Journal of Chemical Neuroanatomy 37 (2009) 141–148

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

# Consequences of different housing conditions on brain morphology in laying hens

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ARTICLE INFO

*Article history:*
Received 11 August 2008
Received in revised form 2 December 2008
Accepted 4 December 2008
Available online 24 December 2008

*Keywords:*
Adult brain plasticity
Environmental influences
Hippocampus
Nidopallium caudolaterale
Serotonin
Social stress

ABSTRACT

The aim of this study was to analyze the impact of physical and social stress on the avian forebrain morphology. Therefore, we used laying hens kept in different housing systems from puberty (approximately 16 weeks old) until the age of 48 weeks: battery cages, small littered ground pen, and free range system. Cell body sizes and catecholaminergic and serotonergic innervation patterns were investigated in brain areas expected to be sensitive to differences in environmental stimulation: hippocampal substructures and the nidopallium caudolaterale (NCL), a functional analogue of the prefrontal cortex. Our analysis shows both structures differing in the affected morphological parameters. Compared to battery cage hens, hens in the free range system developed larger cells in the dorsomedial hippocampus. Only these animals exhibited an asymmetry in the tyrosine hydroxylase density with more fibres in the left dorsomedial hippocampus. We assume that the higher spatial complexity of the free range system is the driving force of these changes. In contrast, in the NCL the housing systems affected only the serotonergic innervation pattern with highest fibre densities in free range hens. Moreover hens of the free range system displayed the worst plumage condition, which most likely is caused by feather pecking causing an altered serotonergic innervation pattern.

Considering the remarkable differences between the three housing conditions, their effects on hippocampal structures and the NCL were surprisingly mild. This observation suggests that the adult brain of laying hens displays limited sensitivity to differences in social and physical environment induced post-puberty, which warrants further studies.



## 1. Introduction

Environmental complexity is well known to affect the morphology, neurochemistry and physiology of the central nervous system (Rosenzweig and Bennett, 1996; Diamond, 2001; Mohammed et al., 2002; Rosenzweig, 2003). While a physically enriched environment can promote brain development, several factors have attenuating effects on neuronal structures and cognitive abilities. The deleterious effects of social deprivation, as a specific kind of impoverished environment in social living animals was first described by Harlow and Harlow (1966) who demonstrated severe impairments in social and sexual behaviour of rhesus monkeys raised in social isolation. Comparably, early globally deprived human orphans exhibit cognitive and social deficits (Kaler and Freeman, 1994), as well as a significantly decreased metabolism in different brain areas (Chugani et al., 2001). Structural effects of social deprivation were also verified by neurochemical and neuroanatomical changes of forebrain areas in rodent pups (Braun et al., 2000; Ziabreva et al., 2003a,b; Poeggel et al., 2003) and young birds (Gruss and Braun, 1997; Leitner and Catchpole, 2007).

It is known that social and physical aspects of the environment can affect dissociable neuronal substrates (Schrijver et al., 2002, 2004). While, e.g. the hippocampus reacts to environmental enrichment, the mammalian prefrontal cortex (PFC) is especially sensitive to social deprivation. However, both areas display morphological and functional changes in response to chronic stress. Morphological changes (McEwen, 1999; Blanchard et al., 2001; Sapolsky, 2003) and suppressed neurogenesis in the hippocampus as a result of stress are accompanied by modified learning and memory abilities (Mirescu and Gould, 2006). Comparably, chronic stress induces atrophy of the PFC and impairs working memory capacities as well as behavioural flexibility (Cerqueira et al., 2007).

Both the hippocampus and the PFC react with changes in activity and organization of the modulatory monoaminergic

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*Abbreviations:* DM, dorsomedial hippocampus; C, caudal; FRS, free range system; BC, battery cages; mHp, medial hippocampus; NCL, nidopallium caudolaterale; PFC, prefrontal cortex; R, rostral; TH, tyrosine hydroxylase; SLGP, small littered ground pen; 5-HT, serotonin.

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doi:10.1016/j.jchemneu.2008.12.005

networks (Fontenot et al., 1995; Braun et al., 2000; Blanchard et al., 2001; Ziabreva et al., 2003a; Vicentic et al., 2006). Early maternal deprivation as a special kind of social stress (Chugani et al., 2001) decreases dopaminergic, but increases serotonergic innervation of the prefrontal cortex in rodents (Winterfeld et al., 1998; Braun et al., 2000). Comparably, social separation also modulates monoaminergic pathways in the chick's forebrain (Gruss and Braun, 1997).

Despite a general decrease of brain plasticity during aging in mammals (Rosenzweig and Bennett, 1996; Frick and Fernandez, 2003; Rosenzweig, 2003), neuronal changes in adaptation to environmental enrichment and stress appear to be preserved throughout the entire lifespan within the hippocampus and the PFC (Rosenzweig and Bennett, 1996; Blanchard et al., 2001). Since birds display comparable social and cognitive capacities as mammals (Jarvis et al., 2005), a similar vulnerability of analogous structures can be assumed for adult bird brains. Like its mammalian counterpart, the avian hippocampus processes and stores spatial information (Colombo and Broadbent, 2000), displays use-dependent plasticity (Clayton and Krebs, 1994) and is able to generate new nerve cells during the whole life-span (Barnea and Nottebohm, 1994). Similarly, the nidopallium caudolaterale (NCL) of the avian forebrain represents a functional equivalent of the mammalian PFC and is involved in the generation of executive functions (Güntürkün, 2005).

