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Research report

Serotonin release in the caudal nidopallium of adult laying hens genetically selected for high and low feather pecking behavior: An *in vivo* microdialysis study

Marjolein S. Kops[a,*], Joergen B. Kjaer[b], Onur Güntürkün[c], Koen G.C. Westphal[a], Gerdien A.H. Korte-Bouws[a], Berend Olivier[a], J. Elizabeth Bolhuis[d], S. Mechiel Korte[a]

[a] Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, Utrecht, The Netherlands
[b] Friedrich Loeffler Institut, Institute for Animal Welfare and Animal Husbandry, Celle, Germany
[c] Department of Psychology, Ruhr-University of Bochum, Bochum, Germany
[d] Adaptation Physiology Group, Wageningen University, Wageningen, The Netherlands

HIGHLIGHTS

- Selection on high and low feather pecking (FP) affects serotonergic brain levels.
- High FP (HFP) had higher serotonin release in the caudal nidopallium than low FP (LFP).
- Serotonin release levels after D-fenfluramine were similar between lines.

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ABSTRACT

Severe feather pecking (FP) is a detrimental behavior causing welfare problems in laying hens. Divergent genetic selection for FP in White Leghorns resulted in strong differences in FP incidences between lines. More recently, it was shown that the high FP (HFP) birds have increased locomotor activity as compared to hens of the low FP (LFP) line, but whether these lines differ in central serotonin (5-hydroxytryptamine, 5-HT) release is unknown. We compared baseline release levels of central 5-HT, and the metabolite 5-HIAA in the limbic and prefrontal subcomponents of the caudal nidopallium by *in vivo* microdialysis in adult HFP and LFP laying hens from the ninth generation of selection. A single subcutaneous D-fenfluramine injection (0.5 mg/kg) was given to release neuronal serotonin in order to investigate presynaptic storage capacity. The present study shows that HFP hens had higher baseline levels of 5-HT in the caudal nidopallium as compared to LFP laying hens. Remarkably, no differences in plasma tryptophan levels (precursor of 5-HT) between the lines were observed. D-fenfluramine increased 5-HT levels in both lines similarly indirectly suggesting that presynaptic storage capacity was the same. The present study shows that HFP hens release more 5-HT under baseline conditions in the caudal nidopallium as compared to the LFP birds. This suggests that HFP hens are characterized by a higher tonic 5-HT release.

1. Introduction

Severe feather pecking (FP) is the pecking at and pulling out of feathers of conspecifics. This detrimental behavior causes welfare problems in laying hens and has multifactorial causes [1–3]. Genetic studies have shown a moderate heritability of FP [4,5] with genetic variations in several genes of the monoaminergic systems that seem to be related to FP behavior [6–8]. From neurobiological and pharmacological studies there is indeed a growing body of evidence on the involvement of brain monoamines such as serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) in the propensity to develop FP [7,9–14]. Comparing brain monoamine levels in young chickens from commercial lines selected on production traits (*e.g.* egg size and egg quality), unintentionally also differed in levels of FP, revealed that the young chickens of the line with higher FP levels had lower 5-HT and DA turnover ratios

* Corresponding author. Tel.: +31 620274192; fax: +31 302537900.
E-mail addresses: m.s.kops@uu.nl (M.S. Kops), Joergen.Kjaer@fli.bund.de (J.B. Kjaer), onur.guentuerkuen@ruhr-uni-bochum.de (O. Güntürkün), K.G.C.Westphal@uu.nl (K.G.C. Westphal), G.A.H.Korte@uu.nl (G.A.H. Korte-Bouws), B.Olivier@uu.nl (B. Olivier), Liesbeth.Bolhuis@wur.nl (J.E. Bolhuis), S.M.Korte@uu.nl (S.M. Korte).

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than the line with lower FP [13]. Treatment of these young chickens with a tryptophan-rich diet [9] or pharmaceutical D2 receptor antagonist such as haloperidol [15] was very effective at reducing gentle FP ratios by increasing brain 5-HT and DA levels. In contrast, more gentle FP incidences were recorded after decreasing 5-HT levels by inhibiting 5-HT release *via* a 5-HT1A autoreceptor agonist [16]. Thus gentle FP may be related to low turnover of central 5-HT and DA as shown in the rostral forebrain of young chickens. It should be noted, though, that severe FP is mostly prevalent at an adult age [1,17]. Differences in brain monoamine levels were inconsistent in adult chickens of commercial lines selected for traits other than FP, that coincidentally also affected FP [18–20]. In 1997, Kjaer and Sørensen [5] started genetically selecting chickens on their individual display of severe FP behavior. This resulted in experimental lines called the high (HFP) and low (LFP) feather pecking selection lines. Next to strong divergences in FP ratios in the third and following generations [21,22], the HFP compared to LFP were more motivated to eat feathers [23] – a behavior related to FP [2,24] – and differed in gut flora [25] and had also increased locomotor activity in their home cage as compared to LFP [26].

