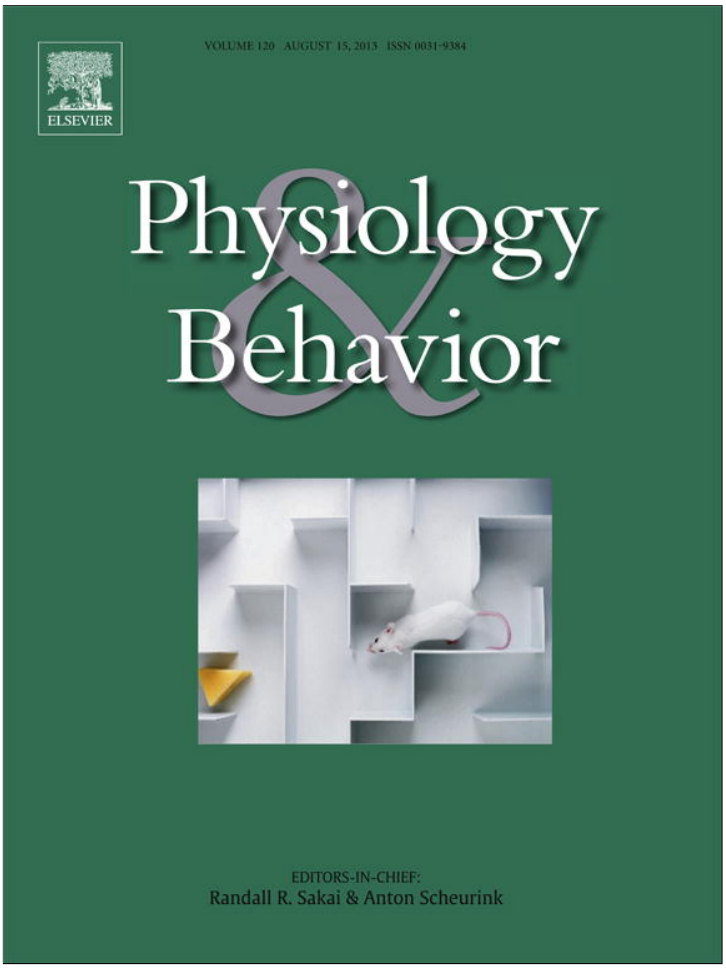

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Effects of feather pecking phenotype (severe feather peckers, victims and non-peckers) on serotonergic and dopaminergic activity in four brain areas of laying hens (*Gallus gallus domesticus*)

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HIGHLIGHTS

- Four brain areas belonging to the basal ganglia-thalamopallial circuit were examined
- Feather peckers and victims had higher 5-HT turnover in thalamus than non-peckers
- These phenotypes also differed in 5-HT levels in medial striatum
- No phenotypic differences for 5-HT were found in arcopallium and hippocampus
- DA turnover levels were not affected by feather pecking phenotype

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ABSTRACT

Severe feather pecking (SFP) in laying hens is a detrimental behavior causing loss of feathers, skin damage and cannibalism. Previously, we have associated changes in frontal brain serotonin (5-HT) turnover and dopamine (DA) turnover with alterations in feather pecking behavior in young pullets (28–60 days). Here, brain monoamine levels were measured in adult laying hens; focusing on four brain areas that are involved in emotional behavior or are part of the basal ganglia-thalamopallial circuit, which is involved in obsessive compulsive disorders. Three behavioral phenotypes were studied: Severe Feather Peckers (SFPs), Victims of SFP, and Non-Peckers (NPs). Hens (33 weeks old) were sacrificed after a 5-min manual restraint test. SFPs had higher 5-HIAA levels and a higher serotonin turnover (5-HIAA/5-HT) in the dorsal thalamus than NPs, with intermediate levels in victims. NPs had higher 5-HT levels in the medial striatum than victims, with levels of SFPs in between. 5-HT turnover levels did not differ between phenotypes in medial striatum, arcopallium and hippocampus. DA turnover levels were not affected by feather pecking phenotype.
These findings indicate that serotonergic neurotransmission in the dorsal thalamus and striatum of adult laying hens depends on differences in behavioral feather pecking phenotype, with, compared to non-pecking hens, changes in both SFP and their victims. Further identification of different SFP phenotypes is needed to elucidate the role of brain monoamines in SFP.