The aim of the present study was to analyze the impact of the post-pubertal physical and social environment onto the neurochemical architecture of the avian hippocampus and NCL. We used commercially reared laying hens that, subsequent to a largely similar early ontogeny in groups without a mother were exposed to three extremely different social and physical environments as adults. We therefore believe that our study provides two different insights: first, it adds knowledge on the environment-induced plasticity of the adult avian hippocampal and 'prefrontal' systems. Second, the methods applied describe a new approach in animal welfare studies of laying hens, and possibly of other production animals, based on objectively measurable parameters that may prove useful in future experiments.

## 2. Method

In the present study, 24 ISA Brown hens were used from three different housing conditions with eight animals per condition: (1) battery cages (BC), (2) small littered ground pen (SLGP) with partially slatted floor, and (3) free range system (FRS) at an organic farm.

Day old chicks were obtained from a commercial hatchery. All hatchlings were vaccinated and gender-sorted before they were transported to two commercial rearing farms. The hens kept in the BC and the SLGP were bred as free range hens in the same stable at one commercial rearing farm. These hens were beak trimmed to reduce the damage due to feather pecking and cannibalism in poultry (Hartini et al., 2002). The FRS hens were bred under similar conditions as the BC and SLGP hens at another commercial rearing farm. They did not undergo beak trimming. Also, the composition of the feed of the FRS hens was different from that of the other hens in that it contained 100% crop products, in accordance with the regulations concerning feed composition in biological farms. At 16 weeks of age, all hens were transported to their respective layer farms, where they were housed for 32 weeks under three different housing conditions.

### 2.1. Housing conditions

#### 2.1.1. Battery cages

The battery cages were wire mesh cubicles, arranged in two tiers. A battery cage measured 45 cm (depth) by 60 cm (width) by 47 cm (height at the front; 43 cm height at the rear) with the floor slightly inclining from the rear to the front. Each cage housed five hens. There was 540 $cm^2$ of floor space per animal. The cages were provided with two drink nipples in the rear and a food trough in front.

#### 2.1.2. Small littered ground pen with partially slatted floor

The small littered ground pen consisted of wire mesh fences, measuring 480 cm (depth) by 300 cm (width) by 200 cm (height). Each pen housed 56 hens. There was approximately 2570 $cm^2$ floor space per animal. The animals in the present study were taken from one pen. The pens were equipped with two food troughs and a central bowel drinker.

Twelve rhomb-shaped group nests were positioned at one side of the pen. The nests were arranged in a triangle, with two nests at the base, a row of four nests at the second story, three nests at the third story, two nests at the fourth story, and one nest on top. All nests were filled for about 1/4 with buckwheat. Along the rear of the pen, a slatted frame, serving as perch, was elevated 45 cm above the floor. The frame measured 200 cm (depth) by 300 cm (width). A board prevented access to the space under the perch. The floor of the pen was covered with a thick layer of wood shavings, for pecking, scratching and dustbathing. The hens had free access to any part of the pen.

Hens in both battery cages and SLGP were fed ad libitum with standard layer mash. Food troughs were replenished twice a day. In addition, the hens in the SLGP were fed once each afternoon with a handful of grains, scattered on the floor.

Both housing systems were situated in the same large stable under natural light conditions. Electric strip lights ensured a light period of at least 16 h (which exceeded 16 h if the natural day length was longer), artificial lights being on from 5:00 to 21:00.

### 2.2. Free range system

A maximum of 12000 hens was group-housed in a stable, measuring 420 $m^2$ floor space, whereas the effective space was approximately 600 $m^2$ through elevated perches. Per animal 18 cm of perch space was available. The stable was sub-divided into four equal-sized compartments, each of them housing 3000 animals. The floor of the stable was covered with a thin layer of sharp, dry sand. Turf-like flooring developed through the continuous mixing of the sand with manure by the foraging hens. Once daily, wheat and broken maize was scattered on the floor. This stimulated intense foraging. Hens were fed with biological layer mash. The food was provided five times a day via a chain system that transported the feed into the food trough. About 14 cm of feeder space was allotted per laying hen. There were seven drink nipples for one hen each that provided water ad libitum. Feeding started at 10:00 in the morning. The stable was equipped with automatic roll-away nests, covered with removable soft AstroTurf® matting. The nest boxes were available for a period of four h, starting at 07:00. Electric strip lights ensured a light period of at least 15 1/2 h (which exceeded 15 1/2 h if the natural day length was longer), artificial lights being on from 5:00 to 20:30. All hens had access to an outdoor run, from 11:00 till the end of the natural light period. The run was covered with mulch and sand. Once a week, a bale of straw was spread over the area. The run extended over an area of 4.8 hectares.

At the age of 48 weeks, after 32 weeks in the respective housing conditions, the hens were transported to the histological laboratory where they were perfused within 4 days. On arrival, a series of photographs was taken from all animals.

### 2.3. Fixation

The animals were injected with 2000 Units heparin, 30 min later they were deeply anesthetised with equithesin (0.9 ml/100 g bodyweight) and perfused though the left ventricle with 0.9% saline (40 °C), followed by 4% paraformaldehyde in 0.12 M PBS (4 °C, pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde in 0.12 M PBS + 30% sucrose for 2 h at 4 °C, cryoprotected in 0.12 M PBS + 30% sucrose at 4 °C for 24 h and cryosectioned in frontal plane (40 μm). The left or right brain side was marked by a hole stuck with a small needle. Slices were collected in ten parallel series and stored in 0.12 M PBS containing 0.1% sodium azide at 4 °C until they were subjected to immunohistochemistry. The study was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