The objective of the current study was to measure the release of monoamines in the extracellular synaptic cleft in the caudal nidopallium of adult HFP and LFP hens by *in vivo* microdialysis. The nidopallium is a large associative area in the chickens' forebrain with a potential role in the guiding of motor actions and decision making [27–29]. The caudolateral nidopallium (NCL) receives, more than the caudocentral nidopallium (NCC), input from monoaminergic systems and serves frontal-like executive functions [30]. The NCC displays a limbic connectivity [31,32]. Both NCL and NCC have reciprocal projections to the arcopallium intermedium, a somatosensory area, and the arcopallium mediale, a limbic region [31,33,34]. Both nidopallic regions contain serotonergic [35] and dopaminergic afferents [36] and receptors [37–39]. In contrast to taking samples of brain tissue [18] or measuring 5-HT blood concentrations (*e.g.* [40,41] *in vivo* microdialysis allows the measurement of the extracellular monoamine release within a short timeframe in a particular brain area of conscious freely moving animals. Appropriate central monoamine release is essential for stimulation of pre- and postsynaptic monoamine receptors corresponding with adequate stimulation of the second messenger systems or target organs (see review on 5-HT metabolism by [42]. Although microdialysis is used to study, for instance, the role of monoamines in imprinting [33,43,44] and feed intake [45] in young chickens, as far as we know, microdialysis has never been performed in adult chickens. A second objective was to compare blood plasma concentrations of tryptophan (precursor of brain 5-HT [46]) between the HFP and LFP lines to establish whether potential line differences in the release of monoamines in the brain might have a peripheral cause (*e.g.* by diet) or whether there is evidence for an altered synthesis and/or release in the brain. Here we investigate whether divergent selection for FP produces differences in serotonergic neurotransmission in the forebrain of adult laying hens.

2. Materials and methods

2.1. Ethical statement

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University, The Netherlands, and found to be in accordance with Dutch legislation on the treatment of experimental animals, the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

2.2. Animals and housing

White Leghorn hens from the 9th generation of divergently selected lines, the HFP and LFP, were used. Details regarding the selection procedure have been described previously [21,22]. Eggs of both HFP and LFP birds were brooded and after hatch, the one-day old female chicks received a health check followed by a neck tag with a color/number combination for identification. In total 84 female chicks were distributed over 12 pens (42 chicks/line; $n = 7$/pen). Birds were not beak-trimmed. The chicks were housed in pens with a concrete floor (1.9 by 1.2 m) covered with paper (first 7 weeks) or sawdust (after week 7). Water and a commercial mash diet were provided *ad libitum*: a starter diet (week 1–5), a grower diet (week 6–16) and a layer diet (from week 17 onwards). Each pen had a 50 cm high perch installed and a lower perch (a block of wood) in the first seven weeks. In week 8, each group was reduced by one chicken (used for another experiment). By that time, three chicks turned out to be male and 4 chicks had died within the first week. Therefore, the total group size was 65 animals (LFP: $n = 32$; HFP: $n = 33$). Continuous light was given the first week, and then 18 h of light (week 2) followed by 13 h (week 2–3), and 10 h of light (week 4–15). From 17 weeks of age onwards, the light period was extended by 1 h per week, until the birds had 16 h of light (2.00 am–6.00 pm) at 23 weeks of age.