1. Introduction

Severe feather pecking (SFP) is a detrimental behavior in laying hens which leads to feather and skin damage and sometimes even death of the recipient due to cannibalism [1]. In search of factors playing a role in increasing the vulnerability for developing into a feather pecker, brain monoamines have become of increasing interest. Several studies investigating the neurobiology of laying hens with high-and low-feather-pecking (FP) incidence, indicated that SFP is linked to lowered

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serotonergic and dopaminergic turnover [2–8]. For instance, young pullets from a high FP line selected for large egg size and good eggshell qualities showed lower serotonin (5-hydroxytryptamine; 5-HT) and dopamine (DA) turnover levels in the rostral part of the forebrain than young pullets from a control line. Also, a 5-HT_{1A} autoreceptor agonist, which inhibits 5-HT release increased the incidence of SFP [6,9], while SFP decreased after chronic dietary supplementation of the 5-HT precursor tryptophan [10]. This high FP line also had a more sensitive DA system, as demonstrated by an increased behavioral response to the DA receptor agonist apomorphine [6]. Pharmacological studies showed that treatment with the DA D_2-receptor antagonist haloperidol reduced feather pecking in adult White Leghorns [2] and feather picking in grey parrots [11]. Thus, FP might be associated with low serotoninergic and dopaminergic neurotransmission. It is important to keep in mind that most studies on 5-HT and DA involvement in FP behavior have been performed in young chicks. These pullets, however, mainly show gentle FP behavior, while SFP behavior usually becomes more pronounced at a later age [4,12]. Gentle FP behavior at a young age, however, is not necessarily predictive for SFP behavior in the same individual at adult life [13]. Remarkably, only a few animals within a flock will start with SFP, while the majority of birds do not [14].

SFP is a very persistent and goal-directed behavior with clear impulsive compulsive characteristics [15]. Feather damaging behavior also can be seen in parrots [16]. In mice, a similar type of maladaptive behavior, i.e. barbering, can be observed [17], and in humans, a specific hair-pulling disorder, i.e. trichotillomania, is known [18,19]. Both impulse control disorders (ICD) and obsessive compulsive disorders (OCD) in humans are associated with lower levels of brain 5-HT [20]. Mice lacking the gene encoding for brain tryptophan hydroxylase 2—resulting in 5-HT depletion—exhibit more compulsive behaviors, like burying, aggressive and motor impulsive behaviors [21]. Deficits and lesions in human frontal–striatal–thalamic brain areas also decrease 5-HT levels and increase the risk for ICD or OCD [20,22,23]. It is unknown whether similar deficits in basal ganglia-thalamopallial circuits underpin SFP in laying hens. Evidence demonstrating functional and structural similarities between avian and mammalian brains is accumulating [24–28], therefore it becomes necessary to focus on specific brain areas, instead of entire frontal brains as in previous studies [4,5].

In the present study, four brain areas were selected that may be involved in emotional behavior or are part of the basal ganglia-thalamopallial circuit which is involved in obsessive compulsive disorders (OCDs): the telencephalic medial striatum, hippocampus, arcopallium, and the diencephalic dorsal thalamus. The avian ventral striatum, a combination of the medial striatum and the nucleus accumbens, plays an important role in reward [29], as does the human caudate nucleus [30]. The hippocampus of both mammals and birds seems to be involved in memory and learning [31]. The arcopallium is a somatomotor region surrounded by the subnuclei that constitute the amygdala [24,28,32]. Functionally, the arcopallium seems also to be involved in anxiety [33]. Anxiety often forms the base of obsessions and attempts to relieve this anxiety might lead to compulsions [20,34,35]. The dorsal thalamus has direct connections with the telencephalic areas [36] and disinhibition of the thalamus will affect goal-directed behavior in humans and animals, with compulsions as risk factor [35].

Measuring 5-HT and DA levels and turnover in these brain areas will increase our knowledge of the neurobiological mechanisms of SFP in laying hens. This study is the first to analyze four brain regions of interest of adult laying hens characterized by their FP phenotype. The phenotypes used are severe feather peckers (SFPs), 'victims', and 'non-feather peckers' (NPs), based on individual observations on giving and receiving SFP. We hypothesize that severe feather peckers show a distinct pattern in monoaminergic levels and turnover, in particular in 5-HT and DA, as compared to the other phenotypes.