### 2.4. Immunohistochemistry

The immunohistochemical detections of serotonin (5-HT, polyclonal rabbit IgG, Immunostar, USA) or tyrosine hydroxylase (TH, polyclonal rabbit IgG, Chemicon, Germany) were performed with free-floating slices according to the immuno-ABC-technique (Hellmann and Güntürkün, 2001). For each antibody staining, we used one complete serial. After each incubation step, the slices were washed three times for 5 min with PBS. Endogenous peroxidases were blocked with 0.3% $H_2O_2$ in de-ionized water for 30 min. Slices were incubated with 10% normal goat serum in 0.12 M PBS + 0.3% Triton X-100 (PBS-X) for 1 h to block non-specific binding-sites in the tissue. Then the slices were incubated with primary antibody solution (5-HT 1/5000, TH 1/1000 in PBS-X + 1% normal goat serum) for 72 h at 4 °C. The secondary antibody reaction was carried out with biotinylated goat anti-rabbit IgG (1/200 in PBS-X; Vectastain Elite kit, Vector, Burlingame, CA), for 1 h at room temperature. Afterwards, the sections were incubated in an avidin–biotin–peroxidase solution (Vectastain ABC-Elite kit, 1/75 in PBS-X), the peroxidase-activity was detected using a heavy metal intensified 3′3-diaminobenzidine (DAB, Sigma) reaction, modified by the use of 1% β D-glucose/glucoseoxidase (Sigma; Hellmann and Güntürkün, 2001). The sections were mounted on gelatinized slides, dehydrated and coverslipped with Permount (Fisher Scientific, New Jersey, USA). For the cell size measurement corresponding serial sets were stained with cresyl violet.

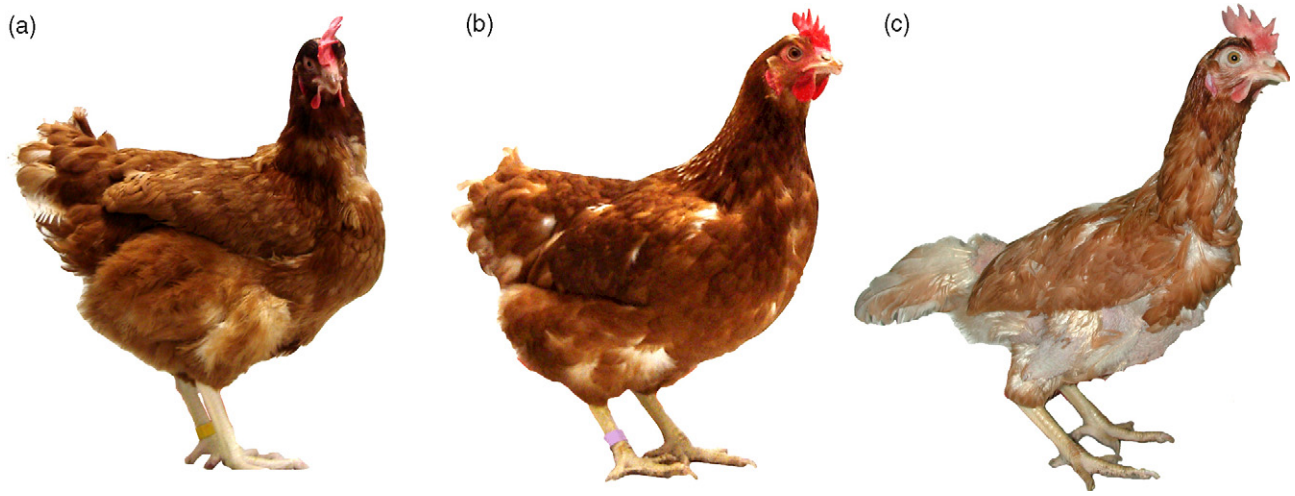


**Fig. 1.** Laying hens from the three different housing conditions: (a) battery cages (BC), (b) small littered ground pen (SLGP) and (c) free range system (FRS).

### 2.5. Plumage condition score

The appearance of the laying hens was evaluated by 21 scorers (age 25.95 ± 4.05) who were blind to the subject of the study. They were shown pictures of each of the 24 hens without knowing about their housing conditions. The scorers had to mark the individual plumages on a questionnaire from 1 (complete) to 7 (strong loss of feathers). To avoid a position effect of the pictures three different picture sets were prepared with each set being randomised by MATLAB R 2006b. For each picture set seven scorers were interviewed.

### 2.6. Data and statistical analysis

To allow blind analysis, slices were coded for group and hemisphere. All analyses were performed in both hemispheres. Hippocampal measurements were conducted in two different areas, the dorsomedial (DM) and in the medial hippocampus (mHp) spanning from the lateral to the medial V-shaped layer including the triangular portion (Atoji and Wild, 2004). The hippocampus was analyzed at A 6.4 (Karten and Hodos, 1967; Fig. 2b). NCL was analyzed in two areas A 5.8 (caudal NCL) and 6.4 (rostral NCL) (Karten and Hodos, 1967; Fig. 3b).

For morphometric analyses, the soma sizes of 50 randomly chosen cells with a clearly visible nucleus were measured in the area of interest of both hemispheres (Manns and Güntürkün, 1999). Fibre densities were determined according to Güntürkün (1998) within two 100 μm × 100 μm$^2$ randomly placed in the area of interest. The squares were subdivided by a micrometer grid with a grid lines distance of 20 μm. All fibres crossing a grid line within the square were counted. All analyses were conducted under 40 × 1.6 magnifications at a Leica DML microscope (DMRE Leica Microsystems, Bensheim, Germany). Sections were scanned into the computer by a video equipped system (TK-C1381 JVC, Friedberg, Germany) which was connected to an image analysis system (analySIS 3.1, Soft Imaging System GmbH, Münster, Germany).

Statistical analysis was carried out with the program Statistica (StatSoft, Tulsa, USA). We compared anatomical data by mixed 2 × 3 ANOVAs with "hemisphere" (left, right) as repeated measure and "housing condition" (BC, SLGP, FRS) as between-subject factors. To account for multiple pair-wise comparisons we used Post hoc Duncan new multiple range tests, which were conducted in case of significant main effects.