2.3. HPLC-ECD determination of large neutral amino acids (LNAA) in blood plasma

At 17 weeks, blood taken from the wing vein was collected in a 4 ml EDTA tube and put on ice. Samples were centrifuged and 200 μl plasma was put in a 1 ml serum tube and stored at −70 °C until analysis. Large neutral amino acids (LNAA), such as tryptophan (TRP), L-valine (Val), I-methionine (Met), leucine (Leu), I-isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), and the internal standard I-norleucine (NLeu) were detected simultaneously using an ultra-high performance liquid chromatography (UHPLC) with electrochemical detection using an Alexys 110 LC-EC analyzer (Antec, Zoeterwoude, The Netherlands). The system consisted of two pumps, one autosampler with a 1.5 μl loop, a column (Acquity UPLC HSS T3 1.0 mm × 50 mm, 1.8 μm particle size, Waters, Milford, USA), a μVT-03 detector flow cell with glassy carbon working electrode (potential setting +0.85 V *vs.* Ag/AgCl). The column and detector cell were kept at 40 °C in a column oven. Stock solutions of the amino acid were prepared in Milli-Q water and stored at −70 °C. To 20 μl of plasma 80 μl 100% methanol was added and subsequently vortex mixed. Then 20 μl 0.5 mM NLeu was added and vortex mixed. After 10 min on ice the samples were centrifuged during 10 min at 15,000 × *g*. Subsequently 20 μl of the supernatant was added to 60 μl of 0.05 M sodium borate buffer pH 10.4, mixed and pipetted into autosampler vials. During analysis the samples were kept at 4 °C in the autosampler. Primary amino acids in the sample were derivatized pre-column [47] using a reagent consisting of 37.5 mM o-Phtalaldehyde (OPA) (Pickering Laboratories, USA), 50 mM sodium sulphite, 90 mM sodium borate buffer pH 10.4. This reagent was prepared by mixing a 0.75 M OPA solution (prepared in methanol) with a 1 M sodium sulphite solution (in Milli-Q water) and a 0.1 M sodium borate buffer pH 10.4 (mixing ratio 1:1:18). The derivatization was performed automatically in-line using the autosampler. A 9 μl sample was mixed with 0.5 μl reagent just prior to the analysis. Separation was achieved using mobile phase A (50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, pH 4.5, 8% acetonitril, 10% methanol). As soon as the compounds of interest were completely detected a step gradient using mobile phase B (50 mM phosphoric acid, 50 mM citric acid, 0.1 mM EDTA, pH 4.5, 60% acetonitril) was applied to rinse the column removing any late eluting compounds. The flow rate was set at

200 μl/min. The chromatogram was recorded and analyzed using a Clarity data system (Antec, Zoeterwoude, The Netherlands). Concentrations of LNAAs in the sample chromatograms were calculated using a calibration curve and corrected for recovery variations using the internal standard. The limit of detection for TRP was 50 nM (signal to noise ratio 1:3). Met and Val peaks overlapped. Concentrations were depicted in μM (is mol/l). The TRP/LNAA ratio was determined by dividing TRP concentrations by the sum of the other LNAA (Tyr + (Met + Val) + Ile + Leu and Phe) [48].

2.4. Chemicals and drugs

TRP, Tyr, Val, Met, Leu, Ile, Phe, NLeu were obtained from Sigma Aldrich, USA. Citric acid, phosphoric acid, ethylenediaminetetraacetic acid disodium salt (EDTA), sodium hydroxide, potassium chloride, and, 1-Octanesulfonic acid sodium salt (OSA) were obtained from Acros Organics, Belgium. Boric acid was obtained from Merck, Germany. Acetonitril and methanol were obtained from Biosolve BV, The Netherlands, and o-Phtalaldehyde (OPA) from Pickering Laboratories, USA. Sevoflurane (SevoFlo, Abbott Animal Health, Chicago, IL, USA) was used to induce a rapid and effective general anesthesia. Carprofen (Rimadyl, 50 mg/mL, Pfizer Animal Health, Capelle a/d IJssel, The Netherlands; 5 mg/kg BW SC) and butorphanol (Dolorex 10 mg/mL, Merck Animal Health, Schiphol-Rijk, The Netherlands; 1 mg/kg BW SC) were used as general analgesia. Local analgesia on the skull was alfacaine (2%) with adrenaline (Alfasan Nederland BV, Woerden, The Netherlands). D-fenfluramine (Sigma Aldrich, USA) was dissolved in saline and administered after baseline measurements during microdialysis, into the inguinal region at a dose of 3.0 mg/kg BW SC (diluted to a volume of 0.5 ml/kg). D-fenfluramine is a serotonin releaser and serotonin reuptake blocker and induces the release of serotonin from the presynaptic neuron and blocks the re-uptake of serotonin [49–51].

