus*) that had been trained in a pool of similar size (Sawrey et al., 1994), they reached about the same final escape latencies (about 10 s) already after 30 trials, as compared to about 40–50 trials in the three North American species. One may notice that the schedule employed here included additionally a reversal of the target platform.

In comparison to laboratory mice, both vole species learned considerably faster. Laboratory mice showed, in the same setup and using the same procedures, an average final escape latency between 20–30 s, depending on strain and genetic background (unpublished data from 600 transgenic control mice). The inferiority of the laboratory mice is mainly based on lower performance during the first day of training, and on greater performance variation during ongoing training. On the other hand, individual and species differences in the wild voles were based on the same three factors which accounted for most of the behavioral variation of mice in the Morris maze, namely, thigmotaxis (associated with long swim paths), swimming speed, and spatial memory scores from the probe trial (Lipp and Wolfer, 1998).

Thigmotaxis in the water maze denotes the tendency of the animals to swim in proximity to the walls. It probably evolves from the instinctive escape response of small mammals placed in a water tank, which leads to many wall contacts during the first trials. It is shown by both wild voles and laboratory mice. Subsequently, these wall approaches are replaced gradually by search strategies. Thigmotaxis in the water maze should not be confounded with thigmotaxis in an open-field arena, where it probably reflects avoidance of open spaces.

In mice, we have identified two main search strategies in Morris water maze learning (Lipp and Wolfer, 1998). One group of mice develops proper navigational behavior, including initial orientation, well-directed swimming paths, and subsequent searching over the old platform position once it has been replaced or removed. Such behavior is usually regarded as "cognitive." The other group adopts a strategy of circular searching ("spiralling in") which is reflected in high scores of swimming in parallel to the walls without making contacts. Both groups eventually succeed, the circular swimmers mostly having longer escape paths. Scores thought to measure spatial memory in the probe trial tend to be poorer in the wall swimmers, which continue their circular search for the vanished platform. This dichotomy is verified only after observing large samples of mice, however, because many animals can shift between these main strategies.

The two species observed here appear to represent the extreme prototypes of normal mouse behavior in the Morris maze: most *C. glareolus* showed the typical spatial search behavior with slow swimming in the start zone, identification of invisible target, straightforward approach, and insistent (but short) searching once the platform had been relocated. On the other hand, *M. oeconomus* started to swim quickly, kept a fast swimming pace along the walls, and identified the platform position obviously by another strategy that resulted in probe trial scores of at chance level. Yet, the prolonged swim paths of *M. oeconomus* after platform reversal indicate that many of them were expecting the platform in a particular position. Thus, their strategy has a spatial component, too. It is clear, however, that they seem to pay much less attention to distal visual cues. Given that blind rats are also able to learn the Morris task (Lindner et al., 1997), it appears likely that this alternative "noncognitive" strategy is based at least partially on some form of kinesthetic spatial mapping.

Finally, we also checked our data base for an answer to the apparent paradox that a naturally good swimmer such as *M. oeconomus* had problems in the water maze. Whishaw and Tomie (1996) had attributed the superiority of rats over mice in the Morris maze to the semiaquatic life-style of *Rattus norvegicus*, as compared to the dry-land life style of *Mus musculus*. For one rat strain, we noted a coincidence with *M. oeconomus*, namely, relatively high swimming speed, possibly characteristic of semiaquatic species. However, the chief comparative deficit of *M. oeconomus* was its inability to suppress inappropriate thigmotactic responses, while the main problem of *Mus musculus* was unstable performance (see above). One may note, however, that a minority of house mice matched the learning performance of *C. glareolus* and *Rattus norvegicus*, and that genetic variation in Morris maze learning in rats is very large. Thus, the dry-land vs. semiaquatic distinction seems barely applicable to our data.