## 3. Results

### 3.1. Plumage condition

The condition of the plumage was affected by the housing conditions ($F(2,21) = 63.11$, $p < .001$). Post hoc comparison confirmed that laying hens kept in the FRS had significantly less feathers than the hens from the other two housing conditions (FRS = 6.17 ± 0.64; SLGP = 2.9 ± 0.57; BC = 3.26 ± 0.69; $p > .001$). There were no significant differences in plumage condition between the SLGP and the BC hens ($p = .29$; Fig. 1).

### 3.2. Hippocampus

Generally, the DM displayed significantly larger cells (113.37 ± 18.35 μm$^2$; ranging from 78.74 μm$^2$ to 155.21 μm$^2$) than the mHp (101.27 ± 23.27 μm$^2$; ranging from 56.05 μm$^2$ to 165.47 μm$^2$) in all three "housing conditions" (*t*-test: $t(94) = -2.83$, $p < .01$; Table 1; Fig. 2a). Statistical analysis revealed no significant main effects in mHp ("housing condition": $F(2,21) = 2.72$, $p = .09$; "hemisphere": $F(1,21) = 1.77$, $p = .20$) as well as no significant interaction between "hemisphere" and "housing condition" ($F(2,21) = 0.07$, $p = .93$). In contrast, cell sizes of the DM differed significantly between the groups ($F(2,21) = 4.47$, $p < .05$). FRS hens displayed significantly larger cells (124.19 ± 18.86 μm$^2$; ranging from 79.99 μm$^2$ to 155.21 μm$^2$) than BC hens (104.06 ± 13.54 μm$^2$; ranging from 78.74 μm$^2$ to 127.56 μm$^2$; Post hoc Duncan new multiple range tests $p < .01$; Table 1; Fig. 2a). Neither significant effect of "hemisphere" ($F(1,21) = 2.43$, $p = .13$) nor significant interaction between

**Table 1**
Results of the hippocampus.

| Brain area | Measured parameter | | FRS | SLGP | BC |
|---|---|---|---|---|---|
| Hippocampus | | | | | |
| DM | Cell size [μm$^2$] | Right | 127.19 ± 15.69 | 112.09 ± 14.55 | 110.19 ± 10.54 |
| | | Left | 121.17 ± 22.26 | 111.63 ± 20.58 | 97.93 ± 13.99 |
| | TH fibre density/10,000 μm$^2$ | Right | 40.88 ± 31.04 | 57.00 ± 24.55 | 64.38 ± 24.84 |
| | | Left | 59.50 ± 23.91 | 50.00 ± 24.42 | 68.88 ± 12.53 |
| | 5-HT fibre density/10,000 μm$^2$ | Right | 20.00 ± 12.35 | 15.63 ± 9.57 | 18.88 ± 17.85 |
| | | Left | 19.00 ± 13.12 | 12.75 ± 11.98 | 17.25 ± 11.41 |
| mHP | Cell size [μm$^2$] | Right | 118.12 ± 21.38 | 96.81 ± 13.81 | 96.10 ± 17.57 |
| | | Left | 111.78 ± 32.83 | 91.78 ± 13.17 | 93.02 ± 27.23 |
| | 5-HT fibre density/10,000 μm$^2$ | Right | 9.75 ± 5.92 | 6.5 ± 4.41 | 7.38 ± 8.26 |
| | | Left | 7.00 ± 4.14 | 5.12 ± 2.75 | 8.63 ± 7.39 |