2.5. Microdialysis

2.5.1. Habituation

At 23 weeks of age, two hens per pen were randomly selected for microdialysis ($n = 12$/line). Prior to surgery, each selected hen was habituated for 3–5 days in the microdialysis room adjacent to the operating room. There, hens were individually kept in wooden boxes ($l \times b \times h$; 60 cm × 30 cm × 40 cm) with steel mesh as rooftop and a Perspex window in the front and one in the partitions allowing visual contact with the neighboring hen. The cages used for microdialysis were of similar design as the habituation cages but did not contain side-windows to allow for undisturbed individual microdialysis measurements and had been customized such that the front window could open to function as a worktable; a slot was cut out for the microdialysis tubing to fit through. The sharp edges of this slot were covered with a rubber hose. Light was controlled by a time switch (2.00 am–6.00 pm). A radio played during light hours and animals also became habituated to humans walking close by their cages. Temperature in the habituation room was on average (±SEM) 18.7 ± 1.4 °C with an average humidity of 60.4 ± 9.2%. In the morning of surgery, the chickens were put on feed restriction [52] and birds were weighed. Water remained continuously available *via* the drinking nipples in the cage.

2.5.2. Surgery

Hens were anesthetized with a mixture of sevoflurane (7–8 l/min) and oxygen (0.8 l/min). When sedated, substances of analgesia were administered subcutaneously in the inguinal region. Body weight was on average 1.2 ± 0.3 kg. Body temperature was taken before and after surgery. Oxygen (O_2) flow was kept on 0.65 l/min for the entire procedure with a lower maintaining sevoflurane gas flow (3–4 l/min) administered *via* an intubation tube. Each hen was stereotaxically implanted directly in the brain with a fixed microdialysis probe (MAB 4.11.2CU, 2 mm membrane length with membrane OD of appr. 0.3 mm OD and shaft OD of 0.18 mm, Microbiotech, Stockholm, Sweden) aimed to cover the area of NCC and NCL as higher-order limbic and executive structures (Fig. 1). The head was fixed in a 6 in. (15.2 cm) raised stereotaxic apparatus (model 902, David Kopf instruments, Tujunga, CA, USA) and a chicken/duck adapter (model 917, David Kopf instruments) with the adaptor slide (model 1246, 45 Degree Adaptor Slide, David Kopf instruments, Tujunga, CA, USA) set to an angle of angle of 43° to bring the skull in the horizontal plane. The stereotaxic coordinates were determined following the brain atlas of a two-week old chick by Puelles et al. [53]. The absence of a brain atlas for adult chickens provided an uncertainty of the probe location, but a pilot study (not described here) provided the following coordinates: A; anterior to interaural line +6.0 mm, L; lateral to the interaural line +7.3 mm, V; ventral from the dura mater −6.3 mm. Probes were anchored on the skull with anchor screws and dental cement (methyl methacrylate dental, Vertex-Detal, Zeist, The Netherlands). To prevent chickens from damaging the probe by head scratching, the probe was also covered by a metal casing (aluminum; ID 8 mm, OD 10 mm; height 20 mm) screwed on an aluminum base (with OD of 9 mm; height 6 mm) that was imbedded in the cement. The inside of the base around the probe was also carefully filled with cement for extra stability of the probe. After implantation, hens were housed individually and placed in