2. Materials and method

2.1. Birds and housing

In this study, 27 female White Leghorns (*Gallus Gallus*) were selected for brain analysis from 260 hens in total of which a subpopulation was previously studied [37]. Eighty hens originated from an unselected control line (C) and hundred and eighty of the fourth generation of a low mortality line (L) aimed at breeding with those candidates of which siblings showed low group mortality [38]. Phenotypes were equally spread among these lines (see 2.5. Statistical analysis). The hens were obtained from ISA, the layer breeder division of Hendrix Genetics, the Netherlands. All non-beak trimmed hens were housed per line (26 pens in total, 10 birds/pen) in 1.9 × 1.2 m pens. Water and a commercial mash diet were provided *ad libitum*. Pens were provided with a perch and floors were covered with sand (1/3) and wood shavings (2/3). For more details on housing conditions, see [37]. The experiment was approved by the Institutional Care and Use Committee of Wageningen University and in accordance with Dutch legislation on the treatment of experimental animals, in conformation with the ETS123 (Council of Europe 1985) and the 86/609/EEC Directive.

2.2. Categorizing behavioral phenotypes

FP behavior was observed using behavior sampling over three weeks for 30 min each week on varying times during the day (9.00 am–16.00 am), at an age of 19, 20 and 21 weeks. We recorded the frequency of severe feather pecking (SFP), i.e. bouts of hard pecks and pulling attempts directed at feathers at the tail, back and wings. A bout of SFP was defined as pecks in a continuous series directed to the same chicken to the same body part. Gentle feather pecks (*nibbling* on feathers) and aggressive pecks (directed at head, see [39]) were also recorded, but not taken into account in the categorization procedure. Hens were categorized as feather pecker (SFPs) when giving a minimum of two SFP bouts over the observations weeks, without receiving any SFP bouts, as victim when receiving at least one SFP bout, but not giving SFP; or as non-pecker if not giving or receiving SFP. For brain analysis, we selected n = 9 birds per behavioral phenotype. To account for pen effects, the selection of phenotypes was balanced over pens, that is, we chose one SPF bird, one non-pecker and one victim per pen. The three FP phenotypes did not differ in aggressive pecks given or received (data not shown).

2.3. Brain tissue preparation

At 33 weeks, chickens were subjected to a 5-min manual restraint test on two consecutive days, using a method previously described [40]. Order of testing was balanced for phenotype and line. Immediately after testing, the hens were sacrificed by cervical dislocation. Brains were removed and immediately deep frozen in n-heptane, put on dry ice and stored at −80 °C [4]. Slicing of brains was executed in a cryostat (Frigocut Jung Mod_700) under cold conditions (−10 °C). Slice thickness was 400 μm. The four regions of interest were located using the brain atlas for 2-week-old chickens [41], thereby taking into account the increased brain size in our hens at 33-weeks of age. Punches were taken from multiple slices, with corresponding figure numbers in the atlas: Medial striatum (MSt; interaural 7.56 – 5.68 mm) including the accumbens (Acb; interaural 8.08 – 7.56 mm), hippocampus (Hi1, Hi2, PHiM, PHil, PHil1, PHil 2, and PHiA; interaural 6.16 – 0.40 mm), and the dorsal thalamus (DPe, DMA, DIA, DLA; interaural 3.04 – 1.36 mm). For the arcopallium, the area referred to as amygdala core by [41] was sampled (interaural 4.24 – 2.08 mm). Brain samples of the left and right hemisphere were taken together and analyzed as one.