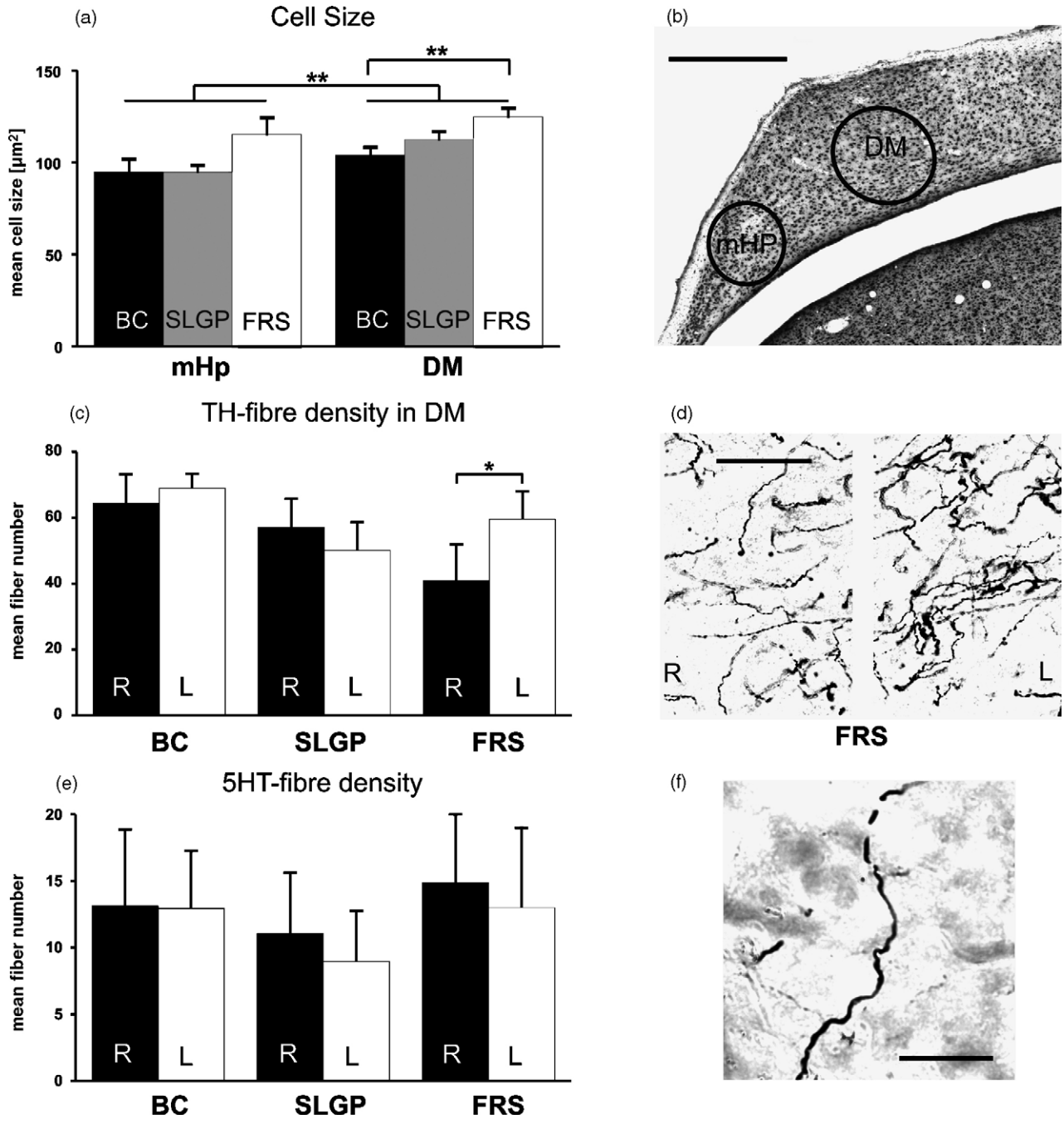


**Fig. 2.** Effects of the three different housing conditions (BC = battery cages; SLGP = small littered ground pen; FRS = free range system) on the hippocampus. (a) Mean cell size in $\mu m^2$ within the mHp and DM. (b) Cresyl violet staining of the hippocampus with the two analyzed areas, dorsomedial hippocampus (DM) and medial hippocampus (mHP). (c) TH fibre density per 10,000 $\mu m^2$ within the right (R) and left (L) DM. (d) TH fibre staining of the FRS DM, right (R) and left (L) side. (e) 5-HT fibre density per 10,000 $\mu m^2$ within the right (R) and left (L) hippocampus. (f) 5-HT fibre staining of the DM. $^*p < .05$, $^{**}p < .01$, scale bar: (b) 500 µm, (d) 50 µm and (f) 20 µm. Arrow bars represent standard errors.

"hemisphere" and "housing condition" ($F(2,21) = 0.72$, $p = .49$) were detected within the DM for cell size.

In the hippocampus, an en-passant type of the TH innervation could be observed with fibres travelling in close vicinity along the somata and dendrites of target-neurons displaying a large number of bouton-like axonal swellings (Fig. 2d). While the TH innervation density was very high in the DM, only a few terminating fibres could be detected in the mHp. Therefore, we confined our quantitative analysis to DM.

There were no main effects of "housing condition" ($F(2,21) = 1.20$, $p = .33$) or "hemisphere" ($F(1,21) = 2.18$, $p = .15$) for the TH fibre density. However, the interaction between "hemisphere" and "housing condition" reached significance ($F(2,21) = 4.14$, $p < .05$). Post hoc comparison revealed that FRS hens exhibited an asymmetry in the density of TH-positive fibres with a stronger innervation of the left DM (Post hoc Duncan new multiple range tests $p < .05$; Table 1; Fig. 2c).

Serotonergic innervation density was likewise higher in DM ($17.25 \pm 12.51$/10,000 $\mu m^2$) compared to mHp ($7.39 \pm 5.68$/10,000 $\mu m^2$; $t$-test: $t(94) = 4.97$, $p < .001$). Statistical analysis neither demonstrates significant effect of "housing condition" (DM $F(2,21) = 0.45$, $p = .64$; mHp $F(2,21) = 0.59$, $p = .56$), "hemispheres" (DM $F(1,21) = 0.59$, $p = .45$; mHp $F(1,21) = 0.71$, $p = .41$) nor significant interactions of "hemispheres" and "housing condition" (DM

**Table 2**
Results of the NCL.

| Brain area | Measured parameter | | FRS | SLGP | BC |
|---|---|---|---|---|---|
| Nidopallium caudolaterale | | | | | |
| Rostral NCL | Cell size [$\mu m^2$] | Right | 97.44 ± 18.63 | 91.60 ± 14.06 | 89.78 ± 14.06 |
| | | Left | 93.04 ± 28.41 | 95.78 ± 12.93 | 95.14 ± 17.77 |
| | TH fibre density/10,000 $\mu m^2$ | Right | 109.31 ± 72.57 | 134.06 ± 65.99 | 92.69 ± 28.06 |
| | | Left | 123.75 ± 46.52 | 112.75 ± 48.43 | 103.13 ± 44.15 |
| | 5-HT fibre density/10,000 $\mu m^2$ | Right | 9.38 ± 14.48 | 3.75 ± 3.01 | 9.63 ± 10.94 |
| | | Left | 6.50 ± 6.88 | 2.88 ± 3.99 | 5.00 ± 4.11 |
| Caudal NCL | Cell size [$\mu m^2$] | Right | 64.80 ± 10.48 | 60.00 ± 7.22 | 64.98 ± 9.17 |
| | | Left | 61.37 ± 7.36 | 5 7.61 ± 4.72 | 60.52 ± 8.32 |
| | TH fibre density/10,000 $\mu m^2$ | Right | 116.44 ± 78.40 | 101.00 ± 53.39 | 76.19 ± 44.46 |
| | | Left | 118.31 ± 59.38 | 109.31 ± 51.47 | 99.88 ± 54.04 |
| | 5-HT fibre density/10,000 $\mu m^2$ | Right | 11.87 ± 10.05 | 2.00 ± 2.33 | 8.63 ± 9.56 |
| | | Left | 8.87 ± 7.47 | 2.88 ± 3.40 | 8.13 ± 6.10 |

$F(2,21) = 0.05$, $p = .95$; mHp $F(2,21) = 1.07$, $p = .36$) in both hippocampal subareas (Table 1; Fig. 2e and f).