Infrapyramidal Mossy Fiber Variations and Water Maze Learning: Similarities in Voles and Mice

The species differences in water maze learning were predicted surprisingly well by the correlative data from two studies on water maze learning and IIP-MF in mice (Schöpke et al., 1991; Bernasconi-Guastalla et al., 1994). Firstly, the two mouse strains

with genetically different IIP-MF projections, C57BL/6 (IIP-MF/SP-MF index about 53%) and DBA/2 (IIP-MF index about 16%), were also equally successful in escape learning. C57BL/6 showed more spatial search behavior, but DBA/2 compensated by faster swimming during acquisition, like the two vole species. Differences emerged during reversal learning, however; the C57BL/6 mice with the larger IIP-MF projections showed more search behavior around the old platform position but were eventually more efficient in finding the new platform position, again similar to *C. glareolus* in comparison to *M. oeconomus*. The same superiority of reversal learning associated with larger IIP-MF projections was also observed in two samples of random-bred mice. In these animals, significant correlations with the IIP-MF projection were observed for the platform reversal phase only. We reanalyzed the digitally stored swim paths of these earlier studies in order to calculate the degree of thigmotaxis which had not been measured at that time. C57BL/6 and DBA/2 showed moderate differences in thigmotaxis during acquisition, in some trial blocks with significantly more wall swimming in DBA/2. On the other hand, we found a moderate yet significant negative correlation between the average degree of thigmotaxis and the IIP-MF projection in a sample of random-bred mice ($\rho = -0.44$, $n = 24$, $P < 0.05$). Again, this parallels the association of very small IIP-MF projections with thigmotactic wall swimming in *M. oeconomus*. One may note that ineffective search strategies of mice with small IIP-MF projections have also been found in complex water mazes with a totally different geometry (Schwegler et al., 1988).

Thus, the species differences in IIP-MF and associated patterns of spatial learning as observed for *C. glareolus* and *M. oeconomus* appear as accentuated within-species differences seen in laboratory mice: individuals with scarce IIP-MF projections show more thigmotaxis, and swim faster but finally succeed in escaping from the water maze almost as quickly as individuals with more extended IIP-MF projections. The latter, however, show in many cases a more careful and controlled search behavior which appears most effective after platform reversal, in spite of the fact that they waste some time in search for the old platform.

On the other hand, our data indicate that these similarities should not be generalized extensively. Many hybrid mice and some inbred mouse strains show IIP-MF projections that are twice as large as observed in *C. glareolus*, but show inferior scores in searching behavior and overall escape performance. Also, both wild species outperformed mice in escaping from the water tank, regardless of the strategies used. It is likely that this superiority reflects, at least partially, the relatively higher brain weight of the wild voles. A higher degree of such encephalization in *C. glareolus* as compared to *M. oeconomus* may contribute to the behavioral species differences. Lastly, there are probably other unrecognized differences in the brains of these two species partially responsible for their differential learning behavior.

To summarize, the behavioral phenotype of the two vole species in the water maze was in accordance with their size of the IIP-MF, as predicted by correlations between IIP-MF and water maze learning in mice. Obviously, the proportion of IIP-MF does not predict overall escape performance but rather the type of search

strategies in the water maze. Larger IIP-MF projections appear to be associated with a preference for “cognitive” search strategies, or with a superior ability to suppress inappropriate coping responses that are likely to result in less efficient “noncognitive” search strategies.