2.4. Central monoamine analysis with HPLC

Brain samples were analyzed using a High Performance Liquid Chromatography (HPLC) method. For that, the tissue samples were homogenized in an ice-cold solution containing 5 μM clorgyline, 5 μg/ml glutathione and 1.2 μM N-methylserotonin (NMET, internal standard) using sonication. To 80 μl homogenate, 20 μl 2 M HClO4 was added and mixed. After 15 min in ice water, the homogenates were centrifuged for 15 min at 15000 g (4 °C). The supernatants were diluted 10 times with water before HPLC analysis. The concentration of serotonin [5-HT] and its metabolite 5-hydroxyindoleacetic acid [5-HIAA], and dopamine [DA] with according metabolites 3-methoxytyramine [3-MT], 3, 4-dihydroxyphenylacetic acid [DOPAC], and homovanillic acid [HVA] in the tissue extracts were measured by HPLC with electro chemical detection (ECD). The HPLC system consisted of a pump model P100, a model AS300 autosampler (both from Thermo Separation Products, Waltham, MA, USA), a ERC-3113 degasser (Erma CR. Inc. Tokyo, Japan), an ESA Coulochem II detector with 5011 analytical cell set at potential +550 mV (ESA Inc. Bedford MA, USA), a data acquisition program (Atlas 2003, Thermo Separation Products) and a column (150 mm × 4.6 mm i.d.) packed with Hypersil BDS C18, 5 μm particle size (Alltech Associates, USA). The mobile phase solution consisted of 50 mM citric acid, 50 mM phosphoric acid, 0.1 mM EDTA, 45 μl/l dibutylamine, 77 mg/l 1-octanesulfonic acid sodium salt, 10% methanol; the pH of the buffer was adjusted to 3.4 with NaOH. Separation was performed at 45 °C using a flow rate of 0.8 ml/min. The concentration of each compound was calculated by comparison with both the internal and the external standards. The protein content of each homogenate sample was determined using the DC protein Assay (Bio-Rad). Monoamine concentrations are expressed as nmol/g protein. Turnover levels of serotonin (5-HIAA/5-HT) and dopamine ((DOPAC + HVA + 3-MT/DA) were calculated as an index for the activity of, respectively, the serotonergic and dopaminergic system [4]; high levels indicate a quicker metabolic pathway due to higher biosynthetic enzyme activity.

2.5. Statistical analysis

Effects of phenotype (SFPs vs. victims vs. NPs) on monoamines were analyzed with a general linear model (GLM) that included effects of line (C and L) and day of sacrifice, using SAS Software 9.2. In a preliminary analysis of the data, no interactions between phenotype and line were found. In case of significant phenotype effects, post-hoc least square means were used to detect pair-wise differences. A log transformation for 5-HT and 5-HIAA in the hippocampus was executed to obtain normality of residuals. Data are presented as mean ± SEM.

3. Results

3.1. Feather pecking phenotypes

In nine out of 26 pens SFP was detected during observations. This led to the categorization of the 'SFPs', 'victims', and 'NPs' phenotypes (n = 9/phenotype). The SFP hens selected gave on average 3.4 ±0.5 bouts of SFP and received none, victims received on average 2.1 ±0.4 bouts of SFP and gave none, and NPs gave zero bouts of SFP, but one out of the nine NP-characterized hens received one bout of SFP (average 0.1 ± 0.1).

3.2. Phenotype effects on dopamine

In the hippocampus, levels of DA and of its metabolites were below detection level for most samples. In the other brain areas, phenotype did not affect DA, DOPAC, HVA, and 3-MT levels (data not shown), except that HVA ($F_{2,22} = 3.63$, $p < 0.05$) in the arcopallium was higher in SFPs (18.1 ± 2.1) and tended to be higher in victims (18.1 ± 3.0) in comparison to NPs (13.1 ± 2.0), without affecting DA turnover. DA turnover levels did not differ between the phenotypes (dorsal thalamus: $p > 0.10$, 1.17 ± 0.08; medial striatum: $p > 0.10$, 0.24 ± 0.01; arcopallium: $p > 0.10$, 1.45 ± 0.17).

3.3. Phenotype effects on serotonin

5-HT levels in the dorsal thalamus (Fig. 1, upper panel) were unaffected by phenotype, but phenotype affected 5-HIAA levels in this brain area ($F_{2,21} = 5.43$, $p < 0.05$) with SFPs having higher levels than NPs ($p < 0.01$). Thalamus 5-HIAA levels of victims were intermediate between SFP and NPs, but tended to be higher than those of NPs ($p < 0.10$). Phenotypes differed in 5-HT turnover levels in the dorsal thalamus ($F_{2,21} = 5.67$, $p < 0.05$). Post-hoc tests revealed that SFPs and victims both had a higher 5-HT turnover than NPs.