### 3.3. NCL

Within all groups, rostral NCL displayed larger cell bodies ($93.80 \pm 17.71\ \mu m^2$; ranging from $61.30\ \mu m^2$ to $141.29\ \mu m^2$) compared to the caudal one ($61.55 \pm 8.08\ \mu m^2$; ranging from $46.48\ \mu m^2$ to $83.89\ \mu m^2$; *t*-test: $t(94) = -11.48$, $p < .001$). There was neither a main effect of "housing condition" (caudal $F(2,21) = 0.99$, $p = .38$; rostral $F(2,21) = 0.06$, $p = .94$), "hemisphere" (caudal $F(1,21) = 3.6$, $p = .07$; rostral $F(1,21) = 0.23$, $p = .64$), nor significant interaction of "hemispheres" and "housing condition" (caudal $F(2,21) = 0.11$, $p = .90$; rostral $F(2,21) = 0.73$, $p = .49$; Table 2; Fig. 3a) for cell sizes.

Apart from some en-passant fibres, TH positive fibres in the NCL showed a so-called basket-type pattern with single fibres densely coiled around the somata and initial dendrites of postsynaptic targets (Fig. 3d; Durstewitz et al., 1998). The TH fibre density was very high in the NCL but did not differ between caudal ($103.52 \pm 56.34/10{,}000\ \mu m^2$) and rostral ($112.61 \pm 51.93/10{,}000\ \mu m^2$) areas (*t*-test: $t(94) = -0.82$, $p = .41$). Statistical analysis neither demonstrates significant effect of "housing conditions" (caudal $F(2,21) = 0.90$, $p = .42$; rostral $F(2,21) = 0.61$, $p = .55$), "hemispheres" (caudal $F(1,21) = 0.55$, $p = .47$; rostral $F(1,21) = 0.02$, $p = .90$) nor significant interactions of "hemispheres" and "housing condition" (caudal $F(2,21) = 1.81$, $p = .84$; rostral $F(2,21) = 1.41$, $p = .27$; Table 2; Fig. 3c).

Compared to the TH fibre density, serotonergic innervation was much lower. Only a few fibres could be detected within the whole telencephalon. No differences were revealed between caudal ($7.06 \pm 7.58/10{,}000\ \mu m^2$) and rostral ($6.19 \pm 8.32/10{,}000\ \mu m^2$) areas (*t*-test: $t(94) = 0.54$, $p = .59$). While no main effects were observed in the rostral NCL ("housing condition" $F(2,21) = 0.96$, $p = .40$; "hemisphere" $F(1,219 = 2.67$, $p = .18$) as well as no significant interaction of "hemispheres" and "housing condition" ($F(2,21) = 0.40$, $p = .67$). In the caudal NCL the main effect of the "housing condition" approached significance ($F(2,21) = 3.29$, $p = .057$) indicating a higher density of 5-HT fibres in the caudal NCL of FRS hens ($10.37 \pm 8.69/10{,}000\ \mu m^2$) compared to the caudal NCL of SLGP hens ($2.44 \pm 2.85/10{,}000\ \mu m^2$; Post hoc Duncan new multiple range tests $p < .05$; Table 2; Fig. 3e and f). Neither a main effect of "hemispheres" in the caudal NCL ($F(1,21) = 0.58$, $p = .48$) nor a significant interactions of "hemispheres" and "housing condition" ($F(2,21) = 0.854$, $p = .44$) were detected for 5-HT fibre density.

## 4. Discussion

Our study shows that the different housing conditions of commercially reared laying hens lead to dissociable neuroanatomical effects in the hippocampus and NCL of their brains. These data indicate that both analyzed areas retain their lifelong neuronal plasticity but react to distinct aspects of the environment.

### 4.1. Morphometric alterations in the hippocampus indicate sensitivity to environmental complexity

Both the avian and the mammalian hippocampus play a central role in the processing of spatial information (Atoji and Wild, 2006; Bingman and Sharp, 2006). The correspondences between different avian and mammalian hippocampal substructures are still a matter of debate. Different studies suggest that the avian V-shaped layer is comparable to the mammalian dentate gyrus, while the avian DM incorporates components comparable to the mammalian Ammons' horn and the subiculum (Atoji and Wild, 2004, 2006). Our morphological analysis demonstrating larger cell sizes, a higher serotonergic innervation density, and a virtually exclusive TH-positive innervation of the DM compared to the mHP underlines the anatomical differences of these two areas. In contrast to most other telencephalic areas of the bird brain, hippocampal TH-immunolabeling mainly represents noradrenergic, but not dopaminergic fibres (Durstewitz et al., 1999). Since the noradrenergic innervation of the mammalian hippocampus is highest within area CA1/3 as well, the TH-immunolabeling pattern further supports that the DM corresponds to the mammalian Ammon's horn (Atoji and Wild, 2006).

Morphometric differences between the groups were confined to the DM. Cell sizes in the DM were significantly larger in FRS compared to BC birds. Hippocampal experience-dependent plasticity to environmental enrichment is preserved throughout the entire lifespan both in mammals (Kempermann et al., 1997; Nilsson et al., 1999) and birds (Clayton and Krebs, 1994). In rats for example, exposure to an enriched environment leads to increased volumes. Chicks, which were reared with visual barriers were found to have improved spatial memory (Freire et al., 2004) and increased hippocampal dendrite lengths (Freire and Cheng, 2004). It should be noted, that the available space per individual was about identical for FRS and BC birds. Thus, the hippocampal difference in cell sizes was not the result of the available space *per se* but of the spatial complexity of the surrounding. In addition, these data support the notion of a life-long plasticity in the avian hippocampus.