Mossy Fiber Variations Appear to Be Related to Lifestyle and Habitat

The differential behavior of bank and root voles in the water maze corresponds to qualitative observations of these animals in their natural habitat. A main lifestyle difference between *C. glareolus* and *M. oeconomus* is based on the different feeding strategies. Russian *C. glareolus* are forced to find seeds which are usually dispersed over a large territory, while the root vole does not need seeds (Gromov and Erbaeva, 1995) and usually has abundant food in a chosen locality. Thus, home ranges are larger in bank voles (Okulova and Ignatova, 1964) and are, moreover, much more complex. It is not clear, however, whether there are fundamental sex differences in *C. glareolus* or *M. oeconomus* with respect to home range size, as has been observed in North American voles (Jacobs et al., 1990; Gaulin and Fitzgerald, 1986; Galea et al., 1994a, 1996). Sex differences in home range size have thus far been observed for breeding females of Russian *C. glareolus* only, these having smaller territories, yet not for young animals as investigated here (Okulova and Ignatova, 1964). In any event, the issue deserves further investigation, given the reports on seasonal variation of brain weight in *C. glareolus* (Yaskin, 1984) and other small rodent species (Jacobs, 1996). In particular, an investigation of seasonal differences in IIP-MF and dentate granule cell proliferation (see below) would seem appropriate.

Given the highly variable and larger habitats of *C. glareolus* as contrasted with the homogenous and small habitats of *M. oeconomus*, one can assume that *C. glareolus* underwent a selection for flexible processing of complex information and probably for superior spatial mapping, while the ecological demands for such capacities are less stringent in *M. oeconomus*. It would seem that this selection process has entailed evolutionary changes of the IIP-MF projection.

Also, unpublished observations from other species suggest that size variations of mossy fibers are related to habitat and lifestyle. Large IIP-MF projections appear to be associated with complex environments and/or activities requiring smooth sensory integration of many stimuli into behavioral actions such as food gathering or hunting. Representative species for such a lifestyle are, besides *C. glareolus*, *Apodemus* spp., and shrews (*Sorex* spp.), all of them showing proportionately large IIP-MF projections. On the other hand, species occupying simply structured and smaller habitats that require less elaborate feeding activities include several vole species (*Microtus* spp., *Terricola subterraneus*). All of them have been found to have much smaller IIP-MF projections (unpublished data). The general properties of hippocampal circuitry may be the same in many of these species, but size variations of their IIP-MF projections might provide a convenient natural regulator for increasing or decreasing stability of intrahippocampal parallel

processing according to the ecological profile of a species or even of a subpopulation of a species.

Caveats for Species Comparisons

A well-known problem in comparative approaches is that brain differences between genera reflect a phylogenetic trend and might thus be independent of life style or spatial learning (Harvey and Pagel, 1991). For example, *M. microtus* might have an extremely reduced IIP-MF projection because it is derived from an ancestral form with almost missing IIP-MF. Thus, whatever the actual environment, its IIP-MF projection remains scarce because once in the evolutionary past, the mossy fiber development was developmentally channeled to a *Microtus*-type pattern. This conundrum is inherent in any comparative approach and usually difficult to cope with. For the data here, however, a solution can be presented. The main point is the difference between macroevolution and microevolution and the underlying genes. Genes underlying phylogenetic trends fall into the category of homeotic genes, i.e., genes which switch developmental pathways. Once a developmental pathway is chosen, the phenotype remains phylogenetically irreversible and is often used for taxonomic classification. For example, some ungulate genera carry horns, while other genera carry antlers. However, there is another class of genes, tentatively labeled variobox genes, which encode for variations in pelage, physiognomic traits, and cognitive abilities (Lipp, 1995). These variobox genes are developmental endpoint genes which are characterized by the following properties: 1) they act late during development, thus influencing only few remaining targets, chiefly coat color patterns, physiognomic traits, or late-maturing brain systems such as higher-order association cortex and dentate gyrus; 2) spontaneous mutations occur frequently and are widespread within many species, because these mutations have no immediate impact on biological fitness; and 3) they respond very quickly to artificial and natural selection, thereby forming the genomic substrate for domestication, local within-species and between-species adaptations, and the breeding of intraspecific variations of form, colors, and behavior by fanciers. In the brain, such genes may be closely related to (and sometimes even identical with) genes subserving adult plasticity.

To summarize, in comparing species for correlated neuroanatomical and behavioral traits, it is first necessary to recognize whether the structural brain trait is differentiating early (in which case it is likely to be regulated by homeotic genes) or whether it is developing at a time when most species-specific characteristics have been established. If the latter is the case, one must check for the presence of intraspecific genetic variation and, possibly, for variation within closely related species. Finally, one can check for the presence of adult plasticity, since late-developing systems are likely to maintain some degree of plasticity.