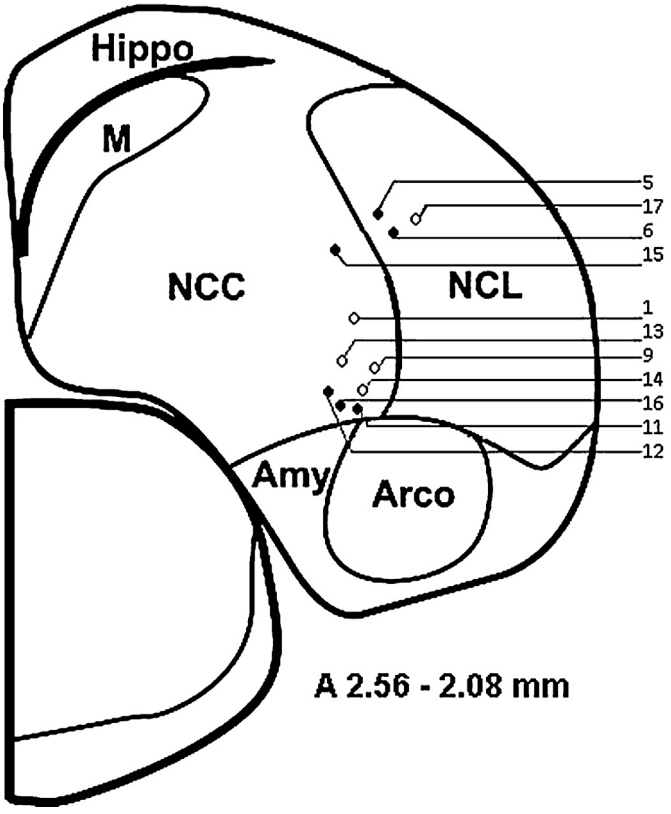

Fig. 1. Probe placements in the left caudal nidopallium illustrated in a schematic coronal section of a 23 week-old chicken brain. The representation of the brain areas in adult chickens is based on tracing and immunohistochemistry studies in chickens and pigeons [27–29,31,33] and on the (young) chicken brain atlas by Puelles et al. [53]. The anterior distance from the zero point is labeled (2.56–2.08 mm following [53]. Probes: open circles, LFP; closed circles, HFP. Abbreviations: Amy, amygdala; Arco, arcopallium; Hipp, hippocampus; M, mesopallium; NCC, caudocentral nidopallium; NCL, caudolateral nidopallium.

the microdialysis room until the end of the experiment. Three hens were subjected to surgery per day.

2.5.3. Microdialysis study

The microdialysis study was performed in conscious freely moving hens, one day after implantation of the microdialysis probe. The microdialysis system consists out of an infusion pump, a swivel with arm and tubing that ran from the pump to the swivel and probe and ended in the vials (to collect the dialysate). This infusion pump (KdScientific Pump 220 series, USA) perfused the system with Ringer solution (147 mM NaCl, 2.3 mM KCl, 2.3 mM $CaCl_2$ and 1 mM $MgCl_2$) at a constant flow rate of 0.33 μl/min. During microdialysis, the flow rate was set at 1.5 μl/min. The PE Tubing (Instech Laboratories, Inc., Plymouth, PA, USA) was attached with connection pieces (Gilson, Villiers-le-Bel, France) to both in- and outlet of the probe with an infusion pump linked to the inlet. Before sampling, a test run determined a delay time of 26 min, *i.e.* the time from probe to vial (length tubing was 65 cm, from swivel to head). At 7.45 am, hens were connected to a channel swivel (type 375/D/22QM, Instech Laboratories, Inc., Plymouth, PA, USA) which allowed them to move freely. At 11.00 am, about three hours after connection, 30-min samples were manually collected in vials containing 15 μl of 0.1 M acetic acid and directly frozen at −70 °C until analysis with HPLC. From 11.00 am until 1.00 pm four baseline samples were collected. Hereafter, D-fenfluramine was injected and, at 17.00 pm, the final sample (12 in total) was taken. Thereafter animals were culled and the brains were dissected and immediately stored in formaldehyde (4%) for later investigation of probe localization.

2.5.4. Chromatographic conditions

Microdialysis samples were stored at −70 °C until analysis. The neurotransmitter serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), respectively, were detected simultaneously by HPLC with electrochemical detection using an Alexys 100 LC-EC system (Antec, Zoeterwoude, The Netherlands). The system consisted of two pumps, one autosampler with a 10 port injection valve, two columns and two detector cells. Column 1 (NeuroSep105 C18 1 mm × 50 mm, 3 μm particle size) in combination with detector cell 1, detected 5-HT. Column 2 (NeuroSep 115 C18 1 mm × 150 mm, 3 μm particle size) in combination with detector cell 2, detected the metabolite. The mobile phase for column 1 consisted of 50 mM phosphoric acid, 8 mM KCl, 0.1 mM EDTA (pH 6.0), 9%MeOH, 5% ACN and 400 mg/l OSA. The mobile phase for column 2 consisted of 50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA (pH 3.25), 19.5% methanol and 700 mg/l OSA. Both mobile phases were pumped at 50 μl/min. Samples were kept at 8 °C during analysis. From each microdialysis sample 5 μl was injected simultaneously onto each column. The neurotransmitter 5-HT was detected electrochemically using μVT-03 flow cells (Antec, Zoeterwoude, The Netherlands) with glassy carbon working electrodes. Potential settings were for 5-HT +0.30 V *vs.* Ag/AgCl and for the metabolite 5-HIAA +0.59 V *vs.* Ag/AgCl. The columns and detector cells were kept at 35 °C in a column oven. The chromatogram was recorded and analyzed using the Alexys data system (Antec, Zoeterwoude, The Netherlands). The limit of detection was 0.05 nM (S/N ratio 3:1). Absolute extracellular monoamine concentrations are expressed as nM.