Only FRS hens displayed an asymmetry of the hippocampal TH-innervation with less TH-immunopositive fibres in the right hippocampus. An accumulating body of evidence indicates that the avian hippocampus is functionally and anatomically lateralized (Tommasi et al., 2003; Freire and Cheng, 2004; Bingman et al., 2006), whereby left and right sides preferentially analyze different aspects of the space (Tommasi et al., 2003; Prior, 2006; Bingman and Sharp, 2006; Nardi and Bingman, 2007). This goes along with lateralized responses to environmental factors such as spatial complexity (Denenberg et al.,

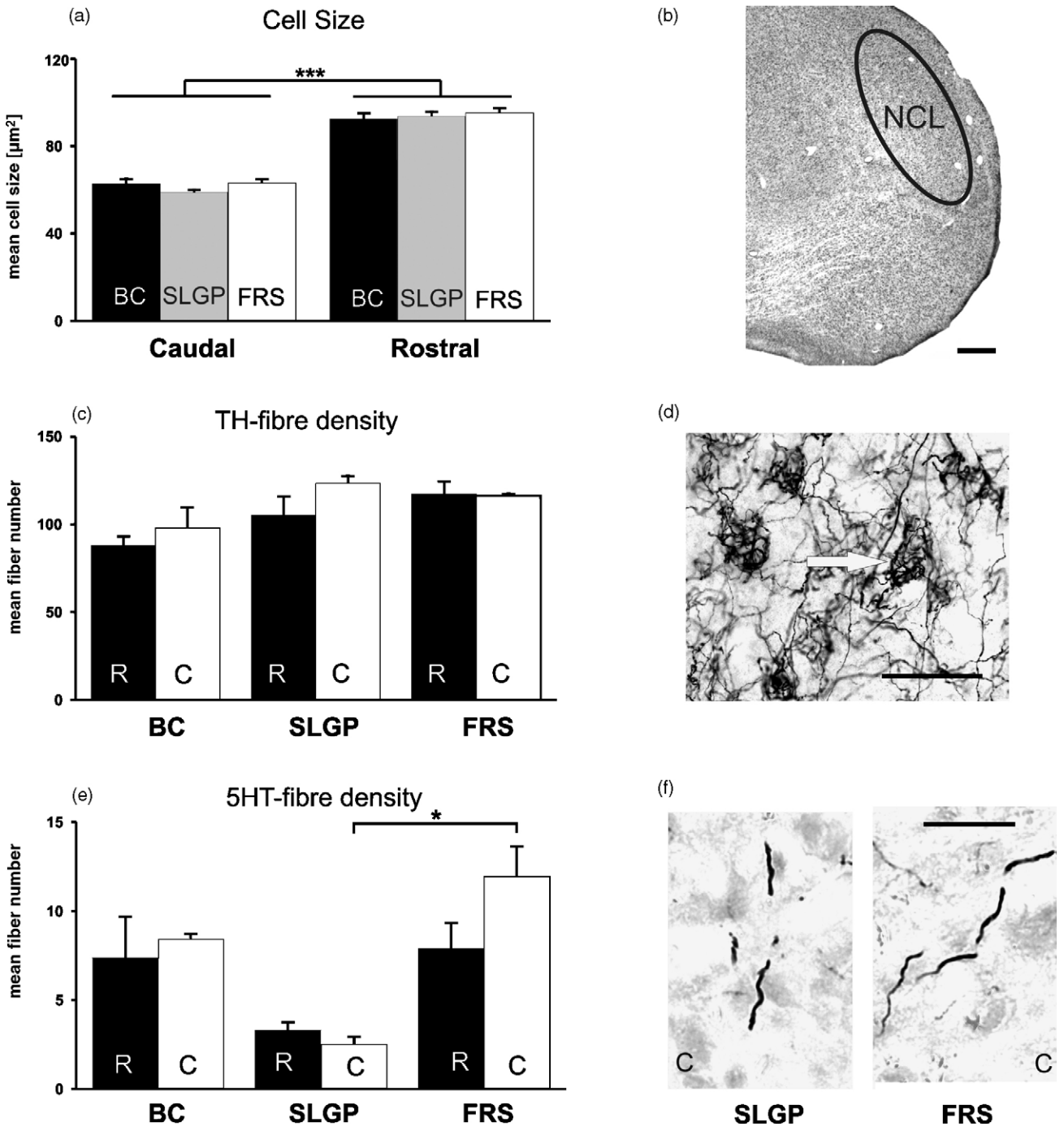


**Fig. 3.** Effects of the three different housing conditions (BC = battery cages; SLGP = small littered ground pen; FRS = free range system) on the NCL. (a) Mean cell size in $\mu m^2$ within the rostral and caudal NCL. (b) Cresyl violet staining, mark indicates the analyzed area. (c) TH fibre density per 10,000 $\mu m^2$ within the rostral (R) and caudal (C) NCL. (d) TH-fibre staining of the NCL, arrow indicates a basket. (e) 5-HT fibre density per 10,000 $m^2$ within the rostral (R) and caudal (C) NCL. (f) 5-HT fibre staining of the NCL. $^*p < .05$, $^{***}p < .001$, scale bar: (b) 1000 µm, (d) 50 µm and (f) 20 µm. Arrow bars represent standard errors.

1978; Miu et al., 2006) or stress (Neveu and Merlot, 2003). We assume that the higher complexity of the enclosure of FRS hens was the reason, why the TH-asymmetry was only visible in this group.