In the medial striatum (Fig. 1, lower panel), phenotype did affect 5-HT levels ($F_{2,22} = 4.72$, $p < 0.05$). Post-hoc tests indicated higher 5-HT levels for NPs compared to victims, with intermediate levels in SFPs. 5-HIAA in the medial striatum tended to be affected by phenotype ($F_{2,22} = 2.89$; $p < 0.10$), with higher levels for SFPs than for victims and a tendency for higher levels in NPs than in victims. 5-HT turnover in the medial striatum was unaffected by phenotype.

In the arcopallium and hippocampus, no significant effects of phenotype on 5-HT, 5-HIAA or 5-HT turnover were found (Table 1).

4. Discussion

Monoaminergic turnover was measured in adult laying hens that differed in phenotype: severe feather pecking (SFPs), victims of SFP, and non-feather peckers/non-victims (NPs). The most pronounced findings were: 1) both SFPs and victims had higher 5-HT turnover in the dorsal thalamus as compared to NPs, 2) in the medial striatum, NPs had higher 5-HT than victims with SFPs in between, 3) no phenotypic differences in 5-HT neurotransmission were found in the arcopallium or hippocampus, and 4) DA turnover did not differ between phenotypes in any of the four brain areas.

4.1. Dopaminergic activity

There were no dopaminergic differences found between phenotypes, with exception of higher HVA levels for SFPs and victims than for NPs in the arcopallium. This single result shares resemblance with higher DOPAC levels, also a DA metabolite, measured in the rostral part of the brain of adult chickens from the White Leghorn line with more feather damage due to FP in comparison with a Rhode Island Red line low in feather damage [42]. Still, the absence of strong dopaminergic phenotypic differences is unexpected as several studies have indicated that modulating dopaminergic activity affects FP behavior [2–4,43]. It has to be noted, though, that due to strong neural and monoaminergic connections, serotonergic abnormalities will affect the dopaminergic system (described by [44]), thus despite the lack of dopaminergic turnover differences between phenotypes the DA system can still be affected although not shown by the used method (punches) in the present study.

4.2. Serotonergic activity

Differences in serotonergic neurotransmission of the different behavioral phenotypes were mainly found in the dorsal thalamus, but not in the hippocampus or arcopallium. In the dorsal thalamus, SFPs and victims had a higher 5-HT turnover than NPs due to higher 5-HIAA levels. 5-HT turnover levels in the medial striatum, albeit numerically higher for SFPs, did not significantly differ between the three phenotypes, despite significant differences for 5-HT and 5-HIAA separately. That is, NPs had higher 5-HT levels than victims with intermediate levels in SFPs, while for 5-HIAA, SFPs tended to have