Besides measuring the absolute extracellular concentrations of 5-HT and 5-HIAA, also the response to D-fenfluramine was measured. It was not possible to measure DA concentrations in the chromatogram, because other molecules in the sample disturbed the DA-peak.

2.6. Histology

Dissected brains were quickly stored in formaldehyde until verification of the probe localization. Two days before brain slicing, the brains were placed in 30% sucrose solution. Probe placements were verified on 60 μm cresyl violet stained sections obtained with the frozen technique.

2.7. Statistical analyses

2.7.1. Microdialysis

Effects of line (LFP *vs.* HFP) on baseline monoamine and metabolite were analyzed by repeated measures ANOVA with time (4 levels: −90 min, −60 min, −30 min, and 0 min) as within-subject factor and line (LFP or HFP) as between-subject factor. Post-injection data were compared in repeated measures ANOVA with time (8 levels: 30 min, 60 min, 90 min, 120 min, 150 min, 180 min, 210 min, and 240 min) as within-subject factor and line (LFP or LFP) as between-subject factor. When the assumption of sphericity was violated, the results were corrected by the Greenhouse–Geisser procedure. All data were analyzed using the SPSS 20 software statistical package. For the analyses, only the data of hens with the probe localized in the area of interest (based on the histological study, see below) were included.

2.7.2. Large neutral amino acids concentrations in blood plasma

Mean levels of the large neutral amino acids (LNAAs) in blood plasma in the LFP and HFP were analyzed with use of independent *t*-tests. Blood was taken when animals were 17 weeks of age. At that age, the group consisted of 65 animals (LFP: $n = 32$; HFP: $n = 33$). A line comparison was made within this former group. Also, a line comparison was made within the subset of selected animals based on the histological study after microdialysis (LFP: $n = 5$; HFP: $n = 6$).

3. Results

3.1. Histology

Data of three hens was excluded before histology took place: two hens (of the LFP line) removed their cement caps before the actual microdialysis started, whereas a third hen (of the HFP line) had to be culled because of surgical complications. Due to this, histology to determine the probe placement was performed in 21 brains (LFP: $n = 10$; HFP: $n = 11$). The probes were aimed at the borderline of the NCC and the NCL (Fig. 1). Birds with a probe localized outside the region of interest (anterior distance from the zero point: 2.56–2.08 mm) were excluded from the dataset. This held for 10 animals in total ($n = 5$ per line). Consequently, the group size was reduced to eleven in total (LFP: $n = 5$; HFP: $n = 6$).

3.2. Serotonergic dialysate concentrations

3.2.1. Baseline serotonin and metabolite levels in the caudal nidopallium

Fig. 2 shows the extracellular levels of 5-HT and 5-HIAA. Repeated measures ANOVAs on the first four baseline measurements revealed significant line differences for 5-HT ($F_{(1,9)} = 17.34$; $P = 0.02$), with HFP having higher 5-HT levels compared to LFP. Lines did not differ in baseline 5-HIAA concentrations ($F_{(1,9)} = 0.19$; $P = 0.68$). There were no significant time x line interactions and no effect of time on the absolute mean baseline values, with the exception of 5-HIAA levels ($F_{(1,3)} = 3.19$; $P = 0.04$) which slightly decreased over time.

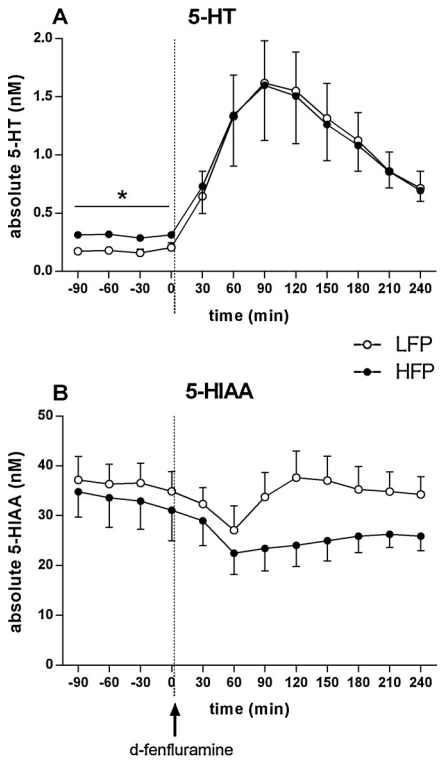

Fig. 2. Measuring 5-HT and 5-HIAA by *in vivo* brain microdialysis in the caudal nidopallium of adult LFP and HFP hens. 5-HT (A) and 5-HIAA (B) in the caudal nidopallium were measured under basal conditions (4 time points, −90 min to 0 min) and after D-fenfluramine injection (8 time points, 30–240 min) (left and right from the dotted line). LFP in open circles ($n = 5$) and HFP in closed circles ($n = 6$). Mean (±SEM) values for monoamines, $^{*}P < 0.05$