### 4.2. Alterations of the nidopallial serotonergic innervation pattern indicate impact of social stress

The NCL of the avian brain is a multisensory associative area, which represents a functional equivalent to the mammalian prefrontal cortex (PFC) (Divac et al., 1985; Güntürkün, 1997; Durstewitz et al., 1998; Kröner and Güntürkün, 1999). Rostral and caudal NCL differ in connectivity (Kröner and Güntürkün, 1999) and, as shown here, in cell size. This rostro-caudal pattern was not affected by the different housing conditions.

Different from the hippocampus, TH-fibres within the NCL mainly represent dopaminergic and not noradrenergic fibres (Durstewitz et al., 1999). No group-specific TH-difference could be observed. However, the 5-HT innervation displayed differences between the groups with significantly higher fibre densities in the NCL of FRS hens. The serotonergic system plays a critical role in neural responses to stress (Sodhi and Sanders-Bush, 2004) and is especially affected by social stress (Blanchard et al., 2001). As a

consequence, chronic stress severely disrupts key processes of the PFC (Cerqueira et al., 2007). Accordingly, the observed differences in serotonergic innervation density may represent stress-related responses.

At first glance, the assumption of higher stress conditions in FRS hens might seem surprising, since these animals are kept in the seemingly most natural housing conditions. Moreover, it has been shown that exposure to an enriched environment attenuates stress responses in mice (Benaroya-Milshtein et al., 2004). However, neither the sex composition (McBride et al., 1969) nor the group size (Collias and Collias, 1967) were normal for FRS hens. Under natural living conditions, chicken live in small groups of 10–30 animals establishing a stable hierarchy, which is based on individual recognition. We do not know if a hierarchy was established in the SLGP condition, but it is more likely than in a group of 3000 animals. Furthermore, FRS hens displayed the worst physical conditions with bad plumage (Fig. 1c) indicating high levels of feather pecking resulting in stress and therefore altered serotonergic levels (Blanchard et al., 2001; van Hierden et al., 2002, 2004).

Changes of the serotonergic system were confined to the nidopallium and were absent in the hippocampus. This indicates that the serotonergic fibre systems within the hippocampus and the NCL react differently. Given the fact that especially the mammalian hippocampus is known to display structural damage or impaired synaptic plasticity in response to chronic stress (Sousa et al., 2000; McEwen, 2001), this finding is rather surprising. However, in contrast to mammals, no glucocorticoid receptor immunoreactive cell nuclei are found in the avian hippocampal region (Kovács et al., 1989) indicating that the bird hippocampus is not a primary target of stress related hormones. Moreover, the PFC of the mammalian brain is directly connected with the hippocampus via axons originating in the subiculum and ventral CA1 region (Jay and Witter, 1991; Tierney et al., 2004), which are strongly involved in learning and memory processes (Wall and Messier, 2001). Chronic stress impairs the synaptic plasticity of this connection inducing the dysfunction/stress related response of the PFC. Such a strong connection between the hippocampus and the NCL does not exist in birds (Kröner and Güntürkün, 1999; Atoji et al., 2002; Atoji and Wild, 2004). Although we cannot exclude that the avian hippocampus displays stress related plasticity at another cellular level, like, e.g. receptor densities, the avian brain might possess stress responsive circuits, which differ from those identified in mammals.

### 4.3. Effects of commercial rearing conditions

Compared to the substantial differences in the housing conditions the investigated laying hens were exposed to, the present study could only detect surprisingly few long-term structural changes in the brain. We cannot exclude that other parameters like dendritic branching or spine density were affected by the differential housing conditions (Rosenzweig and Bennett, 1996). But since we explicitly selected neuronal parameters and areas, which are well known to be influenced by the complexity of the physical and social environment in young and adult mammals as well as in young chicken, our results may suggest that the adult bird brain is less responsive to differences in the environment compared to young animals. On the other hand, it is conceivable that the observed neuronal pattern represents a common and long lasting effect of traumatic experience. All three groups of hens shared a similar, possibly stress-inducing early ontogeny until the age of 16 weeks. It is conceivable that these shared experiences exerted irreversible effects onto the chicken brain that reduced adult plasticity. In fact, laying, brooding and rearing conditions have been shown to have major effects on behavioural development and social behaviour (Rodenburg et al., 2008). Maternal deprivation during early postnatal stage can have serious consequences for social and affective behaviour (Card et al., 2005) and stress response (Mirescu et al., 2004). However as the production systems differed in many aspects (e.g. feed, group size, cage size) it is impossible to assign neuroanatomical differences in the hens kept in the different systems to a single factor. This was not the aim of the study. We wanted to assess whether evidently different housing conditions during the laying period would affect brain anatomy. To assess the effects of different factors on the neuroanatomy of laying hens, strictly controlled experiments are needed which manipulate the factor(s) under study and keep all other factor constant. These experiments, however, will not be able to mimic the production systems, because they differ in too many (sometimes uncontrollable) aspects.

In summary, our results provide evidence for a structure- and hemisphere-specific sensitivity to social and inanimate housing aspects which affect neuronal elements in higher forebrain areas of adult laying hens. The differential effects of the housing conditions on hippocampal and prefrontal alterations were probably related to spatial and stress-related factors, respectively. Since the FRS produces the most crowed social interactions, this rearing condition enhances social stress and hence, does not represent conditions appropriate for the species. All in all, differences of the analyzed morphometric parameters were rather small between the housing conditions. Since this might be caused by impoverished breeding and pre-pubertal rearing conditions, the effects of these conditions on brain development and brain plasticity deserve further investigation.

## Acknowledgements

The authors would like to thank Ernst Beitler and Jan Vroegindeweij for supplying the laying hens used in the present study and to Ariane Schwarz for her great support in the laboratory.

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