Turning to mossy fibers variations, one may note that the dentate gyrus and the mossy fibers differentiate very late (Slomińska and Geneser, 1997), the development of the IIP-MF projection being sensitive to both genetic factors (as indicated by massive strain differences) and developmental manipulations (Lipp et al., 1988). There is also subtle variation of the mossy fiber

system between closely related wood mouse species (Slomianka and West, 1987; Donovan and Slomianka, 1996), and we have observed (unpublished data) that *M. pennsylvanicus* from Canada have much larger IIP-MF projections than the Russian *M. microtus* described here (but still much less than the North American deer mouse, *Peromyscus leucopus*). With respect to adult plasticity, there is lifelong growth of recurrent mossy fiber collaterals into the granule cell layer (Wolfer and Lipp, 1995), and these collaterals show reactive proliferation after injuries (Reprea et al., 1994). Also, the dentate gyrus of many species shows slow adult proliferation of granule cells, and it has been shown that the rate of this proliferation in mice is under genetic control (Kempermann et al., 1997a,b). Finally, one may note again that seasonal variation of brain size and of the hippocampus has been claimed for *C. glareolus* and other small species, yet not for *Microtus* (Yaskin, 1980, 1984).

Taken together, these data strongly suggest that the intraspecific genetic variation and interspecific variation of the mossy fiber system (and perhaps of other hippocampal traits as well) are caused by a set of polymorphic genes widely distributed in mammals. These polymorphic genes appear to be responsible for a rapid microevolution of the mossy fiber system, as evidenced by quick adaptation of the IIP-MF following artificial selection for differential behavior in mice and rats (Schwegler and Lipp, 1983; Crusio et al., 1989a; Sluyter et al., 1994), and, as we recently showed recently for feralized mice, following natural selection over few generations (Wolfer et al., 1999). Hence, it appears unlikely that size variations of the IIP-MF are caused by developmental channeling through species-specific homeotic genes.

Hippocampal Stability vs. Lability: Implications for Studying Transgenic Mice

Water maze learning has become the main tool to assess cognitive deficits after targeted disruption of genes in mice (Lipp and Wolfer, 1998; Wolfer et al., 1998). Frequently, mutation-dependent impairments of water maze learning, in particular low spatial memory scores as assessed in probe trials, are taken as evidence for hippocampal malfunctioning. The fact that a wild small mammal with an intact hippocampus shows spatial memory scores mimicking the most severe memory impairments as reported for transgenic mice clearly demands cautious interpretation of probe trial data from water maze learning. Another caveat is that thigmotaxis, the most important confounding factor in mouse water maze learning, appears to be associated with reduced IIP-MF projections. Several knockout mutants of our data base showed poor probe trial scores. In about half of these mutations, the poor scores could be attributed to increased thigmotaxis. It is unlikely that all these targeted mutations plus mossy fiber variations act through a single mechanism. However, it is conceivable that many mutations affecting large populations of neurons destabilize, among other things, intrahippocampal parallel processing, similarly as with small IIP-MF projections. The result is a stronger penchant towards the instinctive thigmotactic behavior, which often leads to kinesthetic search strategies and

with that, to a reduction of proper orientational behavior as defined by psychologists.

Thus, both molecular biology and ecological brain research appear to converge finally on a common problem, namely, to find the neurophysiological correlates of differential spatial strategies vs. truly impaired spatial behavior. If instability of hippocampal place cells is the cause for poor learning, as suggested by studies in knockout mice showing impaired spatial learning (Rotenberg et al., 1996; McHugh et al., 1996; Cho et al., 1998), it might indeed be of interest to test whether wild voles with intact brains but differential strategies in spatial testing situations will show stable or unstable place cells.

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