3.2.2. *Effect of* D-*fenfluramine on monoamine and metabolite levels in the caudal nidopallium*

The D-fenfluramine-response from time point zero onwards for 5-HT and 5-HIAA is also shown in Fig. 2. Time since administration of D-fenfluramine affected levels of 5-HT ($F_{(1,1.343)} = 12.821$; $P = 0.002$, $\varepsilon = 0.192$) and 5-HIAA ($F_{(1,2.982)} = 4.763$; $P = 0.01$, $\varepsilon = 0.731$), although lines did not differ (5-HT: $F_{(1,9)} = 0.002$, $P = 0.96$; 5-HIAA: $F_{(1,9)} = 2.466$, $P = 0.151$). There was no time x line interaction ($F_{(1,7)} = 0.019$, $P = 1.00$) or line effects ($F_{(1,9)} = 0.002$, $P = 0.963$) for 5-HT.

3.3. *Large neutral amino acids concentrations in blood plasma*

No line effects were found for the other LNAA levels measured or the TRP/LNAA ratio in this subset of animals, see Table 1. When comparing between all HFP and LFP hens (65 in total), lines differed in Tyr ($F_{(1,63)} = 2.33$, $P = 0.000$), with higher levels in LFP (152.12 ± 5.36) compared to HFP (120.47 ± 3.37) Moreover, line differences were found for Phe ($F_{(1,63)} = 0.45$, $P = 0.011$), and lines tended to differ for TRP ($F_{(1,63)} = 1.00$, $P = 0.082$). Compared to HFP, LFP had higher levels for Phe (HFP: 76.5 ± 2.03 *vs.* LFP: 83.6 ± 1.79) and tended to have higher levels for TRP (HFP: 86.31 ± 2.30 *vs.* LFP: 92.36 ± 2.53).

Table 1
LNAA levels (mean and SEM) in blood plasma in LFP ($n = 5$) and HFP ($n = 6$) hens.

LNAA	LFP		HFP		*P* value
	Mean	SEM	Mean	SEM	
TRP/LNAA[a]	0.15	0.01	0.15	0.01	0.775
TRP	82.7	3.90	81.7	4.93	0.763
Met + Val	104.4	10.82	102.0	5.28	0.843
Ile	86.2	14.68	83.90	7.92	0.886
Leu	168.9	15.45	161.2	8.96	0.666
Phe	82.2	2.49	77.1	4.27	0.457
TYR	147.3	11.45	122.0	9.17	0.115

[a] Ratio of tryptophan to the sum of the other LNAA [TRP/(Met + Val + Ile + Leu + Phe + TYR)].

4. Discussion

4.1. *Serotonergic differences in lines selected for and against feather pecking*

This microdialysis study in adult hens from the experimental selection lines demonstrated that HFP hens had a significant higher baseline 5-HT release in the caudal nidopallium as compared to LFP hens. Strikingly, the baseline 5-HT concentration had very little variance within line. Note that the 'punch' method used in most other studies, *i.e.* analysis of brain tissue samples as a whole, does not allow for distinction between the 5-HT and 5-HIAA concentrations in the presynaptic neuron and the extracellular matrix. Here, the release of monoamines within one specific brain area was targeted by *in vivo* microdialysis. To our knowledge, this is the first time that *in vivo* microdialysis is performed in adult laying hens, although *in vivo* microdialysis has been performed in young chickens (25 days of age) [33,43–45].