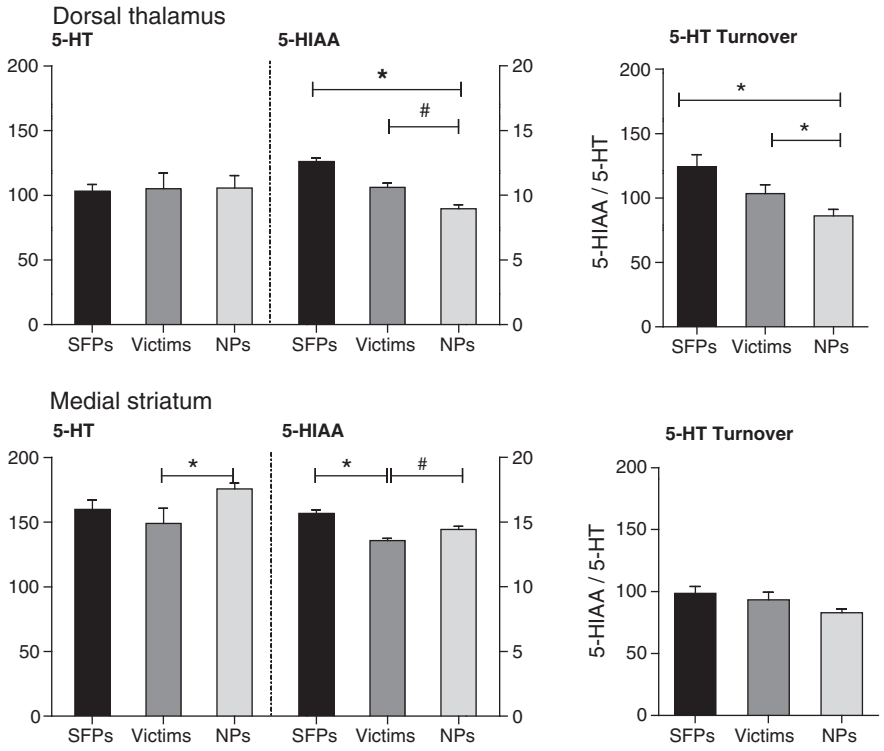

Fig. 1. 5-HT and 5-HIAA (in nmol/g protein), and 5-HT turnover levels in the dorsal thalamus (upper panel) and medial striatum (lower panel) of laying hens characterized as severe feather peckers (SFPs), victims, or non-feather peckers (NPs), n = 9 per phenotype, * $p < 0.05$; # $p < 0.10$.

higher levels than victims with intermediate levels in NPs. In line with other studies [3,4,6,7,10,42], 5-HT neurotransmission is altered in SFPs, but also in their victims.

Stress factors contribute to brain alterations. Captivity in general [45] and more specifically, group size [46], fearfulness of group members [37,47], housing conditions [48], and the absence of maternal care [49] are stress factors that will affect behavior and physiology, including serotonergic structures and their function [48,50]. In general, feather peckers seem to differ in stress responsiveness: Differences in basal and stress-induced CORT were found in lines diverging in FP, although both high [51,52] and low [4,53,54] levels of CORT have been associated with high SFP incidences. Feather peckers also had a higher heart rate during manual restraint than NPs [55] and had altered exploration in an open field [56]. It is known that thalamic and striatal areas as part of the basal ganglia-thalamopallial circuit, are involved in guiding motor actions and decision-making by integrating sensorimotor, motivational, and emotional information from the cortex and limbic areas [24,57,58]. For instance, low 5-HT levels after lesions in the medial striatum of chickens impair the suppression of immediate reward seeking behavior [59], which is considered impulsiveness. It can be speculated that the altered 5-HT neurotransmission reflects increased vulnerability for stressful events in SFPs with corresponding anxiety and increased risk for developing impulsive/compulsive disorders [20,34,35] causing chickens to develop SFP. In birds, the exact role of 5-HT in these brain areas and the development of OCD and anxiety needs further investigation.

Surprisingly, NPs had a decreased 5-HT turnover in the dorsal thalamus as compared to both SFPs and victims. Interestingly, a gene expression study in abnormal tail biting pigs showed that non-biting pigs differed distinctly from tail-biters and their victims [60], similar to these results in chickens. This phenotypic distinction is further supported by the different gene expression patterns in feather pecking chickens that are related to OCD (SFPs, victims, and NPs) [61]. The similarity between SFPs and victims might be explained by victims perceiving attacks of SFP as highly unpredictable and stressful. It has been shown that unpredictable tail-shocks in rats increase 5-HIAA and 5-HIAA/5-HT ratio levels in the thalamus and striatum [62] and that being victimized may lead to long-term structural adaptations in monoaminergic systems [63]. Thus as feather pecking affects both SFPs and victims, NPs seem to remain aloof; showing the importance of phenotypic discrimination.

Our results suggest that both victims and SFPs respond with higher 5-HT activity in the dorsal thalamus. The higher 5-HT turnover levels in SFPs in this study are seemingly in contrast with literature on impulsive and compulsive disorders in humans, rats and mice [18,19,64,65] and in contrast with earlier established negative correlation between 5-HT turnover and time spent on FP behavior in young (28–60 days old) chicks [4,5,10]. Previously, van Hierden and collaborators [4] showed that high FP young (mostly gently FP) pullets have a much higher responsivity towards serotonergic drugs or tryptophan than low FP pullets. It was shown by de Boer and collaborators [64–66] that enhanced inhibitory control of the serotonergic raphe neurons (via 5-HT1A autoreceptor) in trained aggressive male rodents might explain the negative correlation between 5-HIAA/5-HT and aggression. This inverse relationship between tonic (trait-like) 5-HT activity and aggression, however, was only observed in the pathological form of aggression

Table 1
5-HT, 5-HIAA and 5-HT turnover levels in the arcopallium and the hippocampus of hens characterized as severe feather peckers (SPFs), victims, and non-feather peckers (NPs) (in nmol/g protein, Mean ± SEM, n = 9).