The present study demonstrates that HFP hens have higher baseline 5-HT concentrations compared to LFP birds. Several neurobiological factors may be responsible for the observed increased baseline release of 5-HT. For instance, higher 5-HT release could be caused by (1) more tryptophan (TRP) in the blood available (TRP is the precursor for brain 5-HT), (2) a higher enzymatic activity of tryptophan hydroxylase (TPH; metabolizes TRP to 5-HT), (3) a decreased activity of the serotonin transporter (SERT; facilitates the reuptake of released 5-HT into the presynaptic cell), (4) a lowered monoamine oxidase of type A (MAO-A; metabolizes 5-HT to 5-HIAA), or, finally, (5) altered 5-HT_{1A} autoreceptor activity (is part of the short negative feedback loop located on the presynaptic neuron). Peripheral TRP is a precursor for brain 5-HT [42,54]. TRP has to compete with other large neutral amino acids (LNAA) present in the blood to enter the brain *via* the blood–brain-barrier. Here it is shown that HFP and LFP birds do not differ in their TRP concentrations or in TRP/LNAA ratio. This is confirmed by plasma measurements in an earlier generation of these selected lines [41] and suggests that the higher baseline release of 5-HT cannot be attributed to differences in TRP availability. The synthesis of 5-HT from TRP can, however, be affected by the activity of the enzyme TPH [42]. D-fenfluramine induced a dramatic increase in 5-HT concentration in both lines, without differences between the lines. This latter observation demonstrates that the storage capacity in HFP hens probably does not differ from that of LFP hens. Two more factors important for 5-HT metabolism might be affected by selection on FP, namely SERT and MAO-A activity. SERT located on the membrane of presynaptic neurons facilitates the 5-HT clearance from the synaptic cleft [55,56] whereafter 5-HT can be stored again in the vesicles for future release. There are indications for SERT-involvement in FP from commercial selection lines since chickens selected on low mortality (due to low incidences of FP and cannibalism) differed from a control line in peripheral SERT functioning [57]. Peripheral SERT functioning has some predictive value for the activity of the central reuptake system [58]. Besides possible genetic selection effects, the impact of elevated 5-HT levels during life on the receptor activity should not be overlooked. Sustained elevated 5-HT has been recognized, both in the periphery and centrally, to affect not only the receptor sensitivity but also to have a down-regulatory effect on SERT functioning itself (reviews by [59,60]). However, there is no significant difference in the

levels of the 5-HT metabolite 5-HIAA to underpin this hypothesis. Concerning MAO-A, absence of differences in 5-HIAA levels also implies that MAO-A activity is not affected in either of the lines. On the other hand, polymorphisms on the gene coding for the MAO-A have been associated with the susceptibility to receive FP [7], but here, the effect of selection on FP on both SERT and MAO activity remains elusive. The 5-HT_{1A} autoreceptor is part of a short negative feedback loop located on the presynaptic neuron [61]. Under normal conditions, 5-HT molecules are released in the presynaptic cleft and part of these molecules will reach the 5-HT_{1A} autoreceptor, thereby inhibiting neuronal firing and consequently suppressing 5-HT synthesis. A lower serotonergic neurotransmission caused by an underlying hypersensitive 5-HT_{1A} autoreceptor system has been suggested to be a trait-characteristic of violent rodents, showing escalated aggression [61–67]. The relationship between HFP and higher baseline 5-HT neurotransmission found in the present study does not suggest a similar involvement of the 5-HT_{1A} autoreceptors in SFP as for aggression. This supports the hypothesis that feather pecking is different from aggressive pecking [3,68].

4.2. Selection on FP behavior and its neurobiological effects

Recently, it was found that chicks from the HFP line walked a longer distance in their home pen than LFP chicks which lead to the suggestion that HFP chicks suffer from a hyperactivity disorder [26]. These high levels of activity in the HFP may originate from a more activated motor system. Both the NCC and NCL are connected with the medial arcopallium (AM), the n. posterioris amygdalopalii (PoA), the n. taeniae amygdalae (TnA), and intermediate arcopallium (AI) [27,28,31]. Whereas AM, PoA, and TnA are considered limbic and homologous to subcomponents of the amygdala, the AI is a somatomotor area with secondary sensory afferents and projections to rhombencephalic motor areas [69]. Both NCC and NCL contain similar amount of serotonergic fibers and show a high density of 5-HT_{1A} binding sites [35], but the NCL is more densely covered with dopaminergic fibers than the NCC (see pigeon studies by [37,70]. It has been described that both increased and decreased levels of 5-HT may lead to enhanced locomotor activity and enhanced reactivity to a novel stimuli and environmental changes [71]. These behaviors fit the HFP chicks, although these behaviors are usually generated by lower 5-HT levels [71].

5. Conclusion

The present *in vivo* microdialysis experiment clearly shows that adult laying hens of the HFP line are characterized by higher baseline serotonin release in the caudal nidopallium as compared to birds of the LFP line. This suggests that a higher tonic 5-HT neurotransmission activity under baseline conditions is a characteristic of HFP hens. This study illustrates that microdialysis in adult laying hens selected for divergences in feather pecking can provide interesting new perspectives on the role of 5-HT in feather pecking.

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