Phenotype	SFPs	Victims	NPs	*P* value
Arcopallium				
5-HT	247.2 ± 23.8	287.9 ± 43.4	241.3 ± 32.0	0.52
5-HIAA	25.8 ± 1.7	27.3 ± 3.6	20.4 ± 2.6	0.13
5-HT-turnover	107.8 ± 8.3	98.9 ± 8.0	86.0 ± 6.1	0.44
Hippocampus				
5-HT	156.0 ± 16.4	181.1 ± 27.2	164.9 ± 11.7	0.96
5-HIAA	19.8 ± 3.3	21.0 ± 1.7	21.7 ± 1.4	0.55
5-HT-turnover	127.9 ± 19.2	125.9 ± 13.3	131.4 ± 5.7	0.95

(violence). In young pullets, however, stimulation of the 5-HT1A autoreceptor lowered aggression, but increased the time spent on feather pecking behavior [5]. Thus, the motivational drive and neurobiological mechanism involved in high aggression and severe feather pecking behavior probably really differs.

Remarkably, Uitdehaag and colleagues showed higher 5-HIAA and 5-HT turnover levels in adult hens of the White Leghorn line that are characterized by high scores of SFP, compared to adult hens of the brown Rhode Island Red line that do not show much SFP [42]. Thus, multiple factors may contribute to FP at different moments in life probably due to plasticity of the brain and behavioral interactions between birds (e.g. copying behavior or attraction to damaged feathers). This might increase the vulnerability to develop (mostly gentle) FP at a young age, while an increased 5-HT turnover as found here (and in [42]) in adult laying hens may reflect a changed 5-HT signaling in response to being exposed to or executing SFP behavior throughout and later in life (either as victim or as feather pecker). The latter puts emphasis on the importance of separating cause and consequence of FP on brain monoamine levels. Besides indications that FP may arise due to disturbances at a neurochemical level ('cause'), animals can change their own behavior ('consequence') by observing others, called social transmission [67]. Although only the transmission of gentle FP among chickens is confirmed [68], this still asks for carefulness when studying FP phenotypically. That is, if SFP within a group is started by birds with disrupted monoaminergic levels, but is facilitated in others birds, characterizing phenotypes and relating brain-to-behavior becomes complicated. Altogether, it is important to consider both phenotype (i.e. the composite of an organism's observable characteristics) and genotype (i.e. the genetic contribution to the phenotype) when trying to identify causal mechanisms of FP. Hence, we hypothesize the existence of different phenotypes of feather peckers: 'first-order' feather peckers (partly determined by genotype) which may be vulnerable to develop SFP due to (heritable) neurochemical 'deficits', and 'second-order' feather peckers (mainly determined by environment and thus phenotype) in which the feather pecking is socially facilitated or feather pecking is increased because the bird's attention is drawn to the damaged feathers of the victim. Remarkably, (passively coping) chickens with initially low FP incidences are more vulnerable to show 'second-order' feather pecking, because they are more aware of environmental changes [53] and/or might react out of frustration by pecking at damaged or ruffled feathers [69,70]. Therefore, differences in coping strategy or personality may underlie the differences between first- and second-order feather pecking phenotypes. For instance, proactive and reactive (passive) coping style can be distinguished reflecting different ways of coping with stressful circumstances (reviews by [53,71,72]) with behavioral and physiological interspecific variations [4,54,55,73]. Characterizing severe feather peckers as early as possible is the best way to distinguish the different feather pecking phenotypes, e.g. by identifying clear biomarkers with predictive value.

5. Conclusion

To our knowledge, this study is the first to link severe FP behavior in adult laying hens to central 5-HT and DA turnover levels in four brain areas. Unexpectedly, dopaminergic neurotransmission was not affected by phenotype, but SFPs and their victims did have higher 5-HT neurotransmission in the dorsal thalamus and medial striatum. Therefore, it can be concluded serotonergic neurotransmission in the dorsal thalamus and striatum of adult laying hens depends on differences in behavioral phenotype, i.e. severe feather peckers, victims or non-peckers.

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